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THE INTEGRATION OF DIGESTIVE, METABOLIC AND OSMOREGULATORY PROCESSES IN NECTAR-EATING BIRDS

by

Todd Jason McWhorter

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF ECOLOGY AND EVOLUTIONARY BIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2002

THE UNIVERSITY OF ARIZONA ®
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Todd Jason McWhorter

entitled The Integration of Digestive, Metabolic and Osmoregulatory Processes in Nectar-eating Birds

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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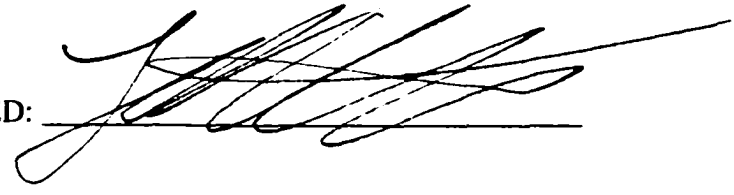
SIGNED: 

ACKNOWLEDGEMENTS

Ask a certain emergency room physician in Laramie, Wyoming, and he'll tell you that Carlos Martínez del Rio's graduate students get a very well rounded education. If you run into me at a conference one day you can ask me what that means. I interviewed to come and work in Carlos' lab not knowing who he was, or really what I wanted to do with my life. By sheer dumb luck, or maybe because of Carlos' exceptional character judging skills, here I am. I think I'm in the right place. I probably don't tell him often enough how lucky I feel to be working with such an exceptional scientist, how proud I am to be part of his legacy, and how much I value his friendship. This dissertation would not have been possible without his guidance and support. I also could not have made it without the constant love and support of my wife, Andrea Rene McWhorter. I don't think that I can ever repay her exceptional patience during my long absences, but I'm sure going to try. So many people have been integral to the successful completion of this work. Berry Pinshow made the research on Palestine sunbirds possible by bringing the U.S.-Israel Binational Science Foundation to my attention, and by being a most gracious host and enthusiastic collaborator. I hope that this is only the beginning of a lifelong friendship. Jorge (Chonito) Schondube, Ana Claudia (Cabeza de Zopilote!) Nepote, Blair Wolf, Don Powers, Lizanne Roxburgh, Klaus Droppelmann, Ian van Tets, Carmi Korine, Juliann Aukema, Ann Danielson-François, Shelli Dubay, Andew McKechnie, Maria-Victoria López-Calleja and Chris Lotz have been constant friends, excellent collaborators and willing co-conspirators. To them I owe much, including probably my sanity. For those not mentioned above that have spent long hours in the lab with me, you have my undying gratitude: Mariela Leiderman, Efrat Elimelich, Itzick Vatnick, Eriquena Bustamante, Nate Miller, Amber Hamilton, Casey Cotant, Jeremy Diaz, and many more. Catherine Hambly generously provided access to unpublished data. Last, but most certainly not least, Eldon Braun, Steve Wright, Jon Harrison, Alex Badyaev, Steven Hopp, and William A. Calder, III (to whose fond memory this dissertation is dedicated) provided resources and invaluable insight along the way.

This research was primarily supported by Grant No. 98-178 from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel to Carlos Martínez del Rio and Berry Pinshow and a NSF grant (IBN-0110416) to Carlos Martínez del Rio. Other sources of funding include: the James R. Silliman Memorial Research Award, the Department of EEB at the U of A, the Department of Biology at George Fox University, and the AMNH's Southwestern Research Station Student Support Fund.

For Bill...

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ABSTRACT

Nectarivorous birds are represented by three major radiations: hummingbirds, honeyeaters, and sunbirds. These lineages share a number of convergent features in ecology, morphology, physiology, and behavior, and have served as important models in the study of foraging strategies and energetics. Because their diet is rich in water and sugar but poor in nitrogen and electrolytes, nectarivores provide a striking opportunity for evaluation of physiological constraints. My research emphasizes a novel aspect of the water-energy interaction: water overingestion in nectar-eating birds. The dual purpose of my dissertation research was to investigate the physiological mechanisms that allow nectar-eating birds to cope with exceedingly high ingestion of water and to elucidate the consequences of ingesting and processing large quantities of water for energy intake and for the maintenance of balance of important metabolites such as glucose. In nectar-eating birds, water overabundance in food has the potential effect of constraining energy procurement by overwhelming osmoregulatory processes and limiting digestive function. My research has allowed the development of an integrated quantitative description of gut and kidney function under the broad range of water loads and hydration conditions that birds can experience in the wild. Understanding limits to water processing will provide general insights into how animals are designed, on how aspects of design constrain their

ecological performance, and into how aspects of design in one physiological system can impose limits on other systems. The osmoregulatory processes of nectar-eating birds highlight the relevance of understanding the impact that events taking place in the gut can have for feeding behavior, and renal and metabolic function. Adopting a broadly comparative approach to understanding the interaction between feeding behavior, digestion, and osmoregulation is pertinent because is unclear whether the many extreme physiological characteristics of hummingbirds that have traditionally been assumed to be associated with a nectar-feeding habit are shared by other nectar-eating birds. In my dissertation research I have begun to examine the similarities, and have found some important differences, in the responses of two major radiations of nectar-eating birds to their sugary and watery diets.

CHAPTER 1 - INTRODUCTION

1.1 – OVERVIEW

Floral nectars are generally dilute aqueous sugar solutions containing trace amounts of amino acids and electrolytes (Baker and Baker 1990). In spite of nectar's simplicity as an energy source for nectarivorous animals, it shows considerable variability in sugar composition and concentration (Pyke and Waser 1981; Baker et al. 1998; Nicolson 2002). The nectars of bird-pollinated plants may contain the disaccharide sucrose, the monosaccharides glucose and fructose, or mixtures of these three sugars (Martínez del Rio et al. 1992), and their sugar concentrations span an order of magnitude in range (from 5% to over 60% sugar wt./vol., Pyke and Waser 1981; Stiles and Freeman 1993). The ecological and evolutionary correlates of sugar composition have received considerable attention over the past decade (Martínez del Rio et al. 1992; Jackson et al. 1998; Herrera M. 1999; Nicolson 2002). The physiological challenges posed for nectarivorous vertebrates by heterogeneity in nectar sugar concentration, however, have been relatively unexplored. The sugars that these animals rely on as energy sources are frequently obtained in nectar with an excess of water (Calder 1979; Calder and Hiebert 1983; Beuchat et al. 1990). The dual purpose of my dissertation research was to investigate the

physiological mechanisms that allow nectarivorous birds to cope with their watery diets and to elucidate the consequences of ingesting and processing large quantities of water for energy intake and for the maintenance of balance of important metabolites such as glucose.

The ability of nectar-feeding birds to use floral nectar as a primary energy source depends on the integration of gastrointestinal and renal function for maintaining energy and water balance. Their physiological capacities to extract the energy and nutrients from nectar solutions and eliminate excess water determine the range of nectar sugar concentrations that they are able to use, and thus influence their food preferences and feeding behavior. Because nectar-feeding birds are members of a mutualistic partnership with bird-pollinated plants, their physiological and behavioral traits can have consequences for these plants (Martínez del Rio et al. 1992). By shaping the food preferences of nectar-feeding birds, their behavioral responses to nectar energy density and their protein requirements have coevolutionary consequences. The unique nutritional, energetic and osmoregulatory problems faced by nectar-feeding birds can be divided into four main areas: 1) How do nectar-feeding birds deal with the water ingested as part of their food once it is in their intestines? 2) How do nectar-feeding birds dispose

of excess water via their kidneys? 3) How do the digestive capacities of nectar-feeding birds shape their feeding behavior? And 4) How do the low nitrogen levels in floral nectars and the high water fluxes experienced by nectar-feeding birds correlate with their nitrogen requirements and nitrogenous waste excretion? In the first section of this introduction, I outline the approaches used to answer these questions and discuss the most significant results of my dissertation research. By focusing on animals that feed on nectar, my research emphasized a novel aspect of the water-energy interaction. In the second section, I outline the usefulness of nectar-feeding birds as model organisms for understanding both mechanisms of physiological function in an integrated perspective and the ecological relevance of physiological characteristics. In the third section, I discuss the challenges that lie ahead. Finally, in the fourth section of this introduction I discuss the format of my dissertation, the contributions of my collaborators, and the co-authorship of papers.

1.1.1 – How do nectar-feeding birds deal with an excess of ingested water?

To fuel their exceptionally high mass-specific energy demands, nectar-feeding birds often experience water fluxes closer to those experienced by amphibians and freshwater fish than to those of endothermic vertebrates (Beuchat et al. 1990). The prodigious amount of

dietary water often ingested by nectar-feeding birds would lead to "water intoxication" in other terrestrial vertebrates (Lumeij and Westerhof 1988; Gebel et al. 1989; Gevaert et al. 1991; de Leon et al. 1994). Beuchat et al. (1990) hypothesized that nectar-feeding birds may deal with the excess water ingested as part of dilute floral nectars by absorbing only a small fraction and leaving the rest to pass quickly through the intestinal tract. This hypothesis would explain the ability of these birds to process such large volumes of water rapidly, but requires the rapid absorption of sugars and electrolytes and strict regulation of transepithelial water flux (Skadhauge 1981; Beuchat et al. 1990). If ingested water is largely absorbed across the intestine, as appears to be the case in most vertebrates (Powell 1987), nectar-feeding birds would be faced with significant renal challenges for water elimination and glucose and electrolyte recovery when feeding on dilute nectar (Beuchat et al. 1990).

The hypothesis of Beuchat et al. (1990) had not been tested until recently because the mixing of gut contents and urine in the cloaca of birds makes the measurement of intestinal water absorption technically challenging. McWhorter and Martínez del Rio (1999) developed a model based on pharmacokinetic techniques to estimate the fractional absorption of ingested water across the gastrointestinal tract of birds. Their model

estimates fractional water absorption as the proportion of ingested water that contributes to body water turnover (McWhorter and Martínez del Rio 1999). The details and assumptions of this model are presented by McWhorter and Martínez del Rio (1999) and in Appendix A of this dissertation. McWhorter and Martínez del Rio (1999) tested and rejected the hypothesis of Beuchat et al. (1990) in broad-tailed hummingbirds; they found that about 80% of ingested water contributed to the turnover of the body water pool, and that fractional water absorption was not correlated with food or water intake rate or diet energy density. In contrast, I found that Palestine sunbirds are able to modulate the absorption of dietary water across their intestines. As in the broad-tailed hummingbirds, fractional and total water turnover increased linearly with water ingestion, but the fraction of ingested water absorbed by sunbirds decreased from 100% to 36% with increasing water intake rate. Palestine sunbirds may therefore avoid absorbing, and thus having to eliminate, up to 64% of their ingested water load when feeding on dilute nectars. To my knowledge, this is the first documentation of regulation of water flux across the gastrointestinal tract to the body. My data suggest that sunbirds regulate transepithelial water flux independently of sugar absorption.

1.1.2 – Renal elimination of excess water by nectar-feeding birds

The study of renal processes in birds has emphasized dehydration over diuresis (see Braun 1993). The form of the relationship between water load and glomerular filtration rate (GFR) has therefore not been described for birds experiencing a large range of water loads (Goldstein and Bradshaw 1998; Goldstein and Skadhauge 2000). Nectar-feeding birds are of special interest because they are capable of ingesting astounding water volumes (reviewed by Martínez del Rio et al. 2001). The renal mechanisms and responses that allow nectar-feeding birds to contend with their watery diets, and the consequences of ingesting and processing large quantities of water for energy intake and the maintenance of metabolite and electrolyte homeostasis remain relatively unexplored.

Nectar-feeding birds are faced with the conflicting demands of eliminating excess water and metabolic by-products while retaining electrolytes, metabolites and substrates for energy metabolism (Yokota et al. 1985). Plasma glucose concentrations of nectar-feeding birds are high and surprisingly variable. In hummingbirds, for example, Beuchat and Chong (1998) found values ranging from 17 mmol L^{-1} glucose in fasted birds to as much as 40 mmol L^{-1} in feeding individuals, resulting in relatively high estimated glucose filtered loads (the product of GFR and the concentration of glucose in plasma). How do

these birds prevent the loss of glucose to urine? Because hummingbirds appear to absorb essentially 100% of the dietary water they ingest (McWhorter and Martínez del Rio 1999), they must have relatively high rates of renal filtration and correspondingly high capacities to recover filtered glucose. S. Medler (unpublished data) estimated that the GFR of the Anna's hummingbirds (*Calypte anna*) was 2.4 ± 0.2 ml h^{-1} (mean ± SE), which is only slightly lower the value predicted based on the relationship between body mass and renal filtration in birds (Williams et al. 1991). Based on this data and predicted GFR values for other species of hummingbirds, it is cleat that these birds must invest a significant amount of energy in recovering filtered glucose. I predicted that Palestine sunbirds would exhibit plasma glucose concentrations similar to those of other nectar-feeding birds. Because these sunbirds modulate water absorption across their intestines (i.e., they get rid of excess dietary water at the supply rather than at the disposal side of the equation), however, I predicted that renal filtration would be low and relatively sensitive to water loading.

The estimation of glomerular filtration rate involves measuring the clearance of a filtration marker from the plasma. This marker, which must be freely filtered, not secreted by the kidney, and not actively transported (e.g., inulin, polyethylene glycol,

iothalamate), is usually delivered either by constant infusion through an external cannula or an implanted osmotic minipump (Goldstein and Braun 1988; Roberts and Dantzler 1989; Rothschild and Goldstein 1990; Williams et al. 1991; Goldstein and Rothschild 1993) or by a single injection (Blaufox and Cohen 1970; Hall et al. 1977; Fettman et al. 1985; Radin et al. 1993). Because of the small body size of Palestine sunbirds and their sensitivity to repeated blood sampling (which most protocols require), we employed a modification of the single-injection slope-intercept method to measure GFR (see Appendix B and Hall et al. 1977 for details). We found that GFR was lower than predicted (approximately 40% of the value predicted based on body mass), and not exceptionally sensitive to water loading. Plasma glucose concentrations were high and varied 1.8 fold between fasted and fed sunbirds, but because GFR was low, glucose filtered load also remained relatively low. Essentially all of the glucose filtered load (98%) was recovered by the kidney. Renal fractional water reabsorption (FWR) decreased from 0.98 to 0.64 with increasing water intake, similar to results reported by Goldstein and Bradshaw (1998) for water-loaded red wattlebirds. The ability of Palestine sunbirds to reduce the absorption of dietary water in the gastrointestinal tract may resolve the potential conflict between filtering a large excess of absorbed water in the kidney and simultaneously retaining filtered metabolites.

1.1.3 – How do the digestive capacities of nectar-feeding birds shape their behavior?

When the sugar concentration in food (i.e., the energy density) is increased, many nectar-feeding birds species decrease their rate of food intake (Collins 1981; Downs 1997; López-Calleja et al. 1997; McWhorter and Martínez del Rio 2000; Martínez del Rio et al. 2001; Nicolson and Fleming 2002). This relationship between intake and food quality has been named the "intake response" (Castle and Wunder 1995). The widespread occurrence of intake responses has often been attributed to compensatory feeding (Simpson et al. 1989). According to this explanation, animals regulate food intake to maintain a relatively constant flux of assimilated energy or nutrients (Montgomery and Baumgardt 1965; Slansky and Wheeler 1992). If the energy or nutrient density of food in decreased, animals compensate by increasing intake. Indeed, although food intake by several species of nectar-feeding birds may vary by nearly 10-fold when these birds are feeding on nectars ranging in concentration from 0.146 to 1.168 mol L^{-1} sucrose, energy intake remains relatively constant (López-Calleja et al. 1997; Levey and Martínez del Rio 1999; McWhorter and Martínez del Rio 1999, 2000). A seemingly alternate hypothesis is that energy assimilation may be constrained by the ability of animals to process the nutrients contained in food. The picture that is emerging from recent studies is that compensatory feeding and physiological constraints to energy assimilation are

complementary processes that shape the feeding behavior of nectar-feeding birds (McWhorter and López-Calleja 2000; McWhorter and Martínez del Rio 2000; Martínez del Rio et al. 2001).

Martínez del Rio et al. (2001) outlined two complementary approaches that have been used to differentiate compensatory feeding from physiological constraint. The first approach involves examining the functional structure of the intake response. The relationship between food intake rate (V) and sugar concentration in food (C) in nectar-feeding birds is best described by a power function:

$$V = aC^{-b}, \qquad (1)$$

where a and b are empirically derived constants (McWhorter and Martínez del Rio 1999, 2000). Perfect compensatory feeding, where food intake rate is regulated to maintain constant energy intake, yields an exponent (b) equal to 1 (Martínez del Rio et al. 2001). Martínez del Rio et al. (2001) used a mathematical model and in vitro sucrose hydrolysis data to predict the maximal possible intake of nectar feeding birds. They found that this model also predicted a power function relationship relating V and C. However, the model consistently predicted an exponent (b) lower than 1. Most intake responses examined in nectarivorous birds to date have exponents that range from 1 to 0.65

(McWhorter and López-Calleja 2000). The presence of numerous examples with exponents less than 1 (reviewed by Martínez del Rio et al. 2001) and the inability of some species to maintain energy balance on very dilute diets (see Nicolson and Fleming 2002) provide circumstantial evidence for physiological constraint(s) to energy assimilation.

The second approach outlined by Martínez del Rio et al. (2001) is experimental and relies on determining the effect of changing energetic demands on the intake response. An endotherm exposed to a sudden drop in ambient temperature, for example, should increase its rate of energy assimilation to match energetic demands unless it is constrained. McWhorter and Martínez del Rio (2000) provided empirical evidence for a physiological constraint to food intake in broad-tailed hummingbirds (*Selasphorus platycercus*). Other experimental studies that have exposed nectar-feeding birds to low ambient temperatures have revealed considerable inter-specific variation (Beuchat et al. 1979; Gass et al. 1999; Lotz 1999; Lotz and Nicolson 1999). The main inference that can be drawn from these studies is that some species of nectarivorous birds are able to compensate for changes in nectar energy density and their metabolic demands over a

much wider range than others by changing their rate of food intake (Martínez del Rio et al. 2001).

In this study, I examined the idea that digestive processes can impose constraints on the rate at which energy can be assimilated by nectar-feeding Palestine sunbirds. I aimed to determine whether energy assimilation is physiologically constrained in sunbirds acclimated to natural ambient conditions. My second objective was to determine how closely the physiological capacities for energy assimilation in this animals match their energetic demands (Diamond 1991; Diamond and Hammond 1992). I employed both of the approaches outlined by Martínez del Rio et al. (2001). I examined the functional structure of the intake response and assessed the effect of acute exposure of sunbirds to low ambient temperatures on feeding rate (McWhorter and Martínez del Rio 2000; Martínez del Rio et al. 2001). I hypothesized that sunbirds would exhibit the typical intake response and compensate for differences in metabolic demands over the range of temperatures to which they were acclimated, but would be unable to do so at ambient temperatures below their acclimation range. I used in vitro measurements of intestinal sucrose hydrolysis and sucrose assimilation efficiency as inputs in a mathematical model, based on chemical reactor theory, that estimates the maximal capacity of sunbirds to

assimilate sucrose (see Appendix C and McWhorter and Martínez del Rio 2000 for details). To assess how close birds were to their potential maximum rates of intake, I compared the results of this model with observed feeding rates (McWhorter and Martínez del Rio 2000).

I fed birds on sucrose solutions of varying concentration and exposed them to two ambient temperatures within their acclimated range (15 and 30°C), and acutely to one temperature well below this range (5 °C). As expected, sunbirds decreased their food intake rates in response to sugar concentration. At 15 and 30 °C, they were able to compensate for differences in food energy density and increased metabolic demands. When exposed to a relatively sudden drop in ambient temperature and, hence, to an acute increase in thermoregulatory and food warming energy expenditures, sunbirds were unable to increase their rate of energy intake. Our simple chemical reactor model predicted observed intake rates at low concentrations (≤ 0.292 mol L^{-1}) and low temperatures (5 and 15 °C) closely. The model indicated that under these conditions, intestinal sucrose hydrolysis rates were near maximal, and thus may have imposed limits to sugar assimilation at 5 °C. However, sunbirds exposed to 5 °C did not lose but rather gained body mass over the day. When faced with a physiological limitation to energy

assimilation, they appeared to balance their energy budgets by reducing their energy expenditures. My results suggest that nectarivorous birds may operate within slim digestive safety margins (i.e., digestive capacity in excess of load). I speculate that slim safety margins may be a trait of animals with the ability to modulate energy expenditures by reduced activity and hypothermia.

1.1.4 – Nitrogen requirements and excretion by nectar-feeding birds

Bird pollinated plants secrete carbohydrate rich nectars with low nitrogen content (Baker and Baker 1982; Gottsberger et al. 1984). The low protein and amino acid levels in floral nectars are believed to be insufficient to meet the nutritional needs of nectarivores (Baker 1977; Baker and Baker 1977; Brice and Grau 1991; Law 1992; Martínez del Rio 1994; Roxburgh and Pinshow 2000; van Tets and Nicolson 2000), although nectar-feeding birds also consume arthropods (Wagner 1946; Paton 1982; Brice and Grau 1991; Brice 1992; van Tets and Nicolson 2000). Because nectar-feeding birds appear to obtain the majority of their energy from floral nectars, however, they must deal with low nitrogen levels and excess water. The nitrogen requirements and endogenous nitrogen losses of nectar-feeding birds measured to date appear to be only a fraction of the value predicted for birds based on body mass (Robbins 1993; Roxburgh and Pinshow 2000). This result is

not unexpected on both evolutionary and physiological grounds. There is an evolutionary necessity to minimize nitrogen loss when specializing on low nitrogen foods, and a liquid, fiber- and lipid-free nectar diet may reduce fecal nitrogen losses (Robbins 1993).

Birds are generally believed to be uricotelic under all circumstances (e.g. Schmidt-Nielsen 1990; Goldstein and Skadhauge 2000). Uricotelic animals are those that excrete more than 50% of their nitrogenous wastes as uric acid and its salts (which I will hereafter refer to as urates). Although urates are energetically expensive to synthesize, they may be favored as nitrogenous waste products in desiccating environments and in animals with cleidoic eggs because they can be excreted with little water and are relatively non-toxic (Willmer et al. 2000). Birds are terrestrial and often need to save water, but some bird species can ingest and excrete prodigious volumes of water. Hummingbirds consuming dilute nectars, for example, can ingest up to 6 times their body mass per day (Beuchat et al. 1990; McWhorter and Martínez del Rio 1999; Martínez del Rio et al. 2001). Until relatively recently nitrogen excretion had not been examined in birds with high rates of water flux.

Preest and Beuchat (1997) exposed Anna's hummingbirds (*Calypte anna*) to low ambient temperatures (10 °C). Birds increased their rates of food and water intake when faced with increased metabolic (i.e., thermoregulatory) demands. Surprisingly, about half of the birds exposed to 10 °C became ammonotelic (Preest and Beuchat 1997). These results are exceptionally significant because: 1) ammonia excretion is believed to occur only as a byproduct of the regulation of acid-base balance in birds (e.g., King and Goldstein 1985), and 2) they suggest that hummingbirds are "facultatively" ammonotelic. Facultatively ammonotelic animals excrete primarily urates, but switch to ammonia under certain conditions (McNab 2002 and references therein). Like uric acid and urea, ammonia is a by-product of amino acid metabolism. Unlike uric acid and urea, however, ammonia does not require additional energy to be synthesized, but it is highly toxic and highly soluble in water (Wright 1995). Preest and Beuchat (1997) speculated that hummingbirds could reduce the metabolic cost of nitrogen excretion by excreting primarily ammonia under conditions of high water flux. Facultative amrnonotely can also be advantageous because it reduces the potential loss of proteins and cations associated with urate excretion (McNabb et al. 1973; Laverty and Wideman 1989; Dawson et al. 1991; Janes and Braun 1997). When nectar-feeding birds are water

limited, either by high temperatures or highly concentrated floral nectars, they could shift back to uricotely (Calder and Hiebert 1983; Preest and Beuchat 1997).

Roxburgh and Pinshow (2002) examined the effects of water, electrolyte and protein intake and ambient temperature on nitrogen excretion by nectarivorous Palestine sunbirds (*Nectarinia osea*). They found that the proportion ammonia in ureteral urine and excreted fluid was independent of water and salt ingestion, but decreased in excreted fluid with increased protein intake. They hypothesized that the ammonotely observed in Palestine sunbirds was "apparent", simply the result of decreasing urate excretion in animals feeding on low protein diets. Therefore, currently available data leaves the question of ammonotely in nectar-feeding birds somewhat unanswered. Is facultative, true ammonotely found in hummingbirds but not in sunbirds? Roxburgh and Pinshow's (2002) study makes a significant point: protein intake may affect the form in which nitrogen is excreted by birds. Nectar feeding birds are unusual because they can ingest large amounts of water, and also because they appear to have low protein intakes and requirements.

In this study I attempted to integrate the insights of Preest and Beuchat (1997) with those of Roxburgh and Pinshow (2002). I manipulated both water and protein intake in captive hummingbirds to test two complementary hypotheses: 1) hummingbirds have low protein requirements, and 2) when ingesting high water loads they increase the fraction of total nitrogen that is excreted as ammonia. I tested these hypotheses under natural ambient conditions using captive individuals of three hummingbird species that are locally sympatric in southeastern Arizona: the magnificent hummingbird (*Eugenes fulgens*), the blue-throated hummingbird (*Lampornis clemenciae*), and the black-chinned hummingbird (*Archilochus alexandri*). Contrary to my hypothesis, no birds excreted more than 50% of nitrogen as ammonia, or more nitrogen as ammonia than urates. Furthermore, ammonia excretion was not influenced by either water or protein intake. The smallest species (*A. alexandri*) excreted a significantly greater proportion (> 25 %) of their nitrogenous wastes as ammonia than the larger hummingbirds (≈ 4 %). My results support the hypothesis that nectar-feeding birds have low protein requirements, but cast doubt on the notion that they are facultatively ammonotelic. My data also hints at a possible size-dependent dichotomy in hummingbirds, with higher ammonia excretion in smaller species. Differences in proportionate water loads and/or post-renal modification of urine may explain this dichotomy.

1.2 – WHY STUDY NECTAR-FEEDING BIRDS?

I investigated the comparative physiological ecology of birds that feed on nectar. Why are these animals worthy of a physiological ecologist's attention? The reasons are manifold. First, these animals feed on exceedingly simple diets that pose special physiological and osmoregulatory challenges. Second, their physiological traits can have consequences for their interactions with mutualistic food plants (Martínez del Rio 1994). Finally, nectar-feeding vertebrates include hummingbirds, which encompass the smallest endothermic vertebrates (Hainsworth and Wolf 1983). Hummingbirds have become a paradigmatic model of life under high energy demands (Hainsworth 1981). The subject of my dissertation is inquiry into the mechanisms by which nectar-eating birds contend with sugary diets that can contain large amounts of water and low amounts of protein and electrolytes. It is a natural extension of previous work on the digestive ecology of hummingbirds and avian frugivores.

Ecological physiologists have been criticized for demonstrating in excruciating detail that animals can live where they do (Bartholomew 1987). This criticism has been leveled especially at research on the physiological mechanism of organisms functioning in extreme environments (Feder 1987). Attempting to understand how nectarivores cope

with their sugary and watery diets is arguably an example of research of function in extreme environments. How can I justify a research project that seems to follow a worn paradigm? The main goal of my dissertation was to use nectar-eating birds to investigate the interaction among several physiological systems. I employed a strategy of developing simple mathematical models to guide my investigation of the integration of digestive, metabolic, and osmoregulatory processes, and then testing them in the laboratory. My research provides an integrated perspective of digestion, metabolism, and renal function in an ecological context. It also tackled one of the significant current challenges of physiology, which is to piece together the mechanisms of physiological function in an integrated perspective (Noble and Boyd 1993).

1.3 – THE CHALLENGES AHEAD

Although in my dissertation research I have made considerable progress in understanding the integration of organ system function for maintaining energy and water balance in birds that experience high water fluxes, many questions remain unanswered. These questions can be divided into two main areas: 1) questions addressing the generality of the physiological and behavioral responses I have documented; and 2) questions addressing the mechanisms and regulation, and possibly the evolution, of the processes that I have described. In this section, I briefly outline these unanswered questions, and the challenges that lie ahead.

1.3.1 – The generality of the physiological and behavioral responses of nectar-feeding birds

The fractional absorption of dietary water across the gastrointestinal tract has been measured in two species of nectar-feeding birds: the broad-tailed hummingbird (McWhorter and Martínez del Rio 1999), and the Palestine sunbird (present study, Appendix A). My research has uncovered important differences in how these species handle excess water ingested as part of their nectar diets. Palestine sunbirds are able to modulate the absorption of water. This response allows them to avoid up to 60% of their

ingested water load when feeding on dilute nectars, while broad-tailed hummingbirds appear to absorb essentially all ingested water. It is unknown if the ability to modulate water absorption is a general pattern among sunbirds or even among other taxa of nectar-feeding or non-nectarivorous passerine birds. The Palestine sunbird is approximately twice the body mass of the broad-tailed hummingbird (approximately 3 vs. 6 g), and hummingbirds have higher mass-specific metabolic rates that passerine birds (Suarez 1992). It is possible that there are body size differences in the ability to modulate water absorption among nectar-feeding birds. Perhaps small hummingbirds, such as the broad-tailed, are reaching the lower limit for endothermy and the large volumes they must consume when feeding on dilute nectars simply overwhelm their osmoregulatory processes and thus limit their rates of food intake. Clearly, additional water absorption measurements are necessary to uncover body size effects and address the generality of the phenomenon I have documented in Palestine sunbirds. These measurements have relevance for understanding the physiology and ecology of these animals, and also for understanding the evolution of nectarivory in birds.

The magnitude of renal filtration and water recovery in nectar-feeding birds is determined by the conflicting demands of eliminating water and nitrogenous wastes

while retaining important metabolites and electrolytes (Yokota et al. 1985). This conflict is magnified when these birds feed on dilute nectars. Glomerular filtration by Palestine sunbirds is considerably lower than predicted by allometry (Williams et al. 1991), which is not surprising given their ability to modulate dietary water absorption. The change in renal fractional water absorption (decreasing from near 1 to approximately 0.64) with increasing water intake, however, indicates that these birds are still experiencing considerable water loads. No published measurements of GFR exist for hummingbirds. S. Medler's unpublished observations (discussed above) suggest that GFR in Anna's hummingbirds is slightly lower than expected based on body mass. At about 5 g, these hummingbirds are similar in body mass to Palestine sunbirds. Because smaller broad-tailed hummingbirds do not appear to be able to modulate dietary water absorption, I would expect their GFR to be higher than predicted based on body mass. The arguments made above regarding body size and taxonomic differences in water absorption across the gastrointestinal tract apply here as well.

The behavioral response of animals to variations in the energy or nutrient density of food that I have described in detail above and in Appendix C (i.e., the "intake-response") appears to be a very general phenomenon. It has been documented in animals ranging

from blowflies to sheep, herbivorous rodents, and nectar-feeding birds (McWhorter and Martínez del Rio 2000 and references therein). In nectar-feeding birds, this behavioral response is shaped by the complementary processes of compensatory feeding to maintain constant energy or nutrient assimilation, and digestive constraints to energy assimilation (Martínez del Rio et al. 2001). The hummingbirds, flower-piercers and sunbirds that have been examined to date appear to have very slim digestive safety margins. It is unclear, however, if slim digestive safety margins are a general pattern among nectar-feeding birds, and whether or not these digestive limitations are correlated with the dominant sugars (i.e., glucose and/or fructose vs. sucrose) present in the nectar of plants preferred by a given species. Experiments are now under way in which hummingbirds (Carlos Martínez del Rio's lab at the University of Wyoming) and sunbirds (Sue Nicolson's lab at the University of Pretoria) are fed equicaloric mixtures of sucrose and glucose plus fructose to determine whether sucrose hydrolysis or the transport of monosaccharides is the rate limiting step for sugar assimilation. Because digestive and absorptive processes are probably closely matched to each other (Diamond and Hammond 1992), however, it is likely that no one step is more limiting than another. Intriguingly, Jorge Schondube and Carlos Martínez del Rio (unpublished data) have found that cinnamon-bellied flower-piercers (*Diglossa baritula*) are able to assimilate

approximately 10% more energy when feeding on a glucose plus fructose mixture than when feeding on a sucrose solution, while magnificent hummingbirds are unable to do so. Perhaps because hummingbirds have phenomenally high mass-specific metabolic rates (Suarez 1992), their ability to digest sucrose is more closely matched with the ability to absorb monosaccharides than in the passerine flower-piercers. It is also possible that there are important ecological correlates to this pattern (e.g., differences is sugar preferences, foraging behavior, or nectar sugar composition) that remain to be discovered.

1.3.2 – The mechanisms and regulation of physiological processes

To my knowledge, my work on Palestine sunbirds is the first to document the adaptive modulation of water absorption across the gastrointestinal tract of a vertebrate. This conclusion has been met with some skepticism by peer reviewers and colleagues at scientific conferences. Although my data suggests that water absorption is modulated independently of sugar assimilation, the mechanisms by which this occurs are unknown. We present several possibilities in Appendix A, including differences is the passive permeability of the intestine, and active water secretion involving recruitable aquaporin water channels. In addition to providing a mechanistic explanation for the ability of

sunbirds to modulate dietary water absorption, uncovering these mechanisms will lead to a greater understanding of how water flux across membranes is regulated.

Although my work is integrative, it leaves many important aspects of osmoregulation in nectar-eating birds untouched. For example, nectar can have exceedingly low levels of electrolytes and low Na^+/K^+ ratios (Hiebert and Calder 1983). Na^+ conservation in the face of high water fluxes and low contents in nectar is a significant challenge that nectar-eating birds must cope with (Calder and Hiebert 1983; Hiebert and Calder 1983). Two other significant and related areas that my research did not tackle are: 1) the role that circulating levels of regulatory hormones (e.g., AVT and aldosterone) play in the regulation of water balance in nectar-eating birds, and 2) the renal mechanisms that determine changes in glomerular filtration. Modulation of GFR in birds appears to result from both changes in single nephron filtration rates of both mammalian and reptilian type nephrons and from changes in the number of reptilian type glomeruli filtering (Dantzler and Braun 1980). These changes appear to be mediated at least in part by the action of arginine vasotocin (Braun 1993). Measurements of regulatory hormones in conjunction with GFR measurements in birds experiencing large water fluxes are certainly necessary.

These measurements will allow us to complete the picture of the regulation of renal function in birds over a wide range of water fluxes.

The nitrogen requirements of nectar-feeding birds are clearly very low, and hummingbirds and Palestine sunbirds do not appear to exhibit true facultative ammonotely (see Appendix D and Roxburgh and Pinshow 2002). Both of these observations suggest significant post-renal modification of urine by nectar-feeding birds. Refluxing of urine into the distal large intestine, possible in birds because the ureters and intestine both open into the cloaca, is known to facilitate significant post-renal modification of urine composition, including uptake of water and electrolytes and break down and recycling of protein in many species of birds (Goldstein and Braun 1986; Braun 1999; Karasawa 1999). The significance of post-renal modification of urine in nectar-feeding birds, and the contribution of gut microbes to nitrogen recycling are almost completely unknown. Measurements of these phenomena will increase understanding of not only the nitrogen balance and excretion of nectar-feeding birds, but also of their foraging ecology. Understanding how nectar-feeding birds are able to manage such low nitrogen requirements, for example, may provide insight into how they decide to switch between foraging for insects and nectar. In addition, understanding

nitrogen recycling may provide clues about the evolution of nectarivory in birds from insectivorous ancestors.

1.4 – CONTRIBUTIONS OF COLLABORATORS

Nature abhors a vacuum, and science does not occur in one. My dissertation represents research that is complementary to work that has previously been done on hummingbirds as part of my M.Sc. thesis at the University of Wyoming (McWhorter 1997; McWhorter and Martínez del Rio 1999, 2000), and previous and current research done in the United States, Mexico and Chile with a number of collaborators (McWhorter and López-Calleja 2000; Martínez del Rio et al. 2001). My research program has developed into a fruitful, rewarding, and hopefully life-long collaboration with my advisor, Carlos Martínez del Rio. The research on Palestine sunbirds, which comprises the majority of the work presented in my dissertation, would have not been possible if Berry Pinshow had not brought the possibility of funding by the U.S.-Israel Binational Science Foundation to our attention. My dissertation consists of two brief introductory chapters, and four appendices containing co-authored research papers to be submitted for publication. The ideas contained therein belong as much to Carlos Martínez del Rio, Berry Pinshow, Don Powers and Lizanne Roxburgh as they do to me. I have, however, been the researcher primarily responsible for conducting all of the experiments described in this document. I have also been the primary author of the manuscripts resulting from this research, and am

therefore primarily responsible for the content as well as any errors, omissions, or oversights contained therein.

CHAPTER 2- PRESENT STUDY

The purpose of my dissertation research was to investigate the physiological mechanisms that allow nectar-eating birds to cope with their watery diets and to elucidate the consequences of ingesting and processing large quantities of water for energy intake and for the maintenance of balance of important metabolites such as glucose. The methods, results, and conclusions of this research are presented in detail in the manuscripts appended to this document (see Appendices A-D). This chapter is a brief summary of the most important findings in these papers.

2.1 – Modulation of Ingested Water Absorption by Palestine Sunbirds: Evidence for Adaptive Regulation (Appendix A)

Co-authors: Carlos Martínez del Rio (Department of Zoology and Physiology, University of Wyoming) and Berry Pinshow (Mitrani Department of Desert Ecology, Jacob Blaustein Institute for Desert Research, and Department of Life Sciences, Ben-Gurion University of the Negev, Israel).

Summary: Nectarivorous birds feed on dilute sugar solutions containing trace amounts of amino acids and electrolytes. To meet their high mass-specific energy demands they must often deal with exceptionally high proportionate water fluxes. Despite nectar intake rates that may reach more than 5 times body mass per day, hummingbirds appear to absorb all ingested water. Here, we report the results of experiments designed to examine the relationship between nectar intake and water turnover in nectar-feeding Palestine sunbirds (*Nectarinia osea*). Like hummingbirds, sunbirds ingested large amounts of water. At the lowest sucrose concentration (292 mmol l^{-1}), food intake rates reached 2.2 times body mass. Fractional and total water turnover increased linearly with water ingestion, but the fraction of ingested water absorbed by sunbirds decreased from 100%

to 36% with increasing water intake rate. Palestine sunbirds may therefore avoid absorbing, and thus having to eliminate, up to 64% of their ingested water load when feeding on dilute nectars. To our knowledge, this is the first documentation of regulation of water flux across the gastrointestinal tract to the body. Our data suggest that sunbirds regulate transepithelial water flux independently of sugar absorption. These intriguing results open the door to many questions about how water transport is regulated in the vertebrate gastrointestinal tract. We suggest that intestinal water and body water form two separate but interacting pools in nectar-feeding birds. Convergence in diet has led to the evolution of many similar traits in hummingbirds and sunbirds. The physiological traits of these two groups that allow the processing of a water and sugary diet, however, may be very different.

2.2 - Renal Function in Palestine Sunbirds: Elimination of Excess Water Does Not Constrain Energy Intake (Appendix B)

Co-authors: Carlos Martínez del Rio (Department of Zoology and Physiology, University of Wyoming), and Berry Pinshow and Lizanne Roxburgh (Mitrani Department of Desert Ecology, Jacob Blaustein Institute for Desert Research, and Department of Life Sciences, Ben-Gurion University of the Negev, Israel).

Summary: Although the renal responses of birds to dehydration have received significant attention, the consequences of ingesting and processing large quantities of water have been less studied. Nectar-feeding birds must often deal with exceptionally high water intake rates in order to meet their high mass-specific energy demands. Birds that ingest large volumes of water may either eliminate excess water in the kidney or they may regulate the volume of water absorbed in the gastrointestinal tract. Because water absorption in the gastrointestinal tract of Palestine sunbirds (*Nectarinia osea*) decreases with increasing water ingestion rate, we predicted that glomerular filtration rate (GFR) in these birds would not be unusually high in spite of large ingested water loads. When feeding on dilute sucrose solutions, sunbirds ingested between 4 and 6 times their body

mass in nectar per day, yet they were able to compensate for varying nectar energy density and increased thermoregulatory energy demands with no apparent difficulty. GFR was lower than predicted (1690.67 ± 148.11 $\mu l\ h^{-1}$, approximately 40% of the value predicted based on body mass), and not exceptionally sensitive to water loading. Plasma glucose concentrations were high and varied 1.8 fold between fasted (16.08 ± 0.75 mmol l^{-1}) and fed (28.18 ± 0.68 mmol l^{-1}) sunbirds, but because GFR was low, glucose filtered load also remained relatively low. The kidney recovered essentially the entire glucose filtered load (98%). Renal fractional water reabsorption (FWR) decreased from 0.98 to 0.64 with increasing water intake. The ability of Palestine sunbirds to reduce the absorption of dietary water in the gastrointestinal tract may resolve the potential conflict between filtering a large excess of absorbed water in the kidney and simultaneously retaining filtered metabolites.

2.3 – Does Digestive Capacity Limit Food Intake? An Experimental Test and a Model (Appendix C)

Co-authors: Carlos Martínez del Rio (Department of Zoology and Physiology, University of Wyoming) and Berry Pinshow (Mitrani Department of Desert Ecology, Jacob Blaustein Institute for Desert Research, and Department of Life Sciences, Ben-Gurion University of the Negev, Israel).

Summary: Many species of animals decrease their intake rate when the energy/nutrient density of food is increased. This intake response to food quality can be explained by two complementary processes: compensatory feeding and physiological constraint. The compensatory feeding hypothesis assumes that animals vary intake to match energy expenditures. Alternately, energy assimilation, and thus food intake, may be constrained by the physiological capacity of animals to process the nutrients contained in food. Both compensatory and constrained feeding predict a negative relationship between the energetic content of food and intake rate. When energy requirements are low animals may adopt a compensatory strategy that allows them to match intake to energy demands. As demands increase, animals may reach their digestive ceiling and hence their rate of

food intake may be determined by their physiological capacity. To examine the interplay between compensatory and constrained feeding we varied both food quality and energy demands and compared the intake responses of Palestine sunbirds (*Nectarinia osea*) with the results of a model that predicts their maximal intake from measurements of intestinal hydrolytic capacity. We fed birds on sucrose solutions of varying concentration and exposed them to two ambient temperatures within their acclimated range (15 and 30°C), and acutely to one temperature well below this range (5 °C). As expected, sunbirds decreased their food intake rates in response to sugar concentration. At 15 and 30 °C, they were able to compensate for differences in food energy density and increased metabolic demands. When exposed to a relatively sudden drop in ambient temperature and, hence, to an acute increase in thermoregulatory and food warming energy expenditures, sunbirds were unable to increase their rate of energy intake. Our simple chemical reactor model predicted observed intake rates at low concentrations (≤ 0.292 mole per liter) and low temperatures (5 and 15 °C) closely. The model indicated that under these conditions, intestinal sucrose hydrolysis rates were near maximal, and thus may have imposed limits to sugar assimilation at 5 °C. However, sunbirds exposed to 5 °C did not lose but rather gained body mass over the day. When faced with a physiological limitation to energy assimilation, they appeared to balance their energy

budgets by reducing their energy expenditures. Our results suggest that nectarivorous birds may operate within slim digestive safety margins. We speculate that slim safety margins may be a trait of animals with the ability to modulate energy expenditures by reduced activity and hypothermia.

2.4 - Are Hummingbirds Facultatively Ammonotelic? Nitrogen Excretion and Requirements as a Function of Body Size (Appendix D)

Co-authors: Donald R. Powers (Department of Biology, George Fox University, Newberg, Oregon) and Carlos Martínez del Rio (Department of Zoology and Physiology, University of Wyoming).

Summary: Most birds are uricotelic. An exception to this rule may be nectar-feeding birds, which excrete significant amounts of ammonia under certain conditions. Although ammonia is toxic, because it is highly water soluble its excretion may be facilitated in animals that ingest and excrete large amounts of water. Bird pollinated plants secrete carbohydrate- and water-rich floral nectars that contain exceedingly little protein. Thus, nectar-feeding birds are faced with the dual challenge of meeting nitrogen requirements while disposing of large amounts of water. The peculiar diet of nectar-feeding birds suggests two hypotheses: 1) these birds must have low protein requirements, and 2) when ingesting large quantities of water their primary nitrogen excretion product may be ammonia. To test these hypotheses, we measured maintenance nitrogen requirements (MNR) and total endogenous nitrogen losses (TENL) in three hummingbird species

(*Archilochus alexandri*, *Eugenes fulgens* and *Lampornis clemenciae*) fed on diets with varying sugar, protein, and water content. We also quantified the form in which the by-products of nitrogen metabolism were excreted. The MNR and TENL of the hummingbirds examined were exceptionally low. However, no birds excreted more than 50% of nitrogen as ammonia, or more nitrogen as ammonia than urates. Furthermore, ammonia excretion was not influenced by either water or protein intake. The smallest species (*A. alexandri*) excreted a significantly greater proportion (> 25 %) of their nitrogenous wastes as ammonia than the larger hummingbirds (≈ 4 %). Our results support the hypothesis that nectar-feeding birds have low protein requirements, but cast doubt on the notion that they are facultatively ammonotelic. Our data also hint at a possible size-dependent dichotomy in hummingbirds, with higher ammonia excretion in smaller species. Differences in proportionate water loads and/or post-renal modification of urine may explain this dichotomy.

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APPENDIX A

MODULATION OF INGESTED WATER ABSORPTION BY PALESTINE SUNBIRDS: EVIDENCE FOR ADAPTIVE REGULATION

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Running Head: *Modulation of ingested water absorption by sunbirds*

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Summary

Nectarivorous birds feed on dilute sugar solutions containing trace amounts of amino acids and electrolytes. To meet their high mass-specific energy demands they must often deal with exceptionally high proportionate water fluxes. Despite nectar intake rates that may reach more than 5 times body mass per day, hummingbirds appear to absorb all ingested water. Here, we report the results of experiments designed to examine the relationship between nectar intake and water turnover in nectar-feeding Palestine sunbirds (*Nectarinia osea*). Like hummingbirds, sunbirds ingested large amounts of water. At the lowest sucrose concentration (292 mmol l^{-1}), food intake rates reached 2.2 times body mass. Fractional and total water turnover increased linearly with water ingestion, but the fraction of ingested water absorbed by sunbirds decreased from 100% to 36% with increasing water intake rate. Palestine sunbirds may therefore avoid absorbing, and thus having to eliminate, up to 64% of their ingested water load when feeding on dilute nectars. To our knowledge, this is the first documentation of regulation of water flux across the gastrointestinal tract to the body. Our data suggest that sunbirds regulate transepithelial water flux independently of sugar absorption. These intriguing results open the door to many questions about how water transport is regulated in the vertebrate gastrointestinal tract. We suggest that intestinal water and body water form

two separate but interacting pools in nectar-feeding birds. Convergence in diet has led to the evolution of many similar traits in hummingbirds and sunbirds. The physiological traits of these two groups that allow the processing of a water and sugary diet, however, may be very different.

Introduction

To fuel their exceptionally high mass-specific energy demands, nectar-feeding birds often experience water fluxes closer to those experienced by amphibians and freshwater fish than to those of endothermic vertebrates (Beuchat et al., 1990). Extremely high water flux rates have been measured in many species of nectarivorous and frugivorous birds (Rooke et al., 1983; Powers and Nagy, 1988; Weathers and Stiles, 1989; Williams, 1993; Powers and Conley, 1994; Goldstein and Bradshaw, 1998; Lotz and Nicolson, 1999; McWhorter and Martínez del Rio, 1999; Nicolson and Fleming, 2002). McWhorter and Martínez del Rio (1999) found that, depending on sugar concentration, broad-tailed hummingbirds (*Selasphorus platycercus*) consumed volumes of nectar ranging from 1.6 to 5.4 times their body mass per day. Beuchat et al. (1990) estimated that Anna's hummingbirds (*Calypte anna*) consume a volume of nectar equal to three times their body mass per day under energetically demanding conditions. Until recently, the physiological challenges associated with the simultaneous regulation of energy intake and water and ion homeostasis by these animals remained relatively unexplored.

Beuchat et al. (1990) hypothesized that when hummingbirds are ingesting large volumes of dilute nectar, perhaps only a small fraction is absorbed in the small intestine, leaving the rest to pass quickly through the intestinal tract. This hypothesis would

explain the ability of these birds to process such large volumes of water rapidly, but requires the rapid absorption of sugars and electrolytes and strict regulation of transepithelial water flux (Skadhauge, 1981; Beuchat et al., 1990). If ingested water is largely absorbed across the intestine, as appears to be the case in most vertebrates (Powell, 1987), nectar-feeding birds would be faced with significant renal challenges for water elimination and glucose and electrolyte recovery when feeding on dilute nectar (Beuchat et al., 1990). McWhorter and Martínez del Rio (1999) developed a model based on pharmacokinetic techniques to estimate the fractional absorption of ingested water across the gastrointestinal tract of birds. Their model estimates fractional water absorption as the proportion of ingested water that contributes to body water turnover (McWhorter and Martínez del Rio, 1999). McWhorter and Martínez del Rio (1999) tested and rejected the hypothesis of Beuchat et al. (1990) in broad-tailed hummingbirds; they found that about 80% of ingested water contributed to the turnover of the body water pool, and that fractional water absorption was not correlated with food or water intake rate or diet energy density.

Although nectar-feeding birds are convergent in diet, and indeed often in appearance and behavior, it is unclear if the physiological mechanisms by which they cope with a nectar diet are also convergent. Nectar poses peculiar problems to the animals that feed

on it because it is a relatively dilute solution of sugars containing trace amounts of amino acids and electrolytes (Baker, 1975, 1977; Baker and Baker, 1983). Here we revisit the hypothesis of Beuchat et al. (1990) in another lineage of nectar-feeding birds. We report the results of experiments designed to examine the relationship between nectar intake, water absorption, and water turnover in the Palestine sunbird [*Nectarinia osea* (Bonaparte, 1856)], an Old World nectarivore in the family Nectariniidae. Based on previous measurements in hummingbirds, we hypothesized that water absorption by sunbirds would be essentially complete at all sucrose concentrations naturally encountered in floral nectars. Alternately, we hypothesized that if water absorption were modulated, fractional absorption would decrease to some obligatory minimum with increasing water intake. This hypothesis was based on the observation that nutrient absorption does not take place without concomitant transport of water, whether via hydration spheres of molecules in nutrient transporters (e.g. Loo et al., 1996; Loo et al., 1998) or paracellular solvent drag (e.g. Pappenheimer and Reiss, 1987; Pappenheimer, 1990). As a corollary to our alternate hypothesis, we predicted that absorbed water loads would be greater when sugar assimilation rates are higher. Because researchers generally assume that water turnover in nectar-feeding animals can be used to approximate nectar intake, given that ingested water comes only from food (von Helversen and Reyer, 1984;

Kunz and Nagy, 1988; Powers and Nagy, 1988; Weathers and Stiles, 1989; Tiebout and Nagy, 1991), our results also test the primary assumption of a significant body of work on the field energetics and water fluxes of nectarivorous animals.

Material and methods

Bird capture and maintenance

Male Palestine sunbirds (body mass 5.74 ± 0.07 g, n = 10) were captured with drop nets on the grounds of Midreshet Ben-Gurion, home of the Sede Boqer Campus of Ben-Gurion University of the Negev, Israel (30° 51' N, 34° 46' E; Israel Nature and National Parks Protection Authority permits 5981 and 7686). The birds were housed individually in outdoor aviaries (1.5 m x 1.5 m x 2.5 m) and fed a maintenance diet of two artificial nectar solutions between experiments. The diets included a 20-25% sucrose equivalent solution and a 15% sucrose solution supplemented with a soy protein infant formula (Isomil™, Abbott Laboratories, Netherlands) diluted to approximately 2.5 g protein per 100 g sucrose. Food and water were available ad libitum. Birds were also offered freshly killed fruit flies (Drosophila spp.) at least twice a week. During experiments, birds were housed individually in opaque Plexiglas® cages (0.3 m x 0.3 m x 0.3 m) with individual light sources. The front of these cages was coated with a reflective Mylar™ polyester

film to create a one-way mirror effect that permitted observation of birds in a darkened room with minimal disturbance. One of the perches in the center of each cage was fitted to hang from an electronic balance (Scout II 200 g x 0.01 g, Ohaus Corporation, Florham Park, NJ) so body mass could be monitored continuously. Birds were allowed to acclimate to cages and experimental temperatures for 2-3 d before experiments began and were left undisturbed in outdoor aviaries for a minimum of 7 days between trials. The study was conducted using light cycles that matched the natural photoperiod (13.25 to 14.5 h light). Birds were fed experimental diets, which consisted of sucrose solutions made with distilled water, for a minimum of 24 h before trials began. The food intake rates of Palestine sunbirds switched among diets of varying energy density stabilize within 4 hours (T.J. McWhorter, C. Martínez del Rio and B. Pinshow, unpublished data).

Experimental design

Experiment 1: Fractional absorption of ingested water as a function water intake rate.

We relied on the behavioral responses of nectar-feeding birds to food of varying energy density in the design of this experiment. Typically, nectar-feeding birds decrease their food intake rate with increasing sugar concentration (Martínez del Rio et al., 2001). Manipulation of sugar concentration therefore leads to a wide range of variation in the

quantity of food ingested. We used a repeated measures design in which we measured water absorption in four sunbirds fed on four dietary sugar concentrations (292, 584, 876, and 1168 mmol l^{-1} sucrose) at one ambient temperature (30 ± 2 °C), randomizing the order in which diets were presented to subjects.

Experiment 2: Fractional absorption of ingested water as a function of sucrose assimilation. When ambient temperatures decrease, birds must consume and assimilate more sugar to meet increased energy demands for thermoregulation. We measured water absorption in six sunbirds feeding on 584 mmol l^{-1} sucrose solutions at both 15 ± 1 °C and 30 ± 2 °C in a repeated measures design to determine the effect of sucrose assimilation rate. We randomized the order in which subjects were exposed to the two temperatures.

Experimental measurements

Water turnover rates were estimated by injecting 1.85×10^5 Bq of ^3H_2O in 15 µl of distilled water into the *pectoralis* of each bird approximately 1.5 h after the lights came on. Injection volumes were verified gravimetrically by weighing syringes (25 µl, Hamilton Company, Reno, NV) to the nearest 0.0001 g before and after injection. Excreted fluid

samples were collected, using glass microcapillary tubes, immediately after excretion and placed in separate scintillation vials. Samples were collected at irregular intervals for approximately 30 h, excluding the dark portion of photoperiod during which sunbirds do not excrete. Sample collection was not initiated until approximately 40 min after injection, allowing sufficient time for complete equilibration of ^{3}H with body water (estimates of equilibration time vary from 15-30 min in small birds, Williams and Nagy, 1984; Speakman, 1997). Liquid scintillation cocktail (ACS II, Amersham, Piscataway, NJ) was added to all excreted fluid and injection samples, which were counted correcting for quench and lumex in a Packard Tri-Carb 1600TR Liquid Scintillation Analyzer. Fractional water turnover rate ($K_{^{3}H}$, h^{-1}) was estimated by fitting negative exponential functions to the relationship between the specific activity of ^{3}H in excreted fluid and time. In most cases, ^{3}H specific activity was high enough on the second day to estimate water turnover and absorption. Because birds were not injected on the second day, these measurements provided a test for the effects of handling and injection on water turnover and absorption during the first day. Food intake rate ($\mu l\ h^{-1}$) was recorded over the course of each experimental trial by measuring the change in food level to the nearest 0.5 mm in a tube of constant internal diameter, correcting for evaporation and food spillage.

Total body water volume (TBW, μL) was estimated using isotope dilution (Nagy, 1983; Speakman, 1997). Briefly, a small blood sample (approximately 50 μL) was taken approximately 4 h after injection by puncturing the brachial vein. The water microdistilled from this sample (Nagy, 1983) was analyzed for specific activity of ^3H as described above. The slope of the relationship between specific activity of ^3H in excreted fluid and time was extrapolated to the zero time concentration of marker in body water. We used this modification of the isotope dilution technique described by Speakman (1997) because of the sensitivity of small birds to repeated blood sampling. We assumed that the rate of disappearance of marker from blood was equal to the rate of appearance in excreted fluid. The specific activity of marker in each fluid would, of course, not be equal because of renal and post-renal modification of urine and the mixing of urine with gut contents. After the final experimental run, one bird was killed with CO_2 and dried to constant mass at 80 °C to confirm TBW estimated by isotope dilution. The TBW of that bird measured by dehydration (3591 μl, or 63.8% of body mass) was 1.6% higher than the average volume for that individual estimated by isotope dilution.

Estimating water absorption in sunbirds

We used the mass balance approach developed by McWhorter and Martínez del Rio (1999) to estimate the fraction of ingested water that was absorbed by sunbirds (f_w).

Simply stated, this method determines the proportion of ingested water that contributes to the turnover of the TBW pool. Assuming that birds were in neutral water balance, f_W was estimated as:

$$f_W = (K_{^3H} \bullet TBW - \dot{V}_M)\ \dot{V}_I^{-1} \qquad (1),$$

where $\dot{V}_I$ (μl h^{-1}) was the rate of water intake, and $\dot{V}_M$ (μl h^{-1}) was the rate of metabolic water production. We assumed that metabolic water production was due only to carbohydrate catabolism. Indeed, the respiratory quotient (RQ) of actively feeding sunbirds and hummingbirds indicates carbohydrate catabolism (RQ = 1, C. Hambly, B. Pinshow, E.J. Harper and J.R. Speakman, unpublished data, Suarez et al., 1990; Powers, 1991). Birds in this study were in mass balance during all experimental trials, so we further assumed that the rate of carbohydrate catabolism was equal to the rate of sucrose assimilation. We calculated the rate of sucrose assimilation as the product of sucrose intake rate and assimilation efficiency. Sucrose assimilation efficiency was estimated as the fraction of ingested sucrose that was assimilated in an independent set of experiments (0.9992 ± 0.0004 SD, n = 8). Sucrose assimilation efficiency was independent of sugar concentration.

Statistical analysis

Experiment 1: To describe the relationship between fractional water absorption (f_w) and water intake rate ($\dot{V}_I$), and to assess differences among subjects and treatment days, we constructed a linear model with f_w as a dependent variable, and the reciprocal of water intake ($\dot{V}_I^{-1}$), individual bird and treatment day as independent variables. We used the reciprocal transformation of $\dot{V}_I$ to obtain a linear relationship (i.e. $f_w = a + b\dot{V}_I^{-1}$) because visual inspection of the relationship between and f_w and $\dot{V}_I$ resembled a hyperbola that tended asymptotically to a constant value for large values of $\dot{V}_I$. Because relationships between volumetric food intake and sugar concentration in nectar-feeding birds are power functions (Martínez del Rio et al., 2001), we determined the effects of subject and treatment day on food, water and sucrose intake rates using linear models of $\log_e$ transformed intake and sucrose concentration data. We similarly used $\log_e$ transformed data to determine the significance of the relationship between water absorbed per mass sucrose assimilated and sucrose concentration. We used linear models on untransformed data to assess significance and subject and treatment day effects in all other cases. Analysis of covariance (ANCOVA) was used to check for differences in the slope of the relationship between water flux and water intake between sunbirds and hummingbirds.

We used the Spearman rank correlation test to check for a correlation between diet sucrose concentration and sucrose intake rate.

Experiment 2: Repeated-measures ANOVA was used to test for differences in food and sucrose intake rates, fractional water absorption, water absorbed per mass of sucrose assimilated, and the total absorbed water load between temperatures.

All values are presented as mean ± SEM.

Results

Experiment 1: Fractional absorption of ingested water as a function water intake rate.

Sunbirds consumed significantly less food as dietary sucrose concentration increased ($F_{1, 29} = 107.0$, $p < 0.0001$; Fig. 1B). There was no significant effect of subject ($F_{3, 29} = 1.7$, $p > 0.1$) or treatment day ($F_{1, 29} = 1.5$, $p > 0.2$) on food intake rate, so we removed these variables from the model. The relationship between food intake and sucrose concentration was adequately described by a power function ($y = 231.77x^{-0.87}$, $r^2 = 0.76$, Fig. 1B). The exponent of this relationship was not significantly different from 1

($t = -1.52$, $df = 33$, $p > 0.1$). Hence, although food, and thus water, intake rate varied approximately 3.5 fold between the lowest and the highest sucrose concentration, sunbirds did not increase their sucrose intake significantly with increasing sucrose concentration ($r_s = 0.12$, $p = 0.49$, $n = 35$; Fig. 1A). Sucrose intake averaged 77.17 ± 3 mg h^{-1} (17.94 ± 0.7 kJ d^{-1}). At low sucrose concentrations, sunbirds consumed between 0.8 and 2.2 times their body mass in food in 14 h of daylight (Fig. 1B).

The relationships between the specific activity of ^3H in excreted fluid (disints min^{-1} μl^{-1}) and time were well described by exponential functions (r^2 ranged from 0.57 to 0.96, $n = 35$). The decline in the specific activity of ^3H in excreted fluid with time therefore seemed to follow one-compartment, first-order kinetics. Fractional water turnover rate ranged from 0.037 to 0.117 h^{-1} and was, linearly correlated with water intake rate ($F_{1,29} = 169.50$, $p < 0.0001$). Because there was no significant effect of subject ($F_{3,29} = 1.4$, $p > 0.2$) or treatment day ($F_{1,29} = 2.0$, $p > 0.1$) on K_{3H} as a function of $\dot{V}_I$, so we removed these variables from the model and estimated a common relationship ($K_{3H} = 1.15 \times 10^{-4} \dot{V}_I + 0.03$, $r^2 = 0.84$). When birds were feeding on the most dilute nectar (292 mmol l^{-1} sucrose), approximately 10% of their TBW pool was turning over each hour. Average

TBW estimated by isotope dilution was 3470 ± 86 μl (or 63.6 ± 0.7 % of body mass, n = 4).

Fractional water absorption (f_w) ranged from 0.33 to 1.02 (averaging 0.59 ± 0.04, n = 35). Because we found no significant effect of subject ($F_{3,29} = 0.29$, $p > 0.8$) or treatment day ($F_{1,29} = 3.1$, $p > 0.08$) on f_w, but a highly significant effect of $\dot{V}_f^{-1}$ ($F_{1,29} = 40.03$, $p < 0.0001$), we estimated a common relationship between fw and $\dot{V}_f^{-1}$. The reciprocal transformation adequately described the relationship between fw and $\dot{V}_f$ (Fig. 2). These results suggest that Palestine sunbirds may avoid absorbing up to 64% (1 - 0.36 = 0.64) of ingested water when feeding on dilute nectars. Fractional water absorption was also positively correlated with sugar concentration in food ($y = 0.32x + 0.37$, $r^2 = 0.34$, $F_{1,29} = 17.13$, $p < 0.0003$), which is not surprising given the negative relationship between water intake rate and sucrose concentration. Because we found no significant effects of subject ($F_{3,29} = 0.43$, $p > 0.7$) or treatment day ($F_{1,29} = 0.86$, $p > 0.3$) on f_w as a function of sucrose concentration, we removed these variables from the model. Water flux estimated from fractional water turnover rate (K_{3H}) and total body water (TBW) measurements ranged from 112.97 to 463.83 μl h^{-1} and increased linearly with water intake rate ($F_{1,29} = 237.29$, $p < 0.0001$). Because we found no effects of subject ($F_{3,29} = 0.2$, $p > 0.8$) or treatment day ($F_{1,29} = 1.95$, $p > 0.1$) we estimated a common relationship between water

flux and water intake rate ($K_{^3H}$ • TBW = 0.42 $\dot{V}_I$ + 81.64, r^2 = 0.89; Fig. 3). The slope of this relationship was significantly less than 1 (0.42 ± 0.03, t = 22.8, df = 33, $p < 0.001$), and significantly lower than that of the same relationship in broad-tailed hummingbirds ($ANCOVA_{slopes}$ $F_{1,35}$ = 27.8, $p < 0.0001$).

The volume of water absorbed per mass of sucrose assimilated ($\mu l\ mg^{-1}$) declined significantly with the sucrose concentration of the diet ($y = 1.47x^{-0.9}$, r^2 = 0.78; $F_{1,29}$ = 106.66, $p < 0.0001$; Fig. 4A). There was no significant effect of subject ($F_{3,29}$ = 0.5, $p > 0.6$) or treatment day ($F_{1,29}$ = 0.44, $p > 0.5$), so we removed these variables from the model. Absorbed water load (f_W • $\dot{V}_I$, μl h-1) was positively correlated with food intake rate ($F_{1,29}$ = 152.53, $p < 0.0001$; Fig. 4B). There was no significant effect of subject ($F_{3,29}$ = 0.64, $p > 0.5$) or treatment day ($F_{1,29}$ = 0.83, $p > 0.3$), so we removed these variables from the model and estimated a common relationship ($y = 0.40x + 25.09$, r^2 = 0.84).

Experiment 2: Fractional absorption of ingested water as a function of sucrose assimilation. Sunbirds feeding on 584 mmol l^{-1} sucrose solutions consumed about 1.3 times more food and sucrose at 15 than at 30 °C (624.52 ± 29.83 vs. 487.23 ± 25.47 $\mu l\ h^{-1}$ and 124.84 ± 5.96 vs. 97.4 ± 5.09 mg h^{-1}, respectively; $F_{1,5}$ = 6.6, p = 0.05 for both variables). These values translate into energy intake rates of 29.01 ± 1.39 and 22.64 ±

1.18 kJ d^{-1}, respectively. Fractional water absorption was not significantly different between temperatures (0.44 ± 0.02 vs. 0.43 ± 0.02 at 15 and 30 °C, respectively, $F_{1,5}$ = 0.22, p = 0.66; Fig. 2). The volume of water absorbed per mass sucrose assimilated did not differ between temperatures (1.94 ± 0.09 vs. 1.88 ± 0.08 μl mg^{-1} at 15 and 30 °C, respectively, $F_{1,5}$ = 0.21, p = 0.67). Although the absorbed water load ($f_w \bullet \dot{V}_I$) was about 1.3 times greater at 15 °C than at 30 °C, it did not differ significantly between treatments (237.34 ± 11.49 vs. 184.72 ± 14.46 μl h^{-1}, respectively, $F_{1,5}$ = 5.06, p = 0.074). We suspect that lack of statistical significance in this case was the result of low power due to small sample sizes.

Discussion

Nectar-feeding birds vary their food intake rate in response to sugar concentration; sunbirds in this study maintained constant rates of energy intake despite a 3.5 fold variation in food intake rate between the lowest and the highest sucrose concentrations (Fig. 1). This behavioral response allowed us to explore their physiological responses to a wide range of ingested water loads. Contrary to our hypothesis, and in contrast with the results reported by McWhorter and Martínez del Rio (1999) for broad-tailed hummingbirds, the fraction of ingested water absorbed (f_w) by Palestine sunbirds

decreased with water intake rate (Fig. 2). The functional relationship between f_w and water intake rate was an asymptotic function that tended towards 0.36 as ingested water load became large. This result implies that sunbirds can avoid absorbing, and thus having to eliminate, up to 64% of their ingested water load when feeding on dilute nectars. To our knowledge, this is the first documentation of apparent regulation of water flux across the gastrointestinal tract (GIT) to the body in vertebrates.

Although fractional absorption decreased with increasing sucrose concentration, the absorbed water load increased with food intake rate (Fig. 4B). The volume of water absorbed per mass sucrose assimilated decreased with sucrose concentration in food (Fig. 4A), despite constant sucrose intake. This suggests that sunbirds can regulate transepithelial water flux independently of sugar absorption. These intriguing results open the door to many questions about how water transport is regulated in the vertebrate GIT. In this discussion, we explore the differences in water turnover and fractional absorption between sunbirds and hummingbirds, members of separate evolutionary radiations of nectar-feeding birds. We posit that differences in mechanisms of sugar absorption and mass-specific food intake rates between these groups may explain the apparent ability of sunbirds to modulate water absorption. We discuss the implications of

our findings for estimating food intake in nectarivorous animals based on water flux rates and suggest that intestinal water and body water form two separate but interacting pools in nectar-feeding birds.

Our results provide empirical support for the hypothesis posed by Beuchat at al. (1990) for nectar-feeding birds. Sunbirds did not absorb all ingested water and the fraction of water absorbed in the intestine decreased with the ingested water load. Our results and those of McWhorter and Martínez del Rio (1999) highlight important differences between sunbirds and hummingbirds. Fractional water turnover rates in Palestine sunbirds ranged from 0.037 to 0.117 h^{-1}, while those in broad-tailed hummingbirds ranged from 0.12 to 0.61 h^{-1} (McWhorter and Martínez del Rio, 1999). In other words, sunbirds feeding on the most dilute nectar (292 mmol l^{-1} sucrose) turned over approximately 10% of their TBW pool each hour, compared to over 50% in hummingbirds. Because daily food intake by broad-tailed hummingbirds may reach 5.4 times their body mass while that of Palestine sunbirds only reaches about 2.2 times body mass in birds feeding on 292 mmol l^{-1} sucrose solutions, this difference may not be surprising. However, when similar rates of water intake are considered, hummingbirds and sunbirds show large differences in fractional and total water turnover rates. The

slope of the linear relationship between water flux and water intake rate (Fig. 3) provides a relative estimate of the fraction of ingested water that contributes to body water turnover. If 100% of ingested water were contributing to the turnover of the TBW pool at all water intake rates, the slope of this relationship would be equal to 1. The slope of this relationship in sunbirds was significantly less than one and shallower than that of the same relationship in hummingbirds. Sunbirds appear to regulate water flux from the GIT to the body, whereas hummingbirds do not. Convergence in diet has led to the evolution of many similar traits in hummingbirds and sunbirds (e.g. elongated bill, small body size, and pugnacity). The physiological traits of these two groups that allow the processing of a water and sugary diet, however, may be very different.

The mechanisms of intestinal water absorption in nectar-feeding birds are unknown but are probably facilitated by sugar uptake. Active transport appears to account for essentially all intestinal glucose absorption in hummingbirds (Karasov et al., 1986). Loo et al (1996) have shown that the translocation of each glucose molecule by the mammalian intestinal Na^+/glucose cotransporter (SGLT1) is coupled with the transport of up to 260 water molecules (potentially transporting 4.8 l of water per mole of glucose). Hummingbirds, which appear to absorb all ingested water, also exhibit the highest rate of

carrier-mediated glucose uptake measured in a vertebrate (Karasov et al., 1986). McWhorter and Martínez del Rio (1999) estimated that the amount of water potentially accompanying mediated glucose absorption in broad-tailed hummingbirds exceeded the water content in food by 1.7- to 5.5-fold, depending on sucrose concentration. Sunbirds in this study assimilated on average $3.1 \times 10^{-3} \pm 1.6 \times 10^{-4}$ mol of glucose in 14 h. The mediated uptake of this quantity of glucose could be responsible for the transport of 15 ml of water. Average daily water intake by sunbirds in this study ranged from 2 to 8.7 ml. As in hummingbirds, this amount exceeds the water ingested in food by a large margin (approximately 1.7 to 7.5-fold, depending on sucrose concentration). This comparison is perplexing because sunbirds appear to be able to modulate water absorption whereas hummingbirds do not.

Modulation of intestinal water absorption requires the rapid absorption of dissolved sugars and efficient extraction of electrolytes and amino acids present at low levels in ingested nectar (Beuchat et al., 1990). It also requires that the permeability of the intestine to transepithelial water flux be regulated. How may sunbirds regulate water flux while rapidly absorbing osmotically active sugars and electrolytes? One possibility is that the permeability of the intestine to transepithelial water flux by solvent drag

increases with sugar concentration. This would require that sunbirds have a low capacity for mediated glucose uptake relative to hummingbirds and significant passive absorption of nutrients at high sugar concentrations. Although passive absorption appears to be insignificant in hummingbirds (Karasov et al., 1986), it is an important route for nutrient absorption in some passerine and psittacine birds (Karasov and Cork, 1994; Caviedes-Vidal and Karasov, 1996; Afik et al., 1997; Chediack et al., 2001). It would be instructive to measure the capacity for mediated glucose uptake and determine whether the magnitude of passive absorption of carbohydrate probes varies with water intake (given constant energy intake) in sunbirds. Another possibility is that water cotransported into enterocytes during mediated nutrient absorption does not contribute to turnover of the TBW pool, but rather is secreted rapidly back into the intestinal lumen (Chang and Rao, 1994). Our estimates of the capacity for water absorption via mediated Na^+-glucose cotransport in birds are based on measurements on the mammalian SGLT1 expressed in the *Xenopus* oocyte made by Loo et al. (1996). Their measurements, however, sought to isolate water transport by that cotransporter and represent one element in a complex membrane system. The links between nutrient absorption, electrolyte balance and the regulation of transepithelial water flux in birds remain unknown.

Beuchat et al. (1990) raised their hypothesis to explain the ability of hummingbirds to cope with extraordinary water fluxes. Daily food intake by broad-tailed hummingbirds may reach 5.4 times their body mass while that of Palestine sunbirds only reaches about 2.2 times body mass in birds feeding on 292 mmol l^{-1} sucrose solutions. Metabolic mass-specific sucrose intake rate (mg h^{-1} $kg^{-0.75}$) is about 3 times higher in hummingbirds than in sunbirds. Why may sunbirds modulate water absorption while hummingbirds do not? Perhaps there are significant physiological differences in nutrient absorption and the regulation of transepithelial water flux between these groups. It is also possible that the extraordinarily high mass-specific energy demands of hummingbirds lead to water intake rates that simply overwhelm their physiological capacities to regulate water absorption. We speculate that water ingestion and subsequent absorption are unlikely to constrain energy intake by sunbirds. The apparent ability of sunbirds to modulate water absorption may allow them to profitably feed on dilute floral nectars by minimizing the metabolic cost of recovering glucose and electrolytes filtered in the kidney. Indeed, we have preliminary data suggesting that glomerular filtration rates in sunbirds are lower than expected based on allometric estimates.

Implications for doubly labeled water studies

Water turnover in nectar-feeding animals has often been used to approximate nectar intake, assuming that ingested water comes only from food (von Helversen and Reyer, 1984; Kunz and Nagy, 1988; Powers and Nagy, 1988; Weathers and Stiles, 1989; Tiebout and Nagy, 1991). These approximations are based on the assumption that isotope concentrations in water leaving the body are the same as those in the body water at the same time (Lifson and McClintock, 1966). Differences in isotope concentrations between these pools can arise from both physical and biological fractionation (Lifson and McClintock, 1966; Speakman, 1997; Visser et al., 2000). Biological fractionation is due to incomplete mixing of the isotope label between the body and ingested water. Although physical fractionation can be accounted for mathematically, the issue of incomplete mixing has received very little attention (Visser et al., 2000). Nagy and Costa (1980) argued that biological fractionation might occur in birds eating bulky, energy dilute foods with consequent high gastrointestinal passage rates, but there is no data to support this argument. Visser et al. (2000) recently determined that ingested water reaches isotopic equilibrium with the body water pool regardless of water intake rate in red knots (*Calidris canutus*), which may have water fluxes up to 17 times greater than predicted for free-living birds. In contrast, our results and those of McWhorter and

Martínez del Rio (1999) suggest that biological fractionation is occurring in nectar-feeding birds, i.e. that intestinal water and body water form two separate but interacting pools. Our model estimates the proportion of ingested water that contributes to the turnover of the TBW pool. We assumed that the rates of appearance of marker in excreted fluid and disappearance from TBW were equal, rather than assuming that the concentrations of markers were equal. If complete equilibration of intestinal water and body water were occurring, our model would estimate f_w as 1 regardless of water flux rate, which was not the case for either sunbirds or hummingbirds. Thus, our results tell a cautionary tale for the estimation of food intake based on water flux rates in nectar-feeding animals: nectar intake will be underestimated if water absorption in not complete. Our data also suggest that additional attention needs to be paid to the issue of biological fractionation when using stable and radioactive hydrogen isotopes to measure whole body rates of water turnover in animals.

Acknowledgements

We are grateful to Lizanne Roxburgh, Mariela Leiderman, Efrat Elimelich and Itzick Vatnick for expert assistance with experiments. Catherine Hambly generously provided access to unpublished data. Two anonymous referees made incisive comments that allowed us to greatly improve upon previous versions of this paper. This research was supported by Grant No. 98-178 from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel to CMR and BP and an NSF grant (IBN-0110416) to CMR. This is publication 365 of the Marco and Louise Mitrani Department of Desert Ecology.

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Legends

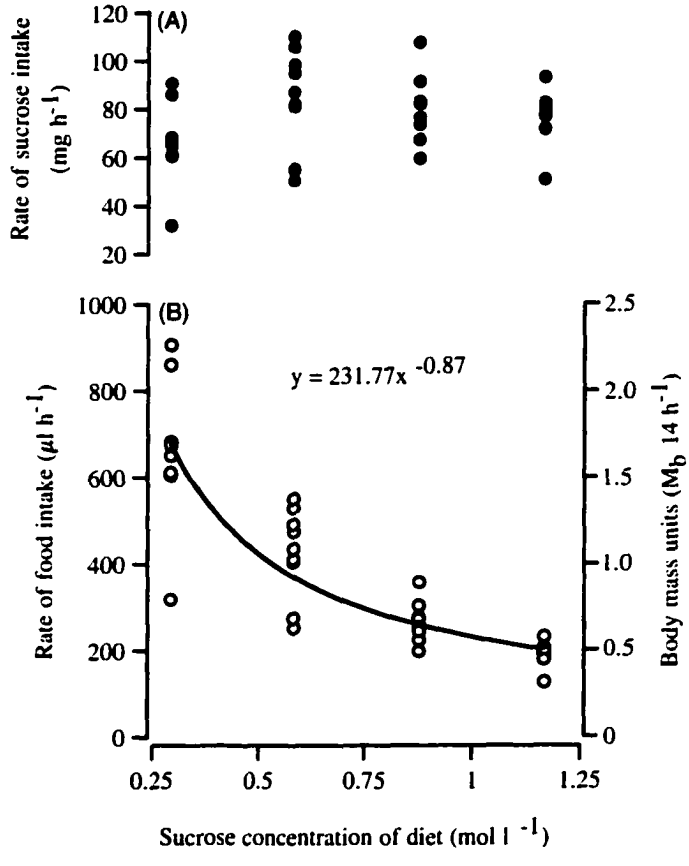
Fig. 1. Behavioral responses of sunbirds to varying sucrose concentration in food. (A) The rate of sugar intake did not increase significantly with sucrose concentration ($r_s = 0.12$, $p = 0.49$, $n = 35$). Average sucrose intake was 77.17 ± 3 mg h^{-1} (17.94 ± 0.7 kJ d^{-1}). (B) Volumetric food intake rate declined significantly with dietary sucrose concentration ($F_{1, 29} = 107.0$, $p < 0.0001$). The relationship was well described by a power function ($r^2 = 0.76$) with an exponent that was not significantly different from 1 ($t = -1.52$, $df = 33$, $p > 0.1$). Changes in food energy density from 0.292 to 1.168 mol l^{-1} sucrose led to an approximately 3.5 fold variation in food (and thus water) intake. The right-hand axis shows food intake in multiples of body mass (5.74 ± 0.07 g, $n = 4$) per 14 hours. At low sucrose concentrations, sunbirds consumed between 0.8 and 2.2 times their body mass in food in 14 h of daylight.

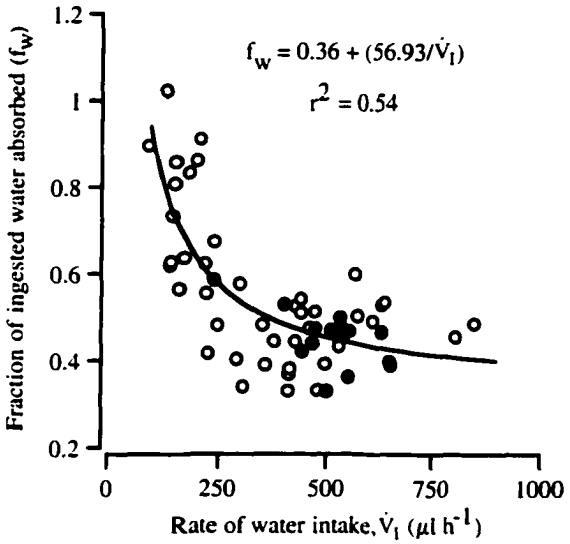
Fig. 2. Fractional absorption of ingested water (f_w) across the gut of Palestine sunbirds ranged from 0.33 to 1.02 (averaging 0.59 ± 0.04, $n = 35$) and declined significantly and non-linearly with water intake rate ($f_W = 0.36 + 56.93\,\dot{V}_I^{-1}$, $F_{1,29} = 40.03$, $p < 0.0001$, $r^2 = 0.54$). Palestine sunbirds may therefore avoid absorbing up to 64 % of ingested water when feeding on dilute nectars. In sunbirds feeding on 584 mmol l^{-1} sucrose

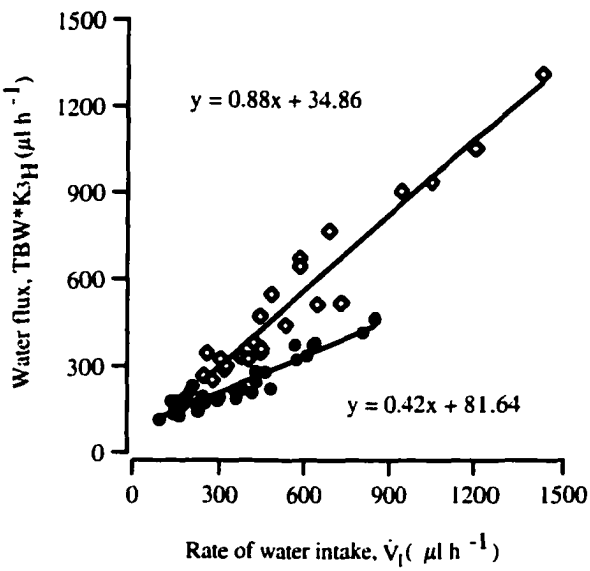
solutions, f_w was not significantly different between 15° C (filled circles) and 30° C (0.44 ± 0.02 vs. 0.43 ± 0.02, respectively, $F_{1,5} = 0.22$, $p = 0.66$).

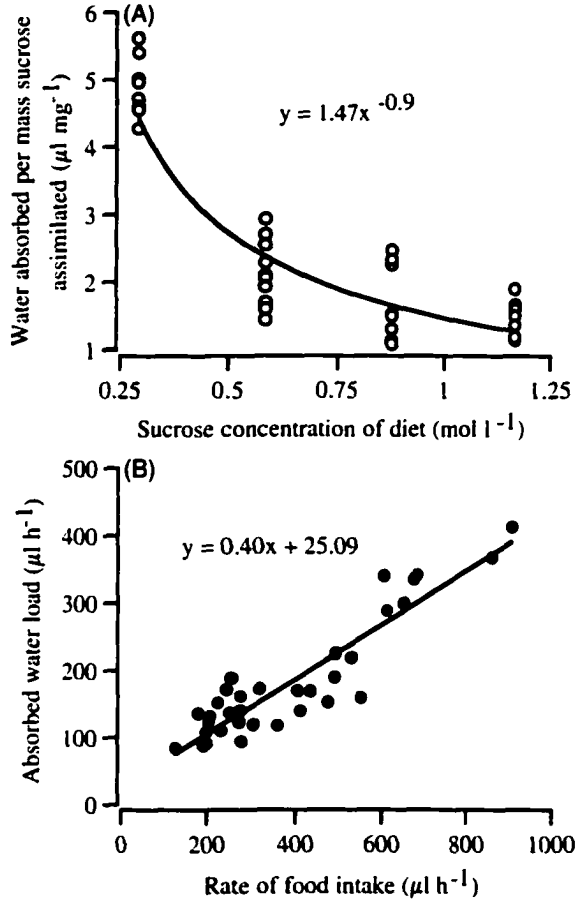
Fig. 3. Water flux in sunbirds (filled circles) estimated from fractional water turnover rate (K_{3H}) and total body water (TBW) measurements ranged from 112.97 to 463.83 μl h^{-1} and increased linearly with water intake rate ($r^2 = 0.89$, $F_{1,29} = 237.29$, $p < 0.0001$). The slope of this relationship was significantly less than 1 (slope ± SE = 0.42 ± 0.03, $t = 22.8$, $df = 33$, $p < 0.001$), and significantly lower than that of the same relationship in broad-tailed hummingbirds (unfilled diamonds; slope ± SE = 0.88 ± 0.05, $ANCOVA_{slopes}$ $F_{1,35} = 27.8$, $p < 0.0001$; data for hummingbirds from McWhorter and Martínez del Rio 1999).

Fig. 4. Water absorption as a function of sucrose assimilation and food intake rate in sunbirds. (A) The volume of water absorbed per mass of sucrose assimilated (μl mg^{-1}) declined significantly with the sucrose concentration of the diet ($r^2 = 0.78$; $F_{1,29} = 106.66$, $p < 0.0001$), despite constant sucrose intake, suggesting that sunbirds regulate transepithelial water flux independently of sugar absorption. (B) The absorbed water load ($f_W \bullet \dot{V}_I$, μl h-1) was positively correlated with food intake rate ($r^2 = 0.84$; $F_{1,29} = 152.53$, $p < 0.0001$).


(A)
Rate of sucrose intake (mg h-1)
120
100
80
60
40
20
(B)
y = 231.77x -0.87
Rate of food intake (µl h-1)
1000
800
600
400
200
0
2.5
2.0
1.5
1.0
0.5
0
Body mass units (Mb 14 h-1)
0.25
0.5
0.75
1
1.25
Sucrose concentration of diet (mol l -1)


$f_w = 0.36 + (56.93/\dot{V}_I)$
$r^2 = 0.54$
Fraction of ingested water absorbed (f_w)
1.2
1
0.8
0.6
0.4
0.2
0
250
500
750
1000
Rate of water intake, $\dot{V}_I$ ($\mu l\ h^{-1}$)


1500
1200
900
600
300
0
0
300
600
900
1200
1500
y = 0.88x + 34.86
y = 0.42x + 81.64
Water flux, TBW*K3H (µl h -1)
Rate of water intake, V̇I (µl h -1)


(A)
Water absorbed per mass sucrose assimilated (μl mg-1)
y = 1.47x -0.9
6
5
4
3
2
1
0.25
0.5
0.75
1
1.25
Sucrose concentration of diet (mol l-1)
(B)
Absorbed water load (μl h-1)
y = 0.40x + 25.09
500
400
300
200
100
0
0
200
400
600
800
1000
Rate of food intake (μl h-1)

APPENDIX B

RENAL FUNCTION IN PALESTINE SUNBIRDS: ELIMINATION OF EXCESS WATER DOES NOT CONSTRAIN ENERGY INTAKE

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Running Head: *Renal function in Palestine sunbirds*

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Summary

Although the renal responses of birds to dehydration have received significant attention, the consequences of ingesting and processing large quantities of water have been less studied. Nectar-feeding birds must often deal with exceptionally high water intake rates in order to meet their high mass-specific energy demands. Birds that ingest large volumes of water may either eliminate excess water in the kidney or they may regulate the volume of water absorbed in the gastrointestinal tract. Because water absorption in the gastrointestinal tract of Palestine sunbirds (*Nectarinia osea*) decreases with increasing water ingestion rate, we predicted that glomerular filtration rate (GFR) in these birds would not be unusually high in spite of large ingested water loads. When feeding on dilute sucrose solutions, sunbirds ingested between 4 and 6 times their body mass in nectar per day, yet they were able to compensate for varying nectar energy density and increased thermoregulatory energy demands with no apparent difficulty. GFR was lower than predicted (1690.67 ± 148.11 μl h^{-1}, approximately 40% of the value predicted based on body mass), and not exceptionally sensitive to water loading. Plasma glucose concentrations were high and varied 1.8 fold between fasted (16.08 ± 0.75 mmol l^{-1}) and fed (28.18 ± 0.68 mmol l^{-1}) sunbirds, but because GFR was low, glucose filtered load also remained relatively low. Essentially all of the glucose filtered load (98%) was recovered

by the kidney. Renal fractional water reabsorption (FWR) decreased from 0.98 to 0.64 with increasing water intake. The ability of Palestine sunbirds to reduce the absorption of dietary water in the gastrointestinal tract may resolve the potential conflict between filtering a large excess of absorbed water in the kidney and simultaneously retaining filtered metabolites.

Introduction

The study of renal processes in birds has emphasized dehydration over diuresis (see Braun, 1993). The form of the relationship between water load and glomerular filtration rate (GFR) has therefore not been described for birds experiencing a large range of water loads (Goldstein and Bradshaw, 1998; Goldstein and Skadhauge, 2000). Nectar-feeding birds are of special interest because they are capable of ingesting astounding water volumes (reviewed by Martínez del Rio et al., 2001). It is generally believed that GFR is more variable and more responsive to water status in birds than in mammals (Williams et al., 1991; Dantzler, 1992; Osono and Nishimura, 1994; Goldstein, 1995). GFR decreases in response to water deprivation in many avian species (Williams et al., 1991; Goldstein and Skadhauge, 2000) and appears to increase only moderately in response to water loading (Skadhauge and Schmidt-Nielsen, 1967; Braun and Dantzler, 1975; Roberts and Dantzler, 1989). The GFR data available, however, are for birds that do not regularly cope with large ingested water loads. The physiological mechanisms that allow nectar-feeding birds to contend with their watery diets, and the consequences of ingesting and processing large quantities of water for energy intake and the maintenance of metabolite and electrolyte homeostasis are relatively unexplored.

Sugar-rich floral nectars provide a ready source of fuel for the high mass-specific energy demands of nectar-feeding birds, but also supply an excess of water in most circumstances (Calder, 1979; Calder and Hiebert, 1983; Beuchat et al., 1990). Indeed, extremely high water flux rates, often reaching as high as 6 times body mass per day, have been measured in many species of nectarivorous and frugivorous birds (Rooke et al., 1983; Powers and Nagy, 1988; Weathers and Stiles, 1989; Williams, 1993; Powers and Conley, 1994; Goldstein and Bradshaw, 1998; Nicolson, 1998; Lotz and Nicolson, 1999; McWhorter and Martínez del Rio, 1999; Nicolson and Fleming, in press). The extraordinary drinking rates exhibited by nectar-feeding birds would lead to pathological consequences in other terrestrial vertebrates. In humans, rats, domestic pigeons and gray parrots, over-ingestion of water results in "water intoxication" (Lumeij and Westerhof, 1988; Gebel et al., 1989; Gevaert et al., 1991; de Leon et al., 1994), and leads to negative effects associated with plasma dilution, hyponatraemia (low plasma sodium), and rupture of erythrocytes due to osmotic swelling (Fanestil, 1977; Gebel et al., 1989).

Nectar-feeding birds are faced with the conflicting demands of eliminating excess water and metabolic by-products while retaining electrolytes, metabolites and substrates for energy metabolism (Yokota et al., 1985). Plasma glucose levels in hummingbirds are high and surprisingly variable (ranging from 17 mmol l^{-1} in fasted birds to as much as 40

mmol l^{-1} in feeding individuals, Beuchat and Chong, 1998), resulting in relatively high estimated glucose filtered loads (the product of GFR and the concentration of glucose in plasma). How do these birds prevent the loss of glucose to urine? In the mammals and birds for which renal glucose recovery has been investigated (summarized in Beyenbach, 1985), the high plasma glucose concentrations found in nectar-feeding birds would lead to severe renal glucose loss and presumably osmotic diuresis. Hummingbirds produce extremely dilute urine (Calder and Hiebert, 1983) and the morphology of their kidneys suggests that they are well suited for water disposal (Johnson and Mugaas, 1970; Casotti et al., 1998; Beuchat et al., 1999). Because hummingbirds also appear to absorb essentially all ingested water (McWhorter and Martínez del Rio, 1999), they probably rely on a large renal capacity for water elimination (and thus energetically expensive renal glucose and electrolyte reabsorption) and on relatively high evaporative water loss rates (Lasiewski, 1964; Powers, 1992) to maintain water balance. The problem of excess ingested water, however, can be handled both from the supply and disposal sides of the equation. Nectar-feeding sunbirds (family Nectariniidae) reduce the fractional absorption of ingested water with increasing water intake rate (McWhorter et al., submitted). Sunbirds may therefore avoid a substantial absorbed water load, and thus the associated

costs of recovering metabolites in the kidney and potential limitations to energy intake, when feeding on dilute nectars.

Here we report the results of experiments designed to examine the relationship between energy and water intake and kidney function in the Palestine sunbird [*Nectarinia osea* (Bonaparte, 1856)], an Old World passerine nectarivore. Despite water intake rates that exceed several times their body mass per day (Lotz and Nicolson, 1999; Nicolson and Fleming, in press; McWhorter et al., submitted), sunbirds, unlike hummingbirds, may not face exceptional renal water loads. We hypothesized that GFR in Palestine sunbirds would not be unusually high (i.e. it would be consistent with the allometric prediction of 4.3 ml h^{-1} for a 5.8 g bird, Yokota et al., 1985; Williams et al., 1991) or remarkably sensitive to water loading (Goldstein and Bradshaw, 1998). As a corollary to this hypothesis, we predicted that sunbirds would have plasma glucose concentrations comparable to those of hummingbirds (Beuchat and Chong, 1998), but relatively low glucose filtered loads and would consequently excrete very little glucose (McWhorter and Martínez del Rio, 2000). We further predicted that fractional water reabsorption (FWR) by the kidney would decrease with increasing water load (Goldstein and Bradshaw, 1998).

Material and methods

Bird capture and maintenance

Male Palestine sunbirds (body mass 5.81 ± 0.19 g, n = 13) were captured with drop nets on the grounds of Midreshet Ben-Gurion, home of the Sede Boqer Campus of Ben-Gurion University of the Negev, Israel (30° 51' N, 34° 46' E; Israel Nature and National Parks Protection Authority permits 5981 and 7686). Birds were housed individually in outdoor aviaries (1.5 m x 1.5 m x 2.5 m) and fed a maintenance diet of two artificial nectar solutions between experiments. The diets included a 20-25 % sucrose equivalent solution and a 15 % sucrose solution supplemented with a soy protein infant formula (Isomil™, Abbott Laboratories, Netherlands) diluted to approximately 2.5 g protein per 100 g sucrose. Food and water were available ad libitum. Birds were also offered freshly killed fruit flies (Drosophila spp.) at least twice a week. During experiments, birds were housed individually in opaque Plexiglas® cages (0.3 m x 0.3 m x 0.3 m) with individual light sources. The front of these cages was coated with a reflective Mylar™ polyester film to create a one-way mirror effect that permitted observation of birds in a darkened room with minimal disturbance. One of the perches in the center of each cage was fitted to hang from an electronic balance (Scout II 200 g x 0.01 g, Ohaus Corporation, Florham Park, NJ) so body mass could be monitored continuously. Birds were allowed to

acclimate to cages for 2-3 d before experiments began and were left undisturbed in outdoor aviaries for a minimum of 7 days between trials. The study was conducted using light cycles that matched the natural photoperiod (13.25 to 14.5 h light). Birds were fed experimental diets, which consisted of sucrose solutions made with distilled water, for a minimum of 24 h before trials began.

Experimental design

We relied on the behavioral responses of birds to nectar of varying energy density in the design of this experiment. Typically, nectar-feeding birds reduce their food intake rate with increasing sugar concentration (López-Calleja et al., 1997; McWhorter and Martínez del Rio, 1999; McWhorter and López-Calleja, 2000; McWhorter and Martínez del Rio, 2000; Martínez del Rio et al., 2001). Manipulation of sugar concentration therefore leads to a wide range of variation in the quantity of nectar (and thus water) ingested. We used a repeated measures design in which we measured GFR and renal FWR in eight sunbirds fed five different sugar solutions (146, 292, 584, 876, and 1168 mmol l^{-1} sucrose) at two ambient temperatures (15 ± 1 and 30 ± 2 °C). In a separate repeated measures experiment, we measured urine and excreta osmotic concentration and glucose concentration in eight sunbirds fed on four sugar solutions (146, 292, 584, and 1168 mmol l^{-1} sucrose) at three ambient temperatures (5 ± 2, 15 ± 1, and 30 ± 2 °C). In both

experiments, we randomized the order in which diets were presented to subjects. Finally, we measured the plasma glucose concentration of nine sunbirds, both when feeding on their normal maintenance diet (described above) and after a 12-hour overnight fast, in a repeated measures design. Birds were randomly assigned to the first treatment (i.e. fed vs. fasted) and all measurements were conducted at 25 ± 2 °C.

Estimating GFR and FWR in sunbirds

GFR was estimated with a single injection of ^{14}C-labeled inulin, using a modification of the slope-intercept method (Hall et al., 1977; Florijn et al., 1994). The only assumption we made in modifying this method was that the rate of marker disappearance from plasma was equal to the rate of appearance in excreta. The specific activity of marker would of course be different among plasma, urine and excreta because of reabsorption of filtered water in the kidney and mixing of urine with gut contents in the cloaca. Our method allowed us to measure renal function in unanesthetized, actively feeding birds with minimal disturbance. GFR (μl h^{-1}) was estimated as:

$$GFR = Q_i \bullet K_{^{14}C} \bullet A^{-1}, \quad (1)$$

where Q_i is the quantity of marker injected (disintegrations min^{-1}), $K_{^{14}C}$ is the fractional inulin turnover rate (h^{-1}), and A is the zero-time intercept concentration of marker in

plasma (disintegrations min^{-1} μl^{-1}). Fractional inulin turnover rate was estimated by fitting negative exponential functions (Hall et al., 1977) to the relationship between the specific activity of ^{14}C in excreta and time. The slope of the fractional inulin turnover curve was then used to extrapolate the plasma marker concentration of a single blood sample, taken 2-3 hours after injection, to the zero-time intercept concentration (and thus also estimate the inulin distribution space). This method was used because of the sensitivity of small birds to repeated blood sampling. Fractional recovery of filtered water in the kidney (FWR) was estimated as one minus the ratio of marker concentration in plasma to that in urine ($FWR = 1 - [P_M \bullet U_M^{-1}]$).

Experimental measurements

GFR and FWR measurements: We injected $4.63x10^4$ Bq of inulin-[^{14}C]-carboxylic acid (molecular weight 5175 ± 95; Amersham, Piscataway, NJ) in 15 μl of distilled water into the *pectoralis* of each bird approximately 1.5 h after the lights came on. Injection volumes were verified gravimetrically by weighing syringes (25 μl, Hamilton Company, Reno, NV) to the nearest 0.0001 g before and after injection. Fresh excreta samples were collected for 2-3 hours, after which a ureteral urine sample was collected with a closed-ended polyethylene cannula (Goldstein and Braun, 1989) and a blood sample

(approximately 50 μl) was collected by puncturing the brachial vein. We separated plasma from blood cells by centrifugation (3 min) before radioisotope analysis. Liquid scintillation cocktail (ACS II, Amersham) was added to all excreta, plasma, urine and injection samples, which were counted correcting for quench and lumex in a Packard Tri-Carb 1600TR Liquid Scintillation Analyzer.

Excreted fluid and ureteral urine glucose and osmotic concentration measurements: Fresh excreta samples were collected from actively feeding sunbirds over a 30-minute period, pooled for each bird separately, and immediately frozen for later analysis. After excreta collections were completed, we captured birds and collected a ureteral urine sample with a closed-ended polyethylene cannula (Goldstein and Braun, 1989). We measured the osmotic concentration of the samples using an Osmette II freezing point depression osmometer (Precision Systems Inc., Natick, MA), and glucose concentration using a clinical diagnostic kit (Procedure No. 315, enzymatic determination by the Trinder reaction; Sigma Chemical, St. Louis, MO).

Plasma glucose measurements: We collected blood samples (approximately 30 μl) by puncturing the brachial vein one hour after the lights came on. Fed birds were allowed to

feed normally for one hour before sampling. Plasma was separated from the blood sample by centrifugation and immediately assayed for glucose concentration as above.

Statistical analysis

Because relationships between food intake rate and sugar concentration in nectar-feeding birds are power functions (López-Calleja et al., 1997; McWhorter and Martínez del Rio, 1999; McWhorter and López-Calleja, 2000; McWhorter and Martínez del Rio, 2000; Martínez del Rio et al., 2001; Nicolson and Fleming, in press), we determined the effects of temperature and individual bird (subject) on food intake rate using linear models of $\log_e$-transformed intake and sucrose concentration data. Analysis of covariance (ANCOVA) was used on $\log_e$-transformed data to compare the slope and intercept of this relationship among experimental temperatures. The relationships between the osmotic and glucose concentrations of ureteral urine and excreted fluid and water intake rate were best described by power functions, so we similarly applied linear models to $\log_e$-transformed data. We used linear models on untransformed data to assess significance and subject and temperature effects in all other cases. Repeated-measures analysis of variance (RM-ANOVA) was used to assess differences in plasma glucose concentration between fed and fasted birds. All values are presented as mean ± SEM.

Results

GFR and FWR measurements: Sunbirds consumed significantly less food as sucrose concentration in the diet increased ($F_{1, 27} = 382.1$, $p < 0.0001$, $n = 37$; Fig. 1B). Food intake rate was significantly higher at 15 than at 30 °C (approximately 1.4 times; $F_{1, 27} = 42.15$, $p < 0.0001$). There was no significant effect of subject ($F_{7, 27} = 2.19$, $p = 0.07$) on food intake rate, so we removed this variable from the model. We described the relationship between food intake and sucrose concentration using a power function for each temperature separately (Fig. 1B). The exponents of these relationships were not significantly different from -1 ($t = 1.87$, $df = 19$, $p > 0.05$ and $t = 0.72$, $df = 16$, $p > 0.05$ for 15 and 30 °C, respectively) or from each other ($ANCOVA_{slopes}$ $F_{1,33} = 2.76$, $p = 0.11$). Sucrose intake rate was 1.6 times greater at 15 than at 30 °C ($F_{1,27} = 42.59$, $p < 0.0001$), but was not correlated with dietary sucrose concentration ($F_{1,27} = 2.13$, $p = 0.16$). Hence, although food, and thus water, intake rate varied from 7.2 to 9.5 fold (for 30 and 15 °C, respectively) between the lowest and the highest sucrose concentrations, sunbirds did not increase their sucrose intake significantly with increasing sucrose concentration (Fig. 1A). Sucrose intake averaged 119.45 ± 5.08 mg h^{-1} (27.76 ± 1.18 kJ d^{-1}) for 15 °C and 75.28 ± 5.17 mg h^{-1} (17.49 ± 1.2 kJ d^{-1}) for 30 °C. When feeding on 0.146 mol l^{-1} sucrose

solutions, sunbirds consumed between 4 and 6 times their body mass in food in 14 h of daylight, depending on temperature.

The relationships between the specific activity of ^{14}C inulin in excreta (disintegrations min^{-1} μl^{-1}) and time were well described by negative exponential functions (r^2 ranged from 0.61 to 0.99, n = 37). The decline in the specific activity of ^{14}C inulin in excreta with time therefore apparently followed one-compartment, first-order kinetics (Fig. 2). Fractional inulin turnover rate (K_{14C}) did not differ significantly between temperatures (ANOVA $F_{1,35} = 2.9$, $p = 0.1$). Inulin distribution space estimated by the intercept method ranged from 3.14 to 7.89 % of body mass (4.7 ± 0.43 %, n = 37).

Glomerular filtration rate (GFR) in Palestine sunbirds ranged from 576.19 to 5203.47 $\mu l\ h^{-1}$ (1690.67 ± 148.11 $\mu l\ h^{-1}$, n = 37; Fig. 3). There was a significant effect of temperature ($F_{1,34} = 6.95$, $p = 0.013$) and water intake rate ($F_{1,27} = 7.68$, $p = 0.01$) on GFR, but no significant effect of subject ($F_{7,27} = 0.78$, $p = 0.61$), so we removed the latter variable from the model. To examine the effects of water intake independently of temperature, we constructed separate linear models for measurements at each temperature. GFR was correlated with water intake rate at 15 °C ($y = 0.58x + 869.72$,

$r^2 = 0.53$, $F_{1,18} = 20.55$, $p = 0.0003$), but not at 30 °C ($F_{1,15} = 0.21$, $p = 0.65$). Mean GFR was significantly higher at the higher temperature (1434.67 ± 153.19 vs. 1991.85 ± 253.28 $\mu l\ h^{-1}$ for 15 and 30 °C, respectively; $ANCOVA_{temperature}$ $F_{1,33} = 6.13$, $p = 0.019$).

Fractional water reabsorption (FWR) in the kidney ranged from 0.98 to 0.64 (0.82 ± 0.02, n = 29) and decreased significantly with water intake rate as predicted ($F_{1,19} = 6.65$, $p = 0.018$; Fig. 4). Because there were no significant effects of subject ($F_{7,19} = 1.21$, $p = 0.34$) or temperature ($F_{1,19} = 0.08$, $p = 0.77$), we removed these variables from the model and estimated a common relationship between FWR and water intake rate ($y = -1.6 \bullet 10^{-4}x + 0.91$, $r^2 = 0.34$).

Excreted fluid and ureteral urine glucose and osmotic concentration measurements:

Osmotic concentration declined significantly with increasing water intake rate ($F_{1,40} = 48.36$, $p < 0.0001$), and was significantly greater in ureteral urine than in excreted fluid ($F_{1,31} = 57.91$, $p < 0.0001$; Fig. 5B). Because there were no effects of subject ($F_{9,31} = 0.91$, $p = 0.53$) or temperature ($F_{2,31} = 1.72$, $p = 0.2$), we removed these variables from the model. We described the relationship between osmotic concentration and water intake rate using separate power functions for ureteral urine and excreted fluid

($y = 18045.61x^{-0.82}$, $r^2 = 0.49$, $F_{1,11} = 10.47$, $p = 0.008$, $n = 13$, and $y = 1101.14x^{-0.57}$, $r^2 = 0.65$, $F_{1,28} = 51.1$, $p < 0.0001$, $n = 32$, respectively; Fig. 5B). Ureteral urine osmotic concentration ranged from 14.96 to 329 mOsm [kg H_2O]$^{-1}$ (115.5 ± 25.28, n = 13), and that of excreted fluid ranged from 12.33 to 95 mOsm [kg H_2O]$^{-1}$ (30.82 ± 3.82, n = 32).

Glucose concentration declined significantly with increasing water intake rate ($F_{1,37} = 13.47$, $p = 0.0008$), and was significantly higher in ureteral urine than in excreted fluid ($F_{1,28} = 17.1$, $p < 0.0003$; Fig. 5A). There were no effects of subject ($F_{9,28} = 0.9$, $p = 0.54$) or temperature ($F_{2,28} = 2.21$, $p = 0.13$), so we removed these variables from the model. Glucose concentration was not significantly correlated with water intake rate when ureteral urine data were considered separately ($F_{1,9} = 0.67$, $p = 0.43$, $n = 11$), probably because of small sample size, particularly at higher rates of water intake. The relationship between glucose concentration and water intake rate in excreted fluid was adequately described by a power function ($y = 26.18x^{-0.62}$, $r^2 = 0.4$, $F_{1,27} = 8.21$, $p = 0.008$, $n = 31$; Fig. 5A). Glucose concentration in ureteral urine ranged from 0.28 to 10.39 mmol l^{-1} (2.97 ± 1.05, n = 11), and that in excreted fluid ranged from 0.12 to 3.52 mmol l^{-1} (0.6 ± 0.12, n = 31).

Plasma glucose measurements: Plasma glucose concentration was significantly greater in fed (28.18 ± 0.68 mmol l^{-1}) than in fasted sunbirds (16.08 ± 0.75 mmol l^{-1}, $F_{1,7}$ = 335.44, $p < 0.0001$, n = 8).

Discussion

The behavioral response of sunbirds to changes in food energy density allowed us to explore their physiological responses to a wide range of ingested water loads. Sunbirds maintained constant rates of energy intake despite water intake rates that varied as much as 9.5 fold between the lowest and highest sucrose concentrations (Fig. 1). They consumed between 4 and 6 times their body mass in food per day when feeding on dilute sucrose solutions, depending on ambient temperature. Such phenomenal water ingestion rates would lead to pathological consequences in many terrestrial vertebrates (Lumeij and Westerhof, 1988; Gebel et al., 1989; Gevaert et al., 1991; de Leon et al., 1994), yet sunbirds were able to compensate for varying food energy density and increased thermoregulatory energy demands with no apparent difficulty. Our results suggest that water processing does not limit energy intake in Palestine sunbirds.

Glomerular filtration rate was lower than expected (40% of the value predicted based on body mass, Yokota et al., 1985; Williams et al., 1991), and not exceptionally sensitive

to water loading (Fig. 3). Plasma glucose concentrations were high and varied 1.8 fold between fasted and fed birds, but because GFR was low, glucose filtered load also remained relatively low (0.05 mmol h^{-1} in fed birds). Essentially all of the glucose filtered load (98%) was recovered by the kidneys. Renal fractional water reabsorption decreased from 0.98 to 0.64 with increasing water load (Fig. 4), comparable to observations in nectar-feeding red wattlebirds (*Anthochaera carunculata*, Goldstein and Bradshaw, 1998). Because the fraction of ingested water absorbed by Palestine sunbirds decreases with water intake rate (McWhorter et al., submitted), however, their low GFR and high proportional renal recovery of glucose is not surprising. They deal with the problem of water over-ingestion by not absorbing all the water that they ingest, rather than by absorbing it and then filtering it in the kidney. In this discussion, we explore the consequences of these adaptations to high water loads for the simultaneous maintenance of water and energy balance. We posit that the metabolic cost of recovering filtered metabolites, and the potential for these processes to limit energy intake, are much lower in sunbirds than in hummingbirds (Nicolson and Fleming, in press).

Water ingestion and subsequent absorption in intestine has the potential to constrain an animal's energy intake rate by exceeding its capacity for water disposal (McWhorter

and Martínez del Rio, 1999; Martínez del Rio et al., 2001). Water loads (preformed water in food plus metabolic water) greater than the sum of evaporative water loss and maximum renal water elimination (GFR minus a minimum fractional water reabsorption necessary to retain filtered metabolites) will overwhelm osmoregulatory processes and lead to water intoxication unless the animal decreases food intake. Food intake by sunbirds in this study increased with no detectable plateau as diet sucrose concentration and ambient temperature decreased (Fig. 1). Indeed, the slopes of the relationships between food intake and diet sugar concentration at both 15 and 30 °C were not significantly different from one, indicating that birds were compensating completely for changes in food energy density (Martínez del Rio et al., 2001). In addition, the 1.6 fold higher food and sucrose intake rates observed at 15 °C correspond almost exactly to the 1.5 fold increase in metabolic rate observed in Palestine sunbirds between ambient temperatures of 15 and 30 °C in the laboratory (C. Hambly, B. Pinshow, E.J. Harper and J.R. Speakman, unpublished data). The sugar concentrations in the diets used in this study span the range of sugar concentrations found in the nectar of bird pollinated plants (Pyke and Waser, 1981; Gryj et al., 1990; Stiles and Freeman, 1993). Our results suggest, therefore, that water processing does not limit energy intake in Palestine sunbirds over the range of sugar concentrations that they encounter naturally.

McWhorter et al. (submitted) found that the fraction of ingested water absorbed (f_w) by Palestine sunbirds decreased from 100% to 36% with increasing water intake rate ($\dot{V}_I$). In addition, Goldstein and Bradshaw (1998) found evidence suggesting that dietary water was not completely absorbed from the gut of nectar-feeding red wattlebirds under conditions of high water intake. Therefore, in spite of water intake rates that exceed several times their body mass per day (Lotz and Nicolson, 1999; Nicolson and Fleming, in press; McWhorter et al., submitted), sunbirds may not face exceptional renal water loads when feeding on dilute nectars. In Figure 6 we compare water intake rate, estimated water load and renal free water clearance (GFR – [GFR • FWR]) as a function of diet sucrose concentration for birds in this study (data for both temperatures combined). Water load was estimated as water absorption rate ($f_w \bullet \dot{V}_I$, where $f_w = 0.36 + [56.93 \bullet \dot{V}_I^{-1}]$, McWhorter et al. submitted) plus metabolic water production (estimated based on sucrose assimilation rate, assuming carbohydrate catabolism). Estimated water load increases much more slowly with decreasing sucrose concentration in the food than does water intake rate, and roughly parallels renal free water clearance rate. The difference between water load and water clearance represents the fraction of water that is lost by evaporation. The ability of sunbirds to modulate the absorption of preformed

water in food substantially reduces the water load that must subsequently be eliminated by the kidney.

Excreted fluid glucose concentrations are comparably low in Palestine sunbirds (0.6 ± 0.12 mmol l^{-1}) and broad-tailed hummingbirds (*Selasphorus platycercus*, 1.3 ± 0.6 mmol l^{-1}, McWhorter and Martínez del Rio, 2000). Does renal glucose processing and conservation differ between sunbirds and hummingbirds? Glucose filtered loads in sunbirds were low (0.05 mmol h^{-1} in fed birds) in spite of plasma glucose concentrations similar to those of hummingbirds (Beuchat and Chong, 1998). Although no published values for GFR in hummingbirds are available, we can make a rough estimate based on an allometric regression derived from several other avian species. For instance, the GFR of a 3.5 g broad-tailed hummingbird should be approximately 3 ml h^{-1} (Williams et al., 1991). Assuming an average plasma glucose concentration of 35 mmol l^{-1} in fed hummingbirds (based on measurements in three species, Beuchat and Chong, 1998), the predicted glucose filtered load would be 0.11 mmol h^{-1}, or more than twice that of the larger sunbird. The glucose filtered load that must be recovered by the kidneys of Palestine sunbirds is 3.4 times lower than that estimated for broad-tailed hummingbirds when corrected to metabolic body mass (2.26 vs. 7.64 mmol h^{-1} $kg^{-0.75}$, respectively).

Although excreta and urine concentrations of other metabolites (e.g. amino acids) and electrolytes were not measured in this study, the above argument may be applied to them as well. The ability of sunbirds to modulate their absorbed water load may therefore resolve the potential conflicts between eliminating excess water and metabolic by-products while retaining electrolytes, metabolites and energy (Yokota et al., 1985).

Palestine sunbirds rely on the integrated function of two organ systems to maintain water balance in spite of highly variable and often extremely high water intake rates: 1) fractional absorption of dietary water is modulated in the gastrointestinal tract (McWhorter et al., submitted); and 2) FWR is modulated in the kidney. GFR in sunbirds appears to be relatively insensitive to water loading. Goldstein and Bradshaw (1998) likewise concluded that changes in urine flow rate in nectar-feeding red wattlebirds were more closely related to modulation of renal FWR than to changes in GFR. The correlation between GFR and water intake rate at 15 °C but not at 30 °C suggests that GFR in sunbirds is more sensitive to water loading at low ambient temperatures (Fig. 3). Estimated water load (absorbed plus metabolic water) was higher at 15 °C, so this is not surprising, however the significantly higher mean GFR at 30 °C is perplexing. It is possible that evaporative water loss was higher at 15 °C because of increased metabolic

demands (Powers, 1992; Williams, 1996) and thus that GFR was modulated in response to water deficit when birds were feeding on concentrated sucrose solutions (Williams et al., 1991). The observed decrease in ureteral urine osmotic concentration with increasing water intake (Fig. 5B) supports our contention that modulation of renal FWR, rather than of GFR determines renal water elimination in sunbirds. The low osmotic glucose concentrations of excreted fluid relative to ureteral urine (Fig. 5) support the idea that sunbirds are relying on modulation of ingested water absorption in their gastrointestinal tract to reduce renal water loads. Sunbirds and hummingbirds lose exceptionally small amounts of glucose and electrolytes in excreted fluid (McWhorter and Martínez del Rio, 2000 and C. Martínez del Rio and C. Lotz unpublished data). We posit that the metabolic cost of recovering filtered metabolites, and the potential for these processes to limit energy intake, are much lower in sunbirds than in hummingbirds (see also Nicolson and Fleming, in press).

Acknowledgements

We are grateful to Mariela Leiderman for expert assistance with experiments. Catherine Hambly generously provided access to unpublished data. This research was supported by Grant No. 98-178 from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel to CMR and BP and a NSF grant (IBN-0110416) to CMR. This is publication XXX of the Marco and Louise Mitrani Department of Desert Ecology.

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Legends

Fig. 1. Palestine sunbirds reduced their food intake rates in response to increased sucrose concentration in food. Energy intake therefore remained relatively constant at a level that appeared to be dictated by ambient temperature, and hence by thermoregulatory demands. (A) Sucrose intake by sunbirds was not significantly correlated with sucrose concentration in food, despite food intake rates that varied 7.2 to 9.5 fold (for 30 and 15 °C, respectively) between the lowest and the highest sucrose concentrations. Sucrose intake was 1.6 times greater at 15 °C (unfilled circles) than at 30 °C (filled circles), averaging 119.45 ± 5.08 mg h^{-1} (27.76 ± 1.18 kJ d^{-1}) for 15 °C and 75.28 ± 5.17 mg h^{-1} (17.49 ± 1.2 kJ d^{-1}) for 30 °C. (B) Sunbirds consumed significantly less food as dietary sucrose concentration increased. Food intake rate was significantly higher at 15 than at 30 °C. We described the relationship between food intake and sucrose concentration using a power function for each temperature separately ($y = 313.24x^{-1.11}$, $r^2 = 0.95$ and $y = 224.63x^{-0.93}$, $r^2 = 0.87$ for 15 and 30 °C, respectively). The exponents of these relationships were not significantly different from -1. When feeding on the most dilute sucrose solution (0.146 mol l^{-1}), sunbirds consumed between 4 and 6 times their body mass in food in 14 h of daylight, depending on ambient temperature. Note that both axes in panel B and the x-axis of panel A are logarithmic scales.

Fig. 2. The relationships between the specific activity of ^{14}C inulin in excreta (disintegrations min^{-1} μl^{-1}) and time were well described by exponential functions (r^2 ranged from 0.61 to 0.99, $n = 37$). The decline in the specific activity of ^{14}C inulin in excreta with time therefore apparently followed one-compartment, first-order kinetics. Data are shown here for three individuals and were semi-log_e transformed for clarity. Analysis was performed on untransformed data (Motulsky and Ransnas, 1987).

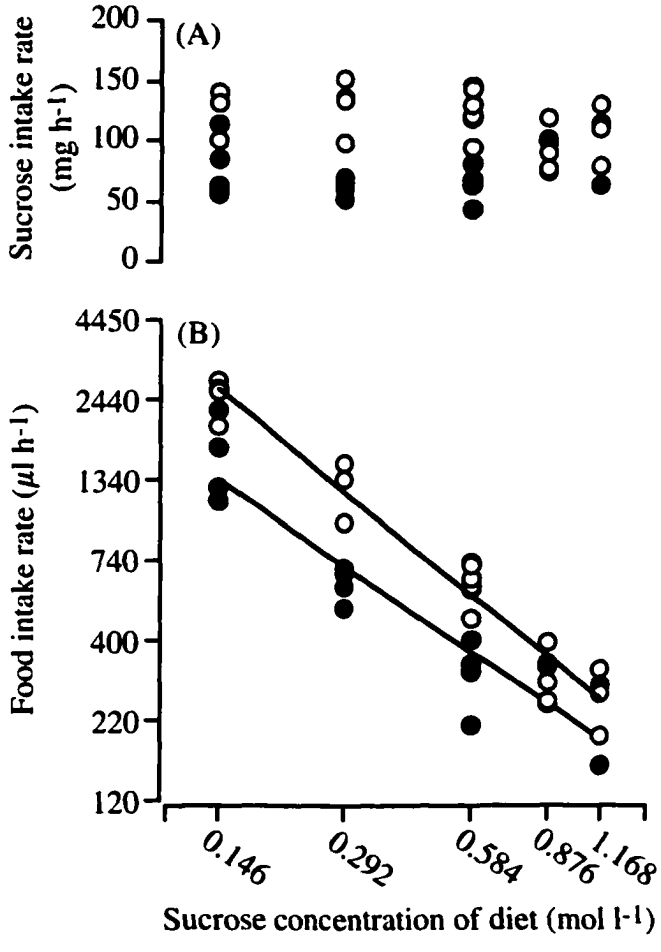
Fig. 3. Glomerular filtration rate (GFR) as a function of rate of water intake and ambient temperature in Palestine sunbirds. GFR ranged from 576.19 to 5203.47 μl h^{-1} and was correlated with water intake rate at 15 °C (unfilled circles; $y = 0.58x + 869.72$, $r^2 = 0.53$), but not at 30 °C (filled circles). Mean GFR was significantly higher at the higher temperature (1434.67 ± 153.19 vs. 1991.85 ± 253.28 μl h^{-1} for 15 and 30 °C, respectively).

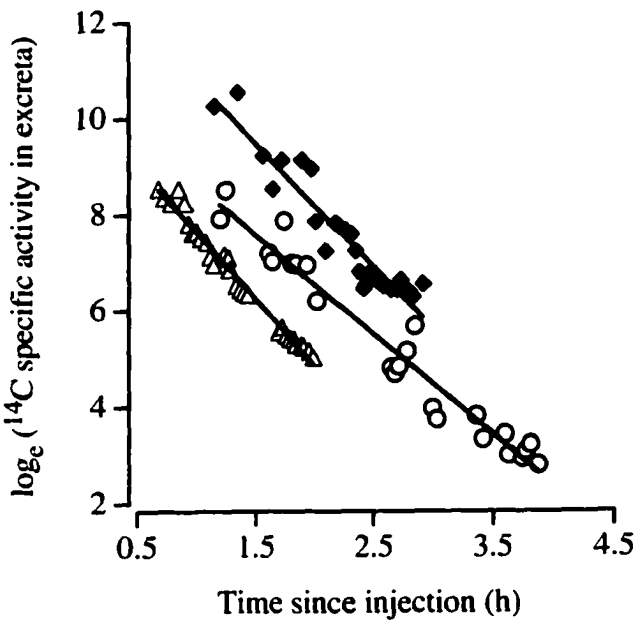
Fig. 4. Fractional water reabsorption (FWR) in the kidney ranged from 0.98 to 0.64 (0.82 ± 0.02, n = 29) and decreased significantly with water intake rate as predicted ($y = -1.6 \cdot 10^{-4}x + 0.91$, $r^2 = 0.34$). There was no significant effect of ambient temperature (15 °C unfilled circles, 30 °C filled circles) on FWR as a function of water intake rate.

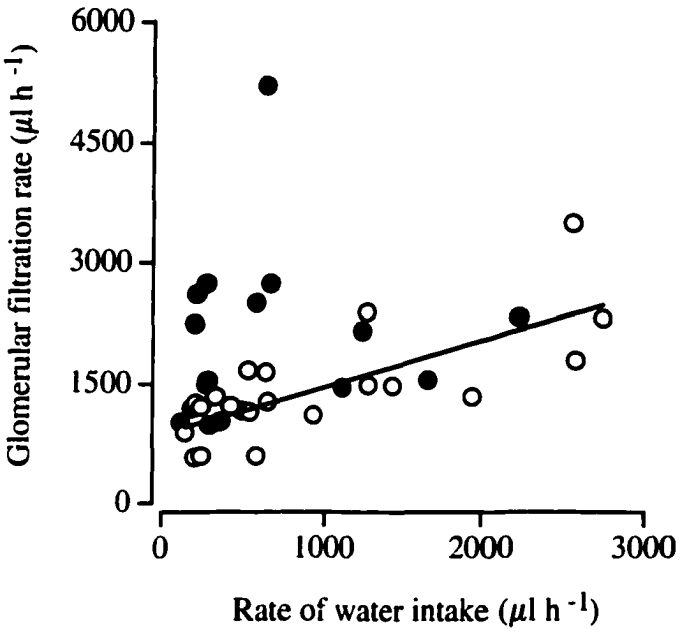
Fig. 5. Glucose and osmotic concentrations in excreted fluid and ureteral urine of Palestine sunbirds varied with rate of water intake. (A) Glucose concentration declined significantly with increasing water intake rate, and was significantly higher in ureteral urine (unfilled squares) than in excreted fluid (filled diamonds). Glucose concentration was not significantly correlated with water intake rate when ureteral urine data were considered separately, probably because of small sample size, particularly at higher rates of water intake. The relationship between glucose concentration in excreted fluid and rate of water intake was adequately described by a power function ($y = 26.18x^{-0.62}$, $r^2 = 0.4$, n = 31). Glucose concentration in ureteral urine ranged from 0.28 to 10.39 mmol l^{-1} (2.97 ± 1.05, n = 11), and that in excreted fluid ranged from 0.12 to 3.52 mmol l^{-1} (0.6 ± 0.12, n = 31). (B) Osmotic concentration declined significantly with increasing water intake rate, and was significantly greater in ureteral urine than in excreted fluid.

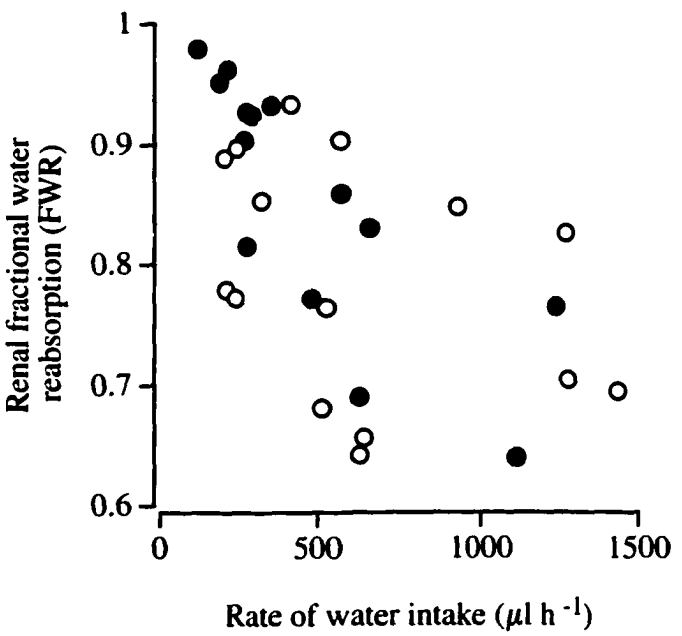
We described the relationship between osmotic concentration and water intake rate using separate power functions for ureteral urine and excreted fluid ($y = 18045.61x^{-0.82}$, $r^2 = 0.49$, $n = 13$, and $y = 1101.14x^{-0.57}$, $r^2 = 0.65$, $n = 32$, respectively). Osmotic concentration of ureteral urine ranged from 14.96 to 329 mOsm [kg H_2O]$^{-1}$ (115.5 ± 25.28, $n = 13$), and that of excreted fluid ranged from 12.33 to 95 mOsm [kg H_2O]$^{-1}$ (30.82 ± 3.82, $n = 32$). Note that the scales of all axes are logarithmic.

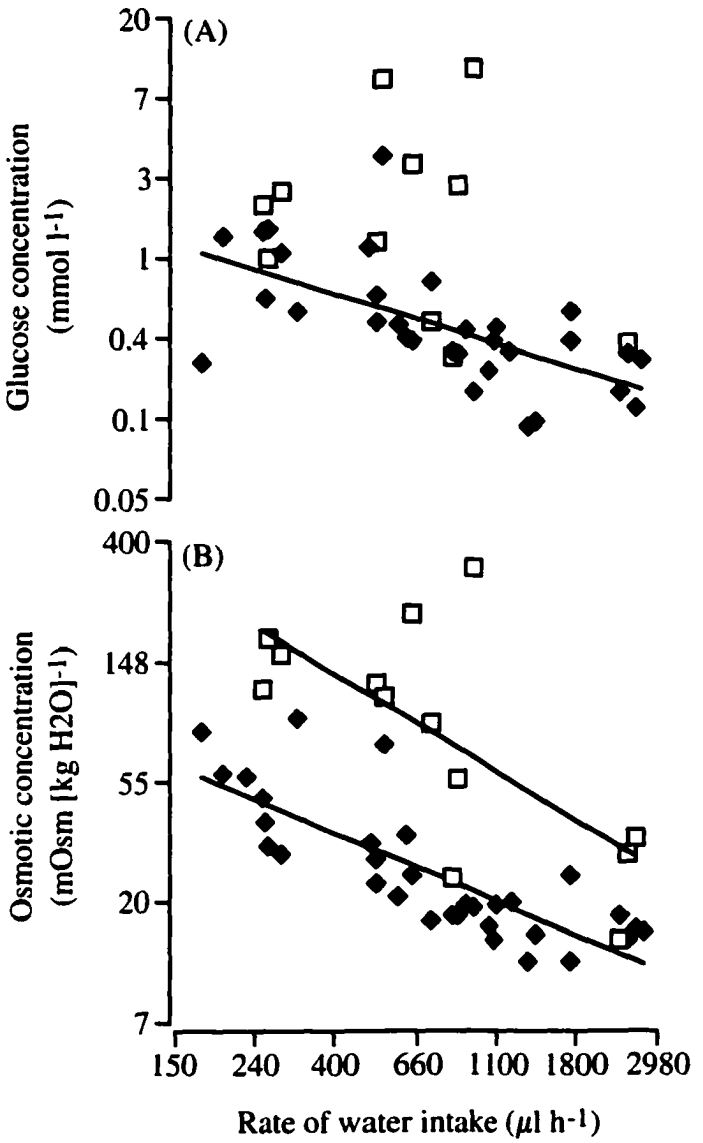
Fig. 6. Water intake rate, estimated water load (ingested water that is absorbed in the gastrointestinal tract plus metabolic water) and renal free water clearance as functions of diet sucrose concentration in Palestine sunbirds. Estimated water load increases much more slowly with decreasing diet sucrose concentration than does water intake rate, and roughly parallels renal free water clearance rate. The ability of sunbirds to modulate the absorption of preformed water in food substantially reduces the water load that must subsequently be eliminated by the kidney. Data for both temperatures were combined; see text for an explanation of how variables were estimated. No inferential statistics were performed on estimated water loads.

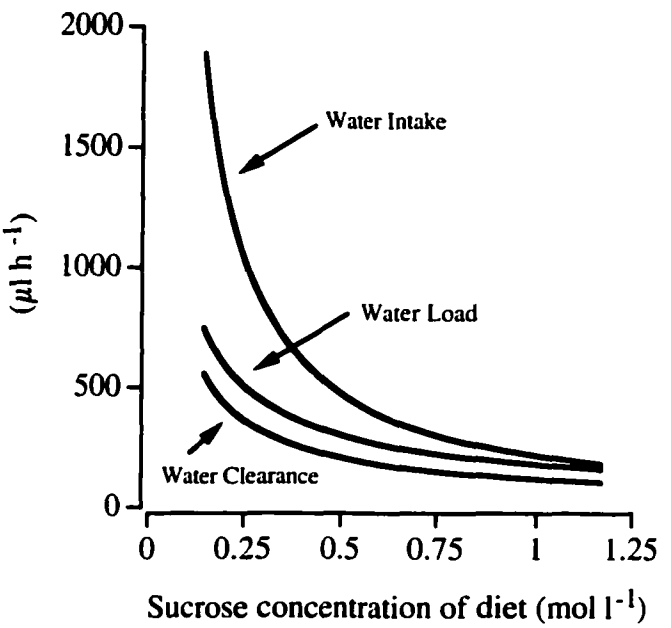

(A)
Sucrose intake rate (mg h-1)
200
150
100
50
0
(B)
Food intake rate (μl h-1)
4450
2440
1340
740
400
220
120
0.146
0.292
0.584
0.876
1.168
Sucrose concentration of diet (mol l-1)


12
10
8
6
4
2
0.5
1.5
2.5
3.5
4.5
log$_e$ (^{14}C specific activity in excreta)
Time since injection (h)


6000
4500
3000
1500
0
0
1000
2000
3000
Glomerular filtration rate (μl h -1)
Rate of water intake (μl h -1)


Renal fractional water reabsorption (FWR)
1
0.9
0.8
0.7
0.6
0
500
1000
1500
Rate of water intake (μl h -1)


(A)
Glucose concentration (mmol l-1)
20
7
3
1
0.4
0.1
0.05
(B)
Osmotic concentration (mOsm [kg H2O]-1)
400
148
55
20
7
150
240
400
660
1100
1800
2980
Rate of water intake (μl h-1)


2000
1500
1000
500
0
(µl h -1)
Water Intake
Water Load
Water Clearance
0
0.25
0.5
0.75
1
1.25
Sucrose concentration of diet (mol l-1)

APPENDIX C

DOES DIGESTIVE CAPACITY LIMIT FOOD INTAKE? AN EXPERIMENTAL TEST AND A MODEL

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Running Head: *Gut Function in Palestine Sunbirds*

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Summary

Many species of animals decrease their intake rate when the energy/nutrient density of food is increased. This intake response to food quality can be explained by two complementary processes: compensatory feeding and physiological constraint. The compensatory feeding hypothesis assumes that animals vary intake to match energy expenditures. Alternately, energy assimilation, and thus food intake, may be constrained by the physiological capacity of animals to process the nutrients contained in food. Both compensatory and constrained feeding predict a negative relationship between the energetic content of food and intake rate. When energy requirements are low animals may adopt a compensatory strategy that allows them to match intake to energy demands. As demands increase, animals may reach their digestive ceiling and hence their rate of food intake may be determined by their physiological capacity. To examine the interplay between compensatory and constrained feeding we varied both food quality and energy demands and compared the intake responses of Palestine sunbirds (*Nectarinia osea*) with the results of a model that predicts their maximal intake from measurements of intestinal hydrolytic capacity. We fed birds on sucrose solutions of varying concentration and exposed them to two ambient temperatures within their acclimated range (15 and 30°C), and acutely to one temperature well below this range (5 °C). As expected, sunbirds

decreased their food intake rates in response to sugar concentration. At 15 and 30 °C, they were able to compensate for differences in food energy density and increased metabolic demands. When exposed to a relatively sudden drop in ambient temperature and, hence, to an acute increase in thermoregulatory and food warming energy expenditures, sunbirds were unable to increase their rate of energy intake. Our simple chemical reactor model predicted observed intake rates at low concentrations (≤ 0.292 mole per liter) and low temperatures (5 and 15 °C) closely. The model indicated that under these conditions, intestinal sucrose hydrolysis rates were near maximal, and thus may have imposed limits to sugar assimilation at 5 °C. However, sunbirds exposed to 5 °C did not lose but rather gained body mass over the day. When faced with a physiological limitation to energy assimilation, they appeared to balance their energy budgets by reducing their energy expenditures. Our results suggest that nectarivorous birds may operate within slim digestive safety margins. We speculate that slim safety margins may be a trait of animals with the ability to modulate energy expenditures by reduced activity and hypothermia.

Introduction

Nectarivorous birds, including hummingbirds and sunbirds, decrease their food intake rate when the sugar concentration in nectar is experimentally increased (Collins 1981; Downs 1997; López-Calleja et al. 1997; Lotz and Nicolson 1999; Martínez del Rio et al. 2001; McWhorter and Martínez del Rio 1999; McWhorter and Martínez del Rio 2000; Nicolson and Fleming in press). This "intake response" relationship (Castle and Wunder 1995) often leads to relatively constant rates of energy assimilation. The occurrence of such an inverse relationship between energy/nutrient density and food intake rate in a wide variety of animals has often been attributed to compensatory feeding (see for example Simpson et al. 1989). According to this explanation, animals regulate food intake to maintain a constant flux of assimilated energy or nutrients, compensating for the composition of food and their metabolic demands (Montgomery and Baumgardt 1965; Slansky and Wheeler 1992). A seemingly alternate hypothesis to compensatory feeding is that food intake is constrained by the ability of animals to process the nutrients contained in food (Levey and Martínez del Rio 1999).

Martínez del Rio et al. (2001) outlined two complementary approaches that have been used to differentiate compensatory feeding from physiological constraint. The first approach involves examining the functional structure of the intake response. The

relationship between food intake rate (V) and sugar concentration in food (C) in nectar-feeding birds is best described a power function:

$$V = aC^{-b}, \qquad (1)$$

where a and b are empirically derived constants (McWhorter and Martínez del Rio 1999; McWhorter and Martínez del Rio 2000). Perfect compensatory feeding, where food intake rate is regulated to maintain constant energy intake, yields an exponent (b) equal to 1 (Martínez del Rio et al. 2001). Martínez del Rio et al. (2001) used a mathematical model and in vitro sucrose hydrolysis data to predict the maximal possible intake of nectar feeding birds. They found that this model also predicted a power function relationship relating V and C. However, the model consistently predicted an exponent (b) lower than 1. Most intake responses examined in nectarivorous birds to date have exponents that range from 0.65 to 1 (McWhorter and López-Calleja 2000). The presence of numerous examples with exponents less than 1 (reviewed by Martínez del Rio et al. 2001) and the inability of some species to maintain energy balance on very dilute diets (see Nicolson and Fleming in press) provide circumstantial evidence for physiological constraint(s) to energy assimilation.

The second approach outlined by Martínez del Rio et al. (2001) is experimental and relies on determining the effect of changing energetic demands on the intake response.

An endotherm exposed to a sudden drop in ambient temperature, for example, should increase its rate of energy assimilation to match energetic demands unless it is constrained. McWhorter and Martínez del Rio (2000) provided empirical evidence for a physiological constraint to food intake in broad-tailed hummingbirds (*Selasphorus platycercus*, Trochilidae). Other experimental studies that have exposed nectar-feeding birds to low ambient temperatures have revealed considerable interspecific variation (Beuchat et al. 1979; Gass et al. 1999; Lotz 1999; Lotz and Nicolson 1999). The main inference that can be drawn from these studies is that some species of nectarivorous birds are able to compensate for changes in nectar energy density and their metabolic demands over a much wider range than others by changing their rate of food intake (Martínez del Rio et al. 2001).

Floral nectars provide sugars to pollinators. Sugars are a readily assimilable source of fuel for the high mass-specific energy demands of nectar-feeding animals (Martínez del Rio 1990b; Martínez del Rio and Karasov 1990). Arguably nectar is among the simplest foods on earth. Bird-pollinated plants secrete relatively dilute aqueous sugar solutions containing trace amounts of amino acids and electrolytes (Baker and Baker 1990). The simplicity of nectar facilitates the modeling of its digestion and investigation of how digestion influences feeding behavior (Jumars and Martínez del Rio 1999). Nectar and

the animals that feed on it appear to be ideally suited to explore physiological limitations and the constraints they may impose on feeding. Here we examine the idea that digestive processes can impose constraints on the rate at which energy can be assimilated by nectar-feeding Palestine sunbirds (*Nectarinia osea*, Nectariniidae). We aimed to determine whether energy assimilation is physiologically constrained in sunbirds acclimated to natural ambient conditions. Our second objective was to determine how closely the physiological capacities for energy assimilation in this animals match their energetic demands (Diamond 1991; Diamond and Hammond 1992).

We employed both of the approaches outlined by Martínez del Rio et al. (2001). We examined the functional structure of the intake response and assessed the effect of acute exposure of sunbirds to low ambient temperatures on feeding rate (Martínez del Rio et al. 2001; McWhorter and Martínez del Rio 2000). We hypothesized that sunbirds would exhibit the typical intake response and compensate for differences in metabolic demands over the range of temperatures to which they were acclimated, but would be unable to do so at ambient temperatures below their acclimation range. We used in vitro measurements of intestinal sucrose hydrolysis and sucrose assimilation efficiency as inputs in a mathematical model that estimates the maximal capacity of sunbirds to assimilate sucrose. To assess how close birds were to their potential intake maximum,

we compared the results of this model with observed feeding rates (McWhorter and Martínez del Rio 2000).

Material and methods

Bird capture and maintenance

Male Palestine sunbirds (body mass 6.07 ± 0.13 g, n = 8) were captured with a drop net on the grounds of Midreshet Ben-Gurion, home of the Sede Boqer Campus of Ben-Gurion University of the Negev, Israel (30° 51' N, 34° 46' E; Israel Nature and National Parks Protection Authority permits 5981 and 7686). The birds were housed individually in outdoor aviaries (1.5 m x 1.5 m x 2.5 m) and fed a maintenance diet of two artificial nectar solutions between experiments. The diets included a 20-25 % sucrose equivalent solution and a 15 % sucrose solution supplemented with a soy protein infant formula (Isomil™, Abbott Laboratories, The Netherlands) diluted to approximately 2.5 g protein per 100 g sucrose. Food and water were available ad libitum. Birds were also offered freshly killed fruit flies (Drosophila spp.) at least twice a week. During experiments, birds were housed individually in opaque Plexiglas® cages (0.3 m x 0.3 m x 0.3 m) with individual light sources. The front of these cages was coated with a reflective Mylar™ polyester film to create a one-way mirror effect that permitted observation of birds in a

darkened room and collection of samples with minimal disturbance. One of the perches in the center of each cage was fitted to hang from an electronic balance (Scout II 200 g x 0.01 g, Ohaus Corporation, Florham Park, NJ) so body mass could be monitored continuously. Birds were allowed to habituate to cages for 2-3 d before experiments began and were left undisturbed in outdoor aviaries for a minimum of 10 days between trials. The study was conducted using light cycles that matched the natural photoperiod (13.25 to 14.5 h light). Birds were fed experimental diets, which consisted of sucrose solutions made with distilled water, for a minimum of 24 h before trials began.

Food intake as a function of sucrose concentration

We measured food intake rate in eight sunbirds fed on four different sucrose solutions (146, 292, 584, and 1168 mmol L^{-1}, two birds per diet) at three ambient temperatures (5 ± 1, 15 ± 1 and 30 ± 2 °C) in a repeated measures design. We subjected sunbirds to 30 and 15 °C because these temperatures are very close to the average maximum and minimum air temperatures (31.4 ± 0.7 and 18 ± 0.7 °C, respectively, summary data for the years 1977-1982, Zangvil and Druian 1983) in Sede Boqer for the months of July, August and September during which we conducted the study. We also subjected sunbirds to 5 °C, which is well below the most extreme nighttime low temperature during the summer

months, but which closely matches the average minimum temperature during December, January and February (5.8 ± 0.7 °C, Zangvil and Druian 1983). Palestine sunbirds are resident in Sede Boqer and thus experience significant circannual and circadian air temperature fluctuations. Daily minimum and maximum air temperatures recorded in the sunbird aviary over the course of the study were very similar to those reported by Zangvil and Druian (1983). The sugar concentrations selected span the range of energy densities naturally found in floral nectars of bird-pollinated plant species (Baker 1975; Nicolson 2002; Pyke and Waser 1981). The assignment of birds to a particular diet and the order in which birds were subjected to temperatures was randomized. In order to assess the responses of birds to acute changes in thermoregulatory energy demands, we changed the ambient temperature from 25 °C (air temperature in the constant environment room during cage habituation) to the experimental temperature at 0600 hours on the day of measurement. Temperature was stable (± 2 °C) for a minimum of one hour before intake measurements were begun at 0730 hours. Food intake rate and body mass were measured at three-hour intervals, ending at 1930 hours. Fresh excreted fluid samples were collected from each bird over a 30 minute period at 30 °C, pooled, and immediately frozen (0 °C) for later analysis. We measured the sucrose, glucose and fructose content

of these samples using clinical diagnostic kits (Catalog Nos. SCA-20, 315 and FA-20, respectively; Sigma Chemical, St. Louis, MO).

Intestinal sucrase activity measurements

After food intake measurements were completed, three sunbirds were euthanized with CO_2. The small intestines were immediately excised, flushed clean with ice-cold 1.02% saline, divided into three sections, snap frozen in liquid nitrogen, and stored at -80 °C. Intestinal sections were thawed at 5 °C and homogenized (30 s at setting 6, Model 5100 homogenizer, OMNI International, Waterbury, CT, USA) in 9 volumes of 350 mmol L^{-1} mannitol in 1 mmol L^{-1} Hepes/KOH, pH 7.5. Sucrase activity was measured according to Dahlqvist (1984) as modified by Martínez del Rio et al. (1995). Briefly, tissue homogenates (100 μL) diluted with 350 mmol L^{-1} mannitol in 1 mmol L^{-1} Hepes/KOH were incubated at 40°C with 100 μL of 56 mmol L^{-1} sucrose solution in 0.1 mol L^{-1} maleate/NaOH buffer, pH 6.5. After a 10-20 min incubation, reactions were arrested by adding 3 mL of a stop/developing Glucose-Trinder (one bottle of Glucose-Trinder 500 reagent [Sigma Chemical, St. Louis, MO] in 250 mL 1.0 mol L^{-1} TRIS/HCl, pH 7 plus 250 mL of 0.5 mol L^{-1} NaH_2PO_4/Na_2HPO_4, pH 7). After 18 minutes at 20 °C, the absorbance of the resulting solution was measured at 505 nm with a spectrophotometer

(Spectronic 20 GENESYS, Spectronic Instruments, Rochester, NY). In our preparation sucrose hydrolyses were linear even after 30 min. The apparent Michaelis constant (Km*) and pH optima for intestinal sucrase activity were 30.3 ± 3 mmol L^{-1} and 6.0, respectively. On the basis of absorbance standards constructed for glucose we calculated total intestinal hydrolytic activities and activities standardized per intestinal length and volume (Biviano et al. 1993). Intestinal volume was estimated from the average circumference of the small intestine of sunbirds, measured at 0.5 cm intervals along the length of the intestine. Because the internal diameter of the intestine in this species tapers distally, we estimated total intestinal volume as that of the sum of a series of cylinders with decreasing radius (McWhorter and Martínez del Rio 2000).

Statistical analysis

We used repeated measures analysis of variance (RM-ANOVA) to assess the significance of differences in food and sucrose intake rates, and in the mean proportions of sugars (sucrose, glucose, and fructose) found in excreted fluid. Relationships between food intake rate and sugar concentration in nectar-feeding birds are best described by power functions (López-Calleja et al. 1997; Martínez del Rio et al. 2001; McWhorter and López-Calleja 2000; McWhorter and Martínez del Rio 1999; McWhorter and Martínez

del Rio 2000; Nicolson and Fleming in press). We therefore used linear models of $\log_e$-transformed food intake and sucrose concentration data, assuming data were independent, to describe the functional relationship between these variables and thus allow comparison of empirical data with predicted food intake rates. We similarly used a linear model on untransformed data to assess the effects of energy assimilation rate and net energy gain on body mass change over the course of the experiment. All values are presented as mean ± SEM.

Results

Feeding trials

Palestine sunbirds consumed significantly less food as diet sucrose concentration increased ($F_{3, 23} = 37.96$, $p = 0.002$) and significantly more food as ambient temperature decreased ($F_{2, 23} = 6.56$, $p = 0.02$; Fig. 1). There was no significant interaction between diet and temperature ($F_{6, 23} = 2.36$, $p = 0.13$). Sunbirds' rate of food intake was significantly higher at both 5 and 15 °C than at 30 °C ($F_{1, 15} = 12.17$, $p = 0.025$ and $F_{1, 15} = 44.7$, $p = 0.003$, respectively), but was not significantly different between 5 and 15 °C ($F_{1, 15} = 0.76$, $p = 0.43$). We therefore described the relationship between food intake and sucrose concentration using a power function for 30 °C separately ($F_{1,7} = 63.35$,

$p = 0.0002$, $r^2 = 0.91$), but we combined food intake data for 5 and 15 °C ($F_{1,15} = 452.77$, $p < 0.0001$, $r^2 = 0.97$; Fig. 1). The exponent of the putative relationship for 5 and 15 °C was not significantly different from 1 (0.92 ± 0.04, $t = 1.95$, $df = 15$, $p > 0.05$), however the exponent of the relationship at 30 °C was significantly lower than 1 (0.76 ± 0.1, $t = 2.56$, $df = 15$, $p < 0.05$). When feeding on 0.146 mol L^{-1} sucrose solutions, sunbirds consumed between 3 and 6.6 times their body mass in food per day, depending on temperature.

Sucrose concentration did not have a significant effect on sucrose intake rate ($F_{3,23} = 1.83$, $p = 0.28$). However, sucrose intake increased significantly with decreasing ambient temperature ($F_{2,23} = 13.12$, $p = 0.003$; Fig. 1). There was no significant interaction between sucrose concentration and temperature ($F_{6,23} = 0.95$, $p = 0.51$). Following the pattern observed in the food intake data, sucrose intake rate was significantly higher at both 5 and 15 °C than at 30 °C ($F_{1,15} = 21.01$, $p = 0.01$ and $F_{1,15} = 44.03$, $p = 0.003$, respectively), but was not significantly different between 5 and 15 °C ($F_{1,15} = 2.26$, $p = 0.21$). Although food intake rate varied approximately 10-fold between the lowest and the highest sucrose concentrations, sunbirds maintained relatively constant rates of energy intake for a given ambient temperature. Sucrose intake averaged 134.63 ± 4.57

mg h^{-1} (31.29 ± 1.06 kJ d^{-1}) for 5 and 15 °C combined, and 105.38 ± 9.05 mg h^{-1} (24.49 ± 2.1 kJ d^{-1}) for 30 °C.

The change in body mass (Mb) of sunbirds over the course of intake measurements ranged from -4.4 to 6.9 % Mb $12h^{-1}$, but did not differ significantly with sucrose concentration in food or with ambient temperature ($F_{3,23} = 2.59$, $p = 0.19$ and $F_{2,23} = 3.19$, $p = 0.096$, respectively). There was, however, a significant positive correlation between the change in body mass and the rate of energy assimilation ($F_{1,23} = 18.27$, $p = 0.0003$, $y = 0.39x - 7.89$, $r^2 = 0.45$, all three temperature combined; Fig. 2). In order to disentangle the effects of temperature on body mass change, we then constructed a linear model for each temperature separately. There was a significant effect of energy assimilation rate on the change in body mass at both 15 and 30 °C ($F_{1,6} = 19.89$, $p = 0.004$, $r^2 = 0.77$ and $F_{1,6} = 12.51$, $p = 0.012$, $r^2 = 0.68$, respectively), but surprisingly there was no significant effect at 5 °C ($F_{1,6} = 0.37$, $p = 0.57$, $r^2 = 0.06$). Because neither the slopes or intercepts of the relationship between the change in body mass and the rate of energy assimilation differed significantly from one another between 15 and 30 °C ($ANCOVA_{slopes}$ $F_{1,12} = 0.2$, $p = 0.66$ and $ANCOVA_{intercepts}$ $F_{1,12} = 0.47$, $p = 0.5$, respectively), we estimated a common relationship for these temperatures ($F_{1,15} = 29.84$,

$p < 0.0001$, $r^2 = 0.68$; Fig. 2).

The proportions of sucrose, glucose and fructose found in sunbird excreted fluid differed significantly from each other ($F_{2,23} = 46.65$, $p < 0.0001$; Fig. 3), but did not vary with sucrose concentration in food ($F_{3,23} = -0.13$, $p > 0.05$). Fructose was the most abundant sugar (60.76 ± 7.14 % of total sugars excreted) and was present in a significantly greater proportion than either sucrose or glucose ($F_{1,15} = 53.68$, $p = 0.002$ and $F_{1,15} = 46.68$, $p = 0.002$, respectively). The proportions of sucrose and glucose present in excreted fluid were not significantly different from each other (21.71 ± 4.39 and 17.53 ± 3.5 %, respectively, $F_{1,15} = 3.75$, $p = 0.13$).

Sucrase activity measurements

Sucrase activity standardized by intestinal length declined sharply (by a factor of approximately 23) from the most proximal to the most distal section of the small intestine (Fig. 4). However, the diameter of the intestine declined from 1.64 ± 0.05 to 0.9 ± 0.05 mm from the duodenum to the intestinal junction with the cloaca. Therefore, sucrase activity standardized by the volume of intestinal contents decreased with positionby a factor of approximately 7 along the length of the intestine (Fig. 4). We calculated total

sucrase activity by summing the activity in each section, and estimated maximal sucrase activity using the Michaelis constant for our preparation. Maximal total intestinal sucrase activity (corrected to the pH optima of 6.0) equaled 12.12 ± 1.89 μmol min^{-1}.

Discussion

Palestine sunbirds inhabiting the Negev Desert in Israel are subjected to relatively large fluctuations in ambient temperature on both a seasonal and a daily basis. These nectarivorous passerine birds are year-round residents at Midreshet Ben-Gurion in Sede Boqer, where air temperatures may reach 40 °C in the summer and plummet to as low as −1 °C during winter nights (Zangvil and Druian 1983). A primary objective of this study was to determine whether energy assimilation is physiologically constrained in sunbirds acclimated to natural ambient conditions. Sunbirds exposed to 15 and 30 °C, temperatures close to the average daily minimum and maximum during the summer months when the study was conducted, increased their food intake rate as hypothesized. These birds exhibited the typical intake response (Fig. 1) and were able to compensate for differences in food energy density and increased metabolic demands with no apparent difficulty.

A second objective of this study was to determine how closely the physiological capacities of Palestine sunbirds for energy assimilation match their energetic demands (Diamond 1991; Diamond and Hammond 1992). Many endotherms increase their food ingestion rates when acclimated to low ambient temperatures (Hammond and Diamond 1997; McWilliams and Karasov 1998). These increases in food intake are frequently accompanied by changes in digestive function, including intestinal hypertrophy and increases in the expression of digestive enzymes and nutrient transporters (Konarzewski and Diamond 1994; McWilliams et al. 1999). To avoid the changes that follow chronic acclimation to low temperatures, and thus differentiate compensatory feeding from physiological constraint, we measured food intake by sunbirds subjected to 5 °C under acute conditions. When exposed to a relatively sudden drop in ambient temperature and, hence to an acute increase in thermoregulatory and food warming energy expenditures, sunbirds were unable to increase their rate of energy intake. This failure to increase energy intake points to the existence of physiological limitations to the rate at which sunbirds can ingest food. However, sunbirds faced with an acute increase in energy demands to which they were not acclimated did not lose but rather gained body mass over the day. When faced with a physiological limitation to energy assimilation,

Palestine sunbirds appeared to balance their energy budgets by reducing the rate at which they used energy.

The picture that is emerging from this and other recent studies is that compensatory feeding and physiological constraints to food assimilation are complementary processes that shape the feeding behavior of nectar-feeding birds (Martínez del Rio et al. 2001; McWhorter and López-Calleja 2000; McWhorter and Martínez del Rio 2000). In the following paragraphs we suggest that the physiological capacities of nectarivorous birds to assimilate energy are closely matched to their energetic demands. We present the results of a mathematical model indicating that intestinal sucrose hydrolysis rates in Palestine sunbirds were operating at near-maximal levels when birds were exposed to 5° C and, thus, were probably imposing limits to the rate at which sucrose was ingested and assimilated. We then discuss the match between sucrose hydrolysis and the uptake of glucose and fructose. Finally, we consider the ecological consequences that limitations imposed by digestive function can have for these small endotherms.

Physiological constraint or compensatory feeding?

Palestine sunbirds decreased their food intake rate with increasing sugar concentration, following the pattern exhibited by many nectar-feeding birds (Collins 1981; Downs 1997; López-Calleja et al. 1997; Lotz and Nicolson 1999; Martínez del Rio et al. 2001; McWhorter and Martínez del Rio 1999; McWhorter and Martínez del Rio 2000; Nicolson and Fleming in press). The exponent of the intake response for 15 °C was not significantly different from 1, indicating that birds were compensating perfectly for variability in food energy density and their metabolic demands. Indeed, the 1.3 fold increase in energy intake observed between 30 and 15 °C closely matches the 1.5 fold increase in metabolic rate measured in Palestine sunbirds between these temperatures (C. Hambly, B. Pinshow, E.J. Harper and J.R. Speakman, unpublished data). Energy intake rate was not significantly different among diets, and there was no significant difference in the ability of birds to maintain or increase their body mass between 15 and 30 °C (Fig. 2), providing additional evidence for compensatory feeding. In spite of the fact that sunbirds were unable to increase their rate of energy intake when subjected acutely to an ambient temperature below their acclimated range (5 °C), these birds were still able to maintain or increase their body mass (Fig. 2).

How does a Palestine sunbird consuming 6 times its body mass of a dilute sucrose solution while exposed to low ambient temperatures manage to maintain or increase body mass over 12 h? Behavioral observations, albeit anecdotal, may provide the answer. Sunbirds at 5 °C exhibited behaviors commonly associated with energy conservation: they spent less time flying and hopping between perches, and exhibited ptiloerection while perching (Gass and Montgomerie 1981). The energy saved by these behavioral responses was presumably sufficient to offset increased energy demands and allow sunbirds to maintain or increase their body mass. Sunbirds balanced their energy budgets by reducing energy expenditures when a physiological constraint prevented them from increasing their rate of energy intake.

Does intestinal capacity limit food intake? A model

The presence of characteristic intake response curves in Palestine sunbirds eliminated two possible limitations to food intake: food harvesting rate and water processing by the kidney (Beuchat et al. 1990). Here we use harvesting rate in the limited sense of ingesting food without processing it in the gut (McWhorter and Martínez del Rio 2000). Food harvesting is potentially limited by the rate at which food can be licked by sunbirds and depends on the viscosity of the sugar solution (see Gass and Roberts 1992 and

references therein). Water processing by the kidney may be limited by the rate at which filtered glucose, metabolites and electrolytes can be recovered (Yokota et al. 1985). Palestine sunbirds are able to modulate the fractional absorption of ingested dietary water, however, and thus they may avoid a substantial renal water load when feeding on dilute nectars (McWhorter et al. submitted). Although food intake rate did not increase significantly between 15 and 5 °C, it increased nearly 10-fold from the highest to lowest sugar concentration. Clearly, at sucrose concentrations higher than 146 mmol L^{-1}, sunbirds were not limited by food harvesting or water processing rates. Water processing by the kidney may limit food intake in sunbirds feeding on extremely dilute sugar solutions, however.

Most of the energy ingested by nectar-feeding birds comes from sugar. Thus, the physiological processes that determine the rate at which ingested sugar is assimilated and metabolized are good candidates for factors limiting food intake. Simple mathematical models of gut function have proven useful for understanding the interaction between energy balance, physiological capacities for sugar assimilation and feeding behavior in nectar-feeding birds (Martínez del Rio et al. 2001; McWhorter and Martínez del Rio 2000). McWhorter and Martínez del Rio (2000) predicted food intake capacity in broad-

tailed hummingbirds based on in vitro measurements of sucrose hydrolysis rates and gut morphology. Their method models the intestine of nectar-feeding birds functions as a plug-flow chemical reactor (Penry and Jumars 1987). The model makes two assumptions: 1) digesta flows unidirectionally (Jumars and Martínez del Rio 1999), and 2) the rate at which sucrose is hydrolyzed in the intestine ($-r_s$) follows simple Michaelis-Menten kinetics:

$$-r_S = S_{max}C_s(K_m + C_s)^{-1}, \qquad (2)$$

where S_{max} equals the rate of hydrolysis along the intestine (μmol min^{-1} μL^{-1}), K_m is the Michaelis constant of sucrase (μmol μL^{-1}), the membrane-bound disaccharidase which hydrolyzes sucrose to glucose and fructose (Martínez del Rio 1990a), and C_s is the concentration of sucrose (μmol μL^{-1}) down the intestine or with time (Jumars and Martínez del Rio 1999). Equation (2) can be integrated to yield the throughput time (τ) required to reduce the initial sucrose concentration (C_{s0}) to a given final value (C_{sf}):

$$\tau = (S_{max})^{-1}[K_m \ln(C_{s0}C_{sf}^{-1}) + (C_{s0} - C_{sf})]. \qquad (3)$$

In plug flow reactors if one knows τ and the volume of gut contents (G in μL), intake rate ($\dot{v}_0$ in μL min^{-1}) can be estimated as:

$$\dot{v}_0 = G\tau^{-1}. \qquad (4)$$

We predicted the food intake capacity of Palestine sunbirds over a range of experimental sucrose concentrations using McWhorter and Martínez del Rio's (2000) model. The parameter values used in the model were: S_{max} averaged along the length of the intestine (0.135 μmol min^{-1} μL^{-1}), K_m (0.0303 μmol μL^{-1}), and G (89.8 μL). Because we found that approximately 99.9% of sucrose was hydrolyzed, we assumed that C_{sf} was equal to 0.001 C_{s0} (Martínez del Rio et al. 2001; McWhorter and Martínez del Rio 2000; McWhorter et al. submitted). Figure 1 compares the food and sucrose intake capacities predicted by the model with actual intake data at four dietary sucrose concentrations. Both the model's output and intake data were well described by power functions. However, the exponent of the predicted intake response (0.75) was significantly lower than that of the observed intake response (0.92 ± 0.04, $t = 3.95$, $p < 0.05$, $n = 4$), suggesting that at 15 °C birds were exhibiting compensatory feeding. The intake rates predicted by the model are remarkably close to actual intake data for 146 and 292 mmol L^{-1} sucrose (Fig. 1), so it is not surprising that birds were unable to increase their rate of food intake at 5 °C.

Martínez del Rio et al (2001) predicted a "broken" intake response for animals experiencing high energy demands. In this scenario, intake rate follows constrained feeding at low concentrations and compensatory feeding at high concentrations (see Figure 3 in Martínez del Rio et al. 2001). Our results hint at such an outcome (Fig. 1). At low concentrations (≤ 0.292 mol/L) and temperatures (5 and 15°C) the observed intake was very similar to that predicted by the model, whereas at higher concentrations and temperatures, the observed values were lower than those predicted. Detecting a change in slope in a biphasic linear relationship demands larger samples than those in our experiments (Neter et al. 1996), yet our results are consistent with the possibility of the broken intake response predicted by Martínez del Rio et al. (2001).

Glucose and fructose uptake by Palestine sunbirds

Near complete assimilation of nectar constituents seems to be characteristic of nectar-feeding birds (Hainsworth 1974; Jackson et al. 1998; Karasov et al. 1986; Martínez del Rio 1990b). Our data, however, revealed subtle differences in the assimilation of sugars. Fructose was present in excreted fluid at approximately 3 times the level of sucrose or glucose. The differences in the uptake efficiency of glucose and fructose in Palestine sunbirds may be the result of differences in their mechanisms of intestinal transport.

Glucose is transported across the luminal membrane of enterocytes by a Na^+-dependent active transporter (SGLT1, Pajor and Wright 1992), whereas fructose is transported by a distinct facilitated transporter (GLUT5, Rand et al. 1993). As in humans, the rate of intestinal transport of fructose appears to be lower in Palestine sunbirds than that of glucose (Gitzelmann et al. 1989; Holdsworth and Dawson 1964). Lower fructose uptake rates may be the result of lower GLUT5 densities or turnover constants relative to SGLT1. Because fructose transport in birds has not been researched in detail, however, this apparent difference in transport rates cannot be explained.

Glucose was present in the excreted fluid of Palestine sunbirds at approximately the same level as sucrose. This result indicates that sucrose hydrolysis and the ability of sunbird intestines to absorb the resulting glucose are closely matched. Indeed, Weiss et al. (1998) reported that glucose transport and sucrase activity remained approximately matched to each other in mice (*Mus musculus*). They concluded that neither sucrase nor the glucose transporter was the rate-limiting step for sucrose digestion but that both steps were equally limiting (see also Hammond and Diamond 1997). The same would seem to apply to Palestine sunbirds, although our conclusion must be tempered by the fact that what is known about glucose and fructose transporter physiology is from studies of

mammals (Gitzelmann et al. 1989; Holdsworth and Dawson 1964; Pajor and Wright 1992; Rand et al. 1993; Weiss et al. 1998).

Is a small digestive spare capacity a trait of animals that can modulate energy costs? Taken together, the patterns of food intake exhibited by Palestine sunbirds exposed to low ambient temperatures and the modeling of their digestive capacities provide compelling evidence for a physiological constraint to food intake. Our results suggest that Palestine sunbirds, like the hummingbirds studied by McWhorter and Martínez del Rio (2000) and Martínez del Rio et al. (2001), operate with small digestive spare capacities. Safety factors for sunbirds feeding at 15° C were modest and ranged from 1.05 to 1.5 from the lowest (146 mmol L^{-1}) to the highest (1168 mmol L^{-1}) sucrose concentration (Fig. 1). At 30° C, safety factors were slightly larger, ranging from 1.62 to 1.66. The relatively modest safety factors that are typically found in nectar feeding birds are noteworthy.

Rapid-exposure experiments, such as the one described here and by McWhorter and Martínez del Rio (2000) are informative because they reveal the immediate or short-term digestive spare capacity of an animal. They allow probing the relative importance of

behavioral changes and physiological mechanisms for balancing the energy budgets of small endotherms over short time scales. Hummingbirds, for example, are known to employ several behavioral strategies and physiological mechanisms, such as adjusting their territory size (Carpenter et al. 1983) and using nocturnal torpor (Carpenter and Hixon 1988), to balance their energy budgets or maximize net energy gain during premigratory fattening. Faced with a digestive constraint to energy assimilation, captive Palestine sunbirds were apparently able to reduce their energy expenditures enough to maintain or increase their body mass. Anecdotal observations discussed previously suggest that behavioral changes played an important role in energy conservation.

The use of nocturnal hypothermia or torpor by sunbirds to reduce thermoregulatory energy expenditures is poorly understood. Belle Leon (unpublished data, cited in Lovegrove 1993) documented a 50% drop in the metabolic rate and a 5 °C drop in the overnight body temperature in lesser double-collared sunbirds (*Nectarinia chalybea*), although passerine birds are not generally thought to use torpor. We did not measure body temperature or metabolic expenditures concurrently with food intake in this study. Such measurements, using standard, readily available techniques (i.e., respirometry or doubly-labeled water), would be very useful in determining the relative contributions of

behavior and physiology in balancing the energy budgets of sunbirds subjected to acute increases in energetic demands.

We emphasize that our results inform the responses of small nectar-feeding birds to acute changes in energy demands. It may be that chronic, long-term, cold exposure leads to up-regulation of digestive capacity. Many animals increase the size of gastrointestinal tract and/or the activity of the enzymes and transporters in it when challenged with a chronic increase in energy expenditures (Dykstra and Karasov 1992; Karasov 1996; Karasov and Hume 1997; McWilliams et al. 1999). Curiously, the only study that we are aware of in which a small nectar-feeding bird was exposed chronically to low temperatures revealed a very different pattern. López-Calleja (1998) exposed green-backed firecrown hummingbirds (*Sepahnoides sephanoides*) to 5 and 30 °C for 16 days. Cold-acclimated birds did not increase food intake over this period, and López-Calleja (1998) found no significant differences in intake between cold and warm-acclimated birds fed on sugar solutions with the same concentration. The only difference between cold and warm acclimated birds was that the former entered torpor more often and maintained lower body masses than the latter. Unlike many other animals that increase digestive capacity in response to increased energy demands, hummingbirds decreased

expenditures. We speculate that animals with the capacity to modulate energy expenditures with either torpor, hypothermia, or reduced activity can have low safety margins in the physiological traits that mediate food acquisition both in the short and the long term. Rather than increase the capacity to process foods in the face of increased demands, hummingbirds and sunbirds can reduce their energy output.

Acknowledgements

We are grateful to Mariela Leiderman and Lizanne Roxburgh for expert assistance with experiments. Catherine Hambly generously provided access to unpublished data. The critical comments of Alex Badyaev and Jorge Schondube allowed us to greatly improve upon previous versions of this paper. Research was supported by Grant No. 98-178 from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel to CMR and BP and a NSF grant (IBN-0110416) to CMR. This is publication XXX of the Marco and Louise Mitrani Department of Desert Ecology.

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Legends

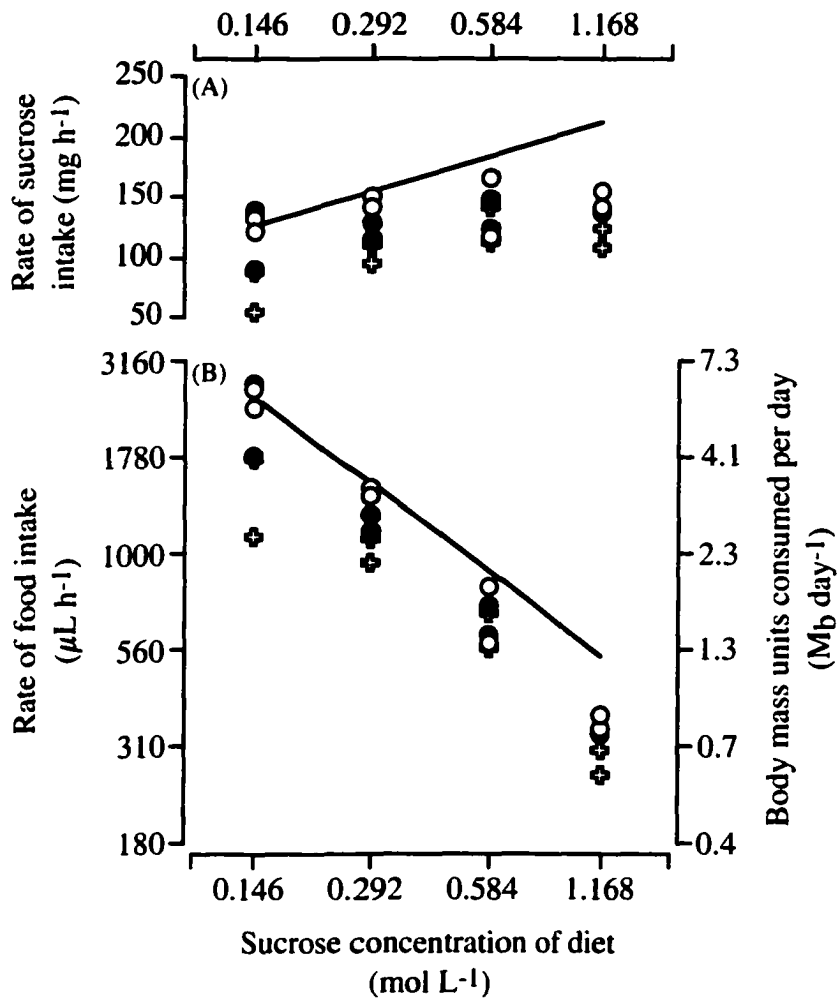
Fig. 1. Palestine sunbirds reduced their food intake rates in response to increased sucrose concentration in food. In spite of a 10-fold variation in food intake rate (panel B), sucrose intake did not vary with with sucrose concentration in food (panel A). Both sucrose and food intake rates were significantly higher at both 5 °C (unfilled circles) and 15 °C (filled circles) than at 30 °C (unfilled crosses), but were not significantly different between 5 and 15 °C. The relationship between food intake and sucrose concentration was well described by power functions ($y = 370.11x^{-0.76}$, $r^2 = 0.91$ at 30 °C and $y = 419.55x^{-0.92}$, $r^2 = 0.97$ at 5 and 15 °C). Because there were no significant differences in intake between 5 and 15 °C, we fitted a common relationship. The right hand y-axis in panel B shows food intake rate in multiples of body mass (Mb) per day. The heavy lines in both panels represent food and sugar intake rates predicted by a model of gut function. Both curves are power functions ($y = 611.84x^{-0.75}$ and $y = 209.43x^{0.26}$, for food and sucrose intake, respectively). Note that all axes in panel B and the x-axis of panel A are logarithmic scales.

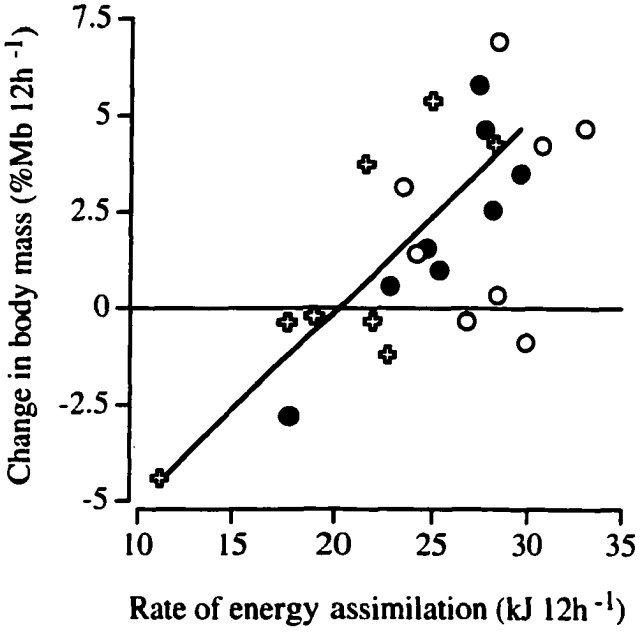
Fig. 2. Sunbirds gained more body mass when they were able to assimilate more energy. The change in body mass of sunbirds over the course of intake measurements ranged

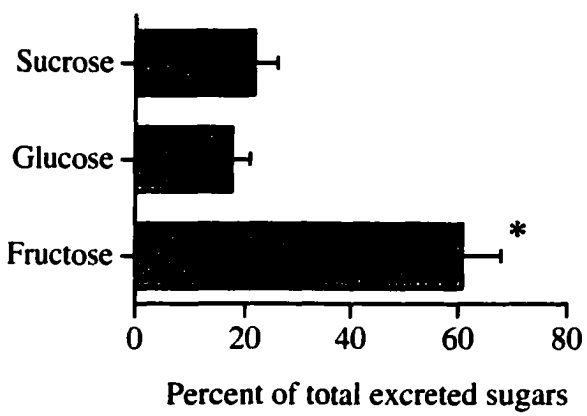
from -4.4 to 6.9 % Mb $12h^{-1}$ and was significantly correlated with the rate of energy assimilation at both 15 and 30 °C (filled circles and unfilled crosses, respectively). Because there were no significant differences in the slope or intercept of this relationship between 15 and 30 °C, we fitted a common relationship for these temperatures ($y = 0.5x - 10.08$, $r^2 = 0.68$).

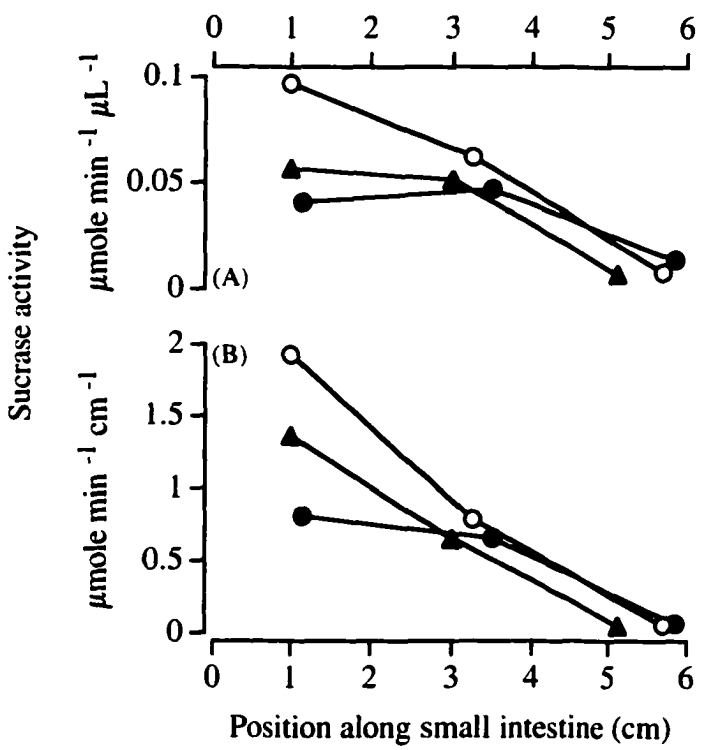
Fig. 3. The proportions of sucrose, glucose and fructose found in sunbird excreted fluid differed significantly from each other, but did not vary with sucrose concentration in food. Fructose was the most abundant sugar (60.76 ± 7.14 % of total sugars excreted) and was approximately 3 times more abundant in excreted fluid than either sucrose or glucose.

Fig. 4. Distribution of sucrase activity along the length of the small intestine of three Palestine sunbirds. Activity was standardized by volume of digesta (A) and intestinal length (B) in each intestinal section. Activity was measured at pH 6.5 and at a sucrose concentration equal to 28 mmol L^{-1}.


0.146
0.292
0.584
1.168
250
200
150
100
50
(A)
Rate of sucrose intake (mg h-1)
3160
1780
1000
560
310
180
(B)
Rate of food intake (µL h-1)
7.3
4.1
2.3
1.3
0.7
0.4
Body mass units consumed per day (Mb day-1)
0.146
0.292
0.584
1.168
Sucrose concentration of diet (mol L-1)


7.5
5
2.5
0
-2.5
-5
10
15
20
25
30
35
Change in body mass (%Mb 12h -1)
Rate of energy assimilation (kJ 12h -1)


Sucrose
Glucose
Fructose
*
0
20
40
60
80
Percent of total excreted sugars


Sucrase activity
μmole min -1 μL -1
0.1
0.05
0
(A)
μmole min -1 cm -1
2
1.5
1
0.5
0
(B)
0 1 2 3 4 5 6
0 1 2 3 4 5 6
Position along small intestine (cm)

APPENDIX D

ARE HUMMINGBIRDS FACULTATIVELY AMMONOTELIC? NITROGEN EXCRETION AND REQUIREMENTS AS A FUNCTION OF BODY SIZE

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Running Head: **Hummingbird Nitrogen Excretion and Requirements**

For submission to: *Physiological and Biochemical Zoology*

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Summary

Most birds are uricotelic. An exception to this rule may be nectar-feeding birds, which excrete significant amounts of ammonia under certain conditions. Although ammonia is toxic, because it is highly water soluble its excretion may be facilitated in animals that ingest and excrete large amounts of water. Bird pollinated plants secrete carbohydrate- and water-rich floral nectars that contain exceedingly little protein. Thus, nectar-feeding birds are faced with the dual challenge of meeting nitrogen requirements while disposing of large amounts of water. The peculiar diet of nectar-feeding birds suggests two hypotheses: 1) these birds must have low protein requirements, and 2) when ingesting large quantities of water their primary nitrogen excretion product may be ammonia. To test these hypotheses, we measured maintenance nitrogen requirements (MNR) and total endogenous nitrogen losses (TENL) in three hummingbird species (*Archilochus alexandri*, *Eugenes fulgens* and *Lampornis clemenciae*) fed on diets with varying sugar, protein, and water content. We also quantified the form in which the by-products of nitrogen metabolism were excreted. The MNR and TENL of the hummingbirds examined were exceptionally low. However, no birds excreted more than 50% of nitrogen as ammonia, or more nitrogen as ammonia than urates. Furthermore, ammonia excretion was not influenced by either water or protein intake. The smallest species (*A.*

alexandri) excreted a significantly greater proportion (> 25 %) of their nitrogenous wastes as ammonia than the larger hummingbirds (≈ 4 %). Our results support the hypothesis that nectar-feeding birds have low protein requirements, but cast doubt on the notion that they are facultatively ammonotelic. Our data also hint at a possible size-dependent dichotomy in hummingbirds, with higher ammonia excretion in smaller species. Differences in proportionate water loads and/or post-renal modification of urine may explain this dichotomy.

Introduction

Birds are generally believed to be uricotelic under all circumstances (e.g. Schmidt-Nielsen 1990; Goldstein and Skadhauge 2000). Uricotelic animals are those that excrete more than 50% of their nitrogenous wastes as uric acid and its salts (which we will hereafter refer to as urates). Urates are relatively non-toxic, have low solubility in water and can be held in colloid solutions with proteins at very high concentrations (Janes and Braun 1997). Although urates are energetically expensive to synthesize, they may be favored as nitrogenous waste products in desiccating environments and in animals with cleidoic eggs because they can be excreted with little water (Willmer et al. 2000). Birds are terrestrial and often need to save water, but some bird species can ingest and excrete prodigious volumes of water. Hummingbirds consuming dilute nectars, for example, can ingest up to 6 times their body mass per day and can show water fluxes similar to those of amphibians and freshwater fish (Beuchat et al. 1990; McWhorter and Martínez del Rio 1999; Martínez del Rio et al. 2001). Until relatively recently nitrogen excretion had not been examined in birds with high rates of water flux.

Preest and Beuchat (1997) exposed Anna's hummingbirds (*Calypte anna*) to low air temperatures (10 °C). Birds increased their rates of food and water intake when faced

with increased metabolic (i.e. thermoregulatory) demands. Surprisingly, about half of the birds exposed to 10 °C became ammonotelic (Preest and Beuchat 1997). These results are exceptionally significant because: 1) ammonia excretion is believed to occur only as a byproduct of the regulation of acid-base balance in birds (e.g. King and Goldstein 1985), and 2) they suggest that hummingbirds are "facultatively" ammonotelic. Facultatively ammonotelic animals excrete primarily urates, but switch to ammonia under certain conditions (McNab 2002 and references therein). Like uric acid and urea, ammonia is a by-product of amino acid metabolism. Unlike uric acid and urea, however, ammonia does not require additional energy to be synthesized, but it is highly toxic and highly soluble in water (Wright 1995). Preest and Beuchat (1997) speculated that hummingbirds could reduce the metabolic cost of nitrogen excretion by excreting primarily ammonia under conditions of high water flux. Facultative ammonotely can also be advantageous because it reduces the potential loss of proteins and cations associated with urate excretion (McNabb et al. 1973; Laverty and Wideman 1989; Dawson et al. 1991; Janes and Braun 1997). When nectar-feeding birds are water limited, either by high temperatures or highly concentrated floral nectars, they could shift back to uricotely (Calder and Hiebert 1983; Preest and Beuchat 1997).

Roxburgh and Pinshow (2002) examined the effects of water, electrolyte and protein intake and ambient temperature on nitrogen excretion by nectarivorous Palestine sunbirds (*Nectarinia osea*). They found that the proportion ammonia in ureteral urine and excreted fluid was independent of water and salt ingestion, but decreased in excreted fluid with increased protein intake. They hypothesized that the ammonotely observed in Palestine sunbirds was "apparent", simply the result of decreasing urate excretion in animals feeding on low protein diets. Therefore, currently available data leaves the question of ammonotely in nectar-feeding birds somewhat unanswered. Is facultative, true ammonotely found in hummingbirds but not in sunbirds? Roxburgh and Pinshow's (2002) study makes a significant point: protein intake may affect the form in which nitrogen is excreted by birds. Nectar feeding birds are unusual because they can ingest large amounts of water, and also because they appear to have low protein intakes and requirements.

Floral nectar diets present animals with a unique set of physiological challenges. Bird pollinated plants secrete carbohydrate rich nectar with low nitrogen content (Baker and Baker 1982; Gottsberger et al. 1984). Under many circumstances, nectar-feeding birds must consume large volumes of excess water to meet their metabolic demands (Beuchat

et al. 1990; Lotz and Nicolson 1999; McWhorter and Martínez del Rio 1999; Martínez del Rio et al. 2001). The mechanics of carbohydrate digestion and gut function, and how these processes affect feeding behavior, are relatively well understood in nectar-feeding birds (Karasov et al. 1986; Martínez del Rio 1990; McWhorter and Martínez del Rio 1999, 2000; Martínez del Rio et al. 2001). Until recently (Roxburgh and Pinshow 2000; van Tets and Nicolson 2000), the nitrogen requirements of nectarivorous birds received little attention aside from the pioneering studies of Paton (1982) and Brice and Grau (Brice and Grau 1989; Brice and Grau 1991; Brice 1992). The low protein and amino acid levels in floral nectars are believed to be insufficient to meet the nutritional needs of nectarivores (Baker 1977; Baker and Baker 1977; Brice and Grau 1991; Law 1992; Martínez del Rio 1994; Roxburgh and Pinshow 2000; van Tets and Nicolson 2000), although nectar-feeding birds also consume arthropods (Wagner 1946; Paton 1982; Brice and Grau 1991; Brice 1992; van Tets and Nicolson 2000). Because they appear to obtain the majority of their energy from floral nectars, however, the protein requirements of these animals are predicted to be extremely low.

Nitrogen requirements have been measured in a handful of representatives of each of the three major radiations of nectar-feeding birds. To date, one species of honeyeater

(Meliphagidae, Paton 1982), one species of hummingbird (Trochilidae, Brice and Grau 1991), and two species of sunbirds (Nectariniidae, Roxburgh and Pinshow 2000; van Tets and Nicolson 2000) have been examined. The nitrogen requirements and endogenous nitrogen losses of nectar-feeding birds appear to be only a fraction of the value predicted for birds based on body mass (Robbins 1993; Roxburgh and Pinshow 2000). This result is not unexpected on both evolutionary and physiological grounds. There is an evolutionary necessity to minimize nitrogen loss when specializing on low nitrogen foods, and a liquid, fiber- and lipid-free nectar diet may reduce fecal nitrogen losses (Robbins 1993).

In this study we attempted to integrate the insights of Preest and Beuchat (1997) with those of Roxburgh and Pinshow (2002). We manipulated both water and protein intake in captive hummingbirds to test two complementary hypotheses: 1) hummingbirds have low protein requirements, and 2) when ingesting high water loads they increase the fraction of total nitrogen that is excreted as ammonia. We tested our hypotheses under natural ambient conditions using captive individuals of three hummingbird species that are locally sympatric in southeastern Arizona: the magnificent hummingbird (*Eugenes fulgens* Gould), the blue-throated hummingbird (*Lampornis clemenciae* Swainson), and

the black-chinned hummingbird (*Archilochus alexandri* Bourcier and Mulsant). Our experiments took advantage of the behavioral response of nectar-feeding birds to varying energy density in food (López-Calleja et al. 1997; McWhorter and Martínez del Rio 1999; McWhorter and López-Calleja 2000; McWhorter and Martínez del Rio 2000; Martínez del Rio et al. 2001). By varying both sugar and protein concentration in food, we were able to elicit a wide range of water and nitrogen intakes while maintaining constant energy intake. We measured the maintenance nitrogen requirements (MNR) and total endogenous nitrogen losses (TENL) in these hummingbirds and also quantified the forms in which their nitrogenous wastes were excreted.

Material and methods

Adult male hummingbirds (*E. fulgens* 7.51 ± 0.45 g, n = 7; *L. clemenciae* 7.91 ± 0.41 g, n = 7; *A. alexandri* 2.66 ± 0.05 g, n = 7, mean Mb ± SD) were captured with mist nets on the grounds of the American Museum of Natural History's Southwestern Research Station in Portal, Arizona, during June and July of 1999 and housed individually in wire mesh cages (40 x 25 x 30 cm) inside an outdoor aviary. The study was conducted under natural ambient temperature and light cycles (photoperiod approximately 15L:9D, maximum temperature averaged 31.14 ± 2.56 °C, minimum temperature averaged 13.12

± 2.7 °C). Birds were allowed to acclimate to experimental cages and diets for 24 to 48 h before the experiments began. During experiments, birds were fed synthetic diets modified from Brice and Grau (1989). Diets contained only sucrose, NaCl, and caesin acid hydrolysate as a nitrogen source (Sigma Chemical, St. Louis, MO). Six different diets were used to obtain a wide range of food, and thus water and nitrogen, intake rates (Fig. 1). Sucrose concentration ranged from 0.292 to 1.168 mol L^{-1} with three nitrogen levels (0, 1.2 and 4.0 g L^{-1} caesin acid hydrolysate). NaCl concentration was held constant at 9.07 mmol L^{-1}.

Individual birds were randomly assigned to a diet, so that each species was tested on each diet at least once. At 0800h on the day of the experiment a galvanized metal pan containing 200 mL of mineral oil was placed under the cage to collect excreta (mixed urinary and fecal materials) without allowing for evaporation. Birds were then left undisturbed for 24 h. Food was available *ad libitum* and intake was measured using a small calibrated glass feeding tube placed through a hole in the wall of the experimental cage. Body mass was measured upon capture and before release, and remained constant (or increased slightly in the case of *A. alexandri*) over the experimental period. Excreta and mineral oil were collected after 24 h, put in Nalgene bottles and frozen at -20 °C for

later analysis. Birds were held for a maximum of three days (including acclimation and experimental periods) and subsequently released unharmed at the site of capture.

Excreta samples were thawed, separated from the mineral oil by centrifugation (5000 rpm for 3 min) and diluted with 1000 mL of distilled water to provide adequate volumes for analysis. One-hundred microliters (100 μL) of 10% (vol./vol.) acetic acid was added to acidify samples and thus prevent the volatilization of ammonia. The pH of samples after the addition of acid ranged from 3.75 to 5.48. Excreta and food samples were analyzed for total nitrogen content in a Carlo Erba NA 1500 elemental analyzer (CE Instruments, Milan, Italy) at the Columbia University Biosphere 2 stable isotope facility in Oracle, Arizona, USA. Liquid samples (15 μL) were pipetted into pre-cleaned tin capsules containing 10 mg of acid washed Chromasorb W® adsorbent (Costech, Valencia, CA). Benzoic acid was used as a standard for the elemental analysis. Nitrogen balance requirements and endogenous losses were determined by regression of apparent nitrogen retention (nitrogen intake minus excretion) on nitrogen intake (Smith and Green 1987; Brice and Grau 1991; Korine et al. 1996; Witmer 1998; Delorme and Thomas 1999; van Tets and Hulbert 1999; Roxburgh and Pinshow 2000) using total nitrogen data from the elemental analysis (Fig. 3).

Clinical diagnostic kits (Sigma Chemical, St. Louis, MO) were used to analyze excreta samples for uric acid (Procedure No. 685), urea (Procedure No. 535), ammonia (Procedure No. 171-UV), creatinine (Procedure No. 555), creatine (modification using Procedure No. 520 without creatine kinase substrate), and bile acids (Procedure No. 450, assuming an equal mix of cholic, deoxycholic and lithocholic acids [mean MW = 392.58 g mol^{-1}] and that each bile acid is bound to either taurine or glycine [mean MW = 100.095 g mol^{-1}, mean 14.925 % N]). Prior to analysis for uric acid content, an aliquot of each excreta sample was diluted 2:1 with a 1.0 mol L^{-1} LiOH solution to dissolve urate precipitates (Laverty and Wideman 1989; Roxburgh and Pinshow 2002). Samples were also analyzed for total soluble protein using the Bio-Rad Protein Assay Kit II (Catalog No. 500-0002, Bio-Rad Laboratories, Hercules, CA). Nitrogen excretion in each of these forms is reported as a percentage of the total excreted nitrogen measured using these biochemical assays. Osmolarity of excreta supernatant was measured using a Wescor model 5500 vapor pressure osmometer (Wescor, Logan, UT).

Statistical analysis

In order to compare the relationship of volumetric intake and food energy density among the three species, we used analysis of covariance (ANCOVA). ANCOVA was performed on log-transformed data because we found that the relationship between intake and food

energy density was best described by a power function (Martínez del Rio et al. 2001). ANCOVA was also used to probe for differences in the slopes and intercepts of nitrogen retention curves between species. Analysis of variance (ANOVA) was used to test for differences in mean nitrogen excreted as a given nitrogenous waste product between species, and least-squares linear regression was used to test for correlations between excretion of all forms of nitrogenous wastes and water or nitrogen intake. Paired t-tests were used to determine the significance of body mass changes over the experimental period. Values are reported as means ± SE, unless otherwise indicated and significance was accepted at $\alpha = 0.05$.

Results

Food intake - energy and nitrogen

Volumetric food intake (I, mL day^{-1}) by hummingbirds decreased significantly with increased sucrose concentration (C, moles L^{-1}, $ANCOVA_{concentration}$ $F_{1,17} = 47.51$, $p < 0.0001$). The relationship between intake and concentration was best described by power functions in all species ($I = 6.72\ (C)^{-0.78}$, $I = 8.93\ (C)^{-0.76}$, and $I = 11.64\ (C)^{-0.71}$, $r^2 \geq 0.83$, for *A. alexandri*, *E. fulgens*, and *L. clemenciae*, respectively; Fig. 2). *Archilochus alexandri* ate less at any given sugar concentration than either of the larger

species ($ANCOVA_{intercepts}$ $F_{2,17} = 17.97$, $p = 0.002$, Tukey's HSD $p < 0.05$ for both species), but *E. fulgens* and *L. clemenciae* had statistically similar concentration corrected volumetric intake rates. There was no significant difference in the slopes of the intake response relationship among the three species ($ANCOVA_{slopes}$, $F_{2,15} = 0.41$, $p = 0.67$). Sucrose intake (g day^{-1}) did not vary significantly with food energy density ($ANCOVA_{concentration}$ $F_{1,17} = 0.66$, $p = 0.43$), but was significantly lower in *A. alexandri* ($ANCOVA_{intercepts}$, $F_{2,17} = 8.98$, $p = 0.002$, Tukey's HSD $p < 0.05$ for both larger species; Fig. 2). Daily sucrose intake averaged 2.06 ± 0.17, 2.68 ± 0.16, and 3.45 ± 0.28 g (mean ± SE) for *A. alexandri*, *E. fulgens*, and *L. clemenciae*, respectively. Assuming 16.6 kJ g^{-1} of sucrose, this translates into relatively constant energy intake rates of 34.2 ± 2.82, 44.49 ± 2.66 and 57.27 ± 4.65 kJ day^{-1} for the three species, respectively. At our experimental concentrations, nitrogen content did not have an effect on intake ($ANCOVA_{nitrogen}$ $F_{1,16} = 0.26$, $p = 0.62$ when diet nitrogen level was added to the food intake model as a covariate).

When food intake rates were scaled to metabolic body mass ($kg^{0.75}$) for interspecific comparisons (Robbins 1993), concentration corrected volumetric intake by *A. alexandri* was significantly higher than that of the larger species, which did not differ from each

other ($ANCOVA_{intercepts}$ $F_{2,15} = 17.08$, $p = 0.0001$, Tukey's HSD $p < 0.05$). Sucrose intake by *A. alexandri* was also significantly higher (one way ANOVA $F_{2,16} = 10.19$, $p = 0.0014$, Tukey's HSD $p < 0.05$ for both larger species). Metabolic mass corrected volumetric food and sucrose intake rates were about 1.5 times higher in *A. alexandri* than in the larger species.

Body mass remained constant ($t = 0.17$, $df = 6$, $p = 0.87$ and $t = -0.57$, $df = 6$, $p = 0.59$ for *E. fulgens*, and *L. clemenciae*, respectively) or increased slightly (mean increase 9.72 ± 2.47 %, $t = -3.81$, $df = 6$, $p = 0.009$ for *A. alexandri*) over the experimental period. There was no significant correlation between percent body mass increase and apparent nitrogen retention in *A. alexandri* ($F_{1,6} = 0.206$, $p = 0.67$).

Nitrogen balance

Daily nitrogen intake ranged from zero to 10.89 mg in *A. alexandri*, zero to 24.44 mg in *E. fulgens* and zero to 15.09 mg in *L. clemenciae*. Apparent nitrogen retention (intake minus excretion) increased significantly with nitrogen intake in all species ($ANCOVA_{nitrogen}$ $F_{1,16} = 154.32$, $p < 0.0001$, Fig. 3). The slopes of these relationships were almost identical (≈ 0.5, $ANCOVA_{slopes}$ $F_{2,14} = 0.06$, $p = 0.95$). Maintenance nitrogen

requirements (MNR) and total endogenous nitrogen losses (TENL) were determined for each species using the x- and y-intercepts of a least-squares linear regression model, respectively (Smith and Green 1987; Brice and Grau 1991; Korine et al. 1996; Witmer 1998; Delorme and Thomas 1999; van Tets and Hulbert 1999; Roxburgh and Pinshow 2000). In *A. alexandri*, the relationship between apparent nitrogen retention and intake was nonlinear at high intakes. Thus, apparent nitrogen retention in *A. alexandri* was lower than expected at the highest observed nitrogen intake rate. We did not use this value for nitrogen balance calculations. MNR and TENL values are reported in Table 1.

Nitrogen excretion

Hummingbirds excreted nitrogen in detectable quantities as ammonia, uric acid (urates), urea, soluble protein, creatine, creatinine and associated with bile acids (i.e. as taurine and/or glycine associated with cholic, deoxycholic and lithocholic acids, see Methods for assumptions). Nitrogenous waste excretion by hummingbirds varied among species. *Archilochus alexandri* excreted the largest proportion of assayed nitrogen as ammonia (ANOVA $F_{2,18} = 61.35$, $p < 0.0001$, Tukey's HSD $p < 0.05$), but there were no significant differences in ammonia excretion between *E. fulgens* and *L. clemenciae* (Fig. 4).

Consequently, *A. alexandri* excreted a smaller proportion of assayed nitrogen as urates than either of the larger species (ANOVA $F_{2,18} = 18.84$, $p < 0.0001$, Tukey's HSD $p < 0.05$), which were again not significantly different from each other. The mean proportion of nitrogen excreted in each other form was not significantly different among species. Excretion of nitrogen associated with bile acids was detectable in trace amounts for all species. No birds excreted > 50% of nitrogen as ammonia, or more nitrogen as ammonia than urates, during any experimental trial. Nitrogen excretion data are summarized in Table 2.

The proportion of assayed nitrogen excreted as ammonia did not increase with water intake rate as hypothesized in any species (Fig. 5). There was a significant negative correlation between ammonia excretion and water intake rate in *L. clemenciae* ($y = -0.16x + 5.74$, $r^2 = 0.67$; $F_{1,6} = 10.16$, $p < 0.03$). In addition, there was a significant negative correlation between urea excretion and water intake rate in *E. fulgens* ($y = -0.17x + 6.4$, $r^2 = 0.89$; $F_{1,5} = 31.79$, $p < 0.005$). There were no additional significant correlations between the proportion of nitrogen excreted in any other form and water intake rate, for any species. Creatinine excretion was positively correlated with nitrogen intake rate in *A. alexandri* ($y = 0.1x - 0.004$, $r^2 = 0.59$; $F_{1,6} = 7.15$, $p < 0.05$). There were

no other significant correlations between the proportion of nitrogen excreted in any form and nitrogen intake rate, for any species.

Excreta osmolality did not vary among species ($ANCOVA_{species}$ $F_{2,15} = 0.06$, $p = 0.94$; $ANCOVA_{slopes}$ $F_{2,13} = 1.81$, $p = 0.2$) but decreased significantly with increasing water intake rate in a nonlinear fashion ($y = 12.93 + (2241.66/x)$, $r^2 = 0.73$; $F_{1,18} = 45.33$, $p < 0.0001$, Fig. 6). Osmolality ranged from 102 to 667.8 mOsm $(kg\ H_2O)^{-1}$ for all species combined.

Discussion

As predicted, we found that all three hummingbird species had extraordinarily low nitrogen requirements. Contrary to our predictions, and in contrast with Preest and Beuchat's (1997) previous observations, we did not find facultative ammonotely in hummingbirds. Although ammonia excretion was significantly higher in *A. alexandri* than in *E. fulgens* and *L. clemenciae*, all three species were uricotelic. Furthermore, the fraction of nitrogen excreted as ammonia was independent of water intake. In the first section of this discussion, we compare the nitrogen requirements of hummingbirds with those of other nectar- and fruit-feeding animals and with predicted values. We examine

both proximate (dietary) and ultimate (evolutionary, adaptation to diet) explanations for the low nitrogen requirements of these animals. We then discuss the apparent nitrogen retention observed in nitrogen balance studies. In the second section we pose two non-mutually exclusive explanations for the observed patterns of ammonia excretion: differences in proportionate water loads and post-renal modification of urine among hummingbirds of different body sizes.

Because hummingbirds modulate volumetric intake to keep sugar intake relatively constant, our experimental design allowed us to manipulate nitrogen intake with minimal changes in energy consumption. Energy intake was not significantly different between dietary sucrose concentrations for any species. *Archilochus alexandri*, *E. fulgens* and *L. clemenciae* maintained constant energy intake rates of 34.2, 44.5 and 57.3 kJ day^{-1}, respectively. Provided with a nectar diet containing artificially high protein levels (nitrogen intake exceeded MNR by up to 11 times in *A. alexandri*, 6.1 times in *E. fulgens* and 4.7 times in *L. clemenciae*) and given no other nitrogen sources (e.g. arthropods), the birds in our study varied volumetric intake based only on food energy density. Roxburgh and Pinshow (2000) report a similar observation in nectarivorous Palestine sunbirds.

Nitrogen requirements of nectar-feeding birds

The maintenance nitrogen requirements (MNR) and total endogenous nitrogen losses (TENL) of the hummingbirds examined in this study were exceptionally low and comparable to values obtained for other nectar-feeding birds (Table 1). Robbins (1993) predicted that birds should have a MNR of 430 mg N $kg^{-0.75}$ d^{-1} and TENL of 270 mg N $kg^{-0.75}$ d^{-1}. *Archilochus alexandri*, *E. fulgens* and *L. clemenciae* had mass-independent MNRs that were 19.9%, 36.7% and 28.5% of this value, respectively. The broad-tailed hummingbird (*Selasphorus platycercus*) had a similarly low MNR that was only 14.5 % of the value predicted by Robbins for birds (McWhorter 1997). TENL values were 17%, 28.8%, 23.6% and 16.7% of the predicted value for these four species of hummingbirds, respectively. Figure 7 compares predicted MNR (nitrogen balance rather than body mass balance requirements) and TENL with actual values for small (less than 100 g in body mass) nectar- and fruit-feeding birds, eutherian mammals and marsupials (see Table 1 for values). The nitrogen requirements and endogenous losses of nectar-feeding animals are strikingly lower than those predicted by body mass, and these low values appear to be a general pattern that is independent of phylogenetic affinities.

Nitrogen requirements in nectar-feeding animals are probably low as a result of two interacting factors: feeding on a low nitrogen diet and physiological adaptation to that diet. When consuming liquid diets low in fiber and lipids, animals should have relatively lower fecal nitrogen losses due to low secretion of protein digesting enzymes and bile acids, reduced sloughing of intestinal epithelial cells and smaller populations of gut microorganisms (Robbins 1993; Roxburgh and Pinshow 2000). Indeed, metabolic fecal nitrogen (MFN) losses in nectar-feeding marsupials are among the lowest reported for mammals (Smith and Green 1987; van Tets and Hulbert 1999). There is also an evolutionary necessity to minimize nitrogen loss when specializing on low nitrogen foods (Robbins 1993). Adaptation to a nectar diet by birds may result in relatively lower rates of endogenous protein turnover and/or nitrogen recycling (facilitated in birds by mixing of urinary and fecal materials in the cloaca). In the following paragraphs, we examine these two possibilities in detail.

Endogenous urinary nitrogen (EUN) losses in nectar-feeding marsupials (Smith and Green 1987; van Tets and Hulbert 1999) and fruit-feeding bats (Delorme and Thomas 1996; Korine et al. 1996; Delorme and Thomas 1999) are comparatively low. One explanation posed for the low EUN losses observed in these animals centers on the

actions of glucose and insulin (Korine et al. 1996). Insulin is antigluconeogenic and therefore may minimize protein turnover by stimulating protein synthesis and decreasing protein degradation (Fukagawa et al. 1985; Florini 1987). Roxburgh and Pinshow (2000) suggest that the extremely high plasma glucose concentrations observed in nectar-feeding birds (reported for one species of sunbird and two species of hummingbirds Beuchat and Chong 1998; Roxburgh and Pinshow 2000) may act in concert with insulin to minimize gluconeogenesis and thus contribute to very low EUN losses in these animals. Mixing of fecal and urinary materials in the cloaca makes simple (i.e. non-surgical, see for example Teekell et al. 1968) separate estimates of 24 hour MFN and EUN difficult to obtain in birds. It is likely that both are low in nectar-feeding birds. To disentangle the effect of diet from that of adaptation to diet, it would be instructive to measure the nitrogen requirements and endogenous losses of non-nectarivorous birds fed on a nectar diet.

The possibility of post-renal nitrogen recycling has also recently been posed to explain the low nitrogen requirements of nectar-feeding birds. Galliform birds have large microbial populations capable of breaking down urates in their cecae (Laverty and Skadhauge 1999), and the recycling of urates has been recently documented in chickens (Clench 1999). Ammonia resulting from the breakdown of urates may be incorporated

into microbial protein or may be used in the enzymatic synthesis of glutamic acid, which is then absorbed by the cecal epithelium (Mortensen and Tindall 1981). Roxburgh and Pinshow (2002) found significant post-renal modification of urine in Palestine sunbirds with low water intake rates, specifically increased ammonia and decreased urate concentrations in excreta relative to ureteral urine. They suggest that microbial nitrogen recycling may be occurring in the distal large intestine of nectar-feeding birds, which do not possess functional cecae. However, except for one unpublished observation (C. A. Beuchat, cited as pers. comm. in Roxburgh and Pinshow 2002), there is no data available on the presence of bacteria with uricase activity in the gastrointestinal tracts of nectar-feeding birds. The capacity of the avian large intestine to absorb amino acids is also unknown, but it has been demonstrated in non-ruminant mammals (see Fuller and Reeds 1998 for a review). The mechanisms and functional significance of nitrogen recycling in birds that do not possess functional cecae remain unknown (Roxburgh and Pinshow 2002).

Apparent nitrogen retention was observed in all three hummingbird species examined in this study (see Fig. 3), and has been observed in all studies of nectar-and fruit-feeding animals to date where nitrogen balance was measured (Smith and Green 1987; Brice and

Grau 1991; Korine et al. 1996; Witmer 1998; Delorme and Thomas 1999; van Tets and Hulbert 1999; Roxburgh and Pinshow 2000). Similar observations of positive nitrogen balance have been made in human nutrition studies (Hegsted 1976; Young 1986). Brice and Grau (1991) found that nitrogen retention by Costa's hummingbirds (*Calypte costae*) increased with nitrogen intake while body mass remained constant. Birds in this study, including those on zero nitrogen diets, maintained or slightly increased body mass over the duration of the study. The slight increase in body mass observed in *A. alexandri* was likely due to increased fat mass, typically found in newly-captive hummingbirds presented with *ad libitum* food (T. J. McWhorter and C. Martínez del Rio, unpublished observations), rather than increased protein mass. The lack of a significant correlation between percent body mass increase and apparent nitrogen retention provides additional evidence in support of this contention. It is possible that nitrogen was actually retained by hummingbirds, but more likely that it was lost as sloughed skin or feathers or as ammonia volatilized from respiratory surfaces (Brice and Grau 1991; Roxburgh and Pinshow 2000). Interestingly, nitrogen retention by *A. alexandri* was considerably lower than expected at the highest nitrogen intake level (about 11 times MNR), suggesting a nitrogen retention plateau in small hummingbirds. This result must be interpreted with

caution, however, because it was only observed in one individual and no indication of such a plateau has been observed in any other species examined (see also Hegsted 1976).

It is clear that the nitrogen requirements of nectar-feeding birds are exceptionally low. Despite these low requirements, floral nectars alone are not an adequate nitrogen source. Brice and Grau (1991) estimated that floral nectars could provide a maximum of about 14 % of the nitrogen requirement of Costa's hummingbirds. Nectar-feeding birds also consume arthropods to varying degrees, depending on their nutritional needs (Wagner 1946; Paton 1982; Brice and Grau 1991; Brice 1992; van Tets and Nicolson 2000). Making the assumptions of 75 % foraging success and adequate insect abundance, they estimated that Costa's hummingbirds could meet their nitrogen requirements by foraging on insects for only 6 minutes per day, or about 12 % of total foraging time. Time budget data for nectar-feeding birds agree very well with this estimate: male Anna's hummingbirds (*Calypte anna*) spent an average of 8 min d^{-1} foraging for arthropods (Stiles 1971), New Holland honeyeaters (*Phylidonyris novaehollandiae*) spent 10 min d^{-1} (only 5 % of their total foraging time, Paton 1982), and Palestine sunbirds spent 12 min d^{-1} (about 10 % of their total foraging time, Roxburgh and Pinshow 2000).

Nitrogen excretion by hummingbirds

Water intake by hummingbirds in this study ranged from 83 to 665 % of body mass per day for *A. alexandri*, 68 to 314 % for *E. fulgens* and 102 to 336 % for *L. clemenciae*, with the largest mass-specific water intake rates observed in birds feeding on 0.292 mol L^{-1} sucrose solutions. The proportion of assayed nitrogen excreted as ammonia was significantly higher and urates significantly lower in *A. alexandri* than in the larger hummingbirds (Fig. 4). Excretion of ammonia and urates were not significantly different between *E. fulgens* and *L. clemenciae*, and the proportion of nitrogen excreted in each other form was not significantly different among species. No birds excreted > 50% of nitrogen as ammonia, or more nitrogen as ammonia than urates, during any experimental trial. Ammonia excretion was not positively correlated with water intake rate in any species. A significant negative correlation between ammonia excretion and water intake in *L. clemenciae* describes a modest decrease in the proportion of ammonia excreted from 5.8 % to 1.8 % with increasing water intake rate. The negative correlation between urea excretion and water intake rate in *E. fulgens* describes a similarly modest decrease from 5.6 % to 2.6 % of total nitrogen excreted. The excretion of all other forms of nitrogenous waste were not significantly correlated with water intake rate. Our results therefore do not support the hypothesis that hummingbirds are facultatively ammonotelic.

When food and sucrose intake were scaled to metabolic body mass, intake by *A. alexandri* was significantly higher than that of the two larger species. We posit that mass-specific water intake (% Mb day $^{-1}$, 1.8 times greater on average in *A. alexandri*, which is 35 % and 33 % of the body mass of *E. fulgens* and *L. clemenciae*, respectively) explains the greater proportion of ammonia excreted by *A. alexandri*. In other words, there may be a size-dependent dichotomy of ammonia excretion in hummingbirds. Indeed, the proportion of assayed nitrogen excreted as ammonia by 3.4 g broad-tailed hummingbirds (*Selasphorus platycercus*) feeding on a nitrogen-free diet (31.13 ± 1.66 %, n = 16) was very similar to that excreted by *A. alexandri* (25.66 ± 2.71 %) and again there was no positive correlation with water intake (T. J. McWhorter and C. Martínez del Rio, unpublished data). Basal metabolic rates of small hummingbirds such as *A. alexandri* are 10 to 15 % higher than predicted based on body mass (Lasiewski 1963; Prinzinger et al. 1981), whereas this is not the case for larger hummingbirds such as *E. fulgens* and *L. clemenciae* (Lasiewski and Lasiewski 1967). Higher metabolic demands necessitate higher energy intake, and as observed in *A. alexandri*, higher mass-specific rates of food and sucrose intake. It is possible that these small hummingbirds are reaching the lower size limit for endothermy. In order to meet their phenomenal metabolic demands they must deal with larger ingested water loads.

Post-renal modification of nitrogenous wastes may also explain the lack of a positive correlation between ammonia excretion and water intake in hummingbirds. Roxburgh and Pinshow (2002) examined the effects of water, electrolyte and protein intake and ambient temperature on nitrogen excretion by Palestine sunbirds. They found that the proportion of ammonia in ureteral urine and excreted fluid was not correlated with ambient temperature, electrolyte intake, or water intake. Although protein intake did not influence nitrogenous wastes in ureteral urine, the proportion of ammonia in excreted fluid was higher when protein intake was reduced. This increase in excreted ammonia was accompanied by a concomitant reduction in urate concentration. The authors suggest that urate was broken down in the distal large intestine, leading to "apparent" ammonotely in sunbirds (Roxburgh and Pinshow 2002). Refluxing of urine into the distal large intestine, possible in birds because the ureters and intestine both open into the cloaca, is known to facilitate significant post-renal modification of urine composition, including uptake of water and electrolytes and break down and recycling of protein in many species of birds (Goldstein and Braun 1986; Braun 1999; Karasawa 1999). As mentioned above, microorganisms with uricase activity have been reported in the distal large intestine of hummingbirds. Roxburgh and Pinshow's (2002) results question the

significance of functional ammonotely as a feature unique to birds that experience high rates of water flux. It is possible that post-renal modification of urine in hummingbirds varies with body size. The exceptionally low maintenance nitrogen requirements of *A. alexandri*, *C. costae* and *S. platycercus* suggest that nitrogen recycling may be occurring to a greater degree in small hummingbirds. Although there was no significant correlation of ammonia excretion and protein intake in any species in this study, a larger proportion of ammonia in excreta would be expected to accompany greater nitrogen recycling.

Specialization to sugary diets in birds may involve differences in the physiological mechanisms of water and protein metabolism between taxa and body sizes. It is clear that the MNR and TENL of nectar-feeding animals are exceptionally low. Smaller hummingbird species excrete a significantly larger proportion of their nitrogenous wastes as ammonia than larger hummingbirds, but our results and those of Roxburgh and Pinshow (2002) and van Tets et al. (2001) do not support the hypothesis of facultative ammonotely with increasing water intake in birds. The physiological mechanisms that account for these differences are unknown, but differences in proportionate water loads and post-renal modification of urine are strong candidates. The mechanisms of nitrogen conservation and recycling in birds that do not possess functional cecae remain unknown.

Additional data on populations of gut microorganisms with uricase activity, the extent of post-renal modification of urine and the protein digestive and absorptive capacities in nectar-feeding birds are necessary in order to solve this mystery.

Acknowledgements

We wish to thank Nathan Miller, Amber Hamilton, Andrea McWhorter, Theo and Sean Powers, Wade and Emily Sherbrook, and the SWRS Staff and volunteers for capable field and lab assistance. Ana Claudia Nepote and Elena Ruiz prepared samples for elemental analysis, and Joost van Haren provided access to analytical facilities at Columbia University's Biosphere 2 Center. Berry Pinshow and Jorge Schöndube provided valuable comments on an earlier draft of this manuscript. This research was funded by the American Museum of Natural History's Southwestern Research Station Student Support Fund, the University of Arizona Department of Ecology and Evolutionary Biology, the George Fox University Department of Biology and Chemistry, and Linfield College (fellowship to N. Miller).

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Table 1. Maintenance nitrogen requirements (MNR) and total endogenous nitrogen losses (TENL) of nectar- and fruit-feeding vertebrates below 100 g in body mass. Values were estimated by regression of apparent N retention on N intake except as otherwise specified. Data are arranged in order of increasing body mass.

Species	**Body Mass (g)**	**MNR mg N $kg^{-0.75}d^{-1}$**	**TENL mg N $kg^{-0.75}d^{-1}$**	**Source**
Black-chinned hummingbird (*Archilochus alexandri*)	2.7	85.5	46	This study
Broad-tailed hummingbird (*Selasphorus platycercus*)	3.4	62.6	45.2	McWhorter 1997
Costa's hummingbird (*Calypte costae*)	3.5	77.1 312.7[a]	77.8	Brice and Grau 1991
Palestine sunbird (*Nectarinia osea*)	6.9	165 213[a]	81	Roxburgh and Pinshow 2000
Magnificent hummingbird (*Eugenes fulgens*)	7.5	158	77.7	This study
Blue-throated hummingbird (*Lampornis clemenciae*)	7.9	122.4	63.8	This study
Lesser double-collared sunbird (*Nectarinia chalybea*)	8	253.2	157	van Tets and Nicolson 2000
Queensland blossom bat (*Syconycteris australis*)	18	337	255.1	Law 1992
New Holland honeyeater (*Phylidonyris novaehollandiae*)	20	93.2	60.1	Paton 1982
Eastern pygmy possum (*Cercartetus nanus*)	25.4[b] 30.9[c]	40.9[b,d] 149.3[c,d]	29.2[b] 62.6[c]	van Tets and Hulbert 1999
Cedar waxwing (*Bombycilla cedrorum*)	34.5	484	196.5	Witmer 1998
phyllostomid fruit-bat (*Artibeus jamaicensis*)	37	213.7[d]	196.6	Delorme and Thomas 1999

[a] Minimum body mass maintenance requirement.

[b] Sugar plus pollen diet.

[c] Sugar plus mealworm diet.

[d] Truly digestible nitrogen.

Table 2. Nitrogen excretion by hummingbirds, reported as a percentage of the total excreted N measured using biochemical assays (mean ± SE).

Species	Ammonia	Urates	Urea	Soluble Protein	Creatine	Creatinine	Bile Acids[a]
Black-chinned hummingbird (*Archilochus alexandri*)	25.7 ± 2.7[b]	64.6 ± 4.5[b]	3 ± 0.9	5 ± 1.4	1.3 ± 0.3	0.4 ± 0.2	0.031 ± 0.012
Magnificent hummingbird (*Eugenes fulgens*)	4.5 ± 0.4	84.6 ± 0.9	4.1 ± 0.4	4.7 ± 0.9	1.5 ± 0.3	0.6 ± 0.3	0.013 ± 0.004
Blue-throated hummingbird (*Lampornis clemenciae*)	3.1 ± 0.5	86.9 ± 1.7	4.2 ± 0.8	4.3 ± 0.9	1.1 ± 0.1	0.3 ± 0.2	0.011 ± 0.004

[a] Nitrogen in taurine and/or glycine associated with cholic, deoxycholic and lithocholic acids, see Methods for assumptions.

[b] Significantly different from mean proportion of assayed N excreted by other species (ANOVA, $p < 0.0001$, Tukey's HSD $p < 0.05$).

Legends

Figure 1. Hummingbirds vary food intake when fed solutions of different sugar concentrations. When fed low sucrose concentrations (0.292 mol L^{-1}) they drink significantly more than when fed high concentrations (1.168 mol L^{-1}). Note that the food intake and sugar concentration axes in the top panel of this figure run in opposite directions. We manipulated protein intake by varying both sugar and protein content of food. The table in the lower panel shows the combinations of sugar concentrations and nitrogen levels used in our experimental design. The lines in the upper panel show predicted nitrogen intake levels for a 7.6 g hummingbird feeding on each nitrogen level when sugar concentration is varied. Each symbol represents a unique combination of sucrose concentration and nitrogen level. We achieved high protein intakes by feeding hummingbirds on low sucrose concentrations (0.292 and 0.584 mol L^{-1}) with high protein contents (1.2 and 4 g L^{-1}). Conversely, we achieved low protein intakes by feeding hummingbirds on high sucrose concentrations (0.876 and 1.168 mol L^{-1}) and low nitrogen contents (0 and 1.2 g L^{-1}). Because hummingbirds modulate volumetric intake to maintain constant sugar intake, we were able to manipulate nitrogen intake with minimal changes in energy consumption.

Figure 2. Volumetric food (lower panel) and sucrose (upper panel) intake as a function of sugar concentration in: black-chinned hummingbirds (*Archilochus alexandri*), unfilled squares; magnificent hummingbirds (*Eugenes fulgens*), filled diamonds; and blue-throated hummingbirds (*Lampornis clemenciae*), unfilled circles. Food Intake (I) decreased significantly with increased sucrose concentration (C) in relationships adequately described by power functions ($I = 6.72\ (C)^{-0.78}$, $I = 8.93\ (C)^{-0.76}$, and $I = 11.64\ (C)^{-0.71}$, $r^2 \geq 0.83$, for the three species, respectively). Concentration corrected volumetric intake by *A. alexandri* was significantly lower than that of both larger species. Note that the scales of both axes in the bottom panel are logarithmic, and that intake has not been standardized by body mass. Sucrose intake per day did not vary with sucrose concentration, but was significantly lower in *A. alexandri*.

Figure 3. Apparent nitrogen retention increased significantly with nitrogen intake in all species. Maintenance nitrogen requirements (MNR) and total endogenous nitrogen losses (TENL) when feeding on a nitrogen-free diet were determined for each species using the x- and y-intercepts of a least-squares linear regression, respectively. In *A. alexandri*, the relationship between apparent nitrogen retention and intake was nonlinear at high intakes. Because apparent nitrogen retention in *A. alexandri* was lower than

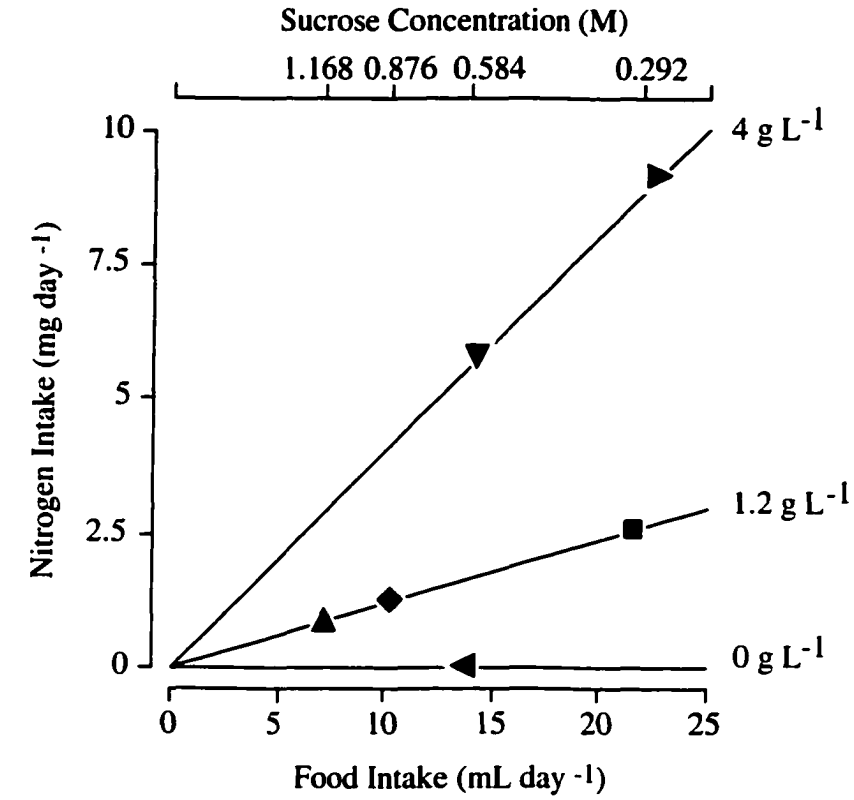
expected at the highest observed nitrogen intake rate we did not use this value for balance calculations. The apparent retention observed at most intake rates can be easily explained by non-excretory losses of skin cells and feather components. Birds maintained body mass throughout the 24 h experimental period. Note that the scales of the axes in this figure differ among species. MNR and TENL values are reported in Table 1.

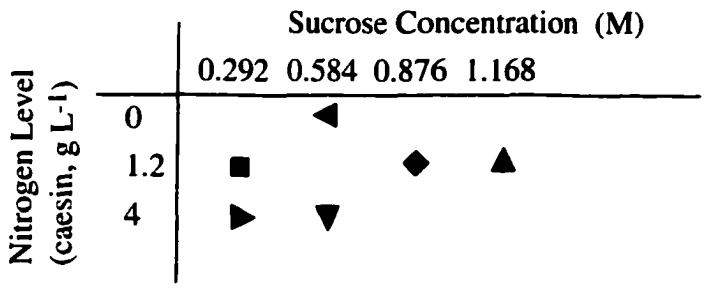
Figure 4. Nitrogenous waste excretion by hummingbirds varied between species. *Archilochus alexandri* (35% and 33% of the body mass of *E. fulgens* and *L. clemenciae*, respectively) excreted a greater proportion of assayed nitrogen as ammonia, and consequently a smaller proportion as urates, than either of the larger species (ANOVA $F_{2,18} = 61.35$ and 18.84, for ammonia and urates, respectively, $p < 0.0001$, Tukey's HSD $p < 0.05$ for comparisons with larger species in both cases). Excretion of ammonia and urates were not significantly different between *E. fulgens* and *L. clemenciae*. The mean proportion of nitrogen excreted in each other form was not significantly different among species. Excretion of N with bile acids was detectable in trace amounts for all species. No birds excreted > 50% of N as ammonia during any experimental trial. N excretion data are summarized in Table 2.

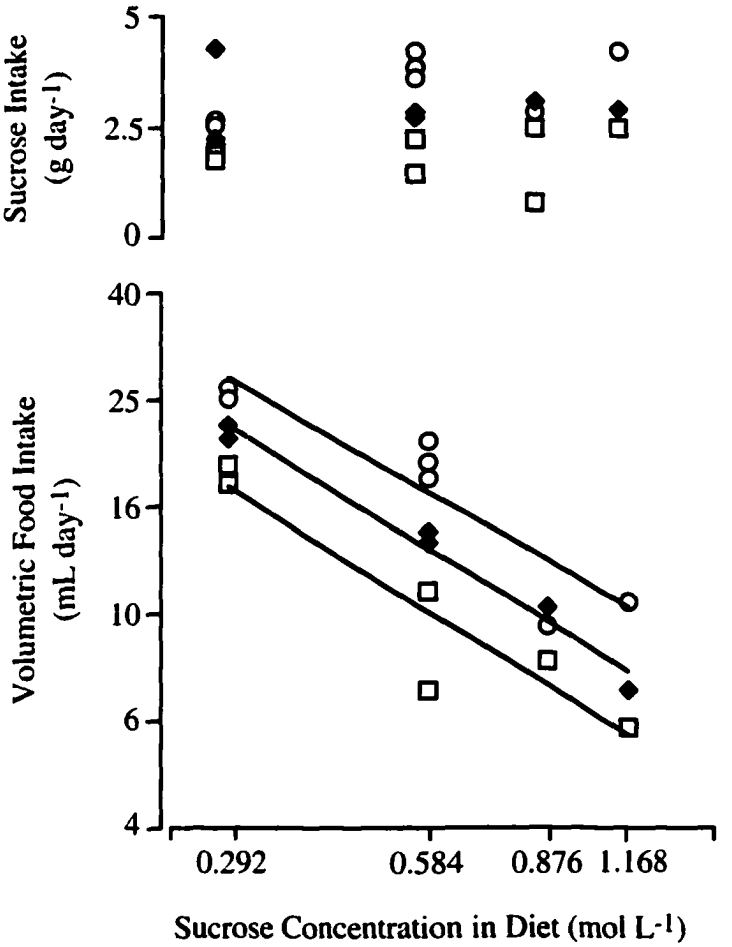
Figure 5. The proportion of assayed nitrogen excreted as ammonia did not increase with water intake rate as hypothesized in any species (*A. alexandri*, unfilled squares; *E. fulgens*, filled diamonds; *L. clemenciae*, unfilled circles). There was a significant negative correlation between ammonia excretion and water intake rate in *L. clemenciae* ($F_{1,6} = 10.16$, $p < 0.03$). In addition, there was a significant negative correlation between urea excretion and water intake rate in *E. fulgens* ($F_{1,5} = 31.79$, $p < 0.005$). There were no additional significant correlations between the proportion of nitrogen excreted in any other form and water intake rate, for any species. Creatinine excretion was positively correlated with nitrogen intake rate in *A. alexandri* ($y = 0.1x - 0.004$, $r^2 = 0.59$; $F_{1,6} = 7.15$, $p < 0.05$). There were no other significant correlations between the proportion of nitrogen excreted in any form and nitrogen intake rate, for any species.

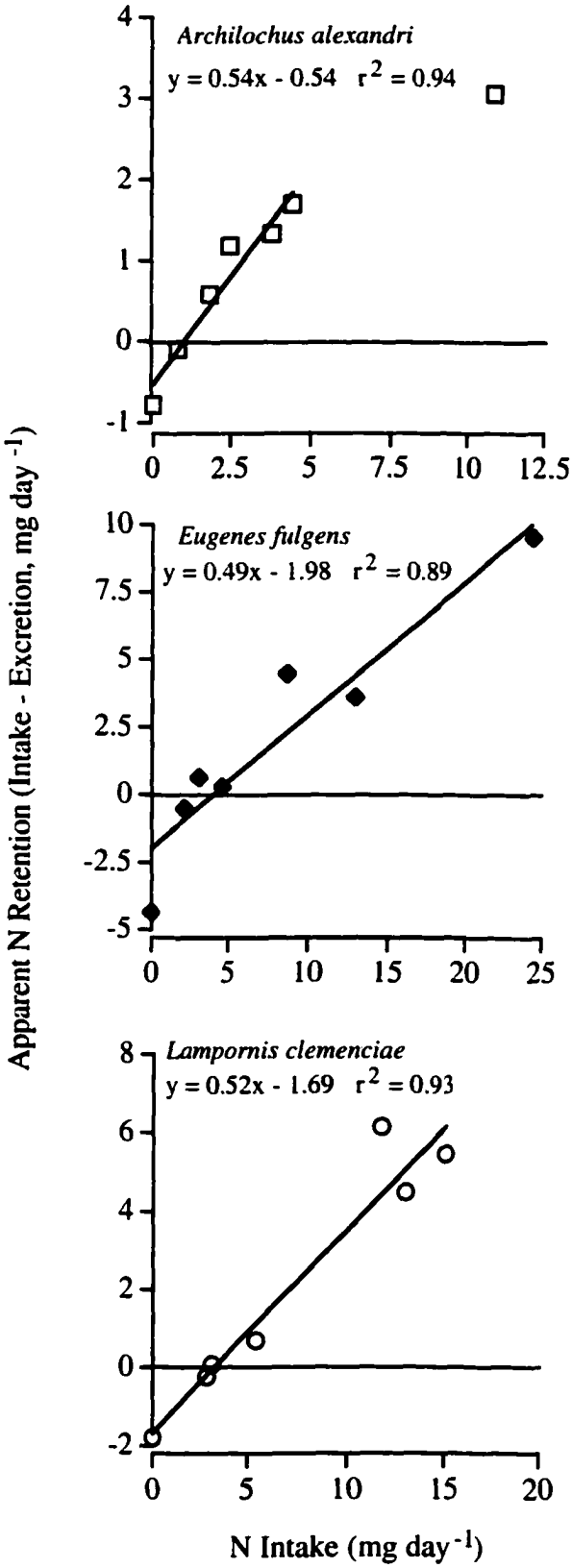
Figure 6. Excreta osmolality did not vary among species (*A. alexandri*, unfilled squares; *E. fulgens*, filled diamonds; *L. clemenciae*, unfilled circles) but decreased significantly with increasing water intake rate in a nonlinear fashion ($F_{1,18} = 45.33$, $p < 0.0001$). Osmolality ranged from 102 to 667.8 mOsm $(kg\ H_2O)^{-1}$ for all species combined.

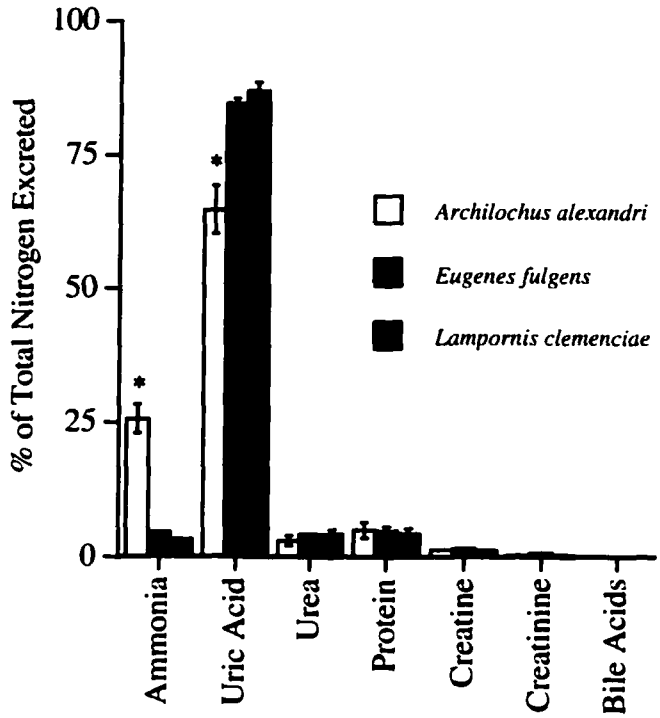
Figure 7. Maintenance nitrogen requirements (MNR, filled symbols) and total endogenous nitrogen losses (TENL, open symbols) of nectar- and fruit-feeding animals are considerably lower than predicted based on body mass (hummingbirds, circles; nectar- and fruit-feeding birds, diamonds; nectar- and fruit-feeding mammals and marsupials, triangles; see Table 1 for data). Data are shown only for animals below 100 g in body mass. The solid lines represent MNR as a function of body mass for eutherian mammals (582 mg N $kg^{-0.75}$ d^{-1}), birds (430 mg N $kg^{-0.75}$ d^{-1}) and marsupials (356 mg N $kg^{-0.75}$ d^{-1}, respectively, from the top). The dashed line represents TENL as a function of body mass for birds (270 mg N $kg^{-0.75}$ d^{-1}), data are not shown for mammals and marsupials because of the difficulty of estimating metabolic fecal nitrogen losses for these animals. Allometric data adapted from Robbins (1993). For nectar- and fruit-feeding birds only, MNR = 0.32 x body $mass^{1.14}$, $r^2 = 0.83$, TENL = 0.31 x body $mass^{0.88}$, $r^2 = 0.81$. All values in this figure were estimated by regression of apparent N retention on N intake and are not based on minimum body mass maintenance requirements. Note that the scales of both axes are logarithmic.

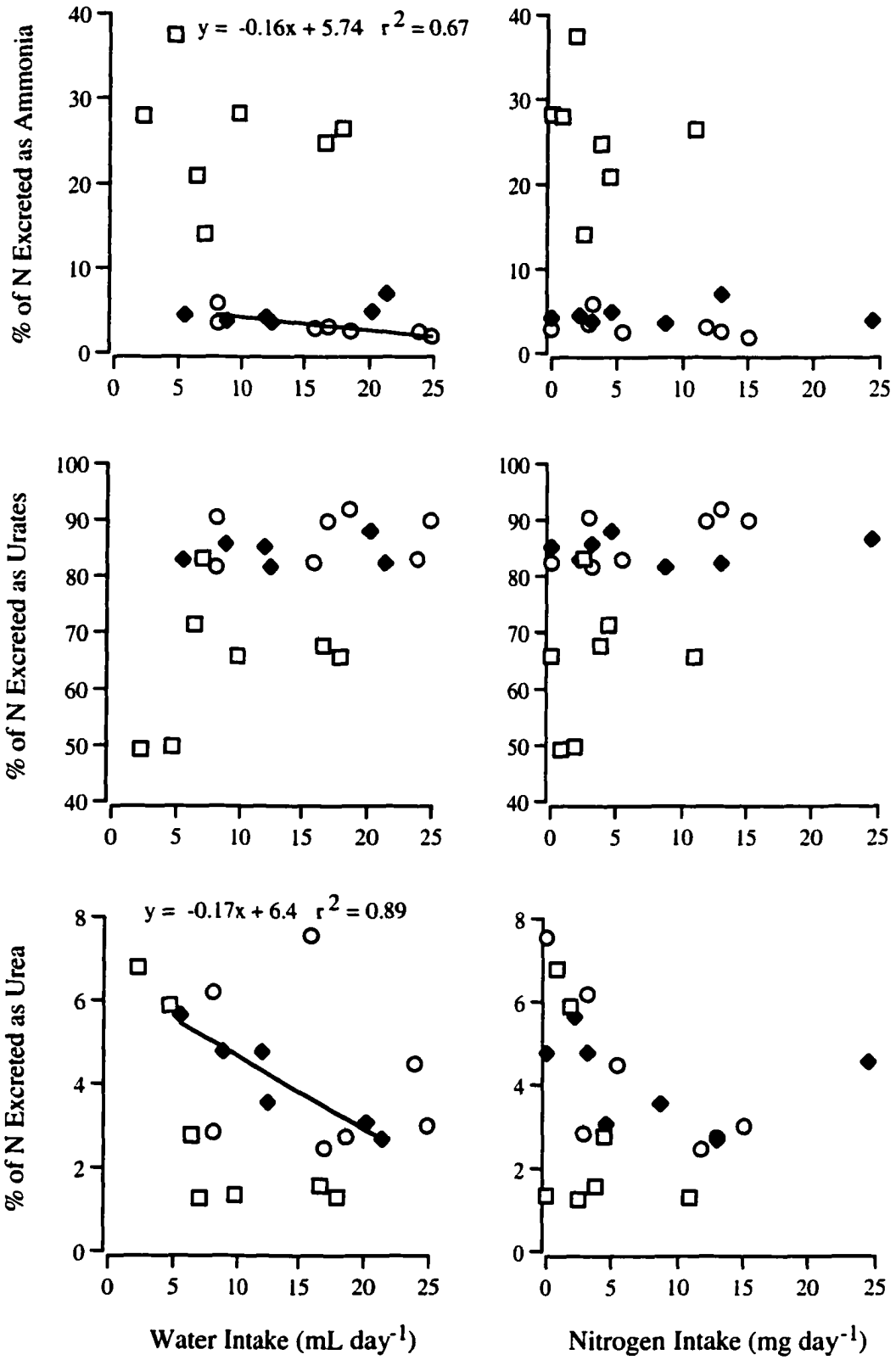

Sucrose Concentration (M)
1.168 0.876 0.584 0.292
10
7.5
5
2.5
0
Nitrogen Intake (mg day -1)
4 g L-1
1.2 g L-1
0 g L-1
0 5 10 15 20 25
Food Intake (mL day -1)

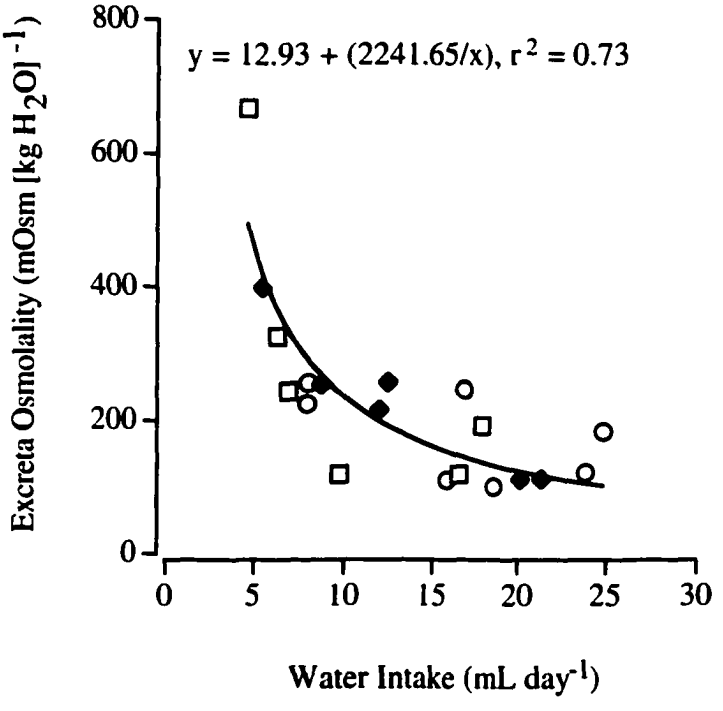

Sucrose Concentration (M)
0.292 0.584 0.876 1.168
Nitrogen Level (caesin, g L-1)
0
1.2
4

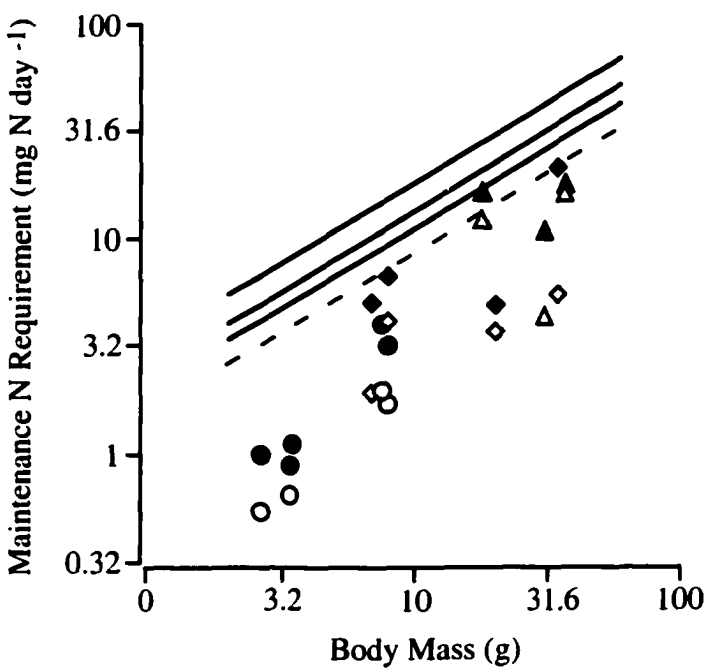

Sucrose Intake
(g day-1)
5
2.5
0
Volumetric Food Intake
(mL day-1)
40
25
16
10
6
4
0.292
0.584
0.876
1.168
Sucrose Concentration in Diet (mol L-1)


Archilochus alexandri
y = 0.54x - 0.54 r2 = 0.94
Eugenes fulgens
y = 0.49x - 1.98 r2 = 0.89
Lampornis clemenciae
y = 0.52x - 1.69 r2 = 0.93
Apparent N Retention (Intake - Excretion, mg day-1)
N Intake (mg day-1)


100
75
50
25
0
% of Total Nitrogen Excreted
*
*
Archilochus alexandri
Eugenes fulgens
Lampornis clemenciae
Ammonia
Uric Acid
Urea
Protein
Creatine
Creatinine
Bile Acids


y = -0.16x + 5.74 r2 = 0.67
y = -0.17x + 6.4 r2 = 0.89
% of N Excreted as Ammonia
% of N Excreted as Urates
% of N Excreted as Urea
Water Intake (mL day-1)
Nitrogen Intake (mg day-1)


y = 12.93 + (2241.65/x), r2 = 0.73
Excreta Osmolality (mOsm [kg H2O]-1)
Water Intake (mL day-1)
800
600
400
200
0
0
5
10
15
20
25
30


Maintenance N Requirement (mg N day -1)
100
31.6
10
3.2
1
0.32
0
3.2
10
31.6
100
Body Mass (g)

APPENDIX E - ANIMAL SUBJECTS APPROVAL

Institutional Animal Care
and Use Committee

THE UNIVERSITY OF
ARIZONA
TUCSON ARIZONA

P.O. Box 210101
Tucson, Arizona 85721-0101

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 -- USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use.
The following listed proposal has been granted ***Final Approval*** according to the review policies of the IACUC

PROTOCOL CONTROL NUMBER/TITLE

#99-103 - "How do Nectar- and Fruit-Eating Birds Cope with a Sugary and Watery Diet? Integration of Metabolic, Digestive, and Osmoregulatory Processes"

PRINCIPAL INVESTIGATOR/DEPARTMENT

Caros Martinez del Rio - Ecology & Evolutionary Biology

GRANTING AGENCY

NSF and BSF

SUBMISSION DATE **July 26, 1999**

APPROVAL DATE. **August 24, 1999** APPROVAL. VALID THROUGH* **August 23, 2002**

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON **August 25, 1999**

REVISIONS (if any)

MINORITY OPINIONS (if any).

Richard C. Powell

Richard C. Powell, PhD, MS
Vice President for Research

DATE August 25, 1999