The integration of osmoregulation and energy balance in nectar-feeding birds

By

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White-bellied sunbird (*Cinnyris talatala*)

New Holland honeyeater (*Phylidonyris novaehollandiae*)

Declaration

The experimental work described in this thesis was carried out in the Department of Zoology and Entomology, University of Pretoria, South Africa, and in the School of Veterinary and Biomedical Sciences, Murdoch University, Western Australia, from 2006-2010. I, Cromwell Purchase, declare that the thesis, which I hereby submit for the degree Doctor of Philosophy (Zoology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Publications and manuscripts in preparation

In the course of this research, several manuscripts were published. All references are in the format for the Journal of Comparative Physiology B, except chapter three that has been submitted to the Journal of Experimental Biology. A list of these manuscripts follows in chronological order:

Journal publications

Purchase C, Nicolson SW, Fleming PA (2010) Added salt helps sunbirds and honeyeaters maintain energy balance on extremely dilute nectar diets. J Comp Physiol B 180:1227-1234

Purchase C, Nicolson SW, Fleming PA (2013) Salt intake and regulation in two passerine nectar drinkers: whitebellied sunbirds and New Holland honeyeaters. J Comp Physiol B 183(4):501-510

Purchase C, Napier KR, Nicolson SW, McWhorter TJ, Fleming PA (2013) Gastrointestinal and renal responses to variable water intake in whitebellied sunbirds and New Holland honeyeaters. J Exp Biol 216(9):1537-1545

And for experiments run in parallel with those described in Chapter 3:

Napier KR, Purchase C, McWhorter TJ, Nicolson SW, Fleming PA (2008) The sweet life: diet sugar concentration influences paracellular glucose absorption. Biol Lett 4:530-533

Disclaimer

This PhD thesis consists of chapters that have been prepared as stand-alone manuscripts. These manuscripts have all been published in peer reviewed journals. As a consequence, there may be some repetition between chapters.

Thesis summary

Nectar-feeding birds must ingest copious amounts of water due to their liquid diet. Large volumes of preformed water in the dilute diet mean that birds feeding on these diets risk the loss of solutes in order to excrete this water. Previous studies have found that on dilute diets (<0.25 $mol.l^{-1}$), white-bellied sunbirds (*Cinnyris talatala*) are unable to maintain energy balance and lose excessive amounts of electrolytes via cloacal fluid. Therefore how these small nectarivores handle water and electrolytes is intricately linked with how they obtain energy from a nectar diet. Understanding the physiological mechanisms for handling water and electrolytes will reveal how nectarivorous birds can deal with a range of nectar diet concentrations. These mechanisms were investigated through a series of experiments that exposed birds to varying electrolyte and water loads through compensatory feeding (requiring birds to ingest greater volumes of energy-dilute diets than energy-concentrated diets).

I tested the effect of adding electrolytes to a 0.1 $mol.l^{-1}$ sucrose diet in white-bellied sunbirds (*Cinnyris talatala*) and New Holland honeyeaters (*Phylidonyris novaehollandiae*). Addition of salts (NaCl and KCl) enabled both species to drink significantly more of the dilute diet than in the absence of salt. On 20 $mmol.l^{-1}$ combined salts, both sunbirds and honeyeaters consumed an extraordinary 8 times their body mass in fluid daily. KCl alone had no effect on consumption but a loss of Na^+ clearly limits consumption of extremely dilute diets. Plasma Na^+ levels, and sucrose assimilation efficiencies confirmed this, leading to the conclusion that Na^+ depletion on very dilute salt-free diets interferes with water excretion or sugar digestion and/or assimilation.

I then evaluated the behavioural responses of these two nectarivore species to salt solutions. Preference tests (simultaneously presenting birds with a range of diets with salt added and repeating this experiment with different sugar concentration base solutions) showed that both species ingested similar amounts of all diets when fed the concentrated

base solutions (i.e. low total intake). However, when the birds had to increase their intake of more dilute sucrose diets to maintain energy balance, they avoided the higher salt concentrations. Through active diet switching, birds maintained constant intakes of both sucrose and sodium.

To test renal concentrating abilities of these two nectarivores, I conducted no choice tests, by feeding them 0.63 $mol.l^{-1}$ sucrose containing 5-200 $mmol.l^{-1}$ NaCl over a 4 h trial. In both species, cloacal fluid osmolalities increased with diet NaCl concentration, but while sunbirds excreted all the Na^+ ingested, honeyeaters retained sodium on the more concentrated diets. The kidneys of sunbirds and honeyeaters, like those of hummingbirds, are well suited to diluting urine; however unlike hummingbirds, sunbirds and honeyeaters also appear to concentrate urine efficiently when necessary.

The final part of this thesis examined how these birds deal with excess preformed water loads on dilute nectar diets. I used the elimination of intramuscular-injected [^{14}C]-L-glucose and ^3H_2O to quantify intestinal and renal water handling on diets varying in sugar concentration. Both species showed significant modulation of intestinal water absorption, allowing excess water to be shunted through the intestine on dilute diets and therefore reducing renal load. During the natural overnight fast, both sunbirds and honeyeaters arrested whole kidney function, shutting down *GFR* as another way of reducing renal load. Both sunbirds and honeyeaters are able to maintain osmotic balance on markedly different diet concentrations and hence preformed water loads, by varying intestinal water absorption as well as excretion via the intestine and kidneys.

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General introduction and outline of the study

Nectar-feeding birds must deal with copious watery diets, deficient in ions and protein, to obtain the bulk of their energy requirements (Köhler et al. 2012). Compensatory feeding, in which birds increase their intake of more dilute nectars in order to maintain energy intake (Martínez del Rio et al. 2001) results in variable and sometimes massive water loading. Nectar-feeding birds are small, with high metabolic rates, requiring the efficient extraction of both energy and nutrients from a dilute food source passing rapidly through the gut (Beuchat et al. 1990). This thesis focuses on two species of nectar-feeding birds, a sunbird and a honeyeater, examining their ingestion and processing of diets of highly variable water and ion content, and the roles of the intestine and kidneys in dealing with excess water. While previous research has focussed extensively on energy regulation of nectar-feeding birds, here the emphasis is on high water loads (coupled with low dietary electrolyte content) and how this affects their digestion and osmoregulation.

Avian nectarivores and their diet

There are three distinct evolutionary lineages of specialised avian nectarivores: hummingbirds (Trochilidae) of the Americas, sunbirds (Nectariniidae) in Africa and Asia, and honeyeaters (Meliphagidae) in Australasia (Nicolson and Fleming 2003b). Adaptations to nectar feeding show convergent evolution in these families: long curved or straight bills, specialised tongues, and an intestinal and renal system adapted to efficiently managing a nectar diet. Hummingbirds are the oldest and most speciose family, and also the smallest birds (Pyke 1980), weighing 2-20 g (Cotton 1996). Sunbirds are slightly larger, weighing 5-22 g (Cheke and Mann 2001), and honeyeaters are the largest specialised nectarivores, weighing 8-250 g (Pyke 1980). There are other families of birds that depend on nectar to a lesser degree. These include the Hawaiian honeycreepers, flower-piercers, tanagers, and

lorikeet parrots, together with many species that feed on nectar opportunistically such as white-eyes, bulbuls, barbets, mousebirds, and starlings (Lotz and Schondube 2006; Nicolson and Fleming 2003b; Symes et al. 2008).

Plant nectars contain simple sugars, easily digested and rich in energy, in the form of sucrose and its components glucose and fructose (Nicolson and Fleming 2003b). Nectar may also contain other sugars, such as xylose, which remains puzzling because most pollinators are averse to this sugar (Jackson and Nicolson 2002). Other minor components of nectar include inorganic ions, proteins, amino acids and lipids (Nicolson and Thornburg 2007). Secondary compounds, such as alkaloids, phenolics and terpenoids, may act as a repellent to some nectar consumers, while attracting others specific to the plants' needs (Adler 2000). Nectar from bird-pollinated plants is a poor source of nitrogen, even allowing for the fact that nectarivorous birds have low nitrogen requirements compared with other bird species (Brice 1992; Roxburgh and Pinshow 2000; Van Tets and Nicolson 2000). However, some South African bird-pollinated plants (species of *Aloe* and *Erythrina*) contain relatively high levels of amino acids (Nicolson 2007). Specialised passerines such as sunbirds and hummingbirds visit plants with low nectar volumes, fairly dilute nectars, and predominantly sucrose as the sugar source, while flowers adapted to generalised bird pollinators are characterised by larger volumes, extremely dilute nectars and low sucrose content (Johnson and Nicolson 2008).

A frequently asked question in pollination ecology is why bird pollinated flowers produce dilute nectar. When comparing nectar concentrations of bird and bee pollinated plants, Pyke and Waser (1981) found that hummingbird and honeyeater pollinated flowers were in the 20-25% sugar range, while bee pollinated flowers had a mean sugar concentration of 36%. Several hypotheses have been proposed to account for the low

concentrations of bird nectars (Johnson and Nicolson 2008; Nicolson 2002; Pyke and Waser 1981). Firstly, because viscosity increases exponentially with increasing sugar concentration, it was suggested (Baker 1975) that low concentrations are necessary for birds to extract the nectar efficiently from the flowers. Using the inert polysaccharide Tylose to increase the viscosity of artificial nectar, Köhler et al. (2010a) found that licking frequencies and tongue loads of sunbirds were reduced at high viscosities, while lick duration increased: the rate of nectar ingestion is determined by viscosity. Other hypotheses are that dilute nectars may discourage bees (Bolten and Feinsinger 1978); that the water needs of the birds might influence the nectar concentration, with Calder (1979) predicting an inverse relationship between ambient temperature and nectar concentration; and that dilute diets are secondary consequences of deep tubular flowers, where nectar is protected from evaporation (Plowright 1987). Lastly, because nectar originates from sucrose-rich phloem sap, Nicolson (2002) suggested that hydrolysis of sucrose increases nectar osmolality and the resulting water influx dilutes the nectar. The interacting chemical and microclimatic factors that influence nectar concentration are discussed by Nicolson and Thornburg (2007).

Although nectar-feeding birds have low nitrogen requirements, they do need more than is available in nectar. They thus need to consume both pollen and especially arthropods to make up the extra nitrogen and salt requirements (Stiles 1995). Insect hawking is energetically expensive, but important for gaining enough nitrogen and ions to survive.

Physiological challenges facing avian nectarivores

Even seemingly small differences in nectar concentration can have substantial effects on water and energy balance in nectarivores (Martínez del Rio et al. 2001; Nicolson 1998). Compensatory feeding, where volumetric intake is adjusted to maintain a consistent energy intake has been shown in a variety of avian nectarivores: sunbirds (Lotz 1999; Nicolson and Fleming 2003a), hummingbirds (López-Calleja et al. 1997; McWhorter and Martínez del Rio 1999), honeyeaters (Collins et al. 1980a; Collins 1981) and lorikeets (Karasov and Cork 1996). When fed a range of sugar concentrations, white-bellied sunbirds *Cinnyris talatala* adjusted their food intake to maintain energy balance, but this compensation was not effective on the most dilute diets (0.07 and 0.1 $mol.l^{-1}$), when sunbirds were water-loaded and unable to maintain energy balance (Nicolson and Fleming 2003a). Nectar concentrations as low as 0.1 $mol.l^{-1}$ are not common in the field; however, in rainy weather where flowers are unprotected from the elements, these low nectar concentrations have been recorded (Nicolson and Thornburg 2007). The large variation in nectar concentration between plant species and in different environmental conditions suggests that nectarivores must be extremely dynamic in their foraging techniques and have an extraordinary ability to absorb nutrients from their dilute nectar source. Past research has shown that sunbirds and hummingbirds have similar apparent sucrose assimilation efficiencies, extracting >99% of ingested sugars even when water fluxes are high (Jackson et al. 1998; Köhler et al. 2010b; McWhorter et al. 2004; Roxburgh and Pinshow 2002). These highly efficient mechanisms of sucrose assimilation involve uptake of the monsaccharides glucose and fructose by both passive and active pathways. Paracellular absorption involves movement of solutes by diffusion or solvent drag through the tight junctions that adjoin cells (Karasov and Cork 1994). This route of absorption is important in birds, including nectar-feeding birds (Caviedes-Vidal et al. 2007; Karasov and Cork 1994; Napier et al. 2008). Mediated glucose absorption may be used more on dilute

diets, probably because the concentration gradient is no longer steep enough for efficient transport of glucose from the gastrointestinal tract (GIT) lumen to the cytosol (Napier et al. 2008).

Ion regulation when electrolytes are low

Calder and Hiebert (1983) showed that rufous hummingbirds (*Selasphorus rufus*) excrete and therefore must replace approximately 14% of their total body electrolytes per day when feeding on dilute nectar sources, even though these birds were able to produce extremely dilute urine with an osmotic concentration, 15-24% of plasma concentration. The large volumes of nectar consumed by avian nectarivores, coupled with the low ionic concentrations typically observed in nectars, require extremely efficient regulation of electrolytes. When they are fed salt-free diets, both hummingbirds and sunbirds can recover all but trace amounts of Na^+ and K^+ from excreted fluid (Calder and Hiebert 1983; Fleming and Nicolson 2003; Lotz 1999; Lotz and Martínez del Rio 2004).

Cloacal fluid volume and osmolality of white-bellied sunbirds was shown to vary substantially on sucrose diets of varying concentration (0.07 to 2.5 $mol.l^{-1}$) (Fleming and Nicolson 2003). On the most dilute diets (0.07 and 0.1 $mol.l^{-1}$) tested, the electrolyte outputs were the highest, and electrolyte outputs increased with increasing cloacal fluid volume. This apparent electrolyte washout combined with the inability of sunbirds to maintain energy balance on extremely dilute diets devoid of electrolytes (Nicolson and Fleming 2003a), warranted further attention.

Ion regulation when electrolytes are high

For birds in general, most ion regulation experiments have involved birds subjected to dehydration. The response of sunbirds to an increase of salts in their diet has not yet been tested. Rufous hummingbirds on high salt diets are unable to excrete all the excess salts, retaining ions when NaCl in their diets exceeds 35 mM (Lotz and Martínez del Rio 2004). Fleming and Nicolson (2003) found that on dilute diets, sunbirds produce cloacal fluid with some of the lowest solute concentrations recorded for birds, but could also shut down water excretion on concentrated diets. Furthermore, on concentrated sucrose diets, sunbirds reduced cloacal fluid production and retained osmolytes, which were excreted only during rehydration (Fleming et al. 2004).

Water regulation under varying water loads

The remarkable ability of avian nectarivores to maintain energy and ion balance on a large range of nectar concentrations is due to an efficient renal system and GIT. On dilute diets, the increased need for mediated glucose absorption as well as processing by the kidneys, creates an energetic problem for avian nectarivores. As a way of saving energy while consuming copious amounts of dilute nectars, Beuchat et al. (1990) proposed the hypothesis of partial kidney bypass or water shunting through the gut in hummingbirds. This hypothesis of modulation of water absorption in the gut has subsequently been tested using pharmacokinetic techniques to estimate the fraction of ingested water that is absorbed. These studies reveal that hummingbirds do not modulate intestinal water absorption in order to bypass the kidneys (Hartman Bakken and Sabat 2006; McWhorter and Martínez del Rio 1999). In contrast, Palestine sunbirds (*Cinnyris osea*) are able to absorb as much as 64% of ingested water load when feeding on dilute diets (McWhorter et al. 2003).

Hummingbird and honeyeater kidneys have few mammalian-type long-looped, fluid concentrating nephrons and a poorly developed renal medulla (Beuchat et al. 1999; Casotti et al. 1993; Casotti and Richardson 1992; Casotti et al. 1998). Their kidney design seems to be more for recovering solutes from large quantities of plasma, which, it has been argued, may limit their urine concentrating ability (Beuchat et al. 1990; Goldstein and Skadhauge 2000; Lotz and Martínez del Rio 2004). Sunbird renal morphology has not been described. Goldstein and Bradshaw (1998) suggested that intestinal modulation of water absorption in honeyeaters might supplement the osmoregulatory roles of water reabsorption in the kidneys and postrenal modification. In order to test the gut and renal capacities in such small nectar-feeding birds, a modification of the single-injection slope-

intercept method was developed to measure glomerular filtration rate (GFR)(McWhorter and Martínez del Rio 1999).

Another potential way of eliminating excess water is through evaporative water loss (EWL). Birds have the ability to modulate EWL in response to heat stress by panting and controlling cutaneous evaporation (McKechnie and Wolf 2004; Wolf and Walsberg 1996). In nectarivorous honeyeaters, EWL is significantly affected by both temperature (Collins et al. 1980b) and diet concentration (Collins 1981). EWL has been estimated gravimetrically in two species of honeyeaters (*Acanthorhynchus superciliosis* and *Lichmera indistinca*) (Collins 1981), southern double collared sunbirds (Lotz 1999), and whitebellied sunbirds (Fleming and Nicolson 2003), increasing when birds consumed a more dilute sucrose diet.

Study species and objectives

My research focuses on two passerine avian nectarivores from different continents: the African white-bellied sunbird *Cinnyris talatala* (previously *Nectarinia talatala*; Nectariniidae), and the Australian New Holland honeyeater *Phylidonyris novaehollandiae* (Meliphagidae). These families were chosen for their similar diets and co-evolutionary characteristics, hummingbirds are a well studied species in this research field and are thus a good for literature comparisons. We felt that important research was lacking in these 2 families (sunbirds and honeyeaters) that warranted our investigation. Due to similar climates and habitats, sunbirds and honeyeaters should have more common characteristics than hummingbirds. All the research described was carried out on both species, with experiments on sunbirds carried out at the University of Pretoria and on honeyeaters at Murdoch University, Perth.

The focus of Chapter 1 is on extremely dilute diets, exploring the use of added salt to test whether this enables nectar-feeding birds to maintain energy balance on such diets. With the knowledge that Na^+ is also needed in the active uptake of glucose, I hypothesised that ion management is the limiting factor when birds are water loaded, due to the extra potential for electrolyte losses, and expected these birds to stop drinking dilute sucrose diets due to their plasma ion levels reaching critical levels.

Chapter 2 focuses on a wider range of sucrose and salt concentrations. The first experiment examined preferences of birds offered a choice of four diets at a time containing 0 - 75 $mmol.l^{-1}$ NaCl. The experiment was repeated using five sucrose concentrations (0.075 - 0.63 $mol.l^{-1}$) as the base solution, to see whether sucrose concentration determines the preferences for salt intake. I hypothesised that both sunbirds and honeyeaters would actively choose diets with added salt on the dilute sucrose solutions, but would avoid the salty diets on more concentrated sucrose solutions. The second experiment was a no choice salt loading test, with birds given 0.63 $mol.l^{-1}$ sucrose containing varying concentrations of NaCl from 5 - 200 $mmol.l^{-1}$. These concentrations were used to enable a direct comparison with the study on rufous hummingbirds (Lotz and Martinez del Rio 2004). Ion regulating abilities of the birds on diets containing high salt concentrations were examined by measuring Na^+ and K^+ concentrations and osmolality of cloacal fluid and ureteral urine. I hypothesised that both sunbirds and honeyeaters would be able to concentrate their urine better than hummingbirds.

In chapter 3, I used pharmacokinetics to examine water handling in the gut and kidney of the two nectarivore species, using intramuscular injections of ^3H_2O and C^{14} L-glucose. I measured the elimination rates of both isotopes and calculated water flux, water absorption in the gut, water turnover rate, GFR, fractional water reabsorption in the kidney

and total evaporative water loss. Of special interest was whether water was shunted through the GIT to avoid the necessity for renal processing. The pharmacokinetic methods also enabled EWL to be estimated. I hypothesised that due to their larger size compared with hummingbirds (which can resort to torpor when energetically challenged), sunbirds and honeyeaters would require more efficient mechanisms in handling excessive water loads and therefore were likely to shunt water through their GIT.

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Chapter 1: Added salt helps sunbirds and honeyeaters maintain energy balance on extremely dilute nectar diets

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Abstract

Nectar-feeding birds ingest excess water and risk loss of solutes when they excrete it. Previous work has shown that nectarivores are unable to maintain energy balance on extremely dilute sucrose diets without salts (e.g. <0.25 mol.l^{-1}), and that they lose more electrolytes (i.e. Na^+ and K^+) via cloacal fluid on these diets than on more concentrated diets. Using white-bellied sunbirds and New Holland honeyeaters (*Phylidonyris novaehollandiae*) we tested the effect of adding electrolytes to a 0.1 mol.l^{-1} sucrose diet, by including equimolar NaCl and KCl at concentrations from 5–40 mmol.l^{-1} and the individual salts at 20 mmol.l^{-1}. Addition of salts enabled both species to drink significantly more of the 0.1 mol.l^{-1} sucrose diet than in the absence of salt, and mass loss during the experiment was reduced when salt was included. On 20 mmol.l^{-1} combined salts, both sunbirds and honeyeaters consumed 8 times their body mass in fluid daily. KCl alone had no effect. Birds are thus limited in their consumption of extremely dilute diets by increasing losses of Na^+. This was confirmed by measuring plasma Na^+ levels, which decreased in both species in the absence of dietary Na^+. In addition, sucrose assimilation efficiencies were significantly lower when sunbirds were fed salt-free diet, while glucose levels in ureteral urine remained extremely low. It is concluded that Na^+ depletion on very dilute salt-free diets does not affect Na^+-glucose transport activity in the kidney, but interferes with sugar digestion and/or assimilation in the intestine.

Key words: nectarivory; low Na^+ diets; Na^+-linked glucose transporter

Introduction

Nectars of bird-pollinated flowers are relatively dilute compared to those of insect-pollinated flowers (Nicolson 2002; Pyke and Waser 1981; Stiles and Freeman 1993). On average, nectars consumed by sunbirds in Africa are similar to those of hummingbird-visited plants in the Americas in volume, concentration and sugar composition, with concentrations lying in the range 15-25 % w/w, or 0.46-0.81 $mol.l^{-1}$ sucrose equivalents (Johnson and Nicolson 2008). However, nectar concentrations may vary dramatically both within and between plant species, due to factors such as flower morphology, evaporation, and plant phylogeny (Nicolson and Fleming 2003b; Nicolson and Thornburg 2007).

Avian nectarivores cope with variations in nectar concentration by compensatory feeding, in which volumetric consumption is adjusted according to diet concentration in order to maintain a stable energy intake. Compensatory feeding has been demonstrated in the three main lineages of nectar-feeding birds: honeyeaters, hummingbirds and sunbirds (Collins et al. 1980; López-Calleja et al. 1997; Martínez del Rio et al. 2001; Nicolson and Fleming 2003a). Consequently, on very dilute diets, these birds must drink extraordinarily large volumes of water: several times their body mass in water daily. When fed sucrose solutions ranging in concentration from 2.5 to 0.25 $mol.l^{-1}$, white-bellied sunbirds *Cinnyris (Nectarinia) talatala* (~ 9 g) adjust their intake from ~ 4 $ml.day^{-1}$ to 32 $ml.day^{-1}$ in order to deal with the increasingly dilute diets (Nicolson and Fleming 2003a). However, when offered more dilute solutions (0.1 and 0.07 $mol.l^{-1}$), although they drink more, the birds can not increase their intake sufficiently to maintain energy balance. When offered such dilute diets, broadtailed hummingbirds *Selasphorus platycercus* respond by going into torpor (Fleming et al. 2004).

As a consequence of high water loads, avian nectarivores produce copious and dilute cloacal fluid compared to other birds (Fleming and Nicolson 2003; Lotz and Martínez del Rio 2004). Renal fractional water reabsorption has been shown to decrease substantially with increasing water load in honeyeaters, sunbirds and hummingbirds (Goldstein and Bradshaw 1998b; Hartman Bakken and Sabat 2006; McWhorter et al. 2004). Recovery of solutes from the excreted fluid is impressive, but the huge volumes of water excreted on extremely dilute diets mean that electrolyte output increases significantly with increasing water flux. For example, on the most dilute diets (0.07 and 0.1 $mol.l^{-1}$ sucrose) tested by Fleming and Nicolson (2003), the total electrolyte outputs of white-bellied sunbirds were higher than when when they were fed more concentrated (0.25 – 2.5 $mol.l^{-1}$) diets. In red wattlebirds *Anthochaera carunculata*, Goldstein and Bradshaw (1998b) demonstrated that higher urine flows lead to increased Na^+ excretion because the fraction of filtered Na^+ does not vary with diet concentration. In addition to this electrolyte depletion, physiological limitations to the intake of dilute nectars may include constraints on digestive processes (especially due to rapid gut transit times), the increased energetic costs of electrolyte and glucose recovery, and steeply increasing costs of warming food to body temperature (Beuchat et al. 1990; Fleming and Nicolson 2003; Lotz et al. 2003).

In this study, we examined the effect of adding salt (NaCl and KCl) to very dilute diets (0.1 $mol.l^{-1}$ sucrose) provided to two nectarivore species belonging to different families: African white-bellied sunbirds (*Cinnyris talatala*, Nectariniidae) and Australian New Holland honeyeaters (*Phylidonyris novaehollandiae*, Meliphagidae). We tested the hypothesis that salt depletion prevents these birds from consuming enough food to maintain their energy balance on extremely dilute sugar diets, by measuring food consumption, changes in body mass, plasma Na^+ levels and sugar assimilation efficiencies.

This study shows constraints upon sugar absorption in sunbirds and honeyeaters consuming dilute nectar diets.

Materials and methods

Bird capture and maintenance

Eight white-bellied sunbirds (body mass, 8.8±1.2 SD g) and eight New Holland honeyeaters (body mass, 20.4±1.5 SD g) were captured by mist netting, in Jan Celliers Park in Pretoria and on Murdoch University campus in Perth, respectively. Birds were housed in individual cages (sunbirds: 45 x 45 x 32 cm; honeyeaters: 46 x 56 x 45 cm) at 20 ± 1 °C with an automatic photophase (sunbirds: 0700 to 1900; honeyeaters: 0600 to 1800). Both species were fed a maintenance diet *ad libitum*. Sunbirds received 0.63 $mol.l^{-1}$ sucrose and 2% Ensure® (Abbott Laboratories, Johannesburg, South Africa); honeyeaters received 0.63 $mol.l^{-1}$ sucrose and 15% Wombaroo® powder (Wombaroo Food Products, Adelaide, Australia). The diet was provided in inverted, stoppered syringes hung on the cage sides, from which the birds could feed *ad libitum*. Water was similarly supplied *ad libitum*.

The Gauteng Directorate of Nature Conservation granted permits to capture and house the sunbirds, and the Australian Department of Environment and Conservation approved our use of honeyeaters. All animal care procedures and experimental protocols adhered to institutional regulations of the University of Pretoria (reference number EC013-07) and Murdoch University (reference number R1137/05).

Experimental procedures

Experimental diets consisted of a 0.1 mol.l^{-1} sucrose solution with no added salts, or solutions that included a 1:1 molar mix of NaCl : KCl made up to total concentrations of 2, 10, 20, and 40 mmol.l^{-1} for sunbirds and 10, 20 and 40 mmol.l^{-1} for honeyeaters. Sunbirds were tested on more diets than honeyeaters as the sunbird trials were performed first and we could not predict the effect of added salt on consumption. NaCl and KCl were also tested separately at 20 mmol.l^{-1} each. The 0.1 mol.l^{-1} sucrose diet was choosen as it had been shown in previous research as the point where sunbirds could not consume enough of the diet to maintain energy balance (Nicolson and Fleming 2003a). Individual birds received each experimental diet in random order. Each diet was given for two consecutive days; the first to acclimate the birds to that diet and the second being the test day. Birds were given at least two recovery days on maintenance diet between trials, in order to recover body mass (since the experimental diet was so dilute and lacked protein, Nicolson and Fleming 2003a). In addition to the trials with and without salts, we also investigated compensatory feeding in New Holland honeyeaters (this has already been done in white-bellied sunbirds: Nicolson and Fleming, 2003a). Four sugar-only diet concentrations (0.25, 0.5, 0.75 and 1 mol.l^{-1} sucrose) were examined under the same conditions as the experimental salt diets.

During trials a drip cup containing liquid paraffin was placed below each feeder to measure any spilt diet. Food consumption was measured by weighing (Mettler Toledo PB602S, ±0.01 g, Microsep Ltd., Johannesburg) the feeders and drip cups before and after the test period. Spillage, subtracted from consumption data, was minimal at 0.21 ± 0.23 ml over the 24 h test period, equivalent to 0.32% of mean consumption. Body mass was

monitored by weighing birds at lights-on (sunbirds: 7:00; honeyeaters: 6:00) every morning.

Measurement of plasma Na^+ and K^+ concentrations

In order to assay plasma electrolyte concentration, a small blood sample was collected in heparinised microcapillary tubes by puncture of the brachial vein (using a 23 gauge needle) after birds had fed on three 0.1 $mol.l^{-1}$ sucrose test diets (no salt, 10 $mmol.l^{-1}$ and 20 $mmol.l^{-1}$ mixed salts). All blood samples were taken directly after the trial period and sample collection was consistent for both species and collected by the same person. Blood samples from sunbirds and honeyeaters (n=8 each) were spun in a microcapillary centrifuge and plasma samples were then analysed by flame photometry (model 420, Sherwood Scientific Ltd., Cambridge, UK).

Assimilation efficiencies and glucose concentrations in ureteral urine

To test the effect of added salt on sugar assimilation, eight white-bellied sunbirds were fed two 0.1 $mol.l^{-1}$ sucrose solutions (no salt, 20 $mmol.l^{-1}$ NaCl) for 6 h. Food consumption during this period was measured by weighing feeders. Cloacal fluid was collected under liquid paraffin, then pooled and its volume measured; a small volume of rinse water was used to aid with collecting solutes from the sample. At the end of the 6 h experimental period, ureteral urine samples were collected using a closed-ended cannula to prevent contamination from the cloacal fluid. A polyethylene flexible tube was melted closed on one end and smoothened to prevent any sharp edges, a small hole (semi-circle) was sliced in one side of the cannula just under 1cm from the closed end. The closed end was inserted into the cloaca to block any cloacal fluid contamination and the hole on the

side of the cannula was aligned with the ureter. Sucrose, fructose and glucose assays were then performed on the cloacal fluid samples, whilst volumes of the ureteral urine samples were sufficient for glucose assays only. Sugar assays were carried out using sucrose assay reagent, glucose (HK) assay reagent and phosphoglucose isomerase for fructose assay kit (Sigma-Aldrich Product Codes S 1299, G 3293 and F 2668). A standard curve dilution series was produced for each assay, and samples were read at 340 nm using a spectrophotometer (Biochrom Libra S12, Biochrom Ltd., Cambridge, England).

Assimilation efficiency (AE) was estimated:

$$AE = (sugar_{in} - sugar_{out}) / (sugar_{in})$$

where $sugar_{in}$ (mg) is the concentration (mg ml^{-1}) of sugar in the ingested diet multiplied by the volume of food ingested (ml), and $sugar_{out}$ (mg) is the sugar concentration (mg ml^{-1}) in the total volume of excreta plus rinse water (ml). For the calculation of AE* of glucose and fructose, $sugar_{in}$ was calculated as:

$$glucose_{in} \text{ or } fructose_{in} = (sucrose_{in} - sucrose_{out}) / 2$$

Statistical analysis

Repeated-measures ANOVA was used to test for effects of salt concentration on food intake, changes in body mass and plasma ion concentrations, as well as to compare sugar assimilation efficiencies, and glucose concentrations in cloacal fluid and ureteral urine, on diets with and without added salt. *Post hoc* comparisons were carried out using Tukey's Honest Significant Difference (HSD) test. For comparison of compensatory feeding data, the total sucrose intake (g sugar per g body mass per 24 h) was calculated for

each diet; these data were also analysed by RM-ANOVA. For all statistical tests, the level of significance was $P \leq 0.05$, and data are presented as means ± 1 SD.

Results

Food consumption

Addition of salt resulted in a significant increase in consumption of a 0.1 mol.l^{-1} sucrose diet by white-bellied sunbirds (Fig. 1.1A; $F_{1,29} = 33.00$, $P < 0.001$). There was no significant difference in the amounts of the higher concentration (10, 20 and 40 mmol.l^{-1}) mixed salt diets consumed, but the sunbirds drank significantly more of these three diets than the no salt diet. The 2 mmol.l^{-1} mixed salt diet was not significantly different from the no salt diet. When salts were tested individually, significantly more of the 20 mmol.l^{-1} NaCl diet was consumed compared with the 20 mmol.l^{-1} KCl diet ($F_{1,13} = 38.30$, $P < 0.001$). Furthermore, there was no significant difference in consumption between the 20 mmol.l^{-1} NaCl and the high concentration (10, 20 and 40 mmol.l^{-1}) mixed salt diets. By contrast, consumption of the 20 mmol.l^{-1} KCl diet was not different from that of the no salt and 2 mmol.l^{-1} mixed salt diets.

Similar results were obtained with New Holland honeyeaters (Fig. 1.1B). There was no significant difference in the amount of the higher concentration (20 and 40 mmol.l^{-1}) mixed salt diets consumed, but the honeyeaters consumed significantly more of these two diets than of the no salt and 10 mmol.l^{-1} mixed salt diets ($F_{1,29} = 32.77$, $P < 0.001$). When salts were tested individually, significantly more of the 20 mmol.l^{-1} NaCl diet was consumed, compared with the 20 mmol.l^{-1} KCl diet ($F_{1,13} = 57.20$, $P < 0.001$) which was not different from the no salt diet.

Mass loss

Sunbirds showed a significant effect of the addition of salt upon change in body mass over 24 h (Fig. 1.2A; $F_{3,48} = 59.89$, $P < 0.001$). Mass loss was significantly greater on no salt and 20 mmol.l^{-1} KCl diets compared to diets with 20 and 40 mmol.l^{-1} mixed salt and 20 mmol.l^{-1} NaCl ($P < 0.05$). Mass loss on the 2 and 10 mmol.l^{-1} mixed salt diets did not differ significantly from that on any other diet.

Honeyeaters also showed a significant effect of salt addition upon change in body mass during these trials (Fig. 1.2B; $F_{5,35} = 15.50$, $P < 0.001$). Honeyeaters lost significantly greater mass over 24 h on diets with no salt and 20 mmol.l^{-1} KCl compared to 20 and 40 mmol.l^{-1} mixed-salt and 20 mmol.l^{-1} NaCl diets ($P = 0.005$). Mass loss on the 10 mmol.l^{-1} mixed salt diet did not differ significantly from that on any other diet.

Role of added salt in compensatory feeding

Daily energy intake for white-bellied sunbirds consuming 0.25-2.5 mol.l^{-1} sucrose solutions averages 2.77 ± 0.42 g sugar (0.313 ± 0.038 g sugar · g body mass^{-1} day^{-1}, Nicolson and Fleming 2003, Fig. 1.3A). These earlier data were collected under similar housing and temperature conditions to those used in the present study. Comparable levels of energy consumption were achieved on a 0.1 mol.l^{-1} sucrose diet only in the presence of 20 mmol.l^{-1} NaCl (Fig. 1.3A). Sunbirds did not consume sufficient volumes of the no salt or 20 mmol.l^{-1} KCl diets to ingest comparable quantities of sugar.

Food intake was similarly measured over a range (0.25 to 1 mol.l^{-1}) of sucrose diets for New Holland honeyeaters (Fig. 1.3B). Over this dietary range, the birds maintained a steady intake of 5.67 ± 0.70 g sugar per day (0.278 ± 0.034 g sugar · g body mass^{-1} day^{-1}), with no significant mass loss. As for the sunbirds, the only 0.1 mol.l^{-1} sucrose diet on which honeyeaters could attain comparable levels of energy intake was that containing 20 mmol.l^{-1} NaCl. On both the no salt and 20 mmol.l^{-1} KCl diets, volumes ingested were insufficient to meet daily energy intake.

Plasma Na^+ and K^+ levels

On dilute (0.1 mol.l^{-1}) sucrose diets, both sunbirds and honeyeaters showed a decrease in plasma Na^+ levels, reflecting NaCl levels in their diet (Fig. 1.4A). In sunbirds ($F_{2,14}$ = 11.17, P = 0.001) plasma Na^+ concentration for birds fed the no salt diet was significantly lower than when the birds were fed 10 mmol.l^{-1} and 20 mmol.l^{-1} NaCl diets; there was no significant difference in Na^+ plasma concentration between the two added salt diets (Fig. 1.4A), due to the amount of fluid excreted on this diet it is reasonable to assume the decrease in plasma Na^+ levels in the blood is not normal and hence a physiological issue for the birds. In honeyeaters ($F_{2,14}$ = 11.08, P = 0.001), plasma Na^+ concentration was significantly lower for birds fed the no salt diet compared with the 20 mmol.l^{-1} NaCl diet; plasma Na^+ concentration was intermediate when the birds were fed on the 10 mmol.l^{-1} NaCl diet. In both sunbirds ($F_{2,14}$ = 2.92, P = 0.087) and honeyeaters ($F_{2,14}$ = 2.37, P = 0.129), there were no significant differences in plasma K^+ concentration across any of the diets (Fig. 1.4B).

Sugar assimilation

Sunbirds showed a significant effect of the addition of salt on sucrose assimilation efficiency ($F_{1,7}$ = 11.20, P < 0.012). Sucrose assimilation efficiency was higher on diets containing added NaCl than on diets devoid of electrolytes (Table 1.1). Glucose and fructose assimilation efficiencies were similarly higher for the salt diets, although the data were not statistically significantly different (concentrations in these samples were extremely low and the data are therefore somewhat variable). Glucose concentrations were significantly higher in cloacal fluid than in ureteral urine ($F_{1,7}$ = 8.40, P = 0.023 on the no salt diet; $F_{1,7}$ = 51.31, P < 0.001 on 20 mmol.l^{-1} NaCl). Glucose was barely detectable in the ureteral urine and was unaffected by the addition of salt: concentrations were 0.18±3.43 µmol·l^{-1} on the no salt diet and 3.04±3.42 µmol·l^{-1} on 20 mmol.l^{-1} NaCl.

Discussion

Addition of salt to a very dilute diet of 0.1 mol.l^{-1} sucrose had a dramatic effect on the volume of food consumed by both sunbirds and honeyeaters, and thus their ability to maintain energy balance and prevent severe mass loss in both species. A small amount of mass loss is expected on the experimental diet due to the lack of protein added, however this weight loss was less than 4 % in both species when 20 mmol.l^{-1} NaCl was added compared to as much as 7 % without NaCl. When fed the 20 mmol.l^{-1} mixed salt diet, sunbirds consumed 73.5±3.3 ml·day^{-1}: this equates to ~ 8.35 times their body mass, higher than for any other nectarivore examined (and possibly the highest food intake recorded for any vertebrate). Honeyeaters on the same diet consumed 160±19 ml·day^{-1} or 7.84 times their body mass. In terms of physiological limitations (mentioned in the introduction), our data do not support the theory that these birds were limited by having to warm the extra food consumed or by digestive constraints due to rapid transit rates (transit rates would

necessarily have increased to accommodate this additional food intake). Clearly, the birds are able to deal with the additional diet due to their improved ability to maintain electrolyte balance. How does the added salt allow sunbirds and honeyeaters to cope with the processing and elimination of such large volumes of water?

Since the addition of KCl alone had no significant effect, the response was obviously not due to an osmotic effect or to the presence of K^+ or Cl^- ions, but due to the presence of Na^+ ions. The mixed salt concentrations can therefore effectively be halved to reflect only Na^+ concentration. Because intake of the 10 mmol.l^{-1} mixed-salt diet (2.21 ± 0.38 g sugar) by sunbirds was not different from that on diets on which these birds were able to maintain energy balance (2.41 ± 0.19 g sugar), a concentration of 5 mmol.l^{-1} Na^+ is sufficient to maintain Na^+ balance in sunbirds on 0.1 mol.l^{-1} sucrose diets. This, however, is not the case for honeyeaters, where 10 mmol.l^{-1} Na^+ is required: consumption of the 20 mmol.l^{-1} mixed salt diet (5.34 ± 0.51 g sugar) by honeyeaters was not different from that of diets on which they were able to maintain energy balance (5.21 ± 0.63 g sugar). The difference in Na^+ concentration needed by sunbirds and honeyeaters may be associated with the relative body sizes of the birds.

In a field study of water and sodium use by Australian honeyeaters (Goldstein and Bradshaw 1998a), plasma Na^+ concentration of free-living New Holland honeyeaters captured in both summer and winter averaged 155 mmol.l^{-1}, while plasma K^+ concentration was 6 mmol.l^{-1}. Similar values were obtained for two other free-living honeyeater species (Goldstein and Bradshaw 1998a), and for both white-bellied sunbirds and New Holland honeyeaters feeding on the 20 mmol.l^{-1} NaCl diet in the present study. Although these data indicate that plasma Na^+ concentration is maintained under a variety of environmental conditions, we found significant declines in both white-bellied sunbirds and New Holland

honeyeaters when NaCl was removed from the diet, as also reported for nectarivorous red wattlebirds on a dilute diet with low Na^+ levels (Goldstein and Bradshaw 1998b). The reduced plasma Na^+ levels in sunbirds and honeyeaters can be interpreted as a result of electrolyte depletion due to huge water fluxes: total electrolyte losses in these birds are higher on dilute diet concentrations than on more moderate concentrations (Fleming and Nicolson 2003; Goldstein and Bradshaw 1998b). Increased aldosterone levels in white-bellied sunbirds fed salt-free diets, measured non-invasively as aldosterone output in the cloacal fluid (Gray et al. 2004), are not sufficient to prevent the hyponatraemia resulting from renal losses of Na^+.

One of the stresses of dealing with a dilute salt-free diet is the challenge of absorbing glucose across epithelia. Sodium ions are involved in the mediated transport of glucose across membranes against a concentration gradient. The sodium-linked glucose transporter 1 (SGLT-1) is located in the brush-border or apical membrane of intestinal enterocytes where it contributes towards glucose absorption from the gut lumen. SGLT-1 transports one glucose molecule along with two Na^+ ions from the intestinal lumen to the cytosol (Scheepers et al. 2004). Renal reabsorption of glucose mainly involves the SGLT-2 transporter, located in the apical membrane of renal tubule epithelial cells, which co-transports one glucose molecule with every Na^+ ion (Scheepers et al. 2004; Wright et al. 2007). This transporter mediates the reabsorption of the bulk (~90%) of the filtered glucose in the proximal convoluted tubule and has the same sodium limitations as SGLT-1 (Wright 2001). However, our data suggest that renal reabsorption of glucose is not a limiting step on low salt diets, since negligible amounts of glucose were present in ureteral urine samples compared with the cloacal fluid samples. Furthermore, glucose concentrations in ureteral urine samples were not affected by the inclusion of salts in the diet, and dietary sodium can not directly affect renal glucose absorption as only filtered sodium can affect

glucose uptake. It is therefore more likely that the intestinal SGLT-1 transporter is involved in the response to added salt.

Sucrose assimilation efficiencies were significantly increased in the presence of additional dietary Na^+. This is an intriguing finding, since we have little understanding of why sodium would improve digestion of sucrose molecules. An indirect effect is possible, however, through product inhibition: the membrane-bound sucrase might be inhibited by the local accumulation of glucose (Gray and Ingelfinger 1966), due to reduced rates of transport via SGLT-1. Another interesting possibility is that sodium may be linked with these birds' abilities to modulate intestinal water absorption on dilute diets [demonstrated in Palestine sunbirds (McWhorter et al. 2003) and greenbacked firecrowns (Hartman Bakken and Sabat 2006)], which reduces the water load upon the kidneys; GFR and renal glucose filtered load in these birds are subsequently relatively low (McWhorter et al. 2004).

In terms of intestinal glucose absorption, we found higher assimilation efficiencies for glucose (and fructose) in the presence of Na^+; however, the differences were not statistically significant due to the high variability of the measurements given the low concentrations we were working with. Although we cannot therefore conclude that salt addition to the diet influenced glucose absorption (and therefore we cannot implicate differences in SGLT-1 efficiency or activity), this area warrants further investigation since there is reasonable evidence from other studies that SGLT-1 transport responds to glucose, Na^+ and water in the diet.

Many mammals show upregulation of SGLT-1 transport in response to changes in dietary glucose concentration, particularly those species that encounter significant and

varying carbohydrate levels in their natural diet (Afik et al. 1995; Ferraris and Diamond 1989). This dietary modulation of mediated glucose transport is less apparent in small passerine birds, where the predominance of passive (paracellular or non-mediated) glucose transport dwarfs any changes in mediated transport (e.g. Caviedes-Vidal and Karasov 1996; Levey and Karasov 1992). In our two study species, the extent of paracellular glucose absorption decreases with increasing diet dilution (Napier et al. 2008). This relationship may be due to changes in retention time of digesta in the intestine with sugar concentration, but the response may also depend on luminal osmolality (Napier et al. 2008). Given that the birds in the present study were drinking extremely dilute diets, the relative importance of mediated glucose uptake may be greater, and the role of Na^+ therefore more evident.

Modulation of SGLT-1 activity by Na^+ concentration should also be considered (Ferraris 2001). In chickens, sodium depletion as a result of a low sodium diet reduces the activity of SGLT-1, with the maximum effect reached after two days of treatment (de La Horra et al. 2001). This down-regulation is rapidly reversed, however: within 4 h of drinking 150 $mmol.l^{-1}$ NaCl, chickens previously fed a low-salt diet show increased intestinal glucose transport (due to an increase in the number of active transporters) and glucose uptake rates that equal those in chickens consuming a high salt diet (Garriga et al. 2000). As well as influencing the expression of glucose transporters on the apical membrane of enterocytes, low luminal Na^+ will directly affect the activity of SGLT-1 transporters in the apical membrane because binding of Na^+ ions to the transporter protein is necessary to induce the conformation change that allows glucose binding (Wright et al. 2007). Finally, plasma AVT in white-bellied sunbirds and red wattlebirds (as in other birds) decreases with decreasing dietary sugar concentration (Goldstein and Bradshaw 1998b; Gray et al. 2004). In chickens SGLT-1 activity is stimulated by the incubation of

intestinal tissue in the presence of AVT, suggesting that reduced SGLT-1 activity due to Na^+ depletion may be linked to reduced AVT levels (de La Horra et al. 2001). Increased aldosterone levels on salt-free diets could also be involved in the modulation of SGLT-1 expression in chicken intestine [(Garriga et al. 2000; Garriga et al. 2001) – but see (de La Horra et al. 2001)].

Clearly sunbirds and honeyeaters have Na^+ retention problems when fed dilute salt-free diets. On such diets, there is a trade-off between energy intake and electrolyte loss: retention of Na^+ is incompatible with processing large volumes of water. With the addition of NaCl to dilute diets, birds are able to consume larger volumes and maintain energy balance. Sodium, water and glucose are absorbed in the intestine and reabsorbed in the kidneys, both organs working together in the same direction to maintain high blood glucose and Na^+ levels, whilst eliminating vast volumes of water. Dilute diets lacking sodium apparently do not limit renal glucose recovery (i.e. via SGLT-2), but there appears to be a Na^+-linked mechanism acting to limit intestinal assimilation of sucrose.

Finally, although our diets were extremely dilute, they are comparable with sugar concentrations recorded for nectar from unprotected flowers after heavy rain (Nicolson and Thornburg 2007). Whilst careful retention of ingested electrolytes may help to maintain osmotic balance (Fleming and Nicolson 2003; Lotz and Martínez del Rio 2004), ions present in floral nectar (Nicolson and Thornburg 2007) or insects contribute to replacement of daily ion losses in avian nectarivores. Even when challenged on a dilute diet completely lacking in electrolytes, however, sunbirds and honeyeaters still manage to maintain extremely high assimilation efficiencies (>99.5%), and appear to be able to cope with extremes of diet dilution.

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Table 1.1 Assimilation efficiencies (AE) of different sugars in white-bellied sunbirds fed 0.1 mol.l^{-1} sucrose diets with and without added NaCl (means±SD, n=8)

	Sucrose AE	Glucose AE	Fructose AE
No salt	99.39±0.34*	99.41±0.64	99.47±0.57
20 mmol.l^{-1} NaCl	99.77±0.12	99.80±0.08	99.80±0.19

* denotes significant difference between diets ($p < 0.05$)

Figures

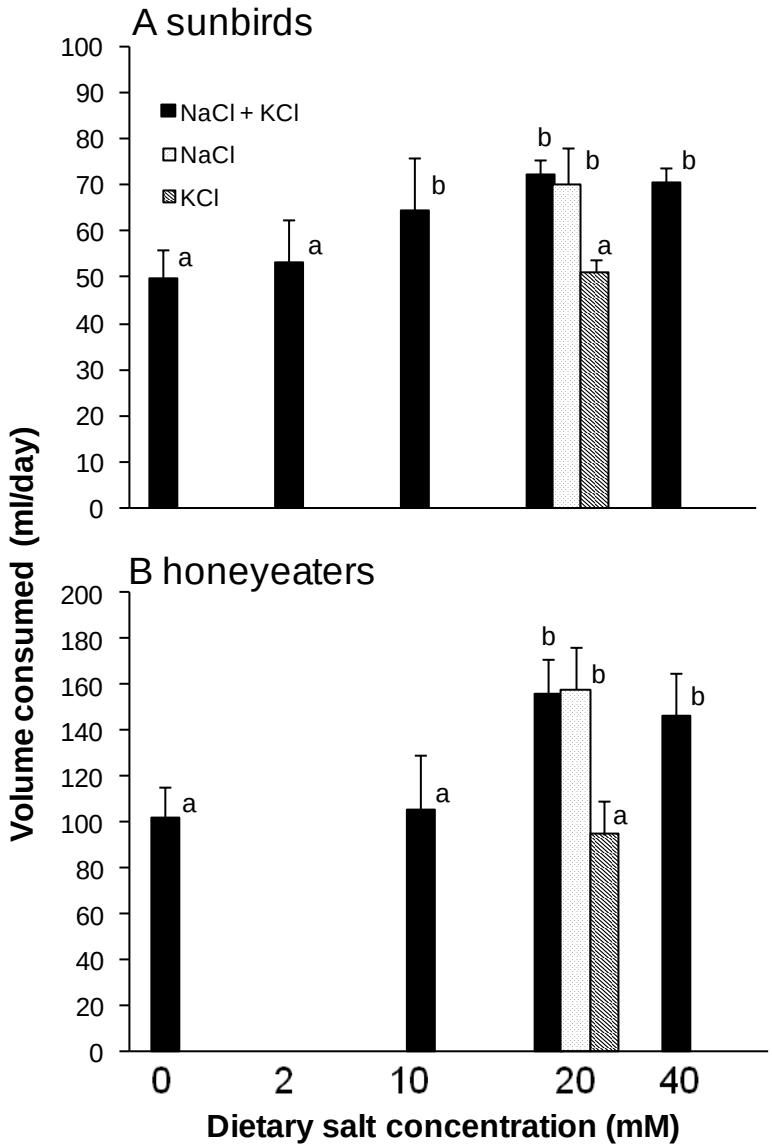

Figure 1.1 Consumption (ml.day^{-1}) by white-bellied sunbirds (A) and New Holland honeyeaters (B) fed 0.1 mol.l^{-1} sucrose solutions with increasing salt concentrations.

Values are means ± SD (n=8). The mixed salt diets have equal molar concentrations of NaCl and KCl made up to the concentration indicated. Statistical significance is annotated by the letters a or b, where no letters in common denote significant differences ($p < 0.001$)

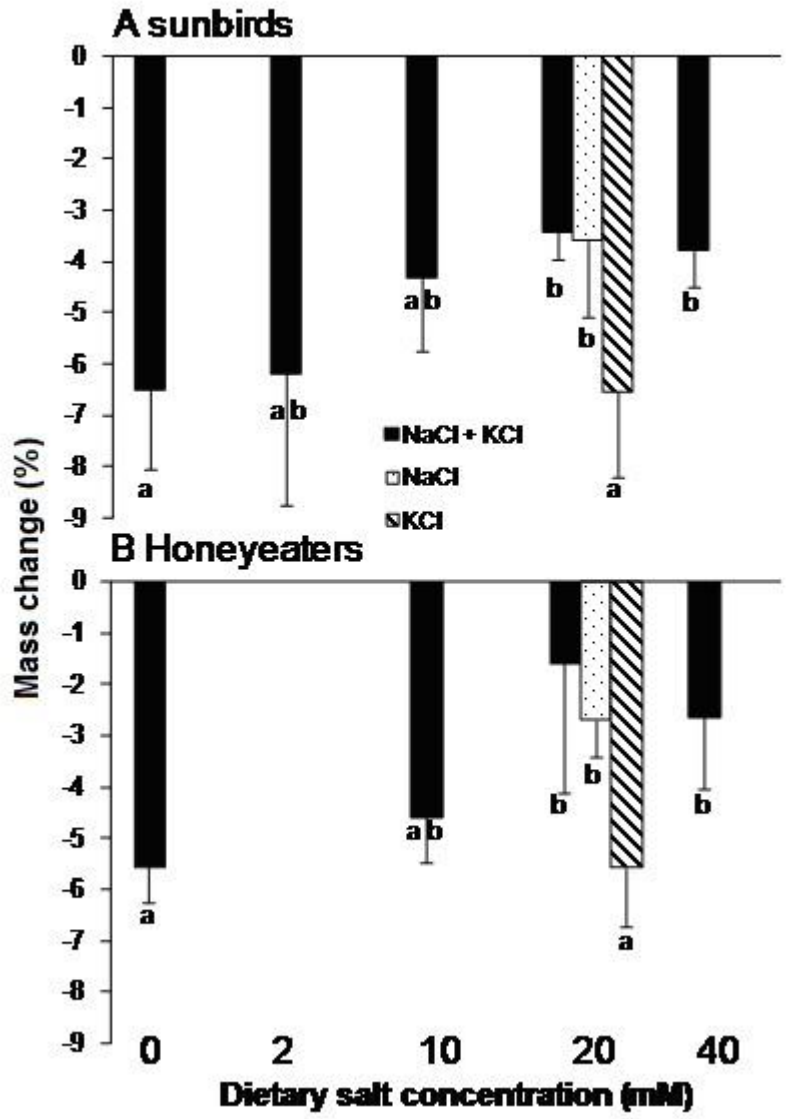

Figure 1.2 Percentage mass loss in white-bellied sunbirds (A) and New Holland honeyeaters (B) consuming 0.1 mol.l^{-1} sucrose solution with increasing salt concentrations. Values are means ± SD (n=8). The mixed salt diets have equal molar concentrations of NaCl and KCl made up to the concentration indicated. Statistical significance is annotated by the letters a or b, where no letters in common denote significant differences ($p < 0.05$)

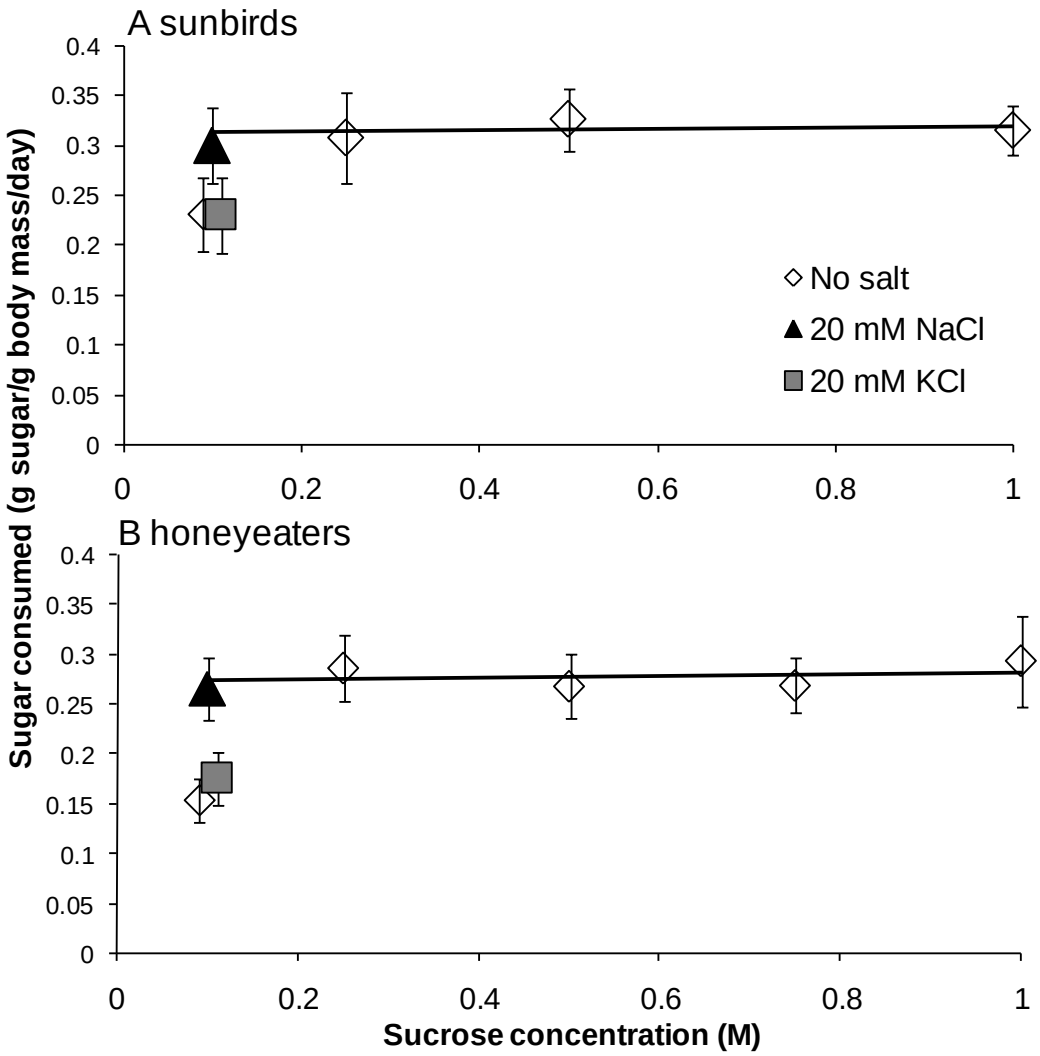

Figure 1.3 Compensatory feeding in white-bellied sunbirds (A, data from Nicolson and Fleming, 2003a), and New Holland honeyeaters (B) compared with data for consumption of 0.1 mmol.l^{-1} sucrose diets with no added salts, 20 mmol.l^{-1} NaCl, or 20 mmol.l^{-1} KCl. The compensatory feeding trend line was fitted for 0.25 – 1 mol.l^{-1} sucrose diets and was extrapolated to estimate energy intake on the lower concentrations. Both sunbirds and honeyeaters consumed sufficient quantities of 20 mmol.l^{-1} NaCl (▲) diet to obtain their daily energy requirement in sucrose, but not when offered the no salt (◊) or 20 mmol.l^{-1} KCl (■) diets. Values are means (± 1SD) for eight individuals for all data on each diet

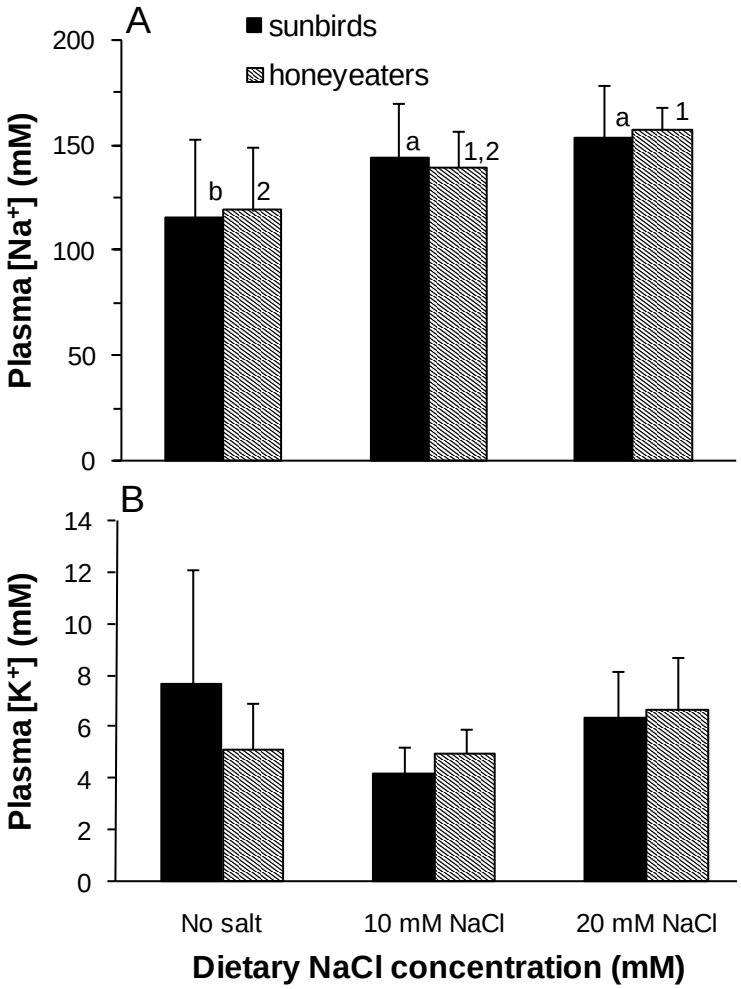

Figure 1.4 Plasma Na^+ (A) and K^+ (B) concentrations (mmol) in white-bellied sunbirds and New Holland honeyeaters fed 0.1 $mol.l^{-1}$ sucrose diets varying in NaCl concentration. Values are means ± SD (n=8). No letters (sunbirds) or numbers (honeyeaters) in common denote significant differences in A ($p < 0.001$). There were no significant differences in plasma K^+ concentration across any of the diets in b

Chapter 2: Salt intake and regulation in two passerine nectar drinkers: white-bellied sunbirds and New Holland honeyeaters

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Abstract

Avian nectarivores face the dilemma of having to conserve salts while consuming large volumes of a dilute, electrolyte-deficient diet. This study evaluates the responses to salt solutions and the regulation of salt intake in white-bellied sunbirds (*Cinnyris talatala*) and New Holland honeyeaters (*Phylidonyris novaehollandiae*). Birds were first offered a choice of four sucrose diets, containing no salt or 25, 50 or 75 mmol.l^{-1} NaCl. The experiment was repeated using five sucrose concentrations (0.075 to 0.63 mol.l^{-1}) as the base solution. Both species ingested similar amounts of all diets when fed the concentrated base solutions. However, when birds had to increase their intake to obtain enough energy on the dilute sucrose diets, there was a general avoidance of the higher salt concentrations. Through this diet switching, birds maintained constant intakes of both sucrose and sodium; the latter may contribute to absorption of their sugar diets. A second, no-choice experiment was designed to elucidate the renal concentrating abilities of these two nectarivores, by feeding them 0.63 mol.l^{-1} sucrose containing 5-200 mmol.l^{-1} NaCl over a 4 h trial. In both species, cloacal fluid osmolalities increased with diet NaCl concentration, but honeyeaters tended to retain ingested Na^+, while sunbirds excreted it. Comparison of Na^+ and K^+ concentrations in ureteral urine and cloacal fluid showed that K^+, but not Na^+, was reabsorbed in the lower intestine of both species. The kidneys of sunbirds and honeyeaters, like those of hummingbirds, are well suited to diluting urine; however they also appear to concentrate urine when necessary.

Key words: salt balance; nectarivores; renal function; osmoregulation

Introduction

Avian nectarivores consume a physiologically challenging diet, high in preformed water and variable in predominant sugar type and concentration (Baker and Baker 1982; Johnson and Nicolson 2008; Nicolson and Fleming 2003b). Nectar ion composition is also highly variable, although there are few data available. Hiebert and Calder (1983) found higher mean K^+ (24.7 $mmol.l^{-1}$) than Na^+ (3.4 $mmol.l^{-1}$) concentrations in the nectar of 19 hummingbird-pollinated plant species. The nectar of some bird-pollinated plants in South Africa, such as *Aloe* and *Erica* species, is generally low in both K^+ (4.2-4.9 $mmol.l^{-1}$) and Na^+ (3.3-3.5 $mmol.l^{-1}$) while in *Protea* species K^+ averages 17.3 $mmol.l^{-1}$ and Na^+ averages 18.0 $mmol.l^{-1}$ (Nicolson and Thornburg 2007). Nectar ion levels are too low to be a good dietary source of ions, but insect feeding supplements the electrolyte intake of avian nectarivores. In wild-caught hummingbirds and honeyeaters, K^+ and Na^+ concentrations measured in excreted fluids were much higher than would be expected from a nectar diet alone (Calder and Hiebert 1983; Goldstein and Bradshaw 1998a) and could be attributed to the insect portion of the diet (Brice 1992; Stiles 1995).

The prioritisation of sugar over water intake is characteristic of specialist avian nectarivores (for review see Köhler et al. 2012). The ability to adjust volumetric intake to maintain a steady energy intake is extremely important for these birds in view of the wide range of nectar concentrations and associated water loads (Fleming et al. 2004a; Nicolson and Fleming 2003a). Electrolyte balance is potentially a major problem for birds that process several times their body mass in water each day (Beuchat et al. 1990; Martínez del Rio et al. 2001; McWhorter and Martínez del Rio 1999; Nicolson and Fleming 2003a). Eight species of hummingbird feeding on dilute nectars were shown to eliminate excess water in chronic diuresis: these hummingbirds conserved solutes by reducing their urine osmolality to a fifth of plasma levels (Calder and Hiebert 1983). Ion concentrations in the

fluid excreted by sunbirds can also be remarkably low. When Lotz and Nicolson (1999) fed southern double-collared sunbirds (*Cinnyris chalybeus*) 0.4 mol.l^{-1} sucrose with no electrolytes, the birds excreted only 0.4 mmol.l^{-1}M K^+ and 1.6 mmol.l^{-1} Na^+ in their cloacal fluid. Similarly, white-bellied sunbirds (*C. talatala*) feeding on artificial diets under laboratory conditions can dramatically dilute their cloacal fluid to only 6.2±2.6 SD mOsmol/kg H_2O when feeding on a 0.25 mol.l^{-1} sucrose diet, thereby minimising their electrolyte losses on this diet (Fleming and Nicolson 2003). However, this same study demonstrated that the osmolality of cloacal fluid and total osmotic excretion (i.e. electrolyte loss) actually increased when sunbirds were challenged on more dilute (0.07 and 0.1 mol.l^{-1} sucrose) diets devoid of electrolytes. The fact that the addition of small amounts of NaCl to extremely dilute diets enables both sunbirds and honeyeaters to increase food intake as seen in chapter 1 of this thesis (Purchase et al. 2010) suggests that salt loss on dilute diets is a serious problem for these birds. Together these data suggest a significant interplay between sodium concentration and the ability of sunbirds and honeyeaters to deal with dilute nectar diets. We therefore predicted that these birds should be acutely sensitive to electrolyte concentrations in their diets.

Very few studies have examined dietary salt preferences in birds. In 1976, Broom observed hummingbirds (ten species) at artificial feeders in the wild. He recorded feeding bout times at feeders containing 0.27 mol.l^{-1} sucrose but differing in salt concentration. At 70 mM NaCl and below, hummingbirds showed no preference for one feeder over another; however they avoided diets containing 125 mmol.l^{-1} NaCl and above (Broom 1976). Another study demonstrated that NaCl and KCl preference of cockatiels (*Nymphicus hollandicus*) varies widely between individuals (Matson et al. 2001). Bob-white quails *Colinus virginianus* demonstrate the ability to distinguish between diets of different salt content: diets with NaCl included were evidently the most palatable, followed by those

with $CaCl_2$ and then KCl (Hamrum 1953). As far back as 1909, the lethal dose of table salt (NaCl) was tested in chickens (*Gallus gallus domesticus*), with little interest in the birds' actual preference (Mitchell et al. 1926).

Limits to salt loading are important when investigating renal abilities and the ability of birds to cope in extreme environments. The urine concentrating ability of birds has been reasonably well documented (Ambrose and Bradshaw 1988; Beuchat 1996; Goldstein et al. 1990; Skadhauge and Bradshaw 1974). However, because water conservation is a major challenge to most terrestrial vertebrates, including the majority of bird species, ion regulation experiments have usually involved birds subjected to dehydration (Goldstein and Bradshaw 1998b; Skadhauge and Bradshaw 1974), where no water is made available to trial birds. The urine concentration ability of birds that have access to sufficient water, in the face of electrolyte loading, has received little attention. Lotz and Martinez del Rio (2004) examined electrolyte excretion in rufous hummingbirds (*Selasphorus rufus*) and found that these nectarivores were unable to excrete all the salt ingested when NaCl concentration in their diets exceeded 35 $mmol.l^{-1}$. The authors argued that this reflected adaptation to generally dilute diets, low in electrolytes, and this is supported by studies of the kidney morphology of hummingbirds and honeyeaters (Casotti et al. 1998; Casotti and Richardson 1992, 1993; Casotti et al. 1993), which suggest limited capacity to deal with high electrolyte loads in these two lineages of nectar-feeding birds. When renal function of red wattlebirds was tested in response to varying fluid intake, Goldstein and Bradshaw (1998b) found that rates of urine flow differed twofold between the most dilute and most concentrated diets, while water fluxes differed sevenfold. This implies that the intestinal tract plays an integral role in the processing of fluid and electrolyte loads, involving either water shunting through the gut or substantial postrenal ion reabsorption by the lower intestine.

The present study examines how African white-bellied sunbirds and Australian New Holland honeyeaters (*Phylidonyris novaehollandiae*) maintain electrolyte balance when they have to adjust their diet intake (and therefore water and electrolyte loading) in order to maintain energy intake. Firstly, we measured the effects of sugar concentration on the relative intake of four simultaneously-offered salt concentration (0 – 75 mmol.l^{-1} NaCl) diets (termed the '*choice*' experiment). These diet preferences were examined over successive trials where the energy value of the diets was adjusted to alter the total food intake required to maintain energy balance, testing the hypothesis that birds would be more likely to avoid salt solutions on more dilute diets. In the second experiment, we measured electrolyte handling (renal and intestinal) on a series of diets where the birds were required to ingest increasing concentrations of NaCl in order to maintain energy balance (termed the '*no-choice*' experiment). This experiment tested the hypothesis of Lotz and Martinez del Rio (2004) that improved diluting ability compromises the concentrating ability of nectar-feeding birds. Together these experiments examine the interplay between electrolyte balance and the regulation of energy intake.

Methods

Bird capture and maintenance

Eight white-bellied sunbirds (body mass 8.8 ± 1.2 SD g) and eight New Holland honeyeaters (body mass 20.4 ± 1.5 g) were captured by mist netting in Jan Celliers Park in Pretoria and on Murdoch University campus in Perth, respectively. Both are common species, ensuring ease of capture and little impact upon local populations. Birds were housed in individual cages (sunbirds: 45 x 45 x 32 cm; honeyeaters: 46 x 56 x 45 cm) at 20 ± 1 °C with an automatic photophase (sunbirds: 0700 to 1900; honeyeaters: 0600 to 1800). Both species were fed a maintenance diet *ad libitum* from inverted stoppered syringes

hanging from the cage sides. Sunbirds received 20% (w/w) sucrose (0.63 mol·l^{-1}) and 5% Ensure® (Abbott Laboratories, Johannesburg, South Africa); honeyeaters received 20% (w/w) sucrose and 15% Wombaroo® powder (Wombaroo Food Products, Adelaide, Australia). These maintenance diets contain low concentrations of Na^+: 3.1 and 2.5 mmol.l^{-1} respectively.

Choice experiment

Birds were offered four sucrose-based diets at a time containing A: 0, B: 25, C: 50 and D: 75 mmol.l^{-1} NaCl. The experiment was repeated using five different sucrose concentrations (0.075, 0.1, 0.15, 0.315 and 0.63 mol.l^{-1}) as the base solution. Trials were carried out over 6 h (commencing 0.5 h after lights on), with the positions of feeders rotated every 1.5 h in order to eliminate side bias. Each bird was randomly assigned to a sucrose concentration and each experimental diet was given over two consecutive days (acclimation and test day), with at least one recovery day between trials, when the maintenance diet was given *ad libitum* (the maintenance diet was also offered for the remaining 6 h of photophase on acclimation and test days). Test syringes were weighed at the beginning and end of each trial, as were paraffin collection jars that were placed under each syringe to collect any spillage. The amount of each diet consumed was calculated by mass difference [(before mass – after trial mass) – spillage]. The salt intake (mmol) over 6 h was calculated by adding the products of volume consumed (V; in litres) and salt concentration (mmol.l^{-1}) for each diet in the trial:

Salt intake (mmol) = [diet B V x 25] + [diet C V x 50] + [diet D V x 75)]

No-choice experiment

In the second experiment, ion intake and excretion were recorded for birds fed diets of varying NaCl concentration. Sunbirds and honeyeaters were fed, in random order, 0.63 $mol.l^{-1}$ sucrose containing the following concentrations of NaCl: 5, 9.9, 19.8, 29.7, 39.7, 59.5, 79.3, 100 and 200 $mmol.l^{-1}$. These concentrations were used to enable direct comparison with the earlier study of rufous hummingbirds (Lotz and Martínez del Rio 2004). Birds were fed the experimental diet from 0700 until 1100. Feeders were weighed hourly to measure intake. Cloacal fluid was collected under liquid paraffin, in trays that were removed and replaced hourly, for determination of its osmolality and Na^+ and K^+ concentrations. Plasma osmolality was not measured because we consider the birds, especially the sunbirds, to be too small for repeated blood sampling. At the end of every experimental session (1100) a ureteral urine sample was collected from each bird for comparison of Na^+ and K^+ levels with the cloacal fluid samples. Ureteral urine and cloacal fluid samples were collected as for chapter 1. All samples of cloacal fluid and ureteral urine were frozen at -20 °C until analysis. The Na^+ and K^+ concentrations were measured by flame photometry (Model 420, Sherwood Scientific Ltd., Cambridge, UK) and osmolality of cloacal fluid was measured with a vapour pressure osmometer (Vapro 5520, Wescor Inc., Utah, USA). At no stage did any bird have to be removed from the trial; both species were able to cope on all diets without any visible ill effects.

Statistical analysis

For the choice experiment, MANOVA was carried out to test whether there was a significant effect of species and sucrose concentration upon the arcsine square root transformed proportions of each diet consumed. MANOVA indicated significant differences in diet preferences between species ($F_{4,67}$= 2.92, P=0.027) and with sucrose

concentration ($F_{16,205}$=3.85, P<0.01), and therefore each diet was analysed for each species separately by one way t-test comparing the transformed proportion data with the arsine square root of 0.25 (i.e. equal consumption of all four diets). A Bonferroni adjustment corrected for the multiple tests within each sucrose concentration.

Data from the no-choice experiment were analysed by repeated-measures ANOVA with consumption on each diet (nine NaCl concentrations) and for each hour included as the repeated dependent measures. *Post hoc* comparisons were carried out by Tukey HSD test. Generalised linear mixed model analyses were used (with individual bird ID included as a random factor to take into account repeated measures on individuals) to detect an association between sodium ingestion, retention, cloacal fluid osmolality and dietary NaCl intake. Even when individual body mass was taken into account, there were significant differences between the species in their ingestion rates and therefore salt ingestion rates ($F_{1,336}$= 170.70, P<0.001), so each species was analysed separately. All data are presented as means ± 1 SD.

Results

Choice experiment

Sunbirds consumed equal amounts of each of the four simultaneously-offered salt solutions on the most concentrated 0.63 mol.l^{-1} sucrose diets (Fig. 2.1a). However, there was increasing avoidance of the high salt concentrations as the sucrose concentration decreased. On the 0.315 mol.l^{-1} sucrose diets, sunbirds showed significant avoidance of the 75 mmol.l^{-1} salt solution (P < 0.05). On the 0.15 mol.l^{-1} sucrose diets, both the 75 mmol.l^{-1} and 50 mmol.l^{-1} salt solutions were avoided (P < 0.01 and 0.05 respectively) and significantly more of the no-salt solution was consumed (P < 0.05). A further decrease to

extremely dilute sucrose diets (0.1 and 0.07 mol.l^{-1} sucrose) resulted in significant avoidance of the 75 mmol.l^{-1} salt solution ($P < 0.05$ and 0.01, respectively).

A similar pattern was observed for the honeyeaters. On the 0.63 mol.l^{-1} sucrose diets, honeyeaters showed significant avoidance of the 75 mmol.l^{-1} salt solution ($P < 0.01$) (Fig. 2.1b). On 0.315 and 0.15 mol.l^{-1} sucrose diets, honeyeaters avoided both the 75 and 50 mmol.l^{-1} salt solutions ($P < 0.001$) and showed significant preference for the no-salt solution ($P < 0.001$). On the 0.1 M sucrose diets, honeyeaters significantly avoided the 75 mmol.l^{-1} salt solution ($P < 0.001$) and preferred the no-salt solution ($P < 0.01$), while on the 0.07 mol.l^{-1} sucrose diet, only an avoidance of the 75 mmol.l^{-1} salt solution was significant ($P < 0.05$).

With the exception of honeyeaters on the most concentrated (0.63 mol.l^{-1}) sucrose diets, the selective feeding shown by both sunbirds and honeyeaters resulted in their maintaining a steady NaCl intake (Fig. 2.2). Sunbirds maintained an intake of 0.408 ± 0.216 mmol NaCl over 6 h (no effect of diet sucrose concentration: $F_{4,28}$=1.99, P=0.124). Honeyeaters maintained an intake of 1.06 ± 0.46 mmol NaCl over 6 h on the 0.07 to 0.315 mol.l^{-1} sucrose diets, but only 0.54 ± 0.05 mmol NaCl on the 0.63 mol.l^{-1} sucrose diets ($F_{4,28}$=3.38, P=0.022).

No-choice experiment

Both species demonstrated significantly greater food intake (g diet.h) in the first hour compared with subsequent hours (sunbirds: $F_{3,21}$= 12.56, P<0.001; honeyeaters: $F_{3,21}$= 102.71, P<0.001). Although there was a significant effect of NaCl concentration on consumption for sunbirds (Fig. 2.3a; $F_{8,56}$= 5.64, P<0.001), there were no trends apparent

in the data, and for honeyeaters, this effect was not statistically significant (Fig. 2.3b; $F_{8,56}$= 1.84, P=0.088). The mass of cloacal fluid collected was approximately half the mass of diet ingested (Fig. 2.3) for both honeyeaters and sunbirds (honeyeaters: $F_{1,8}$= 1927.79, P<0.001; sunbirds: $F_{1,8}$= 2173.41, P<0.001).

The osmolality of sunbird and honeyeater excreta increased linearly with dietary NaCl intake (sunbirds: R^2 = 0.969, $F_{1,64}$=175.90, P<0.001; honeyeaters: R^2 = 0.978, $F_{1,64}$=183.69, P<0.001) (Fig. 2.4). On the most concentrated NaCl diet, cloacal fluid osmolalities of sunbirds averaged 498.6 ± 36.7 mOsmol/kg H_2O and those of honeyeaters averaged 367.5 ± 26.3 mOsmol/kg H_2O (Table 2.1).

In both species, there was a significant correlation between Na^+ intake and Na^+ excretion rates. In white-bellied sunbirds, NaCl excretion closely matched NaCl intake (although there was nevertheless a statistically significant difference between intake and excretion rates; RM-ANOVA with intake and excretion on each of the nine diets as the repeated dependent measures: $F_{1,56}$=15.92, P=0.005). Honeyeaters excreted far less NaCl than they consumed ($F_{1,56}$=284.10, P<0.001). The relationship between Na^+ intake and Na^+ excretion is shown graphically for hour 4 (Fig. 2.5), where excretion in both ureteral urine and cloacal fluid is shown. Sodium excretion in cloacal fluid (sunbirds: $F_{1,67}$=234.37, P<0.001; honeyeaters: $F_{1,64}$=272.16, P<0.001) and ureteral urine (sunbirds: $F_{1,68}$=75.80, P=0.005; honeyeaters: $F_{1,67}$=101.13, P<0.001) in hour 4 were compared with sodium intake (Fig. 2.5). There was no statistically significant difference in Na^+ concentration between cloacal fluid and ureteral urine for either species (sunbirds: $F_{1,71}$=2.08, P=0.154; honeyeaters: $F_{1,71}$=1.64, P=0.204). Maximum cloacal fluid Na^+ concentrations (recorded when birds were fed the 200 mmol.l^{-1} NaCl diet) reached 335 ± 19.37 mmol.l^{-1} Na^+ in sunbirds and 252 ± 38.96 mmol.l^{-1} Na^+ in honeyeaters. Both sunbirds and honeyeaters

showed a linear relationship between Na^+ retention during the 4 h trial and increasing NaCl in the diet (Fig. 2.6). Honeyeaters showed a significant increase in sodium retention with increased dietary load ($F_{1,65}$=1949.38, P<0.001); the pattern was less marked for sunbirds, albeit still statistically significant ($F_{1,64}$=14.26, P<0.001).

Ureteral urine had a significantly higher K^+ concentration than cloacal fluid in both species (RM-ANOVA; sunbirds: $F_{1,71}$=39.40, P<0.001; honeyeaters: $F_{1,71}$=67.87, P<0.001) (Fig. 2.7). For sunbirds, excretion of K^+ in ureteral urine was correlated with sodium intake (sunbirds: $F_{1,65}$=31.25, P<0.001), but honeyeaters did not show an increase in cloacal fluid K^+ excretion with NaCl load ($F_{1,65}$=1.23, P=0.272) (Fig. 2.7).

Discussion

Avian nectarivores maintain a constant, high energy intake despite markedly variable diet concentration and composition. Consequently they may have to switch between water excretion and conservation, as well as dealing with either electrolyte deficiency or loading. In the present study, we took advantage of this compensatory feeding to examine how white-bellied sunbirds and New Holland honeyeaters deal with electrolytes through two experiments. Firstly, we examined diet selection where the birds had a choice between diets that differed in salt concentration, and secondly, we examined salt excretion where the birds did not have choice in their diet. We discuss the findings and implications of these two experiments in terms of our understanding of nectarivore osmoregulation.

Choice experiment

The first set of experiments investigated diet preference when salt was added to sucrose diets. An important finding was that white-bellied sunbirds and New Holland honeyeaters did not avoid all salt in their diets. As a consequence of selective feeding – switching to a low salt concentration when they increased consumption on more dilute diets – these nectarivores maintained a steady salt intake, consuming a total of about 0.41 (sunbirds) and 1.1 mmol NaCl (honeyeaters) during the 6 h trials. This salt may play an important role in glucose absorption and/or osmoregulation in these birds, as discussed below.

While obtaining energy from a dilute and electrolyte-deficient diet, nectarivores are required to ingest and excrete enormous volumes of preformed water, so that electrolyte conservation is vital. Previous research has shown that hummingbirds (Lotz and Martínez del Rio 2004) and sunbirds (Fleming and Nicolson 2003) are able to recover almost all solutes from the excreta. However, on extremely dilute sucrose diets devoid of electrolytes, hummingbirds go into torpor, whilst honeyeaters and sunbirds suffer decreased plasma sodium levels and are unable to maintain energy balance (Fleming et al. 2004b; Goldstein and Bradshaw 1998a; Lotz and Martínez del Rio 2004). We have found that white-bellied sunbirds and New Holland honeyeaters are limited in their intake of extremely dilute diets by increasing losses of sodium, confirmed by a significant decrease in plasma sodium levels in the absence of dietary sodium seen in chapter 1 of this thesis (Purchase et al. 2010). Excessive sodium excretion (natriuresis) and subsequent hyponatremia affect the digestive capacity of nectarivores. Through Na^+/K^+ pumps on the basolateral membrane of intestinal cells, a sodium concentration gradient is established that causes Na^+ ions to enter the cells passively across the apical membrane, accompanied by glucose. The sodium-linked glucose transporter SGLT-1 transports one glucose molecule along with two Na^+

ions from the intestinal lumen to the cytosol (Scheepers et al. 2004). The presence of sodium in the diet therefore aids the uptake of glucose. The addition of even small amounts of sodium (5-10 mmol.l^{-1}) to very dilute sucrose diets enables white-bellied sunbirds and New Holland honeyeaters to increase intake of such diets (Chapter 1-Purchase et al. 2010). Diet switching and modulation of sodium intake, as demonstrated in the present study, allows the birds to maintain sodium intake levels sufficient to assist with sugar digestion/absorption, without wasting energy processing more salt than is required.

Reduction of the sucrose concentration forces the birds to increase volumetric food intake to maintain constant energy intake, thus increasing water intake. However, except on the most concentrated diet of 0.63 mol.l^{-1} sucrose, the sodium intake of white-bellied sunbirds and New Holland honeyeaters in the choice experiment was unaffected by preformed water intake (i.e. sodium intake remained constant over these diets through diet selection, despite a 4.5-fold increase in water intake between 0.315 and 0.07 mol.l^{-1} sucrose diets). Although we recognise that sodium plays an important role in the water balance of every animal, this result suggests that the significance of sodium for homeostasis in these animals is not directly or solely linked to water balance.

We were interested in whether the regulated sodium intake recorded in the choice experiment reflected estimated sodium intake of these birds under wild conditions. A rough calculation of sodium intake from natural nectars for white-bellied sunbirds (these birds consume 0·313 ± 0·038 g sugar per g body mass daily under laboratory conditions and in the field consume, on average, nectars of 20% w/w sucrose and 3.4 mmol.l^{-1} sodium, Nicolson and Fleming 2003a; Nicolson and Thornburg 2007) shows that these birds would naturally consume around 0.0206 mmol of sodium in 6 h. This is about one twentieth of their sodium intake during feeding trials in the present study. If the sodium

preferences apparent in these laboratory experiments can be taken as an indication of the ideal sodium requirements of the birds in the wild, then it is clear that these nectarivores could not meet their sodium requirements from nectar alone. We assume similar values for the New Holland honeyeaters, although we know little about the electrolyte concentrations of Australian nectars. Therefore, if we can assume that their voluntary salt intake in the laboratory reflects sodium requirements, it is likely that arthropods, in addition to being an important source of protein (Paton 1982; Stiles 1995), are also a source of electrolytes for avian nectarivores. If the average insect weighs 10 mg and 11 % is NaCl (Finke 2002), then in order to meet the requirement of 0.0206 $mmol.l^{-1}$ NaCl every 6 h, they would need to consume 19 insects in that time period.

No-choice experiment

The second set of experiments investigated electrolyte handling by white-bellied sunbirds and New Holland honeyeaters when these birds were fed diets with added salt to test the salt loading point. Both species demonstrated the capacity to concentrate their excreta and to modify urine in the lower intestine by recovering potassium on these K^+-free diets. The smaller sunbirds showed greater excreta concentrating ability than the honeyeaters and a better ability to excrete excess dietary sodium. Similarly, when southern double-collared sunbirds were fed 15 $mmol.l^{-1}$ each of K^+ and Na^+ in 0.4 $mol.l^{-1}$ sucrose, they maintained cation balance by producing cloacal fluid with concentrations of each ion around 17 $mmol.l^{-1}$ (Lotz 1999).

Both white-bellied sunbirds and New Holland honeyeaters surpassed the urine concentrating abilities reported for hummingbirds (Table 1). Rufous hummingbirds become salt loaded when feeding on 0.63 $mol.l^{-1}$ sucrose diets with 35 $mmol.l^{-1}$ NaCl

added (Lotz and Martínez del Rio 2004), where their sodium intake would be around 0.0859 mmol over the 3 h trial (calculated from their estimated intake rate of 5.77 ml/day and assuming that they feed for 12 h in the day). Consequently, hummingbirds cannot maintain energy balance on these solutions (Lotz and Martínez del Rio 2004). Similarly, Rooke et al. (1983) found that frugivorous silvereyes (*Zosterops lateralis*) feeding in vineyards during the dry season, where grapes and brackish water were their only water sources, were dehydrated and probably salt loaded. By contrast with rufous hummingbirds and silvereyes, white-bellied sunbirds and New Holland honeyeaters were far more tolerant of salt added to their diet, ingesting reasonable quantities of 200 mmol.l^{-1} NaCl, similar to the salt tolerance of arid-adapted granivores, such as zebra finches and scrubwrens, both of which can tolerate salty solutions of up to 800 mmol.l^{-1} NaCl and show a maximum salt intake of 3.6 mmol in 24 h when drinking 300 mmol.l^{-1} NaCl solutions (Ambrose and Bradshaw 1988; Skadhauge and Bradshaw 1974).

Previous suggestions that, even under conditions of water deficiency, nectarivores cannot produce urine of higher osmolality than plasma (≈ 350 mOsmol/kg H_2O reference value as we did not test plasma osmolality in this trial) may be accurate for some hummingbirds (Beuchat et al. 1990; Lotz and Martínez del Rio 2004). However, under extreme conditions, sunbirds certainly are capable of producing relatively concentrated urine (sunbirds: 499 mOsmol/kg H_2O; present study), while honeyeaters excrete somewhat less concentrated excreta (368 mOsmol/kg H_2O; present study). Some avian nectarivores can therefore produce copious quantities of dilute excreta, but can also concentrate excreta when necessary (Table 2.1).

While the kidney morphology of both hummingbirds and honeyeaters suggests that these birds are adapted to produce dilute rather than concentrated urine, post-renal

modification also plays a role in osmoregulation in birds (Casotti et al. 1998; Casotti and Richardson 1992). Concentration or dilution of excreta can occur in the gastrointestinal tract, with lower intestinal modification of urine described for a variety of bird species, although the focus has been on reabsorption of sodium (Goldstein and Skadhauge 2000). In the present study, postrenal modification was shown for potassium, with more K^+ present in ureteral urine than the excreted cloacal fluid. Conservation of K^+ is important when there is no dietary source (such as on these experimental diets) and has been demonstrated previously for sunbirds on salt-free sucrose diets (Fleming and Nicolson 2003; Lotz and Nicolson 1999). However, the finding that K^+ was reabsorbed, but not Na^+, is an artefact of our experimental design using diets that included Na^+ but not K^+.

Dietary sodium, which is naturally deficient in nectars, clearly plays a significant role in the maintenance of energy balance in nectarivorous birds, and therefore we suspect alternative salt sources may be important for these birds to supplement their nectar diet. This will be especially important under wet or cold conditions, where nectar has been diluted by rain or dew, insects are in short supply, and the birds are required to increase intake to maintain energy balance due to reduced ambient temperatures. There is a dearth of information on nectar ion levels and the extent of arthropod foraging amongst nectarivorous birds. Information on both is required before we can interpret the ecological consequences of varying tolerance to dietary sodium by nectarivorous birds. We also have some way yet to go in terms of understanding the mechanisms of action or role of sodium in the diet of nectarivorous birds; this is compounded by the high level of variation in field water and ion balance shown in these birds (Goldstein and Bradshaw 1998a). Measurements of the Na^+ and K^+ concentrations in excreta of sunbirds in the field would give us a better understanding of the ecological relevance of these data and enable comparison with previous field research on honeyeaters (Goldstein and Bradshaw 1998a).

Acknowledgements.

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Table 2.1. Minimum and maximum osmolality values (mOsmol/kg H_2O; mean ± SD) of cloacal fluid in 4 avian nectarivores.

* estimated from figure

Species	Osmolality (mOsm.kg^{-1})				reference
	min	conditions	max	conditions	
Rufous hummingbird (*Selasphorus rufus*)	16	Feeders and flowers	383	Resource competition stress	Calder & Hiebert 1983
Rufous hummingbird (*Selasphorus rufus*)	60*	0.63 M sucrose, 5 mM NaCl	560*	0.63 M sucrose, 200 mM NaCl	Lotz & Martínez del Rio 2004
White-bellied sunbird (*Cinnyris talatala*)	7.1 ± 4.7	0.25 M sucrose	460.9 ± 253.3	2.5 M sucrose	Fleming & Nicolson 2003
White-bellied sunbird (*Cinnyris talatala*)	62.6 ± 28.2	0.63 M sucrose, 5 mM NaCl	498.6 ± 36.7	0.63 M sucrose, 200 mM NaCl	This study
Palestine sunbird (*Cinnyris oseus*)	47 ± 20	0.29 M sucrose, 3 mg/day NaCl & KCl	754 ± 233	1.46 M sucrose, 30 mg/day NaCl & KCl	Roxburgh & Pinshow 2002
New Holland honeyeater (*Phylidonyris novaehollandiae*)	85.4 ± 70.3	0.63 M sucrose, 5 mM NaCl	367.5 ± 26.3	0.63 M sucrose, 200 mM NaCl	This study

Figures

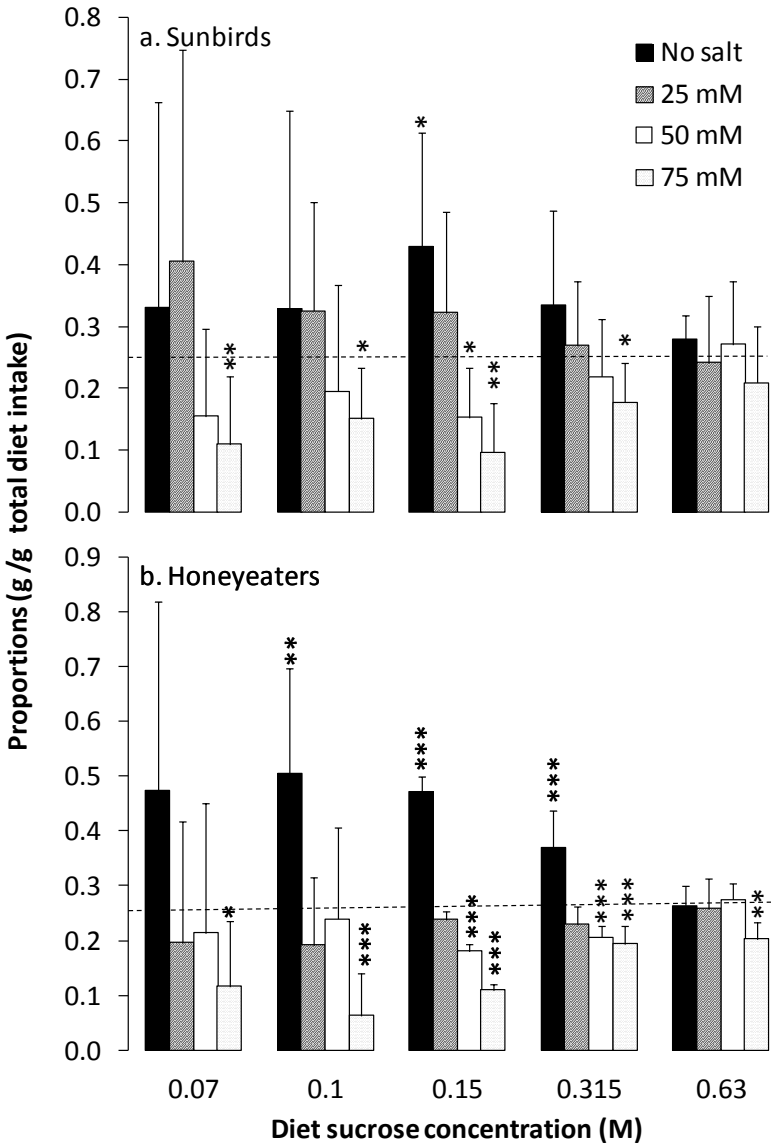

Figure 2.1. Proportions (mean+1 SD) of four simultaneously-offered diets (varying in NaCl concentration) consumed by white-bellied sunbirds (a) and New Holland honeyeaters (b) varied according to the concentration of sucrose in the base solution.

Significant change from an equal proportion of each diet (i.e. 0.25, shown by the horizontal lines) is indicated by asterisks, where (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

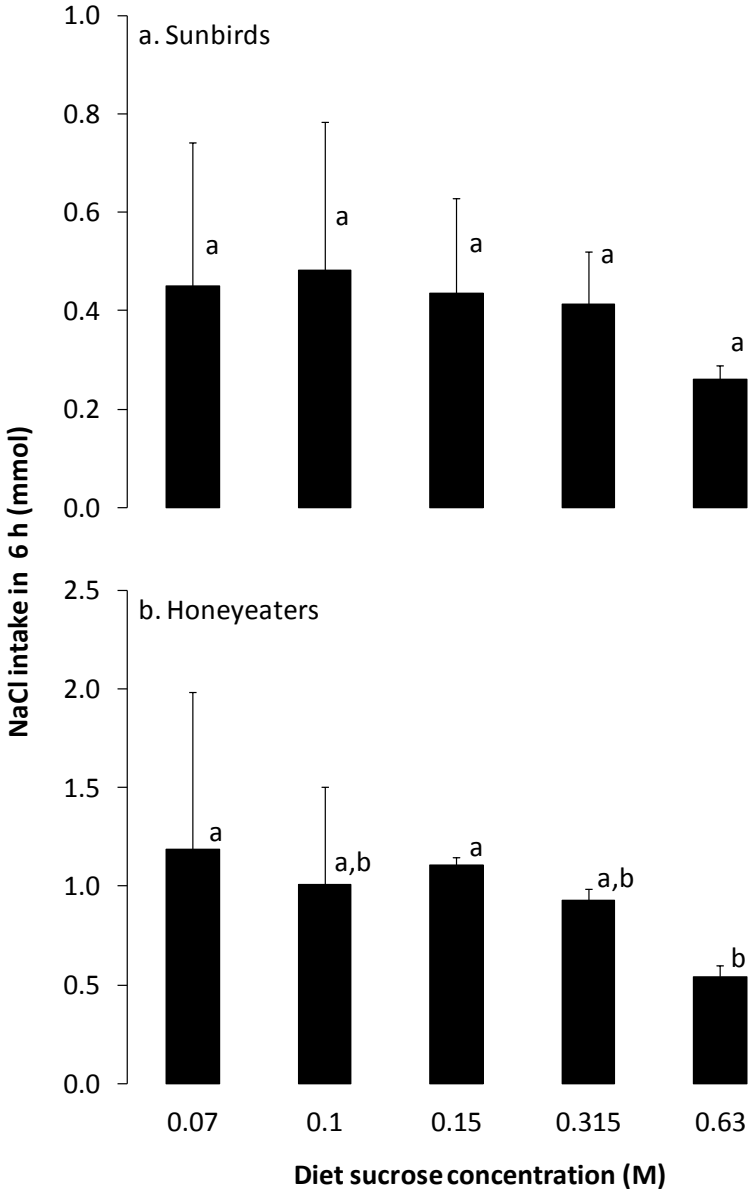

Figure 2.2. NaCl intake over 6 h (mmol NaCl, mean±1 SD) of white-bellied sunbirds (a) and New Holland honeyeaters (b) varied according to the concentration of sucrose in the base solution.

Columns with the same letters were not significantly different (post hoc analyses).

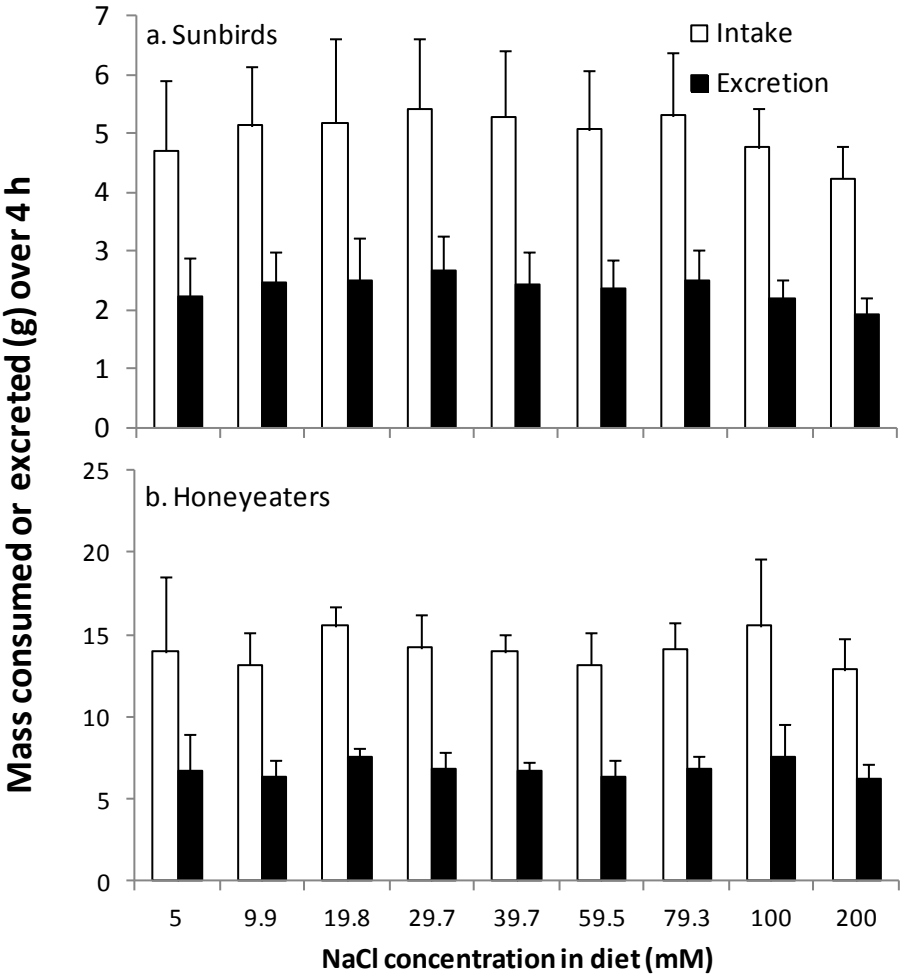

Figure 2.3. Mass of diet consumed and cloacal fluid excreted (g) during the 4 h no-choice trial of white-bellied sunbirds (a) and New Holland honeyeaters (b) across all nine NaCl concentrations in 0.63 mol.l^{-1} sucrose.

The mass of cloacal fluid excreted is about half the mass of the food ingested. Values are means +1 SD.

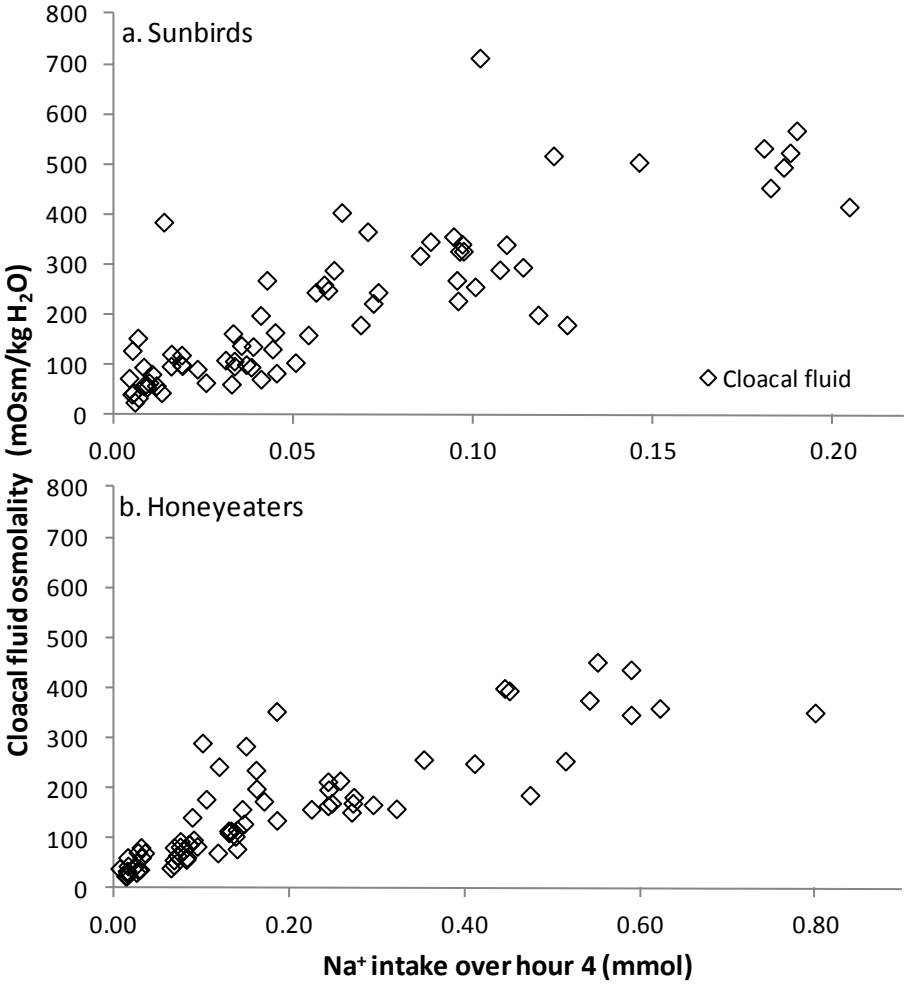

Figure 2.4. Osmolality of cloacal fluid (mOsmol/kg H_2O) over the last hour as a function of Na^+ intake (mmol over 4h) of white-bellied sunbirds (a) and New Holland honeyeaters (b) consuming nine diets of the same sucrose concentration (0.63 $mol.l^{-1}$), but varying in NaCl concentration.

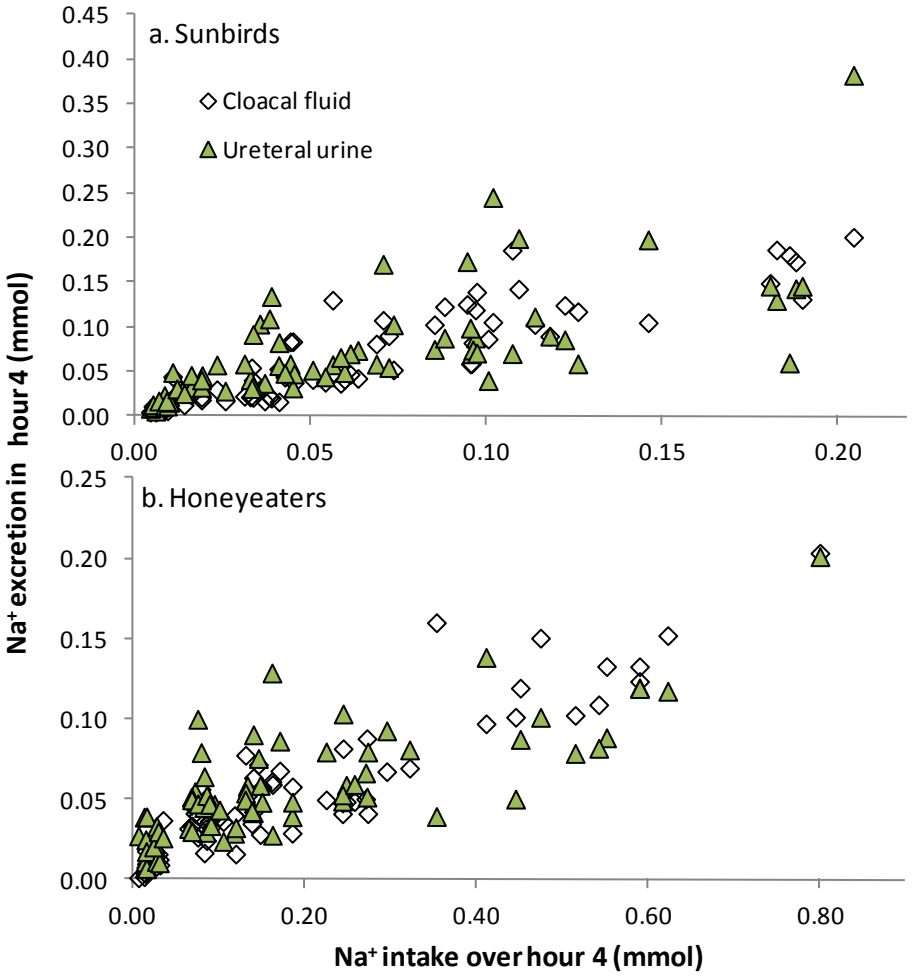

Figure 2.5. Sodium (Na^+) excretion in cloacal fluid (◊) and ureteral urine (▲) over the last hour as a function of Na^+ intake (mmol) of white-bellied sunbirds (a) and New Holland honeyeaters (b) consuming nine diets over 4 h of the same sucrose concentration (0.63 $mol.l^{-1}$), but varying in NaCl concentration.

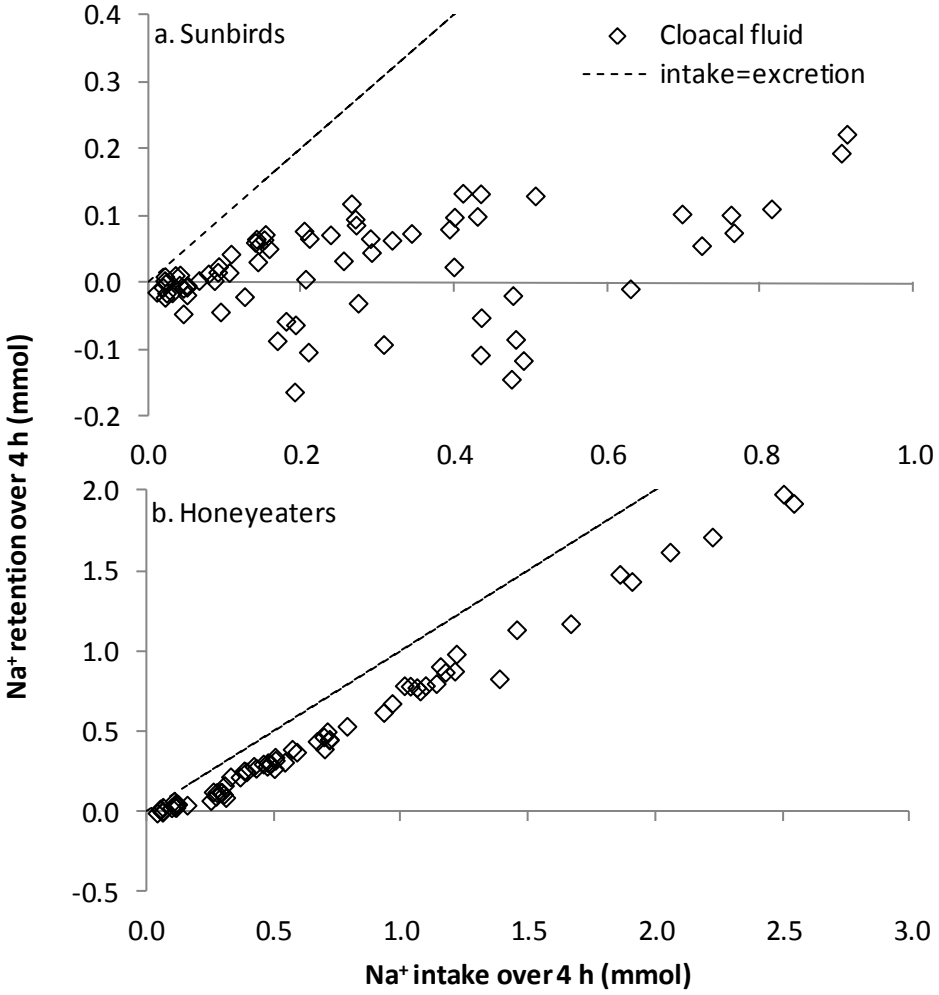

Figure 2.6. Retention rates of sodium compared with NaCl consumption (mmol over 4 h) of white-bellied sunbirds (a) and New Holland honeyeaters (b) consuming nine diets of the same sucrose concentration (0.63 mol.l^{-1}), but varying in NaCl concentration.

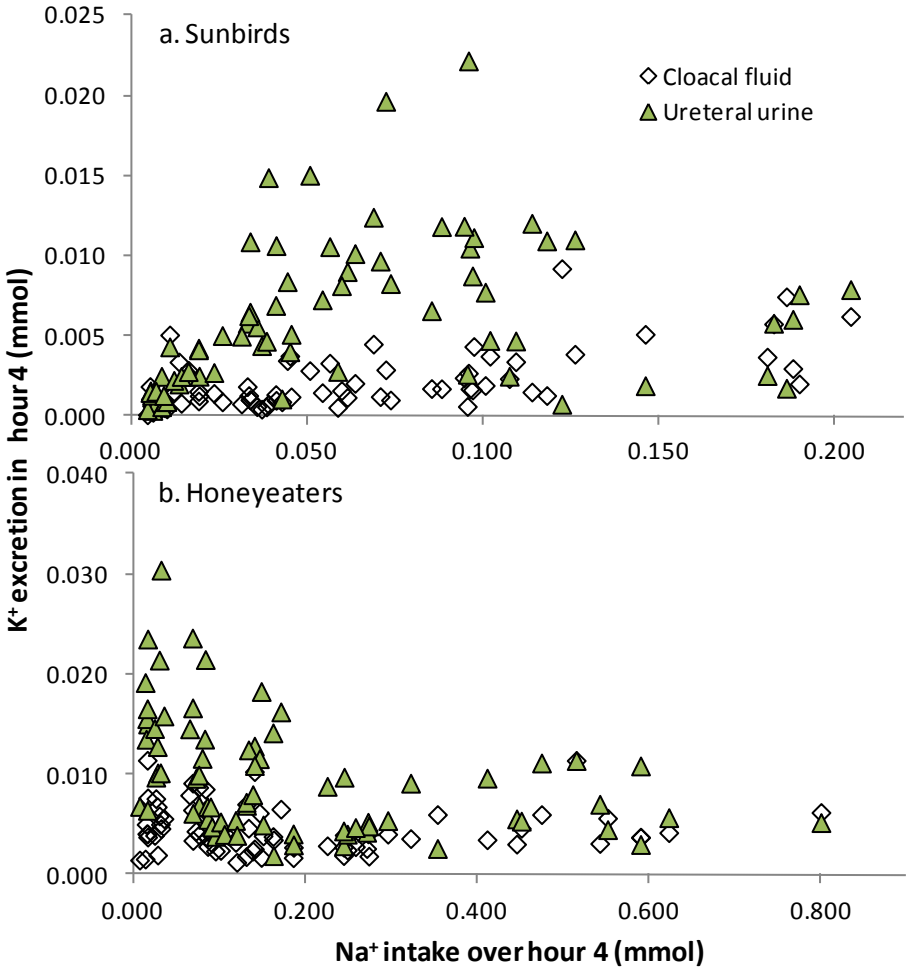

Figure 2.7. Potassium (K^+) excretion in cloacal fluid (◊) and ureteral urine (▲) over the last hour as a function of Na^+ intake (mmol) of white-bellied sunbirds (a) and New Holland honeyeaters (b) consuming nine diets over 4 h of the same sucrose concentration (0.63 $mol.l^{-1}$), but varying in NaCl concentration.

Note different y-axis scale compared with Figure 2.6.

Chapter 3: Gastrointestinal and renal responses to variable water intake in white-bellied sunbirds and New Holland honeyeaters

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List of abbreviations

CF	Cloacal fluid
f_A	Fractional water absorption in the gut
f_T	fractional turnover rate of body water
f_R	Fractional water reabsorption in the kidneys
GFR	Glomerular filtration rate (ml·h^{-1})
GFR'	Estimated overnight *GFR* (ml·h^{-1})
$I^{14}C$	*Time 0* intercept concentration of ^{14}C in plasma (d.p.m.·ml^{-1})
ln-[CF_{3H}]	Log_e-transformed ^3H_2O concentration in cloacal fluid
ln-[CF_{14C}]	Log_e-transformed [^{14}C]-L-glucose concentration in cloacal fluid
K_{el}	Elimination rate constant
K^3H	Fractional water turnover (h^{-1})
$K^{14}C$	Fractional L-glucose turnover (h^{-1})
M_B	Body mass (g)
S	Distribution space
$S^{14}C$	[^{14}C]-L-glucose distribution space (ml)
S^3H	Water distribution space (ml)
$\dot{S}_I$	Sucrose intake rate (g·h^{-1})
TBW	Total body water (ml)
TEWL	Total evaporative water loss (ml·h^{-1})
$\dot{V}_I$	Water intake rate (ml·h^{-1})
$\dot{V}_E$	Water excretion rate (ml·h^{-1})
$\dot{V}_M$	Metabolic water production rate (ml·h^{-1})
$\dot{W}$	Water flux (ml·h^{-1})

Abstract

Nectarivores face a constant challenge in terms of water balance, experiencing water loading or dehydration when switching between food plants or between feeding and fasting. To understand how white-bellied sunbirds and New Holland honeyeaters meet the challenges of varying preformed water load, we used the elimination of intramuscular-injected [^{14}C]-L-glucose and $^{3}H_2O$ to quantify intestinal and renal water handling on diets varying in sugar concentration. Both sunbirds and honeyeaters showed significant modulation of intestinal water absorption, allowing excess water to be shunted through the intestine on dilute diets. Despite reducing their fractional water absorption, both species showed linear increases in water flux and fractional body water turnover as water intake increased (both afternoon and morning), suggesting that the modulation of fractional water absorption was not sufficient to completely offset dietary water loads. In both species, glomerular filtration rate (*GFR*) was independent of water gain (but was higher for the afternoon), as was renal fractional water reabsorption (measured in the afternoon). During the natural overnight fast, both sunbirds and honeyeaters arrested whole kidney function. Evaporative water loss in sunbirds was variable but correlated with water gain. Both sunbirds and honeyeaters appear to modulate intestinal water absorption as an important component of water regulation to help deal with massive preformed water loads. Shutting down *GFR* during the overnight fast is another way of saving energy for osmoregulatory function. Birds maintain osmotic balance on diets varying markedly in preformed water load by varying both intestinal water absorption and excretion through the intestine and kidneys.

Keywords: pharmacokinetics, water balance, osmoregulation, intestinal water absorption, renal function, nectarivore

Introduction

Bird nectars are generally dilute (Baker et al., 1998; Johnson and Nicolson, 2008; Nicolson, 2002; Pyke and Waser, 1981) which dramatically influences the physiology of nectarivores, which must consume large volumes of water to satisfy their energy requirements (Martínez del Rio et al., 2001; Nicolson and Fleming, 2003c). When birds feed on dilute nectar, they can consume up to 5 times their body mass in water daily (Collins, 1981; McWhorter and Martínez del Rio, 1999; Nicolson and Fleming, 2003a). These massive ingested water loads can potentially cause severe disruptions in water balance (Beuchat et al., 1990; McWhorter et al., 2003). Nectarivores also face a constant challenge in terms of fluctuations in water balance, having to switch between avoiding water loading and dehydration as they switch between food plants or between feeding bouts and fasting periods. During fasts (overnight or during disturbance during the day, e.g. due to storms), these birds do not feed and therefore have no water intake. Regulating osmotic balance requires that these birds be able to deal with both extremes (water-loading and dehydration) on a daily basis.

The kidneys are among the most metabolically active tissues in the vertebrate body. They consume a disproportionate amount of a vertebrate's daily energy expenditure to carry out water and waste excretion while ensuring that blood glucose and electrolyte balances are maintained (Silverthorn, 2004). We predict that the metabolic costs of kidney function will be especially high in nectarivorous animals, due to the high preformed water loads of their nectar diet. One way to avoid this high renal metabolic load would be to not absorb all preformed water from the intestine, instead shunting some of the excess water directly through. Beuchat et al. (1990) proposed the 'intestinal shunting hypothesis', predicting that birds feeding on large volumes of dilute nectar could reduce the water load to be processed by the kidneys (renal loading) by reducing intestinal water absorption

(fractional water absorption; f_A). This intestinal shunting hypothesis has been examined for two hummingbird species to date, including broad-tailed hummingbirds, *Selasphorus platycercus* (McWhorter and Martínez del Rio, 1999) and green-backed firecrowns, *Sephanoides sephanoides* (Hartman Bakken and Sabat, 2006). These hummingbird species absorb ~80% and ~90% (respectively) of the water ingested; however, fractional water absorption was not correlated with dietary water intake, as predicted from the intestinal shunting hypothesis (Beuchat et al., 1990). By contrast, a similar study in Palestine sunbirds (*Cinnyris oseus*) demonstrated a significant correlation between fractional water absorption and dietary preformed water intake, suggesting that these birds are able to regulate their absorption of water in relation to the amount of water consumed: as water intake increased, the fraction of ingested water absorbed (f_A) decreased (McWhorter et al., 2003). These data suggest that there may be interesting differences in the handling of water loads between these nectarivore lineages.

A second way to reduce renal metabolic costs of electrolyte and glucose retrieval may be to reduce glomerular filtration rate (*GFR*). Although this has not been found for feeding nectarivorous birds, reduction in renal water reabsorption (f_R) in response to increased water excretion has been recorded (McWhorter et al., 2004). Another way to avoid high renal metabolic load would be to shut down the kidneys when renal processing is not required when the birds are not feeding (i.e. overnight). Both hummingbirds species examined to date apparently arrest kidney glomerular filtration rate (*GFR*) overnight (Hartman Bakken et al., 2004; Hartman Bakken and Sabat, 2006). A similar finding has been recorded for a nectar feeding bat (Pallas's long-tongued bats, *Glossophaga soricina*) during the daytime rest period (Hartman Bakken et al., 2008).

Evaporative water loss (EWL) is a third possible route that could be used to eliminate large volumes of preformed water. In birds, modulation of EWL either through the skin or respiratory surfaces (through panting) has been noted in response to heat stress (Dawson, 1982; Dawson and Whittow, 2000; Skadhauge, 1981; reviewed by Williams et al., 2012) and in relation to hydration state (Arad et al., 1987; Maloney and Dawson, 1998; Williams, 1996). However there are few accounts linking modulation of EWL with water loading (Hartman Bakken and Sabat, 2006). Birds that consume nectar should be capable of higher rates of EWL than those consuming predominantly solid foods. Furthermore, nectarivores consuming dilute nectar should have higher EWL rates than those drinking more concentrated nectars.

In this study, we examined water handling in two nectarivore species: white-bellied sunbirds (*Cinnyris talatala*) and New Holland honeyeaters (*Phylidonyris novaehollandiae*). Based on previous work showing that Palestine sunbirds could modulate their fractional water absorption, we predicted that these two passerines would similarly be able to modulate intestinal water absorption in response to increased preformed water load. We predicted that these nectarivores would also vary renal function in response to diet concentration: *GFR* would increase and renal water reabsorption would decrease with increasing water load, but when these birds were not feeding overnight, we predicted that *GFR* would slow or stop to reduce renal metabolic expenditure. Finally, we predicted that these birds would modulate evaporative water loss in response to increasing water load.

Methods

Animals and maintenance

Eight white-bellied sunbirds were captured in Jan Cilliers Park, Pretoria, and eight New Holland honeyeaters on the Murdoch University campus, Perth, using mist-nets. The birds were housed in individual cages (27 x 31 x 21 cm) in controlled environment rooms maintained at 21 ± 1˚C with an 11 h photoperiod from 0700 to 1800 h. During captivity, sunbirds were fed a maintenance diet consisting of 20% w/w sucrose and 2% Ensure®, a nutritional supplement (Abbott Laboratories, Johannesburg, South Africa); honeyeaters were fed 20% w/w sucrose with 15% Wombaroo® powder (Wombaroo Food Products, Adelaide, Australia). Birds received the maintenance diet in inverted, stoppered syringes. Bird body mass (M_B) at the start of the experiments was 8.07 ± 0.45 g for sunbirds, 22.6 ± 1.65 g for honeyeaters.

During experiments the birds were housed in individual experimental cages (42 x 54 x 50 cm) made of Perspex with a one-way mirror in the front. Birds were fed from inverted syringes fixed to the inside of the back wall of the cage.

The routine animal care procedures and experimental protocols used in this study were reviewed and approved by the University of Pretoria (Animal Use and Care Committee EC013-07) and Murdoch University (Animal Ethics Committee R1137/05). Licenses permitting the possession and use of radiolabelled substances were obtained from the Nuclear Energy Corporation of South Africa (reference number 7710245246084) and from the Radiological Council of Western Australia (license number LS 345/2006).

Experimental method

We varied food intake rate by feeding birds three diet sugar concentrations (0.25, 0.5 and 1 M sucrose solutions) in separate feeding experiments. The order of trials and order of treatment given were both randomly assigned.

Before each trial, birds had fed *ad libitum* from a syringe containing their allocated experimental diet for 15 h. We injected each bird (intramuscular, IM) with a combined dose of ^{14}C-L-glucose and tritiated water ($^{3}H_2O$). At 1600 h, sunbirds were weighed and then injected in the pectoralis muscle with approximately 15 µl of solution containing 140 KBq ^{14}C-L-glucose and 150 KBq of $^{3}H_2O$, while honeyeaters were injected with approximately 50 µl containing 330 KBq of ^{14}C-L-glucose and 360 KBq of $^{3}H_2O$. The mass of solution administered by IM injection was measured by weighing the syringe before and after administration. Aliquots of the IM solutions were saved for radioactivity analysis: samples were transferred to a vial of known mass (±0.00001 g) which was then re-weighed to estimate sample mass.

We examined the elimination of these radiolabelled markers in excreta. Cloacal fluid (CF) samples were collected for 2 h commencing immediately from the time of IM administration (1600 to 1800 h; afternoon samples; PM) and then again the following day (0700 to 0900 h; morning samples; AM). CF samples were collected from wax paper rolled through the cage floor to minimise disturbance, using a pipette immediately after the bird excreted, with the exact time noted. Samples were transferred to a vial of known mass which was then re-weighed to calculate sample mass.

A single ~15 µl blood sample was collected by micro-haematocrit capillary tube from the brachial vein 2 h after IM administration. Microcapillary tubes were sealed with

clay tube sealing compound (Vitrex, Denmark) and centrifuged for 2-3 min at ~9,000 g to separate plasma from blood cells. At the same time as blood sampling, a small sample of ureteral urine was collected by catheter. The plasma and ureteral urine were each transferred to a vial of known mass which was then re-weighed to calculate sample mass.

Injection aliquot, CF, plasma and ureteral urine samples were each mixed with 3 ml of scintillation fluid (sunbirds: Ultima Gold™ XR, Packard Bioscience, Groningen, The Netherlands; honeyeaters: Ecolite+, MP Biomedicals Australasia, Seven Hills, New South Wales) and then counted in a scintillation spectrometer (sunbirds: Packard Tri-Carb Liquid Scintillation Spectrometer; honeyeaters: Beckman LS6500 Liquid Scintillation Counter, Beckman Coulter, Fullerton, CA) for disintegrations per minute (d.p.m.) for ^{3}H and ^{14}C.

Pharmacokinetic calculations

We used the model developed by McWhorter & Martínez del Rio (1999) to measure water handling processes in the intestine and kidney. Total body water (TBW; ml; which can also be expressed as water distribution space, $S_{^{3}H}$) was estimated using the dose-corrected zero-time intercept concentration of $^{3}H_2O$ in body water ($C_{t=0\ ^{3}H}$; d.p.m.·ml^{-1}) as:

$$S_{^{3}H} = \text{TBW} = Q_{i\ ^{3}H} \Big/ \left[\frac{P_{^{3}H}}{e^{(K_{^{3}H} \bullet t)}} \right] \qquad (1)$$

where: $Q_{i\ ^{3}H}$ is the quantity of $^{3}H_2O$ injected (d.p.m.)

$P_{^{3}H}$ is the plasma ^{3}H concentration (d.p.m.·mg^{-1}) in the blood sample taken ~2 h after injection; the actual time of collection was recorded (t ; h).

The elimination rate constant, $K_{^{3}H}$, is the hourly fractional water turnover measured as

^{3}H isotope fractional elimination (h^{-1}) in the CF, estimated from the slope of the relationship between *ln*-[CF^{3}_{H}] vs. time (h) and is mathematically equivalent to the hourly fractional turnover of body water (f_T; Hartman Bakken and Sabat, 2006).

Water flux

Water flux ($\dot{W}$; $ml \cdot h^{-1}$) is a measure of the rate at which ingested water is incorporated into total body water. This was calculated from water elimination data and is thus, strictly speaking, water elimination. However, assuming neutral water balance (assumption correct for afternoon data but not for morning data; see results), the rate of water elimination should equal water incorporation, thus $\dot{W}$ was calculated as:

$$\dot{W} = K_{3H} \bullet \text{TBW} \quad (2)$$

Diet consumption was measured gravimetrically (± 0.001 g; measured at the commencement and end of each experimental phase) and after correcting for leakage (cups of paraffin were placed under each feeder to collect any spilt food which was taken into account in the calculations), these values were used to estimate sucrose ($\dot{S}_I$; $g \cdot h^{-1}$) and water ($\dot{V}_I$; $g \cdot h^{-1}$) intake rates. Intake rates were calculated as a fraction of the actual time spent feeding, since we noted that many individuals would not return to feeding immediately.

As sucrose assimilation efficiency in nectarivores is high and independent of sucrose intake rate ($\dot{S}_I$), we assumed that the fractional assimilation of ingested sucrose is >0.99; this value has been confirmed in sunbirds (Jackson et al., 1998; Köhler et al., 2010; McWhorter et al., 2003). We also assumed that active birds were relying solely on

carbohydrates to fuel metabolism (as has been demonstrated for active hummingbirds which have a respiratory quotient of 1 (Powers, 1992; Suarez et al., 1990; Welch et al., 2006); at night the birds would switch to lipid metabolism. One gram of sucrose was assumed to liberate 0.57 g of water (Schmidt-Nielsen, 1997). Using these assumptions, metabolic water production rate ($\dot{V}_M$; ml·h^{-1}) during steady-state feeding was estimated as:

$$\dot{V}_M = \dot{S}_I \bullet 0.99 \bullet 0.57 \quad (3)$$

Total water gain (ml·h^{-1}) was therefore estimated as:

$$TWG = \dot{V}_M + \dot{V}_I \quad (4)$$

Intestinal function: fractional water absorption

Fractional water absorption in the gut (f_A) was therefore estimated as:

$$f_A = \frac{\dot{W} - \dot{V}_M}{\dot{V}_I} \quad (5)$$

Kidney function: Glomerular filtration rate and renal fractional water reabsorption

To estimate *GFR* (ml·h^{-1}) during feeding, we used a version of the slope-intercept method (Florijn et al., 1994; Hall et al., 1977) that accommodates to small birds that are sensitive to repeated blood sampling, and allows for measurements in non-restrained birds which are therefore able to continue feeding (Napier et al., 2012). The distribution space of [^{14}C]-L-glucose (S^{14}_C; ml) was calculated from the dose-corrected zero-time intercept concentration of [^{14}C]-L-glucose in body water ($C_{t=0\ ^{14}C}$; d.p.m.·ml^{-1}) using the following equation:

$$S^{14}_C = Q^{14}_C \ / \left[\frac{P^{14}_C}{e^{(K^{14}_C \bullet t)}} \right] \quad (6)$$

where: $Q^{14}C$ is the quantity of [^{14}C]-L-glucose injected (d.p.m.)

$P^{14}C$ is the plasma ^{14}C concentration (d.p.m.·mg^{-1}) in the blood sample taken ~2 h after injection; the actual time the blood sample was collected was recorded (*t*; h).

$K^{14}C$ is the fractional elimination of ^{14}C (h^{-1}) in CF, estimated from the slope of the relationship between *ln*-[$CF^{14}C$] vs. time (h).

GFR (ml·h^{-1}) was estimated for feeding periods (McWhorter et al., 2004):

$$GFR = \frac{K^{14}C \bullet Q^{14}C}{I^{14}C} \quad (7)$$

where: $I^{14}C$ is the *time 0* intercept concentration of ^{14}C in plasma (d.p.m.·ml^{-1}) as predicted by $K^{14}C$ from a blood sample taken ~2 h after injection.

Mean estimated *GFR* overnight, when the birds were not feeding (*GFR'*; ml·h^{-1}), was estimated as:

$$GFR' = K'^{14}C \bullet S^{14}C \quad (8)$$

where: the elimination rate constant, $K'^{14}C$, was estimated as the difference in *ln*-[$CF^{14}C$] at lights-out (~1800 h; PM) and lights-on (~0600 h; AM) the following morning (actual times were used for each individual trial). We estimated *ln*-[$CF^{14}C$] by solving the equations for these data for the required time points: the PM value (1800 h) was calculated from the equation representing *ln*-[$CF^{14}C$] over time for the afternoon and the AM value (0600 h) calculated from the equation representing *ln*-[$CF^{14}C$] over time for the morning.

Renal fractional water reabsorption (f_R) was estimated (Goldstein, 1993) as:

$$f_R = 1 - \frac{ln\text{-}[P^{14}_{C}]}{ln\text{-}[U^{14}_{C}]} \quad (9)$$

where: P^{14}_{C} and U^{14}_{C} were the ^{14}C concentrations in plasma and ureteral urine (d.p.m.·ml^{-1}), respectively.

Total evaporative water loss

This experiment allows for the calculation of the water excretion rate ($\dot{V}_E$; ml·h^{-1}):

$$\dot{V}_E = \dot{V}_I\ (1 - f_A) + GFR\ (1 - f_R) \quad (10)$$

With the caveat that there would be no change in total body water, the difference between the rates of water flux and water excretion should equal total evaporative water loss (*TEWL*; ml·h^{-1}):

$$TEWL = (\dot{V}_I + \dot{V}_M) - \dot{V}_E \quad (11)$$

Assumptions of the mass-balance and single injection slope-intercept models and data handling

The first assumption of the pharmacokinetic method used is that the estimates of the elimination rate constant (K_{el}) and distribution space (S) for each probe are derived from correct modelling of the numbers of distribution pools. To test the assumption of a single compartment (as has been found in similar previous pharmacokinetic studies, Napier

et al., 2012), we examined whether isotope concentration and time were linearly related. This was confirmed as statistically significant linear relationships for *ln*-[^{3}H] or *ln*-[^{14}C] against time. Excreta data were also fitted to nonlinear curves by the Marquardt-Levenberg algorithm (SYSTAT Software, SigmaPlot for Windows, San Jose CA; Marquardt, 1963). The following mono- and biexponential models were compared when analysing the curves of concentrations (C) of CF_{3H} and CF_{14C} over time (t), where C_0 is the intercept (d.p.m.·mg plasma^{-1}):

$$C = C_0 e^{-Kelt} \tag{12}$$

$$C = ae^{-\alpha t} + be^{-\beta t} \tag{13}$$

Model fits were then compared by F-tests according to Motulsky and Ransnas (1987), where the residual sum of squares and the numbers of parameters in each model are used to compute the F ratio, which tests for significant differences in the goodness of fit of the two models to the same data. The largest F and smallest P values of each species are reported in each case.

A second assumption of the pharmacokinetic method is that the birds are feeding at a steady rate. Not all birds commenced feeding immediately after they were returned to the cage after injection of the radioisotopes. Napier et al. (2012) have shown that the pharmacokinetic calculations are extremely sensitive to this assumption of steady-state feeding, and any time that the animal is not feeding needs to be taken into account in the calculations, especially for intake rates. To do this, the intake rates were adjusted for actual time spent feeding; this was done by re-setting the $t=0$ to the point when the birds started to defecate regularly (and were thus feeding regularly). In order to handle this data issue objectively, we adjusted the data for each individual separately. While the honeyeaters

would generally return to feeding almost immediately (39 trials; 9 trials had to be adjusted by 18.3 ± 8.8 min, range 10–31), the sunbirds would spend longer before returning to feed (returned to feed immediately for 25 trials, 23 trials had to be adjusted by 22.7 ± 15.4 min, range 4–77).

A third assumption is in regard to data accuracy. Data editing is an important but also very unreliable aspect of handling pharmacokinetic data (Napier et al., 2012). The first excreta samples are likely to have a low concentration of ^{3}H and ^{14}C, because these samples may reflect CF produced before the IM administration of the radioisotope markers, or before the equilibrium from IM (rather than intravenous) administration. Calculations of S and K_{el} are both extremely sensitive to inclusion of these erroneously low values and they do need to be removed (Napier et al., 2012). This method is supported in the pharmacokinetics literature for intravenous injections; even with intravenous injections there is some small lag to complete equilibration (Pappenheimer, 1990). Initial samples where the isotope concentration was <75% of subsequent samples were therefore eliminated from calculations.

Statistical analyses

Two-way repeated-measures analysis of variance (RM-ANOVA) were carried out to examine the effects of diet concentration and time (afternoon: PM or morning: AM) on water intake rate (Statistica, Statsoft Inc. Tulsa OK USA). One-way RM-ANOVA was used to test the effects of time upon *GFR*. Where data were missing for an individual (one white-bellied sunbird), that animal was deleted from the repeated-measures analyses. These analyses were followed by Tukey's Honest Significant Difference test for differences among means. To compare slopes of linear relationships, we used StatistiXL. For all other data, we used a mixed-model linear analysis of effects comparing the

dependent factor (each water handling parameter) against total water gain (independent factor), including bird ID (random factor; these analyses therefore took into account the repeated-measures on each individual), time (fixed factor; AM or PM) and body mass (covariate) in the analysis.

Values are means ± SD throughout. Statistical significance was accepted at $\alpha<0.05$.

Results

For afternoon values, the relationships of *ln*-[CF_{3H}] and *ln*-[CF_{14C}] with time were well described by negative linear functions (Table 3.1; see the example for one honeyeater individual shown in Fig. 3.1), with significant values ($P>0.05$) for the coefficient of determination (r^2) for honeyeaters (^{3}H: $r^2 = 0.88 \pm 0.14$; ^{14}C: $r^2 = 0.87 \pm 0.06$) and sunbirds (^{3}H: $r^2 = 0.73 \pm 0.24$; ^{14}C: $r^2 = 0.89 \pm 0.08$). The afternoon elimination rate of ^3H_2O and [^{14}C]-L-glucose in CF did not violate the assumptions of one-compartment, first order kinetics for either species. In all 24 sunbird ^{3}H trials ($F<0.01$, $P>0.990$), 18 out of 24 honeyeater ^{3}H trials ($F<1.76$, $P>0.185$), 22 out of 24 sunbird ^{14}C trials ($F<3.16$, $P>0.062$), and five out of 24 honeyeater ^{14}C trials ($F<0.47$, $P>0.635$), a biexponential model did not fit elimination significantly better than a monoexponential model.

For morning values, coefficients of determination averaged sunbirds: ^{3}H: $r^2 = 0.90 \pm 0.15$, ^{14}C: $r^2 = 0.60 \pm 0.24$; and honeyeaters: ^{3}H: $r^2 = 0.90 \pm 0.11$, ^{14}C: $r^2 = 0.28 \pm 0.25$. In sunbirds, for 22 of the 23 trials that could be tested, a biexponential model did not fit ^{3}H elimination significantly better than a monoexponential model ($F<0.01$, $P>0.990$). In honeyeaters, for 16 of the 19 trials that could be tested, a biexponential model did not fit elimination significantly better than a monoexponential model ($F<3.708$, $P>0.050$). There were only three ^{14}C trials for sunbirds and five ^{14}C trials for honeyeaters where both the

monoexponential and biexponential relationships were statistically significant; therefore statistical comparison between the different model fits was not valid. The parsimonious option was therefore to use a monoexponential model fit for all data.

The estimate of *TBW* (calculated from $^{3}H_2O$ dilution to estimate distribution space, S^{3}_{H}) for sunbirds was 51 ± 11 % of M_B and for honeyeaters 45 ± 13 % of M_B. The distribution space of ^{14}C-L-glucose (S^{14}_{C}) in sunbirds was 11.25 ± 7.57 % of their M_B while that of honeyeaters was 17.19 ± 1.22 % of their M_B.

Both sunbirds and honeyeaters drank significantly more of the dilute than concentrated diets and consequently water intake rates were higher on the more dilute sucrose diet concentrations (RM-ANOVA diet: sunbirds: $F_{2,20} = 38.77$, $P < 0.001$; honeyeaters: $F_{2,21} = 73.50$, $P < 0.001$). However, there was no significant difference in water intake rates between afternoon and morning (RM-ANOVA time: sunbirds: $F_{7,15} = 0.243$, $P = 0.967$; honeyeaters: $F_{7,16} = 0.134$, $P = 0.994$).

Total body water flux ($\dot{W}$) was positively correlated with total water gain in both sunbirds and honeyeaters (mixed-model linear analysis of effects: $P < 0.001$) for both afternoon and morning data (equations for regression lines shown in Figs 3.2a & 3.3a). There was no significant difference in $\dot{W}$ between afternoon and morning in sunbirds, but honeyeaters showed a different relationship for afternoon and morning data ($P = 0.015$). Comparing $\dot{W}$ between the two species, not surprisingly the intercepts of the $\dot{W}$ data against total water gain were significantly different (PM: $P = 0.001$; AM: $P = 0.032$) which would reflect the greater absolute *TBW* of the honeyeaters compared with the sunbirds. However the slopes comparing $\dot{W}$ and total water gain were not significantly different between the two species ($P > 0.05$).

Fractional intestinal water absorption (f_A) in sunbirds (Fig. 3.2b) did not differ between afternoon and morning ($P > 0.05$), and was significantly correlated with total water gain ($r^2 = 0.78$, $P = 0.002$); sunbirds absorbed all the water ingested on the lowest water gain diets, but only half (average of 50%) the water ingested on the highest water gain diets. New Holland honeyeaters (Fig. 3.3b) had different f_A responses for afternoon and morning ($P = 0.010$): there was a significant correlation between f_A and total water gain for the afternoon ($r^2 = 0.78$, $P = 0.004$), but this relationship did not reach statistical significance for the morning data ($r^2 = 0.06$, $P = 0.057$). f_A in honeyeaters feeding in the afternoon therefore was as low as 0.70 on the highest water gain diets (i.e. these birds were absorbing only 70% of the water in their intestine; up to 30% of the ingested water would pass through the intestine without being absorbed).

Rate of water excretion ($\dot{V}_E$) was not significantly different between afternoon or morning for either species ($P > 0.05$). $\dot{V}_E$ was significantly inversely correlated with total water gain in sunbirds ($P = 0.002$; Fig. 3.2c) and honeyeaters ($P = 0.017$; Fig. 3.3c).

There was a significant effect of time of day on estimates of *GFR* in both sunbirds (RM-ANOVA sunbirds: $F_{1,7} = 124.32$, $P < 0.001$) and honeyeaters ($F_{1,7} = 63.77$, $P < 0.001$). For both bird species, *GFR* was significantly higher in the afternoon than in the morning, and overnight *GFR'* was negligible (Fig. 3.4). For both species, *GFR* was not correlated with total water gain ($P > 0.05$; Figs 3.2d & 3.3d). Estimates of afternoon kidney fractional water reabsorption (f_R) were similarly insensitive to water loading in both sunbirds and honeyeaters (Figs 3.2e & 3.3e).

The estimates of *TEWL* were extremely variable for both species, which may largely be due to the number of pharmacokinetic calculation steps involved in these estimates. The cumulating error was likely to influence the calculations, where even slight differences in estimates of the parameters involved had substantial effects upon calculated values. Many of the estimates were less than zero (Fig. 3.2f, 3.3f). Assuming these values were zero, estimates of *TEWL* for sunbirds (0.56 ± 0.38 ml·h^{-1}, range 0 – 1.55 ml·h^{-1}) were substantial (i.e. 7% of M_B hourly). *TEWL* was significantly positively correlated with total water gain in sunbirds (P = 0.024; Fig. 2f): *TEWL* increased with water loading. The honeyeater data had a high proportion of erroneous values (n=10 of 24 trials yielded *TEWL* estimates <0 ml·h^{-1}) and were highly variable (0.63 ± 0.78 ml·h^{-1}, i.e. 3% of M_B hourly; range 0 – 2.76 ml·h^{-1}, calculated by substituting erroneous data for values with 0 ml·h^{-1}). There was no correlation between *TEWL* and total water gain for honeyeaters (P = 0.216; Fig. 3.3f), but these estimates cannot be considered reliable.

Discussion

We found that sunbirds and honeyeaters handle their water loads similarly for the most part. Both species showed modulation of intestinal water absorption (f_A) but no modulation of *GFR* or renal water reabsorption (f_R) with varying water intake. There were only small differences between these two passerine lineages. Sunbirds were more sensitive to the disruption caused by IM administration and would often not return to feed immediately, but when they did feed, they fed at a fairly steady rate in both the afternoon and morning, with similar water intake, water flux, intestinal absorption, turnover and excretion. Honeyeaters showed a greater range of water gains for morning data, and differences between afternoon and morning data for water flux, intestinal absorption, turnover and excretion. First we will discuss the findings of this study and then assumptions and limitations of the steady-state feeding pharmacokinetics method.

How do sunbirds and honeyeaters deal with water loading?

Body water turnover rate increases linearly with water intake in both sunbirds and honeyeaters. When birds were feeding on the most dilute diets (0.25 $mol.l^{-1}$ is an ecologically-relevant concentration for nectar solutions), sunbirds were turning over up to 80% of their *TBW* every hour, while honeyeaters were turning over up to 50% of their *TBW*. This is a dramatic water turnover rate which is similar to water turnover rates experienced by aquatic vertebrates (Beuchat et al., 1990). How these birds deal with these massive amounts of preformed water is therefore an important aspect of their physiology.

Water loading puts an immense burden on the renal system. The two species of hummingbirds tested to date appear to deal with water loading by relying on their renal system, absorbing the majority of ingested water across the intestine and showing no regulation of intestinal water absorption on dilute diets (Hartman Bakken and Sabat, 2006; McWhorter and Martínez del Rio, 1999). By contrast, Palestine sunbirds regulate their water absorption (f_A), avoiding 64% of ingested water by shunting this water straight through the intestine when intake rates are high (McWhorter et al., 2003), confirming the intestinal shunting hypothesis of Beuchat et al. (1990). Our study supported the findings for Palestine sunbirds, with white-bellied sunbirds also modulating intestinal water absorption, avoiding 50% of the ingested water when water intake rates are high and thereby reducing renal load. New Holland honeyeaters also modulate intestinal water absorption, avoiding up to 30% of ingested water when water intake rates are high in the afternoon. However, in the morning, honeyeaters showed extremely variable responses and, therefore, their f_A was not significantly correlated with total water gain (P = 0.057). This variability is likely due to individual responses to dehydration overnight when the

birds are fasting, thus requiring different levels of rehydration in the mornings, but may also indicate problems with the assumptions of the pharmacokinetic method in this case (i.e. some honeyeaters may not be in a steady feeding state during the morning and may be rehydrating, given that they show lower water flux for corresponding total water gain values measured in the afternoon).

Interestingly, *GFR* did not vary with different levels of water loading for either sunbirds or honeyeaters. A similar lack of response of *GFR* to varying water gain was also recorded in *S. sephanoides* hummingbirds (Hartman Bakken and Sabat, 2006). While the hummingbirds had *GFR* that were 10% lower in the morning compared to the afternoon (Hartman Bakken and Sabat, 2006), this difference between afternoon and morning *GFR* values was even more pronounced for sunbirds (73.5% lower) and honeyeaters (86% lower). The extremely low morning *GFR* values for honeyeaters are especially puzzling, and may be related to rehydration processes.

Neither sunbirds nor honeyeaters showed a relationship between water gain and renal fractional water reabsorption (f_R). This is unexpected, since hummingbirds (*S. sephanoides*) and nectar-feeding bats (*G. soricina*) decrease f_R with increasing water gain as their mechanism of countering water-loading (Hartman Bakken et al., 2008; Hartman Bakken and Sabat, 2006; McWhorter and Martínez del Rio, 1999). The lack of modulation of f_R in sunbirds and honeyeaters supports the suggestion that modulation of intestinal water absorption is likely to be the important physiological mechanism used by these passerines.

When feeding on dilute diets, nectarivores excrete greater volumes of urine (Goldstein and Bradshaw, 1998; Nicolson and Fleming, 2003b), but could potentially also adjust the volume of water that is lost by evaporation. Birds that consume nectar should be capable of higher rates of EWL than those consuming predominantly solid foods, and ideally should be able to modulate their *TEWL* according to their preformed water load. However *TEWL* for *S. sephanoides* was not different than predicted from an allometric expectation and was not affected by water intake (Hartman Bakken and Sabat, 2006). We used the same prediction based on our data and allometric equations (Williams, 1996) and found that the *TEWL* allometric calculations for both sunbirds (2.11 $ml \cdot d^{-1}$ or 0.09 $ml \cdot h^{-1}$) and honeyeaters (3.34 $ml \cdot d^{-1}$ or 0.14 $ml \cdot h^{-1}$) were much lower than the values calculated in the present study (0.56 ± 0.38 $ml \cdot h^{-1}$ and 0.63 ± 0.78 $ml \cdot h^{-1}$ respectively). In sunbirds, two studies have demonstrated a possible link between diet and EWL (Fleming et al., 2004b; Lotz and Nicolson, 1999). Similarly, for two honeyeater species, gravimetrically-measured EWL was affected by diet concentration (Collins, 1981). Pallas's bats (*G. soricina*) increase EWL with increasing water intake (Hartman Bakken et al., 2008). While these data suggest that nectar-feeding animals may respond to increased preformed water load by increasing EWL, it is also important to consider what happens when these animals stop feeding. Hartman Bakken & Sabat (2006) estimated EWL in hummingbirds (*S. sephanoides*) and predicted that these birds would not have any problem replacing the amount of water lost through evaporation (~2% of body water per hour) while feeding, but that, unchecked, this would amount to a loss of ~28% of their total body water when they are not feeding overnight.

Unfortunately, using the pharmacokinetic technique to calculate *TEWL* has proven to be unreliable in this study for sunbirds and honeyeaters. The values needed for the many calculations all include some error in estimation, and minute variations in the components

of final equation may compound to result in large errors. We estimated values for honeyeater *TEWL* which were extremely variable and close to (or below) zero, making it difficult to draw any substantial conclusions. *TEWL* in sunbirds were similarly highly variable, but the *TEWL* estimates were significantly correlated with total water gain.

How do sunbirds and honeyeaters avoid dehydration?

Although *GFR* did not change with varying levels of water loading, it is sensitive to water deprivation: both sunbirds and honeyeaters arrested kidney function at night. Shutting down the kidneys overnight appears to be an important mechanism used by hummingbirds (Hartman Bakken et al., 2004; Hartman Bakken and Sabat, 2006), as well as sunbirds and honeyeaters (present study) to help avoid potential dehydration during the overnight fast as well as energy saving mechanism. Although we recorded no changes in *GFR* with water intake which could be offset by the shunting of water through the GIT, what did change with varying water loads was intestinal water absorption, which was higher for the most concentrated diets and declined with diet dilution for both sunbirds and honeyeaters.

Assumptions and limitations of the steady-state pharmacokinetic model

Certain assumptions are made in the steady-state feeding pharmacokinetic protocol used. While some assumptions are supported by previous studies, others have the potential to cause variations and inconsistencies (Napier et al., 2012).

The first assumption is that the estimates of K_{el} and S are derived from correct modelling of the numbers of distribution pools (i.e. the relationship between isotope

concentration and time reflects dispersal through a single compartment, rather than more than one body compartment). In both species, single compartment, first order kinetics could be applied to $^{3}H_2O$ elimination for both afternoon and morning data. Elimination of [^{14}C]-L-glucose in the afternoon was clearly single compartment; however elimination of [^{14}C]-L-glucose in the morning were less well described by a linear relationship. This may be due to the pattern of CF excretion after fasting overnight - both sunbirds and honeyeaters arrested kidney function at night, and the first excreta samples in the morning, which were smaller in volume and more concentrated than those produced later in the morning, were likely to represent CF that had been retained until the bird recommenced feeding in the morning (Fleming et al., 2004b). Consequently, the relationship with time was lost for these early samples (i.e. the time that the CF was produced was not the time recorded as excreted). This was not observed for $^{3}H_2O$ excretion because water would continue to be reabsorbed and excreted overnight through *EWL* and cloacal reabsorption.

The second assumption is that the animals are feeding at a steady rate. This assumption is valid for the afternoon data but is potentially violated in the morning due to the overnight fast and rapid rehydration and feeding (Fleming et al., 2004a); conclusions about morning data should be made with careful consideration of these potential errors. Additionally, response to the experimental method was also a cause for concern in regard to the assumption of steady state feeding. Because the honeyeaters mostly resumed feeding within minutes, these birds did not confound the assumption of steady-state feeding. However some white-bellied sunbirds did not commence steady-state feeding immediately after being captured and injected, and for half of the experimental trials with sunbirds, the time calculations had to be adjusted accordingly (compared with ~20% of trials with the honeyeaters). Other species differences in feeding and excretion behaviour were also identified. The first excreta after IM administration for the honeyeaters showed higher

[^{14}C]-L-glucose concentrations than subsequent values (Fig. 3.1), while the initial values for the sunbirds were lower than subsequent excreta. This difference suggests that sunbirds probably reduced *GFR* in response to disturbance, but the honeyeaters continued to eliminate [^{14}C]-L-glucose through glomerular filtration and reduced frequency of excretion (i.e. stored cloacal fluid and reabsorbed water in the distal intestine) until they and started feeding normally. When honeyeaters started to feed, the concentration of $^{3}H_2O$ in excreta dropped as urine flow rate increased. But the sunbirds are a different matter; if they retained water then effectively they were a closed system and the pharmacokinetic model would not apply. This is sufficient justification to adjust the intake data by re-setting the *t=0* to the point when the birds started to defecate regularly (and were thus feeding regularly).

The third assumption of the steady-state pharmacokinetic method is in regard to data accuracy, assuming that there is immediate distribution of the marker from the site of injection, that concentrations in the cloacal fluid reflect those in the blood, and that isotope concentrations leaving the body are equal to those in body water at that moment in time (Lifson and McClintock 1966). However previous research has identified differences in isotope concentration between body water and excreted fluids, which occur due to physical and biological fractionation (Lifson and McClintock 1966), a process that is believed to occur in nectar-feeding birds (McWhorter and Martínez del Rio, 1999). Thus, for better accuracy, we estimated the proportion of ingested water contributing to the turnover of TBW following McWhorter et al. (2003). This calculation makes the assumption that the rate of appearance of isotope in the excreted fluid is equal to the disappearance of isotope from TBW. As an aside, although the estimates of TBW (sunbirds: 51 ± 11 %, honeyeaters 45 ± 13 % of M_B) may appear to be lower than would be expected, these values are

marginally lower than values for green-backed firecrowns (56.6 ± 2.0%; Hartman Bakken and Sabat, 2006) or Palestine sunbirds (63.6±0.7%, McWhorter et al., 2003).

Conclusion

In conclusion, this study shows that both sunbirds and honeyeaters use modulation of intestinal water absorption as an important component of water regulation to help deal with massive preformed water loads. Shutting down *GFR* during the natural overnight fast is another way of saving on the energy required by the kidneys and avoiding dehydration. Sunbirds and honeyeaters maintain osmotic balance very effectively on diets that can vary markedly in preformed water load by making use of a combination of mechanisms, varying water absorption and excretion through the intestine, kidneys and EWL.

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Table 3.1. The number of linear relationships between *ln*-[CF^{3}_{H}] and *ln*-[CF^{14}_{C}] against time (n = 8 for each species and each time point) that were statistically significant ($P < 0.05$) by linear regression.

While the data for *ln*-[CF^{3}_{H}] were generally well described by linear relationships with time (particularly for the more dilute diets where high feeding rate resulted in high rates of excretion), the data for *ln*-[CF^{14}_{C}], particularly for concentrated diets in the morning, were less robust.

		Sunbirds		Honeyeaters	
Isotope	Diet	afternoon	morning	afternoon	morning
$^{3}H_2O$	0.25 mol.l^{-1}	8	8	8	8
	0.5 mol.l^{-1}	7	8	8	8
	1 mol.l^{-1}	6	8	7	8
overall		21/24 = 88%	24/24 = 100%	23/24 = 96%	24/24 = 100%
[^{14}C]-L-glucose	0.25 mol.l^{-1}	8	8	8	4
	0.5 mol.l^{-1}	8	6	7	4
	1 mol.l^{-1}	7	6	8	2
overall		23/24 = 96%	20/24 = 83%	23/24 = 96%	10/24 = 42%

Figures

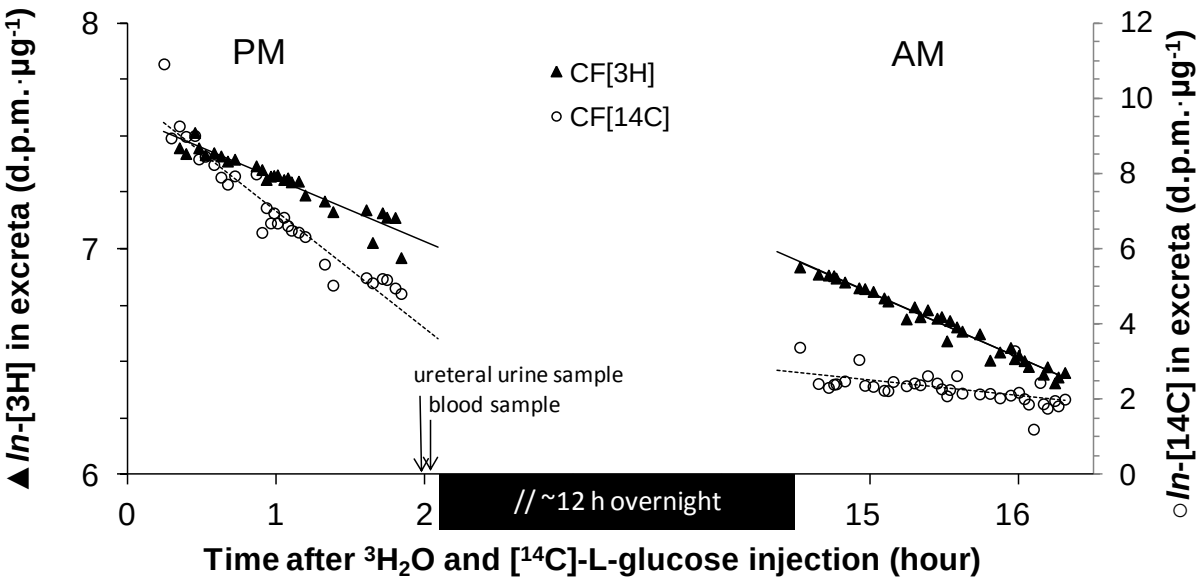

Figure 3.1: Data from a representative New Holland honeyeater individual feeding on 0.5 mol.l^{-1} sucrose illustrating our method of measuring the gastrointestinal and renal function during the afternoon (PM), overnight (black bar) and the following morning (AM).
Each data point represents the *ln*-transformed $^{3}H_2O$ or *ln*-transformed [^{14}C]-L-glucose values in individual cloacal fluid (CF) samples. The timing of the ureteral urine and blood samples is shown (immediately before lights-out). The graph shows that $^{3}H_2O$ appears in CF over time according to single-compartment first order kinetics (confirmed by comparison between mono- and biexponential models); while [^{14}C]-L-glucose adheres to the principles in the afternoon, there was a gentler slope in the morning data [for 17% of sunbird trials and 58% of honeyeater trials, the slopes for these data were not statistically significant (Table 3.1), and only a minority of trials could be compared between mono- and biexponential models].

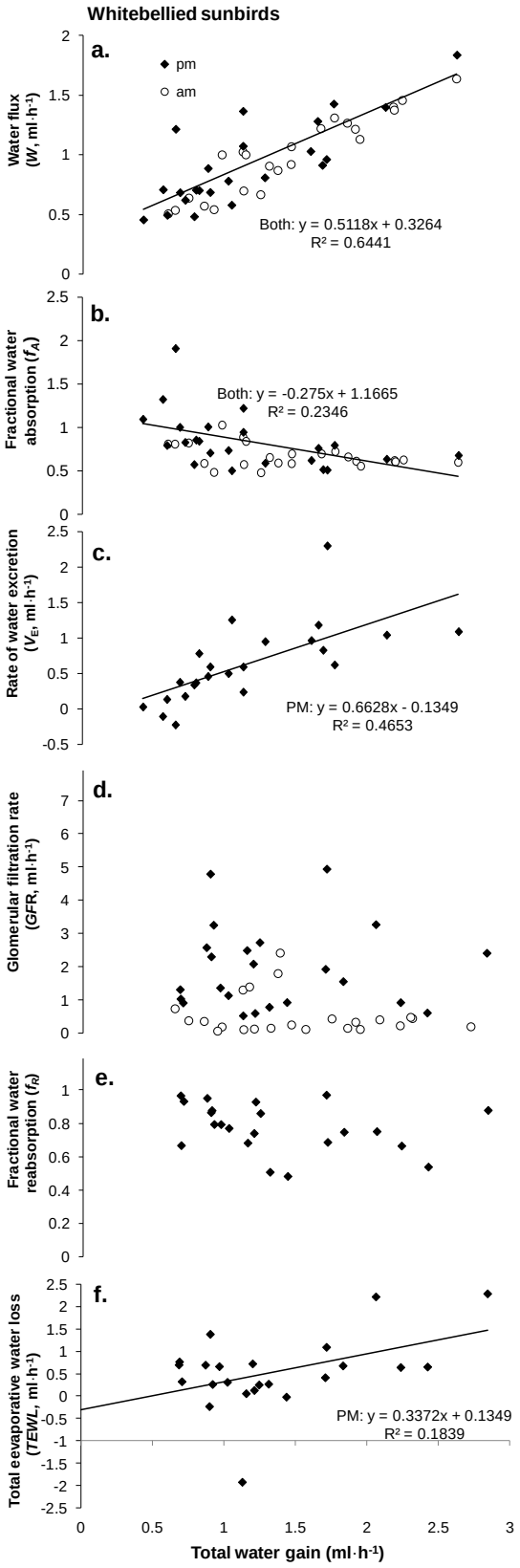

Figure 3.2: The influence of water intake rates (*x*-axes) on the water handling processes during the afternoon (♦) and morning (○) in white-bellied sunbirds.
Rates of (a) Water flux (*W*), (c) water excretion (V_E), and (f) evaporative water loss (*TEWL*) increased linearly with total water gain. (b) Sunbirds modulated gastrointestinal tract fractional water absorption (f_W), shown as an inverse relationship with total water gain. (d) Glomerular filtration rate (*GFR*) and (e) renal fractional water reabsorption (f_R) were not influenced by water intake rate in white-bellied sunbirds.

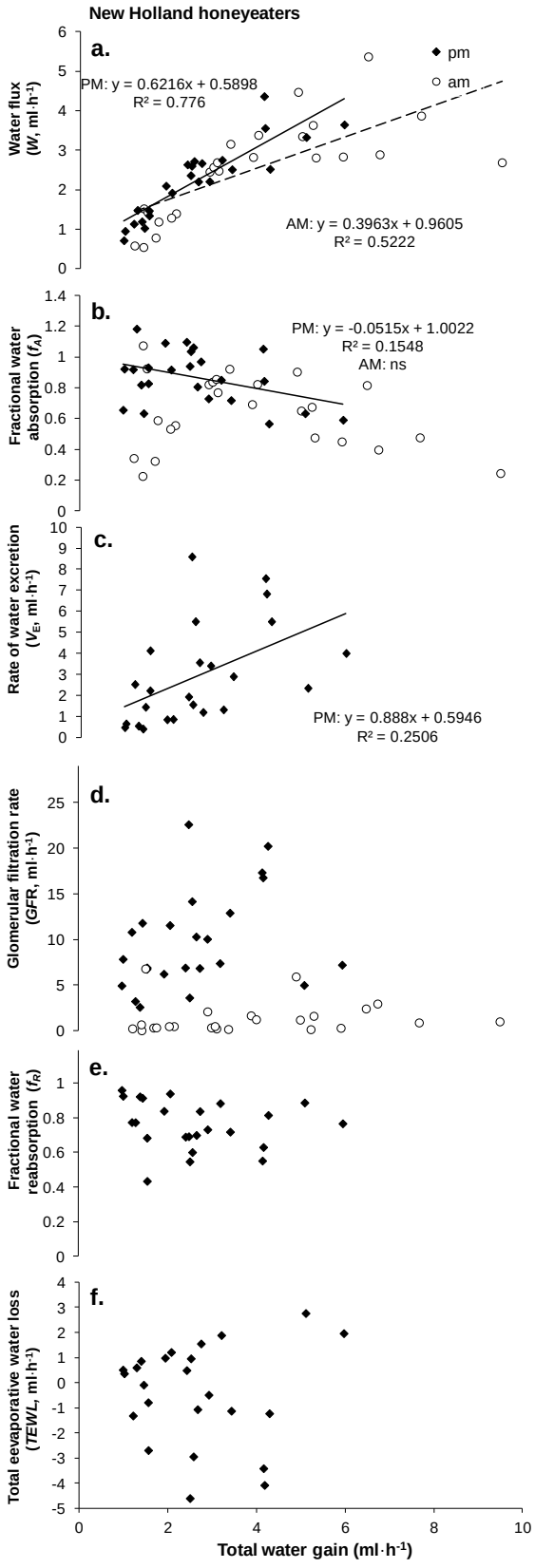

Figure 3.3: The influence of water intake rates on the water handling processes during the afternoon (♦) and morning (○) in New Holland honeyeaters.
Rates of (a) Water flux (*W*) and (c) water excretion (V_E) increased linearly with total water gain. (b) Honeyeaters modulated gastrointestinal tract fractional water absorption (f_W), shown as an inverse relationship with total water gain. There was no relationship between total water gain and (d) Glomerular filtration rate (*GFR*), (e) renal fractional water reabsorption (f_R) or (f) evaporative water loss (*TEWL*) in honeyeaters.

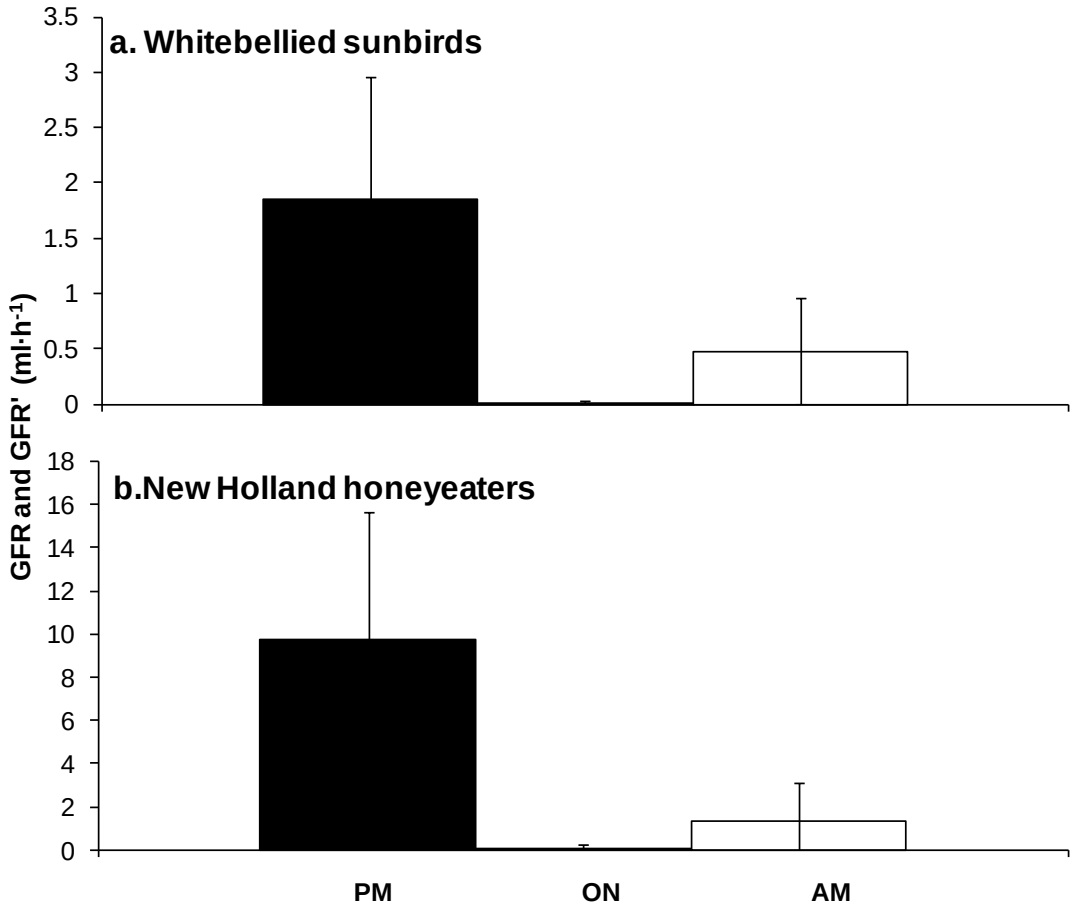

Figure 3.4: Mean (± SD) glomerular filtration rate (daytime: *GFR* or estimated overnight *GFR'*, ml.h^{-1}) in the afternoon (PM), overnight (ON), and early morning (AM) in a) white-bellied sunbirds and b) New Holland honeyeaters.

Both species arrested whole kidney function during the night time fasting periods, with *GFR* values not different from zero, and morning values were significantly lower than afternoon values.

Electronic supplementary appendix:

Adjusted for non-feeding time NOT adjusted for non-feeding time

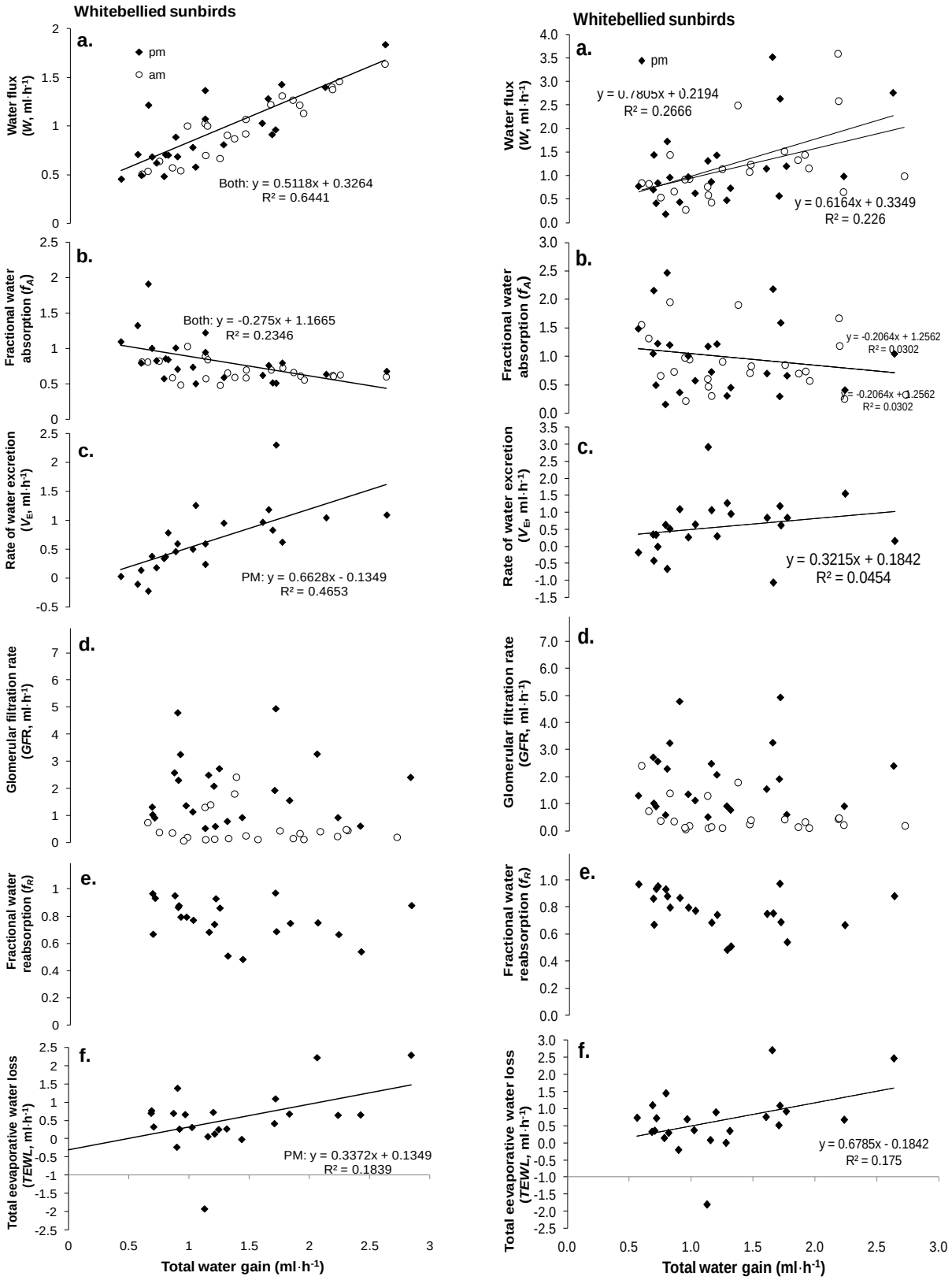

Figure 3.5: The influence of water intake rates (x-axes) on the water handling processes during the afternoon (♦) and morning (○) in white-bellied sunbirds either with (left hand panel) or without (right hand panel) the adjustment for feeding time.

Adjusted for non-feeding time

NOT adjusted for non-feeding time

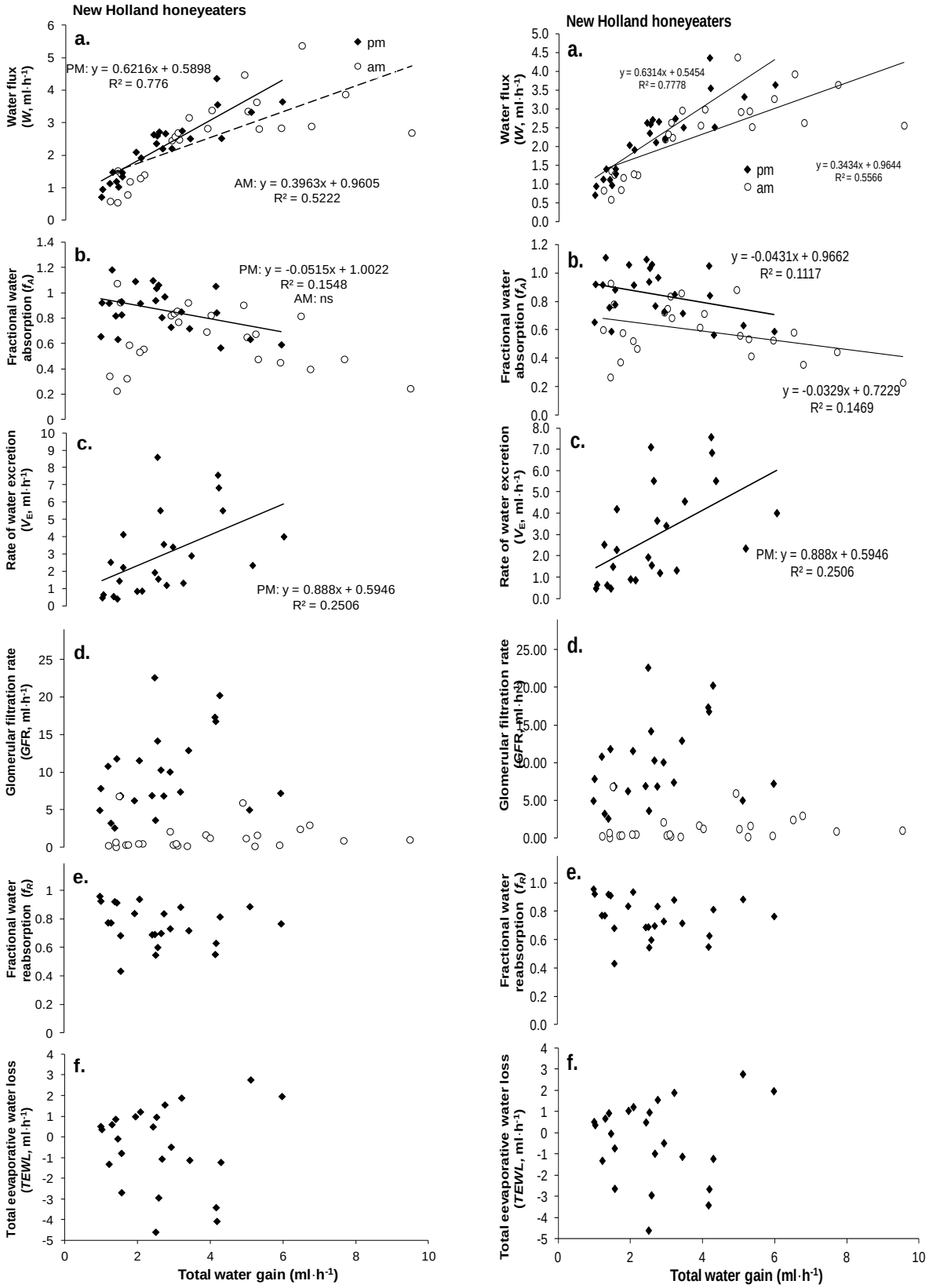

Figure 3.6: The influence of water intake rates on the water handling processes during the afternoon (♦) and morning (○) in New Holland honeyeaters either with (left hand panel) or without (right hand panel) the adjustment for feeding time.

Conclusion

There were many similarities in the responses of white-bellied sunbirds and New Holland honeyeaters to highly variable nectar diets. We found that the addition of NaCl to dilute diets enabled both sunbirds and honeyeaters to increase consumption, both species consuming an extraordinary eight times their body mass in fluid daily. In salt preference experiments, both sunbirds and honeyeaters switched diets to maintain constant intakes of both sucrose and sodium. But when given no choice diets to test their renal concentrating ability, both species increased cloacal fluid osmolalities with diet NaCl concentration; honeyeaters, however, tended to retain ingested sodium while sunbirds excreted it. The pharmacokinetic tests showed that both sunbirds and honeyeaters modulate intestinal water absorption to help deal with high preformed water loads. In addition, both species save energy by arresting whole kidney function during their overnight fast.

This thesis has explored some fundamental physiological abilities that allow nectarivores to consume copious amounts of dilute nectars. The birds are over-ingesting water and under-ingesting salts and nitrogen on these dilute diets in order to obtain energy; they need precise control of water fluxes of many times their body mass daily, while maintaining digestive and osmoregulatory functions. Managing the interactions between energy, water and ion regulation with such precision, on extremely dilute diets, requires a combination of physiological mechanisms and behavioural control. Over 20 years ago, the review by Beuchat et al. (1990) noted that ecological and physiological problems associated with energy balance in hummingbirds had been investigated in some detail, but that at the time there was a lack of knowledge related to hummingbird water and ion homeostasis. There were some early physiological studies on honeyeaters (Collins 1981;

Collins et al. 1980; Collins and Morellini 1979), but little was known about the third lineage of avian nectarivores, the sunbirds. Over the last two decades, our understanding of avian nectarivore physiology has progressed substantially, including numerous studies of sunbirds and honeyeaters (Goldstein and Bradshaw 1998; Lotz 1999; Fleming and Nicolson 2003; Fleming et al. 2004; Gray et al. 2004; Fleming et al. 2008).

Beuchat et al. (1990) hypothesised that nectar-feeding birds would be able to avoid some of their water loading by shunting water through the GIT (i.e. not absorbing all the water ingested), effectively bypassing the kidneys. Two hummingbird species have been investigated to date. Pharmacokinetic tests showed that broad-tailed hummingbirds (*Selasphorus platycercus*) and Chilean green-backed firecrowns (*Sephanoides sephanoides*) are not able to modulate their intestinal water absorption (Hartman Bakken and Sabat 2006; McWhorter and Martínez del Rio 1999), but instead absorb all ingested water. However, under similar conditions of variable water loading, Palestine sunbirds (*Cinnyris osea*) can adaptively regulate water absorption across the gut, shunting up to 64% of ingested water when intake rates are high (McWhorter et al. 2003; McWhorter et al. 2009). In this thesis we present data that supports this finding for a second sunbird species (whitebellied sunbird) as well as the first evidence for a honeyeater species (New Holland honeyeater). Our data shows that both sunbirds and honeyeaters modulate intestinal water uptake, while hummingbirds do not. Passive absorption of glucose is of major importance for nectar-feeding birds (McWhorter et al. 2006; Napier et al. 2008) but paracellular absorption decreases with increasing water load (Napier et al. 2008); hence the more dilute the nectar diet, the more important modulation of water in the gut becomes to control the excessive uptake. The lower intestine is crucial for water and salt regulation (Goldstein and Skadhauge 2000), but this does not conflict with the significant paracellular nutrient absorption (McWhorter et al. 2009).

The integration between total body sodium and fluid intake is interesting. In this thesis we found decreased sodium serum values with increasing fluid intake. The physiological mechanisms associated with a decrease in sodium serum levels is primarily the stimulated release of Aldosterone from the adrenal cortex. Aldosterone acts on renal Na-K ATPase to increase urinary excretion of potassium from the distal tubules in exchange for sodium reabsorption (Goldstein 1993, Skadhauge et al. 1983). As serum sodium increases, water reabsorption increases, following the osmotic gradient. Renal arteriolar blood pressure then increases, helping to maintain GFR. More water and sodium then pass through the distal tubules, overriding the initial effect of aldosterone. The hepato-renal reflex plays an important role in regulating sodium chloride homeostatic balance during food intake. The renal nerve activity is decreased by the hepatic nerves suggesting that the hepatic nerves play an important role in post prandial natriuresis (Morita et al. 1993). In the oral cavity or oesophagus the presence of osmoreceptors stimulated to adjust renal activity or even satiety, by triggering nervous responses in the hypothalamus could play a significant role in fluid intake. The vagus nerve could play an important role but further investigation is necessary to confirm this.

In all nectar-feeding birds tested to date, glomerular filtration rate (GFR) is not influenced by water loading (Hartmann Bakken and Sabat 2006; McWhorter et al. 2004; McWhorter and Martinez del Rio 2003); however, GFR is affected by water deprivation during the nocturnal fast. All three lineages of avian nectarivores arrest whole kidney function during their overnight fasting period, thus saving energy that would be used for reabsorption. However hummingbirds, sunbirds and honeyeaters deal with their water loading differently through modulating GIT water absorption. While fractional water reabsorption (f_R) in both sunbirds and honeyeater is not influenced by water load, in

hummingbirds f_R is reduced on high water loads; this might help hummingbirds to cope in the absence of modulated gut absorption.

White-bellied sunbirds are capable of excreting cloacal fluid that has lower total osmotic and glucose concentrations than their ureteral urine. This shows that, combined with renal regulation, the lower GIT plays an important role in electrolyte and glucose recovery. This was not evident for the New Holland honeyeater. The effects of unabsorbed dietary water shunted through the gut cannot be ignored: this is likely to contribute to the low electrolyte concentrations observed in cloacal fluid of sunbirds (Fleming and Nicolson 2003). Whether the low cloacal fluid solute concentrations are due mainly to modulated intestinal absorption of water, or solute absorption in the lower GIT, the combined effect helps to explain why sunbirds perform better on dilute diets than hummingbirds, with hummingbirds going into torpor when they are unable to maintain energy balance on such diets (Fleming et al. 2004). The ability of hummingbirds to use torpor during times of bad weather and limited diet availability could be the reason why hummingbirds do not have the ability to shunt water through their GIT as the need is negated by torpor (Calder 1994). Attempts have been made to force torpor on sunbirds in captivity with little success (Downs and Brown 2002), while the larger size of honeyeaters on a liquid diet would suggest that they most likely would not use torpor as an energy saving technique.

We found that dietary sodium plays a significant role in the maintenance of energy balance on dilute diets, suggesting that alternative salt sources may be important for these birds to supplement their nectar diet. This supplementation will be especially important under wet or cold conditions, where nectar has been diluted by rain or dew, insects are in short supply, and the birds are required to increase intake to maintain energy balance at low temperatures (Purchase et al. 2010; Köhler et al. 2010). This study also revealed that

the preference of sunbirds and honeyeaters for electrolytes (NaCl) varies according to the sugar concentration of their diet. Both sunbirds and honeyeaters showed remarkable precision in their control of sugar and salt intake by consistently switching diets when offered a variety of sugar and salt concentrations in order to maintain a constant energy and ion intake. This behavioural switching occurs in the wild as nectarivores hawk for insects and drink nectar from flowers.

Future studies

Our knowledge has advanced a great deal over the last two decades, but there are many more questions that await investigation. During this period some research has revealed that hummingbirds have their own unique way of dealing with dilute diets: instead of modulating water absorption, they decrease energy expenditure by entering a form of hibernation (torpor) returning to full function later when diets resume normal concentrations. Sunbirds and honeyeaters, however, modulate water absorption through the gut allowing for significant energy saving, but sunbirds and honeyeaters have different methods of dealing with excess NaCl in their diets. All three lineages arrest whole kidney function during their natural overnight fast. These answers bring about new questions.

The pharmacokinetics experiments used to establish modulation of water absorption have now been carried out on two hummingbird, two sunbird and one honeyeater species. Apart from further pharmacokinetic studies on additional species, the important future questions are what mechanisms are likely to be involved in GFR and intestinal water absorption. This is the next step towards a better understanding of the water handling processes of nectar feeding birds, while endocrine control of these mechanisms is still unknown.

A diet of nectar is very sugar and water-rich, but low in proteins, which are essential for growth, basic body functioning and repair. Hummingbirds, sunbirds and honeyeaters all augment their diet with essential proteins by sporadically eating small arthropods such as insects, spiders and pollen. We have good information on how nectar-feeding birds deal with sugars, water and ions (Köhler et al. 2012); the next step is to learn more about how protein intake is regulated. When offered choices between pairs of complementary liquid diets varying in sucrose and protein content, white-bellied sunbirds defend a mean protein intake of 44 $mg.day^{-1}$ (S. Rodrigues d'Araujo, unpublished data). This confirms the low protein requirements of sunbirds determined in previous studies (Roxburgh and Pinshow 2000; Van Tets and Nicolson 2000). The low activity of aminopeptidase-N in nectarivores is consistent with their exceptionally low nitrogen requirements and relatively low insect and thus protein intake (McWhorter et al. 2009). Less is known about the endogenous enzyme activity (aminopeptidases) in the hindgut and how this could benefit essential amino acid synthesis allowing for more efficient protein absorption. We still have no evidence to explain how birds are capable of exhibiting digestive efficiencies comparable to mammals while consuming relatively more and processing relatively faster, with relatively less intestine.

While compensatory feeding is a physiological behavioural trait that works well for avian nectarivores and is used by most animals, when the composition of the food source available is insufficient to allow for the target nutritional requirement to be reached, the amount of imbalanced diet consumed must be regulated before a critical point is reached (Köhler et al. 2012). In this thesis we showed how sunbirds and honeyeaters can boost their total diet consumption on dilute nectar diets with the addition of NaCl, showing how important ions are when dealing with excessive water loads.

Understanding the role of sodium in the diet of nectarivorous birds still requires much work; this is compounded by the high level of variation in field water and ion balance shown amongst birds (Goldstein and Bradshaw 1998). Measurements of the Na^+ and K^+ concentrations in excreta of sunbirds in the field would give us a better understanding of the ecological relevance of these data and enable comparison with previous field research on honeyeaters (Goldstein and Bradshaw 1998). There is a scarcity of information on nectar ion levels and the extent of arthropod foraging amongst nectarivorous birds. Information on both is required to interpret the ecological consequences of varying tolerance to dietary sodium.

When loaded with NaCl rich solutions, sunbirds and honeyeaters controlled their intake and sodium levels differently. Renal morphological studies on sunbirds are an important next step to compare sunbird kidneys to both hummingbird and honeyeater kidneys and possibly answer some remaining questions regarding the impressive renal capabilities of nectarivorous birds.

Convergence between hummingbirds, sunbirds and honeyeaters, all of which are small and predominantly nectar-feeding birds, is one of the best examples of convergent evolution in birds. Hummingbirds, sunbirds and honeyeaters originated from independent ancestors, although sunbirds and honeyeaters are both passerine lineages (and therefore more closely related to each other than to hummingbirds). Hummingbirds (family Trochilidae) originated in the Old World, and evolved by a transition from tree-dwelling to aerial foraging forms. Sunbirds (family Nectariniidae) can be found throughout Africa and Asia. Honeyeaters (family Meliphagidae) are distributed throughout Australasia (Nicolson and Fleming 2003).

Hummingbirds, sunbirds and honeyeaters are small, light birds, and share beaks that can be highly elongated and either straight or recurved, depending on what type of flower they probe for nectar (Pyke 1980; Cheke 2001). Their tongues are extensible, tipped with brush-like filaments and are either tubular or grooved in order to generate capillary action for drawing nectar. These birds are critical pollinators for a number of flowers, and as an adaptation to the large amount of pollen they are exposed to, their nares have an operculum (Roxburgh and Pinshow 2000).

McWhorter et al. (2003) suggested that sunbirds regulate transepithelial water flux independently of sugar absorption. These results opened the door to many questions about how water transport is regulated in the vertebrate gastrointestinal tract. Results 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, and now we find similar traits in honeyeaters. The physiological traits of these three groups that allow the processing of a water and sugar diet, however, are different. This study shows that the control of large water fluxes on dilute diets is dealt with differently by hummingbirds compared to sunbirds and honeyeaters while salt balance is handled differently by sunbirds and honeyeaters. These different methods of handling the osmoregulatory problems of drinking very dilute diets show the amazing evolutionary changes that have allowed these birds, of different ancestry, to converge on the same nutritional niche on different continents.

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Appendix A: The sweet life: diet sugar concentration influences paracellular glucose absorption

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Short Title: Glucose absorption in a honeyeater and sunbird

Abstract

Small birds and bats face strong selection pressure to digest food rapidly in order to reduce digesta mass carried during flight. One way they may do this is by rapidly absorbing a high proportion of glucose via the paracellular (non-mediated) pathway. Intestinal paracellular permeability to glucose was assessed for two avian nectarivores (the Australian New Holland honeyeater and African white-bellied sunbird) by measuring the bioavailability of radiolabelled, passively absorbed L-glucose. Bioavailability was high in both species and increased with diet sugar concentration (honeyeaters: 37 and 81%; sunbirds: 53 and 71% for 250 and 1000 mmol/L sucrose diets respectively), suggesting that the relative contribution of paracellular to total glucose absorption increases with digesta retention time in the intestine.

Introduction

Paracellular (non-mediated) absorption of glucose in the small intestine accounts for a minimal degree (~5%) of total glucose uptake in non-flying mammals (reviewed by McWhorter, 2005). Birds and flying mammals, however, have less small intestinal surface area and significantly shorter small intestines than non-flying mammals of a similar size, equating to a >50% reduction in intestinal volume (Caviedes-Vidal et al., 2007). As the energetic costs of flight increase with an increase in the load carried, a decrease in the mass of digesta carried is advantageous; yet these animals need to somehow satisfy relatively high energy needs with reduced absorptive surface area (Caviedes-Vidal et al., 2007). Data presented for birds (Karasov and Cork, 1994, Caviedes-Vidal and Karasov, 1996, Levey and Cipollini, 1996, Afik et al., 1997, McWhorter et al., 2006) and bats (Tracy et al., 2007) suggests that enhanced intestinal paracellular absorption of water-soluble nutrients such as glucose and amino acids may compensate for the reduction in intestinal absorptive surface area (Caviedes-Vidal et al., 2007). Paracellular absorption provides a non-saturable absorptive process that automatically compensates for acute changes in dietary nutrient concentrations (Ferraris, 2001). Nectar-feeding birds, with their simple diets, high metabolic demands and extremely rapid behavioural responses to changes in diet energy density (Fleming et al., 2004a, 2004b, McWhorter et al., 2006), may therefore be excellent models in which to study the regulation and mechanisms of nutrient absorption and epithelial permeability.

Along with the Neotropical hummingbirds, two passerine groups (Australian honeyeaters and African sunbirds) make up the three major radiations of nectarivorous birds (Nicolson and Fleming, 2003b). Convergence in diet has led to the evolution of many similar physiological traits between the passerines and hummingbirds, and selective pressure due to their common diet may result in similar mechanisms of intestinal

carbohydrate absorption between these three nectarivores (Nicolson and Fleming, 2003b). For example, all three groups exhibit compensatory feeding, where food intake is adjusted with diet sugar concentration to maintain constant rates of energy intake (Lotz and Nicolson, 1999, McWhorter and Martinez del Rio, 1999, Nicolson and Fleming, 2003a, Schondube and Martinez del Rio, 2003, Fleming et al., 2008). Data presented by McWhorter et al. (2006) in hummingbirds (Trochilidae), novelly suggests that food energy density has an effect on paracellular glucose uptake. These authors found that L-glucose bioavailability, the fraction (F) of an oral dose absorbed into the systemic circulation, varies with food sugar concentration which is inversely related to digesta retention time in hummingbirds (Lopez-Calleja et al., 1997, McWhorter et al., 2006). L-glucose is a biologically inert isomer of D-glucose that is absorbed only via non-mediated mechanisms (Karasov and Cork, 1994, Chang et al., 2004). Our aim was to further investigate the effects of food energy density and intake rate on the bioavailability of radiolabelled L-glucose, at two dietary sugar concentrations (250 and 1000 mmol/L sucrose) in the New Holland honeyeater (Meliphagidae) and the white-bellied sunbird (Nectariniidae). Based on the patterns indicated for hummingbirds (McWhorter et al., 2006), we hypothesised that there would be extensive absorption of orally ingested radiolabelled L-glucose in both species, indicative of significant non-mediated glucose uptake, and that L-glucose bioavailability would increase with diet sugar concentration due to increased digesta retention time.

Materials and methods

Seven New Holland honeyeaters (*Phylidonyris novaehollandiae*, body mass 22.41±0.58 standard error of the mean (s.e.m.) g) and seven white-bellied sunbirds (*Cinnyris talatala*, 8.07±0.17 s.e.m. g) were captured in Murdoch, Western Australia, and Pretoria, South

Africa, respectively, by mist netting. Routine animal husbandry, maintenance diets and experimental housing are detailed in electronic supplementary material A.

The fractional absorption (bioavailability) of L-glucose was measured using [^{14}C]-L-glucose and [^{3}H]-L-glucose, administered orally and by intramuscular (IM) injection to each bird in separate experiments. To vary food intake rate, birds received two different diets (250 and 1000 mmol/L sucrose solutions) in separate feeding experiments. The order of trials and treatments given were both randomly assigned, and followed published protocol (McWhorter et al., 2006). Bioavailability (F) was calculated as:

$$F=(P\cdot S\cdot K_{el})/I$$

where P is the steady state feeding concentration of radiolabelled L-glucose in plasma (dpm/mg of plasma), S is the probe distribution space of [^{14}C]-L-glucose in plasma (mg of plasma), K_{el} is the elimination rate constant for the removal of radiolabelled L-glucose from plasma and its excretion in urine (min^{-1}), and I is the ingestion rate of radiolabelled L-glucose (dpm/min) (Karasov and Cork, 1994, McWhorter et al., 2006).

Results

Birds drank approximately 3 times the volume of the dilute diet (250 mmol/L sucrose) compared with the more concentrated diet (1000 mmol/L, Table 1). The mean steady-state concentration of radiolabelled L-glucose in plasma (P) was relatively high in both species on both diets, indicating significant absorption of the labelled probe; diet treatment did not have a significant effect on P (Table 1).

The elimination of [^{14}C]-L-glucose did not fit a bi-exponential model significantly better than a mono-exponential model for all individual birds of both species (honeyeaters:

F<1.51, p>0.255 and F<3.09, p>0.053; sunbirds: F<0.41, p>0.615 and F<2.63, p>0.092 for 250 and 1000 mmol/L sucrose diets respectively), by the non-linear curve fitting of the concentrations of [^{14}C]-L-glucose in excreta after injection of the probe versus time, indicating single compartment elimination kinetics. Diet treatment did not have a significant effect on the elimination rate constant K_{el} (min^{-1}) or distribution space S (mg plasma) in either species (Table 1). It appears that elimination is quicker in honeyeaters when the half-time to elimination ($T_{1/2}$=0.693/K_{el}) is compared with sunbirds; $T_{1/2}$ in theory should scale with mass and be longer in the heavier honeyeater (Gibaldi and Perrier, 1982). The value of K_{el} for L-glucose is dependent upon renal function (i.e. glomerular filtration rate) which was not measured, and may differ from values predicted based on body size for our study species.

Bioavailability of L-glucose was significantly greater for both species when feeding on the more concentrated diet (honeyeaters: $F_{1,6}$=21.73, p=0.003; sunbirds: $F_{1,6}$=9.22, p=0.023, Table 1, Fig. 1) by repeated-measures-ANOVA. There was no significant interspecific difference in bioavailability on either diet concentration (250 mmol/L sucrose: $F_{1,12}$=2.69, p=0.127; 1000 mmol/L: $F_{1,12}$=0.43, p=0.523) by oneway-ANOVA (Fig. 1).

Discussion

We found extensive absorption of orally ingested radiolabelled L-glucose in the New Holland honeyeater and white-bellied sunbird (Fig. 1), which is indicative of significant non-mediated (paracellular) glucose uptake. The rate of paracellular absorption, in contrast to mediated routes of absorption, varies linearly with solute concentration and does not obey saturation kinetics (Ferraris, 2001). L-glucose bioavailability increases significantly with diet sugar concentration in both honeyeaters and sunbirds, confirming the pattern

suggested for broadtailed hummingbirds (Table 2, McWhorter et al., 2006). Like hummingbirds (Schondube and Martinez del Rio, 2003), New Holland honeyeaters have high D-glucose apparent assimilation efficiency (99.8±0.05% s.e.m. (n=16); T.J.M. & P.A.F. unpublished data) which is independent of diet concentration. D-glucose assimilation efficiency by white-bellied sunbirds has not yet been measured, but we predict is similarly high based on measurements in the congeneric lesser double-collard sunbird, *Cinnyris chalybeus* (97.9%) (Lotz and Nicolson, 1996). As L-glucose bioavailability increases with diet sugar concentration while that of D-glucose does not change measurably, the nutritional significance of paracellular uptake (i.e. relative contribution to total carbohydrate absorption indicated by the ratio of L-glucose to D-glucose bioavailability) must also increase with sugar concentration (McWhorter et al., 2006). Although single values are usually reported for other bird species (Table 2), paracellular absorption is clearly a highly dynamic process and therefore any interspecific comparison therefore needs to account for diet sugar concentration. For example, the nectarivorous rainbow lorikeet apparently absorbs a similar fraction of radiolabelled L-glucose to the granivorous house sparrow, but the comparative significance of this observation is unclear as the sparrows were presented with a glucose diet ~8 times greater in sugar concentration (Table 2).

The relationship between L-glucose bioavailability and sugar concentration is most likely due to the positive correlation between digesta retention time (i.e. contact time with absorptive surfaces in the intestine) and diet energy density as shown in hummingbirds (Lopez-Calleja et al., 1997). Another possibility, which is not mutually exclusive, is that mediated nutrient uptake enhances uptake by the paracellular pathway, either through increased water absorption via the process of solvent drag or modulation of paracellular permeability; the mechanisms by which epithelial permeability might be regulated in

response to the presence of lumenal nutrients are poorly understood (reviewed by Chediack et al., 2003). Understanding why paracellular nutrient uptake changes with diet energy density will require disentangling the effects of digesta retention time, osmolarity, and mediated nutrient transport as modulators of paracellular permeability. This study reveals a new understanding of nutrient absorption in these volant animals, and profoundly demonstrates how digestive physiology is a determinant of feeding behaviour.

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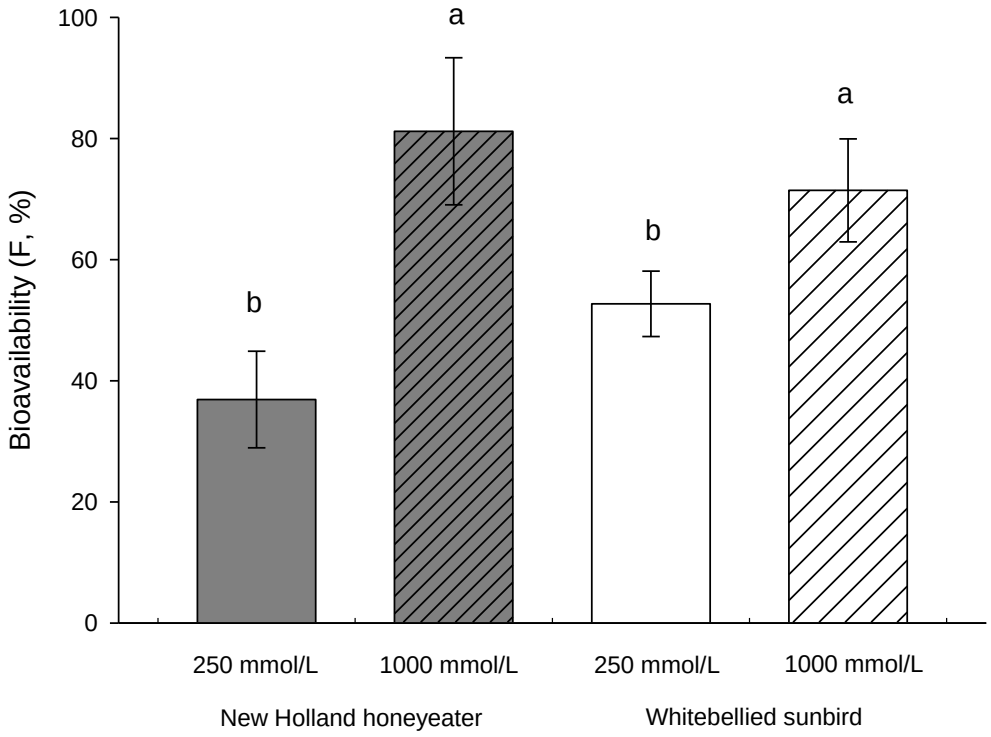

Appendix Figure 1. Bioavailability of radiolabelled L-glucose (F) differed significantly between diet treatment in honeyeaters and sunbirds, but not between the two species on each diet treatment.

Error bars indicate±1 s.e.m., with letters above indicating statistically significant ($P \leq 0.05$) diet or species differences obtained by repeated-measures and oneway-ANOVA, respectively.

Appendix Table 1. Parameters used to determine bioavailability (F) of [^{3}H]-L-glucose in honeyeaters and [^{14}C]-L-glucose in sunbirds.

Values are means±s.e.m. (n=7). Statistical significance determined by repeated-measures-ANOVA, with significant values (P≤0.05) in bold.

Parameter	*New Holland honeyeater*			*White-bellied sunbird*		
	Sucrose Diet		*Comparison of treatment effect*	*Sucrose Diet*		*Comparison of treatment effect*
	250 mmol/L	*1000 mmol/L*		*250 mmol/L*	*1000 mmol/L*	
Drinking rate (ml/min)	58.46±6.48	18.91±1.31	**P<0.001**	40.4±3.09	13.22±1.12	**P<0.001**
Intake rate, I (dpm/min)	122,000±15000	27,000±3000	**P<0.001**	41,400±4500	20,800±1500	**P=0.005**
Steady state plasma, P (dpm/mg of plasma)	538.8±157.9	252.9±29.2	P=0.081	360.7±45. 8	250.5±23.0	P=0.094
Elimination constant, K_{el} (min^{-1})	0.0526±0.0024	0.0523±0.0024	P=0.110	0.0369±0.0021	0.0364±0.0039	P=0.922
Probe distribution space, S (mg of plasma)	1796±435	1641±203	P=0.602	1666±175	1666±159	P=0.984
Bioavailability, F (%)	36.9±8.0	81.2±12.1	**P=0.003**	52.7±5.4	71.4±8.5	**P=0.023**

Appendix Table 2. Bioavailability (F) of experimental radiolabelled L-glucose absorbed via the paracellular route in different avian species. *experimental diet concentration estimated from data provided by authors.

Species	Natural diet	Experimental diet	Bioavailability, F (%)	Reference
Colinus virginianus (northern bobwhite quail)	insectivorous	1800* mmol/L glucose	92±7	(Levey and Cipollini, 1996)
Dendroica coronata (yellow-rumped warbler)	omnivorous	655* mmol/L glucose	91±23	(Afik et al., 1997)
Passer domesticus (house sparrow)	granivorous	3330* mmol/L glucose	80±7	(Caviedes-Vidal and Karasov, 1996)
Trichoglossus haematodus (rainbow lorikeet)	nectarivorous	400mmol/L glucose	80±6	(Karasov and Cork, 1994)
Selasphorus platycercus (broadtailed hummingbird)	nectarivorous	292 mmol/L sucrose 876 mmol/L sucrose	49 74	(McWhorter et al., 2006)
Phylidonyris novaehollandiae (New Holland honeyeater)	nectarivorous	250 mmol/L sucrose 1000 mmol/L sucrose	37 ±8 81±12	Present study
Cinnyris talatala (white-bellied sunbird)	nectarivorous	250 mmol/L sucrose 1000 mmol/L sucrose	53±5 71±8	Present study

Electronic supplementary material A

Materials and methods and statistical details

Birds were housed in individual cages (honeyeaters: 46x56x45 cm; sunbirds: 27x31x21 cm) at 21±1 ° C with an automatic photophase (0600 to 1800; 0700 to 1800 respectively). Both species were fed a maintenance diet *ad libitum* (see Table 1 for nutrient contribution of each diet); honeyeaters: 20% (w/w) sucrose and 15% Wombaroo® powder (Wombaroo Food Products, Adelaide, SA, Australia); sunbirds: 20% (w/w) sucrose and 2% Ensure® (Abbott Laboratories, Johannesburg, South Africa). During experiments, birds were housed individually in opaque plastic cages (42x54x50cm) with an automatic lighting regime as per above, and a one way mirror to minimise disturbance during sample collection. Excreta was collected from wax paper which was rolled through the cage, allowing samples to be collected immediately upon defecation. All animal care procedures and experimental protocols adhered to institutional regulations of Murdoch University (reference number R1137/05) and the University of Pretoria (reference number EC013-07).

The fractional absorption (bioavailability) of L-glucose was measured using [^{14}C]-L-glucose and [^{3}H]-L-glucose, administered orally and by intramuscular (IM) injection to each bird in separate experiments. To vary food intake rate, birds received two different diets (250 and 1000 mmol/L sucrose solutions) in separate feeding experiments. The order of trials and treatments given were both randomly assigned, and followed published protocol (McWhorter et al., 2006). Bioavailability (F) was calculated as:

$$F=(P\cdot S\cdot K_{el})/I$$

where P is the steady state feeding concentration of radiolabelled L-glucose in plasma (dpm/mg of plasma), S is the probe distribution space of [^{14}C]-L-glucose in plasma (mg of plasma), K_{el} is the elimination rate constant for the removal of radiolabelled L-glucose from plasma and its excretion in urine (min^{-1}), and I is the ingestion rate of radiolabelled L-glucose (dpm/min) (Karasov and Cork, 1994, McWhorter et al., 2006). The values of K_{el} and S were obtained from the IM administration trials, and P and I were obtained from the oral administration trials.

For IM administration, each honeyeater was injected into the pectoralis muscle with ~50 µl solution containing 330 KBq of [^{14}C]-L-glucose and 175 mmol/L NaCl, or for sunbirds, ~15 µl of solution containing 140 KBq of [^{14}C]-L-glucose and 175 mmol/L NaCl. The total osmotic pressure of the IM injection solution was controlled at approximately 350mmol/kg, so that the solution was isosmotic with avian blood (Goldstein and Skadhauge, 2000). The parameters for the mono- and bi-exponential models were derived for each individual by non-linear curve fitting of the concentration of [^{14}C]-L-glucose in excreta after IM administration versus time, by use of the Marquardt-Levenberg algorithm (SYSTAT Software, Inc, SigmaPlot for Windows; (SYSTAT Software, Inc, SigmaPlot for Windows, Marquardt, 1963). For oral administration, birds fed from a sucrose solution containing radiolabelled L-glucose *ad libitum* for 3 h (honeyeaters: 37 KBq/ml and 65 KBq/ml [^{3}H]-L-glucose for 250 and 1000 mmol/L sucrose diets

respectively; sunbirds: 17 KBq/ml and 30 KBq/ml [^{14}C]-L-glucose for 250 and 1000 mmol/L sucrose diets respectively), with solutions at an osmotic concentration of ~250 or 1000 mmol/kg. After IM administration, excreta was collected continuously for 2 h, followed by a small blood sample from the brachial vein. One small blood sample was collected 3 h after introduction of the radiolabelled diet during steady-state feeding trials; all birds on all treatments reached steady-state with regard to radiolabel ingestion and excretion by 90 min (data not shown).

Appendix Supplementary material Table 1: Nutritional components of Wombaroo® (Wombaroo Food Products, Adelaide, SA, Australia) and Ensure® (Abbott Laboratories, Johannesburg, South Africa) maintenance diets for honeyeaters and sunbirds.

	Wombaroo®	Ensure®
Protein	13%	15.9%
Fat	5%	14%
Fibre	2%	
Salt	1%	
Carbohydrates (main sugar present: sucrose)	64%	58.5%
FOS (fructooliogsaccharides)		3.6%

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