J Exp Biol Advance Online Articles. First posted online on 24 January 2013 as doi:10.1242/jeb.075176
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.075176


JEB

# Gastrointestinal and renal responses to variable water intake in whitebellied sunbirds and New Holland honeyeaters

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Running title: Water handling in avian nectarivores

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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 |
| $K^3_H$ | 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) |
| $S^3_H$ | 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 whitebellied 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: whitebellied 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 repose 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 whitebellied 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:

$$S\,^{3}H = TBW = Q_i\,^{3}H \Big/ \left[ \frac{P\,^{3}H}{e^{(K\,^{3}H \bullet t)}} \right] \qquad (1)$$

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·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·h$^{-1}$) and water ($\dot{V}_I$; g·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:

$$\text{TWG} = \dot{V}_{\text{M}} + \dot{V}_{I} \quad (4)$$

*Intestinal function: fractional water absorption*

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

$$f_{\text{A}} = \frac{\dot{W} - \dot{V}_{\text{M}}}{\dot{V}_{\text{I}}} \quad (5)$$

*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 ($S^{14}_{\text{C}}$; 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:

$$S^{14}_{\text{C}} = Q^{14}_{\text{C}} \; / \left[ \frac{P^{14}_{\text{C}}}{\text{e}\ (K^{14}_{\text{C}} \cdot t)} \right] \quad (6)$$

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

$P^{14}_{\text{C}}$ 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).

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

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

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

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$^{-1}$):

$$C = C_0 e^{-Kelt} \quad (12)$$

$$C = ae^{-\alpha t} + be^{-\beta t} \quad (13)$$

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 whitebellied 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 ± 1 s.d. throughout. Statistical significance was accepted at $\alpha<0.05$.

## Results

For afternoon values, the relationships of *ln*-[$CF_{^3H}$] and *ln*-[$CF_{^{14}C}$] with time were well described by negative linear functions (Table 1; see the example for one honeyeater individual shown in Fig. 1), with significant values ($P>0.05$) for the coefficient of determination ($r^2$) for honeyeaters ($^3$H: $r^2$ = 0.88 ± 0.14; $^{14}$C: $r^2$ = 0.87 ± 0.06) and sunbirds ($^3$H: $r^2$ = 0.73 ± 0.24; $^{14}$C: $r^2$ = 0.89 ± 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 ± 0.15, $^{14}$C: $r^2$ = 0.60 ± 0.24; and honeyeaters: $^3$H: $r^2$ = 0.90 ± 0.11, $^{14}$C: $r^2$ = 0.28 ± 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 $^3H_2O$ dilution to estimate distribution space, $S_{^3H}$) 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 2a & 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 *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. 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. 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. 2c) and honeyeaters ($P = 0.017$; Fig. 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. 4). For both species, *GFR* was not correlated with total water gain ($P > 0.05$; Figs 2d & 3d). Estimates of afternoon kidney fractional water reabsorption ($f_R$) were similarly insensitive to water loading in both sunbirds and honeyeaters (Figs 2e & 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. 2f, 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. 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 M 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 whitebellied 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. Although we recorded no changes in *GFR* with water intake, 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 whitebellied 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. 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.

**Funding**

This project was funded by the National Research Foundation of South Africa, the University of Pretoria and the Australian Research Council (DP0665730).

**Acknowledgements**

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 Murdoch University (R1137/05) and the University of Pretoria (EC013-07).

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## Tables

**Table 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 M | 8 | 8 | 8 | 8 |
| | 0.5 M | 7 | 8 | 8 | 8 |
| | 1 M | 6 | 8 | 7 | 8 |
| overall | | 21/24 = 88% | 24/24 = 100% | 23/24 = 96% | 24/24 = 100% |
| [$^{14}$C]-L-glucose | 0.25 M | 8 | 8 | 8 | 4 |
| | 0.5 M | 8 | 6 | 7 | 4 |
| | 1 M | 7 | 6 | 8 | 2 |
| overall | | 23/24 = 96% | 20/24 = 83% | 23/24 = 96% | 10/24 = 42% |

## Figures

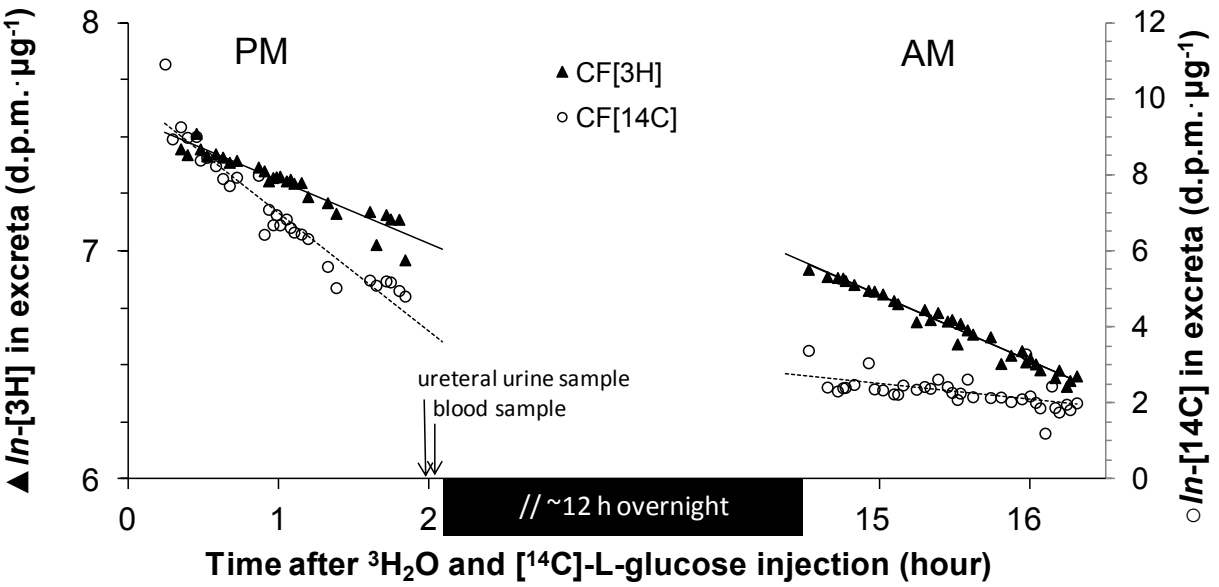



**Figure 1:** Data from a representative New Holland honeyeater individual feeding on 0.5 M sucrose illustrating our method of measuring the gastrointestinal and renal function during the afternoon (PM), overnight (black bar) and the following morning (AM). Each data point represents the *ln*-transformed $^{3}H_2O$ 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 1), and only a minority of trials could be compared between mono- and biexponential models].

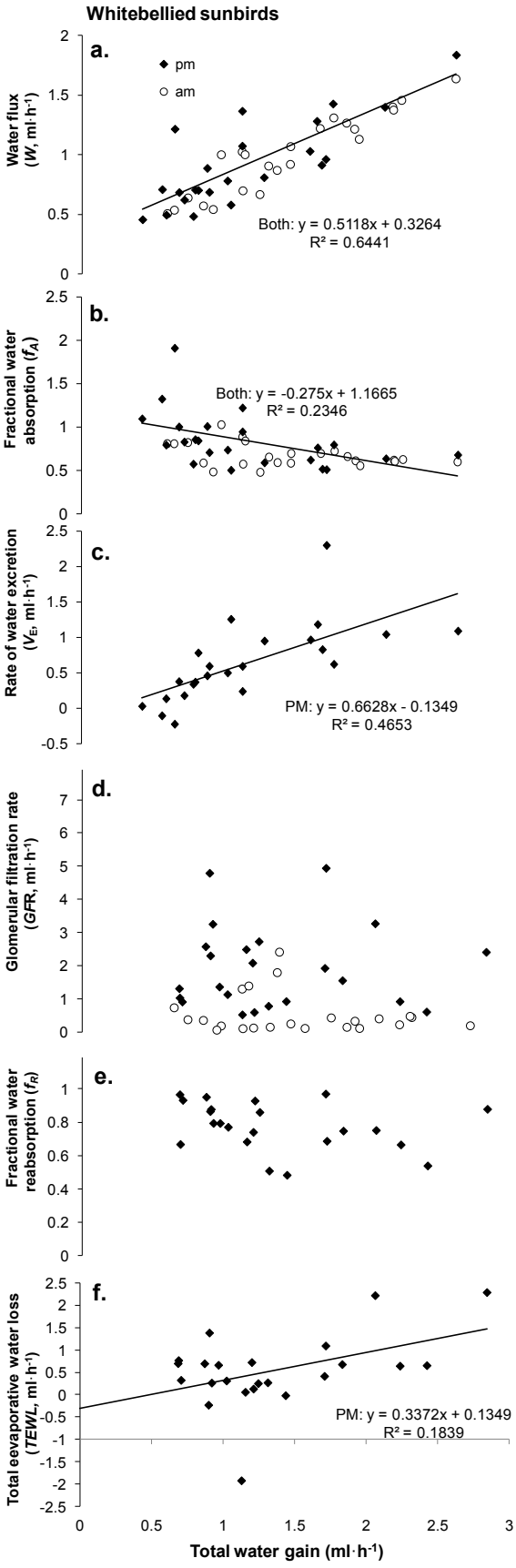



**Figure 2:** The influence of water intake rates (*x*-axes) on the water handling processes during the afternoon (♦) and morning (○) in whitebellied sunbirds. Rates of (a) Water flux (*W*), (c) water excretion ($V_E$), and (f) evaporative water loss (*TEWL*) increased linearly with total water gain. (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 whitebellied sunbirds.

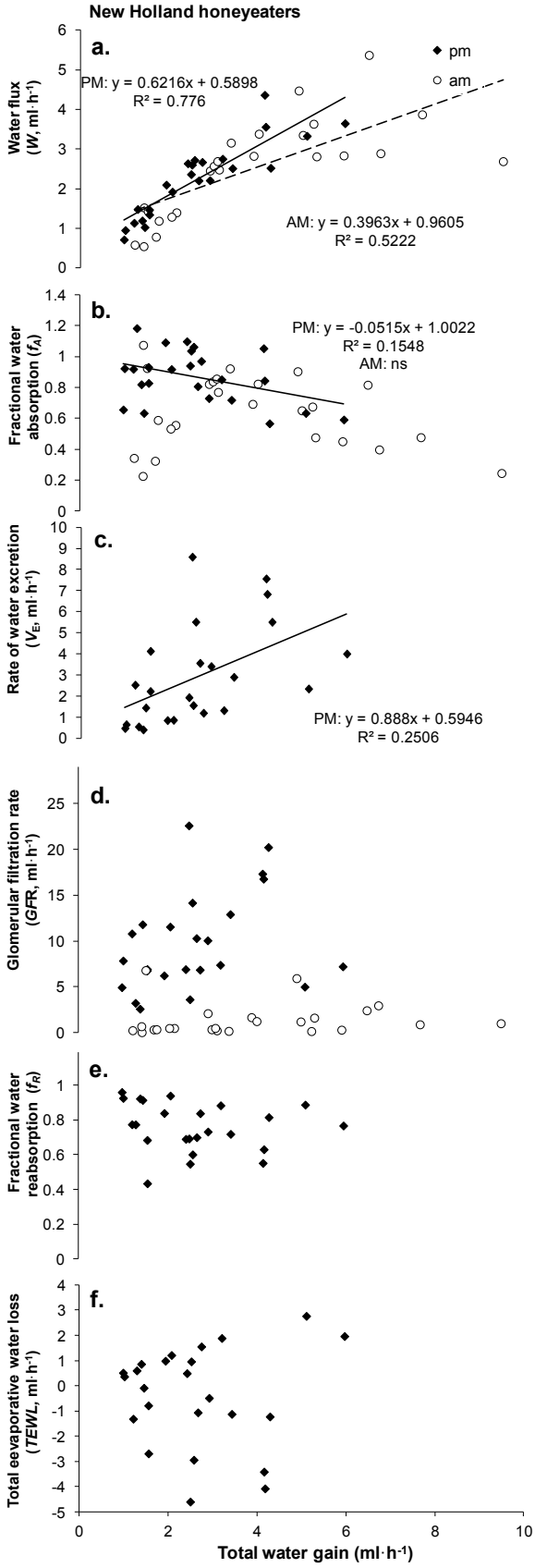



**Figure 3:** The influence of water intake rates on the water handling processes during the afternoon (♦) and morning (○) in New Holland honeyeaters. Rates of (a) Water flux (*W*) and (c) water excretion ($V_E$) increased linearly with total water gain. (b) Honeyeaters modulated gastrointestinal tract 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.

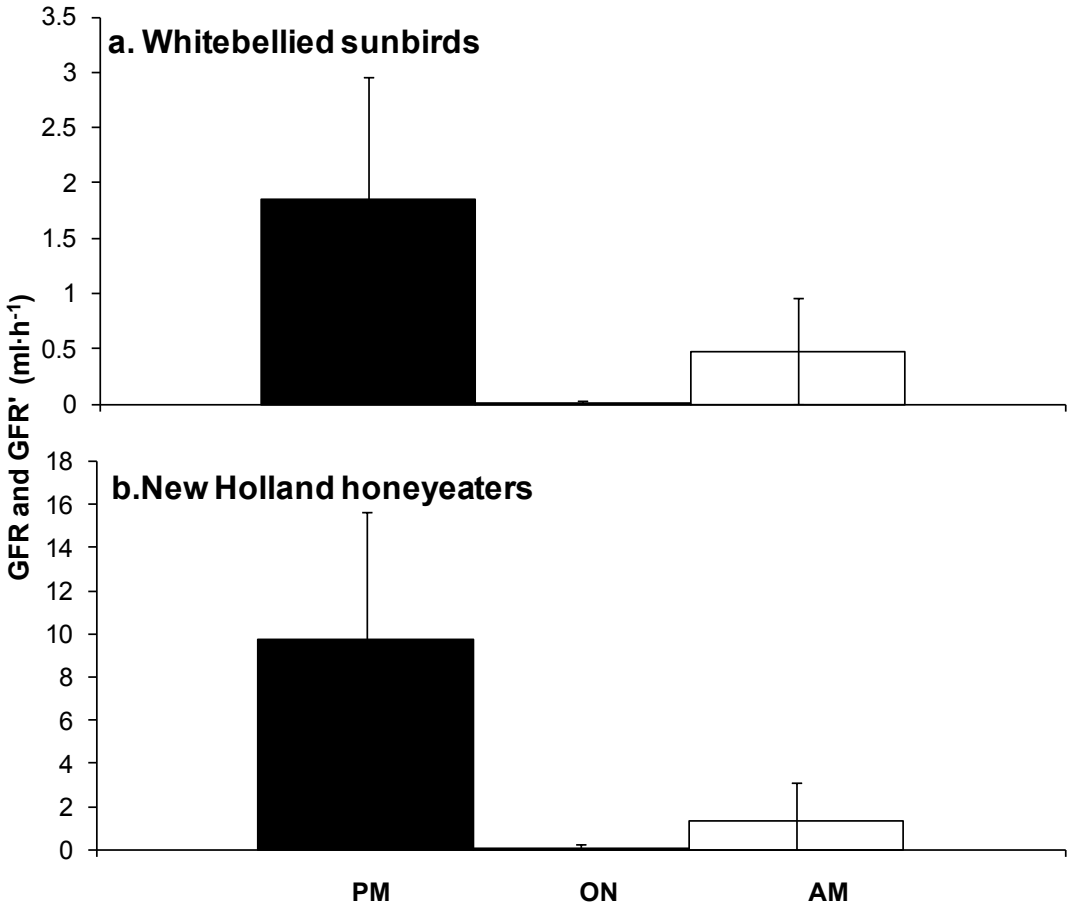
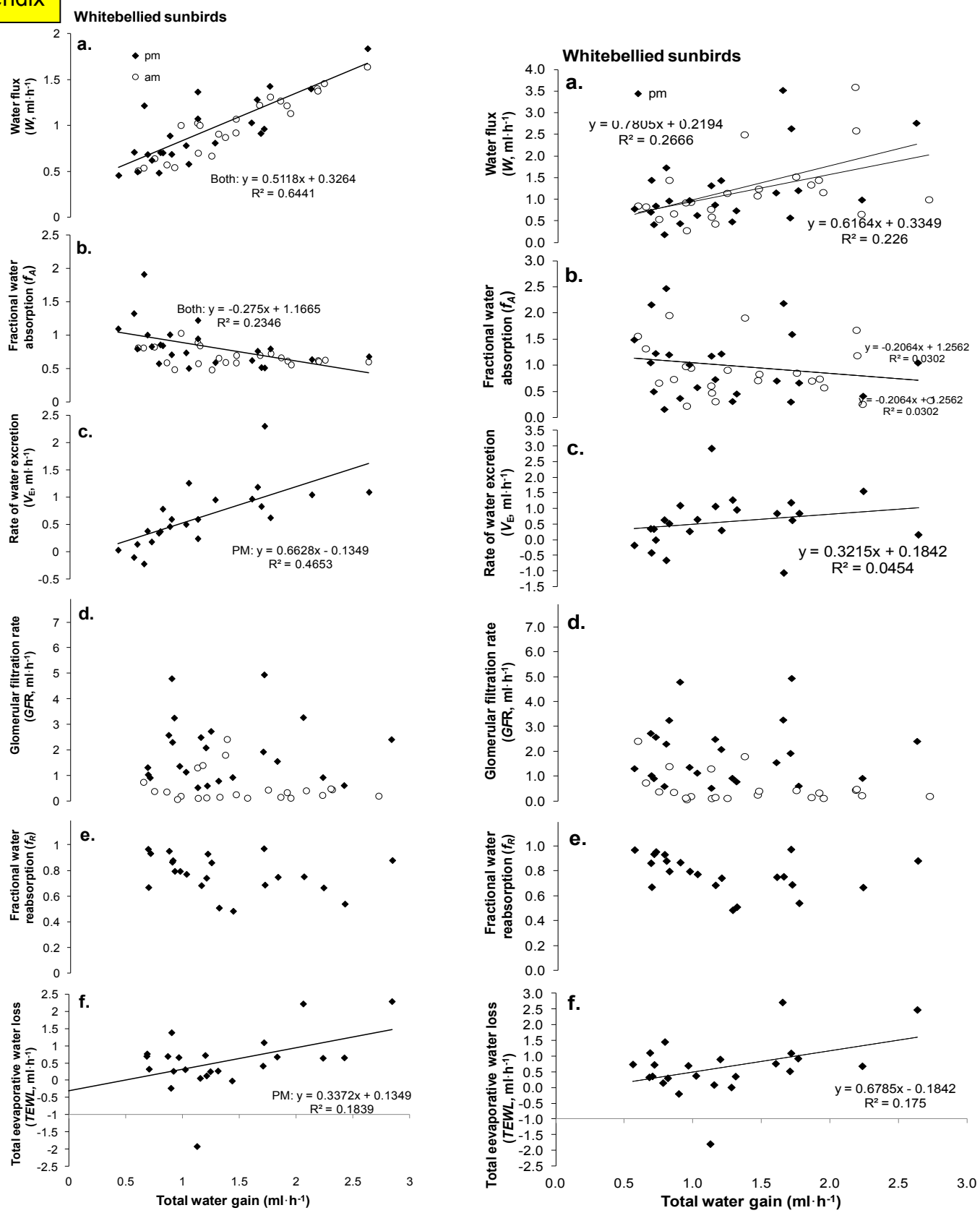


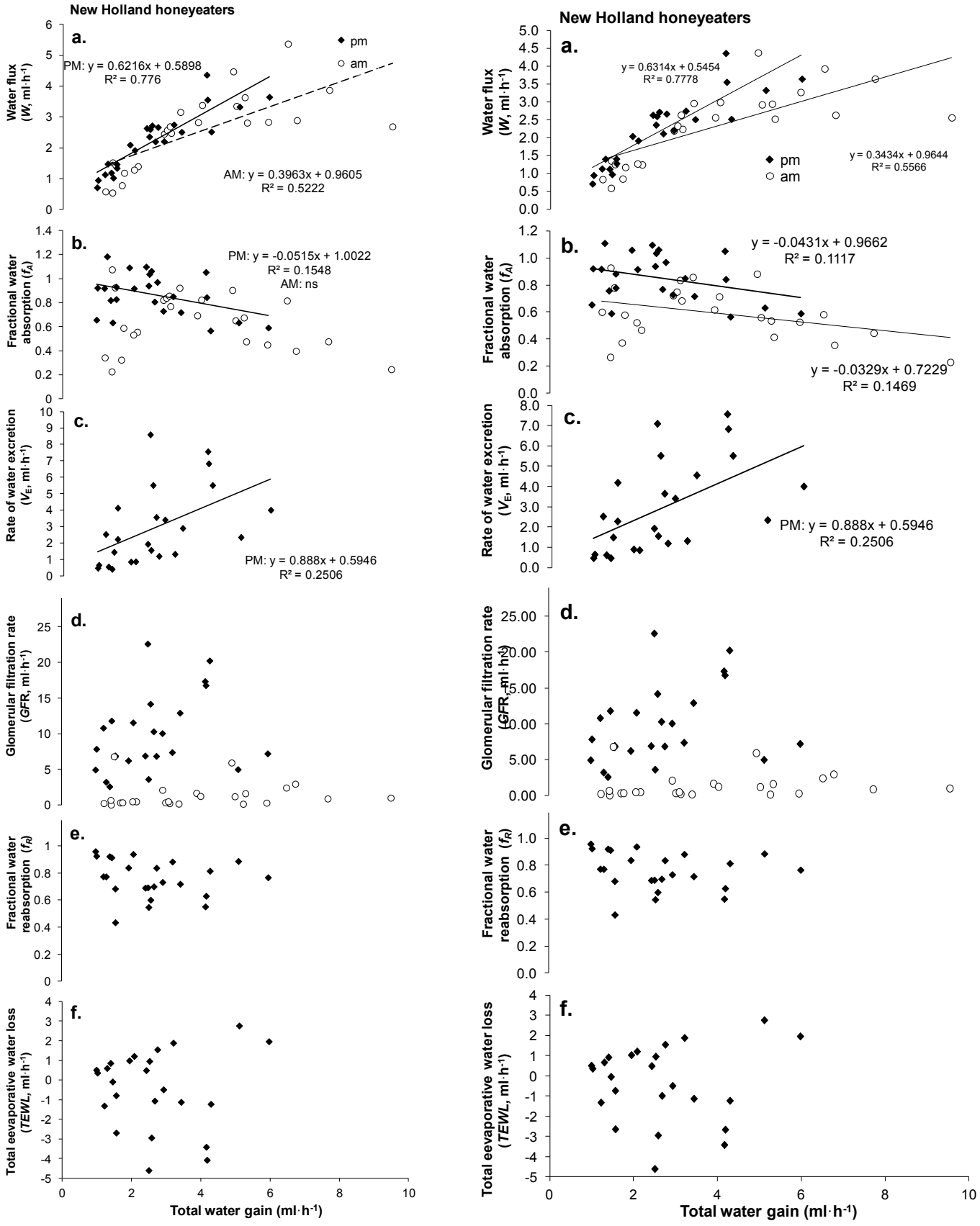

**Figure 4:** Mean ( ± SD) glomerular filtration rate (daytime: *GFR* or estimated overnight *GFR'*, ml/h) in the afternoon (PM), overnight (ON), and early morning (AM) in a) whitebellied sunbirds and b) New Holland honeyeaters. Both species arrested whole kidney function during the night time fasting periods, with *GFR* values not different from zero, and morning values were significantly lower than afternoon values.

Electronic Appendix






**Alternative version of Figure 2:** The influence of water intake rates (*x*-axes) on the water handling processes during the afternoon (♦) and morning (○) in whitebellied sunbirds either with (left hand panel) or without (right hand panel) the adjustment for feeding time.




**Alternative version of Figure 3:** 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.