Osmoregulatory and metabolic costs of salt excretion in the Rufous-collared sparrow *Zonotrichia capensis*

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**A R T I C L E   I N F O**

Article history:
Received 6 July 2012
Received in revised form 20 October 2012
Accepted 20 October 2012
Available online 26 October 2012

Keywords:
Basal metabolic rate
Osmoregulation
Passerines
Renal features
Salt

**A B S T R A C T**

Recent experiments on shorebirds have demonstrated that maintaining an active osmoregulatory machinery is energetically expensive. This may, in part, explain diet and habitat selection in birds with salt glands. However little is known about the osmoregulatory costs in birds lacking functional salt glands. In these birds, osmotic work is done almost exclusively by the kidneys. We investigated the osmoregulatory cost in a bird species lacking functional salt glands, the passerine *Zonotrichia capensis*. After 20 days of acclimation to fresh water (FW) and salt water (200 mM NaCl, SW), SW birds tended to be heavier than FW birds. However, this difference was not statistically significant. Total basal metabolic rate was higher in SW birds as compared with FW birds. Renal and heart masses were also higher in the SW group. We also found greater medullary development and an increase in urine osmolality in the SW group. In spite of *Z. capensis’* ability to tolerate a moderate salt load in the laboratory, we hypothesize that increased cost of maintenance produced by salt consumption may significantly affect energy budget, dietary, and habitat choices in the field.

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**1. Introduction**

Songbirds (Order Passeriformes) have diversified in all continents and now occupy nearly all terrestrial ecosystems (Barker et al., 2004). Nevertheless, few songbird species inhabit marine environments (Wheelwright and Rising, 1993; Martínez del Río et al., 2009). This paucity might be explained by physiological constraints imposed by a habitat that is essentially dehydrating (Sabat, 2000). Generally, birds in the marine environment have only saline water to drink, and almost all available prey (especially invertebrates) are in osmotic equilibrium with this saline water. When birds drink seawater or consume food with high osmolarity, salts are absorbed by the small intestine, and the concentration in the body fluids increases (Purdue and Haines, 1977; Simon, 1982; Holmes and Phillips, 1985). Unless birds can excrete fluids that are more concentrated than ingested water or drink fresh water, the body becomes dehydrated. Feeding on salty marine foods and drinking seawater is especially challenging for passerines because they lack functional salt glands (Shoemaker, 1972) and have a limited ability to concentrate urine (Goldstein and Skadhauge, 2000). This significant osmoregulatory challenge for passerines, and the consequent difficulty of utilizing foods of marine origin in the absence of fresh water, might explain the paucity of truly marine passerine species.

The possibility that the high metabolic costs of osmoregulation in marine environments might be a causative factor in the scarcity of passerine birds in saline environments has, as of yet, not been studied. It is well known that the ingestion of salts and other osmotically active compounds represents a significant energetic cost to animals (Nehls, 1996; Gutiérrez et al., 2011). Any significant additional cost associated to the ingestion of salty prey could also prevent its use and restrict the species distribution. Although these osmoregulatory costs have been extensively studied in ectothermic animals (see Evans, 2009), the topic has received little attention in endothermic vertebrates. To our knowledge, only one study has empirically evaluated the energetic importance of ingesting salts in birds. Gutiérrez et al. (2011) demonstrated experimentally that maintaining an active osmoregulatory machinery is energetically expensive, and suggested that this could, in part, explain diet and/or habitat selection in a migratory shorebird. Such osmoregulatory work is thought to be achieved in marine birds by the salt gland, the size and function of which is affected by habitat salinity, salt load and temperature among other factors (see Gutiérrez et al., 2012 for a review).

The development and function of the salt gland appear to be a limiting factor on the ecology of avian species (Hughes and Winkler, 1990; Gutiérrez et al., 2011), but the metabolic costs associated with its functioning may also affect its choice of diet and habitat of different populations. For those birds that lack functional salt glands, such as passerines, osmoregulatory work lies exclusively in the renal function (Skadhauge, 1981; Braun, 2003). Thus, the objective of this study was to experimentally evaluate the putative effects of ingesting salt on basal rates of energy expenditure along with the osmoregulatory responses in a bird species lacking functional salt glands. Specifically, we evaluated how acclimation to salt and tap water affects the metabolic costs of salt excretion in the Rufous-collared sparrow.
basal rates of energy expenditure, urine and plasma concentration and renal features in the Rufous-collared sparrow (Zonotrichia capensis), a terrestrial passerine species.

2. Material and methods

2.1. Animal collection and experimental design

Rufous-collared sparrows (Z. capensis) are distributed between southeast Mexico and Cabo de Hornos in southern South America (Goodall et al., 1951). In Chile, these omnivorous (Ramirez-Otarola et al., 2011) birds are practically ubiquitous, inhabiting a range of habitats, from deserts to rain forests (Araya and Millie, 2005), over a substantial elevation gradient, from coastal zones to above 4000 m.a.s.l. (Goodall et al., 1946). Birds were captured in Quebrada de la Plata (33° 30′ S, 70° 54′ W), central Chile, a locality characterized by a Mediterranean climate with cool and wet winters and relatively dry and hot summers. Twenty individuals were captured with mist nets in October 2011. Following capture, we transported them to a laboratory in Santiago, Chile (33° 27′ S, 70° 42′ W), where they were held in two outdoor aviaries (200×150×250 cm) under natural temperature and photoperiod (L:D 13:11) regimes. While in captivity, the sparrows consumed mealworms (Tenebrio molitor), birdseed and water, which were available ad libitum. Seeds and water were offered in graduated inverted plastic tubes of 100 mL which allowed birds to eat and drink in a small (ca. 1 cm²) container at the bottom of the tube. Consumed volumes of each solution were determined in a separate experiment using an additional eight individuals. Birds were randomly separated into two groups of four birds. Birds from both groups were maintained in individual cages of 50×50×50 cm at 25±2 °C. L:D 12:12. All birds were fed with mealworms and seeds ad libitum. Birds in one of the treatment groups received fresh drinking water, while the other received saline drinking water. Water treatments were available to birds for 24 h. Fluid intake rates were determined using graduated inverted plastic tubes of 100 mL and corrected for evaporation by using control tubes located outside the experimental cage. After habituating birds to captive conditions for 31 days, we measured the basal metabolic rate (BMR) at 30 °C, which is within the thermoneutral zone for this species (Sabat et al., 2006). Then, each bird was assigned to one of two treatments: one group was housed in an aviary with mealworms, bird seed and tap water (FW acclimated group, n=10) and the other with mealworms, bird seed and salt water (SW acclimated group, n=10). Saltwater osmolality was 200 mM NaCl. Previous experiments have revealed that this solution is the maximum concentration that this species can tolerate without losing weight (Bartholomew and Cade, 1963). After the acclimation period, blood samples of 50 to 100 μL were collected from the humeral vein into heparinized tubes. Blood samples were taken between 08:00 and 10:00 h and centrifuged at 9000 g for 5 min. Following separation, the plasma was kept frozen at −40 °C. Urinary urine was obtained by inserting a small closed-ended cannula into the birds’ cloaca. Urine drained into the cannula via a window placed dorsally over the ureteral orifices; the closed end avoids contamination by intestinal fluids (Goldstein and Braun, 1989). Urine samples from each bird were centrifuged and the supernatant was saved frozen (−40 °C) for later osmometry analysis. The osmolality of the fluids was measured by vapor pressure osmometry (Wescor 5130B). Following fluid sampling, birds were weighed and their BMR was determined.

2.2. Basal metabolic rate

Measurements of BMR were made on the day of capture in post-absorptive (four hour fasted), resting birds during the inactive phase. Measurements were made within the thermoneutral zone of the species using standard flow-through respirometry methods. Briefly, birds were weighed, placed in a dark metabolic chamber (2 L), and then placed in a controlled temperature cabinet (Sable Systems, Henderson, Nevada) at a constant temperature (Tb=30±0.5 °C). The metabolic chamber received air which was dried by passing through a column of Driedrite and CO₂-absorbent granules (Baralyme) before passing through an O₂-analyzer (model Turbo Fox, Sable System, Henderson, Nevada) calibrated with a known mix of oxygen (20%) and nitrogen (80%) that was certified by chromatography (INDURA, Chile). The measurement and calibration protocols we followed were after Williams and Tieleman (2000). Because water vapor and CO₂ were scrubbed before entering the O₂ analyzer, oxygen consumption was calculated as [Withers (1977: p 122)]: VO₂=[FR*60*(Fi O₂−Fe O₂)]/(1−Fi O₂), where FR is the flow rate in mL/min after STP correction, and Fi and Fe are the fractional concentrations of O₂ entering and leaving the metabolic chamber, respectively. Body mass was measured before the metabolic measurements using an electronic balance (±0.1 g), and cloacal body temperature (Tb) was recorded at the end of each measurement with a Cole-Palmer copper–constantan thermocouple attached to a Digisense thermometer (Model 92800-15). We found that all animals were eutermic after the metabolic trials, and we observed no differences in body temperature between drinking water treatment groups (41.73±0.41 °C and 41.34±0.28 °C for SW and FW respectively; t18=1.08, p=0.29). Output from the oxygen analyzer (%) and flow meter was digitalized using a Universal Interface II (Sable Systems) and recorded on computer using EXPEDATA data acquisition software (Sable Systems). Our sampling interval was 5 s. Birds remained in the chamber overnight to achieve steady-state conditions. This typically occurs after 6–8 h for small birds (Page et al., 2011) including Z. capensis (Sabat et al., 2010). We averaged O₂ concentration of the excurrent air stream over a 20 min period after a steady-state was reached (following Tieleman et al., 2002).

2.3. Kidney morphology

Birds were sacrificed by CO₂ exposure, weighed and dissected to remove the organs. Kidneys were removed from symascrum, weighed (±0.0001 g) and preserved in paraformaldehyde glutaraldehyde (4%) until dissection. Medullary cones were dissected from the left kidney using a microscope, and the collection was counted, measured (±0.01 mm) and weighed (±0.1 mg). The percentage of renal medulla was determined as the mass of medullary tissue divided by the mass of the left kidney, assuming a tissue density of 1 g/mL (Sabat and Martinez del Rio, 2002).

2.4. Statistical analysis

Changes in body mass of pre- and post-acclimated birds (time 0 and 20 days) were evaluated using repeated measures ANOVA with acclimation condition as single factor, and both initial and final values of each response variable as repeated measures within each individual. We evaluated the effect of treatment on plasma and urine osmolality using one way ANOVA. The most appropriate analysis for allometric biometric variables is ANCOVA, using body mass as a covariate. However, this is only justified if the correlation between the physiological variable and body mass is significant, given that ANCOVA reduces degrees of freedom in one (reducing power). To test the effect of the acclimation condition on VO₂ we performed first an ANCOVA using body mass (mb) as the covariate using only data of post-acclimated animals. Because, BMR was not affected by body mass (F17, 17 = 0.40, p=0.52), we then performed a repeated measures ANOVA to test whether the BMR of pre- and post-acclimated birds differed. We tested the effect of
the acclimation condition on morphological traits using MANCOVA. When our analyses exhibited a non-significant effect of mb, this term was dropped from the model. To test for specific differences among means in physiological and morphological traits we used a post hoc Fisher test. Characteristics of renal morphology (number of medullary cones by gram of kidney, percentage of kidney comprised by medullary tissue) were examined by ANOVA and by ANCOVA (using kidney mass as a covariate) for medullary tissue. Finally, we evaluated the water content of animals by gravimetric procedures, i.e., by means of drying to constant weight each individual in an oven at 80 °C. Prior to each statistical analysis, data were examined for assumptions of normality and homogeneity of variance, using Kolmogorov–Smirnov and Levene tests, respectively. In all cases, data fulfilled the assumptions necessary for ANOVA.

3. Results

Although SW birds tended to be heavier than those maintained on FW, body mass was not statistically different between drinking water treatment groups (F(1, 18) = 3.89, p = 0.06). On average, mb remained constant during the full experimental course (F(1, 18) = 0.57, p = 0.46) and was not affected by the interaction of treatment and time (F(1, 10) = 0.07, p = 0.77). The a posteriori analyses revealed no significant differences in mb between groups (all p > 0.05), although birds in the SW treatment group showed increases in mb compared with FW birds (p = 0.08). MANCOVA analysis revealed a strong effect of treatment on all morphological variables (Wilks lambda = 0.09, F(5, 11) = 7.56, p = 0.03) but no effect on mb (Wilks lambda = 0.33, F(5, 11) = 1.59, p = 0.33). The a posteriori analyses revealed a significant effect of treatment on heart (p = 0.02) and kidney (p = 0.01) mass but no effect on liver (p = 0.65), intestine (p = 0.10) or gizzard (p = 0.15) mass. Kidney and heart masses were higher in the SW birds than in FW birds (ca. 23% and 20% respectively, Table 1).

At the end of the acclimation period urine osmolality was significantly different between groups. The SW group 23% higher than in the FW group (ANOVA: F(1, 18) = 6.18, p = 0.03; Fig. 1). However, plasma osmolality remained unchanged between groups (F(1, 18) = 0.54, p = 0.48). Water content did not differ between SW and FW acclimated animals (F(1, 18) = 0.01, p = 0.96). The length of medullary cones was greater in SW than FW acclimated animals (F(1, 18) = 29.75, p = 0.01). The number of medullary cones of the right kidney was unaffected by treatment effect (F(1, 18) = 0.19, p = 0.67) nor was the number of medullary cones standardized by kidney mass (F(1, 18) = 2.49, p = 0.14). The mass and percentage of medullary tissue in the kidney was higher in SW acclimated birds than in FW acclimated birds (F(1, 18) = 13.45, p = 0.04 and F(1, 18) = 12.31, p = 0.01; 66% and 40% respectively (Table 1)). Finally, the volume of consumed water was similar in both SW and FW groups (t6 = 0.17; p = 0.87; average 10.62 ± 6.21 and 11.5 ± 8.10 mL/day for each group respectively).

4. Discussion

Recognizing the ways in which organisms allocate energy among various functions competing for limited resources is fundamental to understand the relationship between organism and environment. Thermoregulation, osmoregulation, growth, reproduction, and locomotion require energy and are considered the major activities influencing the energy budgets of birds (McNab, 2002, 2009). Since metabolic rates set the pace of life, assessment of their variability has, and continues to be of, great importance to several contemporary ideas that attempt to link animal energetics to traits such as species distribution, reproductive effort, activity levels and life-history strategies (Kooijman, 2000; Brown et al., 2004; Cruz-Neto and Jones, 2005).

In this vein, our results confirm that salt intake leads to an increase of

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Table 1

<table>
<thead>
<tr>
<th>Body mass, organ masses, and renal features of Zonotrichia capensis acclimated to fresh and salt water (200 mM NaCl); Asterisk denotes significant differences between treatments and the number of (see text for detailed statistics).</th>
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<tbody>
<tr>
<td>Salt water</td>
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<td>Body mass (g)</td>
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<td>Intestine mass (g)</td>
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<td>Kidney mass (g)*</td>
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<td>Cone length (mm)</td>
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<td>Medullary tissue (mg)</td>
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<td>Medullary tissue (%)</td>
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* Values calculated from both kidneys.

** Data from the left kidney.
rates of energy expenditure in birds, and this is likely related to the osmoregulatory costs associated with getting rid of excess electrolytes (Gutiérrez et al., 2011).

Our results suggest that Z. capensis can tolerate moderate salt intake. This is demonstrated by the maintenance of mb throughout the acclimation period. Drinking of salt solutions by Z. capensis is comparable to those found in other terrestrial passнерines, such as Carpodacus mexicanus (Bartholomew and Cade, 1958). However, consumption of 200 mM NaCl is probably not high enough to provoke a decrease in mb, as it is commonly observed in other bird species acclimated to salt water (reviewed in Gutiérrez et al., 2011). In addition, Z. capensis acclimated to salt water appeared to exhibit several physiological modifications that allowed for the maintenance of osmotic homeostasis. In fact, plasma osmolality in SW acclimated birds was similar to those values found in the FW acclimated birds and at concentrations that appear to be normal for passerines (Skadhauge, 1974). As would be expected, osmolality of the aquatic phase of the urine increased by 90% in SW acclimated birds. This phenomenon of elevating urine concentration in order to eliminate excess electrolytes has also been documented in other passerine species consuming high levels of salt in water and food (Sabat and Martínez del Río, 2002; Sabat et al., 2006).

Coupled with physiological adjustments, SW acclimated Z. capensis significantly modified various aspects of kidney morphology. Interspecific studies in birds have suggested that the ability to produce hyperosmotic urine, and hence to cope with aridity and with diets with high salt loads, is correlated with the relative size of the kidney, the fraction of the kidney dedicated to medullary tissue, and with the relative number of medullary cones (Goldstein and Braun, 1986, 1989; Hill et al., 2004; Sabat et al., 2004a; Barceló et al., 2012). Thus, high renal concentrating ability improves the efficiency of renal water conservation and electrolyte and nitrogen waste excretion. Consequently, species with the highest proportion of renal medulla are most effective in water conservation (Wanui, 1989). Some passerines are capable of modifying their kidney morphology in response to salt acclimation. This capacity increases the ability to produce more concentrated urine (Sabat et al., 2004b). Previous results suggest that the phenotypic response of terrestrial birds to salt intake might not be as substantial as the response of true seabirds (Bartholomew and Cade, 1963; Sabat and Martínez del Río, 2002). Our results, however, reveal that Z. capensis exhibits considerable phenotypic plasticity in renal traits associated with changes in salt intake, particularly in kidney mass and medulla development (length of medullary cones and percentage of medullary tissue). Such modifications were already demonstrated in this species as a response to protein intake and water deprivation, which in turn, are related to the increase in the amount of nitrogenous wastes (Sabat et al., 2004a,b; Aldea and Sabat, 2007). Thus, from the osmoregulatory perspective, Z. capensis appears to be a species with a flexible phenotype (Piersma and Van Gils, 2011) in which the plasticity of kidney structure seems to play a crucial role in accommodating moderate osmotic loads.

As previously mentioned, few studies have demonstrated the existence of energetic cost of ingesting salts in birds. Recently, Gutiérrez et al. (2011), observed an increase in the BMR of the dunlin (Calidris alpina) acclimated to saline water. Similarly, an increase in oxygen consumption within minutes of ingestion of a hyperonic solution has been documented in birds with salt glands (Nehls, 1996). Gutiérrez et al. (2011) suggested that the development of the reversible physiological changes exhibited by birds exposed to prolonged osmotic stress (e.g., gland hypertrophy and increased Na+/K+-ATPase activity) is the cause of the observed increases in BMR. Nevertheless, theoretical estimates of added osmoregulatory costs in seabirds consuming seawater approaches ca. 7% (Peaker and Linzell, 1975). This is lower than the 20% increase in BMR observed in saltwater acclimated shorebirds (Gutiérrez et al., 2011). Thus, it seems that thermodynamically-derived estimates of osmotic work tend to underestimate the overall cost of getting rid of salts in birds.

Our results revealed a 30% increase in the total BMR of SW acclimated birds. If we consider that the volume of salt water (200 mM NaCl) intake by Z. capensis is 12 mL per day, we can estimate the energy associated with the elimination of electrolytes (Borsook and Winegarden, 1931) as follows:

$$
\Delta G = -NRT \ln \left( \frac{P}{U} \right)
$$

where $N$ is the number of moles of electrolytes, $R$ the constant of gases, $T$ the corporal temperature in K and $P/U$ is the ratio between the plasma and urine osmolality. Our calculations suggest that the energetic cost of getting rid of salts in SW acclimated birds is approximately 0.20 cal/h. This is nearly 0.40% of the initial BMR. Thus, we suggest that, unlike shorebirds, the increase in BMR cannot be only explained from a thermodynamic perspective. Unlike birds with salt glands, in terrestrial (e.g., passerines) birds, the ingestion of salty prey or saltwater is generally coupled with high urine flow (see McNabb et al., 1972), and the need to eliminate greater amounts of excess electrolytes through the kidney (Braun, 1978). McWhorter et al. (2004) noted that from an economical standpoint, eliminating water by increasing renal filtration rate can be energetically costly for birds. Accordingly, hyperosmotic urine excretion in Z. capensis may have been associated with an increase in BMR coupled with an increase in the mass of metabolically active tissue (i.e., kidney and heart (Daan et al., 1990)). These results suggest that the significant increase in energy expenditure in SW acclimated birds is associated with the increased osmoregulatory demand, which in turn comprises both the energy cost per se (in thermodynamic sense), but also the long-term effect of increasing the mass of osmoregulatory and associated organs, such as kidney and heart. It is also possible that the rate of metabolism in Z. capensis would increase because kidney tissue has a high metabolic intensity and thus higher tissue-specific rates of energy expenditure. For instance, mass and enzymatic activity of metabolically-demanding organs, such as kidney, liver, heart, and gut are thought to be one of the factors underlying changes in BMR in birds (see Vezina and Williams, 2005; Zheng et al., 2008; Swanson, 2010). Thus, further studies are needed to evaluate to what extent the differences of energy demands imposed by osmoregulatory work affect the mass-specific metabolic capabilities of the internal organs.

It has been suggested that the capacity for reversible phenotypic modification is associated with the degree of environmental heterogeneity that a species has experienced through its’ evolutionary history (Schichting and Pigliucci, 1998; Maldonado et al., 2012). Consequently, the ability to modify kidney structure and function would depend on the variation in osmotic load normally experienced by a species. Sabat et al. (2004b) have reported that three songbirds of the genus Circledes differ in the degree to which they demonstrate reversible changes in osmoregulatory traits, such as kidney structure and function. In fact, Sabat et al. (2004b) found that among these species, the altitudinal migrant C. oustaleti has a greater ability to modify osmoregulatory features than other Circledes species that rely on mainly marine prey throughout the year. This flexibility seems to enable C. oustaleti to cope with the winter consumption of marine animals. Our results show that Z. capensis, a truly terrestrial passerine bird, can cope with the moderate ingestion of salts through modifications of osmoregulatory features. This capacity to exhibit physiological flexibility according to salt load is probably associated with the natural variation in osmotic load experienced by this species through the consumption of different types of protein (Sabat et al., 2004a, 2009).

In spite of the ability of Z. capensis to cope with the ingestion of salts in the laboratory, this species has never been a documented consuming marine prey or to drink salt water in the field, although they can inhabit very productive coastal marine environments. In this vein, it is generally accepted that BMR reflects the overall maintenance
energy cost of physiological machinery (Piersma, 2002) which can reach up to 50% of the energy budget of endotherms (Speakman, 2000). We suggest that the increased cost of maintenance produced by salt consumption, as demonstrated in Z. capensis, may significantly affect energy budgets and resultant dietary and habitat choices in terrestrial birds and we recommend further investigation concerning the extent to which the metabolic cost associated with the excretion of electrolytes varies as a function of the ecology of different bird species.

Acknowledgments

This study was funded by FONDECYT 1080007. We thank Nathan Wolf for his useful comments on an earlier version of the manuscript. Birds were captured with permits from SAG, Chile (No 4052/2007). All protocols were approved by the institutional Animal Care Committee of the University of Chile.

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