



# Triple Oxygen Isotope Measurements ( $\Delta^{17}\text{O}$ ) of Body Water Reflect Water Intake, Metabolism, and $\delta^{18}\text{O}$ of Ingested Water in Passerines

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Understanding physiological traits and ecological conditions that influence a species reliance on metabolic water is critical to creating accurate physiological models that can assess their ability to adapt to environmental perturbations (e.g., drought) that impact water availability. However, relatively few studies have examined variation in the sources of water animals use to maintain water balance, and even fewer have focused on the role of metabolic water. A key reason is methodological limitations. Here, we applied a new method that measures the triple oxygen isotopic composition of a single blood sample to estimate the contribution of metabolic water to the body water pool of three passerine species. This approach relies on  $\Delta^{17}\text{O}$ , defined as the residual from the tight linear correlation that naturally exists between  $\delta^{17}\text{O}$  and  $\delta^{18}\text{O}$  values. Importantly,  $\Delta^{17}\text{O}$  is relatively insensitive to key fractionation processes, such as Rayleigh distillation in the water cycle that have hindered previous isotope-based assessments of animal water balance. We evaluated the effects of changes in metabolic rate and water intake on  $\Delta^{17}\text{O}$  values of captive rufous-collared sparrows (*Zonotrichia capensis*) and two invertivorous passerine species in the genus *Cinclodes* from the field. As predicted, colder acclimation temperatures induced increases in metabolic rate, decreases in water intake, and increases in the contribution of metabolic water to the body water pool of *Z. capensis*, causing a consistent change in  $\Delta^{17}\text{O}$ . Measurement of  $\Delta^{17}\text{O}$  also provides an estimate of the  $\delta^{18}\text{O}$  composition of ingested pre-formed (drinking/food) water. Estimated  $\delta^{18}\text{O}$  values of drinking/food water for captive *Z. capensis* were  $\sim -11\text{‰}$ , which is consistent with that of tap water in Santiago, Chile. In contrast,  $\delta^{18}\text{O}$  values of drinking/food water ingested by wild-caught *Cinclodes* were similar to that of seawater, which is consistent with their reliance on marine resources. Our results confirm the utility of this method for quantifying the relative

contribution of metabolic versus pre-formed drinking/food water to the body water pool in birds.

**Keywords:** birds,  $\Delta^{17}\text{O}$ , evaporative water, metabolic rate, metabolic water, stable isotopes

## INTRODUCTION

Understanding the physiological mechanisms that species use to maintain water balance is becoming more relevant as increases in temperature and drought frequency represent significant ecological shifts that are affecting the behavior, distribution, and abundance of animals (McCarty, 2001; Albright et al., 2010; Şekercioğlu et al., 2012; IPCC, 2013; Remeš and Harmáčková, 2018). Because of their diurnal habits and high mass-specific metabolic rates, birds are particularly susceptible to increases in temperature and aridity (Riddell et al., 2021), so better understanding the environmental factors that influence their water balance is an important topic of research. Recent studies highlight that warm temperatures and reduced availability of fresh water impact key aspects of avian physiology, such as energy expenditure, body mass, thermal tolerance/conductance, and evaporative water loss (Carmi et al., 1993; Sabat et al., 2006a, 2009; Barceló et al., 2009; Gerson and Guglielmo, 2011; Smith et al., 2017; McWhorter et al., 2018). In addition to inducing physiological changes, thermal and water stress can also affect behavior, species distribution, and fitness. For instance, using a combination of physiological data, mechanistically informed models and climatic data predicted that the proportion of the ranges of the distribution of avian species with risk of lethal dehydration during heat waves will dramatically increase under future climate scenarios (Albright et al., 2017).

Most metabolic and functional processes of terrestrial animals are sensitive to water balance, where the steady state homeostatic water budget (intake = loss) assumes a constant amount of total body water:

$$WI_D + WI_{PF} + WI_M = WL_C + WL_R + WL_U + WL_{FC} \quad (1)$$

where  $WI_D$  is drinking water;  $WI_{PF}$  is (preformed) water in food;  $WI_M$  is metabolic water formed in the aerobic metabolism of dietary macromolecules;  $WL_C$  is cutaneous loss of water thorough the skin;  $WL_R$  is the loss of water through respiratory surfaces; and  $WL_U$  and  $WL_{FC}$  are the loss of water through urine and feces, respectively. For most birds, the total evaporative water loss (TEWL), which is the sum of cutaneous and respiratory losses ( $WL_C + WL_R$ ), accounts for between 50 and 80% of total losses depending on hydration conditions, while urine and feces ( $WL_U + WL_{FC}$ ) account for only 15–30% of total water losses (Goldstein and Braun, 1986; MacMillen, 1990; Goldstein and Skadhauge, 2000). The relative contribution of  $WI_D$ ,  $WI_{PF}$ , and  $WI_M$  to an animal's total water budget depends on environmental conditions (e.g., temperature, humidity, and water intake), the rate and macromolecular substrate (protein, carbohydrates, and/or lipids) oxidation, and behavioral attributes (e.g., diurnal versus nocturnal activity). Ultimately, an organism's water balance is a function of the interplay between the physical

environment, the physiological and/or behavioral mechanisms for conserving water (minimizing losses), and the production of metabolic water which is directly linked to metabolic rate (Bartholomew and Cade, 1963; MacMillen, 1990; Gerson and Guglielmo, 2011; Rutkowska et al., 2016; Albright et al., 2017).

The contribution of metabolic water to the body water pool is highly variable among birds (MacMillen, 1990; Williams et al., 1993; Sabat et al., 2006a). Because TEWL accounts for the largest proportion of water lost by birds (McKechnie and Wolf, 2004), the  $WI_M/TEWL$  ratio is especially informative: As this ratio increases toward unity, birds rely more heavily on metabolic water to maintain water balance. Importantly, the physiological traits and ecological conditions that constrain this ratio by either favoring or limiting reliance on metabolic water and thus potential independence from environmental water remain largely unknown (Bartholomew and Cade, 1963; MacMillen, 1990). For instance, what role does metabolic water production ( $WI_M$ ) play in maintaining water balance during physiological challenges related to thermoregulation? Does reliance on different water sources vary with thermoregulatory demands? Understanding these mechanisms is critical to creating accurate physiological models that can assess the ability of animals to adapt to potential threats caused by anthropogenic and natural environmental perturbations, especially increases in ambient temperature and drought frequency predicted for many regions over the next century (Walther et al., 2002; Vale and Brito, 2015; Iknayan and Beissinger, 2018). These perturbations are especially relevant for birds from the order Passeriformes because most species are diurnal and have small body masses, high body temperatures, and high mass-specific metabolic rates that make them particularly susceptible to thermal and dehydration stress (McKechnie and Wolf, 2010; Albright et al., 2017).

Several studies have shown that in comparison with their counterparts that occur in more mesic environments, birds inhabiting aridland ecosystems exhibit physiological adjustments to prevent water loss (Casotti and Braun, 2000; Williams and Tieleman, 2005; McKechnie et al., 2016; Gerson et al., 2019). However, few avian studies have examined variation in the use of potential sources of water to maintain water balance (Navarro et al., 2018; Smit et al., 2019), and even fewer studies have focused on the role of metabolic water in the body water budgets of birds (Williams, 2001; Giulivi and Ramsey, 2015). One of the primary limitations is the inability to assess the contribution of metabolic water to the body water pool without the use of injected tracers (e.g.,  $^2\text{H}_2^{18}\text{O}$ ) that require multiple captures of the same individual over short periods of time (Butler et al. 2004). Recently, Whiteman et al. (2019) proposed a new method for estimating the contribution of metabolic water to the body water pool based on the measurement of  $\Delta^{17}\text{O}$ , which is the positive or negative deviation from the

tight linear correlation that naturally exists between  $\delta^{17}\text{O}$  and  $\delta^{18}\text{O}$  values (Sharp et al., 2018; Whiteman et al., 2019). As shown in equation (1), body water inputs primarily include drinking water ( $W_D$ ) and food water ( $W_{PF}$ ), both of which are ultimately derived from meteoric water (i.e., precipitation), and metabolic water ( $W_{IM}$ ). Metabolic water ( $W_{IM}$ ) is assumed to have a  $\Delta^{17}\text{O}$  value of  $-0.44\text{‰}$  reflecting that of inhaled atmospheric oxygen (Liang et al., 2006; Wostbrock et al., 2020). In contrast, the  $\Delta^{17}\text{O}$  value of  $W_D$  and  $W_{PF}$  is that of meteoric water, which is approximately  $+0.03\text{‰}$  regardless of the source (Li et al., 2015; Sharp et al., 2018; Passey and Ji, 2019).  $\Delta^{17}\text{O}$  values of meteoric water have this consistent value because mass-dependent fractionation associated with evaporation and condensation affects all three oxygen isotopes in a similar and predictable fashion (Sharp et al., 2018). By extension, evaporation during physiological processes (e.g., gular fluttering) should have minimal effect on  $\Delta^{17}\text{O}$  values of animal body water.

A linear mixing model can be used to calculate the proportional contribution from drinking/food versus metabolic water (Whiteman et al., 2019). Because drinking/food water and metabolic water together provide 80–99% of the body water of most animals (Bryant and Froelich, 1995; Kohn, 1996), we can ignore the remaining minor contribution (1–20%) from water formed in condensation reactions from the bound oxygen in dietary macromolecules, and model bird body water ( $\Delta^{17}\text{O}_{BW}$ ) as:

$$\Delta^{17}\text{O}_{BW} = F_M \times (-0.44\text{‰}) + (1 - F_M) \times (0.030\text{‰}) \quad (2)$$

where  $F_M$  represents the fractional contribution to body water from metabolic water, and  $(1 - F_M)$  represents the contribution from pre-formed (drinking/food) water. Whiteman et al. (2019) showed that this equation accurately predicted relative changes in  $\Delta^{17}\text{O}$  values of captive deer mice based on their metabolic rate and drinking water intake and that  $\Delta^{17}\text{O}$  measurements in wild mammals appeared to reflect expected variation in relative mass-specific rates of metabolism and water intake. Continued research is required to assess additional potential predictors for this model, such as evaporation-driven variation in  $\Delta^{17}\text{O}$  (as described above), and trophic enrichment in which food water is increasingly influenced by prey metabolic water for higher-trophic level consumers. Another important need is applying this simplified model (equation 2) to non-mammalian taxa.

Assuming a fixed  $\Delta^{17}\text{O}$  value of  $0.03\text{‰}$  for meteoric water is reasonable, but the potential for variation should be noted. Regarding precipitation, patterns emerge at high and low values of  $\delta^{18}\text{O}$ :  $\Delta^{17}\text{O}$  is closer to  $0.01\text{‰}$  if  $\delta^{18}\text{O}$  is above  $-10\text{‰}$  and closer to  $0.04\text{‰}$  if  $\delta^{18}\text{O}$  is below  $-25\text{‰}$  (Passey and Levin, 2021). Unique environmental conditions can alter  $\Delta^{17}\text{O}$  more dramatically: For example, if  $\sim 90\%$  of a closed water body evaporates into air with very low relative humidity, the  $\Delta^{17}\text{O}$  of the remaining water may fall as low as  $-0.20\text{‰}$  (Passey and Levin, 2021); evaporation of water from plants in very dry conditions can have a similar effect (Landais et al., 2006; Li et al., 2017). However, many environmental sources of meteoric water are not subject to the conditions required to cause such variation in meteoric water  $\Delta^{17}\text{O}$ . In addition, while

deviation from  $0.03\text{‰}$  represents important information for hydrological and geochemical studies, the mean  $\Delta^{17}\text{O}$  value for meteoric water ( $0.03\text{‰}$ ) is very distinct from the biological signal of metabolic water ( $-0.44\text{‰}$ ).

Here, we explore  $\Delta^{17}\text{O}$  in birds. We consider the responses of metabolic rate, TEWL, and the contribution of metabolic water to the body water pool in a widely distributed passerine, the rufous-collared sparrow (*Zonotrichia capensis*), with captive experiments of 15-day exposure to cold ( $15^\circ\text{C}$ ) followed by warm ( $30^\circ\text{C}$ ) environmental conditions. We hypothesize that birds acclimated to the cold conditions will have relatively higher resting metabolic rates (RMRs) but will consume less drinking water than when acclimated to warm conditions. We predict that these responses will yield a change in body water  $\Delta^{17}\text{O}$  values that reflect a net increase in the contribution of metabolic water to the body water pool during cold conditions. Unlike previous applications of oxygen isotopes that have focused exclusively on  $\delta^{18}\text{O}$ , our triple isotope approach is much less sensitive to evaporative  $^{18}\text{O}$ -enrichment of body water nor does it require isotopic characterization of all potential water sources. A novel contribution of our study is the application of a new analytical method that estimates the relative contributions drinking and food water ( $W_D + W_{PF}$ ) vs. metabolic water ( $W_{IM}$ ) to the body water pool (Whiteman et al., 2019) based on the analysis of a single blood plasma sample. In addition to using this approach to study water balance in captive sparrows, we also report data on the contribution of metabolic water to the body water pool in two species of wild-caught songbirds in the genus *Cinclodes* (*Cinclodes oustaleti* and the *Cinclodes nigrofumosus*), a coastal group of invertivorous passerines that vary in their ability to use marine resources. Our approach combines phenomenological data collected from the field with results from laboratory experiments designed to identify the physiological mechanisms that constrain how animals respond to environmental conditions (Khaliq et al., 2014). The results improve our understanding of the physiological responses to climate change and the ultimate threats to species' persistence.

## MATERIALS AND METHODS

### Sample Collection

Our captive model species was the omnivorous rufous-collared sparrow, which is widely distributed across a range of habitats in western and southern South America (Araya et al., 2005). We captured 10 individuals using mist nets in the Quebrada de la Plata ( $33^\circ 31'\text{S}$ ,  $70^\circ 5'0\text{W}$ ,  $\sim 500\text{m}$  elevation) in central Chile, a locality with a Mediterranean climate. Following capture, we transported birds to the laboratory for a 2-day habituation period at  $22^\circ\text{C}$ . The birds were maintained in individual cages ( $50 \times 50 \times 50\text{cm}$ ) and were fed *ad libitum* with dried birdseed and water. Water was offered in inverted 100 ml graduated plastic tubes that allowed birds to eat and drink in a small ( $\sim 1\text{cm}^2$ ) container at the bottom of the tube. After the habituation period, birds were maintained at  $15 \pm 0.5^\circ\text{C}$  for 15 days, and then at  $30 \pm 0.5^\circ\text{C}$  for another 15 days (12:12 light:dark

photoperiod). This acclimation period was long enough to ensure complete turnover of the body water pool for a 20–30 g passerine (Bartholomew and Cade, 1963; Smit and Mckechnie, 2015). After each cold or warm acclimation period, we collected samples of blood (50–100 µl) in the morning (09:00–11:00 h) from the humeral vein using hematocrit tubes with anticoagulant (heparine). Blood samples were then centrifuged at 10,000 rpm (relative centrifugal force = 9,250) for 5 min during which plasma was separated from red blood cells, and then, plasma was frozen at  $-80^{\circ}\text{C}$  until isotope analysis. Water intake rates were measured with the inverted graduated plastic tubes and corrected for evaporation by using control tubes located outside each experimental cage.

Wild *C. oustaleti* and *C. nigrofumosus* were collected using mist nets in the austral winter (June 2018) at Los Molles ( $32^{\circ}14'22''\text{S}$   $71^{\circ}30'54''\text{W}$ ) on the central coast of Chile. Blood samples were obtained with heparinized microcapillary tubes from the humeral vein immediately after capture. Blood was centrifuged at 10,000 rpm for 10 min and the plasma was separated from red blood cells and stored at  $-80^{\circ}\text{C}$  until isotope analysis.

## Metabolic Water Analysis

To measure  $\Delta^{17}\text{O}$ , we cryogenically distilled water from 1 to 2 µl blood plasma samples in a vacuum line, then reacted it with  $\text{BrF}_5$  at  $\sim 300^{\circ}\text{C}$  for 5–10 min, quantitatively converting  $\text{H}_2\text{O}$  to  $\text{O}_2$  and other gasses. These other gasses were removed *via* liquid nitrogen traps and the  $\text{O}_2$  was further purified by passing it through a zeolite molecular sieve and a gas chromatography column.  $\text{O}_2$  was then analyzed on a dual-inlet Thermo Scientific 253 isotope ratio mass spectrometer (Bremen, Germany) at the University of New Mexico Center for Stable Isotopes (Albuquerque, NM). The measured values of  $\delta^{17}\text{O}$  and  $\delta^{18}\text{O}$  were used to calculate  $\Delta^{17}\text{O}$  (Sharp et al., 2018; Whiteman et al., 2019). At the beginning of each analytical session, we measured a local water standard (NM2:  $\delta^{18}\text{O} = -13.1\text{‰}$ ,  $\delta^{17}\text{O} = -6.919\text{‰}$ ) that had been calibrated against the international water standards VSMOW2 ( $\delta^{17}\text{O} = \delta^{18}\text{O} = 0.000\text{‰}$ ) and SLAP2 ( $\delta^{18}\text{O} = -55.5\text{‰}$ ,  $\delta^{17}\text{O} = -29.699\text{‰}$ ; Schoenemann et al., 2013; Sharp et al., 2016). The NM2  $\Delta^{17}\text{O}$  values associated with each measurement were then used to calculate a correction factor which we applied to the raw  $\Delta^{17}\text{O}$  values of unknown samples to yield corrected values.

In addition to using  $\Delta^{17}\text{O}$  values to understand reliance upon metabolic water, we used the combination of  $F_M$  values and  $\delta^{18}\text{O}$  values of body water to calculate the  $\delta^{18}\text{O}$  values of the combination of drinking and food water ( $\delta^{18}\text{O}_{\text{D+PF}}$ ) that birds consumed as:

$$\delta^{18}\text{O}_{\text{DFW}} = \left[ \delta^{18}\text{O}_{\text{BW}} - (F_M) \times (\delta^{18}\text{O}_{\text{Air}}) \right] / (1 - F_M) \quad (3)$$

Here, we assumed  $\delta^{18}\text{O}_{\text{Air}}$  was  $19.4\text{‰}$  because of fractionation that occurs during absorption of inhaled atmospheric oxygen. This fractionation depends on the efficiency of oxygen absorption ( $\text{EO}_2$ ; Epstein and Zeiri, 1988); although this efficiency was not measured in our experiment, previous research suggests that an  $\text{EO}_2$  of 0.4 is reasonable for small passerines (Clemens, 1988; Arens and Cooper, 2005), which in humans produces

a fractionation of  $\sim 4.4\text{‰}$  (Epstein and Zeiri, 1988). Although such data are lacking for our study species, applying the plausible range of fractionation values for absorbed oxygen (2–6‰) to equation 3 indicates that the resulting estimate of  $\delta^{18}\text{O}$  of ingested water generally changes by  $< 3\text{‰}$ , which is smaller than much of the naturally occurring variation in  $\delta^{18}\text{O}$  of potential water sources.

## Metabolic Rates and Total Evaporative Water Loss

At the end of the 15-day experimental period at each temperature treatment ( $15^{\circ}\text{C}$  or  $30^{\circ}\text{C}$ ), we measured rates of oxygen consumption ( $\text{VO}_2$ ) and TEWL for sparrows during 3–4 h using standard flow-through respirometry and hygrometry methods that we have previously applied to this species (Sabat et al., 2006a). Measurements were made at ambient temperatures ( $T_a$ ) of  $15.0 \pm 0.5^{\circ}\text{C}$  and  $30.0 \pm 0.5^{\circ}\text{C}$  using an infrared  $\text{O}_2$ - $\text{CO}_2$  analyzer equipped with a hygrometer (FMS, Sable Systems®). All trials were conducted in metallic metabolic chambers (2000 ml). Briefly, birds were placed in metabolic chambers kept at a constant temperature ( $15^{\circ}\text{C}$  or  $30^{\circ}\text{C}$ ) that received air free of water and  $\text{CO}_2$  removed *via* Drierite and  $\text{CO}_2$  absorbent at a flow of 750 ml/min ( $\pm 1\%$ ).  $\text{O}_2$  concentrations in the chamber were recorded during the active period between 06:00 and 18:00. Oxygen consumption was calculated according to the following equation (Lighton, 2018):

$$\text{VO}_2 = \text{FR} \times 60 \times (F_i \text{O}_2 - F_e \text{O}_2) / (1 - F_i \text{O}_2) \quad (4)$$

where FR is the flow rate in  $\text{ml min}^{-1}$ , and  $F_i \text{O}_2$  and  $F_e \text{O}_2$  are the fractional concentrations of inflow and outflow  $\text{O}_2$  in the metabolic chamber, respectively. We calculated absolute humidity ( $\text{kg/m}^3$ ) of air entering and leaving the chamber as  $= P / (T \times R_w)$ , where P is water vapor pressure of the air in Pascal, T is the dewpoint temperature in Kelvin, and  $R_w$  is the gas constant for water vapor ( $461.5 \text{ J/kgK}$ ; Lide, 2001). P was determined using the average value of the vapor pressure of the air entering the empty chamber (i.e., baseline period of 15 min) before and after each experiment with a dewpoint hygrometer located in the FMS. Total evaporative water loss was calculated as  $\text{TEWL} = (V_e \times \rho_{\text{out}} - V_i \times \rho_{\text{in}})$ , where TEWL is in mg/ml,  $\rho_{\text{in}}$  and  $\rho_{\text{out}}$  are the absolute humidity in  $\text{kg/m}^3$  of the inlet air and the outlet air, respectively,  $V_i$  is the flow rate of the air entering the chamber as given by the mass flow controller ( $750 \text{ ml min}^{-1}$ ), and  $V_e$  is the flow of exiting air.  $V_e$  was calculated following (Williams and Tieleman, 2000) as:

$$V_e = V_i - \left[ \text{VO}_2 \times (1 - \text{RQ}) \right] + V_{\text{H}_2\text{O}} \quad (5)$$

$V_{\text{in}}$  and  $\text{VO}_2$  ( $\text{ml min}^{-1}$ ) are known, and we assumed a respiratory quotient (RQ) of 0.71 (Sabat et al., 2006a). Output from the  $\text{H}_2\text{O}$  (kPa) analyzer, the oxygen analyzer (%), and the flow meter was digitalized using a Universal Interface II (Sable Systems, Nevada, United States) and recorded on a personal computer using EXPEDATA data acquisition software (Sable Systems, Nevada, United States). To estimate RMR, we averaged  $\text{O}_2$  concentrations of the excurrent air stream

over a 20-min period after steady state was reached (Tieleman et al., 2002). We estimated the metabolic water production ( $WI_M$ ) of sparrows using the equivalence of 0.567 ml  $H_2O$  per liter  $O_2$  consumed (Schmidt-Nielsen, 1997). We calculated the  $WI_M/TEWL$  ratio at different temperature treatments (15°C or 30°C). We also used equation (1) to calculate sparrow water balance, given that pre-formed water in food was negligible ( $WPF=0$ ); after combining CWL and RWL into TEWL, and combining  $W_U$  and  $W_F$  into  $W_E$  as water losses *via* excreta, equation (1) was simplified to:

$$W_D + WI_M = TEWL + W_E \quad (6)$$

Birds were captured with permits from SAG, Chile (No. 10192/2019). All protocols were approved by the institutional Animal Care Committee of the University of Chile, following the recommendation of the ARRIVE guidelines (Kilkenny et al., 2010).

## Statistical Analysis

We evaluated the effect of thermal acclimation on RMR, TEWL, and water intake using a generalized linear mixed model (GLMM) with body mass as a covariate, acclimation temperature (15°C and 30°C) as fixed factors, and individual identity as a random factor to control for repeated measures. Assumptions of normality and heteroscedasticity in residuals were examined with Q–Q plots and a plot of residuals against fitted values, respectively (Zuur et al., 2009). Dependent variables and covariates were natural log transformed for data normalization. Body mass and isotope values ( $\Delta^{17}O$ ,  $\delta^{18}O$ , and  $\delta^{17}O$ ) were compared between warm (30°C) and cold (15°C) treatments using non-parametric two-sample paired t-tests. The statistical analyses were performed in nlme package (Pinheiro et al., 2013) using the R platform (v4.0.3; R Development Core Team, 2013).

## RESULTS

### Physiological Data of Captive Sparrows

Sparrows acclimated at 15°C exhibited higher RMR ( $93.2 \pm 15.2$  ml  $O_2$   $h^{-1}$ ) and lower daily water intake ( $0.19 \pm 0.05$  ml  $H_2O$   $h^{-1}$ ) in comparison with when they were acclimated at 30°C (RMR:  $70.8 \pm 12.2$  ml  $O_2$   $h^{-1}$  and daily water intake:  $0.26 \pm 0.08$  ml  $H_2O$   $h^{-1}$ ); however, there was no difference in TEWL between temperature treatments (Table 1). In addition, the  $WI_M/TEWL$  ratio, converted to percentage, decreased significantly with increasing  $T_a$  (Figure 1), ranging from ~65% at 15°C to ~55% at 30°C. Using  $WI_M$  and  $W_D$  and the equation (6), metabolic water represented  $22.8 \pm 4.2\%$  and  $14.0 \pm 5.6\%$  of the total body water pool (i.e.,  $WI_M/(WI_M + W_D)$ ) at 15°C and 30°C respectively, while TEWL represented 80% of the total water loss at 15°C and 67% of the total water loss at 30°C.

### Oxygen Isotopes

$\Delta^{17}O$  values were lower for captive sparrows acclimated to 15°C than 30°C (Wilcoxon Sign test =  $-7.5$ ;  $p=0.03$ , Figure 2A).

$\Delta^{17}O$ -based estimates (*via* equation 2) of the proportion of metabolic water in the total body water pool in captive sparrows were 27.2 and 24.1% at 15°C and 30°C, respectively (Figure 2B). Wild *Cinclodes*  $\Delta^{17}O$  values vary both within and among species (Figure 3), and  $\Delta^{17}O$ -based estimates of the metabolic water contribution to the body water pool ranged from 19.6 to 31.0%. Mean ( $\pm SD$ ) metabolic water contributions for *C. nigrofumosus* and *C. oustaleti* were  $23.0 \pm 4.8\%$  and  $27.7 \pm 4.0\%$ , respectively. Intriguingly, two of the three highest measured  $\Delta^{17}O$  values were sourced from *C. nigrofumosus*, suggesting a greater intake of pre-formed drinking/food water than for *C. oustaleti*. This inference is consistent with the general hypothesis of reduced dependence on  $WI_M$  for larger body-sized individuals across birds and mammals (Whiteman et al., 2019), because *C. nigrofumosus* (70–80 g) is more than twice the body mass of *C. oustaleti* (23–28 g; Sabat et al., 2006a). The mean ( $\pm SD$ ) estimated  $\delta^{18}O$  value of the combined pre-formed drinking/food water ingested by captive sparrows was  $\sim -11 \pm 3\%$ , which is within the range for tap water and groundwater in the Santiago Basin of central Chile ( $-15$  to  $-11\%$ ; Iriarte et al., 2004). In contrast, the pre-formed drinking/food water ingested by wild *Cinclodes* was estimated to have mean  $\delta^{18}O$  ( $\pm SD$ ) values near seawater (0‰; LeGrande and Schmidt, 2006):  $0.2 \pm 8.3\%$  for *C. nigrofumosus* and  $-1.4 \pm 0.9\%$  for *C. oustaleti*.

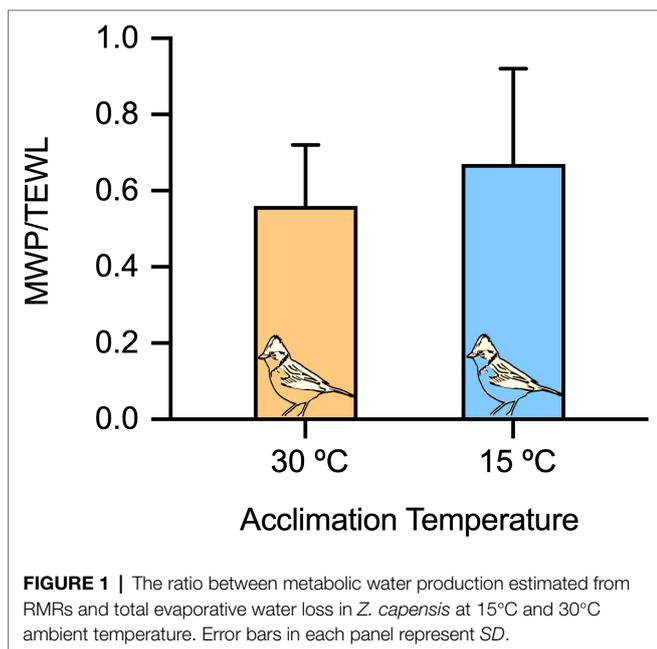
## DISCUSSION

Metabolic water production ( $WI_M$ ) alone is typically insufficient to meet the water requirements of most vertebrates (McNab, 2002), although some species adapted to arid environments or routinely experience long periods of time without food or drinking water can survive solely on metabolic water under certain conditions (Bartholomew and Cade, 1963; MacMillen and Hinds, 1983; Ostrowski et al., 2002). Several factors have been suggested to influence the importance of  $WI_M$  to the water budget of birds, such as the nature of oxidized substrates and environmental temperature. The current study aimed to experimentally assess the effects of changes in metabolic rate and water intake on  $\Delta^{17}O$  values of captive house sparrows, kept in standard housing conditions at 30°C or 15°C for 15 days. As predicted, sparrows acclimated to cooler temperatures increased their RMR by ~31% while decreasing their drinking water intake by ~27% (Table 1). This elevation in metabolic rate suggests that an ambient temperature of 15°C is below their lower critical temperature (Maldonado et al., 2009). Accordingly, plasma  $\Delta^{17}O$  values were lower in individuals housed at 15°C than at 30°C, reflecting the larger contribution of metabolic water relative to drinking/food water to the body water pool (Figure 2). These results support the predictions of a model of the relationship between the  $WI_M:TEWL$  ratio and ambient temperature in granivorous birds (MacMillen, 1990). In this model, the ratio of  $WI_M/TEWL$  increases when temperature declines below the  $T_{LC}$ , as a result of increased  $WI_M$  and dampened TEWL *via* water-recovery adaptations, such as desaturation of exhaled air into nasal surfaces (MacMillen, 1990). In a similar controlled experiment on mammals,  $\Delta^{17}O$

**TABLE 1** | Results of generalized linear mixed model testing the effect of 15 days of thermal acclimation to 15°C (cold) and 30°C (warm) on resting metabolic rate (RMR), total evaporative water loss (TEWL), and water intake in *Zonotrichia capensis*.

Predictors	Coeff. Estimate	95% CI	df	t	Value of p	R <sup>2</sup> marginal/ conditional
<b>RMR (ml O<sub>2</sub> h<sup>-1</sup>)</b>						0.58/0.58
Intercept	0.94	(-1.91-3.79)	9	0.75	0.47	
Mass	1.18	(0.22-2.13)	8	2.84	<b>0.02</b>	
Temperature (30°)	-2.25	(-0.40 to -0.10)	8	-3.85	<b>0.005</b>	
<b>TEWL (mg H<sub>2</sub>O h<sup>-1</sup>)</b>						0.10/0.25
Intercept	2.52	(-2.95-8.00)	9	1.04	0.32	
Mass	0.62	(-1.21-2.45)	8	0.78	0.45	
Temperature (30°)	-0.12	(-0.37-0.13)	8	-1.15	0.28	
<b>Water Intake (ml H<sub>2</sub>O)</b>						0.25/0.66
Intercept	-2.45	(-8.96-4.06)	9	-0.85	0.41	
Mass	0.24	(-1.94-2.42)	8	0.26	0.80	
Temperature (30°)	0.34	(0.13-0.55)	8	3.67	<b>0.006</b>	

Numbers in bold indicate statistical significance ( $p < 0.05$ )



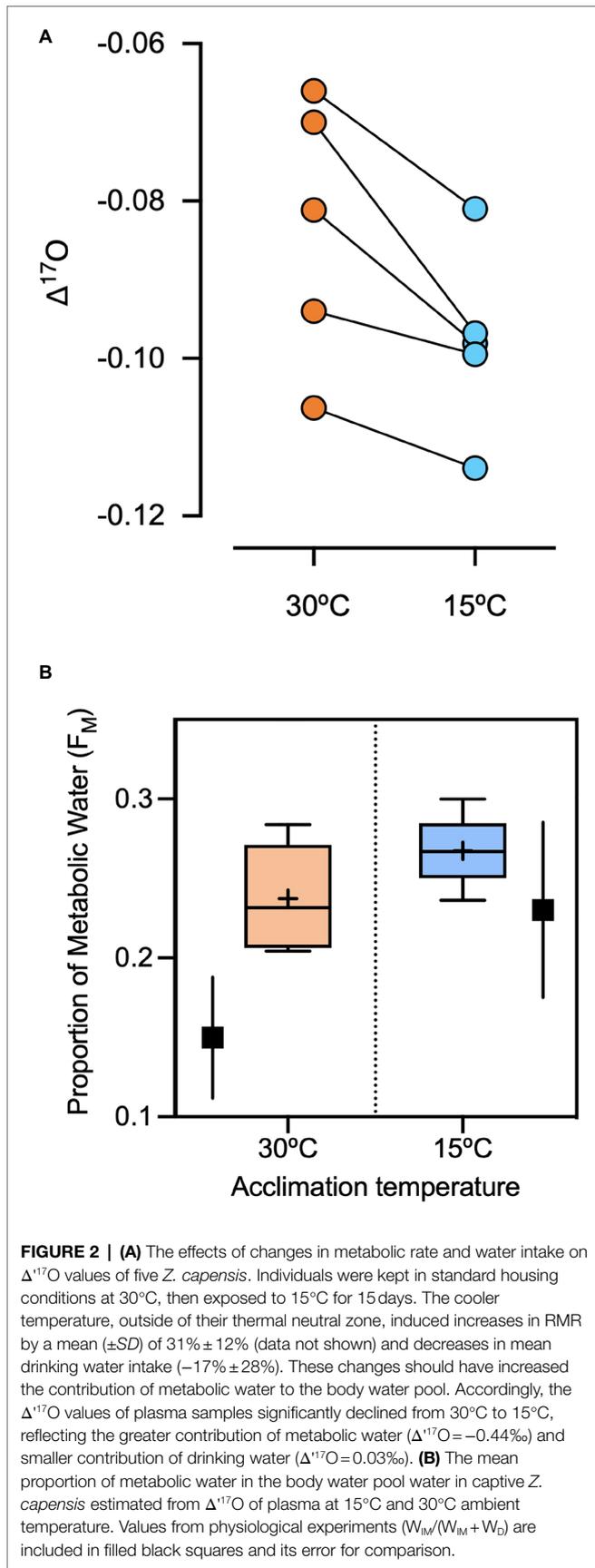
values of plasma from deer mice declined in response to elevated metabolic rate when animals were housed at 5°C rather than 25°C (Whiteman et al., 2019). The magnitude of the decline in  $\Delta^{17}\text{O}$  (0.01–0.03 ‰) in deer mice was similar to that observed here in captive sparrows. Our results confirm that  $W_{I_M}$  in small passerines increases with thermoregulatory demands and highlights the utility of this method for estimating water balance in laboratory conditions.

In a formative study, it was observed that the theoretical production of metabolic water can approach the rate of evaporative water loss in birds with body masses > 60 g, creating the opportunity for these larger birds to be “water-independent” and possibly rely solely on  $W_{I_M}$  (Bartholomew and Cade, 1963). However, MacMillen (1990) suggested that smaller birds (e.g., < 20 g) can also attain favorable states of water balance ( $W_{I_M} > \text{TEWL}$ ) if they are below the lower critical temperature

(~25°C) when experiencing water deprivation. For the captive sparrows (~20 g) in our study, we calculated a mean  $W_{I_M}/\text{TEWL}$  of ~61% (Figure 1), which suggests that sparrows are not capable of relying solely on metabolic water production to maintain water balance, even in conditions that yield increases in metabolic rate (i.e., colder temperatures). Note that  $W_{I_M}/\text{TEWL}$  was 67 and 56% for cold- and warm-acclimated birds, respectively. Nevertheless, our estimates of TEWL come from an experimental setup that controlled the humidity inside the metabolic chambers near zero, which likely does not occur often in nature. In some birds, TEWL appears to vary as a function of absolute humidity ( $\text{mg H}_2\text{O}/\text{m}^3$ ) across a range of environmental temperatures (Powers 1992, Gerson et al., 2014). By using data at comparable temperatures from the literature and climate data available from a local weather station,<sup>1</sup> we calculated that TEWL could be reduced on average by up to 20% at 30°C and 40% at 20°C, which would result in a  $W_{I_M}/\text{TEWL}$  of 76% at 30°C and up to 100% at 15°C.

The traditional approach to measuring  $W_{I_M}$  is to assume a constant equivalence of water production based on oxygen consumption (Schmidt-Nielsen, 1997). Using this approach, we found that  $W_{I_M}$  was 23% of the total water intake (i.e.,  $W_{I_M} + W_D$ ) at 15°C but decreased to 14% at 30°C. These values are slightly lower to the percent contribution of metabolic water to the total body water pool that we estimated from  $\Delta^{17}\text{O}$  data alone, which were 27.5% for cold-acclimated birds and 24.5% for warm-acclimated birds. The similarity in estimates of  $W_{I_M}$  using the two approaches is notable because the  $\Delta^{17}\text{O}$  estimates were based on collection of a single sample and did not rely on any measurements of water intake or loss, highlighting the potential accuracy of this new method (Whiteman et al., 2019). The discrepancy in estimates of  $W_{I_M}$ , which is larger for warm-acclimated sparrows (14.0% vs. 24.5%), may be due to at least two non-exclusive alternatives. First, the calculation for the amount of water consumed per day was obtained under acclimatization conditions when birds were in larger cages that allowed for

<sup>1</sup>www.meteored.cl

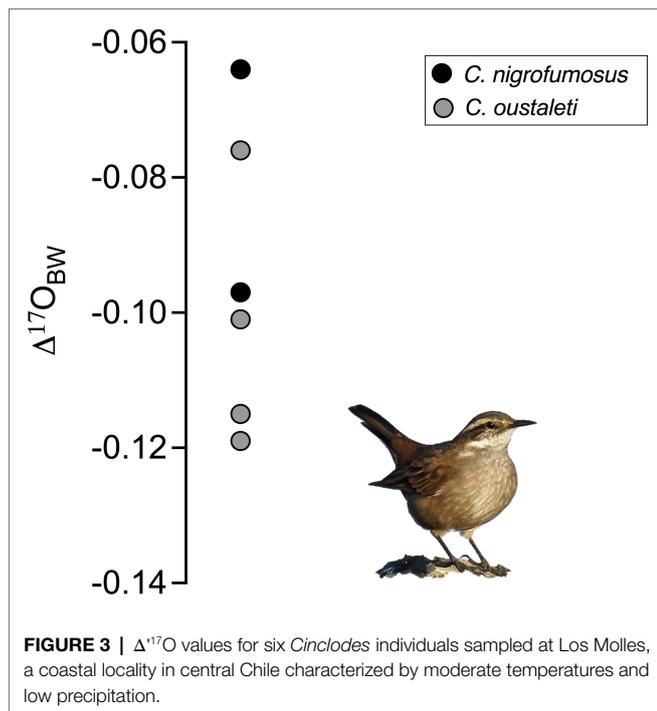


**FIGURE 2 | (A)** The effects of changes in metabolic rate and water intake on  $\Delta^{17}\text{O}$  values of five *Z. capensis*. Individuals were kept in standard housing conditions at 30°C, then exposed to 15°C for 15 days. The cooler temperature, outside of their thermal neutral zone, induced increases in RMR by a mean ( $\pm$ SD) of 31%  $\pm$  12% (data not shown) and decreases in mean drinking water intake ( $-17\% \pm 28\%$ ). These changes should have increased the contribution of metabolic water to the body water pool. Accordingly, the  $\Delta^{17}\text{O}$  values of plasma samples significantly declined from 30°C to 15°C, reflecting the greater contribution of metabolic water ( $\Delta^{17}\text{O} = -0.44\text{‰}$ ) and smaller contribution of drinking water ( $\Delta^{17}\text{O} = 0.03\text{‰}$ ). **(B)** The mean proportion of metabolic water in the body water pool water in captive *Z. capensis* estimated from  $\Delta^{17}\text{O}$  of plasma at 15°C and 30°C ambient temperature. Values from physiological experiments ( $W_M/(W_M + W_D)$ ) are included in filled black squares and its error for comparison.

movement and flight, conditions that yield higher rates of energy consumption than in the more confined conditions when RMR was measured. In addition, the traditional approach of using an equivalency between oxygen consumption and  $WI_M$  (i.e., 0.567 ml  $\text{H}_2\text{O}$  per liter  $\text{O}_2$  consumed) does not distinguish between (1)  $\text{H}_2\text{O}$  that was produced by condensation reactions that occur during the oxidation of food that contains oxygen bound in macromolecules (e.g., protein or lipids) and (2)  $\text{H}_2\text{O}$  that was produced by complex IV of the electron transport chain, and which therefore only contains inhaled atmospheric oxygen (Morrison, 1953). In contrast, the  $\Delta^{17}\text{O}$  approach only estimates the contribution of the latter mitochondrial source of  $\text{H}_2\text{O}$ . It should also be noted that body water exchanges oxygen atoms with dissolved  $\text{CO}_2$  in the blood *via* the bicarbonate buffer system. This  $\text{CO}_2$  is from metabolic decarboxylation (e.g., reactions that occur within the citric acid cycle) and contains oxygen bound in macromolecules (e.g., glucose) as well as phosphate groups (e.g., added by glucokinase). We expect that the influence of dissolved  $\text{CO}_2$  on body water  $\Delta^{17}\text{O}$  is small, because the isotopic fractionation associated with loss of exhaled  $\text{CO}_2$  (Speakman and Racey, 1987; Haggarty et al., 1988) is mass-dependent, and because phosphate groups are likely in isotopic equilibrium with body water (Li et al., 2016). In general, more precise studies of water balance that considers the loss of water through urine and measurements of metabolic rate during longer periods in acclimatization conditions are necessary to establish the precision of the  $\Delta^{17}\text{O}$ -based method.

The contribution of metabolic water to the body water pool is highly variable among birds, ranging from < 10% in some hummingbirds, ~14% in desert-adapted ostriches, and up to 80% in some passerines (MacMillen, 1990; Williams et al., 1993). For example, captive zebra finches (*Taeniopygia guttata*) with *ad libitum* access to drinking water produced only 1 ml metabolic  $\text{H}_2\text{O}$  per ~1.5–1.8 ml of evaporative water lost at temperatures between 15 and 25°C, showing that without drinking water, the birds would have been in negative water balance. However, when birds were dehydrated for 30 days, their TEWL declined and  $WI_M/\text{TEWL}$  increased to one (Cade et al., 1965). The influence of water availability on the relative importance of  $WI_M$  to total water pool in birds is in agreement with our previous work on small mammals. For example, captive mice (*Mus musculus*) that were provided drinking water *ad libitum* had smaller contributions of metabolic water to their body water than did wild desert-adapted small mammals (*Peromyscus leucopus*) of similar body mass (Whiteman et al., 2019). Overall, these results suggest that wild animals lacking *ad libitum* access to water responded by relying more on metabolic water than their captive counterparts.

Birds also have substantial flexibility in their sources of water intake. In wild zebra finches,  $WI_M$  calculated on the basis of field metabolic rate was lower during hot versus cool periods. As a consequence,  $WI_M$  fulfilled 20% of water requirements during hot days and 32% on cold days (Cooper et al., 2019). Because the total water turnover (ml/day) did

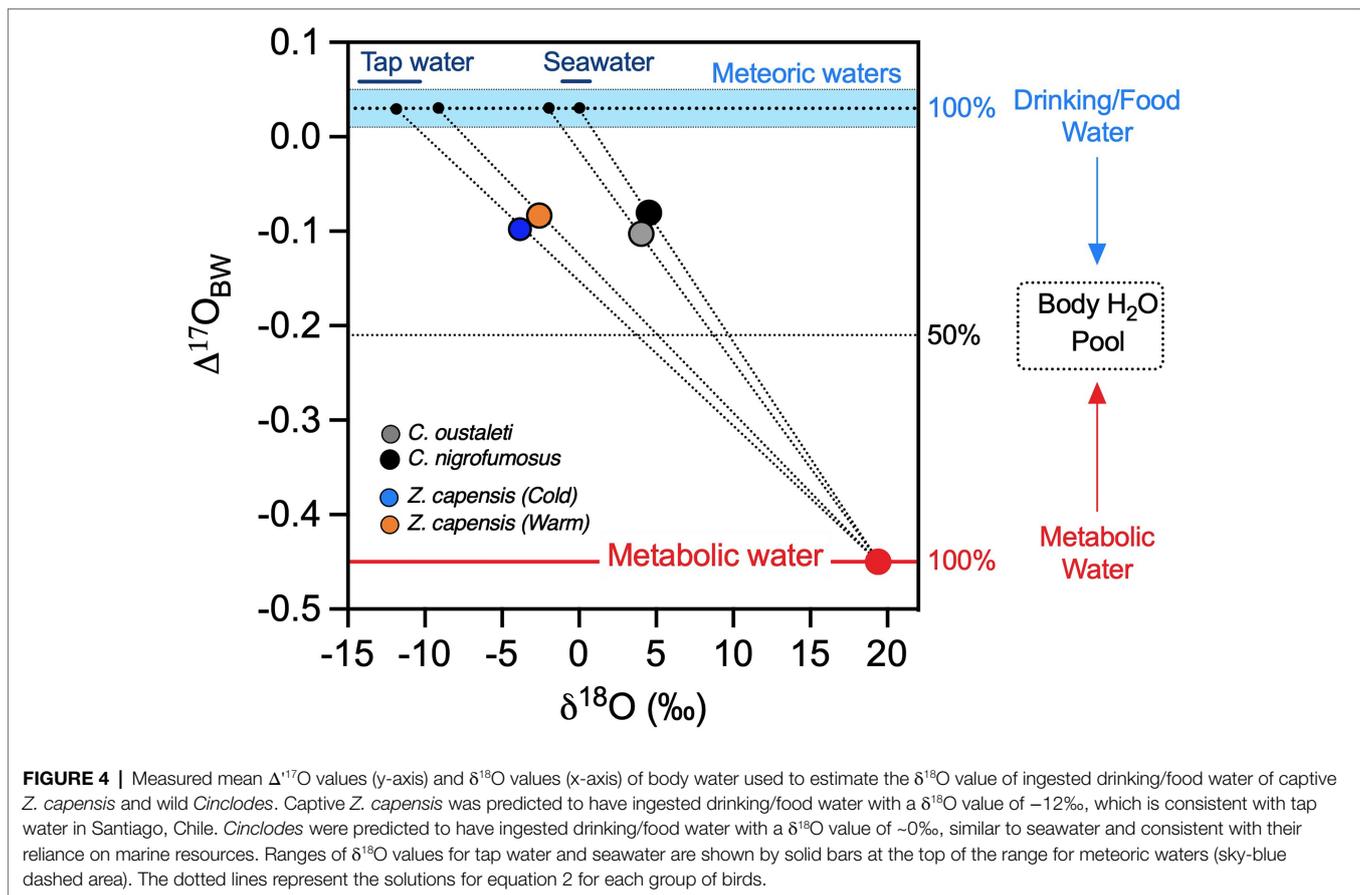


not vary substantially with environmental temperature, the change in the contribution of metabolic water to the total body water pool (i.e.,  $F_M$ ) is likely due to changes in metabolic rates. A question that remains unresolved is whether birds, especially species living in arid seasonal environments, modify their metabolic rate strictly for the purpose of  $WI_M$ . Lastly, another important variable that influences  $WI_M$  is the type of oxidative substrate used for aerobic metabolism. For instance, Zebra finches predictably lose body mass when fasting; however, when they are simultaneously water deprived, they lose substantially more body fat than lean (protein-rich) tissue (Rutkowska et al., 2016). The catabolism of body fat can potentially substantially increase the yield of metabolic water because fat is far more energy dense than other macromolecules oxidized for energy. In contrast, house sparrows (*Passer domesticus*) accelerate protein catabolism during acute dehydration (Gerson and Guglielmo, 2011), presumably to liberate pre-formed water molecules bound in proteinaceous (muscle) tissue (Giulivi and Ramsey, 2015).

We compared the  $\Delta^{17}\text{O}$ -based estimates of the fractional contribution to body water from metabolic water ( $F_M$ ) in captive sparrows with similar estimates for arid- and mesic-adapted wild birds based on allometric equations for water influx rate and field metabolic rate (Williams et al., 1993). We assumed that  $WI_M$  from oxidized substrates is 0.027 ml  $\text{H}_2\text{O}/\text{KJ}$  based on oxidation of carbohydrates; note that this calculation yields the same result for  $WI_M$  as the equivalence method (0.567 ml  $\text{H}_2\text{O}$  per liter  $\text{O}_2$ ) mentioned above (Morrison, 1953). We found that on average, the warm- and cold-acclimated sparrows in our study had  $F_M$  values that were  $\sim 29\%$  and  $\sim 26\%$  higher than the values expected for arid- and mesic-adapted birds of a similar

size. Estimates of  $F_M$  in wild *C. nigrofumosus* were  $\sim 23\%$  or  $\sim 19\%$  higher than expected values for similar-sized birds inhabiting an arid or mesic environment, respectively. Note that  $\Delta^{17}\text{O}$ -based estimates of  $F_M$  for captive sparrows and wild *C. nigrofumosus* are within the 95% confidence interval of those reported for arid- and mesic-adapted birds of similar size. In contrast,  $\Delta^{17}\text{O}$ -based estimates of  $F_M$  for *C. oustaleti* were significantly higher by  $\sim 48\%$  or  $\sim 42\%$  than predicted for birds from arid and mesic environments, respectively. We hypothesize that observed differences in  $F_M$  based on  $\Delta^{17}\text{O}$  versus allometric proxies for *C. oustaleti* may be related to the high energetic and osmoregulatory costs of migrating between coastal (winter) and high elevation (summer) habitats, which may also be why this species has a relatively high BMR in comparison with other *Cinclodes* (Tapia-Monsalve et al., 2018). Overall,  $\Delta^{17}\text{O}$  data revealed that the contribution of metabolic water to body water in captive sparrows and wild *Cinclodes* was similar to or within the same order of magnitude as for other free-ranging birds based on logarithmic allometric relationships. This finding emphasizes the validity of our method to the study of wild birds in natural ecosystems.

As expected, our regression approach based on oxygen isotope analysis shows that captive sparrows consumed drinking/food water that had a  $\delta^{18}\text{O}$  value of  $-11\text{‰}$ , consistent with local tap water in Santiago, Chile (Figure 4). Likewise, isotope data show that wild *Cinclodes* consumed pre-formed drinking/food water with  $\delta^{18}\text{O}$  values of  $\sim 0\text{‰}$ , which is consistent with the oxygen isotope composition of seawater (Figure 4). Intriguingly, one *C. nigrofumosus* individual ingested water with an anomalously positive estimated  $\delta^{18}\text{O}$  value of  $6.3\text{‰}$  (Supplementary Table S1), notably higher than seawater. This enrichment could result from abiotic evaporation occurring in the environment prior to ingestion and/or physiologically mediated evaporative enrichment occurring within the organism. The first explanation suggests seawater ingested as pre-formed drinking/food water in the arid intertidal habitats where *C. nigrofumosus* forages in central and northern Chile may be  $^{18}\text{O}$ -enriched. Alternatively, the relatively high  $\delta^{18}\text{O}_{\text{DFW}}$  value observed for this species may result from isotopic fractionation that occurs during evaporation of body water, which largely depends on environmental temperature and humidity (Kohn, 1996). While *C. nigrofumosus* in this study was sampled in arid central Chile where evaporative  $^{18}\text{O}$ -enrichment of body water could be a factor, this species inhabits humid coastal intertidal ecosystems and has ample access to drinking water in the form of seawater (Sabat et al., 2006a). In contrast to patterns for wild *Cinclodes*, we observed less within-treatment variation in estimates of  $\delta^{18}\text{O}_{\text{DFW}}$  for captive sparrows (Supplementary Table S1), which is expected because (1) their drinking water was sourced from a municipal groundwater-derived aquifer with a relatively constant  $\delta^{18}\text{O}$  value and (2) they were subjected to less variation in temperature and humidity in comparison with wild *Cinclodes* species (Sabat and del Rio, 2002; Sabat et al., 2006a). To better understand the ecological (e.g., habitat use and/or



diet composition) and environmental (e.g., temperature and/or humidity) factors that influence water budgets in birds will require more experiments that assess the effect of physiologically mediated water conservation strategies on the oxygen isotope composition of body water, and additional sampling of birds from a range of environments that span temperature and humidity gradients.

Finally, apparent differences in  $\delta^{18}\text{O}$  of pre-formed drinking/food water between *Cinclodes* species may be related to interspecific variation in the ability to cope with saline-rich prey and drinking water sources (Sabat et al., 2006b; Tapia-Monsalve et al., 2018). For example, the high salinity tolerance of *C. nigrofumosus* (Sabat et al., 2006b), especially at the northern margin of its distribution, may allow this species to rely less on metabolic water because of the ability to obtain and process an abundant source of drinking/food water sourced from the ocean. The ecological conditions that either favor or limit the ability of birds to depend on metabolic water remain largely unknown, while the combined effect of temperature and humidity on a bird's water budget is poorly understood, especially in free-ranging individuals. We predict that a species dependence on metabolic water vs. pre-formed drinking/food water along environmental gradients will depend on the interaction between the ability of different populations/species to retain water and dissipate heat, coupled with the availability of pre-formed water as well as environmental temperature and humidity. For example,

the study of bird species and/or populations that differ in how they evaporatively cool their bodies *via* panting, hyperthermia, or cutaneous evaporation that ultimately influences water loss rates (Gerson et al., 2014) represents a unique opportunity to determine which environmental and physiological variables modulate an animal's use of metabolic versus pre-formed water to maintain water balance.

Overall, our study revealed that the contribution of metabolic water to the total body water pool increased with metabolic rate, consistent with a recent report in small mammals that used a similar  $\Delta^{17}\text{O}$ -based approach (Whiteman et al., 2019). Although these increases may not seem very significant in magnitude, they could account for natural changes in the energy expenditure of animals in the field. More precise studies of water balance that considers all potential sources of water losses during longer periods in acclimatization are necessary to establish the precision of the  $\Delta^{17}\text{O}$ -based method used in this study.  $\Delta^{17}\text{O}$ -based estimates of  $F_M$  in captive sparrows and wild *C. nigrofumosus* were similar to those for free-ranging birds based on independent allometric relationships, while estimates for *C. oustaleti* based on oxygen isotopes were higher than expected, but in the same order of magnitude as predictions based on logarithmic allometric relationships, emphasizing the validity of our method to better understand water balance in wild birds.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and all protocols were approved by the institutional Animal Care Committee of the University of Chile (CICUA), and National Research and Development Agency (ANID).

## AUTHOR CONTRIBUTIONS

PS, SN, and JW designed the research. SP, PS, ZS, and JW performed the research. SP, AG, KM, and PS analyzed the data. PS, SN, RN, JS-H, KM, and JW wrote the paper. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2021.710026/full#supplementary-material>

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