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REVIEW

Access to water affects the condition dependency of nocturnal restlessness in Garden Warblers on a Mediterranean island stopover

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Abstract During migration, many songbirds encounter large ecological barriers, like deserts and seas that require substantial fuel to cross and can lead to dehydration during passage. If muscle is not catabolized to generate metabolic water, birds must seek free water on a subsequent stopover to replenish the water lost. Yet, no work has examined if birds crossing large migration barriers use access to free water in concert with energy or protein stores to make stopover decisions. We captured 61 free-living Garden Warblers (*Sylvia borin*) in spring at a frequently used stopover site in the Mediterranean Sea, housed them with or without drinking water, and measured nocturnal restlessness (Zugunruhe) in relation to energy stores at capture. Both groups lost equivalent flight muscle mass overnight, suggesting that water-deprived birds did not preferentially catabolize this tissue to produce metabolic water. Fat score and body mass, but not flight muscle mass, were positively correlated with nocturnal activity in both treatment groups. However, the slope of the relationship between nocturnal activity and fat score differed between groups, with water-deprived birds of high fat score showing the highest Zugunruhe activity. Our results suggest that birds with large energy stores use access to drinking water to inform their decisions about when to depart from a stopover site. Individuals with higher fat scores might be expected, regardless of flight muscle size, to depart a dry stopover site more readily than a site with freely available water. We suggest follow-up studies to further elucidate the mechanisms of this phenomenon and establish its prevalence in free-living birds.

Keywords Condition dependency · Dehydration · Migration · Stopover · Zugunruhe

Introduction

During migration, many songbirds encounter large ecological barriers, such as deserts and seas, which provide little or no opportunity to feed and drink. Crossing these barriers requires substantial energy, but can also result in dehydration when birds lose water due to evaporation during passage (Fogden 1972; Carmi et al. 1992; Klaassen 1996, 2004; Leberg et al. 1996). Birds replenish the fuel they have catabolized by feeding extensively on stopover. While energy stores and food resources are known to affect stopover duration and the birds' motivation to depart stopover sites (e.g., Fusani et al. 2009, 2011; Goymann et al. 2010; Eikenaar and Bairlein 2014; Smith and McWilliams 2014), the effect of water availability on the birds' stopover behavior has received little attention (Sapir et al. 2004; Tsurim et al. 2008; Mizrahy et al. 2011). Particularly on remote islands in the Mediterranean Sea, or other locations receiving extensive landfall of songbirds

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after barrier crossings, dehydration may influence how body condition determines the timing of the onward migration.

Recent studies of spring-migrating Garden Warblers (*Sylvia borin*) on the islands of Ponza and Ventotene, Italy, have shown that body condition is related to intensity of nocturnal migratory restlessness (Zugunruhe) in caged birds and stopover duration in free-ranging birds outfitted with transmitters (Fusani et al. 2009, 2011; Goymann et al. 2010). These studies used visible fat and pectoral muscle size as an index of condition, but did not consider how water availability may affect condition dependency. Ponza is a particularly interesting study site for investigating the effects of water availability, given that thousands of songbirds stopover on the island after flying for long durations (>10 h) from Africa in spring (Grattarola et al. 1999), and free water on the island is not abundant.

In the experiment presented here, we examined how songbirds respond to water deprivation after crossing a large ecological barrier, whether by preferentially catabolizing flight muscle to replenish body water, or attempting to leave the island due to increasing nocturnal restlessness (Zugunruhe), which has been recently validated as a reliable proxy for departure likelihood (Eikenaar et al. 2014). We captured 61 free-living Garden Warblers on Ponza during spring migration, housed them for 24 h with or without drinking water, and measured Zugunruhe in relation to energy stores at capture. We also used the deuterium dilution method of fat mass estimation (Karasov and Pinshow 1998; McWilliams and Whitman 2013) to measure lipid stores, given that birds with low fat scores may still have visceral fat in significant amounts (Maillet and Weber 2006) and vary considerably in total fat content (McWilliams and Whitman 2013). Past studies (e.g., Fusani et al. 2009, 2011) that found relatively modest correlations between intensity of Zugunruhe and fat score may have benefited from using the deuterium dilution method, which estimates both readily visible and visceral adipose tissue, but the effect of dehydration on this method has not been fully evaluated. Furthermore, we evaluated two intervals of Zugunruhe behavior: (a) the early night period when birds are most likely to depart from a stopover site (e.g., Biebach et al. 2000; Goymann et al. 2010) and, therefore, when condition dependency might be strongest; and (b) all night, when has been traditionally evaluated in previous experiments (e.g., Fusani et al. 2009, 2011).

Our objectives were to (1) determine how access to water affects Zugunruhe activity and overnight changes in flight muscle size, and (2) relate whole-animal fat content estimated using deuterium dilution to visible fat score under conditions of water availability and water deprivation.

Materials and methods

Field techniques

We used mist nets to capture 61 Garden Warblers on the island of Ponza, Italy, between 10 and 13 May 2012. Ponza is a small volcanic island 50 km off the Tyrrhenian coast of Italy (40°55′N, 12°58′E). Within 1 h of capture, we measured subcutaneous fat scored on a 0–8 scale (Kaiser 1993), size of the pectoral muscles scored on a 0–3 scale (Gosler 1991), body mass to 0.1 g, and tarsus length to 0.01 mm. We also measured flight muscle shape at capture to 0.01 mm with a muscle meter (Bauchinger et al. 2011) and used muscle shape, body mass, and tarsus length to calculate total flight muscle mass, using the equation for Garden Warblers developed by Bauchinger et al. (2011): flight muscle mass $= -1.212 + (0.293 \times \text{muscle shape}) + (0.045 \times \text{body mass}) + (0.199 \times \text{tarsus length})$.

Birds were housed individually overnight in $50 \times 25 \times 30$ cm cloth cages so that they were visually isolated from each other. All birds were housed in an indoor room accommodating 20 cages, with natural illumination provided through a large door. No food was provided to reduce the potential for confounding effects of food availability on Zugunruhe (Fusani et al. 2011; Eikenaar and Bairlein 2014). Individuals captured on 10 and 11 May 2012 ($n = 12$ and 17, respectively; total $= 29$) were housed without water or a water dish, whereas birds captured on 12 and 13 May ($n = 15$ and 17, respectively; 32 total) were provided ad libitum water in $11 \times 7.5 \times 3.5$ cm dishes at the time of caging (by 1300 hours). Zugunruhe (migratory restlessness) was measured overnight using an infrared activity sensor connected with an activity recorder on each cage. We counted the number of times the infrared sensor was activated for each 2-min period, and then calculated the average activity during two intervals: civil sundown to midnight and midnight to civil sunrise (GMT + 1) (Fusani et al. 2009, 2011). We used the first interval to represent "early-night" Zugunruhe and averaged the two intervals to represent "all-night" Zugunruhe. In the morning, fat score, body mass, and flight muscle shape were measured again, total flight muscle mass was calculated again as above, and we assessed fat mass using the deuterium dilution method: we injected 50 µL of 99 % deuterium-enriched water (D_2O) into the breast musculature of each bird and drew a 150-µL blood sample after a 60-min equilibration period in a cloth bag [see McWilliams and Whitman (2013) for details]. Birds were subsequently released, and blood samples were flame-sealed and stored at 4 °C until analysis. All work on Ponza was performed under permission number A00785 as of 8/2/2013 from the government of the Regione Lazio according to Italian law.

Determination of fat mass via deuterium dilution

We estimated fat mass of Garden Warblers injected with deuterium given the volume of deuterium injected, the measured concentration of deuterium in plasma, and the predictive models and approach described in McWilliams and Whitman (2013). Briefly, we microdistilled the water from each flame-sealed blood sample and used infrared spectrophotometry to measure deuterium concentration (Karasov et al. 1988). Deuterium concentration was measured at the University of Rhode Island using a FT-IR Spectrometer with a Universal ATR Sampling Accessory, with Spectrum and Spectrum Quant software (PerkinElmer, Waltham, MA, USA). We verified the mass of D_2O injected into birds (0.055 g) by weighing capillary tubes of the same injection volume, randomly selected from samples taken in the field. We then used this injection mass, the molar masses of D_2O (20 g/mol) and unlabeled H_2O (18 g/mol), and the deuterium enrichment of microdistilled blood water as determined by spectrophotometry to calculate estimated water space (Karasov and Pinshow 1998; McWilliams and Whitman 2013). We excluded from the dataset any values of estimated water space that were either <50 or >80 % of body mass, given that these values are biologically unreasonable (McWilliams and Whitman 2013). In total, we excluded 14 samples; ten of 11 exclusions among non-watered birds, and all three exclusions among watered birds, were >80 % of body mass. We used the interspecific predictive model presented in McWilliams and Whitman (2013) to estimate fat mass (g) given estimated water space and measured body mass.

Statistical analyses

All statistical analyses were performed with SAS 9.4 software (SAS Institute 2014). We set statistical significance at $\alpha = 0.05$.

We used correlation analysis to compare estimated fat mass based on the deuterium dilution method with fat scores, both recorded on the morning after all-night captivity, to determine if deprivation of drinking water for $\geq$12 h affected the utility of the deuterium dilution method.

To determine if Garden Warblers that were not provided with water preferentially catabolized protein overnight to release metabolic water, we performed repeated-measures (RM) ANOVA, comparing flight muscle mass pre- and post-overnight captivity (variable = Time), and tested the interaction between water treatment (Water) and time (Water × Time). We also performed RMANOVA with fat score and body mass pre- and post-overnight captivity. We tested residuals for normality and homogenous variance, to ensure satisfaction of model assumptions.

We also used correlation analysis, after Fusani et al. (2009, 2011), to assess the relationship between Zugunruhe and several indicators of body condition (fat score, body mass, flight muscle mass, and muscle score), separately for birds that were or were not provided with water, for both the “early-night” Zugunruhe period and for “all-night.” We used non-parametric Spearman coefficients for all correlation analyses, given that variables of interest were not all normally distributed, even when transformed. In addition, we used ANCOVA to test the contribution of water treatment (categorical variable), condition parameters (covariate = fat score or body mass), and their interaction (Water × Covariate) to “early-night” Zugunruhe behavior. The interaction test indicated whether the slope of the relationship between Zugunruhe and the condition differed with water treatment. We tested residuals for normality and homogenous variance, to ensure satisfaction of model assumptions.

Results

Evaluation of the deuterium dilution method for estimating fat mass

Among Garden Warblers that were provided with water throughout the experiment, fat mass estimated by the deuterium dilution method was strongly correlated with the visible fat score the morning after all-night captivity ($n = 29$, $r_S = 0.73$, $p < 0.0001$), whereas a positive although not significant correlation existed among birds that were not provided with water ($n = 17$, $r_S = 0.45$, $p = 0.073$; Fig. 1). Furthermore, the deuterium dilution method resulted in low deuterium concentrations as measured in microdistilled blood water, and, consequently, there were biased-high water space estimates and biased-

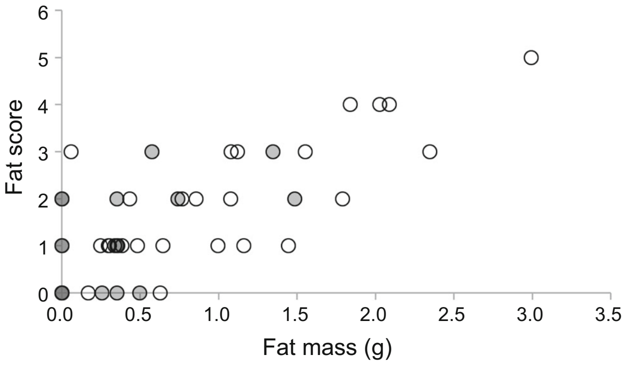

Fig. 1 Fat mass estimated by deuterium dilution and fat score estimated visually were strongly correlated among Garden Warblers provided with water (*open circles*) but only weakly correlated among birds that were not provided with water (*filled circles*)

low fat mass estimates in ten of 29 water-deprived birds as opposed to only three of 32 birds that were provided water. The difference in number of exclusions between treatment groups indicates that non-watered birds were dehydrated whereas watered birds drank the water provided and so were normally hydrated. The lack of significant correlation for non-watered birds is likely related to these many exclusions along with, by chance, several fewer fat birds assigned to the water-deprived group.

Overnight changes in flight muscle, fat score, and body mass

Flight muscle mass decreased overnight for all birds, regardless of water treatment (RMANOVA; Time $F_{1,59} = 134.82$, $p < 0.0001$; Water $F_{1,59} = 1.41$, $p = 0.240$; Water × Time $F_{1,59} = 0.11, p = 0.743$; Fig. 2). Similarly, overall body mass and fat score decreased overnight for all birds, regardless of water treatment (RMANOVA; body mass, Time $F_{1,59} = 611.37$, $p < 0.0001$, Water $F_{1,59} = 0.39$, $p = 0.537$, Water × Time $F_{1,59} = 0.07$, $p = 0.789$; fat score, Time $F_{1,59} = 161.44$, $p < 0.0001$, Water $F_{1,59} = 2.33$, $p = 0.133$, Water × Time $F_{1,59} = 0.65$, $p = 0.422$).

Condition dependency of early-night Zugunruhe (civil sundown to midnight)

During the early night, fat score and body mass at capture were strongly and positively correlated with Zugunruhe among all birds, whether or not they were provided with water, while flight muscle mass was uncorrelated, and muscle score was only weakly correlated with Zugunruhe in both groups (Table 1). Non-watered birds with fat scores of 5 at capture displayed higher Zugunruhe activities than all birds that were provided with water (Fig. 3), despite having average levels of flight muscle mass at capture (Fig. 4). ANCOVA confirmed this condition dependency of Zugunruhe, given that the fat score covariate was statistically significant and interacted with the water treatment (ANCOVA; Water $F_{1,57} = 1.33$, $p = 0.25$; Fat score $F_{1,57} = 23.71, p < 0.0001$; Water × Fat score $F_{1,57} = 4.29$, $p = 0.04$). Therefore, the slope of the relationship between fat score and Zugunruhe differed between watered and non-watered birds. ANCOVA also confirmed that body mass at capture correlated with Zugunruhe, but the body mass covariate did not significantly interact with the water treatment (ANCOVA; Water $F_{1,57} = 2.95$, $p = 0.09$; Body mass $F_{1,57} = 9.02$, $p = 0.004$; Water × Body mass $F_{1,57} = 3.30, p = 0.07$).

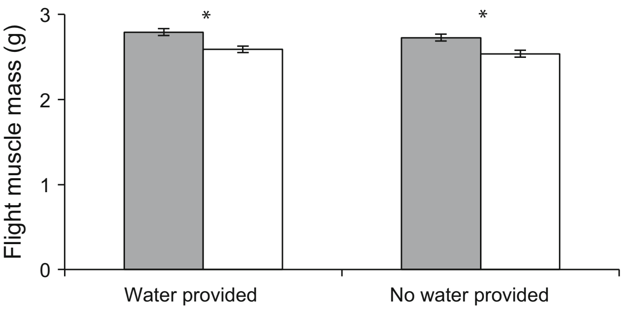

Fig. 2 Values of flight muscle mass (g, LS means ± SE) for Garden Warblers decreased from at capture (*filled bars*) to after an overnight fast (*open bars*) for birds provided water as well as birds not provided water

Table 1 Spearman correlations between two intervals of Zugunruhe and several measures of body condition at capture for Garden Warblers that did not, or did, receive water

	No water provided			Water provided		
	n	*r*	*p* value	*n*	*r*	*p* value
Early-night Zugunruhe						
Body mass	**29**	**0.500**	**0.006**	**32**	**0.358**	**0.044**
Fat score	**29**	**0.495**	**0.006**	**32**	**0.604**	**<0.001**
Muscle score	29	0.350	0.063	**32**	**0.362**	**0.042**
Flight muscle mass[a]	29	−0.054	0.781	32	0.170	0.352
All-night Zugunruhe						
Body mass	**29**	**0.388**	**0.037**	32	0.275	0.128
Fat score	29	0.325	0.086	**32**	**0.559**	**0.001**
Muscle score	29	0.284	0.136	**32**	**0.391**	**0.027**
Flight muscle mass[a]	29	0.028	0.883	32	0.161	0.378

Correlations with $p < 0.05$ are in bold

[a] Calculated from muscle shape, body mass, and tarsus length at capture, after Bauchinger et al. (2011). Equation used: flight muscle mass = −1.212 + (0.293 × muscle shape) + (0.045 × body mass) + (0.199 × tarsus length)

Condition dependency of all-night Zugunruhe (civil sundown to civil sunrise)

Body mass and fat score at capture were less strongly correlated with all-night Zugunruhe activity than they were with early-night activity (Table 1). Fat score no longer correlated with activity for birds that were not provided with water, and body mass no longer correlated with activity for birds that were provided with water. As during early-night Zugunruhe, flight muscle mass was uncorrelated with activity in both groups, and muscle score was only weakly correlated.

Discussion

Access to water affected condition dependency of Zugunruhe

Evidence from our experiment with Garden Warblers suggests that water availability during stopover affects the condition dependency of Zugunruhe, suggesting that free

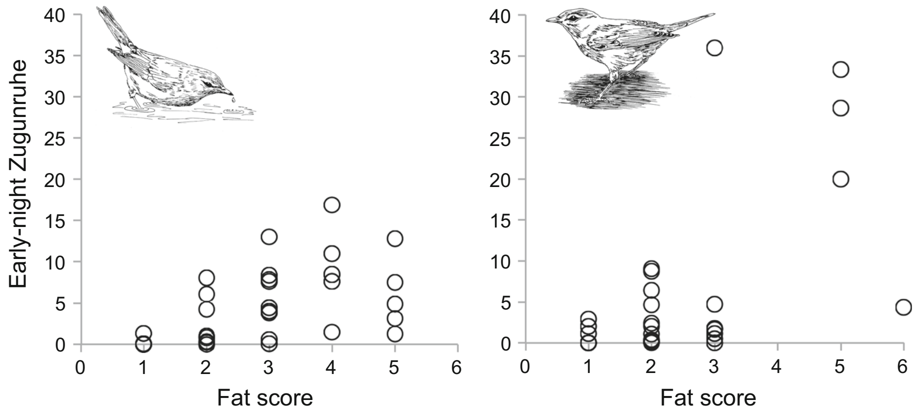

Fig. 3 Scatterplots between fat score at capture and early-night Zugunruhe for Garden Warblers that received water ($n = 32$, *left panel*) versus those that did not receive water ($n = 29$, *right panel*)

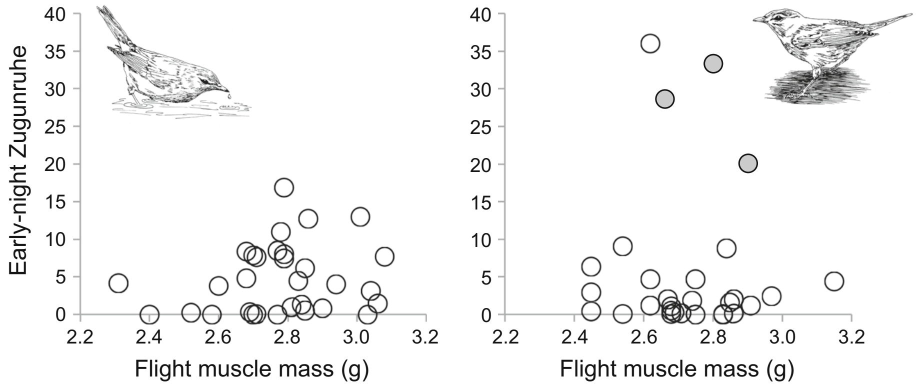

Fig. 4 Scatterplots between flight muscle mass at capture and early-night Zugunruhe for Garden Warblers that received water ($n = 32$, *left panel*) versus those that did not receive water ($n = 29$, *right panel*, with fat scores of 5 *shaded*)

water is important to the behavioral choices of birds after sustained flights. First, we found that the slope of the relationship between fat stores and migratory restlessness differed when birds were or were not provided with drinking water. Previous work by Fusani et al. (2009, 2011) on Ponza showed that energy stores affect migratory restlessness in Garden Warblers and other passerine migrants stopping over on the island. Our study adds that birds having just crossed the Mediterranean Sea are perhaps even more likely to leave the island with large energy stores when they do not encounter free drinking water. Specifically, water-deprived birds with high fat scores in our experiment had higher levels of early-night Zugunruhe than birds with a similar fat score but which had access to drinking water. Second, in a study conducted exclusively with birds provided water, Fusani et al. (2009) found that fat score at capture was the variable most closely correlated with all-night Zugunruhe in Garden Warblers stopping over on Ponza. Interestingly, the relationship was significant in our study only when birds were provided with water (Table 1). Indeed, we found the condition dependency of Zugunruhe to be strongest overall when considered during the early-night period, unsurprising given that birds are most likely to depart a stopover site shortly after sunset (Biebach et al. 2000; Goymann et al. 2010). In short, if an animal cannot find the resource(s) necessary to increase its body condition, it must continue migration as soon as possible (Biebach 1985; Gwinner et al. 1988; Newton 2008).

The "water hypothesis" posits that birds during migratory flight without access to free water catabolize protein to gain water (Klaassen 1996; Bauchinger and Biebach 1998; Jenni and Jenni-Eiermann 1998; Gerson and Guglielmo 2011) and the primary sources of this tissue protein are likely the splanchnic organs and pectoral muscle (see Mizrahy et al. 2011). In our experiment, both treatment groups lost equivalent flight muscle mass overnight, suggesting that water-deprived birds on stopover did not preferentially catabolize this tissue to produce metabolic water. Given the much higher protein turnover rates of splanchnic organs compared to pectoral muscle in songbirds (Bauchinger and McWilliams 2010), catabolism of the digestive tract during migratory flights may have provided Garden Warblers in our study an important source of metabolic water under dehydrating conditions, as also indicated by recent studies of Blackcaps (*Sylvia atricapilla*; Mizrahy et al. 2011). Our results suggest that the strategy of water-deprived birds may be to conserve pectoral mass as if they had access to drinking water, and increase departure likelihood in search of free water elsewhere as long as they also have sufficient fat stores. Indeed, flight muscle mass at capture—a more integrated and quantitative measure of muscle size than muscle score—apparently did not influence the level of Zugunruhe for either water-deprived or watered birds.

There are many physiological reasons why a migrating bird with adequate energy stores should be eager to leave a stopover site that does not offer free water and food, and stay at one that does. Drinking water on stopover is important to rebuilding digestive capacity, gaining lean and fat mass, and maintaining adequate water intake when choosing high-energy, low-water foods (Sapir et al. 2004; Tsurim et al. 2008; Mizrahy et al. 2011). Essentially, birds require preformed water (e.g., drinking water or water in food) to refuel adequately on stopover, and having access to that water may even contribute to the pace of their migration. Both captive studies and those with free-living birds have shown that migrating passerines with access to water exhibit higher fuel deposition rates than individuals without water through several mechanisms: free water hastens the rebuilding of a digestive tract that was catabolized during fasting and long-distance flights (Mizrahy et al. 2011), and the birds' dietary choices are in part constrained by the water content of available food items (Sapir et al. 2004; Tsurim et al. 2008). Our experiment uniquely complements these migratory-stopover feeding studies by showing that the availability of drinking water also affects the condition dependency of Zugunruhe behavior. A bird without access to drinking water at a stopover site may be expected to refuel at a slow rate, in the absence of foods with high water content, and, therefore, would benefit from choosing to leave that site, if able. Fuel deposition rates are critical to the pace of migration and distribution of migratory birds at stopover sites, particularly in spring when birds maximize fuel deposition to minimize migration time to breeding grounds (Hedenström and Alerstam 1997; Schaub and Jenni 2000). Therefore, free water's facilitation of fueling should be an important aspect of stopover site selection.

We expect the relevance of water availability to stopover decisions to be greatest in arid conditions and near ecological barriers (Fogden 1972; Carmi et al. 1992; Leberg et al. 1996). Given that migrants crossing ecological barriers may be water-limited rather than fat-limited (e.g., Leberg et al. 1996), recouping or preventing further water losses should be a focal concern at subsequent stopovers. Choosing stopover sites based on water availability likely falls among the many behavioral strategies that birds adopt to avoid dehydration during desert crossings, e.g., landing in oases, seeking microhabitats with shade, avoiding daytime flight, and choosing flight altitudes that minimize evaporative loss (Biebach 1985; Carmi et al. 1992; Klaassen 1996, 2004).

Using deuterium to estimate body composition of warblers: the problem with dehydration

We used the deuterium dilution method to estimate fat mass of Garden Warblers in addition to measuring the subcutaneous fat score. We expected the two variables to consistently correlate strongly with each other, but instead found that the correlation between estimated fat mass and fat score was strong only among birds that had access to drinking water. Furthermore, the deuterium injection method resulted in low deuterium concentrations as measured in microdistilled blood water, and consequently biased-high water space estimates and biased-low fat mass estimates in ten of 29 water-deprived birds as opposed to only three of 32 birds that were provided water. We suggest the following potential mechanism as the most likely to explain why measurement of fat mass via the deuterium dilution method was ineffective for dehydrated individuals: the deuterium we injected into the birds may not have distributed evenly between blood and tissue in dehydrated individuals. Songbirds, and birds of other orders, that have lost water mass via flight or heat stress continue to maintain high plasma volume despite dehydration of other tissues (Dawson et al. 1983; Carmi et al. 1993). The D_2O we injected into water-deprived animals may have been preferentially absorbed and retained by dehydrated muscle or other tissues, over the plasma, which we sampled. This unequal distribution among sub-pools of the total body-water pool (Lifson and McClintock 1966; Speakman 1997), and failure to equilibrate regardless of adequate time, could account for the low concentration of deuterium that we observed in circulation. We, therefore, caution researchers against using the deuterium dilution method in dehydrated individuals.

Conclusions and future directions

Our data suggest that availability of drinking water after sustained flight over an ecological barrier is an important determinant of departure likelihood from a stopover site. Our experiment supports the notion that fat stores affect Zugunruhe levels in migrating songbirds, but adds that access to drinking water changes the condition dependency of migratory restlessness. Water-deprived birds displayed particularly high nocturnal activity levels when they also had high fat scores. These results suggest that access to drinking water, in addition to energy stores, may significantly impact a migrating bird's decision to depart a stopover site if they are dehydrated. Birds crossing the Mediterranean Sea likely arrive at the volcanic island of Ponza in a dehydrated state, and, consequently, individuals with large fat scores may be expected, regardless of muscle size, to depart this relatively dry site more readily than they would leave a site with more freely available water.

We recommend that future studies take advantage of techniques such as quantitative magnetic resonance and radio-telemetry to establish the physiological mechanisms and prevalence of water-based stopover decisions in free-living birds. Magnetic resonance can be used to quantify the total body water as well as lean and fat mass of free-living songbirds with high accuracy (Guglielmo et al. 2011; McWilliams and Whitman 2013). Researchers might adopt a similar approach to that of Smith and McWilliams (2014) by directly manipulating and quantifying the total body water (as well as lean and fat mass) of birds on stopover and subsequently monitoring their movements and departure dates subsequent to release. Provision of water troughs on the landscape (after Sapir et al. 2004) in concert with this monitoring might further help elucidate the water-based choices of migrants on stopover.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals and animal samples were followed.

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