

# How salinity and temperature combine to affect physiological state and performance in red knots with contrasting non-breeding environments

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**Abstract** Migratory shorebirds inhabit environments that may yield contrasting salinity-temperature regimes—with widely varying osmoregulatory demands, even within a given species—and the question is: by which physiological means and at which organisational level do they show adjustments with respect to these demands? Red knots *Calidris canutus* winter in coastal areas over a range of latitudes. The nominal subspecies winters in salty areas in the tropics, whereas the subspecies *Calidris canutus islandica* winters in north-temperate regions of comparatively lower salinities and temperatures. In this study, both subspecies of red knot were acclimated to different salinity (28/40 ‰)–temperature (5/35 °C) combinations for 2-week periods. We then measured food/salt intakes, basal metabolic rate (BMR), body mass and temperature, fat and salt gland scores, gizzard mass, heat-shock proteins, heterophils/lymphocytes (H/L) ratio and plasma Na<sup>+</sup> to assess

the responses of each taxon to osmoregulatory challenges. High salinity (HS)-warm-acclimated birds reduced food/salt intake, BMR, body mass, fat score and gizzard mass, showing that salt/heat loads constrained energy acquisition rates. Higher salt gland scores in saltier treatments indicated that its size was adjusted to higher osmoregulatory demands. Elevated plasma Na<sup>+</sup> and H/L ratio in high-salinity-warm-acclimated birds indicated that salt/heat loads might have a direct effect on the water-salt balance and stress responses of red knots. Subspecies had little or no effect on most measured parameters, suggesting that most adjustments reflect phenotypic flexibility rather than subspecific adaptations. Our results demonstrate how salinity and temperature affect various phenotypic traits in a migrant shorebird, highlighting the importance of considering these factors jointly when evaluating the environmental tolerances of air-breathing marine taxa.

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## Introduction

Osmotic homeostasis, i.e. the maintenance of a relatively constant salt and water balance in the body fluids, is one of the best-defended physiological states in vertebrates (Evans 2009; Bradley 2009). Amphibians, reptiles, birds, and mammals typically have blood concentrations of around 250–300 mOsm to ensure proper functioning of cells (Bradley 2009). The problem of maintaining the internal environment within fairly well-defined limits varies with the degree of gain or loss of salts and water as a result of environmental pressures (Phillips and Ensor 1972). Therefore, the ability to identify conditions when osmotic

homeostasis is not achieved, or is compromised in the face of conflicting ecological demands, could be a means to identify periods of environmental stress (Gutiérrez 2014).

In marine and other saline environments, organisms may face problems of water conservation because of high salt concentration. Moreover, these problems could be compounded by factors such as temperature, which additionally demands physiological and behavioural adjustments to maintain homeostasis in terms of salt and water balance (e.g. Phillips and Ensor 1972; Skadhauge 1981; Battley et al. 2003; Gutiérrez et al. 2012). In nature, salinity and temperature often vary in concert. In considering the potential impacts of climate-induced changes in these (and other) abiotic factors, it is important to understand their influence on the physiological performance of organisms and, ultimately, on fitness (e.g. Pörtner 2002; Bozinovic et al. 2011). Nevertheless, the combined effects of these abiotic factors have been largely overlooked in aquatic air-breathing vertebrates. By using large-scale biogeographical analyses and environmental tolerances, Brischoux et al. (2012, 2013a) showed that salinity and temperature influence the distribution and dispersal of marine snakes (*Laticauda* spp.), with the species with the more efficient salt glands and lower dehydration rates exploiting more saline oceanic areas.

This may also apply to shorebirds (Charadriiformes, suborders Charadrii and Scolopaci) undertaking seasonal migrations between the far north and further south (e.g. van de Kam et al. 2004), during which they are subject to large changes in the salinity and temperature of their experienced environments. Outside the breeding season, the species that opt to winter in coastal habitats (Piersma 1997) feed on marine invertebrates [typically isosmotic to surrounding seawater (Prusch 1983)] and have limited or no access to freshwater (Gutiérrez 2014). To cope with salt loads, shorebirds—like marine birds—use renal and extrarenal mechanisms which jointly ensure the excretion of excess salt without a net loss of water (Peaker and Linzell 1975; Goldstein and Skadhauge 2000; Gutiérrez 2014). The phenotypic flexibility in osmoregulatory structures (e.g. supraorbital salt glands) is critically important in allowing migratory shorebirds to successfully overcome the osmoregulatory challenges faced in the course of their annual cycles (Gutiérrez et al. 2012). Nevertheless, little is known about the physiological and ecological consequences of living in saline environments and the situations in which osmotic homeostasis is compromised (Gutiérrez 2014).

For example, it has been shown that two shorebird species (red knots *Calidris canutus* and sanderlings *Calidris alba*) wintering at Banc d'Arguin in Mauritania, a coastal wetland on the boundary between the Sahara and the Atlantic Ocean, reduced their food intake when switched from

fresh to seawater under experimental conditions, suggesting that salinity may constrain food intake and thus energy acquisition (Klaassen and Ens 1990). Indeed, recent work on red knots has shown that individuals maintained on seawater have lower food and water consumption rates than those having freshwater available (Oudman et al. 2014). In addition, there is evidence that an increase in salinity substantially increases the basal metabolic rate (BMR) of dunlins *Calidris alpina*, which demonstrates that osmoregulation entails significant energetic costs (Gutiérrez et al. 2011; see also Nelhs 1996 and Peña-Villalobos et al. 2013). Other studies have also shown that shorebirds can suffer adverse effects when living in saline environments (e.g. Purdue and Haines 1977; Hannam et al. 2003; Gutiérrez et al. 2013). However, salinity is not the only factor challenging osmotic homeostasis. By affecting physiological routes of heat/water loss, temperature also exerts a considerable influence on the osmoregulatory performance of birds (e.g. Phillips and Ensor 1972; Skadhauge 1981; Verboven and Piersma 1995; Gutiérrez et al. 2012). At similar salinities, the salt gland mass of red knots increases at both low and high temperatures, which probably reflects increased energy demands at low temperatures (i.e. higher food/salt intake) and elevated water loss at high temperatures (Gutiérrez et al. 2012). Taken together, these results reveal that changes in both salinity and temperature can have significant effects on shorebirds' physiological state and performance.

Long-distance migrant shorebirds breeding in the high Arctic and wintering along tropical coasts may encounter a greater range of conditions (e.g. in terms of salinity and temperature) compared with temperate-wintering birds. An interesting contrast in this respect is provided by two subspecies of red knots: *Calidris canutus islandica* breeds in high arctic Canada and winters in temperate coasts of western Europe, whereas *Calidris canutus canutus* breeds in high arctic Siberia and winters in hot and salty (sub)tropical coasts in West and South Africa (Piersma et al. 2005; Piersma 2007; Piersma and van Gils 2011). In the western Wadden Sea, the main wintering site for *C. canutus islandica*, the average salinity is typically about 28 ‰ (based on daily water salinity recorded throughout this experiment) and winter mean air temperature during the coldest month (February) is about 3 °C [based on monthly ambient temperature recorded over 29 years (see Vézina et al. 2006)]. At the Banc d'Arguin, the main wintering site for *C. canutus canutus*, the average salinity is about 40 ‰ [with values over 50 ‰ in some areas where strong evaporation occurs (Wolff and Smit 1990; Lavaud et al. 2013)] and the average air temperature is around 25 °C [with values over 40 °C during the daylight period (Wolff and Smit 1990)]. Because their osmoregulatory and thermoregulatory demands during the winter must differ widely, birds of these subspecies

might either show ecophysiological adaptations at the level of the subspecies, or ‘just’ show general flexible responses to environmental conditions (Piersma et al. 1996).

In this study, we exposed these two red knot subspecies, under controlled captive conditions, to different salinity–temperature combinations: high salinity (HS)–warm (40 ‰, 35 °C); HS–cold (40 ‰, 5 °C); LS–warm (28 ‰, 35 °C); and LS–cold (28 ‰, 5 °C). Such combinations can be encountered in the wild and should elicit different osmoregulatory (e.g. Gutiérrez et al. 2011, 2012) and thermoregulatory (e.g. Vézina et al. 2006) responses. To capture a broad picture of the physiological state and performance, we measured a suite of physiological and morphological parameters:

1. Food intake has profound implications for the relative intakes of water and salts, and thus, for an animal’s osmoregulatory responses (reviewed by Gutiérrez 2014).
2. The sizes of the gizzard and the salt glands are indicators of relative food intake (van Gils et al. 2003, 2005a, c) and concentrating ability (Staaland 1967; Gutiérrez et al. 2012), respectively.
3. Data on BMR, fat stores, body mass and temperature provide valuable information on an animal’s nutritional and energetic state (Butler and Woakes 2001; McNab 2002); also, changes in BMR may be linked to organ size because organs have high metabolic activities relative to other body structures (Hammond and Diamond 1997; Piersma et al. 2004).
4. Blood parameters such as levels of heat-shock proteins (HSPs; also known as ‘stress proteins’), leukocyte profiles [specifically the heterophil to lymphocyte (H/L) ratio], and natremia (plasma  $\text{Na}^+$  concentration) are recognized as good indicators of stress.

HSPs are molecular chaperones that play key roles in the maintenance of cellular homeostasis under variable environmental conditions (reviewed by Sørensen et al. 2003). In particular HSP70 is considered to be the major HSP family in vertebrates and has been used as an index of a range of stressors including low/high temperatures and salinities (Sørensen et al. 2003). HSPs are not biased by handling stress and, therefore, they are an appropriate measure of short- and long-term stress (Herring and Gawlik 2007). The H/L ratio is positively related to the magnitude of the stressor and to the circulating glucocorticoids (reviewed by Davis et al. 2008; see also Müller et al. 2011). Natremia, an indicator of osmotic and ionic balance, should reflect the outcome of the osmotic challenges faced during the different salinity–temperature regimes (see Brisoux et al. 2013b, 2014).

We hypothesized that red knots will adjust physiological and morphological parameters as a function of their

osmoregulatory and thermoregulatory demands, and that those adjustments might vary within subspecies. On the basis of previous studies, we predict that adjustments (e.g. in BMR, food intake and body composition) would be more pronounced under more osmotically challenging conditions (i.e. HS-warm treatment). We also predict higher BMRs at HS (Gutiérrez et al. 2011) and cold (Vézina et al. 2006); reduced food intake, body mass and gizzard size (Vézina et al. 2006; Oudman et al. 2014), but enlarged salt glands (Gutiérrez et al. 2012), in birds exposed to HS–warm conditions. Second, if the physiological adjustments are partially fixed at the subspecies level, we would predict that tropically wintering *C. canutus canutus* deal with HS–warm better than temperate-wintering *C. canutus islandica*, i.e. they would regulate their natremia more precisely and show a weaker stress response (lower H/L ratio and HSP70 levels). Lastly, we would also predict that *C. canutus islandica* would show a weaker stress response under cold exposure than *C. canutus canutus*.

## Materials and methods

### Birds and housing

Twenty-one adult (non-moulting) red knots were used in this experiment. Of these, 11 individuals (five males, six females) belonged to the subspecies *C. canutus islandica* and ten individuals (seven males, three females) belonged to the subspecies *C. canutus canutus*. All birds were captured with mist nets in the western Dutch Wadden Sea (53°31′N, 6°23′E) in August–September 2013. Birds were aged according to plumage characteristics and sexed using molecular sexing (Baker et al. 1999). They were also assigned to one of the two subspecies co-occurring in the Wadden Sea during southward migration following Nebel et al. (2000). Afterwards, birds were brought into captivity at the Royal Netherlands Institute for Sea Research (NIOZ), Texel, the Netherlands, where they were housed in outdoor cages (4.5-m × 1.5-m surface × 2.5-m height) with unlimited access to water and trout pellets (see Vézina et al. 2006 for details). Each cage held four to six same-subspecies individuals throughout the experiment.

In January 2014, birds were randomly assigned to four indoor identical cages with the same dimensions as the outdoor cages. Then, the birds were fed a diet composed exclusively of 2 to 4 mm mud snails *Peringia ulvae* (formerly *Hydrobia ulvae*) collected by dredging in the Wadden Sea (as in Vézina et al. 2006). Mud snails were presented in two trays (60-cm × 40-cm surface × 5-cm height) with running seawater. This gastropod species is one of the main prey for red knots wintering in the Wadden Sea (van Gils et al. 2003, 2005b) and staging along southern European

coasts during migration (Moreira 1994). Because this prey is isosmotic to seawater (Todd 1964), red knots inevitably consume salt when they ingest them whole. After 2 weeks of acclimation to mud snails, which ensured that red knots had enough time to adjust to a diet of hard-shelled mollusc prey (Piersma et al. 1993), the four groups were exposed to four salinity-temperature combinations: HS–warm (40 ‰, 35 °C), HS–cold (40 ‰, 5 °C), LS–warm (28 ‰, 35 °C), and LS–cold (40 ‰, 5 °C). LS seawater was pumped directly from the sea, whereas HS levels were achieved by adding pure (99.9 %) refined salt (NaCl) to LS seawater. Each treatment lasted 2 weeks. Throughout, birds were held under a 12-h light:12-h dark photoperiod with no access to freshwater.

### Experimental protocol

We employed a staggered design because it was logistically impossible to measure all the physiological parameters in all groups and individuals simultaneously. The choice of a 2-day time lag among groups (i.e. 6-day time lag between the first and the last group) allowed us to do so. On day 12, starting at 1000 hours, we began daily food-intake measurements, which were carried out over 2 consecutive days. On the last day of each treatment (days 14/15 starting at 1000 hours) we collected two to three birds from one cage for blood sampling, salt gland scoring and ultrasound measurements (see below). We then placed the birds in individual holding cages (0.60-m × 0.40-m surface × 0.30-m height) with access to saltwater (salinity of the corresponding treatment) but no food for about 5 h to create a post-absorptive condition prior the onset of the BMR measurements (see below). Prior to metabolic measurements, birds were weighed (to the nearest 0.1 g) and scored for the extent of their subcutaneous fat stores using a semiquantitative scale for shorebirds [0–7, with 7 being the fattest (Meissner 2009)]. We then measured BMR over a period lasting 17 h starting at 1600 hours. At the end of the metabolic trials temperature was monitored with an electronic temperature recorder (Omega 450 ATT; Stanford, CT) attached to a copper constantan thermocouple that was inserted 1.5 cm into the bird's cloaca. Afterwards, we measured body mass for the second time and moved the birds back to the cages to start the following treatment. A random treatment sequence was used. Water salinity and temperature were measured daily with a portable multi-parameter instrument (HD2156.1; Delta Ohm, Benelux). The experimental timeline is shown in Table 1.

### Food and salt intake

We measured overall food intake in all the groups over 2 consecutive days in each treatment (as in Vézina et al.

**Table 1** Experimental timeline designed to examine the effects of salinity and temperature on various physiological traits. The same procedure was repeated for the different salinity-temperature combinations using a 2-day time lag among groups

Time (hours)	Days 1–12	Day 13	Day 14	Day 15	Day 16
0800	Lights on	Lights on	Lights on	Lights on	Lights on
0900	–	–	–	Birds removed from the respirometer, measured for body temperature, weighed, and returned to their cage	Birds removed from the respirometer, measured for body temperature, weighed, and returned to their cage
0930	Temperature and salinity recordings	Temperature and salinity recordings	Temperature and salinity recordings	Temperature and salinity recordings	Temperature and salinity recordings
1000	Placement and withdrawal of food trays	Placement and withdrawal of food trays (FIR 1)	Placement and withdrawal of food trays (FIR 2)	Placement and withdrawal of food trays Two to three remaining birds sampled and fasted for BMR measurements	Placement and withdrawal of food trays Two to three remaining birds sampled and fasted for BMR measurements
1530	–	–	Two to three birds blood sampled and fasted for basal metabolic rate (BMR) measurements	Previous birds weighed and salt gland/fat scored	–
1600	–	–	Previous birds placed in the respirometer	Previous birds placed in the respirometer	–
2000	Lights off	Lights off	Lights off	Lights off	Lights off

2006). Every morning we sieved freshly thawed mud snails to remove all visible water and then took three subsamples (ca. 30 g) of food from this stock. Then we gave a pre-weighed amount of food from the same stock to the birds in a tray containing running saltwater (salinity depending on the treatment) at precisely 1000 hours. The following day, the food trays were removed from the cages at 1000 hours and the remaining food was carefully sieved to remove the water, and weighed again. The subsamples and leftover food were then dried for several days to constant mass in an oven at 60 °C. Following this, the dried samples were burned at 560 °C in a furnace for 5 h to obtain ash mass. Data are presented as the ash-free dry mass (AFDM) of mud snails consumed per bird over 24 h.

We additionally estimated salt (NaCl) concentration of dead mud snails provided as food in both LS and HS treatments using a modified protocol of Mahoney and Jehl (1985) and Blakey et al. (2006). To do this, we repeatedly blotted the surface water from duplicate samples weighing about 1 g each until we felt we could get no more water out without crushing the mud snails. Each sample was then mashed using a mortar, mixed with 0.4 ml Na<sup>+</sup>-free water, and centrifuged for 10 min at 4000g to separate the supernatant solution. A subsample of 30 µl was digested in 30-ml Teflon vials containing 1 ml of concentrated ultrapure HNO<sub>3</sub> and heated at 125 °C in a Savillex hot block during 48 h. After digestion, 3 ml of ultrapure water (MilliQ) was added and 1 ml of subsample was diluted with 9 ml of 0.1 M ultrapure HNO<sub>3</sub>. An additional 10× dilution was necessary for determining Na<sup>+</sup> concentration by inductively coupled plasma mass spectrometry (ICP-MS; Thermo Scientific iCAP Q ICP-MS; Thermo Fisher Scientific, Bremen). Na<sup>+</sup> was then converted to salt intake assuming the 1:1.81 weight ratio of Na<sup>+</sup> and Cl<sup>-</sup> in seawater and osmoconforming marine invertebrates (e.g. Blakey et al. 2006). Salt intake rate (SIR; expressed as grams of salt consumed per bird over 24 h) was estimated for both LS and HS treatments by:

$$SIR = (WM_m \times BW_m \times S_m + WM_m \times AD_m \times S_t) \div 1000$$

where WM<sub>m</sub> is the wet mass of consumed mud snails (in grams), BW<sub>m</sub> is the body water fraction of wet mud snail samples (32.50 %), S<sub>m</sub> is the salinity of mud snails (25.26 and 37.95 g kg<sup>-1</sup> in LS and HS, respectively), AD<sub>m</sub> is the adherent water fraction on wet mud snail samples (26.91 %), and S<sub>t</sub> is the salinity of each treatment (27.04 and 39.80 g kg<sup>-1</sup> in LS and HS, respectively). Note that this is a conservative estimate of salt intake since we did not consider salt ingested by drinking. However, daily focal observations made from behind a one-way mirror suggest that salt intake by drinking was negligible.

## Blood sampling

Blood samples for leukocyte profiles, stress proteins and ions analyses were obtained by puncturing the alar vein using a 26-gauge needle and collecting blood (≈200 µl) into heparinized capillary tubes. For each individual and treatment, blood smears were obtained by transferring a small drop of blood from the capillary tube to a clean air-dried glass slide. The remaining blood was transferred to Eppendorf tubes which were centrifuged for 10 min at 4000g to separate plasma from cells and then stored at -20 °C until assay.

### Leukocyte profiles

Blood smears were sent to the European Veterinary Laboratory, Woerden, the Netherlands, and examined by one person who was blind to the treatments. The slides were air-dried, fixed in absolute methanol and then stained with Wright Giemsa. The first 200 white blood cells per slide were identified and counted as lymphocytes, heterophils, basophils, monocytes or eosinophils. We then calculated the H/L ratio. A subsample of smears (*n* = 20) were scanned twice, and repeatability analyses (details below) showed that there was moderately good agreement between these two scans within individuals [heterophils, *R* = 0.67, 95 % credible intervals (CI) 0.40–0.93, *P* < 0.001; lymphocytes, *R* = 0.56, 95 % CI 0.24–0.89, *P* < 0.001; H/L ratio, *R* = 0.61, 95 % CI 0.31–0.91, *P* < 0.001].

### Heat-shock proteins

We determined HSP70 in blood cells as in Herring et al. (2011) with some modifications. Red blood cells were washed three times with phosphate-buffered saline and after centrifugation the supernatant was aspirated from the final wash and mixed with ×1 extraction reagent and protease inhibitor cocktail (Sigma Aldrich, Spain). Samples were sonicated for 1 min and incubated on ice for 30 min. with occasional mixing. Then, samples were centrifuged again (10 min, 21,000g, 4 °C) and the supernatant was removed and used in posterior analyses. Cell lysates total protein concentration was determined by the Bradford method using bovine serum albumin as the standard. Concentrations of HSP70 were determined from the cell lysates by means of an enzyme-linked immunosorbent assay kit specific for inducible HSP70 (HSP70 EIA kit; Enzo Life Sciences, Lausen, Switzerland). All samples were run in duplicate and the means of duplicates were used in all analyses. All final HSP70 values were standardized by dividing HSP70 concentration by total protein to facilitate comparisons. Based on internal standards run in duplicate, the

mean intra- and inter-assay coefficients of variation were 4.1 and 6.9 %, respectively.

#### Plasma $\text{Na}^+$ concentrations

Plasma  $\text{Na}^+$  concentrations were determined using an electrolyte analyser with ion-specific electrodes (SPOTCHEM EL SE-1510; Menarini, Milan). Briefly, we allowed stored plasma samples and exclusive E-plates to reach room temperature (15–20 °C), stirred the samples without foaming, and then distributed subsamples (22  $\mu\text{l}$ ) on the E-plates before starting the automated measurement.

#### Basal metabolic rate

Measurements of basal rates of  $\text{O}_2$  consumption and  $\text{CO}_2$  production were taken from each individual at the end of each treatment using open-flow respirometry. For details about the respirometry set-up and BMR measurement protocol, see Piersma et al. (2004). Briefly, birds were measured overnight under post-absorptive digestive conditions in dark metabolic chambers at 21.0 °C ( $\pm 0.1$ ), i.e. within the red knot's thermoneutral zone (Piersma et al. 1995). BMR was defined as the lowest 10-min average of  $\text{O}_2$  consumption, which was calculated with the appropriate formulas for our set-up (Piersma et al. 2004).

#### Salt gland scores

We did not use ultrasonography for estimating salt gland size since the head is a uniformly heavily feathered area and the air in the feathers limits the use of such a technique (Dietz et al. 1999). Instead, we estimated salt gland size for each individual using a novel non-invasive approach (Gutiérrez 2014). We used sensory evaluation to score the thickness of the salt glands at the postorbital ridge. This consisted of sliding a finger across a smooth polyvinylchloride plate prepared with five increasing thicknesses (0–0.8 mm; artificially created by overlapping 0.16-mm-thick tape portions) at regular distances from each other, and then comparing these thicknesses with those of the postorbital salt gland ridge. Two trained observers (J. S. G. and A. S.-R.) blind to the treatments independently scored the salt gland ridge on an 11-point ordinal scale (0–5, including half-points). Repeatability analyses (details below) showed that there was good agreement between observers ( $R = 0.71$ – $0.78$ , always  $P < 0.001$ ), so we used the mean of the two scores for the subsequent analyses. Glandular tissue can vary in height and width but rarely in length (Staal and 1967; Siegel-Causey 1990); then, the thickness (i.e. height) of the saltglands at the postorbital ridge might be used a proxy of saltgland size.

#### Ultrasonography

Measurements of the gizzard dimensions (height and width) were done in duplicate by one observer (A. D.) with a Pie 200 ultrasound instrument with a 7.5-MHz linear probe (Pie Medical Benelux, BBV, Maastricht, the Netherlands) as described by Dietz et al. (1999). Prior to the experiment, calibration curves were made for the observer using 20 dead red knots with a wide range of gizzard sizes. As the repeatabilities between the first and the second measurements were high (gizzard height,  $R = 0.90$ – $0.96$ , always  $P < 0.001$ ; gizzard width,  $R = 0.73$ – $0.95$ , always  $P < 0.001$ ), we used the mean values for statistical analyses. The gizzard dimensions were converted to gizzard masses as follows:

$$\text{Gizzard mass (g)} = -1.09 + 3.78 \times (\text{gizzard height} \times \text{gizzard width}).$$

#### Statistical analyses

Analyses were performed using R version 3.1.0 statistical software (R Development Core Team). Throughout, we used  $P < 0.05$  as the level of significance. Data are presented as mean  $\pm$  SEM.

We fitted linear mixed-effects models [package nlme (Pinheiro et al. 2014)] with each parameter (food and salt intake, BMR, body mass, body temperature, fat and salt gland scores, gizzard mass, HSP70, H/L ratio and  $\text{Na}^+$ ) as the response variable; salinity, temperature and subspecies as fixed factors; and group and individual nested within group as random effects (except for food and salt intake where group values are reported). Low sample sizes did not allow a comparison by sex; however, earlier studies did not find sex-dependent differences in various physiological traits, including total body mass and fat stores (Piersma et al. 1999), stress levels (Reneerkens et al. 2002) and immune function (Buehler et al. 2009a, b). We always started with the full model and simplified it using backwards elimination based on ANOVA with  $P < 0.05$  as the selection criterion until reaching the minimal adequate model. Model assumptions were checked using the residuals of the final model. For those traits measured in duplicate for each salinity-temperature regime, we calculated ANOVA-based repeatabilities (the intraclass correlation coefficient) using the package rptR [rpt.aov function (Nakagawa and Schielzeth 2010)]. Three individuals (two *C. canutus canutus* and one *C. canutus islandica*) did not adjust to the combination of HS and high temperature (body mass below the critical threshold of 100 g) so they were returned to outdoor aviaries with unlimited access to freshwater and trout pellets and excluded from further analysis. The sample size for HSP70 differs from that for other response variables because the HSP70 concentration in 12

**Table 2** Statistics and coefficients of minimum adequate models for food and salt intake in two subspecies of red knot acclimated to different salinity-temperature combinations

Response variables	Predictors	Coefficients	SEM	df	t-value	P-value
Food intake (g AFDM)	<i>Intercept</i>	32.79	2.01	24	16.35	<0.001
	<i>Salinity<sup>a</sup></i>	0.00	2.55	24	0.00	0.999
	<i>Temperature<sup>b</sup></i>	-17.99	2.55	24	-7.06	<0.001
	<i>Subspecies<sup>c</sup></i>	-1.07	1.77	2	-0.61	0.606
	<i>Salinity × temperature</i>	13.23	3.54	24	3.74	0.001
Salt intake (g)	<i>Intercept</i>	8.67	0.37	24	23.71	<0.001
	<i>Salinity<sup>a</sup></i>	-2.68	0.37	24	-5.35	<0.001
	<i>Temperature<sup>b</sup></i>	-5.51	0.50	24	-11.01	<0.001
	<i>Salinity × temperature</i>	3.84	0.70	24	5.51	<0.001

Significant terms are depicted in *italic*

AFDM Ash-free dry mass

<sup>a</sup> Reference category is ‘high’

<sup>b</sup> Reference category is ‘cold’

<sup>c</sup> Reference category is ‘*Calidris canutus canutus*’

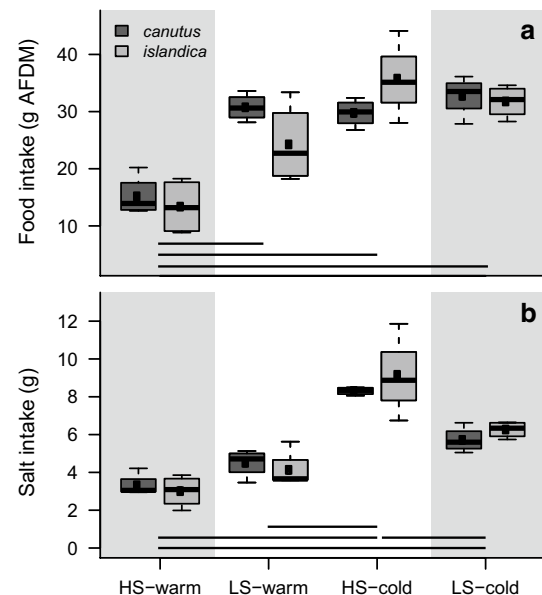
samples was below the detection threshold, independently of the treatment and subspecies. Three extreme outliers (>3 SD) for this response variable were considered as missing values.

## Results

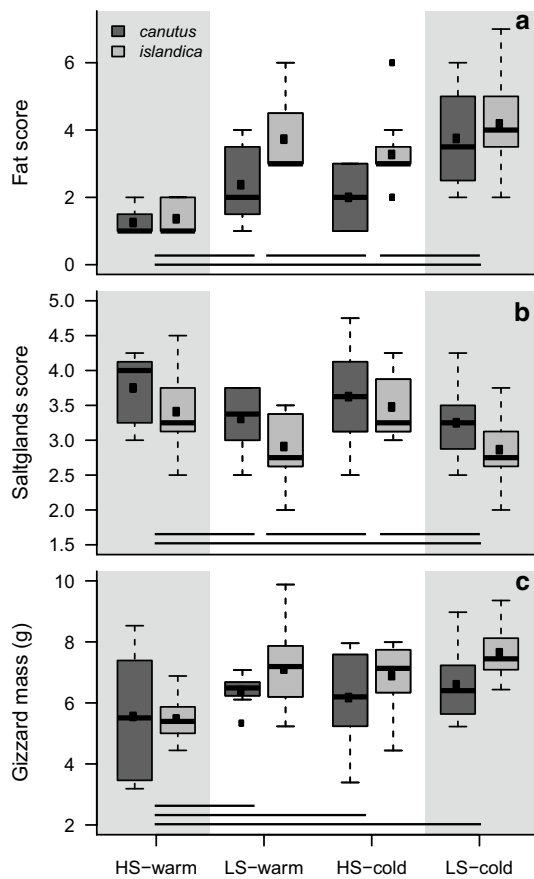
Baseline values, measured prior to the experimental conditions, did not differ significantly between subspecies in any of the 11 parameters measured (always  $P > 0.05$ ). Therefore, actual values for each subspecies were used in all analyses. Because BMR was significantly correlated with body mass (linear regression,  $F_{1,74} = 50.44$ ,  $P < 0.001$ ), we also used residuals of the body mass regressions for this variable in subsequent analyses.

### Responses in food and salt intake

Temperature affected food intake rate with birds acclimated to the cold eating on average 35 % more food ( $32.29 \pm 1.14$  g AFDM bird<sup>-1</sup> day<sup>-1</sup>) than individuals from the warm treatments ( $20.89 \pm 2.11$  g AFDM bird<sup>-1</sup> day<sup>-1</sup>), independently of salinity and subspecies (Table 2; Fig. 1a). We found no significant effect of salinity (Table 2), although birds of both subspecies substantially reduced food intake rates under HS-warm conditions (Fig. 1a). As a result, the interaction term salinity × temperature, but not subspecies, was highly significant (Table 2). Interestingly, the food intake rate of warm-acclimated birds was



**Fig. 1** Food (a) and salt (b) intakes in two red knot subspecies acclimated to different salinity-temperature combinations. *Box-and-whisker plots* give means estimated by the full model (*large filled square*), medians (*horizontal line within plot*), interquartile ranges (*box*), and ranges (*bars*). *Shaded areas* depict wintering salinity-temperature conditions potentially experienced by red knots in West Africa (*Calidris canutus canutus*) and Western Europe (*Calidris canutus islandica*), respectively. Group means that are statistically significantly different from each other (at the 5 % confidence level as indicated by post hoc Tukey tests) are connected by *horizontal bars* (subspecies did not differ within treatments in any case, so we did not include it in the tests). Note that these two variables represent group-specific measurements. *HS* High salinity, *LS* low salinity



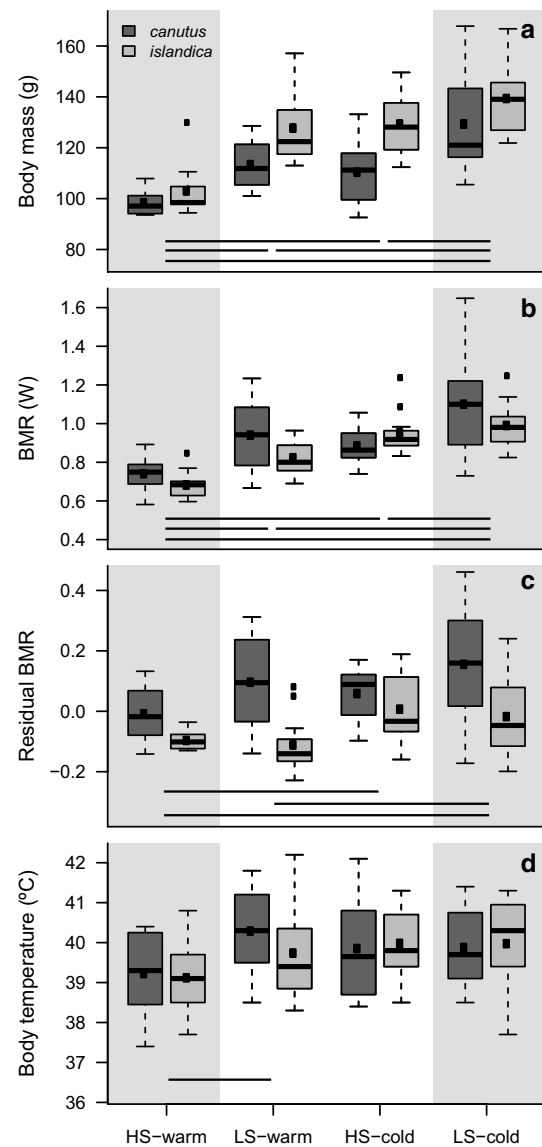
**Fig. 2** Fat score (a), salt-gland score (b), and gizzard mass (c) in two red knot subspecies acclimated to different salinity-temperature combinations. See Fig. 1 for more details and abbreviations

on average 55 % lower when they fed at elevated salinity ( $14.27 \pm 1.45$  g AFDM bird<sup>-1</sup> day<sup>-1</sup> for HS-warm and  $27.50 \pm 2.10$  g AFDM bird<sup>-1</sup> day<sup>-1</sup> for LS-warm).

Salinity, temperature and their interaction significantly affected SIR (Table 2). Birds exposed to HS-warm showed the lowest SIRs ( $3.16 \pm 0.24$  g NaCl bird<sup>-1</sup> day<sup>-1</sup>), whereas those exposed to HS-cold showed the highest SIRs ( $8.67 \pm 0.59$  g NaCl bird<sup>-1</sup> day<sup>-1</sup>) (Fig. 1b). Regardless of the treatment, both subspecies exhibited similar SIRs (subspecies,  $P = 0.704$ ; Fig. 1b).

### Responses in fat stores and digestive organs

Fat score and body mass were similarly affected by explanatory variables (Figs. 2a, 3a; Tables 3, 4, respectively), since they were highly correlated with each other (linear regression,  $F_{1,74} = 451.5$ ,  $P < 0.001$ , subspecies and treatments combined). For fat score, salinity and the three-way interaction (salinity  $\times$  temperature  $\times$  subspecies) were significant (Table 3). HS-acclimated birds had lower fat score,



**Fig. 3** Body mass (a), basal metabolic rate (BMR) (b), residual BMR (c), and body temperature (d) in two red knot subspecies acclimated to different salinity-temperature combinations. See Fig. 1 for more details and other abbreviations

especially under warm conditions (Fig. 2a). At HS-cold, *C. canutus canutus* exhibited lower fat scores than *C. canutus islandica* (Fig. 2a).

Salt gland score (an indicator of concentrating ability) was affected by salinity only (Table 2). Salt gland scores were higher under the HS conditions, regardless of temperature and subspecies (Fig. 2b; Table 3). Overall, salt gland scores were positively correlated with mass-corrected (i.e. residual) BMR (linear regression,  $F_{1,74} = 5.58$ ,  $P = 0.021$ ) and negatively correlated with body mass (linear regression,  $F_{1,74} = 11.87$ ,  $P < 0.001$ ). The variation in gizzard



**Table 3** Statistics and coefficients of minimum adequate models for morphological parameters measured in two subspecies of red knot acclimated to different salinity–temperature combinations

Response variables	Predictors	Coefficients	SEM	df	t-value	P-value
Fat score (0–7)	<i>Intercept</i>	2.00	0.44	51	5.05	<0.001
	<i>Salinity</i> <sup>a</sup>	1.75	0.44	51	4.01	<0.001
	Temperature <sup>b</sup>	−0.75	0.44	51	−1.72	0.091
	Subspecies <sup>c</sup>	1.27	0.52	2	2.44	0.135
	Salinity × temperature	−0.63	0.62	51	−1.01	0.315
	Salinity × subspecies	−0.84	0.57	51	−1.47	0.148
	Temperature × subspecies	−1.16	0.57	51	−2.02	0.048
	Salinity × temperature × subspecies	2.08	0.81	51	2.57	0.013
Salt gland score (0–5)	<i>Intercept</i>	3.71	0.16	55	23.84	<0.001
	<i>Salinity</i> <sup>a</sup>	−0.49	0.09	55	−5.73	<0.001
	Temperature <sup>b</sup>	0.03	0.09	55	0.38	0.704
	Subspecies <sup>c</sup>	−0.32	0.19	2	−1.70	0.232
Gizzard mass (g)	<i>Intercept</i>	6.07	0.29	55	20.65	<0.001
	<i>Salinity</i> <sup>a</sup>	0.96	0.28	55	3.40	0.001
	Temperature <sup>b</sup>	−0.74	0.28	55	−2.62	0.011
	Subspecies <sup>c</sup>	0.60	0.28	2	2.10	0.171

Significant terms are depicted in *italic*

<sup>a</sup> Reference category is ‘high’

<sup>b</sup> Reference category is ‘cold’

<sup>c</sup> Reference category is ‘*C. canutus canutus*’

**Table 4** Statistics and coefficients of minimum adequate models for physiological parameters measured in two subspecies of red knot acclimated to different salinity–temperature combinations

Response variables	Predictors	Coefficients	SEM	df	t-value	P-value
Body mass (g)	<i>Intercept</i>	112.65	4.02	55	28.01	<0.001
	<i>Salinity</i> <sup>a</sup>	17.22	2.28	55	7.55	<0.001
	Temperature <sup>b</sup>	−16.88	2.28	55	−7.39	<0.001
	Subspecies <sup>c</sup>	12.01	4.84	17	2.48	0.024
BMR (W)	<i>Intercept</i>	0.91	0.05	54	19.78	<0.001
	<i>Salinity</i> <sup>a</sup>	0.18	0.04	54	6.02	<0.001
	Temperature <sup>b</sup>	−0.22	0.03	54	−8.49	<0.001
	Subspecies <sup>c</sup>	0.00	0.06	2	0.08	0.945
	Salinity × subspecies	−0.12	0.05	54	−2.60	0.012
Residual BMR	Intercept	0.07	0.04	54	1.69	0.097
	<i>Salinity</i> <sup>a</sup>	0.10	0.03	54	3.98	<0.001
	Temperature <sup>b</sup>	−0.08	0.02	54	−5.11	<0.001
	Subspecies <sup>c</sup>	−0.07	0.05	2	−1.37	0.303
	Salinity × subspecies	−0.12	0.03	54	−3.63	<0.001
Body temperature (°C)	<i>Intercept</i>	39.79	0.30	55	132.13	<0.001
	Salinity <sup>a</sup>	0.41	0.22	55	1.85	0.070
	Temperature <sup>b</sup>	−0.36	0.22	55	−1.65	0.103
	Subspecies <sup>c</sup>	−0.11	0.34	2	−0.32	0.782

Significant terms are depicted in *italic*

<sup>a</sup> Reference category is ‘high’

<sup>b</sup> Reference category is ‘cold’

<sup>c</sup> Reference category is ‘*C. canutus canutus*’

mass (an indicator of relative food intake) was affected by salinity and temperature, but not subspecies (Table 3). HS–warm-acclimated birds showed the smallest gizzards

(5.51 ± 0.26 g), whereas LS–cold-acclimated birds showed the largest gizzards (7.20 ± 0.32 g) (Fig. 2c). Gizzard mass positively correlated with whole-organism BMR (linear

**Table 5** Statistics and coefficients of minimum adequate models for blood parameters measured in two subspecies of red knot acclimated to different salinity-temperature combinations

Response variables	Predictors	Coefficients	SEM	df	t-value	P-value
HSP70 (ng mg <sup>-1</sup> )	Intercept	0.28	0.16	40	1.78	0.082
	Salinity <sup>a</sup>	0.17	0.21	40	1.23	0.226
	Subspecies <sup>c</sup>	0.21	0.22	2	0.97	0.432
	<i>Salinity × subspecies</i>	<i>-0.43</i>	<i>0.19</i>	<i>40</i>	<i>-2.27</i>	<i>0.029</i>
H/L ratio	<i>Intercept</i>	<i>1.60</i>	<i>0.24</i>	<i>53</i>	<i>6.69</i>	<i>&lt;0.001</i>
	<i>Salinity<sup>a</sup></i>	<i>-0.54</i>	<i>0.21</i>	<i>53</i>	<i>-2.54</i>	<i>0.014</i>
	Temperature <sup>b</sup>	0.03	0.21	53	0.14	0.887
	Subspecies <sup>c</sup>	-1.10	0.33	2	-3.39	0.077
	<i>Salinity × subspecies</i>	<i>0.60</i>	<i>0.28</i>	<i>53</i>	<i>2.15</i>	<i>0.036</i>
	Temperature × subspecies	0.51	0.28	53	1.84	0.072
Na <sup>+</sup> (mmol L <sup>-1</sup> )	<i>Intercept</i>	<i>164.01</i>	<i>2.33</i>	<i>53</i>	<i>70.32</i>	<i>&lt;0.001</i>
	<i>Salinity<sup>a</sup></i>	<i>-7.56</i>	<i>3.23</i>	<i>53</i>	<i>-2.34</i>	<i>0.023</i>
	<i>Temperature<sup>b</sup></i>	<i>9.74</i>	<i>2.48</i>	<i>53</i>	<i>3.92</i>	<i>&lt;0.001</i>
	Subspecies <sup>c</sup>	-5.47	2.59	2	-2.11	0.170
	<i>Salinity × temperature</i>	<i>-9.26</i>	<i>3.51</i>	<i>53</i>	<i>-2.26</i>	<i>0.011</i>
	<i>Salinity × subspecies</i>	<i>11.96</i>	<i>3.56</i>	<i>53</i>	<i>3.36</i>	<i>0.001</i>

Significant terms are depicted in *italic*

HSP70 Heat-shock protein 70, H/L heterophils/lymphocytes

<sup>a</sup> Reference category is 'high'

<sup>b</sup> Reference category is 'cold'

<sup>c</sup> Reference category is '*C. canutus canutus*'

regression,  $F_{1,74} = 14.02$ ,  $P < 0.001$ ), but not with mass-corrected BMR (linear regression,  $F_{1,74} = 0.08$ ,  $P = 0.776$ ). We found no correlation between salt gland scores and gizzard mass either when data were pooled (linear regression,  $F_{1,74} = 3.14$ ,  $P = 0.081$ ) or when data were analysed for each of the treatments separately (always  $P > 0.123$ ).

### Responses in body mass, basal metabolism and temperature

Body mass decreased with salinity and temperature in both subspecies (Fig. 3a). Regardless of the salinity and temperature, *C. canutus islandica* overall exhibited higher body masses than *C. canutus canutus* (Fig. 3a; Table 4).

Both whole-organism and mass-corrected BMR were affected by salinity and temperature (Table 4). Overall, BMR tended to be higher in cold treatments (Table 4; Fig. 3b, c). The salinity × subspecies interaction was also significant for both whole-organism and residual BMR (Table 4), indicating that *C. canutus canutus* had higher levels under low salinities.

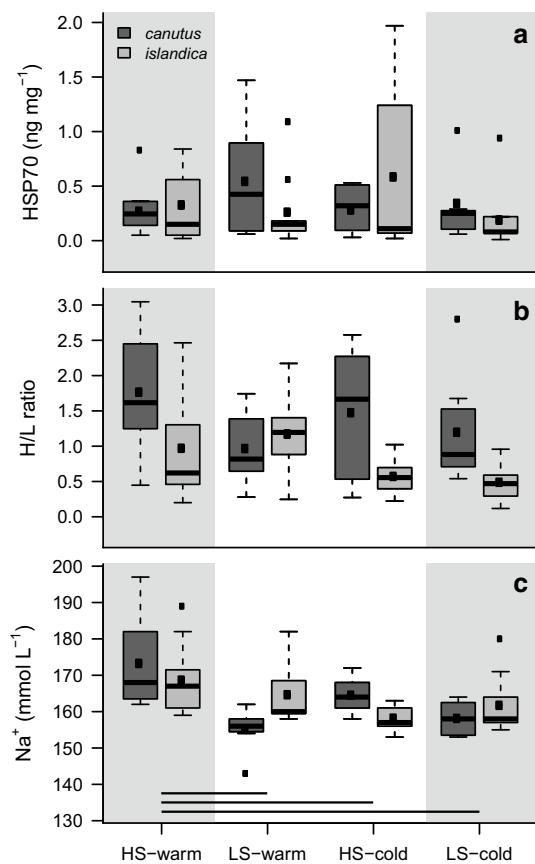
None of the explanatory variables significantly affected body temperature (Table 4; Fig. 3d). We found no relationship between body temperature and body mass or fat scores (linear regressions,  $F_{1,74} = 1.73$ ,  $P = 0.19$ ;  $F_{1,74} = 0.67$ ,  $P = 0.41$ , respectively). However, body temperature positively correlated with both whole-organism (linear

regression,  $F_{1,74} = 10.39$ ,  $P = 0.002$ ) and mass-corrected BMR (linear regression,  $F_{1,74} = 9.074$ ,  $P = 0.004$ ).

### Responses in blood parameters

We found no direct effect of temperature, salinity and subspecies on HSP70 (Table 5; Fig. 4a), although HS-exposed birds had somewhat higher levels of HSP70 ( $0.39 \pm 0.09$  ng mg<sup>-1</sup>) than LS-exposed ones ( $0.35 \pm 0.06$  ng mg<sup>-1</sup>). There was a significant interaction between salinity and subspecies such that *C. canutus islandica* had higher HSP70 levels than *C. canutus canutus* in HS but lower levels in LS (Table 5; Fig. 4a). The variation in H/L ratios was affected by salinity with HS-exposed knots having higher ratios than LS-exposed ones ( $1.12 \pm 0.13$  vs.  $0.93 \pm 0.10$ ) (Table 5; Fig. 4b). The significant interaction between salinity and subspecies indicated that *C. canutus canutus* had higher ratios in HS treatments (Table 5; Fig. 4b). H/L ratio and HSP70 levels were not significantly correlated (linear regression,  $F_{1,62} = 2.43$ ,  $P = 0.123$ ).

Salinity and its interaction with subspecies affected plasma Na<sup>+</sup> levels (Table 5). Plasma Na<sup>+</sup> increased with salinity, with HS-warm-acclimated birds showing the highest levels. Under such conditions, *C. canutus canutus* exhibited higher Na<sup>+</sup> levels ( $173.25 \pm 4.74$  mmol L<sup>-1</sup>) than *C. canutus islandica* knots ( $168.64 \pm 2.90$  mmol L<sup>-1</sup>)



**Fig. 4** Heat-shock protein 70 (*HSP70*) (a), heterophils/lymphocytes (*H/L*) ratio (b), and plasma  $\text{Na}^+$  (c) in two red knot subspecies acclimated to different salinity-temperature combinations. See Fig. 1 legend for more details and other abbreviations

(Fig. 4c).  $\text{Na}^+$  level was not correlated with *H/L* ratio (linear regression,  $F_{1,74} = 2.43$ ,  $P = 0.123$ ) or *HSP70* (linear regression,  $F_{1,74} = 0.04$ ,  $P = 0.85$ ).

## Discussion

In the present study we investigated the physiological adjustments to salinity and temperature in two red knot subspecies with contrasting non-breeding osmoregulatory demands. We found that the simultaneous demands of osmoregulation and thermoregulation yielded changes in energy acquisition and allocation as reflected in phenotypic flexibility of food and salt intake, basal metabolism, body composition and stress responses. Importantly, our results demonstrate that, regardless of the subspecies, the combination of relatively high salinities and temperatures (naturally encountered by shorebirds and other migratory waterbirds) limited the rate of food (energy) acquisition with direct and indirect consequences for the size of metabolically active organs. As a consequence, birds coping with

HS-warm conditions showed several significantly reduced physiological and condition-related traits (body mass, fat stores, gizzard mass and BMR) and augmented stress-related traits (*HSP70*, *H/L* ratio and plasma  $\text{Na}^+$ ).

It is well established that salt loads can have a direct negative effect on the physiology of birds that inhabit saline environments (Sabat 2000; Gutiérrez 2014). Previous studies have shown that salt intake can constrain food intake in captive red knots (Klaassen and Ens 1990; Oudman et al. 2014). In this study we found that red knots feeding on HS mud snails under warm conditions extensively reduced food and energy intake rate. Assuming that 40 % of *Peringia* AFDM constitutes indigestible ballast material (Quaintenne et al. 2011), an energy density of  $22 \text{ kJ g}^{-1}$  AFDM (Zwarts and Wanink 1993) and an assimilation efficiency of 80 % (Kersten and Piersma 1987), the metabolisable energy intake rate of HS-warm-exposed red knots was about  $136 \text{ kJ day}^{-1}$ , in contrast with the  $290 \text{ kJ day}^{-1}$  consumed by LS-warm-exposed ones. These crude estimates represent only a quarter to a half of the upper limit of daily metabolizable energy for a red knot feeding for 12 h  $\text{day}^{-1}$  [ $544 \text{ kJ day}^{-1}$  (van Gils et al. 2005a)], which suggests that managing salt and heat loads may pose a major osmoregulatory challenge for tropical-wintering shorebirds feeding on poor-quality molluscs. This could partly explain why red knots at tropical intertidal sites have lower fuelling rates than birds at higher latitudes (Piersma et al. 2005). Daily food intake, and hence fuelling rates, may be constrained by heat production due to external heat loads in the tropics (Verboven and Piersma 1995; Battley et al. 2003), but also by additional salt loads (Klaassen and Ens 1990; Verboven and Piersma 1995). Our results clearly show that fuelling rates are likely to be constrained by the combination thereof.

Likewise SIR is reduced in warm-acclimated birds, even at relatively low salinities. This suggests that in hyperosmotic environments food/salt intake is probably limited by evaporative water loss rather than by the total amount of ingested salt. That is, water loss could exceed salt gain resulting in water imbalance. Surprisingly, the direct salt intake in captive knots reached up to  $9 \text{ g day}^{-1}$  leading to a mass-specific salt intake of  $71 \text{ mg g}^{-1}$  body mass. This mass-specific salt intake is much higher than reported for other marine birds, including that of wintering common eiders *Somateria mollissima* feeding on whole-shelled bivalves [ $24 \text{ mg g}^{-1}$  (Nelhs 1996)]. Assuming the overall cost of salt turnover at  $1.3 \text{ kJ g}^{-1} \text{ NaCl}$  (Nelhs 1996), HS-cold-acclimated red knots would demand ca.  $11 \text{ kJ day}^{-1}$  to eliminate excess salt, which represents approximately 14 % of their BMR. Such a figure is comparable to the energetic cost of osmoregulation and other physiological demands in waterbirds and probably sufficient to trade-off with other physiological demands (Gutiérrez 2014).

Arguably the experimental conditions described here do not always very closely resemble conditions in the field. However, these results can be used as a proxy for comparative examinations of salt tolerance under different thermal environments. Although our experimental birds were acclimated to the different treatments over relatively short time periods, the fact that three individuals did not adjust to the HS–warm treatment and that they all continued to lose mass under such conditions suggests that we may have come close to their maximum salt tolerance. It is worthwhile to note that birds were exposed to a constant elevated temperature under windless aviary conditions which did not allow them to benefit from the lower night temperatures and the sea breeze they normally experience at tropical intertidal sites. In this sense, it is possible that shorebirds fuelling at the tropical sites avoid heat stress by feeding at night (Zwarts et al. 1990a), and by means of evaporative cooling and cutaneous evaporation (Battley et al. 2003).

The decrease in food intake was accompanied by a reduction in BMR when red knots were simultaneously exposed to HS and heat. This supports earlier findings that red knots downregulate BMR in response to heat acclimation/acclimatization over short periods (e.g. Piersma et al. 1995; Kersten and Piersma 1987; Vézina et al. 2006). However, BMR did not increase with salinity as previously shown in a closely related species (Gutiérrez et al. 2011), possibly because salinity and temperature generated opposite responses in basal metabolism (e.g. Vézina et al. 2006; Gutiérrez et al. 2011), making it impossible to disentangle the effects of the two factors. Nevertheless, birds with higher salt gland scores showed higher BMR after accounting for differences in body mass, indicating that when exposed to saltier conditions birds do pay an additional energetic cost for osmoregulation.

We found no substantial changes in body temperature throughout the experiment. A slight daily or seasonal decrease in body temperature (facultative hypothermia) is shown in many avian species in response to stressful and energy-limiting conditions [e.g. breeding, migratory fuelling, low food supply and low temperature (Butler and Woakes 2001; McKechnie and Lovegrove 2002)]. Although birds exposed to warmth and HS tended to have lower body temperature, it did not differ significantly among the four treatments. Nevertheless, this should be interpreted cautiously since our birds were measured at the end of the metabolic trials, during which they were resting overnight under thermally neutral conditions and thus were not directly exposed to the experimental conditions in the acclimated aviaries. Overall, body temperature was positively correlated with BMR, indicating that individuals modulated these two traits in parallel. The decline of BMR and body temperature during prolonged heat exposure could have prevented the birds from overheating (Kersten and Piersma 1987; Klaassen 1990).

Based on increased HSP70 levels and H/L ratio, it seems that experimental red knots experienced the combination of HS and heat as stressful, resulting in an energy-saving strategy that reduced their food intake rate and BMR. Such metabolic downregulation was also paralleled by a reduction in gizzard mass, suggesting that differences in the size of several digestive organs of red knots could be correlated with differences in BMR (Piersma et al. 1996). In contrast, salt glands measurably increased in size with higher salinity at both high and low temperatures, which suggests that red knots require large salt glands to meet increased osmoregulatory demands (Gutiérrez et al. 2012), despite low food intake. Because salt gland hypertrophy in saline-acclimated birds can occur over short time scales (Peaker and Linzell 1975), it is likely that the size of the salt glands had reached a maximum after 2 weeks of HS acclimation and thus we could not detect differences associated with ambient temperature (see Gutiérrez et al. 2012).

It is interesting to note that red knots, especially those of the subspecies *C. canutus canutus*, exposed to HS showed elevated plasma  $\text{Na}^+$  (up to  $197 \text{ mmol L}^{-1}$ ). One might interpret this as an indication of either osmotic stress or a certain tolerance to hypernatremia over short time periods. The lack of correlation between  $\text{Na}^+$  and stress indicators (HSP70 and H/L ratio) supports the latter interpretation. Indeed, the elevated natremia we recorded in captive red knots is within the range of values reported for other bird species with salt glands [ $135\text{--}216 \text{ mmol L}^{-1}$  (Skadhauge 1981)]. Recent studies that reported elevated natremia in sea snakes (Brischoux et al. 2013b, 2014) suggested that greater tolerance to hypernatremia would be advantageous since active salt excretion would occur only when plasma  $\text{Na}^+$  dangerously exceeds an upper threshold and this, in turn, would substantially decrease energetic costs associated with salt gland functioning. Whether *C. canutus canutus* could have developed a certain degree of tolerance to hypernatremia remains to be further explored. Moreover, the fact that *C. canutus canutus* had somewhat greater H/L ratios and lower body masses and fat scores when exposed to HS provides an interesting avenue for exploring possible differences at the subspecies level. For instance, *C. canutus islandica* held in captivity maintained the schedules of moult and body mass changes of their counterparts in the wild, whereas *C. canutus canutus* showed deviations from normal annual rhythms (Piersma et al. 1996; Piersma and Ramenofsky 1998). Experimental and observational evidence (Piersma et al. 1996; Piersma and Ramenofsky 1998) from red knots that have been kept for research purposes under long-term captive conditions at research facilities at northern temperate latitudes suggests that *C. canutus canutus* are more susceptible to stress than *C. canutus islandica*. One is then tempted to pose the question: do *C. canutus canutus* ‘wintering’ in such an aviary environment

somehow feel that they are at the ‘wrong’ latitude? Birds from different latitudes and with different migratory habits could, for example, have different photosensitive phases (e.g. Berthold 1974; Gwinner and Scheuerlein 1999). Further experiments under photoperiod and temperature regimes resembling those of the red knots’ natural environments are necessary to identify the reason(s) for such sub-specific differences.

In addition to the physiological mechanisms examined here, behavioural strategies leading to a decrease in salt intake are likely crucial in maintaining the osmotic balance (Gutiérrez 2014). Selective feeding on low-salt-load prey is a viable osmoregulatory strategy to avoid salt stress in saline environments (e.g. Purdue and Haines 1977; Nyström and Pehrsson 1988). For example, red knots staging on intertidal areas during the spring migration do not feed extensively on the crustacean brine shrimp *Artemia* spp. at supratidal salinas (100–150 ‰), an observation that has been explained by the avoidance of salt stress (Masero 2002). Instead, the mud snail (the prey used in this study) is the main prey for red knots staging on the mudflats of southern European coasts (Moreira 1994; Masero 2002). Feeding on less salty mud snails—despite their relatively low energy content (van Gils et al. 2005a)—might enable red knots to overcome osmotic stress.

A better understanding of avian adaptations and tolerances to saline environments is important to both basic science and conservation (reviewed in Gutiérrez 2014). In view of the fact that rather inactive red knots exposed to HS and temperature in captivity appeared challenged to even maintain body mass, it remains a big puzzle how hard-working birds in the field that have to commute between feeding and resting areas and may be forced into a lot of evasive flying by aerial predators (see Leyrer et al. 2012; van den Hout et al. 2014) can ever deposit the stores for onward flight in a place like Banc d’Arguin (Zwarts et al. 1990b; Piersma et al. 2005). Such knowledge is rather relevant at a time when fluctuating temperatures and salinity conditions predicted under various climate change scenarios (reviewed in Gutiérrez 2014) will affect the options open to birds and other aquatic air-breathing vertebrates such as sea turtles, snakes, and mammals.

**Author contribution statement** This study was conceived and designed by J. S. G., A. D., J. A. M. and T. P. The experiment was performed by J. S. G., A. S.-R. and A. D. HSP were measured by A. V. Data analyses were performed by J. S. G. and A. S.-R. The manuscript was written by J. S. G., J. A. M., A. V. and T. P.

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