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Corticosterone, food intake and refueling in a long-distance migrant

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ABSTRACT

Elevated baseline corticosterone levels function to mobilize energy in predictable life-history stages, such as bird migration. At the same time, baseline corticosterone has a permissive effect on the accumulation of fat stores (fueling) needed for migratory flight. Most migrants alternate flight bouts with stopovers, during which they replenish the fuel used during the preceding flight (refueling). The role of corticosterone in refueling is currently unclear. In a fasting–re-feeding experiment on northern wheatears (*Oenanthe oenanthe*) in autumn we found that baseline total and free corticosterone levels were negatively related with both food intake and the rate of fuel deposition after fasting. This confirms our earlier findings in wild conspecifics in spring and indicates that corticosterone does not stimulate stopover refueling. Whether the negative relationship between baseline corticosterone level and fuel deposition rate is causal is questionable, because within-individual comparison of corticosterone metabolite levels in droppings did not reveal differences between refueling and control periods. In other words, corticosterone does not appear to be down-regulated during refueling, which would be expected if it directly hampers refueling. We discuss possible correlates of corticosterone level that may explain the negative association between corticosterone and stopover refueling. Additionally, we found that fasting decreases total corticosterone level, which contrasts with previous studies. We propose that the difference is due to the other studies being conducted outside of the migration life-history stage, and provide a possible explanation for the decrease in corticosterone during fasting in migrating birds.

Introduction

Optimal migration theory commonly assumes that birds minimize the overall time of migration (Alerstam, 2011; Alerstam and Lindström, 1990; Hedenström, 2008; Hedenström and Alerstam, 1997). During migration, birds spend most of their time at stopover sites (e.g. Green et al., 2002; Hedenström and Alerstam, 1997; Schmaljohann et al., 2012) where they replenish the fuel (fat) used during flight. Therefore, the rate at which fuel stores are replenished (fuel deposition rate, FDR) may be the most important determinant of the speed of migration. Although an increased assimilation efficiency of food or seasonal shifts in diet may also contribute to fueling (Bairlein, 1985, 2002; Jenni-Eiermann and Jenni, 2003), hyperphagia (over-eating) is thought to be the main driver of fuel deposition (Lindström, 2003). The initial development of hyperphagia and fuel deposition is regulated by the endocrine system (reviewed in Cornelius et al., 2013; Ramenofsky, 2011; Wingfield et al., 1990), with a clear role for the glucocorticoid hormone corticosterone. In Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*), blocking of glucocorticoid receptors suppressed food intake in the pre-migratory phase (Landys et al., 2004). In dark-eyed juncos (*Junco hyemalis*), pharmacological blocking of the increase in corticosterone levels resulting from photo-stimulation inhibited pre-migratory fuel deposition; however, food intake was unaffected (Holberton et al., 2007).

Although these studies show that (baseline) corticosterone is pivotal to the development of migratory fueling, whether corticosterone affects FDR in birds en route is less clear. In red-eyed vireos (*Vireo olivaceus*) caught during autumn stopover and subsequently caged, short-term administration of corticosterone increased the frequency of visits to food bowls (Lõhmus et al., 2006). However, food intake and FDR were not measured. Captive thrush nightingales (*Luscinia luscinia*) subjected in spring to the simulated magnetic field of northern Egypt (so just before barrier crossing) had higher FDR than control birds; however, corticosterone levels did not differ (Henshaw et al., 2009). Currently, only one field study has directly related corticosterone level to FDR during migration; in northern wheatears (*Oenanthe oenanthe*), corticosterone level and FDR were negatively correlated during spring stopover on Helgoland (Eikenaar et al., 2013). In combination with the additional finding that corticosterone level was positively correlated with fuel load (Eikenaar et al., 2013), this may suggest that, at stopover, lean birds had low corticosterone levels because corticosterone impedes

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refueling. However, the sample size was small and environmental factors influencing FDR, such as food availability (Schaub and Jenni, 2000) and predation pressure (Schmaljohann and Dierschke, 2005), were not measured. Nonetheless, if corticosterone indeed hampers refueling, then levels should be down-regulated during stopover. Testing of this idea requires repeated sampling of individuals at different stages of migration which is feasible only in captive birds.

The main aims of the current study were to i) verify that corticosterone level is negatively related with FDR and positively related with fuel load, ii) determine whether FDR is linked to corticosterone through food intake, which is thought to be the main driver of fueling, and iii) determine whether corticosterone levels are down-regulated during refueling. For these purposes, captive birds in migratory condition were subjected to a fasting–re-feeding experiment in autumn. Each bird was subjected to 2 trials, randomized in order. During one trial, birds were fasted for several days after which they were allowed to refuel (e.g. Bauchinger et al., 2008; Biebach, 1985; Gwinner et al., 1988; Totzke et al., 2000). In a control trial, birds had access to unlimited food at all times. Corticosterone levels were determined at the end of fasting and during refueling, and on the corresponding days in the control trial. Northern wheatears are well suited for this study, because they are long-distance migrants, which at stopovers behave like time-minimizers, both in autumn and spring (Delingat et al., 2006; Dierschke et al., 2005; Schmaljohann and Dierschke, 2005). Furthermore, the stopover ecology of this species has been studied in great detail (reviewed in Bairlein et al., 2013).

Methods

Experimental set-up

From August onwards, 29 adult northern wheatears, born in captivity, were housed in two indoor rooms, but in individual cages of 40 × 40 × 50 cm with ad libitum access to food and water. To promote autumnal migratory fueling, on 3 September the photo-period in the rooms was changed from long days (14L:10D) to 12L:12D. This was effective because in the month following the switch to 12L:12D, the mean and SD increase in body mass was 6.5 ± 2.7 g (an increase of 22%, also see Supplementary material). Starting 15 October, after all birds had reached a stable body mass, each bird was subjected to two trials, a fasting–refueling trial and a control trial, randomized in order and separated by three weeks (Fig. 1). In the fasting–refueling trial, we simulated stopover refueling by a fasting–re-feeding protocol, in which the daily amount of food was reduced to 2 g over three consecutive days (days 2–4), followed by three 'refueling days' with ad libitum access to food (days 5–7). In the remainder of the text, we will refer to the reduction in food as 'fasting' and to the fasting–re-feeding protocol as the 'refueling trial'. During the refueling days, food intake and changes in body mass were measured. Daily food intake was determined by subtracting food mass on day X + 1 from food mass on day X. Spilled food, collected by placing the food trays in bird baths, was added to the food mass on day X + 1. Daily changes in body mass were determined by weighing birds to the nearest 0.1 g immediately after lights on (Fig. 1). Daily FDR was calculated as the average daily body mass gain from day 5 to day 8 divided by lean body mass (Delingat et al., 2006). Wing length (maximum chord to the nearest 0.5 mm) was used to calculate lean body mass, employing a linear regression based on 220 'lean' northern wheatears caught on Helgoland in previous years: lean body mass [g] = 0.29 g mm^{-1} × wing length [mm] − 6.85 g (linear regression: $n = 220$, $F_{1,218} = 95.07$, adj-$R^2 = 0.30$, $P < 0.0001$, after Schmaljohann and Naef-Daenzer (2011)). Fuel load was calculated as: (body mass − lean body mass) / lean body mass. At the start of the fast, fat stores were scored according to Kaiser (1993) on a scale ranging from 0 (no fat) to 8 (furcula and abdomen bulging, and breast covered with fat). There was a strong positive correlation between calculated fuel loads and the visual scores of fat stores (Spearman's rho = 0.7, $P < 0.001$, $n = 29$), but since fuel load provides a more objective estimate of fuel stores than visual scoring of fat, we used the former in all analyses.

One of the goals of the experiment was to verify the negative relationship between corticosterone and FDR we observed in the field. In the field, FDRs were calculated from the change in body mass *after* blood-sampling. To make the experiment comparable to our field data, birds were blood-sampled (ca. 70 μl) the day preceding refeeding (day 4 in Fig. 1) at approx. 10:30 AM. To measure baseline corticosterone levels, northern wheatears have to be blood-sampled within 2 min of capture (Eikenaar et al., 2013). To allow instant blood-sampling upon entering the room, birds entered the experiment in five cohorts over five consecutive days (3 birds in both rooms each day). Blood samples were centrifuged immediately after collection and plasma was separated with the samples on a cold block and frozen at −20 °C until assaying (see below). In the plasma, we measured two levels of corticosterone: free (unbound) and total (free corticosterone +corticosterone bound to corticosteroid binding globulin, CBG). To avoid repeated blood-sampling within a few days, on the second day of refueling (day 6 in Fig. 1), glucocorticoid metabolite (GCM) levels were measured in excreta. Birds' excreta were collected on paper sheets placed on the cage bottom. For each bird, all excreta collected in the first 3 h after lights on were put in a 2 ml Eppendorf tube, weighed and homogenized, and frozen at −20 °C until later processing (see below). Earlier on, birds were familiarized with the paper sheets by placing these in the cages on three mornings. The analysis of GCM is a retrospective and integrative measure of plasma corticosterone excreted and metabolized over the last few hours (Goymann, 2005). We therefore used GCM only to make a within-individual comparison between the refueling and control trials (see below), and did not compare GCM levels with plasma corticosterone levels.

In the control trial, the above described procedures were identical with the only difference that at all times birds had ad libitum access to food (Fig. 1). The order of the refueling and control trials was randomized, and trials were separated by three weeks to allow birds to recover from fasting. This was effective, as all birds that were first subjected to

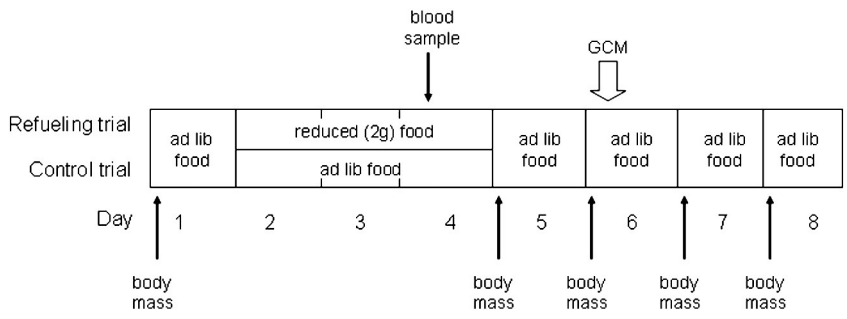

Fig. 1. Schematic representation of the refueling and control trials.

the refueling trial fully re-covered their body mass and fuel load before the start of the control trial. Water was provided ad libitum and the temperature in the rooms was held constant at 20 °C throughout the experiment. During the experiment, in 15 birds, migratory restlessness (Zugunruhe) was measured as part of another study. Briefly, Zugunruhe was measured automatically with motion-sensitive microphones that recorded all movements of the birds (for details, see Maggini and Bairlein, 2010). All 15 birds showed nocturnal activity in each of the nights of the experiment (see Supplementary material), supporting our premise that birds were in a migratory condition. The experiment was conducted at the Institute of Avian Research, Wilhelmshaven, Germany. All procedures were approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Germany.

Corticosterone analysis

Plasma corticosterone concentration was measured using an enzyme-immunoassay (Munro and Lasley, 1988) in the laboratory of the Swiss Ornithological Institute in Sempach. Corticosterone in 20 μl of plasma (diluted 1:10 in H_2O_{bidest}) was extracted with 4 ml dichloromethane, re-dissolved in phosphate buffer (0.1 M, pH 7.0, with 0.1% bovine serum albumin) and analyzed in duplicates. A sheep-anti-corticosterone polyclonal antibody was used in a final concentration of 1:8000 (Chemicon Int; cross reactivity: 11-dehydrocorticosterone 0.35%, progesterone 0.004%, 18-OH-DOC 0.01%, cortisol 0.12%, 18-OH-B 0.02% and aldosterone 0.06%). The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicate on each plate. Plasma pools from chicken (*Gallus domesticus*) with a low and a high corticosterone concentration were used as internal controls on each plate. The intra- and inter-assay variation ranged from 4.14% to 6.38% depending on the internal controls.

Corticosteroid binding globulin

The affinity and capacity of CBG was measured with a radioligand-binding assay with tritiated corticosterone following Breuner et al. (2003). For point sample analyses, 5 μl of plasma was stripped of endogenous steroids with two parts of dextran-coated charcoal solution (0.1% dextran, 1% Norit A charcoal in 50 mM Tris) for 30-min at room-temperature. Outside the stripping procedure, the plasma was maintained below 4 °C. The final assay dilution of the wheatear was 1:720. The binding assay was performed in 50 mM Tris buffer at 4 °C and terminated after 1 h. Glass fiber filters (Whatman) were soaked in 25 nM Tris with 0.3% polyethyleneimine (Sigma®) 40 min before filtering. After filtration, filters were rapidly rinsed with three rinses of 3 ml ice-cold 25 mM Tris.

All assays contained 50 μl [^{3}H] corticosterone (radioligand), 50 μl buffer (total binding) or unlabelled corticosterone (unspecific binding) (1 μM) and 50 μl plasma preparation. Specific binding was determined by subtraction of the unlabelled corticosterone. All samples were run in triplicates. Bound and free radioligands were separated with the use of rapid vacuum filtration over glass fiber filters (Brandel Harvester). After filtration, radioactivity bound to filters was measured by standard liquid scintillation spectroscopy.

Point sample analyses were run on individual plasma samples, whereas saturation analysis was run on pooled samples. For saturation analysis, 0.25–12 nM [^{3}H] corticosterone was incubated with pooled plasma in the presence or absence of 1 μM unlabeled corticosterone. CBG capacity in individual birds was estimated by the use of 20 nM [^{3}H] corticosterone. The K_d found was 2.37 nM and the maximal binding capacity of sites (B_{max}) was 248.0 nM (Fig. 2).

The radioligand-binding assay was performed for 58 plasma samples. In each experiment, CBG values of 17 samples and one control sample (plasma pool of chickens) could be determined, giving a total of 4 experiments. The two samples of a given bird's were always assayed in the same experiment. Intra-assay variation was 4.75% and inter-assay variation 9.7%. Free corticosterone titers were estimated from total corticosterone concentrations and CBG binding parameters by use of the equation of Barsano and Baumann (1989):

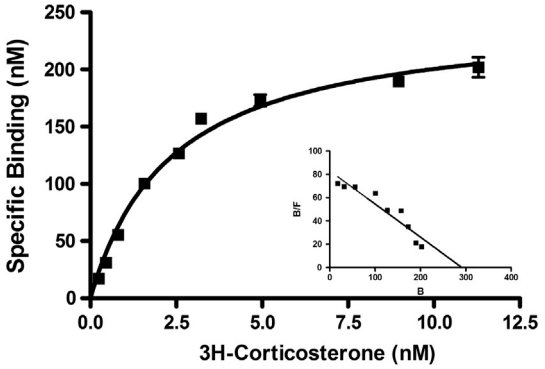

Fig. 2. Equilibrium saturation binding curve demonstrating specific binding of ^{3}H-corticosterone to wheatear plasma as a function of increasing concentrations of radiolabeled corticosterone. Points represent means ± SE. The inset is the Scatchard–Rosenthal re-plot of the data, with B = bound- and F = free-^{3}H corticosterone fraction.

$$H_{free} = 0.5 \times \left[H_{total} - B_{max} - \frac{1}{K_a} \pm \sqrt{\left(B_{max} - H_{total} + \frac{1}{K_a} \right)^2 - 4 \times \left(\frac{H_{total}}{K_a} \right)} \right]$$

where H_{free} is free hormone, H_{total} is total hormone, B_{max} is total binding capacity of CBG, and K_a = 1 / dissociation constant (K_d) (all values in nM).

GCM assay: validation study and sample analysis

To choose the appropriate enzyme immunoassay to measure GCMs in northern wheatears, we performed an ACTH challenge on two male and two female northern wheatears, housed in individual 40 × 40 × 50 cm cages with ad libitum access to food and water. On 30 November at 12:00 AM (lights on at 09:00 AM), all four birds were injected intraperitoneally with 2 μg ACTH dissolved in 100 μl saline. Droppings were collected during ten 30 min periods from 11:00 AM to 4:00 PM. Droppings were collected on paper sheets placed on the bottom of the cages. For each bird, all excreta of each 30 min period were put in a 2 ml Eppendorf tube, homogenized, and frozen at −20 °C until later processing. For GCM extraction, 0.05 g excreta were transferred to new tubes and 0.5 ml 60% methanol was added (Palme et al., 2013). Tubes were shaken for 15 min on a multi-vortex at 400 rpm followed by 1 min shaking at maximum speed on a hand-vortex. Tubes were centrifuged for 1 min and 100 μl of the supernatant was transferred to new tubes, which were subsequently placed in a drying oven at 50 °C until the samples were dried down. Samples were then shipped to Vienna where samples were re-dissolved it in 100 μl of 60% methanol and diluted 1:5 in assay buffer. Group specific antibodies against 3,11-dioxo CM and 3α-ol-11-oxo CM have been shown to be particularly suitable to measure GCMs in birds (Möstl et al., 2005, 3,11-dioxo CM: Lobato et al., 2008; Rettenbacher et al., 2004, 3α-ol-11-oxo CM: Carere et al., 2003; Stöwe et al., 2008). Therefore, we analyzed the samples of the ACTH challenge using an assay with antibodies against 5β-androstane-3α-ol,11,17-dione-17-CMO: bovine serum albumin (Möstl et al, 2002) and a cortisone assay with antibodies against 4-pregnene-17α,21-diol-3,11,20-trione-21-HS bound to bovine serum albumins (Rettenbacher et al., 2004; Stöwe et al., 2013). The group specific antibody of this cortisone assay is "blind" for the upper part (ring D and side chain) of the glucocorticoid molecule, the only position where corticosterone and cortisol differ (the latter having an additional hydroxyl group at C-17). Therefore, the antibody is able to pick up 11-oxo metabolites of both steroids. Since the glucocorticoid secreted by birds is

corticosterone, we are confident that the metabolites detected are corticosterone metabolites. The assay proved to be suitable for measuring GCM levels in adult northern wheatears; in birds of both sexes, the cortisone assay (Stöwe et al., 2013) detected a clear increase in GCMs excreted in response to the ACTH challenge and a decline in GCM levels thereafter (Fig. 3).

Homogenized samples collected in the main experiment were thawed at room temperature, and treated and assayed as described above. All samples were run in duplicates, and the inter-assay variation was 8.4% and 8.5% for a high and low level pool plasma sample, respectively. The two samples of a given bird were always assayed on the same microplate. For each bird, we also calculated the hourly excretion rate of GCMs (Goymann and Trappschuh, 2011) using the GCM level in the 0.05 g portion and total (3 h) sample weight.

Data analysis

To determine whether food intake and FDR after fasting (dependent variables) were related to circulating corticosterone levels just prior to refueling, we performed multilevel generalized linear mixed models (GLMMs) with a normal error structure using MLwiN 2.0 (Rasbash et al., 2004). Separate GLMMs were run for total and free corticosterone. Birds were housed in two rooms and entered the experiment in cohorts (see Experimental set-up). The latter means that during the three day refueling period, the total time we spent inside the bird rooms (and caused disturbance) decreased with cohort, which may have affected food intake and refueling. By entering room and cohort as random factors, we accounted for the non-independence of observations from the same room or same cohort. As a consequence of the randomization of the order of refueling and control trails, FDR was measured during two periods that were three weeks apart. Because FDR may change over the course of the migratory season in both wild and captive birds (reviewed in Jenni and Schaub, 2003), date was entered as a fixed factor (early or late in the season). A similar GLMM was performed to analyze the relationship between food intake and circulating corticosterone levels in the control trial. Sex was not entered into our models, because we had no reason to expect differences between the sexes in the relationships between baseline corticosterone levels, and food intake and FDR.

On the first day after a period of fuel loss, birds may be unable to consume the maximum amount of food (e.g. Hume and Biebach, 1996; Klaassen and Biebach, 1994). We therefore averaged food intake and FDRs over the three refueling days. Note that in the refueling trial, date was weakly correlated with both total and free corticosterone levels (see Table 1). However, because variance inflation factors were low (all < 1.15), this did not cause multicollinearity.

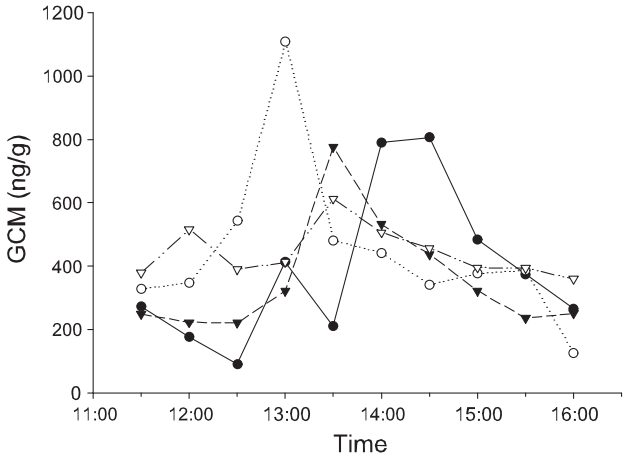

Fig. 3. Amounts of glucocorticoid metabolites in droppings (GCM) measured during 30 min periods in two male (triangles) and two female (circles) northern wheatears that were subjected to an intra-peritoneal injection of 2 μg ACTH at 12:00 h.

The relationship between circulating corticosterone levels (dependent) and fuel load just prior to refueling (day 5) was analyzed with GLMMs with a normal error structure. Separate GLMMs were run for total and free corticosterone in both the control and refueling trails. Cohort and room were entered as random factors. Because corticosterone levels may change over the course of the migratory season (Falsone et al., 2009), date (early/late in the season) was entered as a fixed factor.

Total binding capacity of CBG, and total and free corticosterone levels during fasting and control periods were compared using paired T-tests in SPSS Statistics version 20 (IBM, New York), as were GCM levels and excretion rates during refueling and control periods. To improve normality of residuals, corticosterone (metabolite) levels were $\log_{10}$-transformed prior to all analyses. Results are presented as mean and SD with Cohen's d effect size estimates using each mean's individual SD. All tests were two-tailed.

Results

Fasting, refueling and corticosterone dynamics

Fasting resulted in a reduction in body mass of 3.72 ± 0.75 g and a reduction in fuel load of 0.17 ± 0.04 (meaning that on average birds lost a fuel mass equivalent to 17% of their lean body mass). During the corresponding time period in the control trial (day 1 to day 5), reductions in body mass and fuel load were 0.54 ± 0.78 g and 0.02 ± 0.04, respectively.

Total and free corticosterone levels were strongly correlated, both in the refueling and control trial (Pearson's $r = 0.9$, $P < 0.001$, $N = 29$, and Pearson's $r = 0.92$, $P < 0.001$, $N = 29$, respectively). Total binding capacity of CBG and total corticosterone levels were lower during fasting than during the control period ($T = 13.27$, $P < 0.001$, Cohen's $d = 2.65$, $N = 29$ and $T = 3.17$, $P = 0.004$, Cohen's $d = 0.59$, $N = 29$, respectively). Free corticosterone levels were not different between the fasting and control periods ($T = -0.13$, $P = 0.9$, Cohen's $d = 0$, $N = 29$). Levels of GCMs and hourly GCM excretion rate did not differ between the refueling and control periods ($T = 0.82$, $P = 0.42$, Cohen's $d = 0.27$, $N = 29$ and $T = -1.45$, $P = 0.16$, Cohen's $d = -0.29$, $N = 29$, respectively). Birds were weighed before excreta were collected, which may have increased GCM levels. However, birds were weighed both in the refueling and control trials. Furthermore, GCM levels were comparable to those observed in birds prior to handling for the ACTH challenge, suggesting that the increase in corticosterone secretion as a result of weighing was not very high and/or of long duration.

Just prior to refueling, both free and total corticosterone levels were positively related to fuel load (Table 1, Fig. 4). In the control trial, fuel load and corticosterone levels were not related (Table 1). Date was not related to corticosterone levels in the control trial, but was negatively related to both free and total corticosterone levels in the refueling trial (Table 1).

Corticosterone, food intake and FDR

Daily food intake was 11.16 ± 1.24 g in the refueling days and 9.69 ± 1.44 g in the corresponding control days. Daily FDR was 0.028 ± 0.011, meaning that during a refueling day, birds gained a fuel mass equivalent to 3% of their lean body mass. Daily food intake and daily FDR were higher in the refueling period than in the control period ($T = 4.05$, $P < 0.001$, $N = 29$ and $T = 13.95$, $P < 0.001$, $N = 29$, respectively).

In the refueling trial, daily food intake and daily FDR were positively correlated (Pearson's $r = 0.63$, $P < 0.01$, $N = 29$). Both total and free corticosterone levels were negatively related to daily food intake (Table 2, Fig. 5). The bird with the highest food intake had a strong influence on the negative relationship between corticosterone levels and

Table 1
Relationships between fuel load and total and free corticosterone levels in the control and refueling trials (all N = 29).

		Total corticosterone				Free corticosterone			
Trial	Variable	β ± SE	χ^2	df	P	β ± SE	χ^2	df	P
Control	Fuel load	0.21 ± 0.21	0.98	1	0.32	0.25 ± 0.18	1.87	1	0.17
	Date	−0.07 ± 0.07	0.80	1	0.37	−0.09 ± 0.06	1.83	1	0.18
Refueling	Fuel load	0.95 ± 0.17	32.17	1	<0.001	0.94 ± 0.19	24.4	1	<0.001
	Date	−0.14 ± 0.06	4.99	1	0.025	−0.17 ± 0.07	5.71	1	0.012

food intake (Cook's D > 1 for both total and free corticosterone). However, we have no reason to exclude this bird from the dataset. Moreover, its high FDR indicates that its high food intake was not due to an illness, such as coccidiosis (which, in early stages, increases food intake in northern wheatears, FB, pers. obs.). Similar to food intake, daily FDR was negatively related to both total and free corticosterone levels (Table 2, Fig. 5). Date was not related to food intake, but negatively related to FDR in both the model for total and the model for free corticosterone (Table 2).

In the control trial, corticosterone levels were not related to food intake (model with total corticosterone: β ± SE = 0.32 ± 1.24, χ^2 = 0.07, P = 0.73, N = 29; model with free corticosterone: β ± SE = −0.67 ± 1.38, χ^2 = 0.23, P = 0.63, N = 29). Date was positively related to food intake in the control trial (model with total corticosterone: β ± SE = 1.70 ± 0.50, χ^2 = 11.78, P = 0.001, N = 29; model with free corticosterone: β ± SE = 1.61 ± 0.50, χ^2 = 10.32, P = 0.001, N = 29).

Discussion

Fasting and corticosterone levels

The free hormone hypothesis postulates that only the unbound glucocorticoids are biologically active because only these can interact with target tissues (Mendel, 1989). Therefore, it was claimed that for a meaningful interpretation of GC concentrations, both CBG and free corticosterone have to be measured (Breuner and Orchinik, 2002; Breuner et al., 2013). This hypothesis was recently challenged by presenting the complexity of the assessment of CBG and free hormone levels which is far from understood (Schoech et al., 2013). The interpretation of our results, however, should not be affected considering both views because both total and free corticosterone changed in the same direction in response to the fast. In our captive birds in migratory disposition, the total binding capacity of CBG was lower during fasting than during the control period. Total baseline corticosterone level was also lower during fasting than during the control period, whereas free baseline corticosterone level did not differ between these two periods. Lynn et al. (2003) present two possibilities for a reduction of CBG as a result of their 24 h fast. First, CBG values are decreased as a direct result of energetic demand after 24 h of fasting, in that the fasting birds use the CBG as an energy source. Second, CBG values are reduced indirectly by a fasting-induced increase of corticosterone which might for instance inhibit CBG synthesis or promote CBG breakdown for gluconeogenesis. However, Lynn et al. (2003) concomitantly found an increase of free corticosterone level, whereas in our study free corticosterone level was unchanged. This means that in our experiment on northern wheatears, during fasting, corticosterone secretion is either reduced or its clearance is increased.

The changes in corticosterone levels we observed as a consequence of fasting differ from previous studies on captive birds where, as a result of a similarly moderate fast, free or total corticosterone levels were unchanged or increased (Astheimer et al., 1992; Lynn et al., 2003, 2010; Wall and Cockrem, 2009). An obvious difference between our and these earlier studies is that the latter were done on non-migrants or outside of the migratory period. In migrants, baseline corticosterone levels may be elevated to meet the demands of the increased metabolic rate during flight (Falsone et al., 2009; Jenni et al., 2000; Jenni-Eiermann and Jenni, 2012; Landys et al., 2004, 2006). Baseline corticosterone may also need to be elevated in migrants on the ground when they are close to departure; corticosterone has been proposed to signal migratory readiness in that levels increase when (re)fueling is complete (Landys-Cianelli et al., 2002; Lõhmus et al., 2003; Piersma et al., 2000). In contrast, when fuel stores are low, for example in the first days of stopover refueling, maybe corticosterone decreases to baseline levels to save energy. In our experiment, the period of fasting reduced the birds' fuel stores, thereby perhaps decreasing corticosterone baseline levels. Because corticosterone levels may be lower and less variable in captive populations than in free-living populations (Romero, 2002), this scenario requires testing on wild birds.

Corticosterone and stopover refueling

At baseline levels, corticosterone is known to have a permissive effect on fueling during the early stages of migration (Holberton et al., 2007; Landys et al., 2004). Although this permissive effect may very well persist throughout the migration season, our results clearly show that baseline corticosterone does not stimulate hyperphagia during stopover refueling; food intake after fasting was actually negatively related with baseline total and free corticosterone levels. Interestingly, this negative relationship was only apparent when birds lost significant amounts of fuel, as in the control period, food intake was not related with total or free corticosterone levels. Similarly, in a study on captive

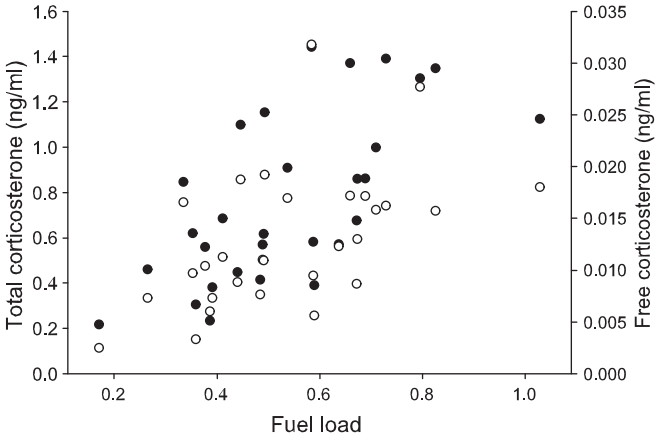

Fig. 4. Relationships between fuel load and total (closed circles) and free (open circles) corticosterone levels in the refueling trial (both N = 29).

Table 2
Relationships between corticosterone levels and both food intake and FDR in the refueling trial. Note that separate models were run for total and free corticosterone. All N = 29.

	Food intake				FDR			
Variable	β ± SE	χ^2	df	P	β ± SE	χ^2	df	P
Total cort	−2.45 ± 0.78	9.82	1	0.002	−0.017 ± 0.007	5.31	1	0.021
Date	−0.15 ± 0.34	0.20	1	0.65	−0.008 ± 0.003	5.44	1	0.020
Free cort	−2.39 ± 0.72	11.13	1	0.001	−0.015 ± 0.007	4.60	1	0.032
Date	−0.22 ± 0.34	0.43	1	0.52	−0.008 ± 0.003	5.86	1	0.015

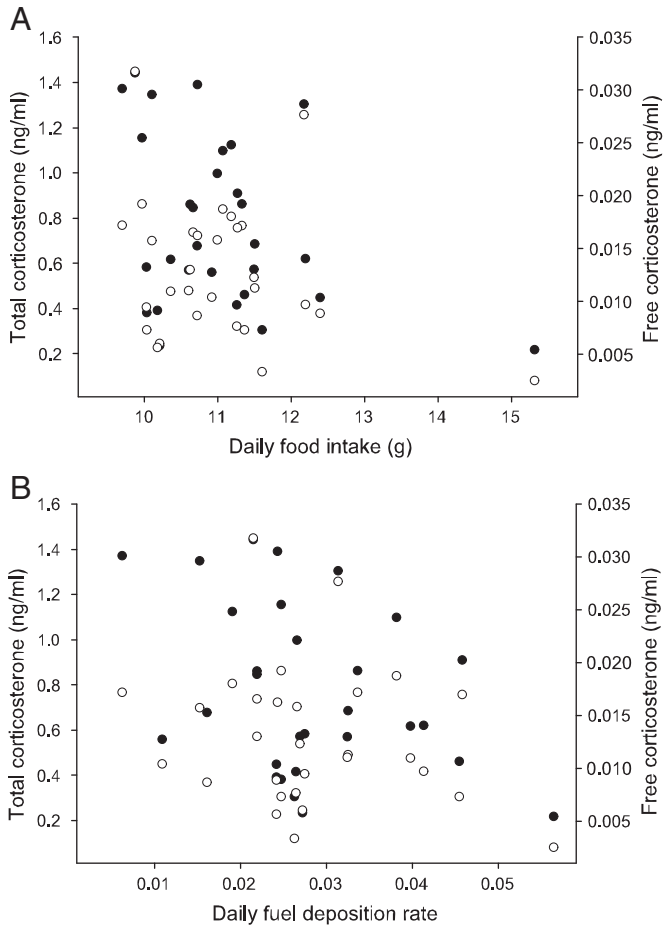

Fig. 5. Relationships between total (closed circles) and free (open circles) corticosterone levels and (A) daily food intake, and (B) daily FDR in the refueling trial (all N = 29).

dark-eyed juncos in spring migratory condition and continuous access to food, baseline total corticosterone was unrelated with food intake (Holberton et al., 2008). This suggests that in migrating birds, corticosterone may only be linked to feeding behavior when there is a considerable change in fuel stores. A reduced food intake with increasing corticosterone levels was most likely the cause of the negative relationship between the FDR and baseline total and free corticosterone levels we observed in both the current study on captive birds in autumn and in an earlier field study on wild northern wheatears in spring (Eikenaar et al., 2013). It may thus appear that corticosterone directly hampers refueling during stopover in migrating birds. Because this could lengthen tenure at stopover sites, resulting in delayed arrival at the breeding or wintering grounds, selection would be expected to favor low corticosterone levels during stopover refueling. In other words, we would expect corticosterone levels to be down-regulated during stopover refueling. This, however, does not seem to be happening; within-individual comparisons showed that GCM levels and GCM excretion rates were not different between the refueling and control periods. Some caution needs to be exerted when interpreting these results, as we currently do not know how exactly the GCM level measured in our cortisone assay relates to plasma corticosterone levels. Nonetheless, there was a clear effect of the ACTH challenge on fecal GCM levels measured using the cortisone assay, indicating that our GCM data are, at least to a certain extent, reflecting plasma corticosterone levels of the last few hours (Möstl et al., 2005). Furthermore, in a different fasting–refueling experiment on captive northern wheatears, plasma baseline corticosterone levels measured on the third day of refueling were not different from levels measured on the corresponding day in control birds (CE, unpublished data).

Perhaps then, corticosterone and stopover refueling are indirectly related because corticosterone levels co-vary with one or more factors that do have a causal relationship with FDR. In the field, multiple intrinsic and environmental factors (e.g. molt, fuel load, food availability, weather, and predation risk) together determine the rate at which birds replenish their fuel stores at stopover sites (reviewed in Jenni and Schaub, 2003). In our controlled experiment on captive birds, most extrinsic factors were absent or were the same for all individuals, and birds were not molting. Therefore, it seems most plausible that intrinsic factors relating to fuel management best explain the variation we observed among individuals in food intake and FDR. Fuel load appears to be a likely candidate because in the current study corticosterone levels were positively correlated with fuel load just prior to refueling, whereas in the control period fuel load was unrelated with corticosterone levels. The idea that the negative relationship between corticosterone and stopover refueling results from corticosterone covarying positively with fuel load fits the proposition that corticosterone signals departure readiness; individuals that have accumulated sufficient fuel for the next flight bout, and thus stop fueling, have elevated baseline corticosterone levels (Landys-Cianelli et al., 2002; Lõhmus et al., 2003; Piersma et al., 2000). However, when 'fuel load' is entered into the GLMMs as a covariate instead of 'corticosterone level', fuel load has no significant relationship with food intake and FDR (results not shown). Similarly, in northern wheatears at stopover on Helgoland, there was no relationship between fuel load at capture and subsequent FDR (Eikenaar et al., 2013). In other bird species studied at stopover sites, fuel load has been found to be positively, negatively or not related with FDR (reviewed in Jenni and Schaub, 2003). Similarly, fuel load has been found to be positively related (e.g. Holberton et al., 1996), negatively related (e.g. Jenni et al., 2000) or not related (e.g. Falsone et al., 2009) with baseline corticosterone level. In conclusion, it is currently unclear whether fuel load can explain the negative relationship we have observed in the field as well as in captivity between baseline corticosterone level and FDR.

It is also possible that corticosterone is indirectly related with FDR because other hormones or neurotransmitters that stimulate or inhibit food intake are involved in the hypothalamo-pituitary-adrenal axis. A possible candidate is ghrelin, a hormone produced in the gut and gastro-intestinal tract. In (poultry) birds, plasma ghrelin levels increase after fasting (Kaiya et al., 2007; Ocloń and Pietras, 2011; Shousha et al., 2005), and exogenous ghrelin stimulates corticosterone release (Kaiya et al., 2002; Saito et al., 2005) and generally inhibits food intake (reviewed in Kaiya et al., 2007, 2009). Maybe therefore, in our experiment, the reduced food intake and FDR in birds with high baseline corticosterone levels was (partly) due to these birds having high ghrelin levels. It should be noted, however, that our current knowledge on the relationships between ghrelin, corticosterone and food intake derives exclusively from studies on domesticated, non-migratory birds.

Effects of date

Because the order of the refueling and control trials was randomized, and trials were separated by three weeks, temporal variation existed in the measurements of corticosterone levels, food intake and FDR. This resulted in effects of date on these variables which are discussed here. Date was not related to corticosterone levels during the control trial, but was negatively related to both free and total corticosterone levels during the refueling trial, meaning that corticosterone levels were lower earlier in the migratory season, but only after fasting. This corresponds to field studies, where, in birds at stopover, total and free corticosterone levels increased over the migratory season (Falsone et al., 2009).

In the control trials, date was positively related to food intake, meaning that birds that had already experienced a fast ate more during control trials. This may be explained by the finding of Totzke et al. (2000) that repeated fasting accelerates migratory fattening. Food intake in

the refueling trials was unaffected by date. Date did, however, affect FDR in that this was lower in birds that were subjected to the refueling trial later in the experiment. This suggests that later in the migratory season, the assimilation of food into fuel (fat) was less efficient. Northern wheatears start arriving on their sub-Saharan wintering grounds in October (Schmaljohann et al., 2012). Shortly after arriving at the wintering grounds, there is no need for the rapid accumulation of fuel stores, typical for stopover refueling. Perhaps, in our experiment, birds refueling in November display an already lowered need to rapidly convert food into fuel for migratory flight compared to birds refueling in October. It should be noted that during the experiment, the birds did not experience a change in day length (they were kept on 12L:12D after the start of the experiment). Therefore, the effects of date most likely were attributable to the strong endogenous rhythms of migratory fueling and Zugunruhe existing in our captive population of northern wheatears (Maggini and Bairlein, 2010).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2014.03.015.

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