



## Regular article

## Corticosterone predicts nocturnal restlessness in a long-distance migrant

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## ARTICLE INFO

## Article history:

Received 31 March 2014

Revised 11 June 2014

Accepted 13 June 2014

Available online 21 June 2014

## Keywords:

Migration

Corticosterone

Nocturnal restlessness

Glucocorticoid metabolites

Stopover

Departure

Radio-telemetry

Wheatear

*Oenanthe*

## ABSTRACT

The decision made by migrating birds to stop refueling and to depart from stopover depends on cues from innate rhythms, intrinsic factors such as fuel reserves, and extrinsic factors such as weather conditions. The physiological mechanism behind this decision, however, is largely unexplored. The transition from refueling to flight involves an increase in both locomotion and energetic demands. Because, at baseline levels, corticosterone stimulates locomotion and is involved in the mobilization of energy, this hormone could encourage departure of migrants. We collected field data on baseline corticosterone, migratory restlessness, and actual departure in northern wheatears at stopover. Additionally, in refueling long-term captive conspecifics, we measured migratory restlessness while simultaneously collecting droppings to determine glucocorticoid metabolite (GCM) levels. We found that migratory restlessness at stopover was positively correlated with corticosterone level. Similarly, in refueling long-term captive birds, migratory restlessness was positively correlated with concurrently measured GCM levels in droppings. To our best knowledge, our study is the first to simultaneously measure a hormonal signal and migratory restlessness. In conclusion, our results are consistent with the hypothesis that, by increasing locomotor activity, baseline corticosterone is involved in the regulation of departure of migrants at stopover. Future studies could reveal how corticosterone is up-regulated in migrants that are ready to depart.

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## Introduction

To support migratory flight, birds accumulate large amounts of fuel in the form of fat. Fuel accumulation occurs before departure from the breeding and wintering grounds (pre-migratory fueling) as well as during stopovers between flight bouts (refueling). Because the time spent refueling at stopover sites by far exceeds the time in actual flight (e.g. Green et al., 2002; Schmaljohann et al., 2012), the time required to complete migration is mainly determined by the duration of stopovers. Consequently, many studies, both empirical and theoretical, have investigated which factors influence departure decisions of migrants at stopover sites and hence stopover duration (reviewed in Chernetsov, 2012; Jenni and Schaub, 2003). It is well established that departure decisions depend on cues from innate rhythms, intrinsic factors such as fuel reserves, and extrinsic factors such as weather conditions (Chernetsov, 2012; Jenni and Schaub, 2003). In contrast, our understanding, and even identification, of the physiological mechanism(s) involved in the regulation of departure is very limited (reviewed in Cornelius et al., 2013). The transition from stopover refueling to active flight involves an increase in both locomotion and energetic demands. Corticosterone, at baseline and moderately elevated levels (i.e. not the levels observed in response to a stressor), is involved in the mobilization of energy during the predictable life-history cycle, and has a stimulatory effect

on locomotion (reviewed in Landys et al., 2006). Therefore, at baseline levels, corticosterone perhaps stimulates departure of migrating birds. Observations from several studies are in line with this idea. In migratory active captive garden warblers (*Sylvia borin*), a nocturnal migrant, autumn baseline corticosterone levels were higher at night than during the day (Schwabl et al., 1991). Observations on captive pre-migratory fueling red knots (*Calidris canutus*) and wild refueling bar-tailed godwits (*Limosa lapponica*) suggest that baseline corticosterone levels are low in the early stages of (re)fueling but rise in preparation for flight (Landys-Cianelli et al., 2002; Piersma et al., 2000). In captive white-crowned sparrows (*Zonotrichia leucophrys gambelii*) and captive dark-eyed juncos (*Junco hyemalis*) photo-stimulated into migratory condition, the expression of migratory restlessness is accompanied by an increase in baseline corticosterone level (Holberton et al., 2008; Landys et al., 2004). Migratory restlessness occurs when birds in migratory disposition are confined to a limited space, i.e. caged, and mainly consists of wing fluttering while perched (Berthold et al., 2000; Biebach et al., 1985). Importantly, it was recently shown that migratory restlessness measured in birds at stopover is an accurate proxy for a bird's likelihood to depart; birds that showed little restlessness were less likely to depart from stopover than birds that showed much restlessness (Eikenaar et al., 2014a). In red-eyed vireos (*Vireo olivaceus*) captured and caged at a stopover site, nocturnal activity in the first hour after sunset was positively correlated with corticosterone level (Löhmus et al., 2003). Unfortunately, as birds were not blood-sampled immediately after capture, corticosterone levels in this study may

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have reflected handling stress (Löhmus et al., 2003). Finally, fecal glucocorticoid metabolite levels in wild blue tits (*Cyanistes caeruleus*) were higher in migratory than in resident individuals (Nilsson and Sandell, 2011).

The aim of the current study was to further characterize the relationship between baseline corticosterone and departure in migrant birds at stopover. For this purpose we took a dual approach by combining a study on wild birds at stopover with a study on captive birds. We used the northern wheatear (*Oenanthe oenanthe*), a nocturnal long-distance migrant, as a model species. In the field birds were caught, immediately blood-sampled for baseline plasma corticosterone, and subsequently caged to measure nocturnal migratory restlessness. Additionally, at release part of these birds was equipped with a radio-transmitter in order to determine their departure latency. Using long-term captive birds, in a fasting–refueling experiment we measured migratory restlessness and simultaneously collected their droppings to determine baseline glucocorticoid metabolite (GCM) level. Our study is the first to link baseline corticosterone level to actual stopover departure, and to simultaneously measure a hormonal signal (GCMs) and migratory restlessness. We expected positive relationships between migratory restlessness and both baseline corticosterone level and GCM level. Furthermore, we expected birds with high baseline corticosterone levels to depart earlier from stopover than birds with low levels.

## Methods

### Field study

The study was conducted on Helgoland (54°11'N, 07°55'E), a small island ca. 50 km off the German North Sea coastline. Northern wheatears rarely breed on Helgoland (Dierschke et al., 2011). In the 2013 spring (May) and autumn (August and September) migration seasons, northern wheatears were caught using mealworm-baited spring traps ( $n = 39$  in spring and  $n = 83$  in autumn). Most (56) individuals trapped in autumn were first-year birds. All birds were trapped between 8 am and 7 pm, well after sunrise and well before sunset. Corticosterone level was not correlated with time of capture (Spearman's  $\rho = -0.05$ ,  $p = 0.55$ ,  $n = 122$ ). Traps were monitored continuously and when a bird was caught it was taken from the trap and blood-sampled (ca. 80  $\mu$ l) immediately from the wing vein. All samples were taken within 2 min from closing of the trap and thus represent true baseline levels (Eikenaar et al., 2013). The plasma was separated within 2 h of blood-sampling and frozen at  $-20$  °C until assaying. After blood-sampling, birds were ringed, wing length (maximum chord) was measured to the nearest 0.5 mm, and body mass to the nearest 0.1 g. Wing length was used to calculate lean body mass (LBM), employing a linear regression based on 220 'lean' northern wheatears caught on Helgoland in previous years:  $LBM [g] = 0.29 \text{ g mm}^{-1} \times \text{wing length [mm]} - 6.85 \text{ g}$  (linear regression:  $n = 220$ ,  $F_{1,218} = 95.07$ ,  $adj-R^2 = 0.30$ ,  $p < 0.0001$ , after Schmaljohann and Naef-Daenzer, 2011). LBM was used to calculate fuel reserves: fuel load = (body mass [g] – LBM [g]) / LBM [g]. Fuel load thus represents the amount of fuel a bird carries relative to its lean body mass. Approx. 1 h after trapping, birds were put in individual cages (40 × 40 × 30 cm) set up in a single indoor room with artificial lighting. Throughout the experiment the lights in the room were switched on at 7 am and off at 9 pm CET, the temperature was held constant at approx. 20 °C, and birds had ad libitum access to water. Each morning, immediately after lights were switched on, each bird was weighed to the nearest 0.1 g, after which it received a food tray with 30 g of mealworms. Food trays were removed immediately before lights-off. Birds were released after the third night in captivity. Migratory restlessness was recorded automatically with motion-sensitive microphones (Piezo-Scheibe 27 mm, Conrad Electronics SE, Hirschau, Germany), that detect vibrations caused by sound or movement. Microphones were attached to the right wall of the cages, which were acoustically separated from

each other by placing them on polystyrene. Each movement of a bird generated an impulse that was transmitted to a recording device. To avoid the recording of occasional non-migratory activity, we set a threshold of three impulses per second before it was recorded as an activity count. Another device created a CSV file summarizing the activity counts over 15 min periods. Individual tracking of radio-tagged birds revealed that northern wheatears leave Helgoland exclusively at night, between 1 and 5.5 h after sunset (Schmaljohann et al., 2013). Therefore, migratory restlessness was defined as the number of 15 min periods between 1 and 5.5 h after lights-off, during which a bird showed at least five activity counts (after Eikenaar et al., 2014a).

On the afternoon of 21 August, 16 birds, caught over the previous four days, were taken from their cages, fitted with a 0.42 g Holohil BD-2N radio-transmitter and released between 3 and 4 pm. Using a Yaesu FT-290RII receiver and a Yagi hand-held antenna we searched for all tagged birds from 16 fixed points distributed to cover the whole island. Daily searches, starting the day after release, lasted from approx. 10 to 12 am. To exclude temporary misses, each bird was searched for at least two days after the radio-signal had been received for the last time. No tag-losses occurred while the birds were on Helgoland. All procedures were approved by the Ministry for Agriculture, the Environment and Rural Areas, Schleswig-Holstein, Germany.

### Fasting–refueling experiment

From August 2013 onwards, 24 adult northern wheatears, hatched in captivity, were housed indoors in individual cages of 40 × 40 × 50 cm in three separate rooms. They all had ad libitum access to food and water, and the temperature in the rooms was maintained at approx. 20 °C. To promote migratory fueling, on 1 September (when all birds had completed molt) the photo-period in the bird rooms was changed from long days (14 L:10 D) to short days (12 L:12 D). Starting 22 October, when all birds had accumulated large fuel stores (mean and SD fuel load =  $0.67 \pm 0.19$ ), the birds were subjected to a fasting–re-feeding trial to simulate stopover refueling (water was provided ad libitum at all times). Birds were fasted by reducing the daily amount of food to 2 g until they were a few grams above their individual LBM. At that point, they were allowed to refuel by increasing the daily amount of food to 20 g. On the 11th night of refueling, when the birds had replenished a considerable part of their fuel stores (mean and SD fuel load =  $0.55 \pm 0.18$ ), excreta were collected on paper sheets placed on the cage bottom. Sheet were placed in the cages 5 min prior to lights-off and again removed the next morning at lights-on. For each bird, all excreta collected in the entire night were put in a 2 ml Eppendorf tube, weighed and homogenized, and frozen at  $-20$  °C until later processing (see below). Each day of the trial, birds were weighed to the nearest 0.1 g immediately after lights had turned on. Migratory restlessness was recorded as described above, but was defined as the number of 15 min periods in the entire night during which a bird showed at least five activity counts. The reason for using the full night is that, unlike on Helgoland, we do not know the 'time window of departure' for long-term captive northern wheatears. The experiment was conducted at the Institute of Avian Research, Wilhelmshaven, Germany. All procedures were approved by the Lower Saxony State Office for Consumer Protection and Food Safety, Germany.

### Corticosterone assay

Corticosterone levels in plasma were determined using enzyme immunoassay kits (Enzo Life Sciences, Inc., former Assay Designs). Corticosterone in 15  $\mu$ l of plasma (diluted in 200  $\mu$ l double-distilled water) was extracted with 4 ml dichloromethane. The dichloromethane containing the extracted corticosterone was aspirated with a disposable Pasteur pipette and evaporated in a water bath at 48 °C. The remaining corticosterone was re-dissolved in 250  $\mu$ l assay buffer and analyzed in duplicates following the kit manufacturer's protocol (with the only difference

that we used a 6 point standard curve with a range of 20,000 to 15.63 pg/ml). An external standard was run in duplicate on each of the five plates for calculation of intra- and inter-assay variation. To determine extraction efficiency, pooled wheatear plasma samples were spiked with corticosterone standard from the kit. Recoveries of the low (1 ng/ml), intermediate (2.5 ng/ml) and high (10 ng/ml) spikes were 87%, 93% and 70%, respectively. The intra-assay variation ranged from 0.25% to 4.35%, and the inter-assay variation was 16.88%. The lower limit of detection in our assay was 0.83 ng/ml and was assigned to the five undetectable samples in our study.

#### GCM assay

Homogenized samples were thawed at room temperature. To extract GCMs, 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 1 min at maximum speed on a hand-vortex followed by 15 min shaking on a multi-vortex at 400 rpm. Tubes were centrifuged for 1 min and 100  $\mu$ l 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 (Vetmeduni) where samples were re-dissolved in 100  $\mu$ l of 60% methanol and diluted 1:5 in assay buffer. Samples were assayed using antibodies against 4-pregnene-17 $\alpha$ ,21-diol-3,11,20-trione-21-HS bound to bovine serum albumin (Rettenbacher et al., 2004; Stöwe et al., 2013). This assay has previously been validated for male and female northern wheatears with an ACTH-challenge (Eikenaar et al., 2014b). Samples were assayed in duplicate on three plates with an intra-assay variation of 4.2%, an inter-assay variation of 3.8%, and a sensitivity of 2.5 ng/g. 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 (entire night) sample weight.

#### Data analysis

##### Field study

Despite immediate blood-sampling, two birds had extremely high corticosterone levels (>5 SD above the mean). These were excluded from the dataset, because their levels unlikely represented baseline corticosterone. Using SPSS version 21.0 (IBM, New York), we performed generalized linear models (GLM) with a negative binomial distribution (count data with SD > mean) and log link function to analyze our data. Current fuel load during stopover affects departure likelihood (e.g. Eikenaar and Schläfke, 2013; Fusani et al., 2009). Therefore, in the analysis of migratory restlessness, we entered fuel load at capture together with corticosterone level and season into the GLM. Corticosterone level was not correlated with fuel load (Pearson's  $r = 0.11$ ,  $p = 0.22$ ,  $n = 122$ ). We checked for an effect of sex on migratory restlessness, but as there was none ( $\beta \pm SE = 0.134 \pm 0.22$ , Wald = 0.37,  $P = 0.54$ ), sex was removed from the model. We also checked for two-way interactions between independent variables. Since none was significant (season \* fuel load:  $\beta \pm SE = 3.74 \pm 2.35$ , Wald = 2.52,  $P = 0.11$ ; season \* corticosterone:  $\beta \pm SE = -0.11 \pm 0.93$ , Wald = 0.014,  $P = 0.91$ ; fuel load \* corticosterone:  $\beta \pm SE = -0.99 \pm 4.06$ , Wald = 0.06,  $P = 0.81$ ) these were deleted from the model.

The time that the radio-tagged birds spent in captivity before release ranged from 1 to 4 days, meaning that some birds accumulated more fuel than others. Because next to current fuel load, the change in fuel load at stopover also affects departure likelihood (Eikenaar and Schläfke, 2013; Schaub et al., 2008), "change in fuel load during confinement" and "fuel load at release" had to be taken into account in the analysis on the radio-tagged birds. Because of small sample size, principle components analysis was used to collapse these two measures into a single metric that was then, together with corticosterone level, entered into the GLM to explain variation in departure latency. This first principle component (PC1) explained 74% of the variance in fuel dynamics;

birds with a high PC1 score had a high fuel load at release (eigenvector = 0.86) and accumulated much fuel while confined (eigenvector = 0.86). Plasma corticosterone levels were log<sub>10</sub>-transformed prior to analyses to increase normality of the data.

##### Fasting-refueling experiment

To determine whether migratory restlessness was related to GCM level and GCM excretion rate during refueling, we performed multilevel generalized linear mixed models (GLMMs) with a negative binomial error structure using MLwiN 2.0 (Rasbash et al., 2004). Birds were housed in three rooms. By entering room as a random factor, we accounted for the non-independence of observations from the same room. In the fasting-refueling experiment, the change in fuel load during refueling was almost identical to fuel load on the day of dropping collection (Pearson's  $r = 0.89$ ,  $p < 0.001$ ,  $N = 24$ ). Therefore, we entered only the latter into the GLMM together with GCM level, or with hourly GCM excretion rate. Fuel load on the day of GCM measurement was neither correlated with GCM level (Pearson's  $r = -0.24$ ,  $P = 0.26$ ,  $n = 24$ ) nor with hourly GCM excretion rate (Pearson's  $r = -0.18$ ,  $P = 0.41$ ,  $n = 24$ ).

## Results

### Corticosterone and migratory restlessness

Migratory restlessness during the night after capture was positively related with baseline corticosterone level at capture (Table 1, Fig. 1). Fuel load at capture had a positive effect on migratory restlessness (Table 1), meaning that fat birds showed more migratory restlessness during the first night after capture than lean birds. Migratory restlessness was higher in spring than in autumn (Table 1, Fig. 1).

### Corticosterone and departure likelihood

Recapture probability of the radio-tagged birds was 1, meaning that no bird was ever missed and again observed in a later search. The number of nights that the birds stayed on Helgoland after being released tended to decrease with corticosterone level at capture ( $\beta \pm SE = -5.22 \pm 3.07$ , Wald = 2.89,  $P = 0.089$ ,  $n = 16$ ). Birds with high corticosterone level thus tended to depart sooner after being released than birds with low corticosterone level (Fig. 2). The PC1 of "change in fuel load during confinement" and "fuel load at release" had a significant negative effect on the number of nights stayed ( $\beta \pm SE = -1.90 \pm 0.86$ , Wald = 4.87,  $P = 0.027$ ,  $n = 16$ ). This shows that birds that gained much fuel during confinement and that were released with a high fuel load were more likely to depart sooner.

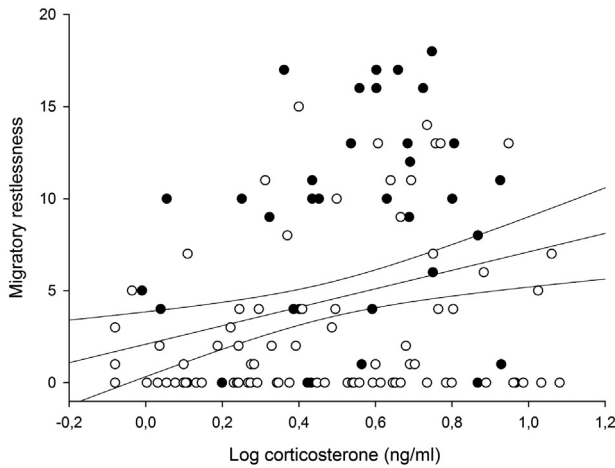
### Fecal GCMs and migratory restlessness

There was a positive relationship between concurrently measured GCM level and migratory restlessness ( $\beta \pm SE = 0.99 \pm 0.33$ ,  $\chi^2 = 8.88$ ,  $P = 0.003$ ,  $n = 24$ , Fig. 3). Fuel load was unrelated with migratory restlessness ( $\beta \pm SE = 1.17 \pm 1.16$ ,  $\chi^2 = 1.02$ ,  $P = 0.31$ ,  $n = 24$ ). Results were very similar when, instead of GCM level, hourly GCM excretion rate was entered into the model (GCM excretion rate:  $\beta \pm SE = 0.014 \pm 0.004$ ,  $\chi^2 = 9.30$ ,  $P = 0.002$ ,  $n = 24$ ; fuel load:  $\beta \pm SE = 0.85 \pm 1.15$ ,  $\chi^2 = 0.55$ ,  $P = 0.46$ ,  $n = 24$ ).

**Table 1**

Relationships between migratory restlessness and baseline plasma corticosterone level, season and fuel load at capture in northern wheatears during stopover ( $n = 122$ ).

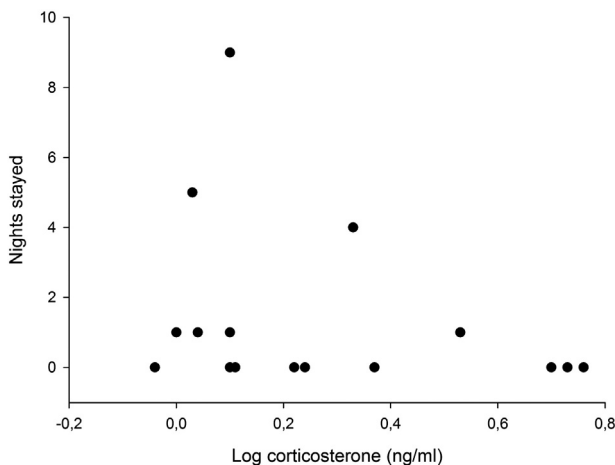
Variable	$\beta \pm SE$	Wald	p
Corticosterone	1.35 $\pm$ 0.39	12.12	0.001
Season (spring)	0.80 $\pm$ 0.23	11.70	0.001
Fuel load	2.24 $\pm$ 1.03	4.72	0.030



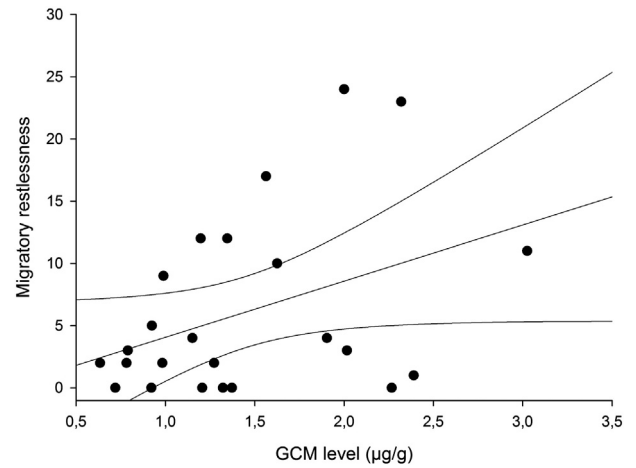
**Fig. 1.** The relationship between baseline plasma corticosterone level and migratory restlessness in northern wheatears during autumn (open circles) and spring (closed circles) stopover on Helgoland ( $n = 122$ ). The linear regression line with 95% confidence limits is plotted.

## Discussion

Our results are consistent with the hypothesis that, at baseline levels, corticosterone is involved in the regulation of departure of migrants at stopover. First, migratory restlessness in northern wheatears caught at stopover was positively correlated with corticosterone level. Part of the variation in migratory restlessness unexplained by corticosterone was attributable to the fuel reserves the birds carried and to the season in which they were measured (Table 1). The former confirms previous findings in this species (Eikenaar and Schläpffe, 2013), whereas the latter perhaps reflects the fact that migratory birds generally travel faster in spring than in autumn (Nilsson et al., 2013). Second, in a fasting–refueling experiment on long-term captive northern wheatears, migratory restlessness was positively correlated with concurrently measured fecal GCM level. It should be noted that because northern wheatears have a minimum gut passage time of approx. 30 min (Franz Bairlein, unpublished data), the plasma corticosterone excreted and metabolized over the last hour(s) before lights went off could, to a small extent, be reflected in the GCM levels. Third, the likelihood of departure from stopover seemed to increase with increasing corticosterone level. Our results solidify the observations from previous studies that already suggested a role for baseline corticosterone in regulating departure during



**Fig. 2.** The effect of baseline plasma corticosterone at capture on the number of nights the northern wheatears stayed on Helgoland after release from temporary confinement in autumn ( $n = 16$ ).



**Fig. 3.** The relationship between concurrently measured dropping glucocorticoid metabolite level (GCM) and migratory restlessness in refueling long-term captive northern wheatears ( $n = 24$ ). The linear regression line with 95% confidence limits is plotted.

migration (Holberton et al., 2008; Landys et al., 2004; Landys-Cianelli et al., 2002; Löhmus et al., 2003; Nilsson and Sandell, 2011; Piersma et al., 2000; Schwabl et al., 1991).

We propose the following role for corticosterone in departure from stopover. Migrants that are ready to start a flight bout appear to up-regulate their corticosterone level to be able to deal with metabolic and behavioral challenges of the coming flight bout (Landys-Cianelli et al., 2002; Piersma et al., 2000). For example, corticosterone could mobilize stored energy, e.g. increase free fatty acids, needed for the oncoming flight bout (Landys-Cianelli et al., 2002). As corticosterone is known to stimulate locomotor activity (reviewed in Landys et al., 2006), the up-regulation of corticosterone may increase the probability of take-off. Such a scenario fits our observation that birds with low corticosterone (metabolite) level both showed little migratory restlessness, and were unlikely to depart. In the field study, birds were blood-sampled immediately after capture which resulted in a time-lag between the hormonal and behavioral (restlessness) measurements. This may have introduced some noise to our data, because day- and night-time corticosterone levels could have differed (e.g. Breuner et al., 1999), and caging could have affected corticosterone level (Wingfield et al., 1982). However, the noise was probably small because the positive association of migratory restlessness with corticosterone persisted in the absence of a time-lag (GCM measurements). Moreover, the likelihood that birds departed from stopover was affected by corticosterone, even though the hormonal signal was measured one to several days before birds were given the opportunity to depart. Besides corticosterone, other hormones, such as tri-iodothyronine and melatonin, have been linked to migratory restlessness (reviewed in Cornelius et al., 2013). Most likely, the collective signals of multiple hormones are integrated in specific regions in the central nervous system to influence departure decisions in migrating birds.

Fluctuations in circulating hormone levels affect the likelihood that an animal shows a particular behavior, but behavior can also affect circulating hormone levels. For example, and relevant to the current study, exercise has been shown to increase circulating corticosterone levels in captive birds (e.g. Harvey and Phillips, 1982; Rees et al., 1984). Therefore, we cannot exclude the possibility that in our study the increased corticosterone (metabolites) levels were a consequence, and not the cause, of increased locomotor activity (measured as migratory restlessness). This, however, seems unlikely for several reasons. First, red knots performing 10 h non-stop flights in wind-tunnels during their natural migration period did not increase their baseline corticosterone level (Jenni-Eiermann et al., 2009). Second, exogenous corticosterone enhanced feeding frequency in red-eyed vireos caught and temporarily caged at stopover (Löhmus et al., 2006). Together

these studies suggest that in migrating birds corticosterone affects locomotion and not, or to a lesser extent, vice versa. Future manipulative studies, i.e. corticosterone treatment, could elucidate whether the positive relationship between baseline corticosterone and migratory restlessness is a causal one.

A question remaining is why, if corticosterone is up-regulated to increase locomotor activity in birds that are ready for departure, did part of the birds with high corticosterone level show very little migratory restlessness? One possible explanation is that, whereas at baseline levels, corticosterone stimulates locomotor activity, at stress-related levels, corticosterone does not have this effect (Breuner et al., 1998). Maybe caging stressed some birds, including part of those caught with high baseline corticosterone level, to such an extent that they showed little or no migratory restlessness. Or perhaps these birds had elevated corticosterone levels because they had exhausted their fuel reserves (Jenni et al., 2000). Out of the 14 birds with the highest corticosterone levels, eight showed no or very little migratory restlessness, whereas six did show restlessness (Fig. 1). Fuel stores were indeed smaller in the former (mean and SD:  $0.022 \pm 0.056$ ) than in the latter (mean and SD:  $0.086 \pm 0.098$ ), but not significantly so ( $t = 1.52$ ,  $p = 0.15$ ,  $N = 14$ ). Another possible explanation is that some birds may have had relatively high corticosterone levels at capture for reasons that have nothing to do with departure readiness. For example, some birds may have experienced a stressful event prior to being caught, e.g. escape from a sparrowhawk, an abundant predator on Helgoland during migration seasons (Schmaljohann and Dierschke, 2005; CE pers. obs.). Such stressful events are reflected in the corticosterone level some time later, and could possibly explain some of the highest corticosterone levels. The highest levels in the current study indeed correspond to levels measured in northern wheatears subjected to a standard capture-stress protocol (sampled 30 min after capture, CE unpubl. data). By the time we measured these birds' (nocturnal) migratory restlessness, their corticosterone would have returned to much lower baseline levels, at which less migratory restlessness is expected to occur.

We can only speculate on how corticosterone is up-regulated in migrants that are ready to depart, but likely other hormones are involved. In several bird species, including northern wheatears, the speed at which fuel reserves are replenished (fuel deposition rate) at stopover decreases towards departure and may even be negative the day prior to departure (Fransson, 1998; Delingat et al., 2006; Bayly, 2007, but see Bayly, 2006). As fuel deposition rate mainly depends on food intake (Eikenaar et al., 2014b; Lindström, 2003), migrants that are ready to depart have a low food intake, which probably affects levels of hormones secreted by the gut and gastro-intestinal tract. One such hormone is ghrelin, of which plasma level in birds indeed increases after fasting (Kaiya et al., 2007; Ocloń and Pietras, 2011; Shousha et al., 2005). It is also known that in birds exogenous ghrelin stimulates corticosterone release within minutes after administration (Kaiya et al., 2002; Saito et al., 2005). In summary, low food intake in birds ready to depart may increase ghrelin level, which in turn increases corticosterone level. This mechanism could operate even for small birds employing a stop-and-go strategy (single day stopovers), because maximum retention time of food in such species is only a few hours (e.g. Hume and Biebach, 1996). It should be noted that birds with low food intake are not in a total fasted state and that low food intake may not have the same effect on ghrelin levels as complete fasting. Nonetheless, some support for this scenario already exists in the observations that, in northern wheatears, corticosterone level is negatively correlated with both fuel deposition rate and food intake while refueling (Eikenaar et al., 2013, 2014b). Concurrent measurement of corticosterone and ghrelin levels in birds at stopover would be a next step to test the validity of this idea.

In contrast to the field study, fuel load did not predict migratory restlessness in the fasting-refueling experiment on captive birds. One possible explanation for the absence of a relationship between fuel

reserves and restlessness is that imposed fasting induced an emergency life-history stage in (some of the) birds, which may have stimulated them to accumulate fat as a risk aversion response rather than as pre-departure fattening per se. Indeed, several studies on captive birds have found an increase in corticosterone level as a consequence of a 24 h fast (e.g. Lynn et al., 2003, 2010). However, the birds in these studies were not in a migratory state, and in contrast to these results, a three day fast in migratory active northern wheatears resulted in a reduction of corticosterone level (Eikenaar et al., 2014b). Clearly, more research is required to understand under what circumstances fasting could induce an emergency life-history stage.

## Acknowledgments

We thank Jochen Dierschke and Klaus Müller for logistical support on Helgoland, Tessina de Lille, Ulrich Meyer and Adolf Völk for assistance with the captive birds, and Samy El Makarem for help with the GCM analysis. We thank two anonymous reviewers for their useful suggestions.

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