Research Article

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Migratory Species Show Distinct Patterns in Corticosterone Levels during Spring and Autumn Migrations

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Abstract: Twice a year billions of birds migrate between breeding and wintering grounds. To facilitate migrations, birds develop migratory disposition, a complex suite of behavioral and physiological adjustments. Glucocorticoid hormone corticosterone is involved in the regulation of migratory behavior and physiology, however no consensus on its exact role in controlling avian migration exists. Using a large dataset on seven songbird species (long- and short-distance migrants) obtained during eleven consecutive migratory seasons on the Courish Spit of the Baltic Sea, we showed the general tendency of similar baseline corticosterone concentrations during both migrations, although stress-induced levels were generally much higher during spring. No difference between long- and short-distance migrants was found in either baseline or stress-induced levels, while there was substantial between-species variation, especially in baseline concentrations. The distinct patterns of corticosterone secretion during seasonal migrations even in ecologically similar species indicate that it is likely to be a species-specific trait. Thus, our study corroborates the inconsistency found in earlier studies and demonstrates how scientific understanding of the role of corticosterone during migration is still evolving. Rather low baseline corticosterone concentrations observed in this study emphasize that birds in both migratory seasons were not in a “stressed” state before capture.

Keywords: Birds, migrations, corticosterone, endocrine regulation, migratory strategies

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1 Introduction

Twice a year billions of birds migrate between breeding and wintering grounds [1]. During both migratory seasons birds cover large distances and often follow similar routes and expend similar amounts of energy each way [2, 3]. To facilitate migrations, birds develop a complex suite of behavioral, physiological and often morphological adjustments known as migratory disposition [4-6].

At the same time, according to the finite state machine theory [6-8], spring and autumn migrations are independent life-history stages occupying unique positions within an annual cycle that enables specifics in regulatory mechanisms. There are many behavioral, physiological and environmental differences between migratory seasons [9]. The overall speed of spring migration is often higher, and its total duration is shorter than in autumn [10-12]. Environmental factors, such as temperature, wind conditions, and food abundance, also differ significantly [13]. Spring migration coincides with development of the reproductive system and increasing level of sex hormones [14-17]. During autumn migration, the reproductive system is inactive and the level of sex hormones is low. Some data indicate that concentrations of other hormones highly likely to be involved in the regulation of migratory disposition can also differ between migratory seasons, e.g. triiodothyronine (T3) [14] and prolactin [18]. All these discrepancies could potentially lead to the development of specific regulatory mechanisms of spring and autumn migratory disposition [9, 19]. Supporting this idea, Sharma et al. [14] recently reported seasonal difference in hypothalamic expression of genes associated with the control of avian migration.

Like other behaviors and physiology, components of migratory disposition are under the control of the endocrine system. Among many hormones, the role of corticosterone in the control of avian migration has been intensively studied during the last three decades [9, 20-22]. There are numerous indications that baseline level

of corticosterone is involved in regulation of migratory features such as migratory feeding behavior and fueling [22-25], level of migratory restlessness [26-28], migratory orientation [27] and even such complex behavior as actual departure from stopover sites [21, 29]. Moderately elevated baseline corticosterone levels (i.e., not the levels observed in stress response) were associated with energy mobilization during migratory flight [23, 30, 31]. The other general function of glucocorticoids is modulation of the organism's response to environmental changes [32]. As birds *en route* often occur in places with an unknown distribution of resources and threats, stress-induced corticosterone levels are likely involved in solving various emergency tasks [33]. However, current knowledge on the exact role of corticosterone in the regulation of migratory behavior and physiology is still controversial, e.g. there is no consensus on the relative concentrations of corticosterone during spring and autumn migrations.

There is only a very limited number of field studies where corticosterone concentrations in spring and autumn are directly compared. Romero et al. [34, 35] observed a distinct plasma corticosterone levels in white-crowned sparrows (*Zonotrichia leucophrys gambelii*) in North America with spring concentrations much higher than autumn levels. Raja-aho et al. [36] reported high baseline corticosterone levels in barn swallows (*Hirundo rustica*) captured just before northward departure from African winter quarters in spring versus low levels in captures just before southward departure from Finland in autumn. However, Eikenaar et al. [37] did not observe a seasonal difference in baseline corticosterone levels in migratory blackbirds (*Turdus merula*) and northern wheatears (*Oenanthe oenanthe*) on the German island Helgoland. Similarly, Bauer et al. [38] reported similar baseline and stress-induced corticosterone concentrations in dark-eyed juncos (*Junco hyemalis*) captured during seasonal migrations in USA. The patterns found in migratory waders were also inconclusive [39, 40]. Most of these studies were performed during only one pair of migratory seasons and no information was provided regarding reproducibility of the observed pattern in subsequent seasons. Loshchagina et al. [41] showed in the analysis of data obtained during seven consecutive migratory seasons that in European robins (*Erithacus rubecula*) captured in the south-eastern Baltic, baseline and stress-induced corticosterone concentrations were nearly twice as high during spring migration as compared with autumn passage. Summarizing, published results do not provide a uniform picture on the difference in corticosterone concentrations between spring and autumn migrations, probably due to environmental variation between sites and seasons.

Moreover, published data concern only a limited number of avian species which vary in ecology, travel various distances and can be conventionally separated into short- or long-distance migrants. It is well established that the expression of many migratory traits is dependent on migratory strategy [19, 42, 43, 44]. Most long-distance migrants cross ecological barriers (e.g. deserts, seas or mountains) and perform long non-stop flights which set specific requirements on fueling strategy in contrast to short-distance migrants, which can safely fuel up during the entire journey [45, 46]. Due to the longer distance the annual cycle of long-distance migrants is more time-constrained and as a result, the expression of many behavioral aspects and physiology is under strict endogenous control [47, 48]. Short-distance migrants, in contrast, rely less on endogenous programmes and environmental factors contribute much more in the regulation of their behavior and physiology [42, 49, 50]. Therefore employing migratory strategy can be reflected in peculiarities of regulatory mechanisms [8, 42].

Our main aim was to compare baseline and stress-induced corticosterone concentrations between spring and autumn migratory seasons in seven songbird species (both short- and long-distance migrants) captured on the Courish Spit in the Baltic Sea. Collecting data during the same seasons and at the same site enabled us to exclude the potential effects of environmental conditions on corticosterone concentrations. We expected that: (1) based on our results in European robin [41], and assuming that baseline corticosterone orchestrates migratory disposition independently of migratory season, baseline corticosterone concentrations should be higher in spring in all species irrespective of their migratory strategy; (2) relatively higher baseline corticosterone level in long-distance compared to short-distance migrants due to a longer migratory distance and associated higher energetic demands and the need for intensification of fat catabolism in long-distance migrants [46]; (3) all species should respond to stress with a marked elevation of corticosterone concentrations in both migratory seasons assuming that the appropriate stress response is essential for survival [32, 33]; (4) a higher stress response in spring than autumn migrants [51] since they might experience more stressful situations due to less predictable environmental conditions in spring [9, 52, 53]; (5) adrenocortical response to stress is modulated by energetic condition with lean (but not exhausted) individuals expressing higher stress-response than fat birds [41, 54]. We have no specific predictions on the dependence of stress-induced corticosterone concentrations on migratory strategy, as we know of no *a priori* reasons why short- and long-distance

migrants should differently respond to various stressful situations during migrations.

As we did not observe behavior and physiology in sampled birds, our main purpose was to provide reliable estimates of baseline and stress-induced corticosterone concentrations during seasonal migrations without testing any causal relationships.

2 Methods

2.1 Study site and model species

The study was performed at the Biological Station Rybachy of the Zoological Institute RAS on the Courish Spit in the Baltic Sea in eleven consecutive migratory seasons (autumn 2013 – autumn 2018). It is widely assumed that geographic features influence migratory behavior, e.g. propensity to initiate migratory flight and migratory orientation [55-57]. As corticosterone is likely involved in the regulation of such behaviors [21, 27, 29], accounting for geographic context is crucial. The Courish Spit is located along to the main migratory direction of birds in

the Eastern Baltic, as suggested by the distribution of ring recoveries (Fig.1A) [58-60]. Tracking of birds in nocturnal migratory flight at the study site revealed that birds use the Courish spit as a leading line and follow it even in crosswinds [61]. Analysis of ring recoveries in the geographic area adjacent to the Courish Spit showed that in the Baltic region birds rarely perform long non-stop flights, with median distance of single flight of only ca. 250 km [62]. This evidence leads us to believe that most birds passing through the Courish Spit or making a stopover here neither prepare for barrier crossing, no recover from previous long-distance flight. However, we suppose that birds may differ in their behavior, distance of single migratory flight, fueling performance etc. according to species-specific or individual migratory tactics.

Trapping of birds was conducted at two field sites located 11 km apart (Fig. 1B). At Rybachy field site (55°09'N, 20°51'E) birds were captured in mist nets; at Fringilla field site (55°05'N, 20°44'E) birds were captured in Rybachy-type funnel traps and mist nets. The funnel traps were located in the open area at the border between pine forest and sand dunes. Mist nets at Fringilla were placed in a deciduous woodland patch around a small

Figure 1. Map of Europe (A) and location of Courish spit (B) with two field sites. Arrows indicate the common flyways of populations passing the Courish spit during seasonal migrations (arrows indicate autumn directions).

artificial pond. Vegetation within the trapping area at Rybachy included two primary habitats: (1) reed bed (predominantly *Phragmites australis*) and (2) shrubs with deciduous trees [63]. In Rybachy-type funnel traps birds were captured 1) during actual migratory flight at daytime, 2) while and shortly after landing at dawn and 3) during pre-departure activity at dusk. In mist nets, birds were captured during the entire day; these captures represent individuals at different stages of stopover (from newly landed birds to those ready for a new migratory flight). In both seasons we captured birds during the standard time-scheme adopted at the Biological Station Rybachy: spring (late March – early June), autumn (last days of August – end of October). For analysis we selected seven species with at least 4–5 blood samples per species collected in spring and autumn during the entire study. According to analysis of ring recoveries of birds captured on the Courish Spit [58], transit populations of song thrush (*Turdus philomelos*) and blackbird (*T. merula*) are short-distance nocturnal migrants; chaffinch (*Fringilla coelebs*) is short-distance diurnal migrant and blackcap (*Sylvia atricapilla*), garden warbler (*S. borin*), reed warbler (*Acrocephalus scirpaceus*) and sedge warbler (*A. schoenobaenus*) are long-distance nocturnal migrants. For short-distance migrants we assume that bird species/populations winter within Europe and migrate from hundreds to nearly three thousand kilometers from the study site. Long-distance migrants winter in Africa and cross the Sahara desert with a travel distance of at least 4.5 thousand kilometers.

As the Courish Spit is located within the breeding range for these species, we selected only samples collected within the main passage time of each species to ensure that birds were actively migrating. The latter was supported by the fact that all but three (one blackcap and two reed warblers were ringed some days before sampling, all in spring) sampled birds were newly captured which were never recaptured during the same or subsequent seasons. The rather large numbers of captured birds in comparison with the number of pairs breeding in the surroundings of the trapping sites along with criteria presented above make the capturing probability of local birds very low. Additionally, to exclude local birds in spring, we selected only individuals without brood patches. In autumn, only birds which had completed or nearly completed post-breeding or post-juvenile molt were selected for corticosterone analysis. Trapping and animal handling comply with the animal care laws of the Russian Federation and were approved by the ethical committee of the Zoological Institute of Russian Academy of Sciences (permit #2013-01-03 and #2015-01-04).

2.2 Blood sampling

We followed a standard stress-restraint protocol to measure baseline and stress-induced corticosterone concentrations [33]. We used a stopwatch to measure the time between the bird colliding with the net (or when it crossed the entrance of the funnel trap) and the end of the first blood sampling. This time varied between 58 s and 5 min 52 s (2 min 24 s, on average). After the first sampling, the birds were kept in a cloth bag, and we tried to take the second sample within 20 min from capture. However, because a new bird was sometimes captured shortly before time of the second blood sample, and because obtaining the baseline corticosterone concentration was a priority, the total time for stress-induced samples to be taken varied between 18 and 40 min after the capture (22 min, on average). It was not always possible to collect the second sample; therefore, the sample size for baseline and stress-induced corticosterone concentrations were not equal. As recommended by Romero [64], in our study we reported absolute stress-induced corticosterone concentrations rather than change from baseline. Blood was taken from the brachial vein into heparinized microtubes; the volume of each sample did not exceed 75 µl. After blood sampling, the birds were ringed, weighed to the nearest 0.1 g, and the wing length was measured to the nearest 0.5 mm. After all manipulations, the birds were released into the wild. Blood samples were centrifuged in a hematocrit centrifuge Elmi CM-70 (Elmi Ltd., Latvia) within an hour after sampling. The plasma was transferred into 0.5 ml Eppendorf tubes and frozen at -20°C for further analysis.

2.3 Corticosterone assay

Laboratory analysis was conducted at A.N. Severtsov Institute of Ecology and Evolution, Moscow, at the Joint Usage Centre “Live collection of wild mammalian species.” Plasma was diluted two-fold with phosphate-buffered saline. Corticosterone concentrations were analyzed directly in the diluted plasma by ELISA kits (EIA-4164, DRG International Inc., United States) following the manufacturer guidelines. According to the specification, corticosterone antibody cross-reactivity to other steroids was 7.4% to progesterone, 3.4% to deoxycorticosterone, 1.6% to 11-dehydrocorticosterone, 0.3% to cortisol and pregnenolone, and less than 0.1% to other steroids. Previously this ELISA kit was validated in the measurement of plasma corticosterone in a short-distance migrant,

European robin [41]. Here we performed a parallelism test for long-distance migrant, the garden warbler. For this, we serially diluted a sample with an initial corticosterone concentration of 86 ng/ml. The standard curve followed the plasma dilution curve in a parallel manner, indicating that the ELISA kits measured corticosterone concentrations precisely. The intra assay coefficient of variation was 2.97% ($n = 402$); the inter assay coefficient of variation was 18.5% ($n = 30$). The detection range of the standard curve varied from 0.56 to 83.16 ng/ml. Thirteen samples fell outside the lower limit. There was not enough plasma to re-assay these samples without dilution, so we excluded them from further analysis. The samples that fell outside the upper limit were re-assayed at a higher dilution. In two cases, there was not enough plasma for re-assaying, and we excluded these samples as well. Most samples were analyzed in duplicates ($n=402$), but in some cases, because of small blood volume (especially for re-assayed samples), we had to measure them in single wells ($n=66$). In total, for seasonal comparisons, we analyzed 245 samples for baseline and 223 samples for stress-induced corticosterone concentrations (Tab. 1).

2.4 Statistical analyses

Statistical analyses were performed using R software version 3.5.0 [65]. We used *dotchart* plots for visual analysis of data structure and identifying outliers [66]. Corticosterone concentrations that exceeded the respective species' means (baseline and stress-induced separately) by more than 3.5 standard deviations were treated as outliers and excluded from further analysis (Table 1). Retaining such high values in the analysis yielded similar

results, but inflated model parameter estimates. To analyze seasonal differences in corticosterone levels we applied a two-step approach. First, we compared baseline and stress-induced corticosterone concentrations in spring and autumn separately for each species. Second, to reveal general patterns in seasonal difference in corticosterone concentrations, we built joint models for baseline and stress-induced corticosterone concentrations incorporating all species. The same models allowed us to check if employing migratory strategy (long- or short-distance migrations) contributes to species-specific corticosterone levels and to the presence of the seasonal difference in hormone levels (see below).

To compare plasma corticosterone concentrations between spring and autumn in three species with the largest sample sizes (blackbird, song thrush and blackcap, Table 1), we used linear mixed effects models [67] implemented in *lme4* package [68]. Separate models were constructed for each species and for baseline and stress-induced corticosterone levels. To normalize distributions, corticosterone concentrations were log10 transformed. Based on the results found in European robins [41], in addition to *season* (spring vs. autumn), we included the following fixed factors into the final models for baseline corticosterone level: *sampling time_A* (time between capture and the end of the first blood sampling, min) and *body condition index* (body mass *g* divided by wing length *mm*). Before building the final models, by means of general linear models, we also checked for the possible effects of *date of capture* (expressed as deviation of capture date from the median date of passage of a particular species in the respective season), *time of capture* (relative to sunrise, min), *age* and *sex* (the later in blackbirds and blackcaps only). As there was no indication on the effects of these

Table 1. Sample size, range and mean values ($\pm$ SD) of corticosterone concentrations in seven model passerine species captured on the Courish Spit of the Baltic Sea in 2013-2018. The outliers, i.e. values which stand for more than 3.5 standard deviations from the mean, are given in brackets.

	Baseline			Stress-induced		
	n	range (outliers)	mean	n	range (outliers)	mean
Blackbird	71	2.2 – 39.2 (58.8)	12.1 $\pm$ 9.6	70	23.4 – 172.1	75.9 $\pm$ 32.8
Song thrush	60	1.5 – 63.7	16.6 $\pm$ 13.6	58	5.9 – 189.9	75.2 $\pm$ 45.2
Blackcap	58	2.3 – 29.5 (86.6, 132.0)	13.0 $\pm$ 19.9	56	6.9 – 165.7	42.6 $\pm$ 36.9
Garden warbler	22	1.6 – 13.2 (43.2)	8.9 $\pm$ 8.4	14	15.5 – 121.5	64.2 $\pm$ 37.6
Reed warbler	11	6.0 – 46.1	21.2 $\pm$ 10.8	10	32.2 – 347.3	131.9 $\pm$ 85.8
Sedge warbler	8	8.1 – 19.5	11.6 $\pm$ 3.7	7	27.7 – 95.8	62.8 $\pm$ 26.0
Chaffinch	15	2.9 – 55.1	22.2 $\pm$ 14.3	15	22.8 – 104.4	64.1 $\pm$ 26.9

variables, we did not include them into the final models. *Year* and *field site* were included as random factors for thrush models, and only *year* was included in models for blackcaps as all but one individual were captured at Rybachy field site. The same variables were included in the final models for stress-induced corticosterone levels, with *sampling time_B* representing the time between capture and the end of the second blood sampling. We also checked whether slopes of the relationships between corticosterone concentrations (separately for baseline and stress-induced levels) and continuous variables differed between spring and autumn migratory seasons. In all but one case (see below) these relationships (if present) did not differ between spring and autumn (lm, $\text{season} \times \text{covariate}$ interactions, $p > 0.05$); therefore, the main effects only were included into the final models. In song thrush, the slope between stress-induced corticosterone and *body condition index* showed opposite relationships for spring and autumn (lm, $\text{season} \times \text{body condition index}$ interaction: $t = -2.17$, $p = 0.04$); therefore, for song thrush we included this interaction into the final model for stress-induced corticosterone concentration. The significance of the difference in corticosterone concentrations between spring and autumn was tested using the *summary* function of the *lmerTest* package [69]. The model fit was tested by visual estimation of residual plots [67, 70], which had no signs of violation of underlying assumptions. For simplicity, model results were recalculated in absolute values of predicted corticosterone concentrations. Predicted values account for effects of covariates included in the analysis, which in our case allows for the possible imbalance in the distribution of covariates between the migratory seasons.

To compare seasonal corticosterone levels in other four species with moderate sample sizes (reed and sedge warblers, garden warbler and chaffinch, Table 1) we used non-parametric Wilcoxon rank sum test (implemented in *wilcox.test* R function) on non-transformed corticosterone concentrations.

Finally, to check for a general pattern in the seasonal difference in corticosterone levels in model bird species, we constructed linear mixed effects models (separately for baseline and stress-induced corticosterone concentrations) where we used all hormone measurements and treated *species* as a random factor [70]. Corticosterone concentrations were log₁₀ transformed. In addition to *season*, we included migratory *strategy* (with garden, reed and sedge warblers, and blackcaps treated as long-distance migrants; and blackbird, song thrush and chaffinch treated as short-distance migrants) as a second fixed factor in the models. Not all species were captured in every year and at each field site. Therefore, to correct

for environmental variance attributed to year of capture and field site, we included a single random factor called *environment* with all possible year-field site combinations as factor levels, as it was suggested for crossed random effects [70]. As separate analysis for each species revealed a significant increase of baseline corticosterone levels with sampling time in some but not other species (see Results), we included *sampling time_A* as a random slope for *species* to account for this between-species variation. Significance testing and assessing the model assumptions were performed as described above.

3 Results

For both baseline and stress-induced corticosterone levels the most representative samples for seasonal comparisons were obtained for blackbirds, song thrushes and blackcaps (Table 1). There were only a few samples with high baseline corticosterone levels (Table 1). We assume that individuals with high baseline corticosterone levels could have been stressed before capture, and the corresponding corticosterone values were unlikely to represent baseline hormone levels, i.e. are outliers. All stress-induced corticosterone samples fall within 3.5 SD from the respective means.

3.1 Baseline concentrations

Neither of the three species with large sample sizes showed a difference in baseline corticosterone levels between spring and autumn migratory seasons (lmm, *season* effect: blackbird, $t = 0.63$, $p = 0.54$; song thrush, $t = -0.24$, $p = 0.81$; blackcap, $t = -1.2$, $p = 0.24$; Table 2, Fig. 2). Mean values predicted by the models were 10.8 ± 1.4 (SE) ng/ml for spring and 9.5 ± 0.4 ng/ml for autumn migrations in blackbird, 12.1 ± 0.7 ng/ml and 12.1 ± 0.3 ng/ml in song thrush, and 8.9 ± 0.8 ng/ml and 8.4 ± 0.4 ng/ml in blackcap, respectively (Fig. 2). In blackbirds, there was an inverse relationship between baseline corticosterone levels and *body condition index*, i.e. in both seasons lean individuals had elevated baseline corticosterone concentration (Table 2, Fig. 3). In song thrushes, no single variable affected baseline hormone levels (Table 2). In blackcaps, we observed a positive trend of baseline corticosterone concentrations with sampling time (Table 2, Fig 4), which we assume is partly attributed to the longest mean sampling time compared with the other two species (2.49 ± 0.91 (SD) min versus 1.95 ± 0.58 in blackbird and 2.36 ± 0.78 in song thrush). The *year* of

sampling and the *field site* had substantial effect on the baseline corticosterone concentrations in blackbirds, and *year* of sampling in blackcaps (Table 2).

There was no significant difference in the baseline corticosterone level between migratory seasons in four species with moderate sample sizes (Table 3, Fig. 5). In two species, reed and sedge warblers, median values in spring were less than median values in autumn (Fig. 5). In chaffinch, spring baseline hormone concentrations were twice as high compared with autumn migration and this difference nearly reached significance (Table 3, Fig. 5).

Figure 2. Predicted baseline corticosterone concentrations (ng/ml) in three model passerine species (blackbird, song thrush and blackcap) captured on the Courish Spit during seasonal migrations in 2013 - 2018. Plotted are the median (horizontal line in the box), 25th and 75th percentiles (horizontal box boundaries), and 10th and 90th percentiles (whiskers). Open dots indicate outliers.

Figure 3. Relationship between body condition index and baseline corticosterone concentrations (log10 transformed) in blackbirds trapped on the Courish Spit during autumn and spring migratory seasons in 2013-2018. A regression line is shown.

Combined analysis that incorporates all model species revealed no general tendency in seasonal difference in baseline corticosterone levels (lmm, *season* effect, $\beta = -0.009 \pm 0.05$ (SE), $t = -0.2$, $p = 0.9$, Table 4). The employed migratory strategy did not affect the baseline hormone level either (lmm, *migratory strategy* effect, $\beta = 0.1 \pm 0.07$ (SE), $t = 1.5$, $p = 0.18$, Table 4). Predicted baseline corticosterone concentration averaged by species and migratory seasons was 6.3 ± 1.2 (SE) ng/ml. Analysis of random effects showed that between-year and between-field site variation in environmental conditions (pooled

Figure 4. Relationship between sampling time and baseline corticosterone concentrations (log10 transformed) in blackcaps trapped on the Courish Spit during autumn (red circles) and spring (blue squares) migratory seasons in 2013-2018. Regression lines for both seasons are shown.

Figure 5. Raw baseline corticosterone concentrations (ng/ml) in four model passerine species (garden warbler, reed warbler, sedge warbler and chaffinch) captured on the Courish Spit during seasonal migrations in 2013 - 2018. Plotted are the median (horizontal line in the box), 25th and 75th percentiles (horizontal box boundaries), and 10th and 90th percentiles (whiskers). Open dots indicate outliers.

Table 2. Effects of season, sampling time and body condition index on baseline and stress-induced corticosterone concentrations (both log10 transformed) in blackbirds, song thrushes and blackcaps captured on the Courish spit during seasonal migrations in 2013-2018. Results of linear mixed effects models are shown. β refers to the model estimate. Significant effects are marked **bold**.

	Baseline				Stress-induced			
	β	SE	t	p	β	SE	t	p
BLACKBIRD								
Fixed effects								
<i>Season (spring)</i>	0.07	0.12	0.63	0.54	0.003	0.06	0.05	0.96
<i>Sampling time</i>	0.07	0.07	1.03	0.31	0.01	0.01	1.49	0.14
<i>Body condition index</i>	-1.26	0.60	-2.10	0.04	-0.43	0.42	-1.04	0.30
Random effects (SD)								
<i>Year</i>	0.13				<0.001			
<i>Field site</i>	0.24				0.09			
<i>Residual</i>	0.24				0.18			
SONG THRUSH								
Fixed effects								
<i>Season (spring)</i>	-0.03	0.13	-0.24	0.81	3.78	1.77	2.13	0.04
<i>Sampling time</i>	0.11	0.07	1.46	0.15	-0.01	0.02	-0.28	0.78
<i>Body condition index</i>	-0.14	1.26	-0.11	0.91	-	-	-	-
<i>Bod.con.ind (spring)</i>	-	-	-	-	-4.43	2.94	-1.51	0.14
<i>Bod.con.ind (autumn)</i>	-	-	-	-	2.38	1.26	1.90	0.06
Random effects (SD)								
<i>Year</i>	0				<0.001			
<i>Field site</i>	0				0.03			
<i>Residual</i>	0.37				0.34			
BLACKCAP								
Fixed effects								
<i>Season (spring)</i>	-0.1	0.08	-1.20	0.24	0.45	0.08	5.50	<0.0001
<i>Sampling time</i>	0.17	0.04	4.19	0.0001	0.002	0.01	0.15	0.88
<i>Body condition index</i>	-1.79	2.10	-0.86	0.40	-2.69	2.01	-1.34	0.19
Random effects (SD)								
<i>Year</i>	0.11				<0.001			
<i>Field site</i>	-				-			
<i>Residual</i>	0.23				0.24			

Table 3. Median baseline and stress-induced corticosterone concentrations (ng/ml) for four species with moderate sample sizes, captured on the Courish Spit during spring and autumn migrations in 2013-2018. The difference between migratory seasons was tested by Wilcoxon rank sum test. Significant difference is marked **bold**

	Baseline			Stress-induced		
	Spring	Autumn	Wilcoxon	Spring	Autumn	Wilcoxon
Garden warbler	8.1	6.0	W=25, p=0.46	96.1	44.1	W=9, p=0.14
Reed warbler	19.9	24.0	W=21, p=0.33	138.3	92.5	W=1, p=0.02
Sedge warbler	10.1	12.0	W=11, p=0.49	58.0	68.3	W=6, p=1.0
Chaffinch	20.9	11.1	W=7, p=0.06	80.6	41.6	W=9, p=0.1

in random factor *environment*, see above) had smaller influence on baseline corticosterone levels in model passerines compared with between-species differences (Table 4).

3.2 Stress-induced concentrations

A highly significant seasonal difference in stress-induced corticosterone levels was observed only in blackcaps (Imm, effect of the *season*: $t = 5.50$, $p < 0.0001$), whereas there were no difference in blackbirds ($t = 0.05$, $p = 0.96$) and marginally significant increase in stress response during spring migration in song thrushes ($t = 2.13$, $p = 0.04$, Table 2, Fig. 6). Accordingly, in blackcaps mean values predicted by the models were three times higher for spring migration (72.4 ± 1.8 (SE) ng/ml) than for autumn migration (23.4 ± 0.4 ng/ml; Fig. 6). In blackbirds, predicted values were 70.2 ± 2.2 ng/ml for spring and 69.6 ± 1.1 ng/ml for autumn (Fig. 6), in song thrush 64.2 ± 6.2 ng/ml and 60.2 ± 1.9 ng/ml, respectively (Fig. 6). There were no effects of *sampling time* on stress response in all three species. *Body condition index* positively affected stress-induced corticosterone levels in autumn and negatively in spring in song thrush and had no effect in blackbird and blackcap (Table 2). The *year* of sampling and the *field site* had a negligible effect on the stress-induced corticosterone concentrations in all species (Table 2).

There was a general tendency of an increased stress response during spring migration in three species with moderate sample sizes, with the exception of sedge

warbler (Table 3, Fig. 7). However, the difference was significant in reed warbler only (Table 3, Fig. 7). Owing to a nearly two-fold numerical difference in stress-induced hormone levels between migratory seasons in garden warbler and chaffinch, we assume that a lack of statistical power was partly due to the insufficient sample size.

Combined analysis accounting for all model species revealed the general tendency of much higher stress-induced corticosterone concentrations during spring migration compared with autumn ones (Imm, *season* effect, $\beta = 0.17 \pm 0.04$ (SE), $t = 4.0$, $p = 0.0001$, Table 4). Migratory strategy had no effect on stress response (Imm, migratory *strategy* effect, $\beta = 0.05 \pm 0.13$ (SE), $t = 0.4$, $p = 0.70$, Table 4). Mean stress-induced corticosterone concentrations predicted by the model were 70.8 ± 1.2 ng/ml (SE) for spring and 47.9 ± 1.1 ng/ml (SE) for autumn migration. Analysis of random effects showed that stress response was relatively constant between species (variation attributed to between-species difference was much smaller than for baseline levels) and nearly independent of year of capture and field site (pooled in random factor *environment*, see above) (Table 4).

4 Discussion

4.1 Comparison between seasons

In this study we showed a general tendency for similar baseline corticosterone levels during spring and autumn migration in seven songbird species and using a large

Table 4. Results of linear mixed-effects models explaining the effects of season (spring vs. autumn) and migratory strategy (long- vs. short-distance migrations) on baseline and stress-induced corticosterone concentrations (both log10 transformed) in seven model passerines captured on the Courish Spit during seasonal migrations in 2013–2018. *Species* are treated as a random factor. *Environment* is a random factor that accounts for between-year and between-field site variation with each year-field site combinations as factor levels (see Methods). β refers to the models estimate. Significant effects are marked **bold**.

	Baseline				Stress-induced			
	β	SE	t	p	β	SE	t	p
Fixed effects								
<i>Intercept</i>	0.80	0.07	12.2	<0.0001	1.68	0.09	18.7	<0.0001
<i>Season (spring)</i>	-0.009	0.05	-0.2	0.9	0.17	0.04	4.0	0.0001
<i>Strategy (short)</i>	0.10	0.07	1.5	0.18	0.05	0.13	0.4	0.70
Random effects (SD)								
<i>Environment</i>	0.10				0.04			
<i>Species</i>	0.28				0.15			
<i>Sampling time_A</i>	0.19				-			
<i>Residual</i>	0.28				0.27			

dataset obtained during eleven consecutive migratory seasons. This tendency was obvious when comparing each species separately and when analyzing hormone measurements in the pooled sample. This finding contradicts our previous results on European robins obtained at the same field sites and during the same migratory seasons, where we found the nearly two-fold increase in baseline corticosterone concentration during spring migration [41]. Only in chaffinches was there a tendency of higher baseline hormone concentrations in spring compared with autumn; the difference was not statistically significant, probably due to the small sample size. Hence, our results are in line with the inconsistency reported in previous field [34, 36-38, 40, 41] and experimental [14, 35] studies and suggest that seasonal difference in baseline corticosterone concentrations probably represents genuine species-specific variation which is independent of environmental conditions.

The range of baseline corticosterone concentrations obtained in this study (1.5-63.7 ng/ml for all species combined, Table 1) is in agreement with baseline values reported in other studies for birds captured during seasonal migrations [e.g. 21, 30, 31, 34, 54, 71]. Even assuming issues with comparing of absolute values of corticosterone concentrations from different studies [72], our results emphasize that birds were not “stressed” before capture. This is in line with the idea that birds are well adapted to elevated energetic demands of seasonal migrations and do not increase baseline corticosterone level above the normal range of its baseline variation under predictable conditions [30, 32, 51].

In line with our initial prediction, we found a general tendency of increased stress response during spring migration compared with autumn passage. In three species (song thrush, blackcap and reed warbler) this difference was significant, reaching three-fold in blackcaps. In two more species (garden warbler and chaffinch) seasonal difference in stress response was not significant, most likely due to small sample sizes. These results corroborate our earlier finding in European robins, where stress response in spring was much higher than in autumn [41]. Many species are more “stressed” during spring migration because to arrive on the breeding ground in time they fly with higher speed and spend less time on stopovers, simultaneously traveling under more unpredictable environmental conditions than in autumn [52, 53]. As a result, enhanced adrenocortical response in spring probably enables flexibility in physiological adjustments and behavioral responses to the more unpredictable conditions together with greater time pressure in spring. On the other hand, most birds in the autumn sample

Figure 6. Predicted stress-induced corticosterone concentrations (ng/ml) in three model passerine species (blackbird, song thrush and blackcap) captured on the Courish Spit during seasonal migrations in 2013 - 2018. Plotted are the median (horizontal line in the box), 25th and 75th percentiles (horizontal box boundaries), and 10th and 90th percentiles (whiskers). Open dots indicate outliers.

Figure 7. Raw stress-induced corticosterone concentrations (ng/ml) in four model passerine species (garden warbler, reed warbler, sedge warbler, and chaffinch) captured on the Courish Spit during seasonal migrations in 2013 - 2018. Plotted are the median (horizontal line in the box), 25th and 75th percentiles (horizontal box boundaries), and 10th and 90th percentiles (whiskers). One case in reed warbler with corticosterone concentration 347.3 ng/ml (in spring) is not shown. Open dots indicate outliers.

were first-year individuals. As stress response probably depends on previous experience [64], one can assume an elevated stress response in young birds migrating for the first time in their lives and continuously encountering novel situations. Therefore, the functional significance of a higher stress response in spring still needs to be elucidated. It is interesting that two closely related species with similar ecology, blackbird and song thrush, differ in their seasonal pattern of stress-induced corticosterone

concentrations. This indicates that seasonal difference in stress-induced corticosterone levels might be species-specific trait, the issue just recently raised by Romero and Gormally [73].

The findings that in the majority of the studied species baseline corticosterone levels did not differ between migratory seasons, while the stress-induced concentrations did differ, support the hypothesis that baseline and stress-induced concentrations are partly independent hormone systems and probably have their own regulatory mechanisms [64, 74, 75]. Three hypotheses (*Energy mobilization*, *Behavior hypothesis* and *Preparative hypothesis*) have been proposed to explain seasonal changes in glucocorticoid secretion and possible difference in both baseline and stress-induced corticosterone concentrations between migratory seasons [51, 76]. Romero et al. [51] concluded that existing evidence generally showed elevated corticosterone levels in birds captured during spring migration which matches the *Preparative* hypothesis. It predicts that corticosterone is highest during times of year when predictable stressors are more likely. If during spring migration birds indeed have a higher chance of encountering poorer environmental conditions than in autumn, it should be reflected in higher corticosterone levels during spring stopovers. However, we observed similar baseline corticosterone levels in all but one species (chaffinch). Stress-induced corticosterone concentrations were indeed higher in spring in most but not all species. For our study we do not know of *a priori* reasons why a chance of encountering poor conditions in spring should be valid for one species but not for others (even after accounting for migratory strategy). Therefore, we conclude that *Preparative* hypothesis is only partially matched by our results.

4.2 Comparison between species

We did not find that long- and short-distance migrants exhibit any difference in baseline as well as in stress-induced corticosterone concentrations. Migration distance, various energetic needs and time pressures are not reflected in corticosterone levels. This finding is in line with observations of Falsone et al. [31] who reported similar baseline and stress-induced corticosterone levels in birds using different migratory strategies, including irruptive species. Present study and Falsone et al. [31] results are also similar in reporting significant between-species variation in baseline as well as in stress-induced corticosterone concentrations (Table 4, Table 2 in [31]), which clearly shows that migratory species actually differ

in corticosterone levels. The reasons for such variation are currently unclear. Additionally, our data indicated that between-species variation was larger for baseline than for stress-induced concentration. In part this result agreed with data on migrating bar-tailed godwits, which showed quite different baseline levels and similar adrenocortical stress response after having completed a non-stop bout of approximately 4500 km and during refueling [78].

According to our prediction and in line with results obtained in European robins [41], all species showed substantial stress response in spring and autumn. This was also observed in some earlier studies [34, 79], but see [80]. Stress-induced glucocorticoid concentrations reflect the animal's response to various stressful situations and are assumed to be a measure of the ability of the hypothalamic-pituitary-adrenal (HPA) axis to respond to a stressor [64]. Low between-species variation in stress-induced corticosterone concentrations (see above) and similar stress response in long- and short-distance migrants may indicate that the ability to mount the appropriate stress response is essential for survival [32, 74, 77].

Our results do not corroborate the particular observation reported by Falsone et al. [31] that chaffinches in autumn had the lowest baseline corticosterone levels compared with other species. In our study chaffinches had a baseline corticosterone level within the range for other species in autumn and much higher levels in spring (Fig. 5). However, we cannot directly compare these two datasets. Chaffinches in the Falsone et al. [31] study were caught directly out of migratory flight, while our birds were partly caught out of flight, and partly after landing while refueling. Therefore, birds captured on the Courish Spit were in two metabolic states: exercising while fasting and resting/refeeding which might have had an impact on baseline corticosterone levels [30, 46].

4.3 Factors affecting corticosterone levels

Quite surprisingly, we found a significant impact of sampling time on baseline corticosterone concentrations only in blackcaps, while there was no relationship in two thrush species. Partly we can explain this result by the longest mean sampling time in blackcaps. However, the difference in mean sampling time between blackcap and song thrush, the species without a relationship between baseline corticosterone and sampling time, was only 13 s. Based on this result, we can suppose that stress-induced corticosterone release begins after 2.5 min, i.e. earlier than the commonly assumed threshold of 3 min [81, 82],

which was also noticed in another study incorporating various migratory species [31]. Eikenaar et al. [37] reported that in blackbirds there was no relationship between baseline corticosterone and sampling time in the samples collected within 2 min from capture. There are indications of substantial variation in the timing of stress-induced corticosterone release even within species [82]. Therefore, it is not surprising that species can differ in time it taken for stress-induced increase of corticosterone concentration to start [73]. Similar to European robins [41], in blackcaps the slope of increase of baseline corticosterone with sampling time did not differ between migratory seasons.

In blackbirds, in both migratory seasons we observed an inverse relationship between baseline corticosterone concentration and body condition index, i.e. lean individuals had elevated baseline corticosterone levels. This finding agrees with our data on European robins [41]. At the same time, body condition index did not affect baseline corticosterone levels in song thrushes and blackcaps. Other studies also report some disagreement in this respect. Jenni et al. [30] and Raja-aho et al. [83] observed elevated baseline corticosterone in lean birds. Whereas Landys-Cianelly et al. [78] and Eikenaar et al. [71] reported the opposite pattern, i.e. baseline corticosterone increased with body mass. One possible explanation for such discrepancies is the metabolic state of captured birds. In the former case birds were sampled directly out of migratory flight or shortly afterwards; baseline corticosterone can increase in exhausted and fasted individuals causing the inverse relationship with body mass [30, 54, 84]. In the latter case birds were captured while refueling at a stopover site and preparing for departure; in such a situation baseline corticosterone level is often positively correlated with body mass or fat stores [71, 78].

Overall we found no support for the prediction that stress response is modulated by energetic condition. Stress-induced corticosterone concentration was affected by body condition index only in song thrush and this relationship had different slopes in spring and autumn. However, more consistency was reported earlier with fat individuals which generally exhibited lower response, e.g. European robins [31], barn swallows [36, 83], hermit thrushes (*Catharus guttatus*) [54] and house sparrows [85]. We propose that the dependence of stress response on energetic condition might be context-dependent, i.e. might change between and within migratory seasons, along migratory route and depend on resource availability and the environmental conditions of a particular season [see also: 86, 87].

5 Conclusions

Overall our study reveals that the occurrence of seasonal difference in baseline and stress-induced corticosterone levels is mainly a species-specific trait, which does not depend on migratory strategy. Long- and short-distance migrants have well established differences in regulatory mechanisms of migratory behavior and physiology including the relative reliance on annual rhythms and environmental cues [42, 43]. However, this variation was not reflected in baseline corticosterone concentrations, the levels most likely involved in regulation of migratory activity and fueling [reviewed in: 9, 22, 25]. We cannot exclude the possibility that regulation of components of migratory behavior and physiology can be arranged by subtle variation in baseline corticosterone within the baseline range of the species, which can hardly be detected by between-species comparison [21, 22, 29]. The general tendency of higher stress-induced corticosterone levels in spring is probably attributed to the time deficit and more unpredictable environmental conditions in this season. However, the absence of seasonal difference in stress response in some species questions the general utility of this interpretation. To understand the biological relevance of the observed seasonal and between-species variation in corticosterone levels, one should consider that corticosterone concentration is only one aspect of glucocorticoid physiology; its action is also affected by transport protein (CBG), receptor numbers in tissues and activity of respective enzymes [51]. Seasonal and species-specific variation in these components could potentially enhance or relax seasonal difference in corticosterone concentrations.

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