Research Article

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Migratory state is not associated with differences in neural glucocorticoid or mineralocorticoid receptor expression in pine siskins

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Abstract: Although the endocrine system likely plays an important role in orchestrating the transition to a migratory state, the specific mechanisms by which this occurs remain poorly understood. Changes in glucocorticoid signaling are one proposed mechanism that may be important in migratory transitions. Although previous work has focused on the role of changes in circulating glucocorticoids, another potential mechanism is changes in the expression of its cognate receptors. Here, we test this hypothesis by comparing mRNA expression of the genes for the mineralocorticoid receptor (*MR*) and glucocorticoid receptor (*GR*) in two brain regions implicated in the regulation of migratory behavior (the hippocampus and hypothalamus) in pine siskins (*Spinus pinus*) sampled before or after the transition to a spring nomadic migratory state. Compared to pre-migratory birds, migratory birds had body conditions more indicative of physiological preparations for migration (e.g., larger body mass), and greater levels of nocturnal migratory restlessness. However, we found no differences between pre-migratory and migratory birds in the expression of *GR* or *MR* mRNA in either the hippocampus or hypothalamus. Thus, differences in expression of receptors for glucocorticoids do not appear to underly the observed differences in physiology and behavior across a migratory transition. Taken together with previous results showing no change in circulating corticosterone levels during this transition, our findings provide no evidence for a role of glucocorticoid signaling in the spring migratory transition of this species.

Keywords: avian, birds, corticosterone, migration, physiology

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1 Introduction

The lives of many vertebrates are organized into annual cycles, made up of different life history stages such as breeding, migration, and wintering. Across vertebrates, the endocrine system plays a central role in orchestrating transitions between these life history stages by coordinating changes in behavior, morphology and physiology [1]. These life history stage transitions provide rich opportunities to resolve the role of endocrine mechanisms in regulating behavior and physiology by comparing endocrine function among animals at different stages. Whereas the role of the endocrine system in regulating the transition to breeding has been well-described [e.g., 2, 3, 4], its role in the migratory stage is not as well understood. The transition to a migratory stage can occur under varying circumstances. On one hand, migration may occur relatively predictably at an anticipated time for which an animal can prepare. This is the case for spring migration in many species of birds, for example. On the other hand, migration may occur in response to relatively unpredictable conditions – either adverse conditions, such as severe storms, or advantageous conditions, such as unpredictable resource pulses; these less predictable migrations are often termed facultative migrations. Although the endocrine mechanisms underpinning these different forms of migration may differ [5], it has been hypothesized that glucocorticoids may play a role in both forms of migration [6-10].

Corticosterone, the predominant glucocorticoid in circulation and the brain of birds, has several

potential functions during the migratory stage of birds. Corticosterone may stimulate or permit physiological preparations for migration (e.g., hyperphagia and fat deposition) [11-13], stimulate migratory departure [10, 14], facilitate metabolic processes needed to meet the energetic demands of flight [15, 16], and facilitate cognitive processes necessary for migration [17, 18]. Most studies investigating the role of corticosterone in migration have focused on changes in circulating hormone levels as a mechanism by which the effects of corticosterone might be mediated. Under this model, increasing levels of circulating corticosterone around the time of migration stimulate changes associated with the migratory transition. However, the mechanisms governing the action of corticosterone are complex, involving many potential points of regulation [reviewed in 19, 20]. Some of these points of regulation ultimately exert their effects by altering circulating glucocorticoid levels (e.g., changes at the level of the anterior pituitary or adrenal gland that lead to changes in glucocorticoid secretion). Other mechanisms, however, can operate independently of changes in circulating glucocorticoid levels. Mechanisms in the latter category include transport mechanisms that can alter delivery of glucocorticoids to target cells and changes to the target cells themselves, such as in the availability of receptors for glucocorticoids and local conversion between active and inactive forms. Differences in sensitivity of the brain to hormones, mediated by variation in hormone receptor availability, are thought to be an important mechanism underpinning variation in the expression of behavior [e.g., 21, 22, 23]. Thus, a likely mechanism by which the effects of corticosterone might be regulated in the context of migration is through changes in expression of receptors for corticosterone. In birds, corticosterone can bind to intracellular mineralocorticoid and glucocorticoid receptors (MRs and GRs, respectively), as well as to less well-described membrane bound receptors [24, 25]. Intracellular MRs are high-affinity genomic receptors for corticosterone that are activated when corticosterone levels are low, whereas intracellular GRs are low-affinity, high-capacity genomic receptors that are primarily activated when corticosterone levels are elevated [24, 26, 27]. Evidence from mammals suggests that both MRs and GRs may also function as membrane-bound receptors, mediating rapid non-genomic effects of corticosterone [28]. It is expected that the effects of corticosterone in the context of migration may occur through activation of MRs and/or GRs [27].

Although the neural mechanisms underpinning migration are not well understood, both the hippocampus and hypothalamus are thought to be important in avian migration. The hippocampus is important in navigation and processing of spatial information, and several studies suggest a role for this brain region in migration [29-32]. The ventromedial area of the hypothalamus is indicated by lesion experiments to be important in the regulation of pre-migratory hyperphagia and fattening [33], as well as in migratory restlessness [34]. Additionally, the mediobasal area of the hypothalamus is hypothesized to be involved in pre-migratory hyperphagia and fattening [9] and in changes in activity patterns associated with migration [35]. The avian hippocampus and hypothalamus are both sensitive to corticosterone, expressing MRs and GRs [36-40]. Although we know little about the action of corticosterone in these brain regions with respect to migratory functions in birds, differences in *MR* mRNA expression in the hippocampus are associated with differences in spatial cognition in selected lines of zebra finches (*Taeniopygia guttata*) [41].

To advance our understanding of glucocorticoid signaling in migration, we compared *MR* and *GR* mRNA expression across a migratory transition in pine siskins (*Spinus pinus*; Figure 1). Pine siskins are facultative nomadic migrants – they have low site fidelity and their movements do not have a strong directional orientation or precise timing [42-44]. Although movements can occur at other times of the year, pine siskins most frequently migrate in the spring and fall [43], with spring nomadism driven in part by increasing spring daylengths [45]. In captivity, the spring nomadic migratory transition in pine siskins is characterized by physiological preparations for migration (e.g., fat deposition) and the expression of migratory restlessness [45, 46]. The spring nomadism of pine siskins likely reflects a form of migration that is intermediate between more predictable and unpredictable forms of migration. We have focused on a nomadic migrant for this study because this form of migration is poorly understood, and it has been proposed that glucocorticoid signaling in response to local environmental conditions may be particularly important in nomadic migration [5]. Yet, we previously found that there is no change in circulating corticosterone levels in association with the spring migratory transition in pine siskins [45]. Thus, if changes in glucocorticoid signaling are important in this transition, they are likely mediated by mechanisms other than those that alter circulating levels of the ligand. One candidate mechanism by which this could occur is via changes in the expression of MRs and/or GRs. If changes in either receptor are important in the migratory transition, then we would expect to find differences in their expression in key brain regions associated with migration among birds in different migratory states. To

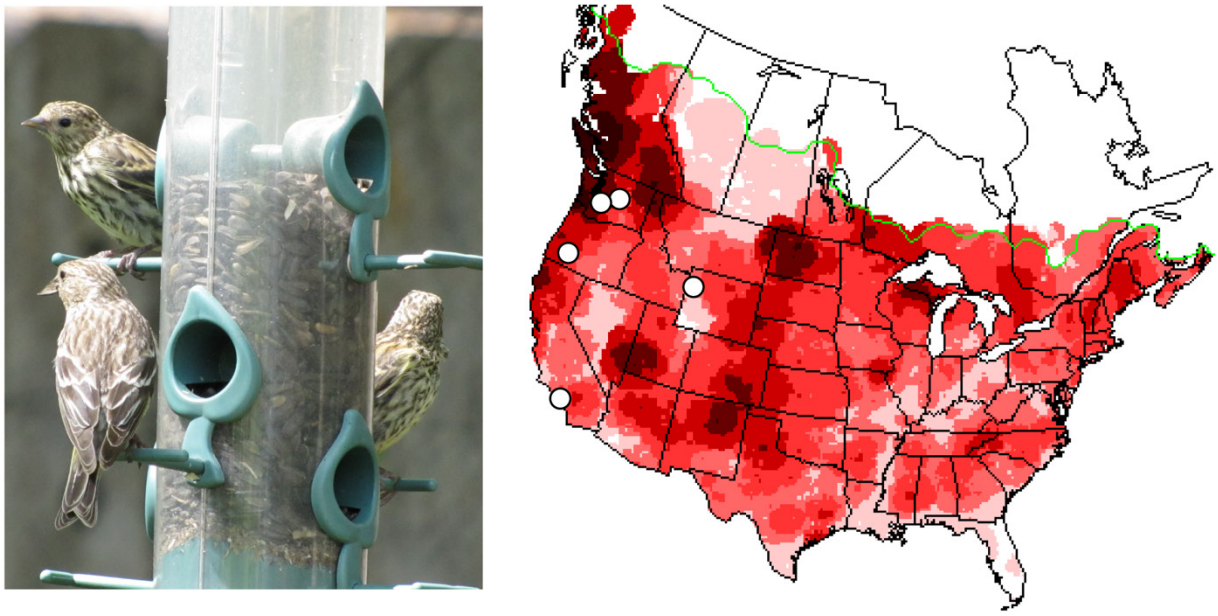

Figure 1. Left: Pine siskins at a feeder. Right: Map of wintering range of pine siskins from the United States Geological Service Patuxent Wildlife Research Center [65] based on data from the Audubon Christmas Bird Count (CBC). Darker colors indicate greater abundances of pine siskins; the green line indicates the limit of CBC data. Open circles indicate capture locations of birds for this study.

test these predictions, we measured *MR* and *GR* mRNA expression in the hippocampus and hypothalamus of pine siskins sampled before or after the transition to a migratory state.

2 Methods

2.1 Animals and experimental design

Birds were collected from sites in the western USA between August 2015 and July 2016 (Figure 1): Eagle Point, OR (42°30′N, 122°49′W; December 2015); Los Angeles, CA (34°07′N, 118°12′W; January 2016); Leavenworth, WA (47°36′N, 120°50′W; July 2016); Randle, WA (46°18′N, 121°32′W; August 2015); and Jackson, WY (43°28′N, 110°49′W; September 2015). Birds were captured in mist nets or funnel traps and transported by vehicle to Loyola Marymount University in Los Angeles, CA where they were housed indoors. Some of these birds (n = 8) were subjects in other experiments [45, 47] before the present study. Birds used in other experiments were held for at least 9 months between the previous experiment and the current experiment as a washout period to minimize any potential carryover effects; these birds were either maintained throughout their time in captivity on a changing photoperiod that mimicked natural changes in day length or returned to such a photoperiod in the fall of 2016 following photostimulation. Thus, all birds in the present experiment had gone through molt in captivity in the summer/fall of 2016 and experienced a natural decline in photoperiod leading up to winter solstice before the experiment. Birds captured at different sites (and different dates), as well those that were subjects in previous experiments, were balanced between the pre-migratory and migratory sampling time points (described below). All birds were after-hatch-year at the time of the experiment.

During the experiment, birds were held in individual cages on a photoperiod that mimicked natural changes in photoperiod at a latitude of 42°N. Birds had ad libitum access to Roudybush Small Bird Maintenance Diet (Woodland, CA), a mixture of nyjer thistle and sunflower seed hearts, grit, and water. We sampled birds at two different time points: late winter, when it was expected that birds had not yet transitioned to a spring migratory state (hereafter, "pre-migratory"), and spring, when birds were expected to be in a migratory state (hereafter, "migratory"). Tissues were collected from pre-migratory birds on March 3, 2017 (n = 7; 5 females and 2 males), and from migratory birds on April 21, 2017 (n = 7; 5 females and 2 males). In advance of tissue sampling, body condition was measured to assess physiological indicators of migratory state (Feb 27, 2017 for pre-migratory birds and Apr 18, 2017 for migratory birds). Birds were also video-recorded for the 2 nights preceding tissue sampling to

assess behavioral state (Mar 1-2, 2017 for pre-migratory birds and April 19-20, 2017 for migratory birds).

2.2 Migratory indicators

Birds were recorded at night using the infrared capabilities of a Sony FDR-AX33 Handycam (New York, NY). We used instantaneous sampling [48] to quantify the behavior of each bird at one-minute intervals between 01:00 and 02:30 hours, a period when pine siskins expressing migratory restlessness are typically active [46]. At each sampling point we categorized behavior [following 46] as stationary, fast wing-beating, flying, jumping (including climbing), feeding, preening, or "other" for behaviors outside these categories (e.g., beak wiping). If the bird was out view, no behavior was recorded, though this was rare. The mean proportion of time spent engaged in locomotor activity (fast wing-beating, flying, and jumping) across the two nights was then used as our measure of migratory restlessness.

We examined four measures of body condition associated with physiological preparation for spring migration: body mass, body fat, and both the size and color of flight (pectoralis) muscles. Increases in body mass, body fat, and flight muscles size are all associated with migratory preparation in pine siskins, as is lightening in the color of flight muscles [45]. We measured body mass to within 0.1 g on an electronic balance. We visually scored furcular and abdominal fat on a scale from 0 (no fat) to 5 [bulging fat; 49] and summed these values to generate a total fat score. Similarly, we visually scored flight muscle size on a scale from 0 (sharp keel with concave muscle) to 3 [muscle extended past keel; 50]. Flight muscle color was scored visually on scale from 1 (darkest) to 3 (lightest) using color standards as previously described [45]. We failed to record body fat for one bird in the non-migratory group, leading to a smaller sample size for this measure in this group (n = 6).

2.3 Tissue collection and quantification of receptor mRNA expression

To collect tissues, birds were first euthanized with an overdose of isoflurane, then the brain was rapidly dissected from the skull and flash frozen whole in liquid nitrogen. Sampling occurred between 1130-1500 hours, and all brains were collected and placed in liquid nitrogen within 4 minutes of euthanasia. Frozen brains were stored at −80°C until tissue punches were collected. Tissue punches were collected in a manner similar to Sewall et al. [51]. Specifically, brains were first sectioned at 300 µm in the coronal plane using a cryostat. Sections were thaw-mounted onto glass microscope slides and rapidly refrozen on dry ice. Using 1-mm diameter biopsy punches, two punches were collected (one from each hemisphere) from each of two brain regions: the hypothalamus and the hippocampus. Thus, for each bird, two punches for a given brain region were pooled for RNA preparation. We chose brain sections containing each region based on comparison with a canary brain atlas [52] and made use of the fact that both brain regions are bounded by neuroanatomical markers that are visible in fresh frozen tissue. By using punches with a diameter smaller than the diameters of the brain regions of interest we could ensure that only tissue that was within the targeted brain region was included. Tissue punches were stored at −80°C until RNA preparation.

Primers for *MR* and *GR*, as well as for the normalizing genes *GAPDH* and *YWHAZ*, were designed based on gene sequences from the white throated sparrow (*Zonotrichia albicollis*; NCBI XM_014271312, XM_005485310, XM_005486061, XM_005479464) using Integrated DNA Technologies' Primer Quest (Coralville, IA). Primers were designed to span an exon junction and a long amplicon (300-800 bp) that would include any sequence amplified by potential qPCR primers. These long amplicon primers were then used with pine siskin cDNA to generate pine siskin amplicons, which were sequenced at the Biocomplexity Institute at Virginia Tech. Using these amplicon sequences final qPCR primers (Table 1) were chosen by modifying primers when differences in the sequence were present. An initial test qPCR with melt-curve was performed to see that a single product was formed by primer amplification. This was followed up by a qPCR standard curve to determine primer efficiency.

RNA was prepared from frozen brain tissue samples using Qiazol (Qiagen, Germantown, MD) and purified on the RNeasy UCP Micro Kit (Qiagen). Samples were disrupted in RULT (with B-mercaptoethanol) using a Bullet Blender (Next Advance, Troy, NY) with 1.0-mm Zirconia/Silica beads (BioSpec, Bartlesville, OK). Samples were subjected to on-column digestion with DNase. RNA was eluted from the column in 15 µl RNase free water and recycled once to re-elute the column. Using 100 ng of tissue, RNA was transcribed to cDNA using the Transcriptor first-strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN) using both anchored-dT and random primers. A quantity of 200 ng was used in a reaction to synthesize cDNA. qRT-PCR was performed on a QuantStudio-6 Flex (Applied Biosystems, Foster City, CA) with Quant Studio

Table 1. Pine siskin primer sequences

Gene	Direction	Sequence (5'-3')	Amplicon length (bp)
MR (NR3C2)	Forward	CTTCCAAAGTGTGTTTGGTGTG	117
	Reverse	GGTAATTGTGCTGGCCTTCTA	
GR (NR3C1)	Forward	ACTCTATGCATGATGTGGTTGAAAATC	93
	Reverse	CCAACATTTCTGGGAACTCAATACTCA	
GAPDH	Forward	GGTGGTGCCAAGCGTGTGAT	117
	Reverse	GCAGGAAGCATTGCTGACAATT	
YWHAZ	Forward	GCAACCAACACATCCCATCAGA	120
	Reverse	ATTGCTTCATCAAAAGCCGTTTT	

Real-Time PCR software. qRT-PCR was performed with an annealing temperature of 60˚C, using Power SYBR Green master mix (Applied Biosystems). Samples were run in triplicate, and reaction efficiencies ranged from 95.9% to 97.8%. Data were analyzed using the standard curve method to measure relative expression of the receptor gene of interest. Thus, relative expression is the quantity of the gene of interest divided by the mean quantity of the normalizing genes (*YWHAZ* and *GAPDH*) for a sample. We verified that the expression of both *YWHAZ* and *GAPDH* were stable between the pre-migratory and migratory period (*YWHAZ*: hippocampus: $p = 0.86$, hypothalamus: $p = 0.91$; *GAPDH*: hippocampus: $p = 0.92$, hypothalamus: $p = 0.39$). For one bird (a female) in the migratory group, we were unable to extract sufficient RNA from the hypothalamus sample leading to a smaller sample size (n = 6) for this tissue in this group.

2.4 Statistical analysis

Statistical analyses were performed in R 3.5.0 [53]. Migratory indicators and mRNA expression were compared between sampling groups (pre-migratory vs. migratory) using *t*-tests when data met the assumption of normality or Mann-Whitney U tests when data were not normally distributed. Body mass was log transformed to meet the assumption of normality. We did not test for effects of sex because sample sizes were not sufficient. We provide estimates of effects size as Cohen's *d* for *t*-tests and *r* for Mann -Whitney U tests. Effect size estimates were generated using the lsr [54] and rcompanion [55] packages in R. The significance threshold was set at $\alpha = 0.05$.

3 Results

3.1 Migratory indicators

Migratory birds were significantly heavier in body mass than pre-migratory birds (Figure 2; $t = 5.14$, n = 7, 7, $p < 0.001$, $d = 2.52$). Migratory birds also had more body fat ($U = 40$, n = 7, 6, $p = 0.006$, $r = 0.75$), larger flight muscles ($U = 46$, n = 7, 7, $p = 0.005$, $r = 0.76$), and lighter colored flight muscles ($U = 38.5$, n = 7, 7, $p = 0.049$, $r = 0.54$) compared to pre-migratory birds.

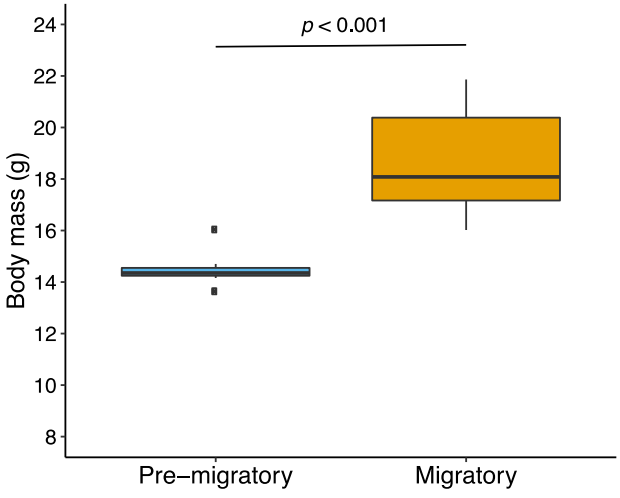

Figure 2. Body mass of pine siskins sampled during the pre-migratory (n = 7) and migratory period (n = 7). Date are shown as box-plots with box limits indicating the interquartile range (IQR; 25-75th percentile), the line within the box indicating the median, and whiskers indicating the minimum value within 1.5*IQR below the 25th percentile and the maximum value within 1.5*IQR above the 75th percentile. Data points with values beyond whiskers are shown as dots.

Migratory birds spent a significantly larger proportion of their time at night engaged in locomotor activity (i.e., they had higher levels of nocturnal migratory restlessness) than did pre-migratory birds (Figure 3; U = 49, n = 7, 7, p = 0.002, r = 0.86). Pre-migratory birds had low levels of nocturnal activity, spending less than 0.5% of observation time engaged in locomotor activity on average.

3.2 Receptor expression

There was no difference in *MR* mRNA expression between pre-migratory and migratory birds in the hippocampus (Figure 4; U = 22, n = 7, 7, p = 0.80, r = 0.09) or hypothalamus (Figure 4; t = -0.01, n = 7, 6, p = 0.99, d = 0.006). Similarly, there was no difference in *GR* mRNA expression between pre-migratory and migratory birds in either brain region (Figure 5; hippocampus: U = 35, n = 7, 7, p = 0.21, r = 0.36; hypothalamus U = 13, n = 7, 6, p = 0.29, r = 0.32).

4 Discussion

We found no evidence for a role of changing sensitivity of the brain to corticosterone in the transition to a spring migratory state in a nomadic migrant. In two brain regions implicated in migration, the hypothalamus and the hippocampus, there was no difference in mRNA expression for receptors for corticosterone (*MR* and *GR*) between pine siskins in a pre-migratory state and those in a migratory state.

Although there were no differences in receptor expression between the two sampling groups, we did find considerable differences in body condition and behavior. Consistent with physiological preparation for migration, migratory birds had larger fat deposits, larger pectoralis muscles, and heavier body masses compared to pre-migratory birds. Migratory birds also expressed nocturnal migratory restlessness, indicative of migratory readiness [56], which was absent in pre-migratory birds. Thus, we are confident that the two groups were sampled in different life history stages. We cannot rule out the possibility that *MR* and *GR* expression changed in preparation for migration, but further in advance of the time point we sampled here (early March), such that changes in receptor expression had already occurred at the time of our pre-migratory sampling. However, even if this is the case, such

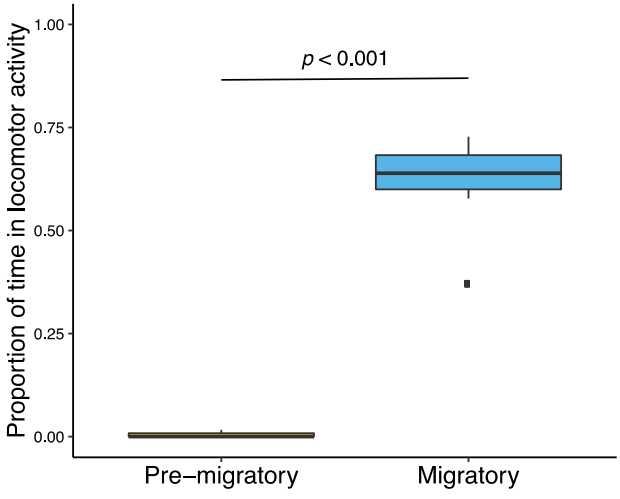

Figure 3. Nocturnal locomotor activity of pine siskins sampled during the pre-migratory (n = 7) and migratory period (n = 7). Data are shown as box-plots; see Figure 2 for a description.

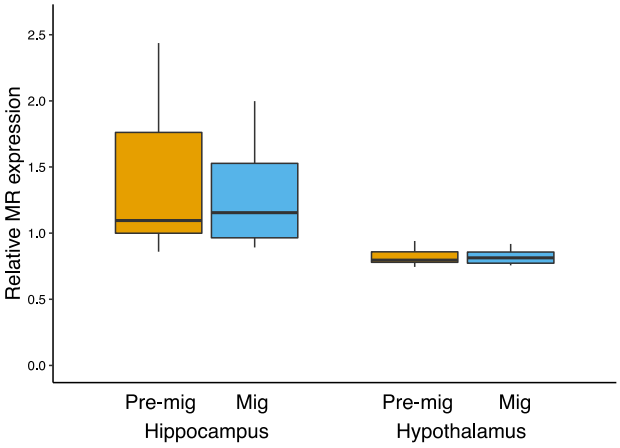

Figure 4. Relative expression of mineralocorticoid receptor (*MR*) mRNA in the hippocampus and hypothalamus of pine siskins sampled during the pre-migratory ("pre-mig"; n = 7) and migratory period ("mig"; n = 7 for hippocampus, n = 6 for hypothalamus). Data are shown as box-plots; see Figure 2 for a description.

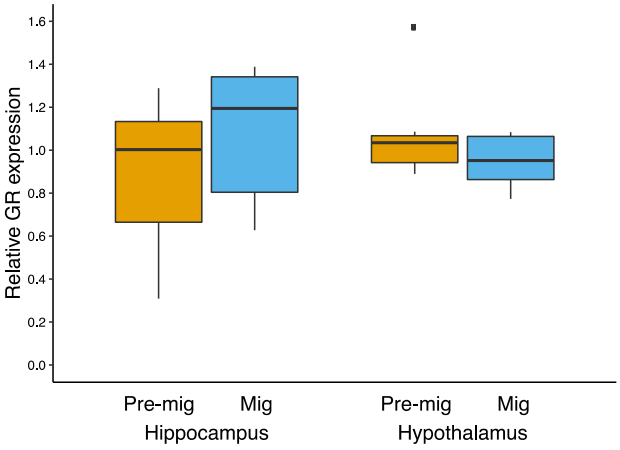

Figure 5. Relative expression of glucocorticoid receptor (*GR*) mRNA in the hippocampus and hypothalamus of pine siskins sampled during the pre-migratory ("pre-mig"; n = 7) and migratory ("mig"; n = 7 for hippocampus, n = 6 for hypothalamus) period. Data are shown as box-plots; see Figure 2 for a description.

changes in expression could not explain the differences in physiology and behavior that we observed between our two sampling points. Another possibility is that there was a transient change in expression around the time of transition that we did not capture; if this is the case it would suggest that different mechanisms are involved in the initiation and maintenance of a migratory state.

We previously found that pine siskins do not elevate circulating corticosterone levels around the spring migratory transition [45]. Thus, taken together with the results of the present study, we find no evidence for a role of changes in glucocorticoid signaling in the spring migratory transition of the pine siskin. However, it would be premature to rule out this possibility entirely. Firstly, mRNA expression does not always reflect protein-level expression [57]; thus, it is possible that there are differences in levels of receptor proteins that we could not detect here. Secondly, there are other mechanisms that could alter glucocorticoid signaling that we did not investigate; these include changes in corticosteroid binding-globulin [58, 59] and activity of enzymes that activate or deactivate corticosterone (e.g., 11β hydroxysteroid dehydrogenases) [20]. Finally, the hypothalamus is a heterogenous structure, made up of numerous functionally distinct nuclei [60]. Consequently, our hypothalamus-level approach may have obscured underlying variation within specific nuclei, though studies of songbirds in other contexts have found hypothalamic-level variation in these receptors [37, 61]. Future work could take a more fine-scale approach to examine variation at the level of different hypothalamic nuclei.

To our knowledge, this is the first study to test for differences in GR and MR receptor expression across the transition to a migratory state. But, in a comparison of sympatric subspecies of dark-eyed juncos (*Junco hyemalis*) Bauer et al. [61] found that compared to the resident subspecies, the migrant subspecies had significantly higher levels of *MR* mRNA expression in hypothalamus at the time just prior to migratory departure, and there was a similar trend in *GR* mRNA expression. It remains to be determined if this result reflects an increase in *MR* and *GR* expression in the migratory subspecies prior to departure, rather than a subspecies difference unrelated to migration. However, such an increase would indicate differences in regulation of glucocorticoid signaling between facultatively migrant pine siskins and more strictly seasonally migrant dark-eyed juncos.

MRs and GRs in the hypothalamus and hippocampus may also function in glucocorticoid negative feedback [62-64]. Seasonal patterns of *MR* and *GR* expression in these tissues could reflect this function. Thus, the absence of differences in *MR* and *GR* expression in pine siskins as they transition to a migratory state could reflect a stable level of sensitivity to glucocorticoid negative feedback across this transition. As facultative migrants, pine siskins are thought to be highly sensitive to local conditions to time their migration [5]. Accordingly, one possibility is that birds may maintain an undampened glucocorticoid response (i.e., weak negative feedback) to facilitate appropriate reaction to environmental cues that drive migratory movements; however, additional work will be necessary to evaluate whether this might be the case.

5 Conclusion

This study is the first to test for differences in the expression of receptors for corticosterone in association with the transition to a migratory state. Using a nomadic migrant as a model, we found no differences in either *MR* or *GR* mRNA expression in the hippocampus or hypothalamus between pre-migratory and migratory birds. Taken together with our earlier findings that circulating corticosterone levels also show no change during this transition, our results do not support the hypothesis that changes in glucocorticoid signaling are an important mechanism mediating the transition to a migratory state in this species.

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