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Intracellular glucocorticoid receptors in spleen, but not skin, vary seasonally in wild house sparrows (*Passer domesticus*)

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Over the short-term and at physiological doses, acute increases in corticosterone (CORT) titres can enhance immune function. There are predictable seasonal patterns in both circulating CORT and immune function across many animal species, but whether CORT receptor density in immune tissues varies seasonally is currently unknown. Using radioligand binding assays, we examined changes in concentrations of glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) in spleen and skin in wild-caught house sparrows in Massachusetts during six different life-history stages: moult, early winter, late winter, pre-egg-laying, breeding and late breeding. Splenic GR and MR binding were highest during the pre-laying period. This may help animals respond to immune threats through increased lymphocyte proliferation and/or an increase in delayed-type hypersensitivity reactions, both of which CORT can stimulate and in which spleen is involved. A decrease in splenic GR and MR during the late breeding period coincides with low baseline and stress-induced CORT, suggesting immune function in spleen may be relatively CORT-independent during this period. We saw no seasonal patterns in GR or MR in skin, suggesting skin's response to CORT is modulated primarily via changes in circulating CORT titres and/or via local production of CORT in response to wounding and other noxious stimuli.

1. Introduction

Glucocorticoid hormones, such as corticosterone (CORT), the primary glucocorticoid in birds and rodents, help regulate metabolism and activity levels as well as immune function [1,2]. CORT secretion increases in response to environmental stressors. In addition, increases in plasma CORT can activate the immune response, as well as immune activation resulting in increases in plasma CORT, creating bidirectional effects between CORT and the immune system [3,4]. Acute increases in plasma CORT titres were traditionally thought to be primarily immunosuppressive, but they can also play immune-enhancing roles [2]. The effect seen may depend on which component of the immune system is being examined [5], seasonal variation in circulating CORT titres [6] and the time scale of CORT activation [7]. For example, a short-term rise in CORT due to acute stress can increase delayed-type hypersensitivity (DTH) reactions, enhancing the reallocation of immune cells from spleen and bone marrow into more peripheral locations, such as skin, whereas sustained high CORT can suppress DTH [8,9].

In fact, because CORT's effects on immune function are so heterogeneous, Spencer *et al*. [5] suggest that it is not helpful to try to describe a single unitary role for CORT on the immune system. Rather, they encourage thinking about CORT function at a number of different physiological levels, such as at the level of different immune tissues. This fits with a general principle of endocrinology, that differential tissue sensitivity, and thus different physiological effects, result from tissue-specific expression of receptors [10]. However, this principle has rarely been tested in free-living animals. As with other hormones, CORT's ability to act on different immune tissues depends on the presence of CORT

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Table 1. Summary of specific immune risks associated with different life-history stages in free-living birds.

life-history stage	specific immune risks	sources
early spring/ pre-egg-laying	increased ectoparasite risk	[27]
	increased blood parasite risk	[27–29]
	increased wounding and/or injury due to fighting with conspecifics over mates or territory	[30,31]
late breeding	increased infectious disease risks due to influx of immune-naive juveniles into population and increased flocking behaviour	[29,32]
moult	increased infection risks due to broken feathers?	[33]

receptors. Many immune tissues, including thymus, spleen, skin and leucocytes, contain high concentrations of one or both of the two intracellular CORT receptors [11,12]. The type I (or mineralocorticoid receptor (MR)) binds CORT with subnanomolar affinity, and the type II (or glucocorticoid receptor (GR)) binds CORT with nanomolar affinity. Because of these affinity differences, it is generally thought that the effects of baseline CORT concentrations are primarily mediated via the MR, whereas the GR becomes more important in conditions of acute or chronic stress [1]. This interpretation is unresolved, however [13], primarily resulting from a lack of detailed information on CORT receptors. The use of GR- or MR-specific agonists supports the idea that receptor expression is related to CORT sensitivity, and in some cases GR and MR appear to mediate different effects on the immune system [3].

Specific receptors for CORT have recently been characterized in house sparrow (*Passer domesticus*) spleen and skin [14]. These two tissues serve crucial roles in the immune system and interact with CORT in complex ways. Specifically, spleen is an important site for lymphocyte mitogenesis and proliferation [15,16], and plays a role in re-allocation of immune cells during DTH [9,17]; these processes are both enhanced by short-term increases in CORT and inhibited by long-term CORT activation. Skin is a major target for immune cells during DTH [9]. Although GR and MR are present in other immune tissues in birds [18], these tissues are either too small to individually assay in sparrows using receptor binding techniques (e.g. thymus), or only important during certain developmental stages (i.e. bursa of Fabricius, which only plays a role in juvenile immune function).

Another critical factor affecting interactions between the CORT and the immune system is the seasonal modulation of both. Immune function varies seasonally in many wild animal species, with different components stronger or weaker at different times of year [19–21]. Klasing [22] suggests all components of immune function cannot be strong at all times of year because of risks associated with autoimmunity and possible trade-offs with other energetically expensive processes (see [19]). CORT secretion also shows seasonal modulation—baseline CORT, stress-induced CORT, adrenal sensitivity and negative feedback of the hypothalamus–pituitary–adrenal axis have all been shown to vary with life-history stage [6,23]. On the receptor side, seasonal changes in CORT receptor binding in brain have also been reported in wild house sparrows [24]. Seasonal changes in CORT secretion and/or receptor concentrations could underlie differences in how CORT affects immune function at different times of year. For example, acute stress augmented DTH [25] and wound healing [26] in Siberian hamsters kept on short days, but not those kept on long days. It is not currently known if free-living wild animals seasonally modulate the CORT sensitivity of different immune tissues.

We used radioligand binding assays to quantify skin and spleen GR and MR in wild house sparrows at six ecologically relevant time points: early winter, late winter, before egg-laying, during breeding, late in the breeding season and in autumn during annual moult. We hypothesized that CORT receptor concentrations in spleen and skin would show seasonal modulation distinct from broader trends in plasma CORT titres. Specifically, because CORT can augment DTH and lymphocyte mitogenesis, at least in the short term, we expected to see increased spleen and skin sensitivity to CORT during times of year associated with increased immune challenges (table 1): the pre-laying period, the late breeding period and moult.

2. Material and methods

(a) Study subjects and chemical adrenalectomy

Wild house sparrows were caught at six times of year corresponding to important life-history stages in New England: moult (6–19 September 2010, $n = 12$), early winter (12–15 December 2010, $n = 12$), late winter (1–14 February 2011, $n = 12$), pre-egg-laying (31 March–5 April 2011, $n = 12$), breeding (23–24 May 2011, $n = 12$) and late breeding (12–18 July 2011, $n = 12$). We caught equal numbers of males and females during each stage. Sparrow age was unknown, but we excluded fledgling sparrows from sampling during breeding and late breeding. For each individual, life-history stages were confirmed by inspecting cloacal protuberances and beak colour (in males) and brood patches (in females); gonads were also removed and weighed at the time of sacrifice. All moulting birds were moulting primary feathers (P3–P9). Additional information on breeding stage in these birds and in another group of birds caught at the same times for a different study has been published previously [23].

Sparrows were caught at bird feeders in Medford and Somerville MA using mist nets and Potter traps. In the laboratory, birds were housed two per cage under day length conditions corresponding to their capture date. To reduce endogenous CORT which would otherwise interfere with receptor binding assays, sparrows received two intramuscular injections of mitotane (*ortho*, *para*-DDD; 180 mg kg^{-1} body weight; Sigma Aldrich, St Louis, MO, USA) approximately 36 and 24 h prior to sacrifice [14,24]. Mitotane appears to be quite specific in reducing circulating CORT [34]; 3 days of mitotane treatment did not affect circulating testosterone or testicular weight in house sparrows [35].

(b) Blood sample processing and radioimmunoassays

To measure the success of mitotane treatment, 36 h after the first injection birds were restrained in cloth bags for 30 min, and

approximately 30 µl of blood was taken in heparinized capillary tubes. Blood samples were centrifuged and plasma removed and stored at −20°C. We determined CORT concentrations in each sample using radioimmunoassay following the methods of Wingfield *et al*. [36]. Samples were assayed in duplicate using antibody B3-163 (Esoterix, Calabasas Hills, CA, USA), and assay values corrected for individual recoveries following extraction. For birds at all life-history stages, mitotane reduced stress-induced CORT; mean CORT was 1.7 ± 3.5 ng ml^{-1}, compared with approximately 20–30 ng ml^{-1} for house sparrows not treated with mitotane [37].

(c) Tissue processing

Birds were anaesthetized with intramuscular injections of ketamine (approx. 80 mg kg^{-1} body weight; Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (approx. 20 mg kg^{-1} body weight; Akorn, Inc., Decatur, IL, USA) [38]. Once deeply anaesthetized, sparrows were transcardially perfused with ice-cold heparinized saline, and spleen, belly skin (the ventral patch of skin beginning halfway down the pectoralis muscle and ending above the cloaca) and back skin (the dorsal patch of skin beginning at the nape and ending above the tail) were removed, plucked of all feathers (in the case of skin) and flash-frozen on dry ice. Tissues were always taken in the same order, and the time to take all tissues was recorded for each bird (mean time = 13.5 ± 1.3 min). Tissues were stored at −80°C until assayed.

(d) Receptor binding assays

Receptor binding assays were done following Breuner & Orchinik [24] and have been described in detail elsewhere [14]. We used homogenization techniques, tissue to buffer ratios, incubation times and temperatures optimized for house sparrow spleen, belly and back skin [14]. Briefly, on the day of the assay, tissue was homogenized in ice-cold buffer and spun at 104 000*g* for 1 h at 4°C in an ultracentrifuge to separate soluble proteins (including MR and GR) from nuclear, mitochondrial and microsomal proteins.

Cytosol was incubated with 10 nM [^{3}H]CORT (PerkinElmer, Waltham, MA, USA) and either (i) buffer, to measure total binding; (ii) 1 µM unlabelled CORT (Sigma Aldrich), to measure non-specific binding; or (iii) 1 µM RU486 (mifepristone; Tocris Bioscience, Minneapolis, MN, USA), which only binds low-affinity GR. After subtracting out non-specific binding, MR binding can be calculated directly from test tubes containing RU486; GR binding can be calculated by subtracting MR binding from total binding. Although RU486 also binds with high affinity to progesterone receptors (PR), CORT only binds avian PR at very high concentrations (about 1 µM [39]). Based on affinity estimates derived from previous equilibrium saturation analyses [14], mass action predicts that 10 nM [^{3}H]CORT should occupy more than 95% of MR and approximately 63% of GR.

Incubations were terminated by rapid filtration and filter paper was mixed with scintillation fluid, vortexed and run on a scintillation counter. We standardized binding per milligram protein in individual samples using Bradford [40] assays. All samples used for analysis contained 1–10 mg protein ml^{-1} buffer, a range shown to produce accurate results for intracellular GR binding assays [41]. Each sample was run in triplicate, and for each tissue, receptor number for all individuals was determined in the same assay to avoid inter-assay variation.

(e) Data analysis

All statistical analyses were run using JMP v. 9.0 (SAS Institute Inc., 2010). Tissue mass (for spleen) and GR and MR binding (for spleen and the two skin areas) were compared among all six life-history stages using analysis of variance (ANOVA). Because sex has been shown to influence immune function in this species [20], we also looked for sex differences. We found no sex differences for any variable ($p \geq 0.45$) except for a marginally significant effect of sex on spleen GR ($p = 0.053$). Because of this, we included sex and sex × season interactions in the analysis for spleen GR but excluded sex from all other analyses.

With equal sample sizes, ANOVA is fairly robust to violations of normality assumptions, but not to violations of homogeneity of variances [42]. Thus, for each analysis, we made sure that data met the homogeneity of variances assumption using Levene's test. In situations where variances among groups were not homogeneous, we ran a Welch's ANOVA [42]. In cases where we found a significant difference among groups, we ran Tukey's Honestly Significant Difference test as a multiple comparison procedure, as recommended by Quinn & Keough [43].

One male in the early winter group had a greatly enlarged spleen compared with other individuals in his group (87.7 mg compared with a mean of 20.8 ± 8.3 mg). This enlarged spleen could indicate some kind of recent immune challenge, such as parasite infection [44,45], independent of any seasonal variation in spleen size, so we chose to exclude this individual from the spleen mass analysis. Several individuals were also excluded from receptor analyses for spleen (one female each during moult and late winter, and one male during pre-laying) and back skin (two females each during early winter and late winter, two males during pre-laying, and one male and one female during breeding) because of low protein concentrations in cytosol (below the 1 mg ml^{-1} threshold, see above). Data are available at the Dryad repository: doi:10.5061/dryad.6mb78.

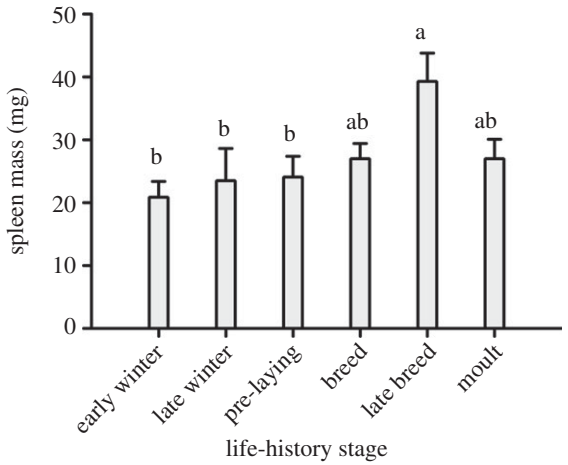

Figure 1. Spleen mass of wild house sparrows caught in Massachusetts at six different life-history stages ($n = 12$ at each life-history stage, except for early winter, where $n = 11$). Different letters represent statistical differences as indicated by post hoc tests. All values are presented as means ± s.e.m.

3. Results

(a) Spleen

Mean mass of house sparrow spleen varied significantly depending on life-history stage (figure 1, $F_{5,65} = 3.10$, $p = 0.014$). Post hoc analysis revealed that spleen mass was greater in the late breeding period when compared with early winter, late winter and pre-laying. GR binding in spleen also varied by life-history stage (figure 2*a*, $F_{11,57} = 2.49$, $p = 0.042$), but not by sex ($F_{11,57} = 3.42$, $p = 0.070$) or sex × life-history stage ($F_{1,5} = 1.73$, $p = 0.14$). Post hoc analysis revealed that GR binding was greatest in the pre-laying period, lowest in the late breeding period and intermediate

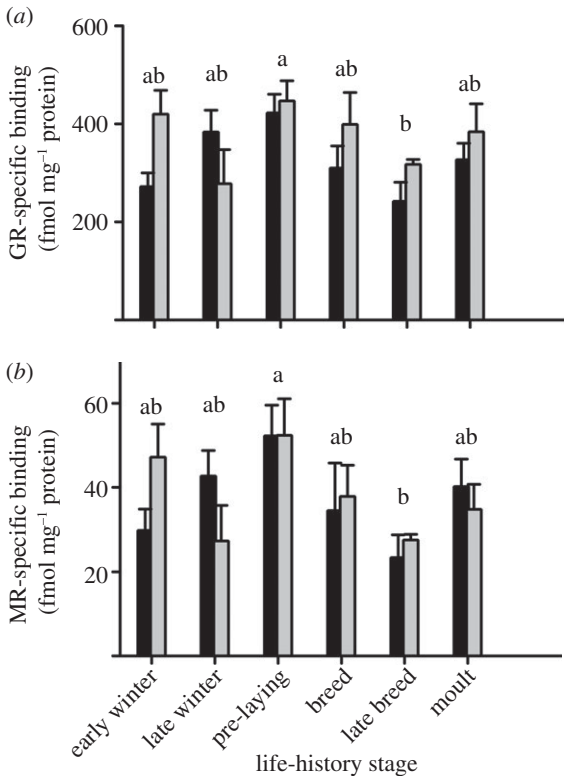

Figure 2. Point sample analysis of (*a*) GR-like and (*b*) MR-like receptors in the spleen of wild house sparrows caught in Massachusetts at six different life-history stages ($n = 6$ males and 6 females at each life-history stage, except for one female excluded during moult and late winter and one male excluded during pre-laying because of low protein concentrations in cytosol). Data represent means $\pm$ s.e.m. of specific binding of 10 nM [^{3}H]CORT to house sparrow cytosol, standardized by protein concentration. MR receptor capacity was determined by adding 1 µM of the GR-specific antagonist RU486 to tubes. GR receptor capacity was determined by subtracting MR capacity from total specific binding. Different letters represent statistical differences among life-history stages as indicated by post hoc tests. Black bars, males; grey bars, females.

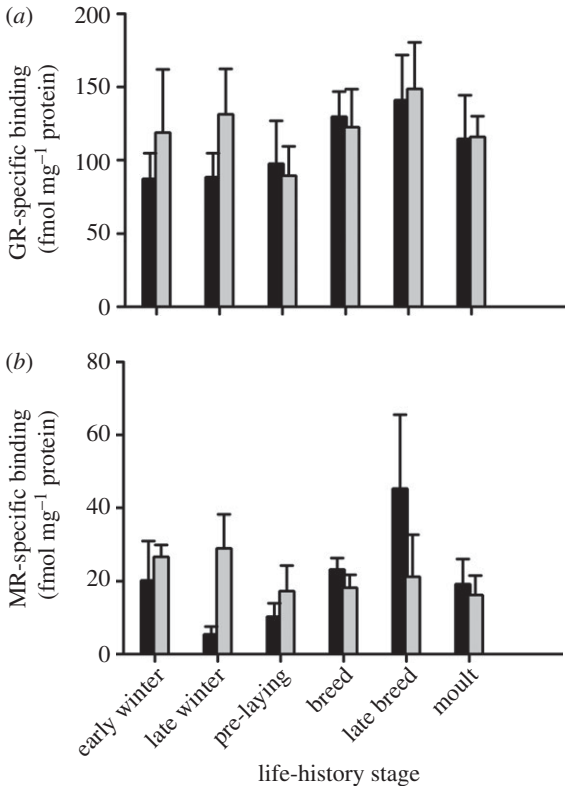

Figure 3. Point sample analysis of (*a*) GR-like and (*b*) MR-like receptors in the belly skin of wild house sparrows caught in Massachusetts at six different life-history stages ($n = 6$ males and 6 females at each life-history stage). Data represent means $\pm$ s.e.m. of specific binding of 10 nM [^{3}H]CORT to house sparrow cytosol, standardized by protein concentration. For more information, see caption of figure 2. Black bars, males; grey bars, females.

during other stages. MR binding in spleen also varied by life-history stage (figure 2*b*, $F_{5,64} = 2.85$, $p = 0.022$). Post hoc analysis revealed the same pattern as in GR—higher MR binding in the spleens of pre-laying birds compared with late breeding birds.

(b) Skin

After subtracting out non-specific binding, we were still able to detect low levels of CORT binding in the presence of RU486, potentially indicating low concentrations of MR. However, in belly skin, neither GR binding (figure 3*a*, $F_{5,66} = 1.02$, $p = 0.41$) nor MR binding (figure 3*b*, $F_{5,30} = 0.92$, $p = 0.48$) varied by life-history stage. Similarly, in back skin, neither GR binding (figure 4*a*, $F_{5,25} = 2.39$, $p = 0.067$) nor MR binding (figure 4*b*, $F_{5,58} = 0.92$, $p = 0.47$) varied by life-history stage.

4. Discussion

This study shows seasonal modulation of CORT receptor binding in immune tissues in a wild bird. To our knowledge, this is the first time this has been shown in any species. We predicted that we would see patterns of seasonal modulation of CORT receptors in spleen and skin that were distinct from broader trends in plasma CORT titres. This was true in spleen—we saw the highest CORT receptor binding during the pre-laying period and the lowest binding during the late breeding period, which did not track baseline or stress-induced CORT titres of house sparrows from the same population caught at the same points in time [23]. However, we saw no significant seasonal trend in CORT receptor binding in skin. An endocrine effect results from the amount of signal (hormone titres), signal availability (transport and/or buffering in the blood via binding proteins) and signal reception (receptor availability). Consequently, seasonal changes in receptors will also impact seasonal changes in the stress response.

Spleen is a critical site for lymphocyte recirculation, the phagocytosis of antigens and production of various immune components, such as parts of the complement system and antibodies [46,47]. Although it is not known if avian spleen serves the same roles as mammalian spleen, John [46] suggests that this organ may in fact be more important in birds because they have fewer lymph nodes overall than mammals do. Because short-term rises in CORT can augment DTH and lymphocyte proliferation, two immune functions in which spleen is involved, we expected to see increased spleen sensitivity to CORT during times of year associated with increased immune risks: pre-laying, late breeding and moult. In keeping with this hypothesis, we did see increased GR and MR binding in spleen during the pre-laying period, a time of year that has been associated with increased blood parasites [27–29], increased ectoparasites [27] and the potential for increased wounding due to

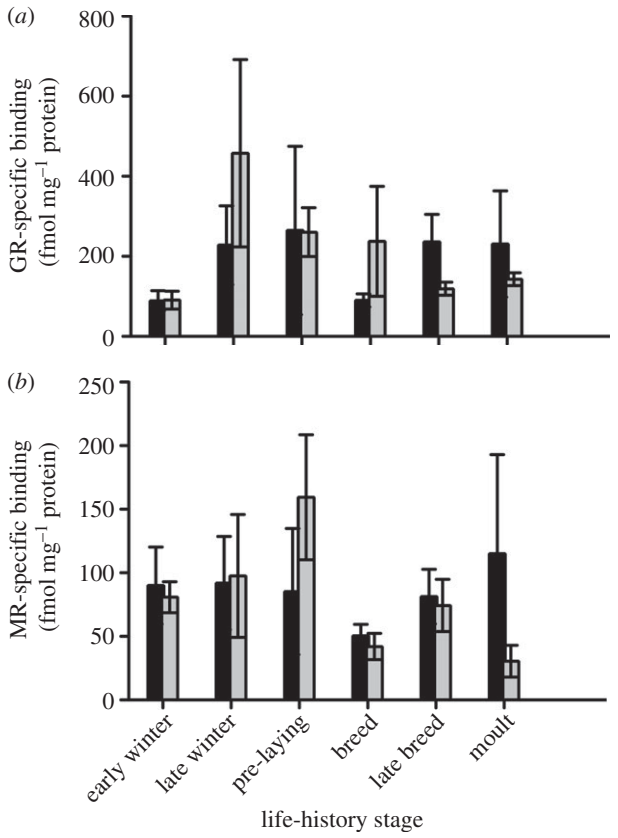

Figure 4. Point sample analysis of (*a*) GR-like and (*b*) MR-like receptors in the back skin of wild house sparrows caught in Massachusetts at six different life-history stages ($n = 6$ males and 6 females at each life-history stage except for two females excluded during early winter, two females excluded during late winter, two males excluded during pre-laying and one male and one female excluded during breeding because of low protein concentrations in cytosol). Data represent means $\pm$ s.e.m. of specific binding of 10 nM [^{3}H]CORT to house sparrow cytosol, standardized by protein concentration. For more information, see caption of figure 2. Black bars, males; grey bars, females.

fights with conspecifics over mates and territory [30,31]. During this life-history stage, a short-term rise in CORT either due to an agonistic encounter [48] or immune activation [3] might facilitate re-allocation of leucocytes to the periphery and/or cause increased lymphocyte proliferation. In wild-caught house sparrows in Florida, an acute restraint stressor did not augment DTH [49], but those birds were caught several weeks later than our pre-laying birds in Massachusetts, and may have been at a different breeding stage. It remains to be tested whether spleen activation by CORT during this time period could increase lymphocyte mitogenesis in wild birds.

Despite our predictions, however, we saw no increase in spleen sensitivity to CORT during moult, despite potentially increased immune risks because of broken blood feathers [33]. Furthermore, we saw a decrease in spleen sensitivity during the late breeding period, despite the fact that this time of year is associated with increased infectious disease owing to a population influx of naive-immune juveniles and increased flocking behaviour [29,32]. It should be noted, however, that baseline CORT, stress-induced CORT and adrenal sensitivity are all low in birds immediately prior to [23] and during moult [6,23]. Because of this, immune function during these time periods might be relatively CORT-independent.

Interestingly, there is some evidence that acute stress activates splenic MR rather than GR [50,51], which may be related to tissue-embedded corticosterone binding globulin (CBG) and/or the presence of the enzyme 11β-hydroxysteroid dehydrogenase (HSD), which converts CORT to an inactive metabolite [3,52]. However, we found remarkably similar patterns in spleen GR and MR binding, despite the fact that MR binding was an order of magnitude lower than GR. MR may regulate GR expression, which might be one reason we see this pattern [11]. Whether splenic MR or GR is the relevant receptor in situations of acute stress, and how seasonal fluctuations in CBG [37], or 11β-HSD activity [53] might amplify or dampen the patterns we see in receptor concentrations, remain to be determined. However, because spleen does not possess all of the necessary enzymes for CORT production [54], splenic GR and MR seem to be a target for circulating rather than locally produced CORT.

Skin serves as an important physical barrier against the entry of pathogens [47]. We saw no seasonal modulation of skin sensitivity to CORT in either the back or the belly region. Not only can systemic CORT affect skin immune processes, but there is also evidence of local glucocorticoid synthesis in mammalian skin [55], which can be induced by wounding [56]. This suggests that skin's response to CORT is modulated primarily via changes in circulating CORT titres and/or via local production of CORT in response to wounding and other noxious stimuli. Interestingly, in contrast with earlier work [14], our point sample assays in house sparrow skin detected both GR and smaller amounts of an MR-like receptor. MR has also been detected in mammalian skin, although its function is not completely clear [57]. We did see large individual variation in GR and MR binding within seasons, which could be related to individual differences in wounding, ectoparasites, exposure to UV damage and other assaults to skin. Future studies should explore the relationship between wounds, ectoparasites and CORT receptors in skin to see whether receptors might be up- or downregulated by tissue damage and local CORT production.

In keeping with past studies of spleen mass in wild animals, we saw smaller spleens in winter (and early spring/pre-laying) compared with breeding [58–60]. House sparrow spleens were largest during the late breeding period, a life-history stage that, as discussed earlier, coincides with increased flocking behaviour and a population influx of juveniles, which might increase the prevalence of infectious disease [32]. Therefore, the size of spleens at this time might reflect increased immune activation. John [46] has also suggested that there could be a link between seasonal peaks in spleen mass and periodic tissue regression, based on the observation that large numbers of lymphocytes infiltrate the testes during testes regression [61]. House sparrows in the late breeding period are beginning the process of gonadal regression [23], so this could be related to size of spleen during this life-history stage. In any case, the peak in spleen mass at this time of year is intriguing, and further investigation may uncover which of spleen's many functions explains this annual growth in size.

In conclusion, this study shows seasonal modulation of CORT receptor binding in immune tissues of a wild bird. Furthermore, the sensitivity of different tissues to CORT appeared to be regulated independently, which helps explain how CORT can have so many effects not just on immune function, but also on metabolism, feeding behaviour,

reproduction and locomotor activity. This study emphasizes the importance of considering seasonal variation in tissue sensitivity to CORT in understanding CORT's actions.

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