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Seasonality of the Stress Response in House Sparrows (Passer domesticus).

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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by

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Abstract

Seasonal changes in plasma corticosterone (CORT) levels indicate that birds modify their stress response through the year. Although this has been well documented, the method by which birds achieve this seasonality is not well understood. In this study I used house sparrows to determine if changes in glucocorticoid receptor (GR) immunoreactivity in several stress-related brain nuclei showed seasonal variation. The house sparrows showed seasonal variation in their stress response with baseline CORT levels being highest during the breeding season and lowest during winter. There was also significant change in plasma CORT post-dexamethasone during breeding, but not during other times of the year. In spite of the seasonal changes in CORT regulation there was no seasonal variation in the GR-immunoreactivity of brain regions involved in the stress response, such as the hypothalamus, nucleus taeniae of the amygdala, or hippocampus. These findings add to the growing research to understand the stress response.

Keywords

Passer domesticus, birds, stress response, seasonality, GR-immunoreactivity, corticosterone

Co-Authorship Statement

I plan to publish data from this thesis in the future. I will be first author on any publications with my supervisor Dr. Scott A. MacDougall-Shackleton being the second author. Dr. Scott A. MacDougall-Shackleton contributed to the experimental design, provided funding and equipment necessary for my experiments. I was involved in the conceptualization and experimental design. I was responsible for all bird care, collected all of the samples and performed all the immunohistochemistry, microscope work, image analysis and statistics during the experiment. I wrote the paper with revisions from my supervisor.

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List of Abbreviations

HPA axis – Hypothalamus Pituitary Adrenal axis

POM – medial preoptic nucleus

PVN – paraventricular nucleus

CRH – corticotrpoin-releasing hormone

AVT – arginine vasotocin

ACTH – adrenocorticotropin-releasing hormone

CORT – corticosteroid (cortisol/corticosterone)

CBG – corticosterone binding globulin

MR – mineralocorticoid receptor

GR – glucocorticoid receptor

mCR – membrane corticosterone receptor

DEX – dexamethasone

PBS – Phosphate-buffered solution

PBS/T – phosphate-buffered solution containing triton X-100

TnA – nucleus taeniae of the amygdala

RT – room temperature

GLv – lateral geniculate nucleus

TeO – Optic tectum

Imc – magnocellular part of the isthmic nucleus

LSD – Fisher's least significant difference

1 Introduction

1.1 What is stress?

Stress is a broad ranging concept that has been applied at the ecosystem, organism and cellular levels. The term stress is very broadly defined and can lead to confusion as to its use and intent. Stress can be used to describe the stimuli, the response to the stimuli, or the pathological consequences to chronic activation of the responses to stress stimuli (Romero et al., 2009). There is a variety of definitions of stress, and none are universally accepted. However, one widely accepted definition is that a stressor is anything that an organism recognizes as a threat to its current homeostasis (Kino et al., 2010). The stressor can be a real or perceived threat to the animal. The general response to a stressor has been termed the stress response, and it is present in all vertebrates. The stress response allows an animal to cope with a constantly changing and unpredictable environment, and the hazards it can contain. Numerous physiological changes are involved in the acute stress response including increased energy availability, improved immune function, inhibition of digestion, growth and reproduction, increased respiration, and changes in behavior (Sapolsky, 2000).

The function of the stress response is to increase the chances of survival for the animal when challenged by a stressor. This usually involves a shift of resources from long-term processes, such as growth and reproduction, to short-term survival. An example would be birds nesting in high elevation habitats encountering a storm. If the storm is severe the birds may abandon their nests and fly to lower elevations until the storm has passed (Breuner and Hahn, 2003). Thus the birds forgo the long-term process of raising young, and instead invest in immediate survival. After the passage of the storm

the birds will return to the nesting ground and may resume nesting. Thus, the severe storm initiates a short emergency life history event that interrupts the current life history stage through activation of the stress response.

1.2 Biological Components of Stress Response

In vertebrate animals the activation of the stress response includes several biological systems. Upon experiencing a stressor the parasympathetic nervous system initiates a fight or flight response that helps the animal escape from the stressor. This fight or flight response is due to the release of epinephrine and norepinephrine into circulation from the medulla of the adrenal glands. These catecholamines cause an increase in heart rate and respiration, an increase in blood glucose levels, and increased alertness as a means to help the animal survive the immediate stressor. This rapid response happens within seconds of a stressor, and helps the animal survive the immediate threat. Following the rapid stimulation of the sympathetic nervous system, there is a longer-term neuroendocrine response involving the hypothalamus-pituitary-adrenal (HPA) axis, the topic of most stress biology research in vertebrate animals. The HPA axis works on a longer timescale, full activation of the system takes minutes, and its effects can last for hours or longer. The HPA axis is thought to be responsible for restoring the animal to homeostasis after a stressful event (Chrousos, 1997; Rich and Romero, 2005).

1.3 HPA axis

The biological response to a stressor begins with the recognition of the stressor, and thus requires information processing by the brain, including the hippocampus and amygdala (Figure 1.1).

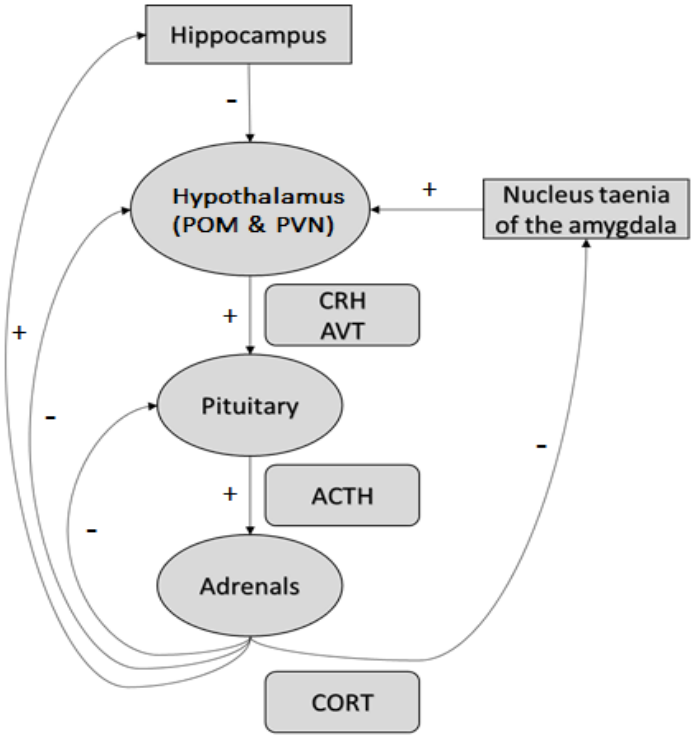

Figure 1-1 Hypothalamic-pituitary-adrenal (HPA) axis. Hippocampus and nucleus taenia (TnA) have inputs into the hypothalamus. Hippocampus inputs inhibit the hypothalamus and through release of this inhibition allow activation of the hypothalamus. Activation of the TnA leads to activation of the hypothalamus. The release of inhibition and/or activation of the paraventricular nucleus (PVN) and medial preoptic nucleus (POM) of the hypothalamus initiates an endocrine response. The Hypothalamus releases corticotropin-releasing hormone (CRH) and arginine vasotocin (AVT). These hormones activate the pituitary which releases adrenocorticotropin-releasing hormone (ACTH) into general circulation. ACTH travels to the adrenals to stimulates the release of corticosterone (CORT). CORT travels through the blood to act on many targets. CORT feedbacks onto every point of the HPA axis to initiate its own negative feedback after a stressor. + indicate activation and – indicate inhibition.

These brain regions initiate a neuroendocrine cascade through the hypothalamus. The disinhibition of the paraventricular nucleus (PVN) of the hypothalamus causes the release of corticotropin-releasing hormone (CRH) and arginine vasotocin (AVT), the avian homolog to the mammalian vasopressin, into the hypophyseal portal blood. CRH and AVT then travel to the anterior pituitary, which in turn releases adrenocorticotropin-releasing hormone (ACTH). ACTH travels through the systemic blood and acts on the adrenal cortex to cause the release of glucocorticoid hormones (corticosterone for birds and reptiles or cortisol in primates; Rich & Romero 2005). CORT has varied and widespread effects on several functions including memory, learning, energy metabolism, protein catabolism and reproduction (Joels et al., 2012; Tasker et al., 2006). CORT is argued to be the primary hormone responsible for behavioral responses to stressors as it can readily cross the blood-brain barrier, unlike epinephrine and norepinephrine (Joels et al., 2012). CORT action in the brain is not only important in terms of behavioral responses, but it also provides negative feedback to the HPA axis. Negative feedback is thought to occur by inhibitory input to the PVN by the hypothalamus' and hippocampus' (Herman et al., 2012).

As a steroid hormone CORT is hydrophobic, can readily cross plasma membranes, and must be bound to carrier proteins to be maintained at high concentrations in the circulatory system (Breuner & Orchinik 2002). For birds CORT is carried in plasma by corticosterone binding globulin (CBG), and it is estimated that over 90 % of the CORT in plasma is bound to CBG (Breuner and Orchinik, 2002). There are two different views on the function of CBG as it relates to CORT. The first view is that the approximately 10 % of CORT that is unbound to CBG is free to move into cells. This

'free CORT' hypothesis has led to the proposal that only the hormone not bound to its carrier protein should be considered biologically active (Breuner and Orchinik, 2002). In contrast, is the view that CBG acts as a carrier for CORT, such that CBG could act as a reserve pool of CORT that could be available after a stressor. CBG itself has also been shown to be important for the delivery of CORT in possibly a tissue-specific manner as cells exhibit a CBG-CORT complex receptor (Schoech et al., 2013). Thus the exact role of CBG in regulation of CORT and the HPA axis is still unclear.

There are two receptor subtypes for CORT in the brain, the mineralocorticoid receptor (MR, Type I) and glucocorticoid receptor (GR, Type II). These receptors are named after their respective effects in the periphery of the body: salt balance and glucose mobilization, respectively. In the brain these receptors are thought to have many effects on cell signaling. In house sparrows (*Passer domesticus*) it was shown that these receptors are located in the cytosol (both GR and MR) and on the cell membrane (mCR)(Breuner and Orchinik, 2001). In mammals CORT receptors in the cytosol act as gene regulators while the cell membrane receptor (mCR) acts on neuron excitability (Joels et al., 2012). This gives CORT effects a timeframe of minutes to hours depending on which receptors are present in the cell. GR and MR belong to the same family of steroid receptors, and thus have similar structures, however cytosolic MR has a 5-10 fold greater binding affinity for CORT than cytosolic GR (Breuner and Orchinik, 2009). This means that cytosolic MR are almost fully occupied at baseline homeostatic levels of circulating CORT, while GR is not extensively occupied except during times of stress and the circadian peak when CORT concentrations are higher (Meijer, Kloet, & Mcewen, 2010).

The observations of different binding affinities of MR and GR, along with studies involving antagonists and agonists to each receptor, have led to the conclusion that cytosolic MR and GR serve very different functions in the brain (Meijer, Kloet, & Mcewen 2010). Cytosolic MR is thought to be responsible for maintaining the stress system as well as determining basal levels of circulating CORT. Cytosolic GR is thought to be responsible for negative feedback following a stress response, and bringing the HPA axis back to homeostasis (Patchev et al., 1994). The affinity for CORT of the membrane bound mCR is lower than the cytosolic MR, making it similar to the GR (Breuner and Orchinik, 2009). This allows mCR to respond to stress-induced levels of CORT, and is hypothesized to contribute to the rapid behavioral changes seen in response to CORT (Meijer et al., 2010). mRNA expression studies indicate that the distribution of these receptors is not even throughout the brain (Suzuki et al., 2011). GR is expressed throughout most of the brain with high levels found in the hippocampus and hypothalamus. MR is more limited in its distribution in the brain and high expression levels have been found primarily in the hippocampus and other discrete limbic structures. Though mCR has been characterized in birds its function is still unknown (Breuner and Orchinik, 2009; Meijer et al., 2010).

1.4 Concepts of HPA regulation

As noted above, the term stress can be used to describe the stimuli, the response to the stimuli or the pathological consequences to chronic activation of the responses to stress stimuli (Romero et al., 2009). These broad and confusing uses of stress along with further understanding of the stress response system, have led to several models being proposed to explain and model the stress response system and more specifically the HPA

axis. The stress response functions to help the animal deal with a short-term stressor, and is thus beneficial in the short term. Repeated or chronic activation of the system can have long-term negative effects. To respond to stressors CORT diverts energy from systems such as immune function and digestion. Repeated suppression of these systems leads to decreased cell division which cause an increased susceptibility to stomach ulcers and a suppressed immune system (Chrousos, 1997). Since the CORT stress response can be viewed from a costly/pathological viewpoint or an adaptive/beneficial viewpoint, two different models of stress have been proposed: allostasis and the reactive scope model.

1.5 Allostasis

The definition of allostasis is the achieving of stability through change (McEwen and Wingfield, 2003; Wingfield, 2005). The allostasis model was developed to help explain seasonal changes to several important life-sustaining parameters that are kept in a state of homeostasis. In the allostasis model the meaning of homeostasis is the maintaining of these important life parameters within set points, while allostasis refers to the systems responsible for maintaining homeostatic balance. With these definitions we can begin to see how an animal will be able to respond to both predictable and unpredictable events in their environment. The maintenance of these homeostatic systems incurs a cost, termed allostatic load. In normal conditions this energy load is easily maintained by the animal. Unpredictable stressors, such as storms, increase the energy necessary to maintain homeostasis, which then increases the allostatic load on the animal. If the allostatic load becomes too high, such that there is an inadequate energy supply to maintain these systems, the animal enters allostatic overload. This can move the animal from its current life history stage, such as breeding, into an emergency life history stage

(McEwen and Wingfield, 2003; Romero et al., 2009). This emergency life history stage is an attempt by the animal to reset its energy balance so that it can maintain homeostasis. If the animal spends too much time in allostatic overload than pathological consequences can ensue (McEwen and Wingfield, 2003).

The allostasis model gives us a way to view the various interactions of the environment and homeostasis. The model gives rise to the idea of three distinct physiological states that can be used to describe how glucocorticoids can adjust physiological processes to match the current environment (Landys et al., 2006; McEwen and Wingfield, 2003). These three states (A, B, C) correspond to basal levels (A), changes due to predictable life events (B), and changes due to unpredictable life events (C). A is the minimum level required to maintain homeostasis for the resting animal. B represents the changes to the levels of glucocorticoids to maintain homeostasis during predictable life history stages such as breeding, migration or molt. C occurs when encountering a stressor or unpredictable event, and glucocorticoids rise to allostatic overload levels as the animal diverts into an emergency life history stage until the stressor is over (Figure 1.2).

Allostasis is an important concept when conceptualizing stress. An animal experiences predictable changes in its environment including molting, breeding and wintering. These have been called stress, such as references to the stress of breeding

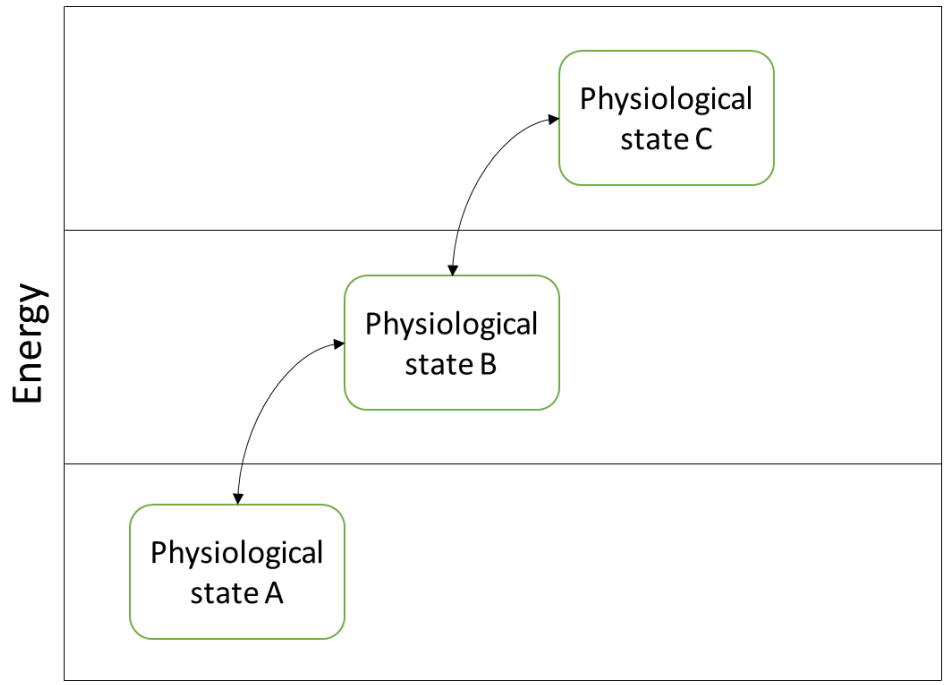

Figure 1-2. Allostatic Model. An animal will be in one of three states (A, B or C) depending on their current situation. State A is the energy required to maintain homeostatic balance in an animal at rest. State B is the normal heightened energy range that is required for the animal to maintain homestatic balance during life history events such as raising young. If an unpredictable event such as a severe storm that is life threatening is encountered the animal will move to state C, an emergency life history stage, to cope with these stressors. After the stressor passes the animal will drop back to state B and resume its current life history stage.

(Wingfield, 2005). Although these challenging activities (breeding, migration) are sometimes referred to as stress they are not the same as an unpredictable stressor, such as encountering a predator. An unpredictable stressor gives rise to a stress response (HPA axis) while the first does not. The concept of allostasis takes into account the different effects of predictable and unpredictable events. By viewing these as separate we can also include in the model the fact that animals can anticipate and prepare for changes in life history stages. The allostasis framework also includes the idea of allostatic load, the concept that a stressor puts a load on the system and that additional stressors could put the animal into allostatic overload even though each stressor by themselves is not enough to do so. While the allostasis framework adds to our understanding of the stress response it does not completely model all aspects of the stress response such as to repeated stressors.

1.6 Reactive Scope Model

The reactive scope model has been recently proposed in an attempt to build upon and refine the allostasis model (Romero et al., 2009). The reactive scope model goes back to using the term homeostasis rather than adding a new term (allostasis) to the stress literature. The reactive scope model is more adaptable to ecological research as well as being applicable to laboratory work.

The reactive scope model adds another level of interaction between hormones or other mediators and responses to changes in homeostasis. Predictive homeostasis is the response range due to daily changes in hormones as well as changes in predictable life history stages such as breeding or migration. When encountering a stressor, the response of the hormone moves the system into reactive homeostasis. These two ranges (predictive

homeostasis and reactive homeostasis) encompass the normal reactive scope for an animal and healthy responses are constrained to within these levels. If a response leads to levels above the reactive homeostasis range the animal enters into homeostatic overload and long-term pathological consequences can ensue. The final level is termed homeostatic failure and refers to hormone levels that fall below those necessary to maintain homeostasis and animals that fall into this level usually die (Figure 1.3).
Not only does the reactive scope model include a range in which the mediators can no longer maintain the basal levels necessary for life, but it also includes ways to model the effects of repeated stressors or developmental stressors on the system through the concept of wear and tear. Repeated stressors on the system could reduce the reactive homeostasis range by decreasing the threshold at which the mediator (hormone) will induce pathological consequences, as repeatedly entering the reactive homeostatic range induces wear and tear. The same idea can be used to model the effects of developmental stress on the system. The point at which homeostatic overload is reached could be moved up or down depending on previous stress history of the animal.

The reactive scope model builds upon some of the strengths of the allostatis model such as the differentiation of predictable and unpredictable stressors in the environment. It goes beyond the allostasis model by introducing the concept of wear and tear. This allows us to view stress as a continuum and not each stressor as an independent event. This makes the model useful in ecology as we can incorporate developmental stress, as well as repeated stressors on the system. One common feature of the allostasis and reactive scope models is that both draw attention to predictable seasonal changes in the stress response and HPA function, a concept I elaborate below.

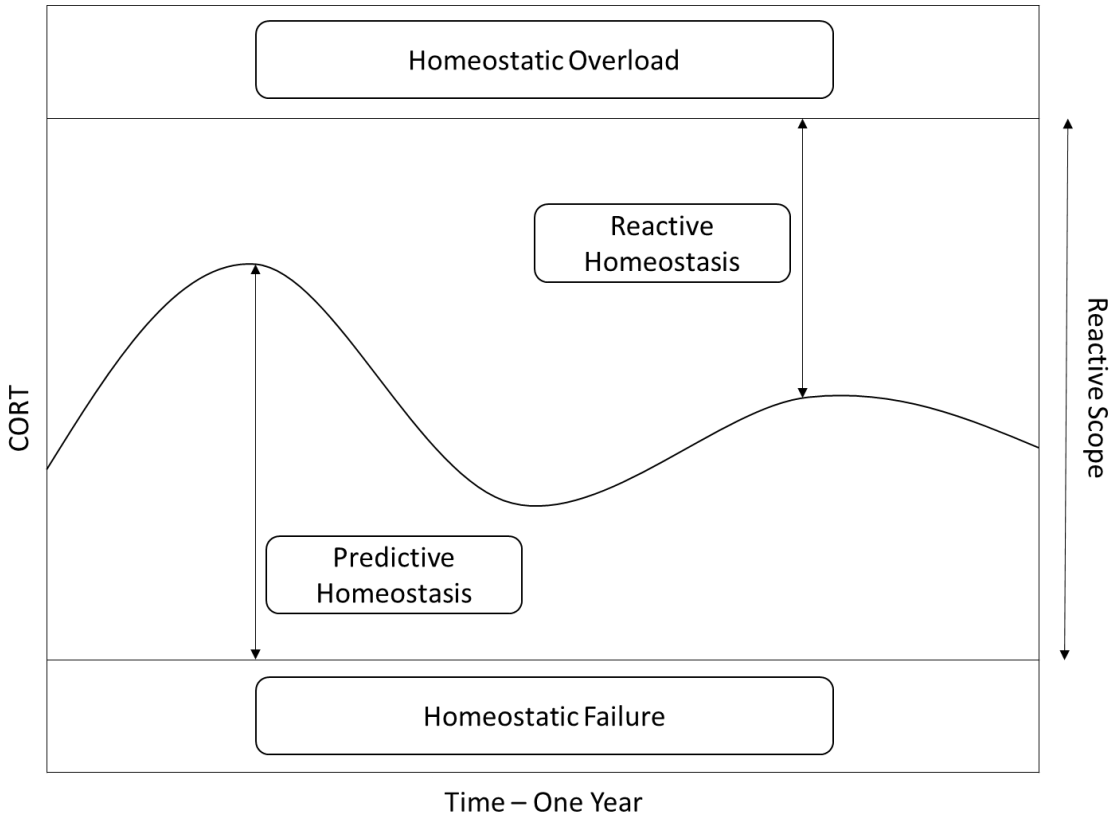

Figure 1-3. Reactive Scope Model. Within a year an animal will experience seasonal changes in any of the physiological mediators such as CORT. These seasonal changes in the mediators encompass the range of the predictive homeostasis. When a stressor is encountered the mediator will move into the reactive homeostasis until it has passed. This is the reactive scope for the mediator and as long as it stays within this range the mediator is beneficial. When the mediator moves outside this range into homeostatic failure or homeostatic overload then pathological consequences can ensue (Romero et al., 2009).

1.7 Seasonality of the HPA axis in songbirds

As animals progress through the seasons of the year, the environment changes. This seasonal change results in changes in the frequency, types, and severity, of the stressors an animal may encounter. In addition, the costs and benefits of mounting a stress response may also change with seasons. Because the stressors an animal must respond to change seasonally, the stress response system is also observed to undergo seasonal changes. Glucocorticoid concentrations appear to be seasonally variable in most species of reptiles, amphibians and birds (Romero, 2002). This seasonal variation in glucocorticoid concentrations typically involves baseline CORT levels being highest during the breeding season for most bird species (Romero, 2002). In addition to changes in baseline CORT concentrations, any or all of the above mentioned components of the HPA axis might change seasonally.

Most studies of birds have shown significant seasonal differences in both baseline CORT and CORT levels in response to stress, with CORT levels during molting (replacing of the feathers) being lower than CORT levels during breeding (Breuner and Orchinik, 2001; Liebl et al., 2013; Romero, 2002; Romero and Remage-Healey, 2000; Romero et al., 1997). CORT is thought to be down-regulated by birds during prebasic molt because elevated CORT has been shown to decrease feather quality and performance (DesRochers et al., 2009). Molting is also a dangerous time for birds because the loss of flight feathers can increase risk of predation, and high levels of CORT have been shown to increase the length of time a bird spends in molt (Romero et al., 2005).

Several studies have also looked at the effect of higher CORT during the breeding season as compared to fall and winter. Higher levels of CORT could have two effects on a bird's behavior during the breeding season. CORT could cause an increase in feeding behavior during the breeding season. Adrenalectomized animals show a reduced food intake that is reversed with administration of low levels of CORT (Landys et al., 2006). In two studies on mourning doves and Macaroni penguins increased levels of baseline CORT have been correlated to increased foraging rates and nestling feedings (Crossin et al., 2012; Miller et al., 2009). To explore the mechanisms by which baseline and stress-induced CORT concentrations change seasonally, researchers have examined species differences in which components of the HPA axis change. Studies looking to see if the sensitivity of the adrenals or pituitary to ACTH or CRH/AVT change seasonally have found species differences (Romero & Wingfield 1998, Romero et al. 1998). In these studies, free-living birds were injected with ACTH or CRH/AVT to see if exogenous hormones would increase 30 minute restraint stress CORT above the levels seen in controls. This hormone challenge determines if the adrenals or pituitary are the rate-limiting steps in the CORT output of HPA axis. House sparrows (*Passer domesticus*) showed seasonal changes in the sensitivity of both the adrenal and pituitary to exogenous hormones, but the exogenous hormones did not increase CORT output across all seasons (Romero, 2006). Thus, neither the adrenals nor pituitary limit CORT release. In contrast to house sparrows, the adrenals and hypothalamus are the limiting step in different seasons in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*). During the winter and fall seasons exogenous ACTH failed to increase CORT output, suggesting that the adrenals limit CORT release during these seasons (Romero and Wingfield,

1998). Two other arctic-breeding songbirds, Lapland longspurs (*Calcarius lapponicus*) and redpolls (*Carduelis flammea*), showed increased adrenal secretion of CORT in response to exogenous ACTH during molt, but they did not reach breeding levels of CORT, indicating that capacity, and not sensitivity, might explain decreased CORT in these species (Romero et al. 1998; Romero et al. 1998). Compared to other arctic birds the snow bunting (*Plectrophenax nivalis*) showed that the pituitary's secretion was maximal during molt, and thus the pituitary is important in controlling CORT release during molt in these birds (Romero et al. 1998). It appears that different species of birds modulate different steps in the HPA axis to regulate CORT output in different seasons.

CBG could also play a role in how birds vary their stress response. Independent of CORT regulation, varying CBG levels could change the levels of free and bound CORT (Breuner, 2002). In the same arctic species used in the studies described above, Lapland longspurs, redpolls, Gambel's white-crowned sparrow and snow buntings, the levels and affinity of CBG appear to not have an effect on the amount of free CORT as during breeding the stress induced CORT levels exceed CBG binding levels, but they do not during molt (Romero et al. 1998; Romero et al. 1998; Romero et al. 1998; Romero & Wingfield 1998). In contrast, house sparrows seasonally alter their CBG levels in a similar manner to CORT, and the estimates of free CORT are seasonally static (Breuner and Orchinik, 2001). Though free CORT is static there is a doubling of CORT bound to CBG in circulation, and this could have its own influence on the organism, such as increasing the pool of available CORT for metabolic needs during breeding, or CBG actively delivering CORT to specific tissues (Breuner, 2002). Not only does there seem to again be species differences in CBG action, but there are also population differences in

CBG levels. Breuner et al. (2003) found that three different populations of white-crowned sparrows that breed at different elevations and latitudes show similar CORT levels, but different CBG levels. These differences in CBG included quantity and affinity for CORT that when accounted for showed a population difference in free CORT. The birds that bred at a higher elevation and latitude had a shorter breeding season, as they can only produce one clutch, and their free CORT was the lowest. This is in contrast to the other two populations that can raise 2 or 3 clutches in a breeding season. There was a linear relationship between length of breeding season and amount of free CORT among these populations (Breuner and Orchinik, 2001). These studies indicate that there are several ways for birds to seasonally regulate the amount of CORT in circulation. There are fewer studies that have looked at any seasonal changes in other aspects of the HPA axis including negative feedback, receptor expression, and receptor distribution. The few studies that have attempted to fill in this gap have been done primarily with house sparrows.

1.8 House Sparrows

Several investigations into the seasonal plasticity of the HPA axis have used house sparrows as a study species, giving rise to a more complete understanding of how the individual components interact in this species. Free-living house sparrows showed seasonal changes in the sensitivities of both the adrenal and pituitary glands to ACTH and CRH respectively, but these results did not fully explain seasonal changes in CORT release (Romero, 2006). A follow-up study using captive house sparrows showed that the adrenals were maximally stimulated by ACTH in molting birds, and this could explain the decreased CORT seen during molt (Romero and Rich, 2007). The seasonal changes in

the sensitivities of both the adrenals and pituitary could allow for additional fine-tuning of the HPA axis to further environmental events. In addition, captivity has an effect on adrenal sensitivity. House sparrows had significantly greater adrenal sensitivity during late breeding and molting due to five days of captivity when they would be undergoing a chronic stress response (Lattin et al., 2012). House sparrows do show seasonal changes in adrenal/pituitary sensitivities but these do not explain the seasonal changes in CORT output.

In addition to seasonal changes in adrenal/pituitary sensitivity, seasonal changes in HPA function likely involve changes in negative feedback. Several studies have used binding assays on homogenized brain tissue to characterize the properties of CORT receptors in the house sparrow brains (Breuner & Orchinik 2009). Further studies then looked at seasonal changes in corticosterone receptors in the house sparrow brain and showed that these receptors undergo seasonal fluctuations in expression in brain tissue (Breuner and Orchinik, 2001). Membrane CORT receptors in the whole brain were lowest during breeding compared to molt and winter, while cytosolic CORT receptors in the whole brain were lowest during winter compared to molt and breeding (Breuner and Orchinik, 2001). A further study using receptor binding assays looked at each of these seasons more in depth, and found lowest GR binding in whole brain during breeding compared to molt, early winter, late winter, pre-egg laying or late breeding (Lattin and Romero, 2013). No significant differences in MR binding in whole brain were found, as well as no difference in hippocampus GR or MR receptors (Lattin and Romero, 2013). Not only have differences in receptor binding been found, but seasonal differences in receptor mRNA expression have also been observed. Using qPCR on homogenized

hippocampus tissue Liebl et al. (2013) found differences in the ratio of MR:GR mRNAs with a higher ratio during molt compared to breeding. Increases in MR mRNA drove this change in ratio; no seasonal change in GR mRNA was observed (Liebl et al., 2013). Though important, these studies need to be interpreted with caution as there was no correlation found between mRNA and protein abundance for either GR or MR in house sparrows (Medina et al., 2013). It thus remains unknown if seasonal changes observed in GR and MR mRNA translate into seasonal changes in receptor protein.

Current research has examined seasonal changes in circulating CORT concentrations, circulating CBG concentrations, and changes in receptor levels in the brain on a global scale. However, a critical gap in our understanding is how the CORT receptors in specific brain regions are changing. Several brain regions are important for the function of the HPA axis. Changes in receptor amount or distribution in particular regions could play a critical role in seasonal modulation of the HPA axis.

1.9 Research Question

I hypothesized that the suppression in the HPA axis during molting in songbirds is due, in part, to an increase in GR protein levels in the hippocampus and hypothalamus, resulting in a higher inhibitory effect on CORT release and a strengthened negative feedback loop. The focus of my research was on GRs as they are only activated in large numbers during a stress response; MRs are thought to be maximally occupied at basal levels of CORT and are thought to be less important for negative feedback of a stress response (Meijer et al., 2010).

Based on the above hypothesis, I predicted that during molting, when basal and

stress-induced levels of CORT are the lowest, that I would observe: i) decreased concentrations of baseline CORT, ii) lower increases in CORT following a stressor, iii) the greatest decrease in CORT following a negative feedback challenge (dexamethasone injection), and iv) changes in GR immunoreactivity of the hippocampus and/or hypothalamus. To test these predictions I captured adult house sparrows and used changes in photoperiod to bring them into breeding condition, molt, wintering condition, and back in to breeding condition. During these four life history stages I measured baseline CORT, CORT following restraint, and CORT following a negative feedback challenge. In addition I collected brains from a subset of birds to measure GR using immunohistochemistry.

2 Methods

All procedures complied with guidelines set out by the Canadian Council on Animal Care (CCAC), and were approved under protocol 2007-089 by the Animal Use Subcommittee of the University of Western Ontario.

2.1 Bird Capture and Care

I captured all birds used in this study at the University of Western Ontario campus in London, Ontario between April 14, 2014 and May 23, 2014. Birds were captured in Potter traps and were housed in individual cages (46 cm x 46 cm x 46 cm) in the Advanced Facility for Avian Research at the University of Western Ontario. Birds were fed a mixture of Living World Premium for Budgies seed (Hagen) and ground Small Bird Maintenance diet (Mazuri, catalogue 56A6) for small birds. Food was given *ad libitum* and changed every day. Each bird was given two meal worms every other day. The birds

were also provided with grit and cuttlebones for maintenance. Every 3 to 4 weeks birds were given spray millet.

2.2 Photoperiod manipulation

Birds were initially maintained on a natural light cycle as they acclimated to captivity. After two weeks in captivity the first breeding group (n = 8) was euthanized. Two birds, in a day, underwent a stress test where three blood samples were collected. Birds were then euthanized and their brains collected and preserved for later immunohistochemistry analysis. After collection of the first breeding group (breeding1) the birds were moved to a constant long day photoperiod (18L:6D). Birds maintained on long days will become photorefractory and will regress their gonads and begin molting their feathers (Dawson, 1991).

Molting progress was assessed using the wing feathers. I euthanized birds for the molting group (n = 15) in mid-August when they were replacing their 4^{th} primary flight feather on either wing. Primary molt was monitored every few days, and I collected blood samples and brains at the same stage of molt over a period of 24 days, rather than on the same calendar date.

The remaining birds were maintained on the long day photoperiod until molting was completed in over 90% of individuals. Birds were then gradually moved to a short day, winter light cycle (8L:16D) over a week, in order to induce photosensitivity. After 10 weeks on the short light cycle I collected blood samples, euthanizedand removed brains from the wintering group birds (n = 15).

The remaining 8 birds were then exposed to a summer, long day, photoperiod (15L:9D) to induce breeding condition (photostimulation). Testosterone increases in

house sparrows when in they are in breeding condition and this causes an increase in melanin deposition into their beaks, turning them black (Keck, 1934). I visually monitored beak color to assess the bird's reproductive condition and I collected blood samples, euthanized and removed brains from the birds (breeding2) when their beaks were completely black (Figure 2.1). The breeding2 group was used to control for any effect of captivity on the function of the HPA axis. I expect to see a decrease in the strength of the stress response in molting and wintering birds as compared to breeding birds. To determine if this is indeed the reason and not that the birds are undergoing a chronic stress response a second breeding group collected after the molting and wintering birds was included.

2.3 HPA axis characterization/ blood collection

I collected blood samples from two birds per day for no more than three consecutive days to minimize stress of the experimenter being in the room to the remaining birds. I started blood collection a third of the way through their respective light part of the day to control for daily fluctuation in CORT levels. Blood was drawn via brachial venipuncture with a 26 gauge needle and collected in microhematocrit capillary tubes. Samples were kept on ice and all samples were centrifuged at 13,000 g for 10

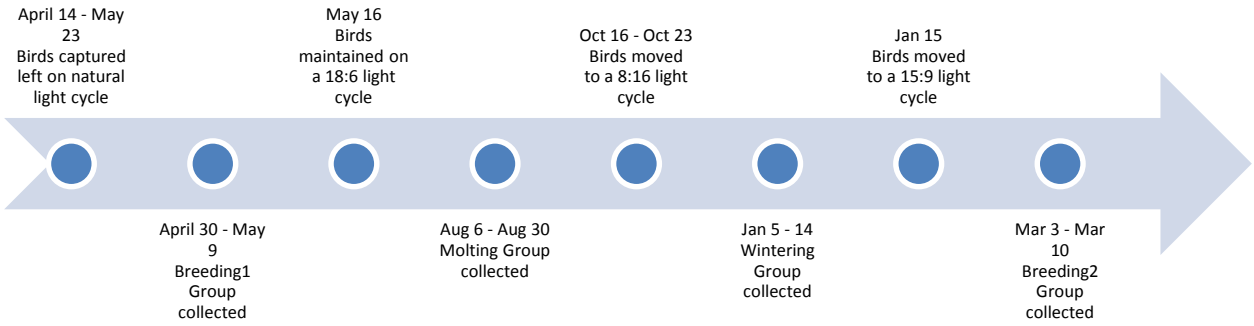

Figure 2-1. Experimental Timeline. House sparrows were captured during April and May 2014, brought into captivity and were maintained on a natural breeding light cycle (18L:6D). Birds were maintained on this long day length until 90% had completed molt around mid-October. Birds were then moved to a short day length to mimic winter (8L:16D). Birds were maintained on this short day length for 10 weeks and then moved to a long day length to bring them back into breeding condition (15L:9D). A subset of birds underwent a stress series where blood was collected, the birds were euthanized and their brains were removed during each of the seasonal time points (Breeding1 n = 8, Molting n = 15, Wintering n = 15, Breeding2 n = 8). . Light cycle changes are shown along the top, blood sample and brain collection dates for each experimental group shown along the bottom.

minutes. Plasma was collected and stored at -20 °C. I collected the first blood sample within 3 minutes of entering the room to obtain a baseline CORT sample (Romero and Reed, 2005). Birds were then placed in an opaque cloth bag for 30 minutes. At the end of the 30 minutes of restraint I collected the second (stress condition) blood sample. I then immediately injected the birds with dexamethasone (Omega, CAT# 02204266) and placed them back in the cloth bag for one hour (1h). Dexamethasone is a synthetic glucocorticoid that maximally stimulates negative feedback. Dexamethasone was injected into the pectoralis muscle at a 1mg/kg dose (Schmidt et al., 2012). I collected a final blood sample (post dexamethasone/negative feedback) at the end of the hour, and the bird was returned to the bag briefly before being euthanized for brain collection (see below).

2.4 Hormone Assay

CORT levels were measured using a CORT radioimmunoassay (ImmuChem Double-Antibody Corticosterone ^{125}I RIA kit 07-120103; MP Biomedicals LLC) in plasma without an extraction. This assay has been previously validated in a variety of songbird species including song sparrows (Newman et al., 2010). The sensitivity of the corticosterone assay was 2 ng/mL. Intraassay variation was 3.35%, and interassay variation was 6.85% (low control) and 0.5% (high control; n = 2 assays).

2.5 Brain Collection

After the final blood collection birds were placed back into a cloth bag until euthanized using an overdose of isoflurane (Baxter, CAT# CA2L9100). Birds were perfused with chilled phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were then removed and placed in 4% paraformaldehyde for 24 hours. Brains were then cryoprotected in 30% sucrose in PBS for 48 hours. After the 48

hours brains were rapidly frozen using dry ice and placed at -80 °C until immunohistochemistry was performed. Brain extraction occurred within 3 hours of the initial blood draw to limit any gene expression changes to receptor expression.

2.6 Immunohistochemistry

Brains were sliced at 40 µm section thickness in the coronal plane using a Microm HM505N cryostat. Sections including the hypothalamus and dorsal medial subdivision of the hippocampus were collected in a single series starting at the splitting of the septomesencephalic tract and stopping after the anterior commissure. These landmarks were observed using a zebra finch atlas (Nixdorf-Bergweiler, B.E. & Bischof, H.J., 2007). After this, sections were collected in alternating series until after the collection of the nucleus taeniae (TnA). Sections were placed free floating in 24-well tissue culture plates containing 0.1M phosphate-buffer solution (PBS, pH 7.5). One series was mounted onto microscope slides (VWR Superfrost Plus) coated with gelatin, Nissl-stained with thionin, serially dehydrated in ethanol, cleared in solvent (NeoClear, EMD Chemicals), and protected with coverslips affixed with Permount (Fisher). These Nissl-stained sections were later used to anatomically identify brain regions in the adjacent sections processed with immunohistochemistry. The remaining series and hypothalamus sections were used for immunohistochemistry.

2.7 GR-immunoreactivity

Cells were stained with an anti-GR antibody (Pierce catalogue #PA1-510A) previously validated in zebra finches (*Taeniopygia guttata*) using western blot and neutralizing peptide (Shahbazi et al., 2011). Staining of zebra finch and house sparrow

brains showed similar distribution patterns, and omission of the primary antibody eliminated all immunoreactivity (data not shown).

Free floating sections were washed 3 times in PBS for 5 minutes on an agitator at room temperature (RT). Sections were then incubated in 0.5% H_2O_2 in PBS for 15 minutes to remove endogenous peroxidases. Sections were subsequently washed three times in PBS for 5 minutes followed by incubation in 10 % normal goat serum (Vector, CAT# S-1000) in PBS containing 0.3 % v/v Triton X-100 (PBS/T) overnight at 4 °C. Sections were then incubated in GR primary antibody (1:500 dilution) and 1 % normal goat serum, in 0.3 % PBS/T for a full day at 4 °C. Sections were washed 3 times in 0.1 % PBS/T for 5 minutes per wash and incubated in biotinylated goat anti-rabbit IgG secondary antibody (1:400 dilution, Vector) in 0.3 % PBS/T for 1 hour at RT. Sections were then washed 3 times in 0.1 % PBS/T for 5 minutes per wash and incubated in avadin-biotin horseradish-peroxidase complex (1:200 dilution, Vectastain ABC Elite Kit, Vector) in 0.3 % PBS/T for 1 hour at RT. Sections were washed twice in 0.1 % PBS/T and visualized using 0.04 % 3,3'-diaminobenzidine tetrahydrochloride (SigmaFAST DAB, Sigma). Sections were then rinsed a final four times in PBS, mounted on microscope slides, serially dehydrated in increasing concentrations of ethanol, and cleared of lipids using an organic solvent (NeoClear, EMD Chemicals). Coverslips were applied to the microscope with the use of Permount (Fisher).

2.8 Image and data Collection

Images were obtained for 8 anatomical areas of the house sparrow brain (Table 2.1). Locations of these nuclei were verified using previously developed brain atlases for

Table 2-1. List of brain regions analyzed for this study. Shown in the left column are the eight brain regions that were analyzed in the study. The right column shows the basic function of each nuclei. The first four regions are involved in the HPA axis and have previously been shown to contain GRs. The second four regions contain high levels of GRs and are not implicated in any function in the HPA axis. These regions are included to determine if there was a global change in GRs seasonally.

Brain Region	**Function**
Medial preoptic nucleus (POM)	HPA Regulation
paraventricular nucleus (PVN)	HPA Regulation
Hippocampus	HPA Regulation
Nucleus taeniae of the amygdala (TnA)	HPA Regulation
Lateral geniculate nucleus (GLv)	Visual sensory nucleus
Optic tectum (TeO)	Visual sensory nucleus
magnocellular part of the isthmic nucleus (Imc)	Sensory integration nucleus
HVC	Song control nucleus

the jungle crow and zebra finch (Izawa and Watanabe, 2007; Nixdorf-Bergweiler and Bischof, 2007). The Nissl stained series was used to identify and localize song area HVC (not an acronym used as a proper name). For the hippocampus analysis, images were taken in slices that also contained the paraventricular nucleus (PVN). For the optic tectum (TeO) analysis, images were taken in slices that also contained the magnocellular part of the isthmic nucleus (Imc). If possible five coronal images using both hemispheres were obtained for each area analyzed. Images were captured using a Spot Idea 5-megapixel digital camera (Diagnostics Instruments) mounted on a Zeiss Axiophot microscope using a 20x objective lens. An experimenter blind to the treatment groups completed all percent coverage or cell count measurements using ImageJ software (NIH). Images were first split between color channels and then the blue channel was converted into 32 bit greyscale images to enhance definition of GR-immunoreactive cells. Cell counts were done using the cell counter plugin for ImageJ. An area measurement was obtained using the selection tool and the area outside was removed. Cells showing a darker nucleus or clear cell body were then counted. In areas where cell counting was not possible, PVN and HVC, a percent coverage was obtained. An area measurement was obtained using the selection tool. The area outside of this area measurement was removed. The image was then subjected to adjustment of the threshold to determine percentage of area covered by GR-immunoreactivity. The threshold was adjusted until a majority of the immunoreactively stained regions were included (Figure 2.2).

2.9 Statistics

All statistics were run using IBM SPSS version 23 software. A Levene's test for homogeneity of variances was conducted for each analysis. Sex was not a significant

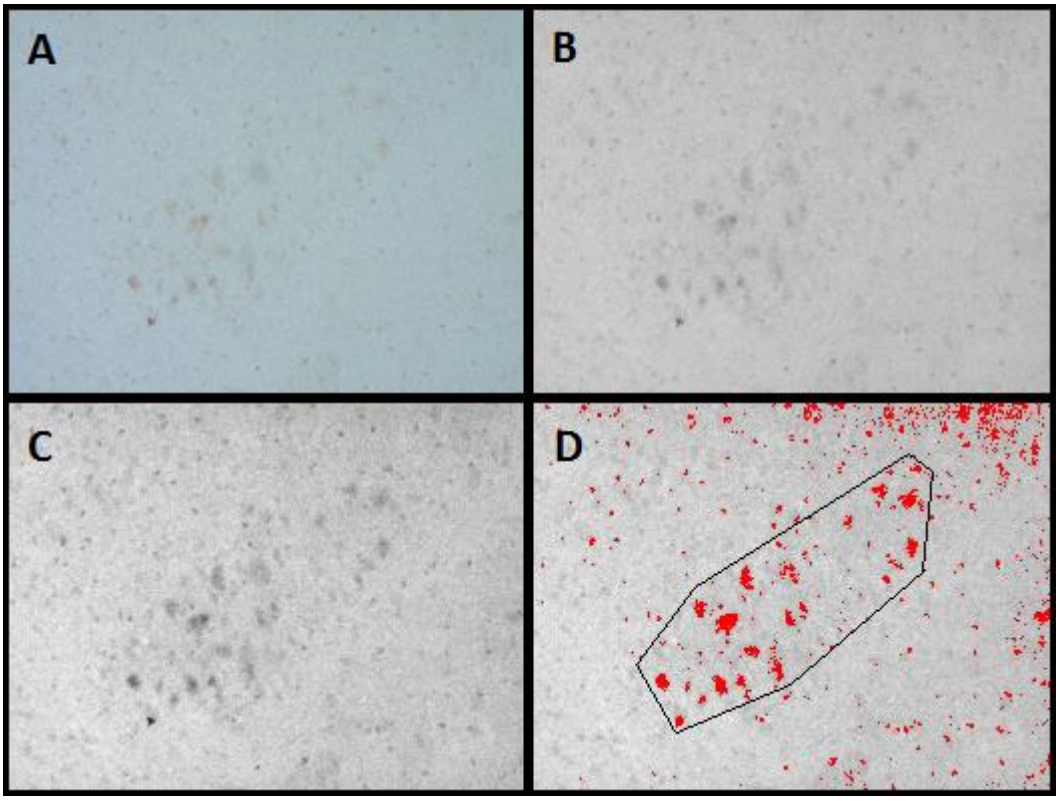

Figure 2-2. Image Threshold Procedure. To quantify percent coverage or cell count measurements original images were processed using ImageJ software (NIH). The original image captured (A) is split along color channels and the blue channel is kept (B). This image is then converted to 32 bit grey scale (C). The image was then subjected to adjustment of the threshold to determine percentage of area covered by GR-immunoreactivity (D).

variable and was collapsed across all groups. One-way repeated measures ANOVAs were used to determine blood sampling differences in plasma CORT with a Greenhouse-Geisser corrected values used. One-way ANOVAs were used to determine seasonal differences in plasma CORT. One-way ANOVAs were used to determine if percent coverage or cell counts varied between seasons for various brain regions. If significant, a post hoc test using Fisher's least significant difference (LSD) was used to determine seasonal differences. Fisher's LSD was used as it increases power and as I have only four groups there is only a small inflation of type I error.

3 Results

3.1 Sex Differences

There were no effect of sex on any of the plasma CORT measurements, or for GR-immunoreactivity in any of the brain regions measured (Table 3.1). Further, there were no significant effects of sex, or significant interactions with sex, in any of the ANOVAs (data not shown). Thus, the data from males and females were pooled for all results below.

3.2 Corticosterone

House sparrows showed a typical stress response and dexamethasone response similar to that reported in other studies (Figure 3.1). A two-way repeated measures ANOVA indicated that there were significant differences in CORT levels among the three bleeding times ($F_{2, 48} = 62.030$, $p < 0.001$). Post-hoc tests showed that the baseline bleed was significantly different from the stress bleed ($p < 0.001$) and post-dexamethasone bleed ($p < 0.001$). The stress bleed was also significantly different from

the post-dexamethasone bleed ($p < 0.001$). There was no significant main effect of season ($F_{3, 49} = 1.075$, $p = 0.368$). However, there was a significant interaction between season

Table 3-1. Sex difference t-tests. T-tests found no significance differences in sex found in either blood sample CORT values at baseline, stressed or post-dexamethasone. There was also no sex difference for GR-immunoreactivity in any of the eight brain regions.

	t value	**Df**	**Significance (p value)**
CORT Values			
Baseline	-0.367	49	0.715
Stress	-0.339	49	0.736
Post-dexamethasone	0.160	49	0.874
GR-immunoreactivity			
Medial preoptic nucleus (POM)	-1.416	40	0.165
Paraventricular nucleus (PVN)	-1.207	40	0.235
Nucleus taeniae of the amygdala (TnA)	-0.773	38	0.444
Lateral geniculate nucleus (GLv)	-1.122	42	0.268
Dorsal medial subdivision of Hippocampus	-0.860	43	0.394
HVC	-1.760	41	0.086
Optic Tectum (TeO)	1.456	39	0.153
Magnocellular part of the isthmic nucleus (Imc)	1.413	39	0.121

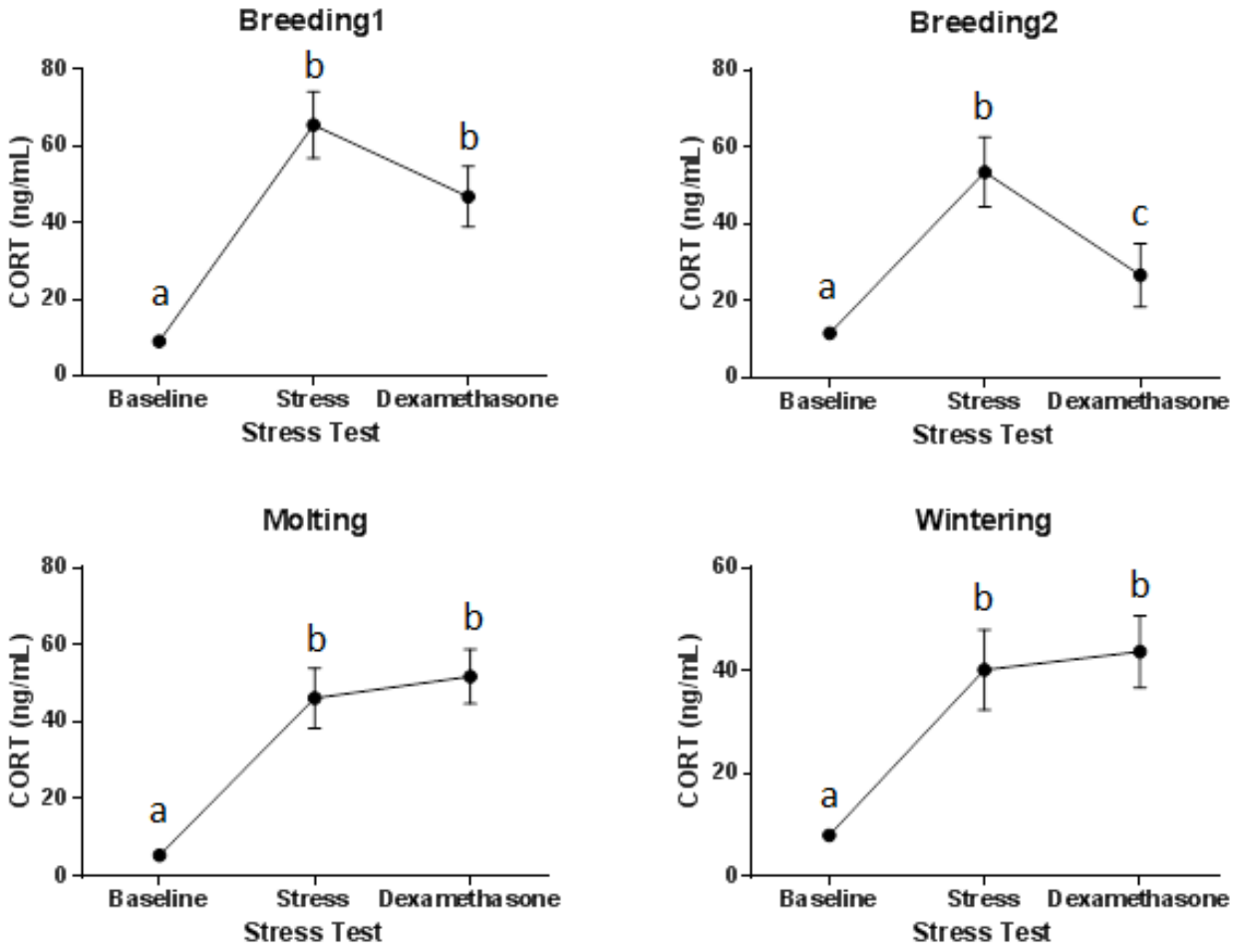

Figure 3-1 Seasonal stress series. HPA axis characterization of house sparrows sampled at four different time points: breeding1 (n = 12), molting (n = 15), wintering (n = 15), breeding2 (n = 11). For each of the four groups mean (± SE) CORT levels are shown for 3 blood samples time points: i) baseline, or unstressed birds; ii) stressed samples collected after 30 minutes of restraint in a cloth bag; iii) dexamethasone samples collected 1 hour after injection with dexamethasone. Error bars for Baseline were too small to be visible.

and bleed time ($F_{6, 98} = 2.371$, $p < 0.05$). To explore this interaction I ran one-way ANOVAs for each season separately, and also used a one-way ANOVA to compare season for each blood sampling time.

A one-way repeated measures ANOVA conducted on data from the first breeding birds (breeding1) to compare CORT levels across bleeding times indicated a significant difference between blood samples ($F_{1.6, 17.2} = 14.915$, $p < 0.001$). Post hoc tests showed that the baseline sample had significantly lower CORT than the stress sample ($p = 0.001$) and post-dexamethasone sample ($p = 0.001$). The stress sample did not have significantly higher CORT than the post-dexamethasone sample ($p = 0.087$, Figure 3.1a). Thus, for birds in the breeding1 condition CORT increased following restraint, but did not decrease following dexamethasone injection.

A one-way repeated measures ANOVA conducted on data from breeding2 birds similarly indicated a significant difference between blood samples ($F_{1.3, 13.3} = 10.239$, $p < 0.001$). Post hoc tests showed that the baseline sample had significantly lower CORT than the stress sample ($p < 0.001$) and post-dexamethasone sample ($p < 0.05$). The stress sample had significantly higher CORT than the post-dexamethasone sample ($p < 0.05$, Figure 3.1b). Thus, in this group CORT levels rose following restraint and then decreased following dexamethasone injection.

A one-way repeated measures ANOVA conducted on data from molting birds indicated a significant difference between blood samples ($F_{1.4, 20.2} = 18.771$, $p < 0.001$). Post hoc tests showed that the baseline sample had significantly lower CORT than the stress sample ($p = 0.001$) and post-dexamethasone sample ($p = 0.001$). The stress sample did not have significantly higher CORT than the post-dexamethasone sample ($p = 0.523$,

Figure 3.1c). Thus, in this group CORT levels rose following restraint, but did not decrease following dexamethasone injection.

A one-way repeated measures ANOVA conducted on data from wintering birds similarly indicated a significant difference between the blood samples ($F_{1.6, 21.7} = 24.483$, $p < 0.001$). Post hoc tests showed that the baseline sample had significantly lower CORT than the stress sample ($p = 0.001$) and post-dexamethasone sample ($p = 0.001$). The stress sample did not have significantly higher CORT than the post-dexamethasone sample ($p = 0.618$, Figure 3.1d). Thus, in this group CORT levels rose following restrain, but did not decrease following dexamethasone injection.

I also determined whether there were significant differences in CORT among the 4 treatment groups for each of the baseline, stress, and post-dexamethasone blood samples. A one-way ANOVA indicated a significant difference in baseline CORT between the seasonal groups ($F_{3, 49} = 4.206$, $p = 0.01$). Post hoc tests showed that the molt group had significantly lower baseline CORT than the breeding1 group ($p < 0.05$) and breeding2 group ($p = 0.001$). The wintering group had significantly lower CORT than the breeding2 group ($p < 0.05$). A one-way ANOVA indicated no significant difference between the seasonal groups in post-restraint stress levels of CORT ($F_{3, 49} = 1.717$, $p = 0.176$). Finally, a one-way ANOVA indicated no significant difference between the seasonal groups in post-dexamethasone levels of CORT ($F_{3, 49} = 1.914$, $p = 0.140$).

Taken together, the above analyses suggest that significant interaction between blood sampling and season resulted from differences in baseline CORT among the four groups, and differences in the change in CORT following dexamethasone injection. To

further explore this I reanalyzed the data to explore changes in CORT rather than absolute levels of CORT.

3.3 Seasonal changes in CORT stress response

In addition to assessing absolute levels of CORT, I also analyzed the change in CORT following restraint and following dexamethasone treatment. There was no significant difference between groups for the change in CORT from baseline to stressed ($F_{3,49} = 1.370$, $p = 0.263$). However, there was significant variation between groups in the response to dexamethasone. A one-way ANOVA indicated that the change in CORT from stress to post-dexamethasone treatment was significantly different among the seasonal groups ($F_{3, 53} = 3.185$, $p < 0.05$). Post hoc tests showed that the breeding2 group had a significantly larger decrease in CORT after the dexamethasone than did the molting ($p < 0.05$) and wintering groups ($p < 0.05$, Figure 3.2).

3.4 GR-Immunohistochemistry

There was no significant variation between groups in the amount of GR-immunoreactivity in the brain regions associated with the HPA axis. One-way ANOVAs indicated no significant effects of season for the hippocampus ($F_{3, 41} = 1.205$, $p = 0.320$), the TnA ($F_{3, 36} = 1.855$, $p = 0.155$), the PVN ($F_{3, 38} = 0.660$, $p = 0.582$), and the POM ($F_{3, 38} = 0.278$, $p = 0.841$). Thus GR levels do not appear to vary seasonally in these brain regions (Figure 3.3).

Four additional areas outside the HPA axis were analyzed to see if any seasonal change in GR levels were seen globally. Several sensory nuclei showed significant variation between groups in the amount of GR-immunoreactivity expressed. A one-way ANOVA conducted on the GR-immunoreactivity of the visual sensory nuclei GLv

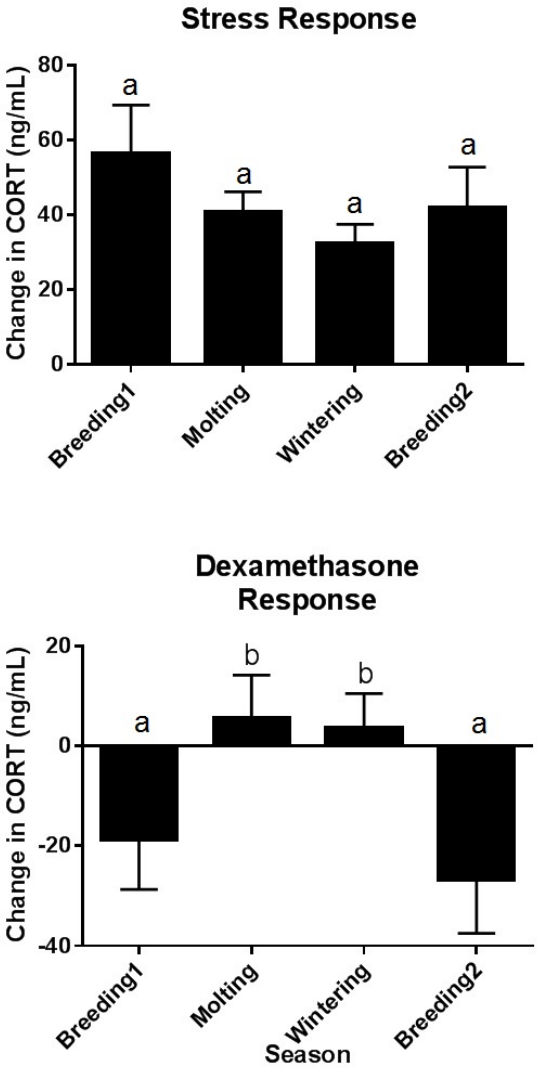

Figure 3-2. CORT Responses. Changes in CORT after 30 minutes of restraint (Stress Response) and post-dexamethasone injection (Dexamethasone Response). For each of the four groups mean (± SE**)** CORT levels are shown.

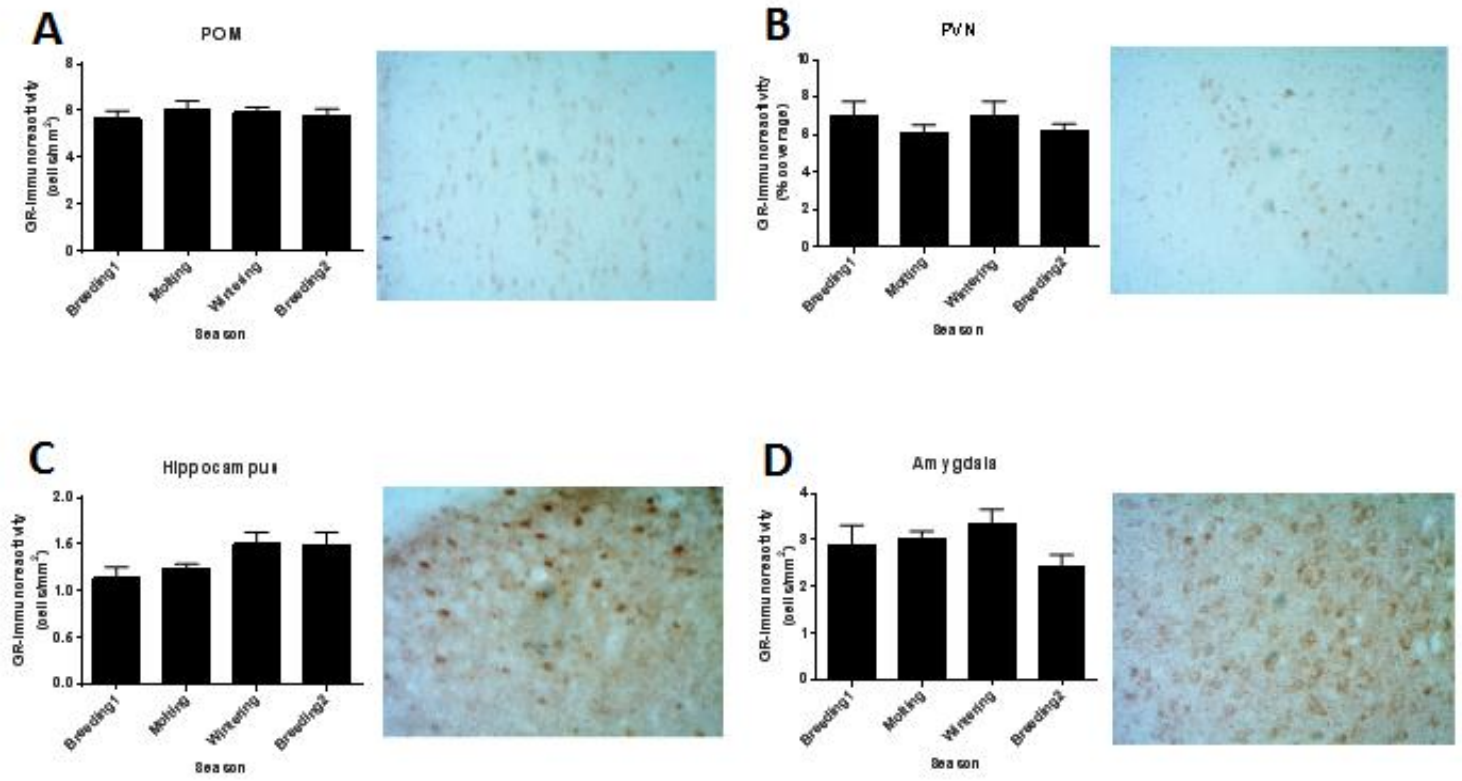

Figure 3-3 GR-immunoreactivity in the HPA axis. GR-immunoreactivity was measured using percent coverage or cell counts in several brain regions associated with the HPA axis. No differences in immunoreactivity was seen in the Medial preoptic nucleus POM (A), Paraventricular nucleus PVN (B), Hippocampus (C) and Amygdala (D).

indicated a significant difference between the seasonal groups ($F_{3, 44} = 3.481$, $p < 0.05$). Post hoc tests showed that the breeding1 group had significantly fewer cells showing GR-immunoreactivity than the wintering group ($p < 0.05$) and breeding2 group ($p < 0.05$). The molting group had significantly fewer cells showing GR-immunoreactivity than the wintering group ($p < 0.05$) and showed a trend towards having fewer cells showing GR-immunoreactivity than breeding2 ($p < 0.07$, Figure 3.4a).

A One-way ANOVA conducted on the GR-immunoreactivity of the visual sensory nuclei TeO indicated a significant difference between the seasonal groups ($F_{3,41} = 3.290$, $p < 0.05$). Post hoc tests showed that the breeding1 group had significantly fewer cells showing GR-immunoreactivity than the wintering group ($p < 0.01$) and molting group ($p < 0.05$, Figure 3.4b). Changes in GR levels appear to be due to captivity rather than seasonality

A further set of brain nuclei expressing GR did not show significant variation between groups in the amount of GR-immunoreactivity. A One-way ANOVA indicated no significant differences between the seasonal groups conducted on the GR-immunoreactivity of the Imc ($F_{3, 37} = 1.088$, $p = 0.336$) and HVC ($F_{3, 39} = 0.132$, $p = 0.936$) (Figure 3.4cd). Thus cells expressing GR do not appear to vary seasonally in the whole brain.

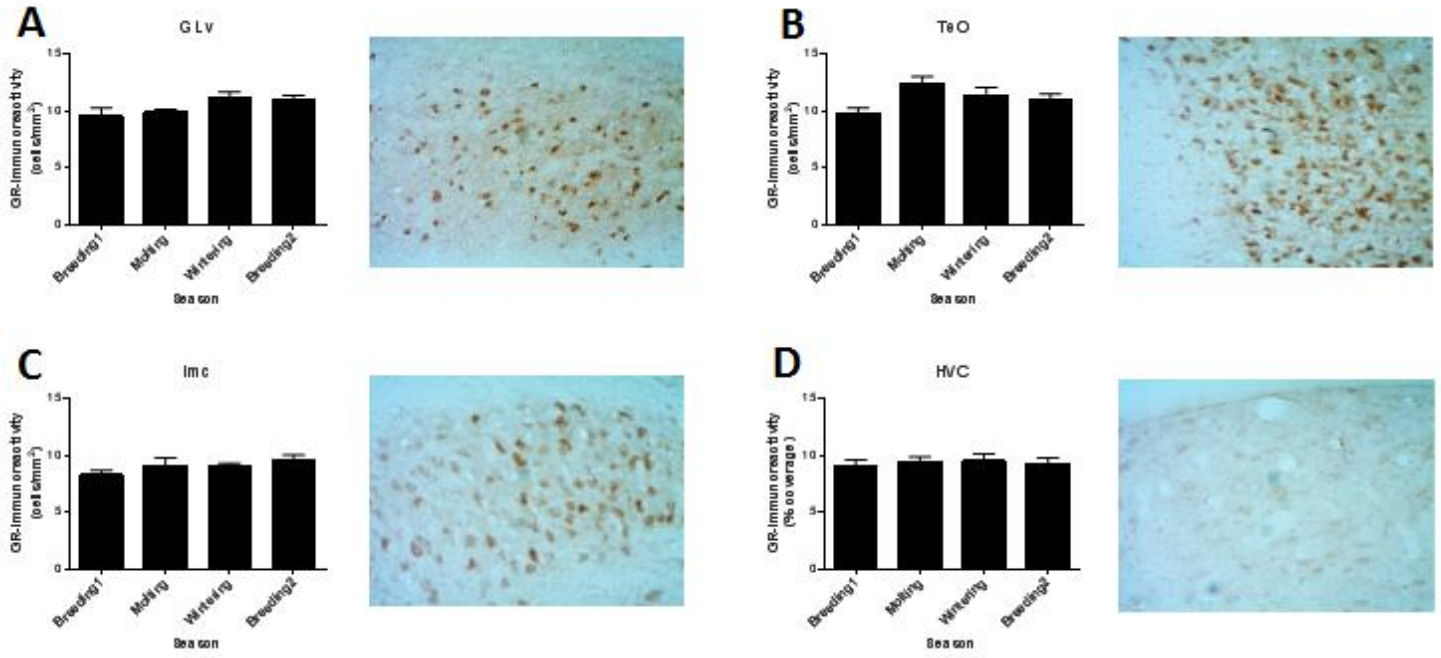

Figure 3-4. GR-immunoreactivity of various brain regions. GR-immunoreactivity was measured using percent coverage or cell counts in several brain regions including the lateral geniculate nucleus GLv (A), optic tectum TeO (B), magnocellular part of the isthmic nucleus Imc (C) and HVC (D). No global changes in brain GR-immunoreactivity across seasons was observed.

4 Discussion

House sparrows showed seasonal variation in several measures of the HPA axis. These included differences in baseline CORT with molting birds having lower baseline CORT than breeding birds. Breeding birds also decreased CORT following treatment with dexamethasone, whereas molting and wintering birds did not. In contrast, I did not find evidence for the predicted seasonal changes in GR-immunoreactivity in the brain. There was no seasonal variation in gr-immunoreactivity in any HPA axis brain regions that I analyzed. The TeO and GLv showed significant differences in GR-immunoreactivity though this appeared to possibly be an artifact of captivity. The Imc and HVC did not show any seasonal differences in GR-immunoreactivity indicating that there were no global changes in GR-immunoreactivity in the house sparrow brain.

4.1 Seasonality of Negative Feedback

Dexamethasone(DEX) is a synthetic glucocorticoid that will maximally induce CORT negative feedback (Schmidt et al., 2012). With several species of birds showing a suppressed baseline and stressed CORT levels during molt I hypothesized that molting birds would show the strongest negative feedback. In contrast breeding house sparrows were able to decrease CORT output in the presence of an ongoing stressor after injection with DEX while molting and wintering birds were unable to decrease CORT (Figure 3.2).

Breeding birds might decrease CORT levels in the presence of a persistent stressor for two reasons. High levels of CORT as seen during a persistent stress event could move an animal into homeostatic overload (Figure 1.3) and have deleterious effects. These effects can include weight loss, suppressed immune system and decreased

reproductive ability. As breeding birds are already exposed to increased levels of CORT naturally they could be closer to this threshold then during other times of year (Figure 1.3). In order to maintain themselves in reactive homeostasis breeding birds use an increased negative feedback ability to decrease CORT levels even in the presence of a persistent stressor. As molting and wintering birds do not have as high CORT levels they maintain a stress response until the stressor is eliminated.

Another explanation for the observed increased negative feedback in breeding birds has to do with raising young. Increased CORT levels are important for survival of the individual but interferes with reproduction. This leads to a trade-off between survival and reproduction (Lendvai et al., 2007). Increased levels of baseline CORT have been shown to increase foraging rates and nestling feedings but parents with a higher stress CORT response had lower weight young (Lendvai et al., 2007; Miller et al., 2009). This creates an issue where increased CORT is beneficial to raising young (baseline) but a heightened stress response is harmful. Breeding birds might attempt to navigate this trade-off by increasing their negative feedback. This would allow them to initiate a stress response but to quickly temper it to decrease any negative effects on their offspring.

4.2 Stress Changes in CORT levels

In all seasons house sparrows showed an increase in CORT to being restrained in a bag, indicative of a stress response. There were no significant differences in any of the three time points in the stress series between the two breeding groups (breeding1 and breeding2). This indicates that length of time in captivity probably did not have a

significant effect on the HPA axis. The lack of an attenuated stress response in breeding2 also indicates the birds were not undergoing chronic stress.

Baseline CORT during molting was lower than baseline CORT in both breeding groups, and wintering birds had lower CORT than the second breeding group (Figure 3.1). This result is consistent with previous research that has shown free living birds have the lowest CORT of the year during molt and the highest during the breeding season (Breuner and Wingfield, 2000; Romero et al., 1997). Reduced baseline CORT during molt is thought to function to reduce the negative effects of CORT on feather growth and performance (more information presented below; DesRochers et al. 2009; Romero et al. 2005).

Unlike previous studies I did not find a seasonal change in stress-induced CORT levels. However, there was a similar trend as in previous research (Figure 3.1) such that wintering and molting birds tended to have a lower CORT response to stress then either of the breeding groups, though this was not statistically significant. Most of the research into the seasonality of the stress response in birds have used free-living birds and collected samples in the field, so captivity could be having either a direct or indirect effect on the seasonal changes in the stress response.

Several possible mechanisms could explain the lack of significant seasonal change in the stressed CORT levels and stress response. One possibility is that because house sparrows are non-migratory they do not have the same need to complete their molt in a timely manner as other species that have to migrate, such as the Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii;* Romero et al. 1997). Increased CORT has been shown to have a detrimental effect on the performance of feathers as

well as to increase the amount of time necessary to grow feathers (DesRochers et al., 2009; Romero et al., 2005; Strochlic and Romero, 2008). Increased CORT in molting birds resulted in lower mass of the rectrices, weaker barbicel hooking strength and more barbules (DesRochers et al., 2009). These effects can cause the feathers to be weaker, have poorer aerodynamic properties, and lower insulative value (DesRochers et al., 2009). As many migratory birds must complete the molt of their flight feathers before they can start migration they have a more urgent need to finish molt quickly despite other environmental challenges, and so they likely suppress their CORT response during molt more strongly than non-migratory birds.

Although house sparrows are not migratory, seasonal changes in CORT responses to stress have been previously observed. Cornelius et al. (2011) recently showed a negative relationship between the amount of time spent molting and percent suppression of CORT in 13 bird species (Cornelius et al., 2011). This study showed a suppression of CORT during molt in house sparrows and a significant difference in stressed CORT levels have been previously observed between breeding and molting in house sparrows (Rich, E.L. & Romero, L.M., 2001; Liebl et al. 2013). Decreasing the length of time in prebasic molt is not just important for migrants but for all birds as during molt they show decreased thermoregulation, decreased flight performance and increased risk of predation (Romero et al., 2005). As house sparrows must still deal with these other risks during the prebasic molt in Ontario and have been shown to previously to have a suppression of CORT this explanation does not seem to fit.

Another possible explanation for the lack of a significant seasonal change in the stressed CORT levels in my study is that the birds were undergoing chronic stress and

had a decreased ability to modulate their stress response. As stated earlier, when birds are undergoing chronic stress they show an attenuated stress response and fail to increase CORT during a 30 minute restraint. This is also accompanied by weight loss and general decrease in body condition (Rich and Romero, 2005). However, in my study birds in all conditions showed a robust stress response to 30 minutes of restraint (Figure 3.3). Most birds had only a small weight loss for the first few weeks in captivity but this stabilized for the remainder of the experiment (data not shown). This again suggests that the birds were not experiencing chronic stress while in captivity. However, this does not preclude captivity from having a transient effect on the function of the HPA axis. In chukars (*Alectoris chukar*) the first five days in captivity had significant effects on the HPA axis but these effects were diminishing by day nine (Dickens et al., 2009), thus, it is possible that captivity had a transient effect on HPA axis function in my study.

A third explanation for the lack of a seasonal stress response is that being in captivity had an indirect effect on the bird's seasonal modulation of the stress response. This indirect effect could be related to the removal of additional external environmental cues. These cues could include variable temperature, storms, or food availability. Photoperiod is the main environmental cue used in captivity to simulate different seasons, but it may not be the only important cue required to cause an effect (Bridge et al., 2009). Previous research in our lab has shown that in breeding male white-throated sparrows (*Zonotrichia albicollis*) both testosterone and photoperiod, independent of each other, can significantly increase the size of the song-control nucleus HVC (Robertson et al., 2014). Photoperiod and testosterone by themselves had an intermediate effect on the size of HVC, but have a synergistic effect when presented together (Robertson et al., 2014). This

integration of multiple cues are important to help the animal time seasonal changes. Thus it may be important for the HPA axis to respond to several environmental cues to adjust to the current environment. The HPA axis responds to unpredictable events with a transient increase in CORT. These responses could be incorporated into the control of the HPA axis such that it can be adapted to the current environment. Predictable life history changes through photoperiod prepare the HPA axis for gross changes in environmental stressors while the frequency of unpredictable stressors can fine tune the HPA axis to their current environment. The fine tuning of the HPA axis could help the bird prepare for future events. If a bird is experiencing unseasonably cold weather at the end of breeding it could recognize that it could be a colder winter and could cause an increase in CORT. This increase could lead to increased CORT in response to further stressors such as storms. This increased CORT could lead to increases in activities such as foraging which would allow the bird to better survive a stormy winter.

Captive birds are often held at a constant temperature and have free access to food. Though they would experience the low pressure from storms they would not experience the resulting winds or precipitation that accompany such storms. This would decrease most common forms of environmental stress experienced by the birds. This decreased stress would result in a decreased stress response to restraint. The birds experience less unpredictable stress while in captivity, and this decreases the HPAs responsiveness to the current environment. Thus, further work is required to determine if the lack of a significant seasonal change in stressed CORT levels in my study are a result of the indirect effects of captivity and a reduced range of environmental cues.

Dexamethasone injection is used to measure the efficacy of negative feedback of the HPA axis (Astheimer et al., 1993; Watson et al., 2006). It has recently been used in song sparrows (Schmidt et al., 2012) and house sparrows (Liebl et al., 2013). The rapid and efficient suppression of CORT after a stressor is important to minimize the long-term deleterious effects of CORT. Previous research has shown that dexamethasone given to breeding song sparrows causes a decrease in CORT (Schmidt et al., 2012). Similarly we saw a decrease in CORT of house sparrows in breeding condition. However we did not see a decrease in CORT levels post dexamethasone treatment in birds in the molting or wintering groups, so birds in molting or wintering did not show the ability to shut down CORT release in the presence of a persistent stressor unlike during breeding. Thus, breeding birds showed an increased efficacy of negative feedback compared to molting and wintering.

Breeding birds have higher levels of CORT than at other times of the year. As shown in the reactive scope model a higher level of basal CORT would mean that a stressor during that time of year would have an increased risk of moving the animal into homeostatic overload. Repeated exposure to stress would compound this risk as it would decrease the animal's ability to deal with the next stressor without moving into homeostatic overload. An increase in negative feedback could be an effort by the birds to reduce CORT even in the face of a stressor to protect against moving into homeostatic overload of repeatedly high CORT. A second consideration is that the ability of breeding sparrows to decrease CORT during a chronic stressor could be an adaptation for raising young. Decreasing CORT levels in the presence of a chronic stressor could reduce nest abandonment.

Liebl et al. (2013) found a significant difference in post-dexamethasone CORT between breeding and molting house sparrows, but no difference in the change in CORT between the two conditions. In my study I did not find any differences in CORT post-dexamethasone for any of my groups (Figure 3.3). I did find a significant difference in the ΔCORT between breeding and molting/wintering birds. There are two differences between the studies in that Liebl et al. (2013) used free-living sparrows and these birds were caught in Tampa, Florida. The differences seen in the dexamethasone data could be due to a captivity induced change in the negative feedback of the HPA axis, but if this were the case the birds lost the ability to induce negative feedback with dexamethasone during molt/winter and regained the ability in the breeding2 condition all while in captivity. This seems unlikely, and a more plausible explanation is the difference in the capture sites of the birds. The birds captured in Ontario have to deal with a harsher winter than those in Tampa, and could prioritize more than their counterparts on successful breeding. The decreased CORT seen in the Ontario breeding birds could be to decrease the chances of nest abandonment that can occur with severe conditions (Breuner et al., 2003; Romero et al., 1997).

The principal hypothesis for why birds suppress their CORT response during molt is related to the catabolic activity of CORT and its incompatibility with protein deposition required for feather growth (Medina et al., 2013; Romero et al., 2005). It is a physiological trade-off in that birds shut down their ability to respond to current acute stressors in favor of feather growth for future fitness (Cornelius et al., 2011). Birds such as the Gambel's white-crowned sparrow have a short molting period (1.8 months) compared to a zebra finch (8 months) (Cornelius et al., 2011). Since white-crowned

sparrows have a shorter time to molt the influences of CORT can have an exaggerated effect on the survival of the individual. Although birds are not molting year round there could be several other benefits to modulating the strength of their stress response throughout the year including raising young.

The observed changes in the baseline CORT sample is the result of the HPA axis adjusting in response to a predictable event (photoperiod). This adjustment allows the HPA axis to have seasonal variability to a stressor. The seasonal variation in the stressed CORT seen in wild birds could be due to the influence of unpredictable events experienced leading up to the season. The roles of both predictable and unpredictable events are important in regulation of the seasonality of the HPA axis.

4.3 Seasonal Changes in Glucocorticoid Receptors

Changes in baseline CORT and changes in efficacy in negative feedback show that there are seasonal changes in CORT output by the HPA axis. Were these changes in CORT output due to changes in GRs in the brain? Glucocorticoid receptors are the main receptor responsible for responding to stressors (Breuner et al., 2003; Meijer et al., 2010). Any changes to the distribution of GR-immunoreactive cells in the hypothalamus or its inputs, hippocampus and amygdala, could lead to a change in the CORT output.

I observed no differences in GR-immunoreactivity in any of the hypothalamus, hippocampus or amygdala (TnA). There were no trends seen in any season. Thus, seasonal changes in CORT output do not seem to be controlled through a change in number of cells expressing GR in the neural portion of the HPA axis.

There are two possible explanations for these lack of differences in the HPA axis. First, it is possible that there was a difference in the distribution of GR, but that it was not

statistically observed as captivity decreased the variability in the HPA axis similar to the non-significant of the stress samples. The lack of unpredictable stressors could have altered the number of cells expressing CORT.

Second, it is possible that there was no real difference in the distribution of GR-immunoreactive cells in different seasons. With no differences seen in the GR-immunoreactivity in the HPA axis it appears that the birds are using another method to modulate their HPA axis. Breuner et al (2001) measured total GR expression in the entire house sparrow brain and found seasonal changes in cytosolic and membrane bound receptor numbers. Cytosolic receptors were lowest during the winter while the membrane receptor was lowest during nesting. These do not coincide with the highest and lowest seasons of CORT output and they found no correlation between basal CORT levels and receptor numbers. Their analysis was across the whole brain and involved competition binding assays (Breuner and Orchinik, 2001). When CORT is at its lowest during molt they did not find a significant change in GR in the whole brain. Another recent study looked at whole brain GR at 6 different life history stages. They found that GR numbers were different between prior to egg-laying and breeding, but that there was no difference between these and the other seasons (Lattin and Romero, 2013). These findings along with my current study showing no seasonal differences in GR-immunoreactivity in the HPA axis suggest that the birds are not changing receptor numbers or cell numbers expressing GR to seasonally regulate their stress response.

I also analyzed several lower sensory nuclei that have an abundant number of cells showing GR-immunoreactivity. These regions have not previously been studied and it is unknown if they undergo a seasonal change. These regions were included as control

regions to see if there was a global change in the expression of GR-immunoreactivity as a function of season. The GLv and TeO showed significant group differences. They had a significant, though slight, increase in GR in the winter and breeding2 groups compared to breeding1 and molting groups. As the birds held in captivity longer had higher levels of GR-immunoreactive cells this increase in GR-immunoreactivity could be due to captivity. As the captive birds were being held in a visually bland environment this could have induced changes within these visual nuclei. The Imc which is involved in integration of several sensory modalities did not show any seasonal differences in GR-immunoreactivity. My data adds to a growing number of studies of the HPA axis in songbirds and especially house sparrows. As there appears to be various ways in which different species regulate their HPA axis it would be important to continue research not only by looking at different species, but specifically with the house sparrow to try and build a complete picture of the seasonality of the HPA axis. Previous studies have shown that the house sparrow seasonally regulates the sensitivity of its adrenals and pituitary as well as the number of receptors seen in whole brains. While these components of the HPA axis experience seasonal changes, they do not account for the seasonal changes in baseline and stressed CORT. Seasonal changes in CBG quantity seem to make the amount of free CORT static throughout the season, but still begs the question as to why the birds alter their CORT output and still does not explain how. I found no seasonal change in the number of cells showing GR-immunoreactivity in the HPA axis. With this investigation into each component of the HPA axis we are closer to understanding how this stress system is modulated. This understanding will be helpful to our understanding of how species can cope with changes in habitat.

As we still do not know fully how house sparrows regulate CORT output, further research should evaluate the possible role of MRs in the seasonal fluctuation of the HPA axis. MR is typically overlooked as they are 90 % occupied at baseline concentrations of CORT (Meijer et al., 2010). They can form heterodimers with GR and this could have a different gene expression pattern then either receptor as a homodimer. Two studies have shown that avian brains contain a 50/50 ratio of GR to MR compared to mammalian brains of 90/10 ratio (Breuner and Orchinik, 2001, 2009). Thus, MRs could be responsible for the adaptive seasonal changes in CORT levels by regulating the response of the HPA axis. Current research indicates that the number of GRs in the house sparrow brain as well as cells showing GR-immunoreactivity in the HPA axis do not contribute to the seasonal changes in CORT output. This does not completely rule out GRs as being involved in the seasonal variation in CORT. These receptors are under tight regulation and are associated with several chaperone proteins while in the cytosol. There could be seasonal changes in these components that could change their affinity for or their interaction with DNA that could lead to the changes we see in CORT output.

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