

**CORTISOL AND CORTICOSTERONE IN THE IMMUNE SYSTEM AND BRAIN
OF DEVELOPING SONGBIRDS**

by

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B.A. Hons., University of British Columbia, 2007

A THESIS SUBMITTED IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

March, 2009

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Abstract

Glucocorticoids (GCs) are primarily produced by the adrenal glands, however GCs can also be synthesized in extra-adrenal tissues. For example, immune and brain tissues contain all the enzymes necessary to synthesize GCs *in vitro* suggesting local GC synthesis. However, few studies have measured endogenous GC levels in neural or immune tissues. The primary objectives of this thesis were: (1) to determine if GCs are synthesized in immune and brain tissue of developing songbirds by measuring endogenous GC levels, (2) to determine how plasma and tissue GC levels change with age and (3) to determine how plasma and tissue GC levels are affected by restraint stress. In Chapter 2, baseline corticosterone and cortisol levels were measured in plasma, immune and brain tissue of zebra finches on the day of hatch (P0), P3, and P30. Cortisol levels in immune organs were higher than cortisol levels in plasma at all ages, suggesting local cortisol synthesis. Interestingly, corticosterone was the predominant plasma GC but cortisol was the predominant GC in immune tissues. In Chapter 3.1 corticosterone and cortisol levels were measured in wild European starlings on P0 and P10 (at baseline and after restraint). At P0, neither GC increased with restraint in plasma or any of the tissues studied. At P10, restraint increased corticosterone in plasma and all tissues, and cortisol in plasma, diencephalon and thymus. In contrast to Chapter 2, local GC levels were low at both ages suggesting that there may be a difference in local GC levels between starlings and zebra finches. To test this hypothesis, in Chapter 3.2, local GC levels in P4 zebra finches and starlings were compared. Again, in zebra finches, cortisol levels in the immune system were higher than cortisol levels in plasma. In starlings, cortisol levels in the immune system were similar to cortisol levels in plasma. These data suggest a difference in immunosteroid synthesis between zebra finches

and starlings. These studies suggest that circulating GC levels are not always representative of tissue GC levels. Measuring GC levels in tissues can provide important insights into how GCs are regulated in age-specific, tissue-specific, and species-specific manners.

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Abbreviations

11 β -HSD: 11 β -hydroxysteroid dehydrogenase	3 β -HSD: 3 β -hydroxysteroid dehydrogenase/isomerase
ACTH: adrenocorticotropin hormone	P450c11: cytochrome P450
bursa: bursa of Fabricius	11 β -hydroxylase
CBG: corticosteroid-binding-globulin	P450c21: cytochrome P450 21 α -hydroxylase
CRH: corticotropin-releasing-hormone	P450scc: cytochrome P450 side chain cleavage
cTel: caudal telencephalon	P: post-hatch day
DHEA: dehydroepiandrosterone	Pgp: P-glycoprotein
dTel: dorsal telencephalon	PROG: progesterone
E ₂ : 17 β -estradiol	rTel: rostral telencephalon
GC: glucocorticoid	SHRP: stress hyporesponsive period
GnRH: gonadotropin-releasing-hormone	T: testosterone
GR: glucocorticoid receptor	TCR: T cell receptor
HPA: hypothalamic-pituitary-adrenal	T cell: thymocyte cell
HPG: hypothalamic-pituitary-gonadal	
LH: luteinizing hormone	

Acknowledgements

First, I would like to thank Amy Newman and Eunice Chin for being patient and thorough teachers and more importantly for being great friends. I would also like to thank other past and present members of the Soma lab including Devaleena Pradhan, Amit Shah, Thierry Charlier, Matt Taves, Sarah Heimovics, Loretta Lau, Roeveena Sequeira, Yan Yu, Ilan Ruhr, Mark Huang, and Kelvin Po for help with research and moral support. I am grateful to Kyle for making everyday life so much easier and enjoyable. I would also like to thank my parents and sister for their love and support. I would like to express my gratitude to NSERC for providing me with funds that allowed me to invest my time and energy into research. Thank-you to my committee members: Drs. Liisa Galea, Greg Miller, and Joanne Weinberg for their useful suggestions and insight, and for providing me with access to equipment necessary to complete my research. Lastly, thank-you to Dr. Kiran Soma for all the opportunities you have given me to do science. I am grateful to have had the opportunity to learn from you in the past four years and am thankful for your mentorship and friendship.

Co-authorship statement

The first manuscript, “Neurosteroids, Immunosteroids and the Balkanization of endocrinology” is a review paper written by myself and my colleagues: Devaleena Pradhan, Amit Shah, Thierry Charlier, Eunice Chin, and Dr. Kiran Soma. For the second manuscript, “Cortisol and corticosterone in the songbird immune and nervous systems: local versus systemic levels during development” I collected plasma and tissue samples, extracted steroids, measured steroid levels, analyzed data, and with the help of Dr. Soma, wrote the manuscript for publication. For the third manuscript “Cortisol and corticosterone in immune organs and brain of European starlings: developmental changes, effects of restraint stress, comparison with zebra finches” I collected plasma and tissue samples, extracted steroids, measured steroid levels, analyzed data, and with the help of Dr. Soma, wrote the manuscript. Eunice Chin and Amit Shah helped with the collection of plasma and tissue samples in the field.

1. Neurosteroids, immunosteroids, and the Balkanization of endocrinology¹

1.1 Introduction

1.1.1 HPG and HPA axes

Traditionally the hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-adrenal (HPA) axes have been viewed as the sole sources of sex steroids and glucocorticoids (GCs), respectively. In the HPG axis, hypothalamic gonadotropin-releasing hormone (GnRH) stimulates the anterior pituitary to secrete gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), into the general circulation (97, 32). In the gonads, gonadotropins stimulate synthesis of sex steroids such as testosterone (T), 17 β -estradiol (E₂) and progesterone (PROG) (Fig. 1.1), which are secreted into the general circulation (74).

Similarly, in the HPA axis, hypothalamic corticotropin-releasing-hormone (CRH) stimulates the anterior pituitary to release adrenocorticotropin hormone (ACTH) into the general circulation (97, 32). In the adrenal glands, ACTH stimulates synthesis of GCs (e.g., cortisol, corticosterone) (Fig. 1.1), which are also secreted into the general circulation (74).

Thus, studies of sex steroids and GCs typically focus on levels in the blood (70). Steroids are small, lipophilic molecules that can cross the plasma membrane and blood-brain barrier, and steroids in the blood reach cells throughout the body. Spatial specificity of steroid action has traditionally been thought to be determined by steroid receptor expression by target cells.

¹ A version of this chapter has been published. Schmidt KL, Pradhan DS, Shah AH, Charlier TD, Chin EH, Soma KK Neurosteroids, immunosteroids, and the Balkanization of Endocrinology. *Gen Comp Endocrinol* 157: 266-274, 2008.

1.1.2 Local steroid synthesis

Steroids can also be synthesized locally, in at least three general ways. First, steroidogenic enzymes in target tissues can convert circulating hormones to more potent hormones locally (Fig. 1.2). For example, in the brain and other tissues, 5α -reduction of gonadal T produces a more potent androgen, 5α -dihydrotestosterone (18). Gonadal T can also be converted to E_2 by aromatase in the brain (24, 69).

Second, steroids can be synthesized locally from circulating inactive precursors (prohormones) (Fig. 1.2). For example, dehydroepiandrosterone (DHEA) is a sex steroid precursor that does not bind with high affinity to any intracellular steroid receptor (124). DHEA can be secreted into the circulation by the gonads or adrenals and then converted to active sex steroids locally (52, 92). Similarly, deoxycorticosterone and deoxycortisol can be locally converted to corticosterone or cortisol, respectively (19, 100).

Third, steroids can be locally synthesized *de novo* from cholesterol (Fig. 1.2). For example, E_2 and cortisol may be synthesized *de novo* in the retina (15, 135). All the GC-synthetic enzymes are also expressed in the skin (101), lung (82), heart (49), and intestine (21). Here, we focus on two examples: (1) steroids synthesized in the nervous system (neurosteroids) (25, 118) and (2) steroids synthesized in the immune system (immunosteroids) (119, 61).

1.2 Neurosteroids

1.2.1 Definition

Baulieu and colleagues detected high levels of DHEA and its esters in the brains of male rats, which have very low levels of DHEA in the blood (25). High levels of DHEA

remained in the brain, even 15 days after gonadectomy and adrenalectomy (25). These results raised the hypothesis that DHEA is a “neurosteroid,” synthesized locally in the brain (25). A strict definition of the term neurosteroid would require the steroid to be synthesized in the brain *de novo* from cholesterol. A less strict definition would also include steroids that are synthesized in the brain from circulating precursors (67, 65).

1.2.2 Sex steroid synthesis in the brain: nonbreeding season

High circulating T levels are generally associated with aggression in the breeding season, particularly during social instability (127). However, some species are territorial year-round, even during the nonbreeding season when the gonads are regressed and plasma T levels are low (26, 110, 105). For example, male song sparrows (*Melospiza melodia morphna*) display similar territorial behavior in response to simulated territorial intrusions during the breeding and nonbreeding seasons (126). Castration has no effect on nonbreeding aggression (125). In contrast, aromatase inhibitors decrease nonbreeding aggression (110, 107, 109). These results suggest that the steroids (particularly estrogens) regulating nonbreeding aggression are of non-gonadal origin (106, 108).

The prohormone DHEA is secreted by the adrenal glands in some vertebrates (52). In nonbreeding song sparrows, circulating DHEA levels are elevated, and DHEA levels in the adrenals and gonads are very high (111, 71, 29). DHEA treatment during the nonbreeding season increases territorial singing behavior (112). Song sparrow and zebra finch (*Taeniopygia guttata*) brain tissue metabolize DHEA to active sex steroids *in vitro* (104, 80). Interestingly, in song sparrows, brain 3 β -HSD activity is upregulated during the nonbreeding season (unpublished results). Thus, systemic DHEA may be metabolized to active sex

steroids in the brain to regulate aggression outside of the breeding season (Fig. 1.2A, Fig. 1.3A). Similar mechanisms may operate in other species (36, 103). Alternatively, DHEA may be synthesized *de novo* from cholesterol in the brain during the nonbreeding season (Fig. 1.2A).

1.2.3 Sex steroid synthesis in the brain: development

Neurosteroid synthesis is pronounced during early development (Fig. 1.3B). In rats, brain expression of P450c17, which metabolizes pregnenolone to DHEA, is highest in the embryo and decreases postnatally (22). Similarly, brain 3 β -HSD mRNA in rodents is highest during early development (42, 63).

Several steroidogenic enzymes are expressed at very high levels in the developing avian brain (30, 118, 58). In rodent brain, these enzymes are typically expressed at much lower levels and thus more difficult to quantify. Neurosteroids may regulate sexual differentiation of the songbird brain. In nestling European starlings (*Sturnus vulgaris*), neural metabolism of DHEA and E₂ may be greater in males than females at specific ages (20). In zebra finches, E₂ synthesized *de novo* in cultured male brain slices triggers development of an important projection in the song control circuit (41). Female brain slices developed this projection only when co-cultured with male slices, suggesting a sex difference in neural E₂ synthesis during development (41, 93).

PROG is also synthesized in the brain during development (136, 68) and promotes myelination (51, 95). Lastly, there is some evidence that GCs are synthesized in the developing and adult brain (64, 60) and regulated by stress (10,11, 71).

1.3 Immunosteroids

1.3.1 Definition

Immunosteroids are steroids produced by immune cells (61). A strict definition of this term would require the steroid to be synthesized in the immune system *de novo* from cholesterol. A less strict definition would also include steroids that are synthesized in immune tissues from circulating precursors (e.g. 17). There is evidence that GCs (119) and sex steroids (4477) are synthesized by immune cells, although the evidence is far greater for GCs.

1.3.2 Glucocorticoid synthesis in the immune system

GCs can be synthesized *de novo* from cholesterol in the immune system (Fig. 1.2B). The developing murine thymus synthesizes GCs *in vitro*. In culture, mouse thymic non-T cells produce pregnenolone and deoxycorticosterone after incubation with 22R-hydroxycholesterol (119). Furthermore, all the steroidogenic enzymes required for corticosterone synthesis are expressed in murine thymus (119, 79, 54). In mice, GC-synthetic enzymes are expressed in thymic epithelial cells (119, 79) and thymocytes (83). In humans, P450c21 and P450c17 are also present in fetal thymus (17, 16).

There is also evidence for *de novo* GC synthesis in the avian thymus and bursa of Fabricius (hereafter bursa). The bursa is a primary immune organ that produces B-lymphocytes and is functionally equivalent to bone marrow in mammals (1, 23, 28). In developing chickens, *in vitro* studies suggest that the thymus and bursa express all the enzymes necessary to synthesize cortisol from cholesterol, in contrast to the avian adrenal

glands, which synthesize corticosterone (53). In developing zebra finches, cortisol levels in the thymus, bursa, and spleen are higher than cortisol levels in the plasma (94). Moreover, in immune tissues, cortisol levels are higher than corticosterone levels (94).

1.3.3 Glucocorticoid synthesis from precursors or metabolites

In addition, circulating inactive precursors, such as deoxycorticosterone or deoxycortisol, might be converted to active GCs within immune tissues (Fig. 1.2B). For example, circulating deoxycorticosterone is elevated in human fetuses (100, 73). If P450c11 is expressed in the developing thymus in humans, as in mice and birds (53, 54), then plasma deoxycorticosterone could be locally converted to corticosterone in human fetuses.

Deoxycorticosterone is synthesized by the adrenals of herring gulls (19) and may be converted to corticosterone in target tissues.

Another mechanism for local production of GCs is via 11 β -HSD (Fig 1). 11 β -HSD1 converts inactive GC metabolites (cortisone and dehydrocorticosterone) back to cortisol and corticosterone, respectively (95, 48). 11 β -HSD1 is expressed in murine splenocytes (134). In contrast, 11 β -HSD2 inactivates cortisol and corticosterone (95) and is also present in murine thymocytes and splenocytes (38).

1.3.4 Developmental changes in glucocorticoid synthesis in the immune system

In general, local production of GCs in the immune system declines with age (Fig. 1.3B). In mice, GC synthesis in the thymus declines after birth, whereas corticosterone production in the adrenal glands increases with age (119). Similarly, in developing zebra

finches, cortisol levels in the immune system decline after hatch, but corticosterone levels in the general circulation increase with age (94).

In vertebrates (particularly altricial species), systemic levels of GCs are low early in development and show little, if any, response to restraint (10, 89). This has been termed the stress hyporesponsive period (SHRP). The SHRP lasts approximately two weeks in rodents (33, 56) and one week in songbirds (99, 120). After the SHRP, systemic GC levels increase. Taken together, these data raise the hypothesis that local synthesis of GCs in the immune system is greatest during the SHRP and is reduced when systemic GC levels rise (Fig 1.3B).

1.3.5 Function of glucocorticoid synthesis in the immune system

GCs are involved in thymocyte selection during development (39, 46). What is less clear is the role of locally-produced GCs in thymocyte selection. When transgenic mice that over-express glucocorticoid receptors (GR) specifically in thymocytes are adrenalectomized, GR overexpression still leads to a decrease in thymocyte number, even though systemic GCs have been removed (78). These data suggest a role for locally-synthesized GCs in lymphocyte selection (78). High local GC levels may also serve to suppress the development of the costly adaptive immune system, thus shunting resources to rapid body growth during early development in species with a fast pace of life (55).

1.4 The Balkanization of endocrinology

1.4.1 Definition

The balance between systemic and local steroid signaling may depend on life history stage, species adaptations, and the costs and benefits of systemic signals (Fig. 1.3). For

example, in nonbreeding song sparrows, there is a shift from systemic to local sex steroid signaling for the regulation of territorial behavior (105; Fig. 1.3A). Similarly, in molting song sparrows, there may be a shift from systemic to local GC signaling (71).

We propose that such shifts from systemic to local synthesis and regulation of steroids within target tissues represent a “Balkanization” of the endocrine system. The term Balkanization was originally used to describe the compartmentalization or division of a state into smaller states, but the term is used more broadly now (see www.oed.com). The result of this process is that individual tissues become capable of autonomously synthesizing and regulating local steroid signals, perhaps independently of the HPG and HPA axes. Such Balkanization can be reversible. For example, when nonbreeding song sparrows transition to the breeding life history stage, there is a corresponding shift from local to systemic sex steroid signaling (Fig. 1.3A). Note that terms exist to describe local steroid synthesis and action (autocrine, paracrine, intracrine) (74, 52). We use the term Balkanization to refer to the *shift* from systemic signals to local signals and local regulation.

1.4.2 Identity of local versus systemic steroid signals

The identities of locally-produced steroids can differ from those of systemic steroids in the circulation. For example, 17β -estradiol is typically considered the principal estrogen in the blood, but 17α -estradiol seems more abundant in the mouse brain and binds with higher affinity to a membrane-associated estrogen receptor in the brain (115). Similarly, corticosterone is considered the principal GC in the blood of birds (130), but cortisol may be synthesized in the immune system of chickens (53) and is present at higher levels than corticosterone in the immune system of developing zebra finches (94). In chickens, bursa

GRs bind cortisol with higher affinity than corticosterone (114). Thus, steroid profiles may differ greatly between blood and target tissues.

1.4.3 Regulation of local steroid synthesis

All the mediators of the HPG and HPA axes may be locally expressed in tissues where local steroid synthesis is predominant. That is, local steroid production may be locally regulated by miniature HPG and HPA axis “homologs” (Fig. 1.4; 77, 101). For example, the rodent hippocampus can synthesize E_2 *de novo* from cholesterol (40, 88). In addition to expressing all the E_2 -synthetic enzymes, the hippocampus also contains GnRH, GnRH receptors, LH, and LH receptors (81, 44, 57). Preliminary data suggest that GnRH treatment of hippocampal slice cultures increases E_2 levels in the medium (81). These data raise the intriguing hypothesis that a “HPG axis homolog” is expressed entirely within the rodent hippocampus. Such considerations may be relevant for songbirds and amphibians as well (e.g., 5, 133).

Similarly, there is local expression of CRH and ACTH in immune tissues (Fig. 1.4; 6, 2, 76). Moreover, injection of a virus into hypophysectomized mice increases ACTH+ lymphocytes in the spleen (102). In human skin, there is evidence for local expression of CRH, CRH receptor, ACTH, ACTH receptor, and all the GC-synthetic enzymes (43). CRH treatment increases production of ACTH, corticosterone, and cortisol in melanocytes (101). Thus, there may be “HPA axis homologs” in the thymus (Fig. 4) and skin.

1.4.4 Mechanisms of action: local versus systemic steroids

Traditionally, steroids have been thought to bind to intracellular receptors and act as transcriptional regulators over hours to days. However, steroids can also bind to plasma membrane-associated receptors and act non-genomically within seconds to minutes (59, 113). E₂ has numerous rapid effects on the brain and behavior (24, 132). For example, E₂ rapidly increases mouse aggression under short but not long photoperiods (116, 117). Many of these rapid effects require doses of E₂ that surpass systemic E₂ concentrations in the blood (132, 80) but that might approximate local E₂ concentrations in specific brain regions or synapses (24). Local E₂ levels could reach high concentrations quickly, because aromatase and other steroidogenic enzymes are rapidly regulated in the brain (3, 80, 104) and are enriched in subcellular compartments such as the presynaptic bouton (91, 40, 85). Thus, neurally-synthesized E₂ might act as a neurotransmitter (4).

Similarly, GCs have rapid effects on various systems, including the immune system (12). GCs induce apoptosis in thymocytes via non-genomic and genomic mechanisms (9). Many rapid effects of GCs on immune tissues require high doses, exceeding stress-induced systemic GC levels (12). In general, locally-synthesized steroids can be produced quickly, reach high concentrations, and rapidly bind to nearby receptors. Taken together, the data raise the hypothesis that local steroids are more likely to act via non-genomic mechanisms than systemic steroids.

1.4.5 Steroid binding globulins and local steroid action

Spatial specificity of steroid action may also be achieved via steroid binding globulins. In addition to low-affinity, high-capacity steroid carriers (albumin and alpha-glycoprotein,

122), there are high-affinity, low-capacity and high-specificity steroid binding globulins: sex hormone binding globulin (SHBG) and corticosteroid binding globulin (CBG) (8, 98). In some species (e.g., humans), both globulins are mainly produced by the liver and secreted into the blood. However, SHBG is not detectable in the plasma of adult laboratory rodents (84) or birds (129). Steroid binding globulins modulate steroid bioavailability and the metabolic clearance of circulating steroids (123, 66). Recent data suggest that binding globulins can also alter local concentrations of steroids. Circulating SHBG and CBG accumulate in specific tissues via interactions with the extracellular matrix (72) or via active internalization into cells (13). There is also evidence for local synthesis of SHBG and CBG in the brain and other organs (121, 45). High levels of binding globulins in specific tissues may either increase local steroid action or serve as a buffer against steroid action. In addition, CBG can be cleaved at target sites to increase local levels of free GCs (34).

1.4.6 Costs and benefits of local versus systemic steroid signals

The balance between systemic and local steroid signals may reflect their relative costs and benefits. For example, territorial aggression in the nonbreeding season may be dissociated from high systemic levels of T because of the costs of circulating T at this time, which include increased energy expenditure (metabolic rate) and reduced fat stores (128, 131, 103, 35). Unlike systemic T, systemic DHEA treatment does not suppress immunity or stimulate the growth of secondary sexual characteristics in nonbreeding song sparrows (112, 75). In the breeding season, high systemic T levels may be useful for coordinating multiple behaviors with multiple physiological systems ('hormonal pleiotropy'; 50). Similarly, in

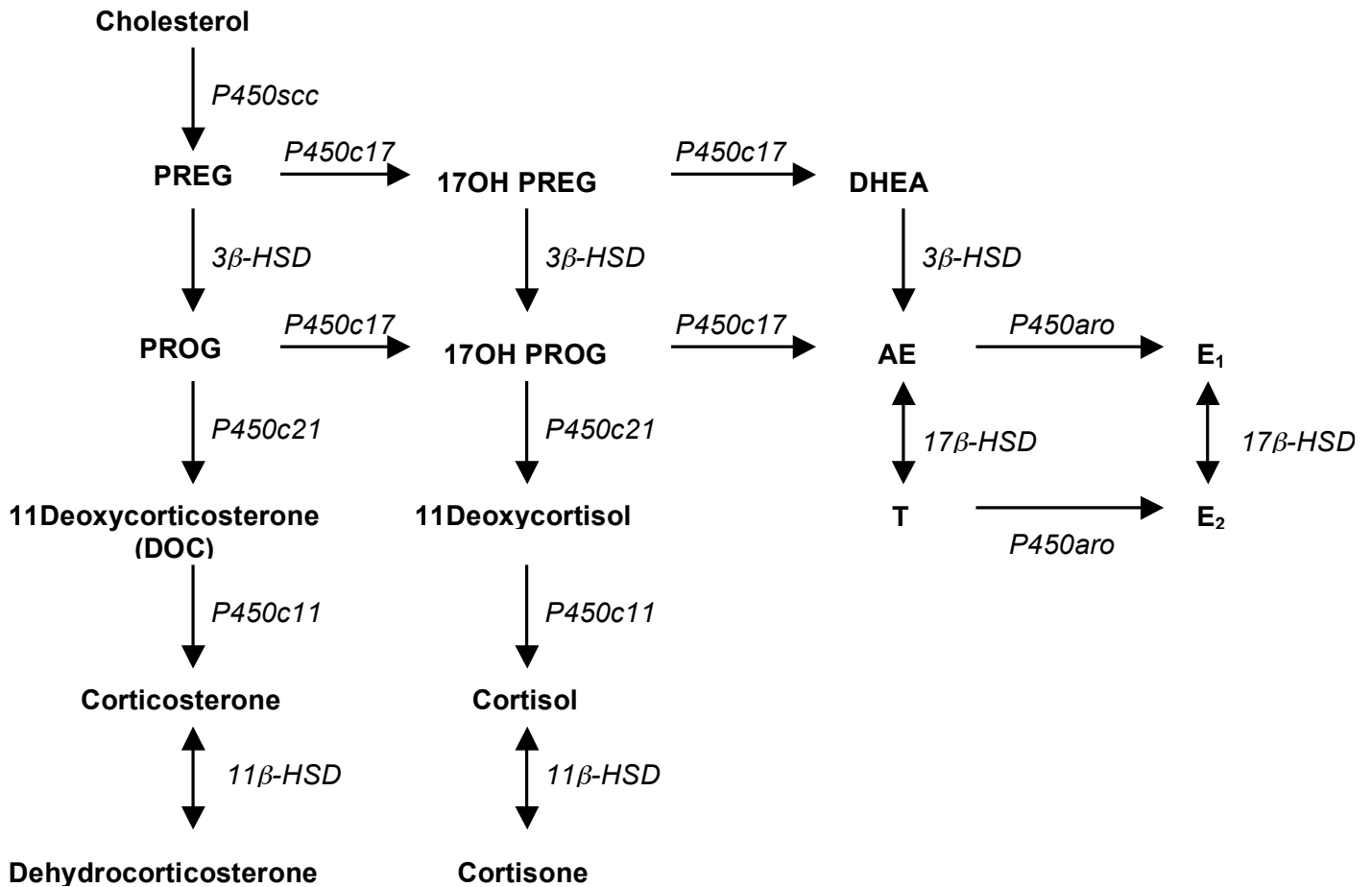
nestling birds, high systemic T can suppress immunocompetence and body growth, which are major costs during this life history stage (31, 27).

Systemic GCs also have benefits and costs (62). Acute increases in systemic GCs regulate many physiological systems (e.g., hepatic glucose mobilization, cardiovascular tone, digestion, growth, and immune function) and this may be adaptive in the short-term (90). However, chronic elevation of systemic GCs can result in “allostatic overload” (62) and detrimental effects on body growth (37), feather growth (86), or immune function (87). One strategy to avoid the costs of high systemic GCs might be local synthesis of GCs. Local GC production in the thymus may regulate thymocyte selection during development (119, 78), when high systemic GCs can have long-lasting negative effects on body growth.

1.5 Conclusions

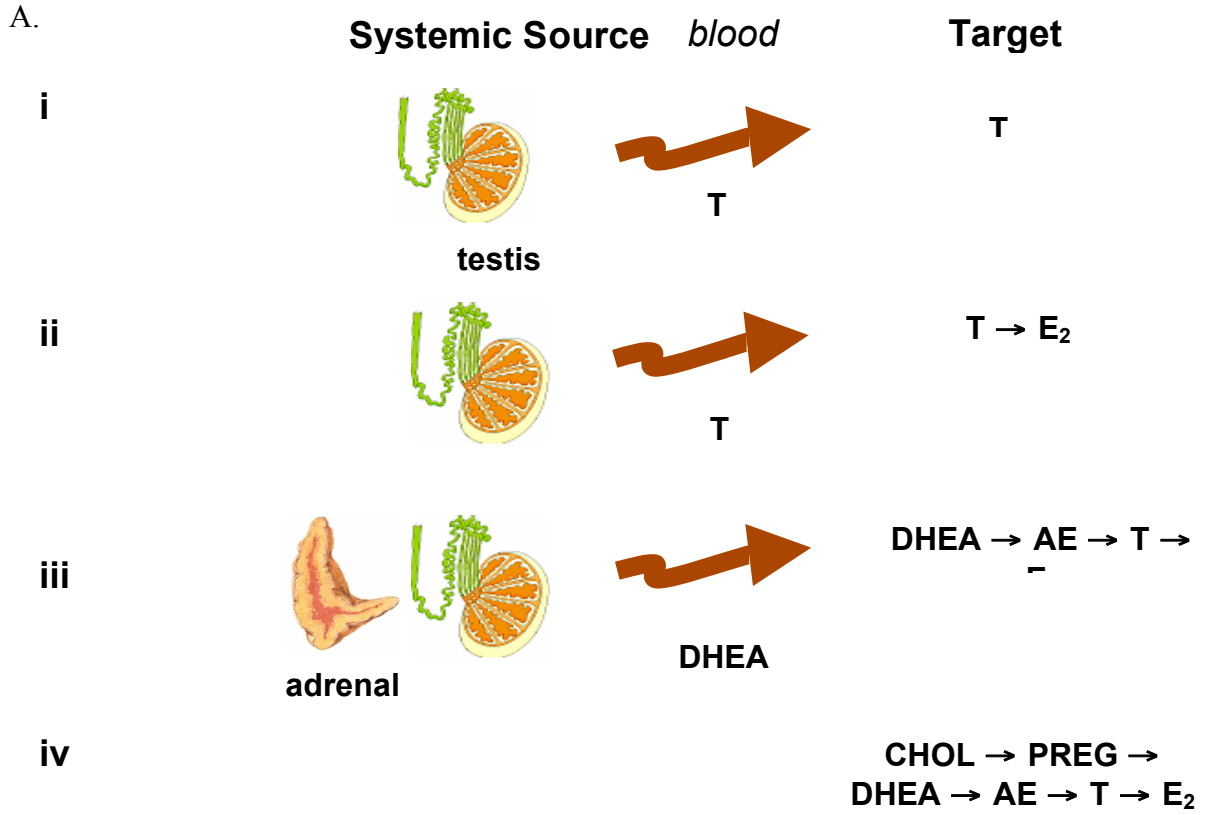
Local steroid synthesis has important implications for comparative endocrinologists, who have historically focused on systemic steroid levels in the general circulation. Studies of birds are likely to provide fundamental insights into this issue because birds show high expression of steroidogenic enzymes in the brain (29, 58, 118), as well as in the thymus and bursa (53). Moreover, comparative and field studies can elucidate the function and evolution of local steroid synthesis (14). Local steroid synthesis is a characteristic of vertebrates, including humans, and may be particularly important when systemic steroid levels are low, such as in the nonbreeding season, early development, post-menopause, Addison’s disease, and Kallman’s syndrome.

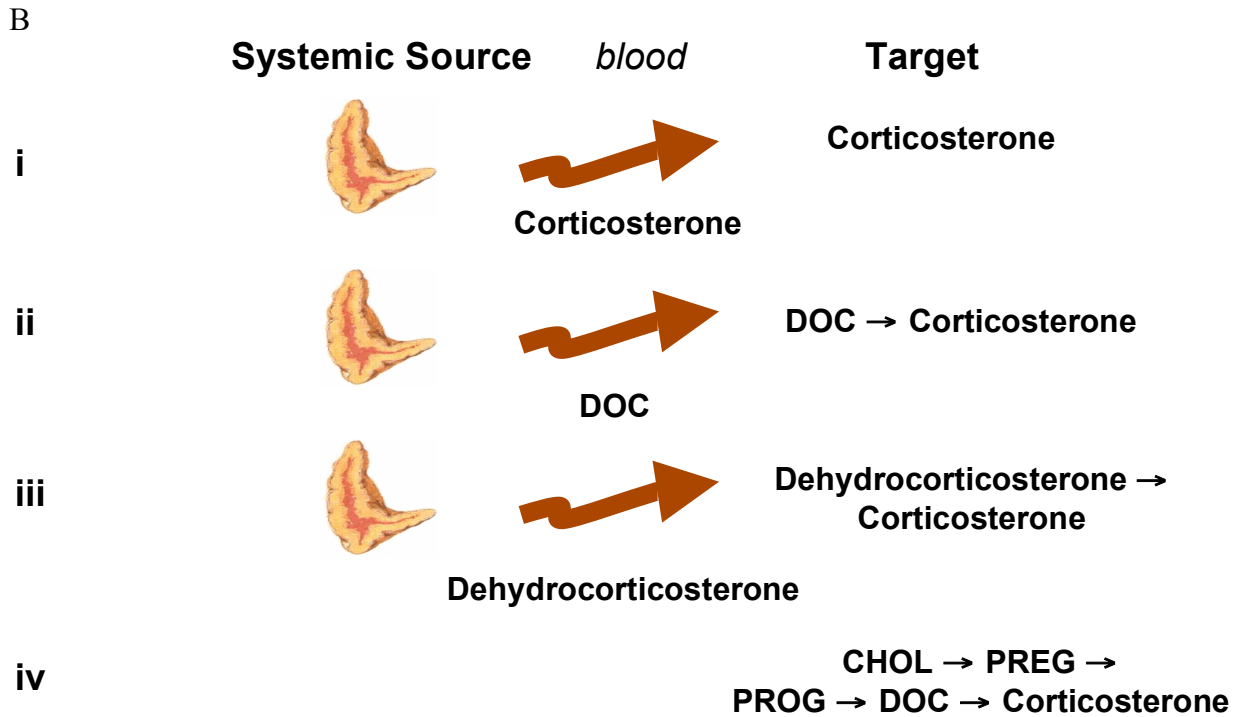
Figure 1.1 Simplified diagram of steroid synthesis



Steroids are shown in bold and enzymes in italics. Steroids: PREG, pregnenolone; 17OH PREG, 17 α -hydroxy-pregnenolone; DHEA, dehydroepiandrosterone; PROG, progesterone, 17OH PROG, 17 α -hydroxy-progesterone; AE, androstenedione; T, testosterone; E₂, 17 β -estradiol; E₁, estrone. Enzymes: P450scc, cytochrome P450 side chain cleavage; P450c17, cytochrome P450 17 α -hydroxylase/17,20 lysase; 3 β -HSD, 3 β -hydroxysteroid dehydrogenase/isomerase; 17 β -HSD, 17 β -hydroxysteroid dehydrogenase; P450aro, cytochrome P450 aromatase; P450c21, cytochrome P450 21 α -hydroxylase; P450c11, cytochrome P450 11 β -hydroxylase; 11 β -HSD, 11 β -hydroxysteroid dehydrogenase.

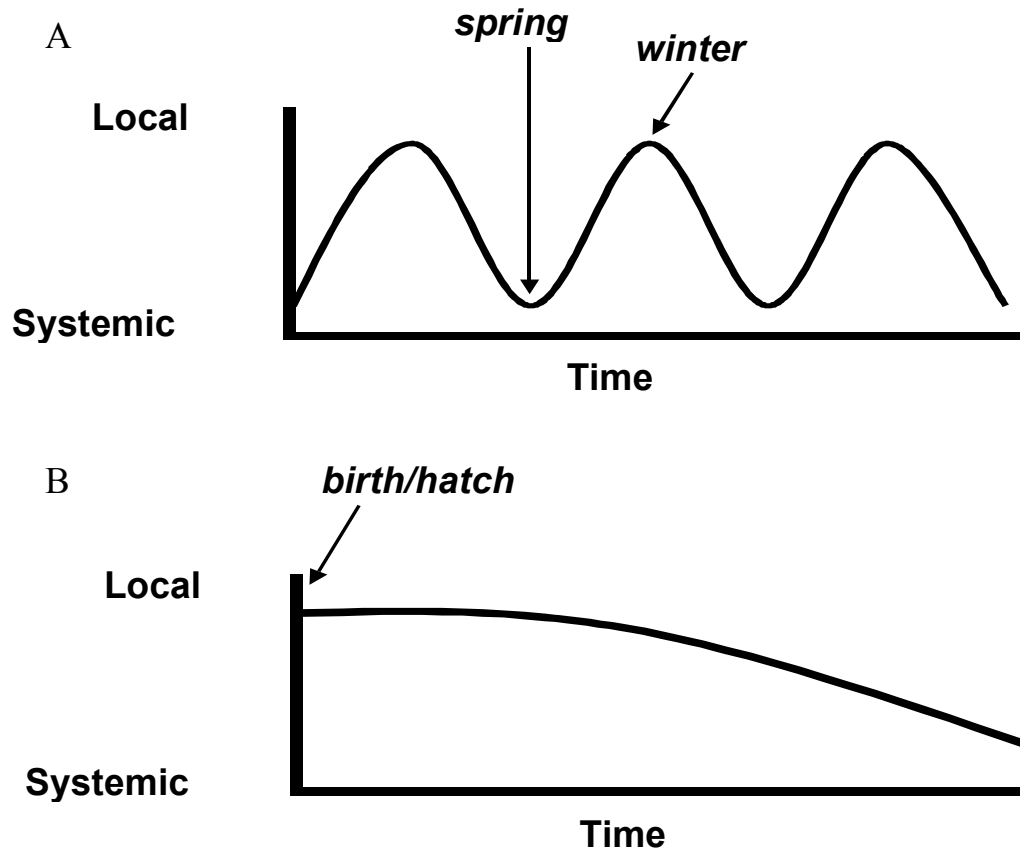
Figure 1.2 Potential mechanisms of steroid synthesis





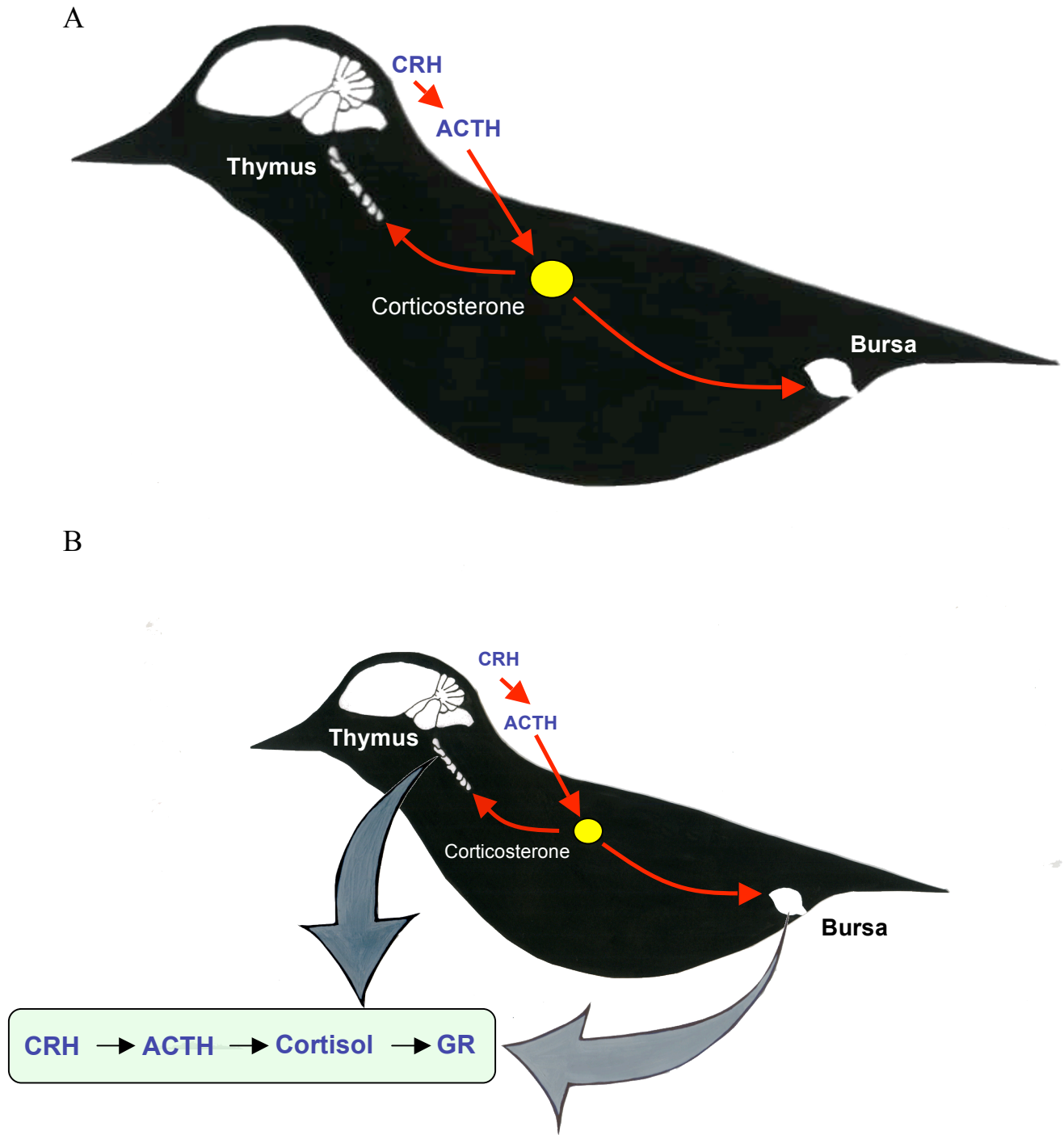
Potential mechanisms of (A) sex steroid synthesis and (B) glucocorticoid (GC) synthesis. (A) Sex steroids can be synthesized in the gonads, released into the blood, and travel to distant target organs and (i) bind to steroid receptors directly or (ii) be locally converted into a more potent steroid. Alternatively, (iii) a circulating prohormone (e.g. DHEA) can be locally metabolized to active sex steroids in the target organ. Lastly, (iv) the sex steroid can be locally synthesized *de novo* from cholesterol. (B) GCs can be (i) synthesized in the adrenals and travel via the blood to distant target organs. Alternatively, (ii) a circulating prohormone (e.g. 11-deoxycorticosterone, DOC) can be locally metabolized to an active GC in the target organ. Also, (iii) an inactive GC metabolite (e.g. dehydrocorticosterone) can be secreted from the adrenals and locally metabolized to an active GC in target tissues. Lastly, (iv) the GC can be locally synthesized *de novo* from cholesterol. T, testosterone; E₂, 17β-estradiol; DHEA, dehydroepiandrosterone; AE, androstenedione; CHOL, cholesterol; PREG, pregnenolone; PROG, progesterone; DOC, 11-deoxycorticosterone.

Figure 1.3 Hypothetical shifts between systemic and local steroid signals



(A) Possible seasonal changes in sex steroid signaling in adult song sparrows. During the spring (breeding season), systemic T levels are high and support high expression of territorial behavior. During the winter (nonbreeding season), systemic T levels are low and local sex steroid synthesis in the brain supports high expression of territorial behavior. We propose that such shifts from systemic signals to local signals represent a “Balkanization” of the endocrine system. (B) Possible developmental changes in sex steroid and glucocorticoid (GC) signaling. Both sex steroid synthesis in the brain and GC synthesis in the immune system appear highest during early development and decline with age, as systemic steroid levels increase.

Figure 1.4 Possible hypothalamic-pituitary-adrenal (HPA) axis “homolog” in the developing thymus and bursa.



(A) In adult birds, corticosterone is secreted from the adrenals and travels via the bloodstream to distant target tissues, such as the thymus and bursa of Fabricius (bursa). (B) In contrast, during development, cortisol (but not corticosterone) may be locally synthesized and regulated by a miniature HPA axis “homolog” within immune organs, such as the thymus and bursa. CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropin; GR, glucocorticoid receptor.

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2. Cortisol and corticosterone in the songbird immune and nervous systems: local versus systemic levels during development²

2.1 Introduction

Glucocorticoids (GCs) affect many systems during development, including the immune and nervous systems. For example, GCs regulate apoptosis and selection of T-cells in the thymus and B-cells in bone marrow (16, 21, 26). Although high GC concentrations decrease thymocyte survival, lower GC levels can antagonize T (thymocyte) cell receptor-signaling and increase thymocyte survival (71). In addition, GCs have important and long-lasting effects on the developing brain, such as regulation of neurotransmitter levels (36) and neurogenesis in the hippocampus (42, 29).

Despite the profound effects of GCs and stress on the immune system and brain during development, circulating GC levels are low and show only a slight, if any, response to stress neonatally. This has been coined the stress hyporesponsive period (SHRP). In rats, the SHRP lasts from post-natal day 3 (PN3) to about PN12 (38, 56, 19). In mice the SHRP lasts from PN1 to about PN12 (60). In birds, plasma corticosterone levels are low during the first few days of life and show little or no increase after restraint stress (61, 64, 73).

It remains a paradox that GCs have profound effects on the immune and nervous systems during development, even though circulating GC levels are low. One hypothesis that may explain this discrepancy is that GCs are produced locally in immune organs and/or brain tissue early in development. The capacity for the developing murine thymus to synthesize GCs *in vitro* has been well-established. In culture, developing mouse thymic non-T cells

² A version of this chapter has been published. Schmidt KL and Soma KK Cortisol and corticosterone in the songbird immune and nervous systems: local versus systemic levels during development. *Am J Physiol Reg Integr Comp Physiol* 295: 103-110, 2008.

produce pregnenolone and deoxycorticosterone after incubation with 22R-hydroxycholesterol (72). GC production in the thymus declines with age, but GC production in the adrenals increases with age (72). Furthermore, steroidogenic enzymes, including P450_{scc}, 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSD), P450_{c21}, and P450_{c11}, are expressed in murine thymus (34, 72).

In chickens, *in vitro* studies suggest that the thymus and bursa of Fabricius (hereafter bursa) express all the enzymes necessary to synthesize cortisol from cholesterol, in contrast to the adrenal glands, which synthesize corticosterone (33). The bursa is a primary immune organ in birds that produces B-lymphocytes and is functionally equivalent to bone marrow in mammals (1, 9). The suggestion that the chicken immune system synthesizes cortisol, not corticosterone, is surprising. Traditionally, the primary adrenal GC in birds is thought to be corticosterone, and cortisol is typically not measured in birds. Importantly, no studies have measured endogenous GC levels in immune tissues, in any species. This is a major gap in our knowledge.

There is also strong evidence that steroids are produced *de novo* from cholesterol in the developing and adult brain (neurosteroids). Studies in rats suggest that dehydroepiandrosterone (DHEA) and progesterone are synthesized in the brain (3, 52, 67). Neural DHEA synthesis declines during development (8). There is also evidence that estrogens are neurosteroids in songbirds, with declining synthesis during development (22, 39). There is little evidence thus far that GCs are neurosteroids. However, GC-synthetic enzymes are expressed in the adult rat brain (43). Aldosterone may be synthesized in the adult rat brain (17). Since corticosterone is a precursor to aldosterone, these data indirectly suggest neural corticosterone synthesis.

Here, we measured endogenous GCs in plasma, immune tissues, and brain of a developing songbird, the zebra finch (*Taeniopygia guttata*). Songbirds are excellent animal models for studying local steroid synthesis in the immune and nervous systems. The avian immune system is well-characterized (9, 12) and the thymus and bursa can both be readily examined. Moreover, the avian brain robustly expresses steroidogenic enzymes (18, 20, 50, 58).

We measured corticosterone and cortisol at the day of hatch (P0), P3 and P30. First, we predicted that GC levels in plasma would be low early in development, but GC levels in immune organs and brain would be high due to the local synthesis of GCs. This result would indicate that GCs can have important effects on immune system and brain development, even though systemic GC levels are low. Second, we predicted that GC levels in plasma would increase with age as the SHRP ended, while local GC levels would decline with age. Third, we predicted that the primary GC in plasma and adrenal glands would be corticosterone, and the primary GC in the immune organs would be cortisol. This result would raise the possibility that corticosterone and cortisol have different functions, despite the prevailing belief that the two GCs are interchangeable.

2.2 Materials and methods

2.2.1 Subjects

Research was carried out under a University of British Columbia Animal Care permit (A06-0408) and procedures were approved by the Canadian Council on Animal Care. Breeding pairs of adult zebra finches were housed separately and given millet seeds, water, grit, and cuttlefish bone *ad libitum*. Breeding pairs were also given a food supplement

consisting of boiled chicken eggs, cornmeal and bread daily. The light cycle provided 14 hours of light and ten hours of dark (lights on at 8:00 AM). Temperature was held constant at 23°C and relative humidity at ~50%.

Subjects were developing male and female zebra finches at P0 (n=16), P3 (n=16) or P30 (n=8) (n = 40 subjects total). Zebra finches are altricial. At P0 and P3, the chicks' eyes were closed, and chicks could not thermoregulate well (14, 78). At P30, chicks had fledged from the nest but remained in the same cage as their parents. At P0 and P3, samples were pooled from two birds of the same sex and age. In total, 136 plasma and tissue samples were analyzed for both corticosterone and cortisol.

2.2.2 Sample collection

All subjects were sampled between 10:00 and 11:00 AM to control for possible diel changes in GCs. A blood sample was collected within 3 min (mean \pm SEM = 2.35 ± 0.10 min) of opening the cage. GC levels are generally at baseline if animals are sampled within 3 min of disturbance (53, 77). At P30, blood samples were taken from the brachial vein by puncturing the vein with a 26-gauge needle and collecting blood into heparinized micro-hematocrit tubes. At P0 and P3, due to the difficulty of collecting a sufficient quantity of blood from the brachial vein, blood was collected via cardiac puncture with heparinized 0.5 mL syringes with 28-gauge fixed needles. Blood was centrifuged at 10,000 rpm for 10 min. Plasma was collected with a Hamilton syringe and stored at -20°C until analysis.

Immediately after blood collection, birds were rapidly decapitated. The period from opening the cage to sacrifice was 2.52 ± 0.11 min. The brains and bodies were immediately chilled at -20°C. After two min, brains were dissected. Brain regions collected include the rostral

telencephalon (rTel), caudal telencephalon (cTel), and cerebellum. First, the cerebellum was collected. Then, the telencephalon was bisected midway to separate the rostral and caudal sections. The rTel and cTel were then separated into the left and right hemispheres. Next, the body was dissected. The bursa was collected at all three ages. The thymus and spleen were collected only at P30, due to the difficulty of collecting these organs in the younger animals. Adrenal glands and breast muscle were also collected at P30 (n=4), as positive and negative controls, respectively. Tissues were immediately frozen on dry ice and stored at -80°C.

2.2.3 Steroid extraction

Steroids were extracted from tissue and plasma using solid phase extraction (SPE) with C18 columns as previously described (45). This extraction procedure results in high and consistent steroid recoveries and effectively removes interfering substances from lipid-rich samples (45). Briefly, tissue samples were homogenized in ice-cold deionized water (dH₂O). Then HPLC-grade methanol was immediately added, and samples were incubated overnight at 4°C. C18 columns were primed with 3 mL HPLC-grade ethanol and equilibrated with dH₂O. Tissue samples were centrifuged at 3000 x g, the supernatant was brought up to 10 mL with dH₂O, and samples were loaded onto C18 columns. Plasma samples were also brought up to 10 mL with dH₂O and loaded onto C18 columns. Columns were then washed with dH₂O, and steroids were eluted with 5 mL 90% HPLC-grade methanol. Samples were evaporated under N₂ at ~40°C. Dried extracts were resuspended in 70 µL assay buffer (phosphate-buffered diluent provided with the cortisol assay). We used absolute ethanol (5% of resuspension volume) to aid in resuspension of steroids (45).

Steroid recovery was determined by spiking plasma and tissue samples with known

amounts of radioinert corticosterone and cortisol and comparing spiked samples to unspiked samples (n=3 pairs for each of plasma, immune tissue, and brain tissue). Recovery of corticosterone was $108 \pm 4.23\%$ in plasma, $92 \pm 4.62\%$ in immune tissue, and $95 \pm 3.14\%$ in brain tissue. Recovery of cortisol was $110 \pm 1.90\%$ in plasma, $84 \pm 3.35\%$ in immune tissue, and $110 \pm 2.68\%$ in brain tissue. Samples were corrected for recovery where applicable.

2.2.4 Corticosterone radioimmunoassay

Levels of corticosterone were determined using a sensitive and specific double-antibody ^{125}I radioimmunoassay (RIA) (MP Biomedicals, 07-120103), which has been validated for songbird plasma and tissue samples (45, 46, 74). The detection limit was 3.12 pg corticosterone per tube. The corticosterone antibody was highly specific (Table 2.1). The cross-reactivities of the corticosterone antibody to the metabolites and precursors of cortisol and corticosterone were determined by us, if the information was not provided by the manufacturer. Specifically, we determined the cross-reactivity of the corticosterone antibody to cortisone and dehydrocorticosterone (Table 2.1).

Of the 70 μL resuspension, 20 μL was used for the corticosterone assay (29% of the sample). This 20 μL was brought up to 100 μL with the phosphate-buffered diluent provided with the corticosterone assay, and 50 μL was assayed in duplicate for corticosterone. Average coefficient of variation between duplicates was 1.8%.

Because samples were resuspended in 5% ethanol, we ensured that the ethanol had no effect on the RIA by comparing a 50 pg standard containing 5% ethanol to a 50 pg standard without ethanol (n = 3 pairs). The standard with ethanol was similar to the standard without ethanol (t = 1.35, p = 0.25). Water blanks and known standards were analyzed in each assay.

All extracted water blanks (n=10) were nondetectable for corticosterone (<3.12 pg corticosterone). For corticosterone, a 30 pg control was analyzed in each corticosterone assay (n=4). We obtained on average 34 ± 2.15 pg. Inter-assay coefficient of variation was 12%.

2.2.5 Cortisol enzyme immunoassay

Levels of cortisol were determined using a sensitive and specific enzyme immunoassay (EIA) (Salimetrics, 1-3012). Several cortisol assays were compared, and this assay was the most sensitive and highly specific. The cortisol assay was similar to the corticosterone assay in several respects. Both assays were highly specific, had similar detection limits, and had similar intra- and inter-assay variation. The detection limit was 3 pg cortisol per well. The antibody was highly specific for cortisol (Table 2.1). The cross-reactivities of the cortisol antibody to the metabolites and precursors of cortisol and corticosterone were determined by us, if the information was not provided by the manufacturer. Specifically, we determined the cross-reactivity of the cortisol antibody to 11-deoxycorticosterone and dehydrocorticosterone (Table 2.1). For the cortisol antibody, the manufacturer reported a low cross-reactivity to corticosterone (0.20%). Nonetheless, we verified this by testing, in duplicate, 1000 pg, 100 pg, and 10 pg of corticosterone in the cortisol EIA, all of which were nondetectable. These results verify that cross-reactivity of the cortisol antibody to corticosterone was <0.30%.

The remaining 50 μ L of resuspension (71% of the sample) was brought up to 70 μ L with the phosphate-buffered diluent provided with the cortisol EIA, and 25 μ L was assayed in duplicate for cortisol. Average coefficient of variation between duplicates was 1.6%. A plate washer was used to ensure that wells were washed consistently (Tecan Columbus

washer: I 109011). Absorbance was measured using a plate reader (Sunrise remote: f039300) at 450 nm, with a 620 nm background correction.

Because samples were resuspended in 5% ethanol, we ensured that the ethanol had no effect on the EIA by comparing a 100 pg standard containing 5% ethanol to a 100 pg standard without ethanol ($n = 3$ pairs). The standard with ethanol was similar to the standard without ethanol ($t = 0.94$, $p = 0.40$). Water blanks and known standards were analyzed in each assay. For cortisol, 8 of 10 water blanks were non-detectable (<3 pg cortisol). The average amount of cortisol detected in the water blanks was 0.65 ± 0.44 pg. For cortisol, a 25 pg control was analyzed in each cortisol EIA ($n=5$). We obtained on average 24.18 ± 1.33 pg. Inter-assay coefficient of variation was 12% for cortisol.

2.2.6 Statistics

Nondetectable samples (below the lowest standard on the standard curve) were conservatively set to zero for both assays. Data were analyzed in SPSS (v. 11 for Mac OS X).

Data were analyzed with mixed-design three-way analysis of variance (ANOVA) tests on each tissue and plasma, with Age and Sex as between-subjects variables and Steroid (corticosterone vs. cortisol) as a within-subjects variable. The Sex factor was not significant (main effects or interactions with another factor) in any of the analyses, and thus data from males and females were combined. The lack of sex differences is consistent with data on other songbird species (73, K. Schmidt, E. Chin, K. Soma, unpublished results). Where applicable, significant interactions between Age and Steroid were broken down into simple main effects using paired t-tests. If the interaction was not significant, significant main effects of Age were broken down using Tukey's honestly significant difference (HSD) test.

Steroid levels in tissues were compared to steroid levels in plasma (as in 6, 10, 17). Note that 1mL of songbird plasma weighs 1.005 ± 0.17 g ($n = 3$; personal observation). Mixed-design two-way ANOVA tests were performed to compare levels of GCs in plasma and tissue using Age as a between-subjects variable and Sample (tissue vs. plasma) as a within-subjects variable. In addition, within individuals, we subtracted plasma GC levels from tissue GC levels (Table 2.2).

For tissues that were only collected in P30 animals, levels of corticosterone and cortisol were compared using paired t-tests, and plasma levels were compared to tissue levels using paired t-tests. Tests were two-tailed. Test results were considered significant for $p < 0.05$. Results are presented as mean \pm standard error of the mean (SEM).

2.3 Results

2.3.1 GC levels in plasma

In the plasma, levels of corticosterone were lowest at P0 and increased with age (Fig 2.1). Cortisol levels showed the opposite pattern and were highest at P0 and decreased with age (Fig 2.1). The interaction between Age and Steroid was significant ($F_{2,20} = 9.22$, $p = 0.002$). A break-down of the interaction into simple main effects revealed that corticosterone was the predominant GC in plasma at P3 ($t_7 = 2.73$, $p = 0.03$) and P30 ($t_7 = 6.06$, $p < 0.001$); however, corticosterone and cortisol were present at similar levels in P0 plasma ($t_7 = 0.24$, $p = 0.82$).

2.3.2 GC levels in the immune system

Local GC levels in the immune system were high at P0 and decreased with age (Fig

2.2). Furthermore, cortisol, not corticosterone, was the predominant GC in the immune system (Fig 2.2). In the bursa, there was a significant main effect of Steroid, which revealed that cortisol levels were significantly higher than corticosterone levels at all three ages ($F_{1,20} = 13.92$, $p = 0.002$). Furthermore, there was a significant main effect of Age ($F_{2,20} = 8.51$, $p = 0.003$). *Post hoc* tests revealed that GC levels in the bursa were higher at P0 than at P3 and P30. GC levels in the bursa were not significantly different between P3 and P30, suggesting that there is a rapid decrease in GC levels in the bursa soon after hatch. A comparison between cortisol levels in the bursa and plasma revealed a significant main effect of Sample, indicating that cortisol levels in the bursa were higher than cortisol levels in the plasma at all three ages ($F_{1,20} = 20.29$, $p < 0.001$) (Table 2.2). In contrast, corticosterone levels in the bursa were not significantly different from corticosterone levels in the plasma ($F_{1,20} = 0.80$, $p = 0.38$) (Table 2.2).

GC levels in the thymus and spleen were analyzed at P30 only (Fig 2.2). As in the bursa, cortisol was the primary GC in the thymus and spleen. Cortisol levels were significantly higher than corticosterone levels in the thymus and spleen ($t_7 = 2.50$, $p = 0.04$ and $t_7 = 2.45$, $p = 0.04$, respectively). Furthermore, cortisol levels in the thymus and spleen were significantly higher than cortisol levels in the plasma ($t_7 = 3.77$, $p = 0.007$ and $t_7 = 4.29$, $p = 0.003$, respectively) (Table 2.2). In contrast, corticosterone levels in thymus and spleen were lower than corticosterone levels in the plasma ($t_7 = 7.57$, $p < 0.001$ and $t_7 = 3.60$, $p = 0.008$, respectively) (Table 2.2).

2.3.3 GC levels in the brain

GC levels in the brain were low, and many samples were nondetectable for

corticosterone and cortisol (Fig 3). In the rTel (Fig 2.3A), there was a significant interaction between Steroid and Age ($F_{2,20} = 7.83$, $p = 0.004$). Decomposition of this interaction indicated that at P30, corticosterone levels were significantly higher than cortisol levels ($t_7 = 3.19$, $p = 0.02$). At P0 ($t_7 = 2.25$, $p = 0.07$) and P3 ($t_7 = 0.36$, $p = 0.75$), corticosterone and cortisol levels did not differ significantly in the rTel (Fig 2.3A). Corticosterone levels in the rTel were significantly lower than corticosterone levels in the plasma at all three ages ($F_{1,20} = 55.84$, $p < 0.001$) (Table 2.2). However, cortisol levels in the rTel and plasma did not differ significantly at any age ($F_{1,20} = 0.71$, $p = 0.41$) (Table 2.2).

In the cTel (Fig 2.3B), there were no significant effects of Age ($F_{2,20} = 2.59$, $p = 0.11$) or Steroid ($F_{1,20} = 2.54$, $p = 0.13$), indicating that corticosterone and cortisol levels were similar and did not change with age. Although cortisol levels in the cTel at P0 appear higher than cortisol levels in the cTel at the older ages and appear to be higher than corticosterone levels in the cTel at P0, the interaction between Age and Steroid failed to reach significance ($F_{2,20} = 1.96$, $p = 0.17$). A *post hoc* power calculation revealed that power for this comparison was 0.75. It is possible that larger sample sizes would result in a significant interaction between Age and Steroid. Corticosterone levels in the cTel were significantly lower than corticosterone levels in the plasma at all three ages ($F_{1,20} = 17.20$, $p = 0.001$) (Table 2.2). Cortisol levels in the cTel and plasma did not differ significantly at any age ($F_{1,20} = 1.60$, $p = 0.22$) (Table 2.2).

In the cerebellum (Fig 3C), there was a significant interaction between Age and Steroid ($F_{2,20} = 4.07$, $p = 0.04$). Analysis of this interaction revealed that corticosterone levels in the cerebellum were significantly higher than cortisol levels at P30 ($t_7 = 2.59$, $p = 0.04$) (Fig 3C). At P0 ($t_7 = 1.51$, $p = 0.18$) and P3 ($t_7 = 2.11$, $p = 0.07$), corticosterone and cortisol

levels did not differ significantly. Corticosterone levels in the cerebellum were significantly lower than corticosterone levels in the plasma at all three ages ($F_{1,20} = 56.52$, $p < 0.001$) (Table 2.2). Cortisol levels in the cerebellum and plasma did not differ significantly at any age ($F_{1,20} = 0.39$, $p = 0.54$).

2.3.4 GC levels in the adrenal glands and breast muscle

Analysis of GC levels in adrenal glands of P30 birds revealed that, as expected, corticosterone levels in the adrenals (2605.03 ± 747.88 ng/g, 100% of samples detectable) were significantly higher than corticosterone levels in the plasma ($t_3 = 3.48$, $p = 0.04$). Cortisol was also present in P30 adrenals, but at lower levels than corticosterone ($t_3 = 3.46$, $p = 0.04$). Cortisol levels in the adrenals (68.07 ± 17.21 ng/g, 100% of samples detectable) were significantly higher than cortisol levels in the plasma ($t_3 = 3.93$, $p = 0.03$).

Both corticosterone and cortisol levels were low in breast muscle, and levels of the two GCs did not differ from one another ($t_3 = 0.49$, $p = 0.66$). Corticosterone levels in the breast muscle (1.38 ± 0.38 ng/g, 75% of samples detectable) were significantly lower than corticosterone levels in the plasma ($t_3 = 7.93$, $p = 0.004$). Cortisol levels in the breast muscle (0.91 ± 0.46 ng/g, 75% of samples detectable) were not different from cortisol levels in the plasma ($t_3 = 1.57$, $p = 0.22$).

2.4 Discussion

To our knowledge, this is the first study to measure (1) cortisol in the plasma of songbirds, (2) corticosterone or cortisol in the nervous system of songbirds, and (3) endogenous corticosterone or cortisol levels in the immune system of any species. In plasma,

the ratio of corticosterone to cortisol increased with age. On P0, plasma corticosterone and cortisol levels were similar; however, within three days, corticosterone was more abundant. In contrast, cortisol was more abundant than corticosterone in the immune system at all three ages. Furthermore, cortisol levels in the immune organs were higher than cortisol levels in the plasma. In the brain, corticosterone and cortisol levels were generally low. Brain corticosterone and cortisol levels were not different at P0 and P3, but at P30, corticosterone levels were higher than cortisol levels.

2.4.1 GC levels in the brain

GC levels in the brain were generally low throughout development. At P0 and P3, corticosterone and cortisol levels were not significantly different in the three regions examined, raising the interesting possibility that both GCs play important, but perhaps different, roles in early brain development. At P30, corticosterone was generally the most abundant GC in the brain, as in the plasma.

In all three regions studied, neural corticosterone levels were lower than plasma corticosterone levels. In contrast, neural cortisol levels were similar to plasma cortisol levels. These data suggest differential metabolism of circulating GCs by neural 11β -hydroxysteroid dehydrogenase (23). Alternatively, there may be differential active transport of corticosterone and cortisol out of the brain. For example, the multidrug resistance P-glycoprotein (Pgp) at the blood-brain barrier transports cortisol, but not corticosterone, out of the brain in mice and humans (30).

These results provide little evidence for local GC synthesis in the developing brain. However, there is some evidence that GC-synthetic enzymes are expressed in the developing

and adult rat brain (17, 43), so more studies are needed before this possibility can be excluded. For example, it is possible that the brain synthesizes GCs only after stress or only in specific regions.

2.4.2 GC levels in the immune system

In the immune system, cortisol levels in the bursa are higher than corticosterone levels in the bursa at all three ages. In addition, cortisol levels in the bursa are higher than cortisol levels in the plasma at all three ages. Very similar results were obtained for the thymus and spleen at P30. With age, cortisol levels in the bursa decreased, while corticosterone levels in the plasma increased, suggesting that local cortisol synthesis in the immune system declines as systemic corticosterone levels increase. The abundance of cortisol in avian immune tissues is quite surprising because traditionally the primary GC in birds has been thought to be corticosterone (24, 44, 77). Nonetheless, in birds, corticosteroid binding globulin binds both corticosterone and cortisol with high affinity (76), and an intracellular glucocorticoid receptor (GR) binds both corticosterone and cortisol with high affinity (68, Dr. C. Breuner, personal communication).

These data suggest local cortisol synthesis in the developing immune system. The present results are consistent with *in vitro* data demonstrating that the chicken bursa and thymus contain all the steroidogenic enzymes, including P450c17, to synthesize cortisol *de novo* from cholesterol (33). Also, studies in mice have measured steroidogenic enzyme proteins and activities in the thymus (34, 72). While these previous studies examined enzyme activities *in vitro* or cultured thymic cells (34, 72), the present study fills an important gap by measuring endogenous corticosterone and cortisol levels in uncultured tissue. Taken together,

the data raise the hypothesis that cortisol is synthesized *de novo* in the avian immune system as an “immunosteroid” (40), analogous to neurosteroids synthesized in the nervous system (10).

It is possible that high local cortisol levels are due to the sequestration of cortisol in these organs, rather than local synthesis. However, previous studies that demonstrate steroidogenic enzymes in the bursa and thymus support the hypothesis that high levels are due to synthesis and not just sequestration (33, 34, 72). Also, in preliminary studies, we have performed short-term (24 hr) incubations of bursa and thymus *in vitro* and measured GCs in conditioned media. We detected high levels of cortisol, but low levels of corticosterone, in the conditioned media (K. Schmidt, Y. Yu, K. Soma, unpublished results).

The functional significance of local GC synthesis in the immune system is an important issue. GCs play an important role in the regulation of lymphocyte development. The mutual antagonism model postulates that signaling through either the GR or the T cell receptor alone induces thymocyte apoptosis. However, when both receptors are simultaneously stimulated, their signals oppose one another, leading to positive selection and thymocyte survival (21, 70). In this model, GC levels that are either too high or too low can induce lymphocyte apoptosis (26). Interestingly, when transgenic mice that over-express GRs specifically in thymocytes are adrenalectomized, GR overexpression still leads to a decrease in thymocyte number, even though systemic GCs have been removed (49). These data suggest a role for local GCs in thymocyte selection.

Because high levels of GCs can induce lymphocyte apoptosis, one possibility is that high local levels of GCs in the zebra finch immune system suppress the development of the costly adaptive immune system, thus shunting resources to rapid body growth during early

development. Zebra finches exhibit a very fast “pace of life” and reach reproductive maturity at an early age (~P90; 78). This life history strategy may favor reduced allocation of resources to the development of costly adaptive immune defenses (35). Thus, a critical question is whether local GC production results in “high” or “intermediate” local GC levels in zebra finches. Comparative studies (35, 41) and experimental hormone manipulations (15) will be useful for resolving this issue.

2.4.3 Roles of corticosterone and cortisol?

Corticosterone and cortisol are often viewed as interchangeable, and a common belief is that a species has just one predominant GC, corticosterone or cortisol. However, there are species that have similar levels of the two GCs in circulation, for example New Zealand white rabbits (69). Another study of rabbits found that at baseline, corticosterone was the predominant adrenal GC, but after chronic ACTH treatment, cortisol became the predominant adrenal GC (31). Thus, the primary circulating GC may be context-specific.

The present results suggest that the primary GC can also be organ-specific and age-specific. Our finding that cortisol levels are highest in early development is consistent with studies showing that the adrenal glands in embryonic and newly-hatched chickens produce corticosterone and cortisol at similar levels, but cortisol production by the adrenals declines soon after hatch (25, 27, 28). Our results suggest that cortisol may have unique effects during early immune system development. Both cortisol and corticosterone administration decrease the weight of the bursa in young chickens, but cortisol causes more vacuolar spaces within the lymph follicle of the bursa, suggesting that cortisol has a stronger effect on bursa

histology than corticosterone (14). Interestingly, in young chickens, GRs in the bursa have a higher affinity for cortisol than corticosterone (68).

At the molecular level, steroid transporters and receptors can differentiate between corticosterone and cortisol. In mice and humans, P-glycoprotein at the blood-brain barrier transports cortisol, but not corticosterone, out of the brain (30). Moreover, in rainbow trout, two GRs are present, and both GRs bind cortisol, the primary circulating GC in fish. However, only one GR (rainbow trout GR 2, rtGR2) binds 11-deoxycorticosterone and corticosterone, indicating that multiple GRs with differential affinities to corticosterone and cortisol can exist within a single organism (5).

2.4.4 Local versus systemic signals

Prolonged high levels of GCs in the circulation can suppress body growth (20, 55), bone deposition, digestion, immune function (57), and feather growth (54). Low systemic GC levels in developing animals could be beneficial by minimizing the negative effects of high circulating GCs (37, 56, 60). Local GC production may provide GCs where they are needed, while avoiding the “costs” of high circulating GCs.

A similar hypothesis states that circulating testosterone (T) levels are low in songbirds during the non-breeding season to avoid the costs of high circulating T (65, 75). In non-breeding songbirds, sex steroids can be locally synthesized in the brain, in order to support the expression of aggression for defense of winter territories (66).

2.4.5 Extra-adrenal GC production

In addition to the immune system, evidence suggests extra-adrenal production of GCs in many organ systems (11). The genes for GC-synthetic enzymes are expressed in the intestinal mucosa (7), heart (32), fetal lung (51), and skin (62).

Interestingly, corticotropin releasing hormone (CRH) and ACTH are also locally expressed in the immune system, skin, and other tissues, potentially providing a mechanism for regulating local GC synthesis. In the immune system, ACTH is expressed in lymphocytes (4), and injection of a virus into hypophysectomized mice increases ACTH-immunoreactive lymphocytes in the spleen (63). CRH is also expressed in the thymus and spleen (2, 47). In the skin, there is evidence for local expression of CRH, CRH receptor, ACTH, ACTH receptor, and all the GC-synthetic enzymes. This has been described as a “miniature HPA axis” or “HPA axis homolog” in the skin (48, 62, 79). Future studies will examine CRH and ACTH expression in bursa, thymus and spleen and the effects of immune challenges.

2.4.6 Perspectives and significance

The present data indicate that cortisol is present in the general circulation, immune system and nervous system of developing zebra finches. Cortisol concentrations can be equal to or greater than corticosterone concentrations. Further, circulating GC levels may not be indicative of local GC levels. Lastly, the ratio of corticosterone to cortisol is organ-specific and age-specific. Our results support the hypothesis that cortisol is an “immunosteroid” synthesized *de novo* from cholesterol in the developing avian immune system. Importantly, our results suggest that the identity of locally produced steroids may differ from the identity of systemic steroids. Future studies should measure mRNA for enzymes involved in GC

synthesis in the developing songbird immune system and confirm high cortisol levels in the immune system using LCMS or GCMS.

Table 2.1 Specificity of immunoassay antibodies

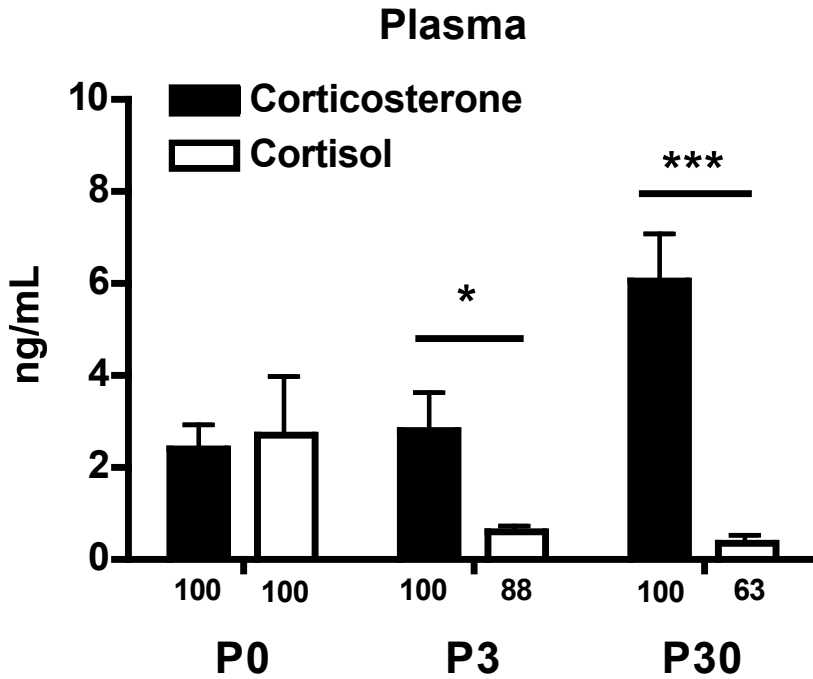
Steroid	Corticosterone Antibody	Cortisol Antibody
Corticosterone	100%	0.20%
Cortisol	0.05%	100%
11-Deoxycorticosterone	0.34%	2.24%
11-Deoxycortisol	<0.30%	0.16%
Dehydrocorticosterone	0.50%	2.03%
Cortisone	<0.30%	0.13%

Table 2.2 Plasma glucocorticoid levels subtracted from tissue glucocorticoid levels

Age	P0		P3		P30	
	Corticosterone	Cortisol	Corticosterone	Cortisol	Corticosterone	Cortisol
Bursa	2.49 ± 4.72	46.27 ± 14.23*	-1.69 ± 1.72	15.63 ± 4.79*	-5.64 ± 0.99	2.68 ± 0.82*
Thymus	-	-	-	-	<i>-5.13 ± 0.59*</i>	2.66 ± 0.70*
Spleen	-	-	-	-	<i>-3.55 ± 0.99*</i>	5.92 ± 1.43*
RTel	<i>-2.04 ± 0.42*</i>	-0.95 ± 1.10	<i>-2.35 ± 0.53*</i>	-0.02 ± 0.35	<i>-4.70 ± 0.82*</i>	-0.10 ± 0.14
CTel	<i>-0.58 ± 1.69*</i>	8.51 ± 6.60	<i>-2.79 ± 0.79*</i>	-0.05 ± 0.26	<i>-4.61 ± 0.77*</i>	0.93 ± 0.20
Cerebellum	<i>-2.42 ± 0.51*</i>	-1.81 ± 1.56	<i>-2.79 ± 0.79*</i>	0.74 ± 0.69	<i>-5.11 ± 0.85*</i>	-0.05 ± 0.19

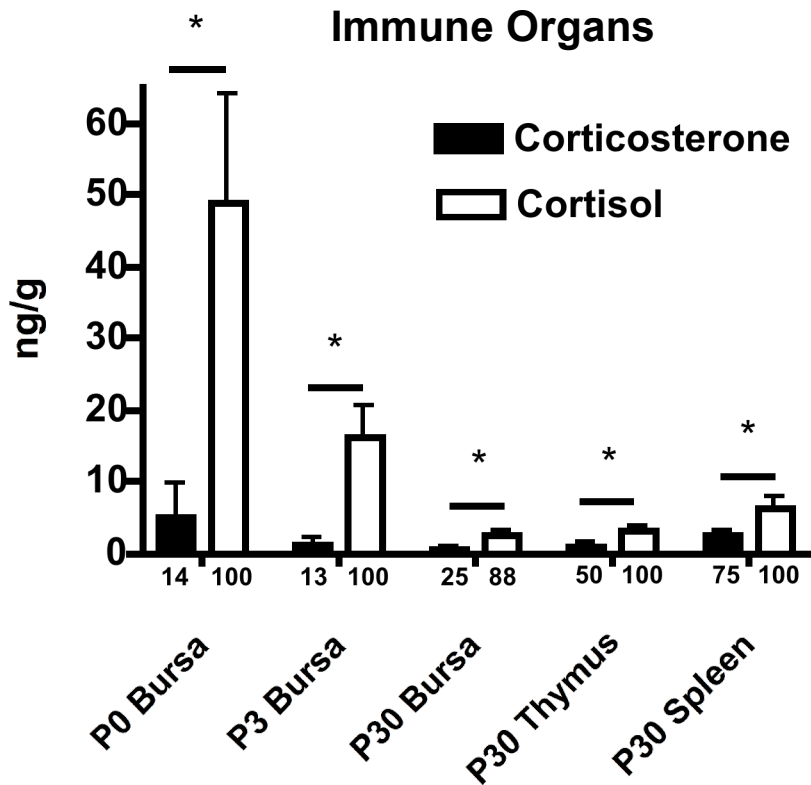
Note: Dashes (-) indicate where data are not available. rTel = rostral telencephalon; cTel = caudal telencephalon. Asterisk indicates a significant difference between plasma GC levels and tissue GC levels. **Bolded** values indicate that tissue levels were significantly higher than plasma levels. *Italicized* values indicate that tissue levels were significantly lower than plasma levels.

Figure 2.1 Plasma levels of corticosterone and cortisol in zebra finches



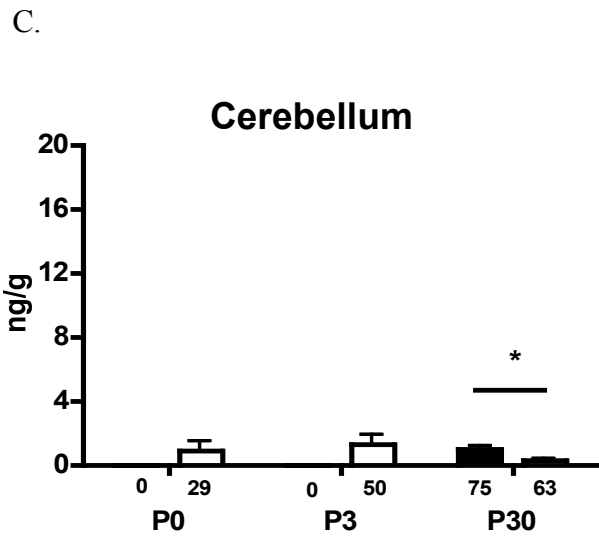
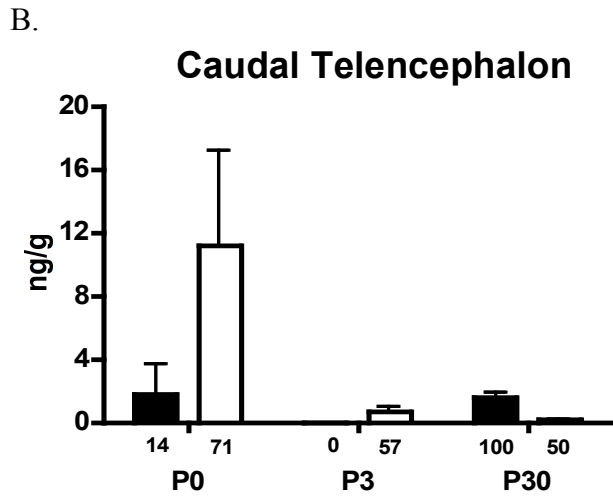
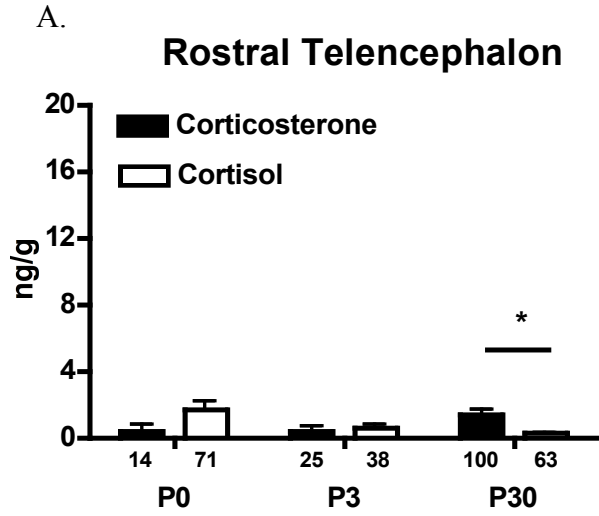
Plasma levels of corticosterone and cortisol in zebra finch chicks on the day of hatch (P0), P3, and P30. Plasma corticosterone and cortisol levels are similar at P0. In contrast, plasma corticosterone levels are significantly higher than plasma cortisol levels at P3 and P30. Numbers along the X axis, below the vertical bars, indicate the percentage of detectable samples. Nondetectable samples were set to zero. Data are means \pm SEM. * $p < 0.05$, *** $p < 0.001$

Figure 2.2 Corticosterone and cortisol levels in immune organs of zebra finches



Levels of corticosterone and cortisol in immune organs of zebra finches on the day of hatch (P0), P3, and P30. In the bursa of Fabricius (bursa), cortisol levels were higher than corticosterone levels at all three ages. Glucocorticoid levels in the bursa were highest at P0 and decreased with age. In the thymus and spleen, cortisol was also the predominant glucocorticoid at P30. Numbers along the X axis, below the vertical bars, indicate the percentage of detectable samples. Nondetectable samples were set to zero. Data are means \pm SEM. * $p < 0.05$

Figure 2.3 Corticosterone and cortisol levels in the brain of zebra finches



Corticosterone and cortisol levels in the brain of zebra finches on the day of hatch (P0), P3, and P30. (A) In the rostral telencephalon, corticosterone and cortisol levels were similar at P0 and P3, but corticosterone was the predominant glucocorticoid at P30. (B) In the caudal telencephalon, corticosterone and cortisol levels were not significantly different at any age. (C) In the cerebellum, corticosterone and cortisol levels were similar at P0 and P3, but corticosterone was the predominant glucocorticoid at P30. Numbers along the X axis, below the vertical bars, indicate the percentage of detectable samples. Nondetectable samples were set to zero. Data are mean \pm SEM. * $p < 0.05$

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3. Cortisol and corticosterone in immune organs and brain of European starlings: developmental changes, effects of restraint stress, comparison with zebra finches³

3.1 Introduction

Glucocorticoids (GC) regulate the development of many physiological systems, including the immune and nervous systems. For example, GCs regulate lymphocyte selection in the thymus (11). High GC levels can induce thymocyte apoptosis, whereas intermediate GC levels decrease thymocyte apoptosis by antagonizing T cell receptor signaling (45). GCs also regulate brain development, including neurotransmitter systems (16), cell birth in the hippocampus (10), and glucocorticoid receptor expression in the hypothalamus (18).

Traditionally, GCs have been thought to be synthesized only in the adrenal glands. However, GCs also can be synthesized in extra-adrenal tissues, including the immune system and brain (6, 37). For example, mouse thymic epithelial cells produce pregnenolone and deoxycorticosterone from 22R-hydroxycholesterol (46), and mouse thymocytes express all the glucocorticoid-synthetic enzymes (32). In chickens, *in vitro* studies suggest that the thymus and bursa of Fabricius (hereafter bursa) express all the enzymes necessary to synthesize cortisol from cholesterol, in contrast to the adrenal glands, which synthesize corticosterone (14). In developing zebra finches (*Taeniopygia guttata*), cortisol levels in immune organs are higher than cortisol levels in plasma, also suggesting local cortisol synthesis (38). Moreover, in immune organs, cortisol levels are higher than corticosterone levels (38). In mice and birds, immune GC synthesis declines with age, as adrenal GC synthesis begins to increase (46, 38).

³ A version of this chapter has been submitted for publication. Schmidt KL, Chin EH, Shah AH, Soma KK Cortisol and corticosterone in immune organs and brain of European starlings: developmental changes, effects of restraint stress, comparison with zebra finches.

In the brain, mRNAs for the steroidogenic enzymes P450_{scc} and P450_{c11β} have been detected in the cortex, amygdala, hippocampus, and cerebellum of developing and adult rats (26, 44, 21). Expression of P450_{c11β} mRNA in the rat brain increases in response to ACTH treatment and adrenalectomy (49). Cultures of rat brain tissue convert 11-deoxycorticosterone to corticosterone, demonstrating P450_{c11β} activity (9, 22). The mRNA for P450_{c21}, which converts progesterone and 17-hydroxyprogesterone to 11-deoxycorticosterone and 11-deoxycortisol, respectively, has been detected in the brains of mice (43) and humans (1). Also, corticosterone may be synthesized in the songbird brain in response to restraint during molt, when circulating levels of corticosterone are low (30). Although it is evident that GCs are produced locally in extra-adrenal tissues, the regulation of local GC synthesis, particularly by stress, remains unclear.

It is also unclear how local GC synthesis varies among species. With over 4000 species, songbirds provide an excellent opportunity for comparative studies. For example, species differences in local GC synthesis may be related to life history strategies, such as “pace of life,” which refers to the rate of reproduction, age at maturity, and longevity of a species (33). Zebra finches exhibit a fast pace of life, and high levels of GCs in the immune system may suppress development of the costly adaptive immune system, thus shunting resources to rapid reproductive development. In species with a slower pace of life, such as the European starling (*Sturnus vulgaris*), there may be a greater investment in adaptive immunity to aid in long-term survival (15, 23).

Here, in Study 1, we determined endogenous corticosterone and cortisol levels in plasma, immune organs and brain of wild European starlings at post-hatch day 0 (P0) and P10 and effects of acute restraint stress. Starlings are an excellent animal model because they

can be readily studied in the field under natural conditions. Furthermore, their plasma corticosterone levels have been well-characterized (35, 19, 7). Songbirds are altricial and are hyporesponsive to stressors during the first week of life. During this time, plasma corticosterone levels are low and show a slight, or no increase in response to restraint (40, 47). However, only one study has measured GC levels in tissues after restraint during development to see if local GC levels follow the same developmental profile (2). In Study 2, we compared corticosterone and cortisol levels in the immune system and brain of starlings and zebra finches. In total, 462 plasma and tissue samples from 70 animals were analyzed for corticosterone and cortisol.

3.2 Materials and methods

3.2.1 Study 1: Effects of acute restraint stress on GC levels in developing European starlings

3.2.1.1 Subjects and field sites

Research was carried out under a University of British Columbia (UBC) permit, following the guidelines of the Canadian Council on Animal Care. Subjects were wild male and female European starling nestlings sampled at P0 (n = 9 baseline and 9 stressed) or P10 (n = 11 baseline and 11 stressed). At P0, chicks' eyes are closed and their ability to thermoregulate and move around the nest is minimal (3). By P10, chicks' eyes are open and their ability to thermoregulate and move around the nest is increasing (3). At P0 and P10, half of the subjects were sampled within 3 min of disturbance to examine baseline levels of GCs (34) and half were sampled after 45 min of restraint stress, which induces maximal plasma corticosterone levels in adult starlings (35). For restraint, chicks were placed in an

opaque cloth bag and kept warm. Samples were collected from the Davistead Dairy Farm in Langley, British Columbia and from the UBC Farm in Vancouver in 2006 and 2007. At both field sites, wooden nest boxes were mounted on posts or buildings. Nest boxes were checked daily to determine clutch initiation and completion as well as hatch dates (4).

3.2.1.2 Sample collection

Subjects were sampled between 12:00 h and 16:00 h to minimize possible diel changes in GCs. Two nestlings were taken from the same nest at the same time. One nestling (randomly chosen) was sampled immediately and the other was sampled after 45 min of restraint. First, a blood sample was collected via cardiac puncture with a heparinized syringe (2.03 ± 0.10 min or 46.99 ± 0.23 min after disturbance). Trunk blood was also collected at sacrifice, and cardiac and trunk blood were pooled. Blood was kept on ice in the field and centrifuged at 10,000 rpm for 10 min once transported back to the lab. Plasma was collected with a Hamilton syringe and stored at -20°C until analysis.

Immediately after cardiac puncture, birds were rapidly decapitated (2.40 ± 0.10 min or 47.38 ± 0.23 min after disturbance). The brains and bodies were immediately chilled on ice for 5 min. Brains were dissected first, and regions collected include the cerebellum, rostral telencephalon (rTel), caudal telencephalon (cTel), dorsal telencephalon (dTel, in P10 birds only), diencephalon, and optic lobes. First, the cerebellum was collected. Next, the telencephalon was dissected. To dissect out the dTel in P10 birds, cuts were made on the telencephalon in the following places: halfway between rostral and caudal edges, halfway between dorsal and ventral edges, and halfway between the midline and lateral edges. The dTel contained the song nuclei HVC and NCM as well as the hippocampus. To dissect out

the rTel, after the dTel was collected, tissue from the rostral edge to halfway between the rostral and caudal edges was collected. To dissect out the cTel, tissue from halfway between the rostral and caudal edges to the caudal edge of the brain was collected. In P0 birds, the telencephalon was dissected into rTel and cTel only. Next the optic lobes were collected. Lastly, the diencephalon was dissected to the depth of the anterior commissure. The body was dissected next. Peripheral tissues collected include the bursa, thymus, and spleen. All tissues were immediately frozen with liquid nitrogen or dry ice in the field. After transport to the lab, tissues were stored at -80°C until analysis.

3.2.1.3 Steroid extraction

Steroids were extracted from tissue and plasma using solid phase extraction (SPE) with C18 columns as previously described (29, 38, 4). This extraction procedure results in high and consistent steroid recoveries and effectively removes interfering substances from lipid-rich samples (29). All samples from P0 birds were extracted and assayed together and all samples from P10 birds were extracted and assayed together. Here, steroid recovery was determined by spiking plasma and tissue pools with 60 pg of corticosterone and 150 pg of cortisol and comparing spiked samples to unspiked samples (n=3 spiked and unspiked pools). For P0 samples, recovery of corticosterone was 94% in plasma and 95% in tissue, and recovery of cortisol was 110% in plasma and 105% in tissue. For P10 samples, recovery of corticosterone was 88% in plasma and 76% in tissue, and recovery of cortisol was 96% in plasma and 84% in tissue. Samples were corrected for recovery where applicable. Samples were resuspended in 70 µL of phosphate-buffered diluent provided with the cortisol enzyme immunoassay (EIA) and 5% absolute ethanol.

3.2.1.4 Corticosterone radioimmunoassay

Of the initial 70 μL resuspension, 20 μL was used for the corticosterone assay (29% of the sample). This 20 μL was brought up to 100 μL with the phosphate-buffered diluent provided with the corticosterone radioimmunoassay (RIA), and 50 μL was assayed in duplicate for corticosterone. Average coefficient of variation between duplicates was 1.29% for the P0 assays and 1.37% for the P10 assays. Final concentration of ethanol in the RIA was 1%, which does not affect this assay (29, 38).

Levels of corticosterone were determined using a sensitive and specific double-antibody ^{125}I -RIA (MP Biomedicals, 07-120103) that has been validated for songbird plasma and tissue samples (29, 38). The detection limit was 3.12 pg corticosterone per tube. The corticosterone antibody is highly specific and has a low cross-reactivity to cortisol (0.05%) and the precursors (11-deoxycorticosterone = 0.34%; 11-deoxycortisol < 0.30%) and metabolites (dehydrocorticosterone = 0.50%; cortisone < 0.30%) of corticosterone and cortisol (38).

Water blanks and controls were analyzed in each assay. For the P0 assays, five of the six extracted water blanks were nondetectable (<3.12 pg corticosterone) and one was slightly above the detection limit and for the P10 assays all six water blanks were nondetectable for corticosterone. A low and high control provided with the RIA kit were analyzed in each assay. For the P0 assays, inter-assay coefficient of variation was 11.70% for the low control and 7.28% for the high control. For the P10 assays, inter-assay coefficient of variation was 10.36% for the low control and 7.64% for the high control.

3.2.1.5 Cortisol enzyme immunoassay

Levels of cortisol were determined using a sensitive and specific EIA (Salimetrics, 1-3012). The detection limit was 3 pg cortisol per well. The cortisol antibody is highly specific and has a low cross-reactivity to corticosterone (0.20%) and the precursors (11-deoxycorticosterone = 2.24%; 11-deoxycortisol = 0.16%) and metabolites (dehydrocorticosterone = 2.03%; cortisone = 0.13%) of corticosterone and cortisol (38). The remaining 50 μ L of resuspension (71% of the sample) was brought up to 70 μ L with the phosphate-buffered diluent provided with the cortisol EIA, and 25 μ L was assayed in duplicate for cortisol. Average coefficient of variation between duplicates was 1.54% for the P0 assays and 2.50% for the P10 assays. Final concentration of ethanol in the EIA was 3.6%, which does not affect this assay (38). A plate washer was used to ensure that wells were washed consistently (Tecan Columbus washer: I 109011). Absorbance was measured using a plate reader (Sunrise remote: f039300) at 450 nm, with a 620 nm background correction.

Water blanks and controls were analyzed in each assay. For the P0 assays five of the six extracted water blanks were non-detectable (<3 pg cortisol) and one was slightly above the detection limit and for the P10 assays all six water blanks were nondetectable for cortisol. A 25 pg control was analyzed in each assay (P0 assays = 26.33 ± 0.43 pg; P10 assays = 23.06 ± 0.67 pg). Interassay coefficient of variation was 3.66% for the P0 assays and 8.19% for the P10 assays.

3.2.1.6 Statistics

Nondetectable samples (below the lowest standard on the standard curve) were conservatively set to zero for both assays (38). Data were analyzed in SPSS (v. 11 for Mac

OS X).

Data were transformed ($\log(x+1)$) to achieve homogeneity of variance. At each age, data were first analyzed with mixed-design three-way analysis of variance (ANOVA) tests on each tissue and plasma, with Stress (baseline vs. stressed) and Sex as between-subjects variables and Steroid (corticosterone vs. cortisol) as a within-subjects variable. There were no sex differences (as in 47, 38), and data from males and females were combined and two-way ANOVAs (Stress x Steroid) were run on each tissue and plasma.

At each age, steroid levels in tissues were compared to steroid levels in plasma (as in 5, 38). Mixed-design two-way ANOVAs were used, with Stress as a between-subjects variable and Sample (tissue vs. plasma) as a within-subjects variable. Since the effect of Stress in this analysis partially overlaps with the preceding analysis (see above), only the effect of Sample is reported. Test results were considered significant for $p \leq 0.05$. Results are presented as mean \pm standard error of the mean (SEM).

3.2.2 Study 2: GC levels in developing European starlings and zebra finches

3.2.2.1 Subjects

Samples from wild P4 European starlings were collected from the UBC Farm in Spring 2007. Samples were also collected from captive P4 zebra finches. Breeding pairs of adult zebra finches were given millet seeds, water, grit, and cuttlefish bone *ad libitum*. Breeding pairs were also given a food supplement consisting of boiled chicken eggs, cornmeal and bread daily. The light cycle was 14L:10D (lights on at 08:00). Temperature was held at $\sim 23^{\circ}\text{C}$ and relative humidity at $\sim 50\%$. Both starlings and zebra finches typically lay 4-6 eggs per clutch and incubate eggs for ~ 10 days (3, 50). Nestlings of both species

fledge the nest at ~P20 (3, 50). In this study, male and female starlings (n = 10) and zebra finches (n = 20) were sampled at P4 within 3 min of disturbance. For zebra finches, plasma and tissue samples from two individuals of the same sex were pooled to increase the chance of reaching detectability in the immunoassays.

3.2.2.2 Sample collection

Procedures were exactly the same as in Study 1. Subjects were sampled between 12:00 h and 16:00 h. A blood sample was collected via cardiac puncture (within 3 min), and pooled with trunk blood collected at sacrifice. Plasma was collected and stored at -20°C. After collection of cardiac blood, nestlings were rapidly decapitated (within 3 min of disturbance). The brain and body were chilled on ice for 5 min. The brain was dissected first. The telencephalon was bisected into rostral and caudal sections. The rTel and cTel were further divided into left and right hemispheres. Only the rTel was used in this study. We then collected the bursa and spleen. Thymus could not be collected in zebra finches at this age.

3.2.2.3 Steroid extraction and immunoassays

Procedures were exactly the same as in Study 1. Recovery of corticosterone was 84% in plasma and 95% in tissue. Recovery of cortisol was 89% in plasma and 90% in tissue. Samples were corrected for recovery where applicable. For corticosterone, all extracted water blanks (n = 5) were nondetectable (<3.12 pg corticosterone). For cortisol, four of the five extracted water blanks were nondetectable (< 3 pg cortisol) and one was slightly above the detection limit. We included a 10 pg and a 83 pg control in each cortisol assay (n = 5 each).

We obtained on average 8.48 ± 0.87 for the 10 pg control and 83.69 ± 3.69 pg for the 83 pg control.

3.2.2.4 Statistics

Local GC levels in starlings and zebra finches were directly compared. Within each subject, plasma corticosterone levels were subtracted from tissue corticosterone levels, and plasma cortisol levels were subtracted from tissue cortisol levels (referred to as “difference scores”). Difference scores were used because starlings and zebra finches have different systemic GC levels, and we wanted to compare local GC levels in tissues, irrespective of plasma GC levels. A negative difference score indicates that plasma corticosterone or cortisol levels were higher than tissue corticosterone or cortisol levels, respectively. A positive difference score indicates the opposite. Difference scores were analyzed in a three-way ANOVA for each tissue, with Species and Sex as between-subjects variables and Steroid (cortisol vs. corticosterone) as a within-subjects variable. There were no sex differences, and data from males and females were pooled and two-way ANOVAs (Species x Steroid) were run for each tissue.

3.3 Results

3.3.1 Study 1: Effects of acute restraint stress on GC levels in developing European starlings

3.3.1.1 Post-hatch day 0 (P0)

P0 plasma

In plasma (Fig 3.1a and 3.1d), there was a significant main effect of Steroid ($F_{1,16} = 199.25$, $p < 0.001$) (corticosterone > cortisol). The main effect of Stress was not significant ($F_{1,16} = 0.07$, $p = 0.79$) nor was the interaction between Steroid and Stress ($F_{1,16} = 0.24$, $p = 0.63$).

P0 immune organs

In the bursa (Fig 3.1b and 3.1e), there was a main effect of Steroid ($F_{1,16} = 21.82$, $p < 0.001$) (corticosterone > cortisol). The main effect of Stress was not significant ($F_{1,16} = 0.22$, $p = 0.64$); however, the percent of samples detectable for corticosterone and cortisol tended to increase with restraint (Fig 3.1b and 3.1e). The interaction between Steroid and Stress was not significant ($F_{1,16} = 0.32$, $p = 0.58$). Corticosterone levels in the bursa were lower than corticosterone levels in the plasma ($F_{1,16} = 8.87$, $p = 0.01$). Similarly, cortisol levels in the bursa were lower than cortisol levels in the plasma ($F_{1,16} = 20.12$, $p = 0.001$).

In the thymus (Fig 3.1b and 3.1e), there was a main effect of Steroid ($F_{1,16} = 14.94$, $p = 0.002$) (corticosterone > cortisol). The main effect of Stress was not significant ($F_{1,16} = 1.27$, $p = 0.28$), but the percent of samples detectable for corticosterone and cortisol tended to increase with restraint (Fig 3.1b and 3.1e). The interaction between Steroid and Stress was not significant ($F_{1,16} = 0.15$, $p = 0.70$). There were no significant differences between corticosterone levels in the thymus and plasma ($F_{1,16} = 2.05$, $p = 0.18$) or between cortisol levels in the thymus and plasma ($F_{1,16} = 0.55$, $p = 0.47$).

In the spleen (Fig 1b and 1e), there was also a main effect of Steroid ($F_{1,16} = 13.10$, $p = 0.003$) (corticosterone > cortisol). The main effect of Stress was not significant ($F_{1,16} = 0.90$, $p = 0.36$). However, similar to the bursa and thymus, the percent of samples detectable

for corticosterone and cortisol tended to increase with restraint (Fig 3.1b and 3.1e). The interaction between Steroid and Stress was not significant ($F_{1,16} = 0.15$, $p = 0.70$). There were no significant differences between corticosterone levels in the spleen and plasma ($F_{1,16} = 0.80$, $p = 0.39$) or between cortisol levels in the spleen and plasma ($F_{1,16} = 2.28$, $p = 0.15$).

P0 brain tissue

For all brain regions, there was a main effect of Steroid (Table 3.1, Fig 3.1c and 3.1f) (corticosterone > cortisol). The main effect of Stress was not significant in any of the brain regions, nor was the interaction between Steroid and Stress (Table 3.1, Fig 3.1c and 3.1f). Corticosterone levels in all brain regions were lower than corticosterone levels in the plasma (Table 3.2). Similarly, cortisol levels in all brain regions were lower than cortisol levels in the plasma (Table 3.2).

3.3.1.2 Post-hatch day 10 (P10)

P10 plasma

At P10, in plasma (Fig 3.2a and 3.2d) there was a significant main effect of Steroid ($F_{1,20} = 250.07$, $p < 0.001$) (corticosterone > cortisol). The main effect of Stress was also significant ($F_{1,20} = 5.17$, $p = 0.04$). The interaction between Steroid and Stress was not significant ($F_{1,20} = 3.34$, $p = 0.08$).

P10 immune organs

In the bursa (Fig 3.2b and 3.2e), there was a main effect of Steroid ($F_{1,20} = 121.32$, $p < 0.001$). The main effect of Stress was not significant ($F_{1,20} = 1.58$, $p = 0.27$). There was a

significant interaction between Steroid and Stress ($F_{1,20} = 26.59$, $p < 0.001$). Restraint increased bursal corticosterone levels but not bursal cortisol levels. Corticosterone levels in the bursa were lower than corticosterone levels in the plasma ($F_{1,20} = 67.91$, $p < 0.001$). Cortisol levels in the bursa did not differ significantly from cortisol levels in the plasma ($F_{1,20} = 0.82$, $p = 0.38$).

In the thymus (Fig 3.2b and 3.2e), there was a main effect of Steroid ($F_{1,20} = 374.52$, $p < 0.001$). The main effect of Stress was also significant ($F_{1,20} = 25.04$, $p < 0.001$). Moreover, the interaction between Steroid and Stress was significant ($F_{1,20} = 26.10$, $p < 0.001$). The effect of restraint was greater on thymic corticosterone levels than thymic cortisol levels. Corticosterone levels in the thymus were lower than corticosterone levels in the plasma ($F_{1,20} = 19.07$, $p < 0.001$). Similarly, cortisol levels in the thymus were lower than cortisol levels in the plasma ($F_{1,20} = 6.15$, $p = 0.02$).

In the spleen (Fig 3.2b and 3.2e), there was a main effect of Steroid ($F_{1,20} = 20.03$, $p < 0.001$). The main effect of Stress was not significant ($F_{1,20} = 1.44$, $p = 0.24$). There was a significant interaction between Steroid and Stress ($F_{1,20} = 10.47$, $p = 0.01$). Restraint increased splenic corticosterone levels but not splenic cortisol levels. Corticosterone levels in the spleen were lower than corticosterone levels in the plasma ($F_{1,20} = 19.07$, $p < 0.001$). Cortisol levels in the spleen did not differ significantly from cortisol levels in the plasma ($F_{1,20} = 0.94$, $p = 0.35$).

P10 brain tissue

For all brain regions (Fig 3.2c and 3.2f), except the diencephalon, the interaction between Steroid and Stress was significant (Table 3.3). In all cases restraint increased

corticosterone levels but not cortisol levels. In the diencephalon, the main effect of stress was significant indicating that restraint increased both corticosterone and cortisol in this region (Fig 3.2c and 3.2f, Table 3.3). The main effect of Steroid was significant in all brain regions (Table 3.3). Corticosterone levels in all brain regions were lower than corticosterone levels in the plasma (Table 3.4). In contrast, cortisol levels in all brain regions did not differ significantly from cortisol levels in the plasma (Table 3.4).

3.3.2 Study 2: GC levels in developing European starlings and zebra finches

In order to compare local GC levels in starlings and zebra finches, we subtracted plasma corticosterone and cortisol levels from tissue corticosterone and cortisol levels (within each subject) to create “difference scores” (Fig 3.3a and 3.3b). We then compared the difference scores between starlings and zebra finches for each tissue.

In the bursa, there was a significant main effect of Steroid ($F_{1,18} = 11.60$, $p = 0.004$). The difference scores were lower for corticosterone than cortisol (i.e., relative to plasma, bursal corticosterone levels are lower than bursal cortisol levels). There was also a main effect of Species ($F_{1,18} = 13.47$, $p = 0.002$); difference scores were lower for starlings than zebra finches. The interaction between Steroid and Species was not significant ($F_{1,18} = 2.49$, $p = 0.13$).

In the spleen, there was also a significant main effect of Steroid ($F_{1,18} = 10.78$, $p = 0.005$). Difference scores were lower for corticosterone than cortisol. There was a trend for an effect of Species ($F_{1,18} = 3.31$, $p = 0.09$); difference scores tended to be lower for starlings. The interaction between Steroid and Species was not significant ($F_{1,18} = 0.40$, $p = 0.54$).

In the rTel, there was a significant main effect of Steroid ($F_{1,18} = 14.05$, $p = 0.002$). The difference scores were lower for corticosterone. The effect of Species was not significant ($F_{1,18} = 2.80$, $p = 0.12$) and there was a trend for an interaction between Steroid and Species ($F_{1,18} = 3.41$, $p = 0.09$).

3.4 Discussion

Our results reveal differential regulation of local and systemic GCs by acute restraint stress, as well as developmental and species differences. First, restraint had no significant effects on GCs in P0 starlings but did in P10 starlings. Second, in P10 starlings, local corticosterone levels were lower than systemic corticosterone levels, but local cortisol levels did not differ from systemic cortisol levels (except in thymus). Third, local GC levels (after correcting for systemic GC levels) differed between wild starlings and captive zebra finches, raising the possibility of a species difference or effect of captivity. This is the first study to measure immune GC levels in wild animals exposed to parasites under natural conditions.

3.4.1 P0 starlings

At P0, corticosterone was the predominant GC in the plasma of starlings. Corticosterone was also the predominant GC in immune and brain tissues. In contrast, previous studies suggest that cortisol, not corticosterone, is synthesized in the developing immune system of chickens and zebra finches (14, 38). The data suggest a species difference in cortisol synthesis in the immune system.

At P0, restraint stress (45 min) had no effect on plasma corticosterone or cortisol levels. Similarly, other altricial animals experience a stress hyporesponsive period, in which

systemic GC levels show little response to restraint (36, 17, 47). However, it has been unclear whether restraint affects local GC levels during this period. If GCs are produced locally, restraint could increase GC levels in tissues but not in plasma (2). Here, restraint did not significantly increase corticosterone or cortisol levels in any of the tissues at P0. However, both corticosterone and cortisol levels showed a trend to increase after restraint in the bursa, thymus and spleen. This trend was not observed in brain tissue. Interestingly, ACTH increases pregnenolone synthesis in thymic cells (46). Thus, it is possible that GC synthesis by immune cells increases with restraint. Future studies should examine local GC levels after other time points of restraint (e.g., 15, 120 min) and the effects of immune stressors (e.g., lipopolysaccharide, keyhole limpet hemocyanin).

We also directly compared local and systemic GC levels at P0. Both corticosterone and cortisol levels were lower in the brain and bursa than in the plasma. In contrast, corticosterone and cortisol levels in the thymus and spleen did not differ from levels in the plasma. These data suggest tissue-specific regulation of GC levels, which could be achieved by several mechanisms. First, 11 β -hydroxysteroid dehydrogenase (11 β -HSD) is a key regulator of local GC levels (39). One isoform (11 β -HSD2) inactivates corticosterone and cortisol, and another isoform (11 β -HSD1) regenerates corticosterone and cortisol from their inactive metabolites (13). Second, multidrug resistance P-glycoprotein (Pgp) actively transports GCs and is present in the brain (12) and immune tissues (31). Third, high expression of glucocorticoid receptors (GRs) or CBG might cause high local GC levels in specific tissues (37). Lastly, differential local GC synthesis could produce tissue-specific GC levels. These mechanisms could keep GC levels elevated where they are required and keep GC levels low where they are detrimental. For example, both early life stress and

corticosterone treatment can have long-term detrimental effects on brain development (20) and cognition and social behavior (41).

3.4.2 P10 starlings

At P10, corticosterone was again the predominant GC in the plasma, immune tissues and brain tissues. In contrast, in developing zebra finches, cortisol is the most abundant GC in immune tissues (38).

In contrast to P0 starlings, restraint significantly increased plasma corticosterone and cortisol levels in P10 starlings. Similarly, in other songbirds, the stress hyporesponsive period only lasts for the first week of life (47, 40). Restraint also increased corticosterone in all the immune and brain tissues studied. In contrast, restraint increased cortisol only in the thymus and diencephalon. It is possible that cortisol is synthesized in the thymus and diencephalon in response to restraint. In support of this, expression of P450c11 β mRNA in the hypothalamus increases with ACTH treatment (49).

Corticosterone levels in immune and brain tissues were lower than corticosterone levels in the plasma. In contrast, cortisol levels in immune and brain tissues did not differ significantly from cortisol levels in the plasma (except for the thymus). These data suggest differential regulation of corticosterone and cortisol in tissues. As mentioned above, this could be achieved by several mechanisms.

3.4.3 Local GC levels in starlings and zebra finches

In Study 2, we directly compared local corticosterone and cortisol levels in wild P4 European starlings and captive P4 zebra finches. In zebra finches, cortisol levels in the bursa

and spleen were higher than cortisol levels in the plasma, suggesting that the immune system of developing zebra finches synthesizes cortisol (38). GC “difference scores” (tissue level – plasma level) were lower in starlings than zebra finches in the bursa, with a similar trend in the spleen. These data suggest a difference between starlings and zebra finches in local GC synthesis or other factors (e.g., 11β -HSD, Pgp).

Differences in immunosteroid levels may be due to differences in life history strategy. Zebra finches exhibit a fast pace of life and reach sexual maturity by ~3 months of age (50). Starlings exhibit a slower pace of life and begin breeding at ~10 months of age (8). The life history strategy of zebra finches may favor reduced investment into costly adaptive immune defenses, with a greater reliance on innate immunity, and greater investment into reproductive development (15, 24). In contrast, starlings may invest more resources into adaptive immunity to increase the chance of long-term survival. Future studies of immunosteroids should compare closely related species in the field that differ in pace of life.

Alternatively, differences in immunosteroid levels may be the result of experiential factors, such as parasite exposure. Early parasite exposure is positively correlated with investment in immune defenses (25, 27). In the wild, nearly all starling nestlings have endoparasites and ectoparasites (3, personal observation). High parasite exposure in starling nestlings may be due to the fact that starlings nest in cavities that have been used previously (3). In contrast, captive zebra finches lay eggs in clean nests that harbor fewer parasites. Future experiments should manipulate parasite exposure and measure immunosteroid levels. Increasing parasite exposure in zebra finches may decrease immunosteroid levels, whereas decreasing parasite exposure in starlings may increase immunosteroid levels.

As an initial step in understanding whether starlings invest more in the adaptive immune system than zebra finches, we calculated the bursa to body weight (B-BW) ratio. B-BW ratio is used as an indicator of immune function in chickens (48, 42). Since we did not have body weights of subjects in Study 2, we divided the P4 bursa weights by the average body weight of P4 starlings (25.48 ± 1.00 g, $n = 37, 4$) and P4 zebra finches (3.15 ± 0.08 g, $n = 86$; A. Shah et al., unpublished data). The B-BW ratio was ~3 times higher in starlings (0.001922 ± 0.000232 , $n = 10$) than in zebra finches (0.000657 ± 0.000029 , $n = 10$; $t(18) = 5.41$, $p < 0.0001$). Similarly, the spleen to body weight ratio was ~2 times higher in starlings (0.001159 ± 0.000152 , $n = 10$) than in zebra finches (0.000595 ± 0.0000745 , $n = 10$; $t(18) = 3.08$, $p = 0.007$). These preliminary data suggest that wild starlings invest more resources into adaptive immunity than captive zebra finches.

3.4.4 Perspectives and significance

These results show a robust difference in the regulation of circulating and local GCs by restraint at P0 and P10. Furthermore, our results suggest that plasma GC levels are not always representative of local GC levels. Determining local GC levels provides important insights into tissue-specific regulation of GCs. Lastly, this is the first study to measure immune GC levels in wild animals under natural conditions. Future comparative studies should examine immune GC levels in tropical vs. temperate birds (27) or colonial vs. non-colonial birds (28) to determine how immunosteroid levels vary with adaptive immunity. Future studies should also determine the effect of other stressors, such as food restriction, during development.

Table 3.1 Effects of Steroid (corticosterone vs. cortisol) and Stress on glucocorticoid levels in the brain of European starlings on the day of hatch.

Variable	Rostral Telencephalon			Caudal Telencephalon			Diencephalon			Cerebellum			Optic Lobe		
	df	F ratio	p	df	F ratio	p	df	F ratio	p	df	F ratio	p	df	F ratio	p
Steroid	1,16	34.13	<0.001	1,16	7.39	0.02	1,16	19.79	0.001	1,16	10.29	0.007	1,16	10.72	0.006
Stress	1,16	0.18	0.68	1,16	0.02	0.88	1,16	0.00	1.00	1,16	0.49	0.50	1,16	4.48	0.053
Steroid x Stress	1,16	0.65	0.43	1,16	0.04	0.85	1,16	0.06	0.82	1,16	0.02	0.90	1,16	0.80	0.39

Note: significant p values are in bold.

Table 3.2 Effect of Sample (plasma vs. tissue) on glucocorticoid levels in the brain of European starlings on the day of hatch.

Variable	Rostral Telencephalon			Caudal Telencephalon			Diencephalon			Cerebellum			Optic Lobe		
	df	F ratio	p	df	F ratio	p	df	F ratio	p	df	F ratio	p	df	F ratio	p
Corticosterone	1,16	22.16	<0.001	1,16	41.47	<0.001	1,16	41.89	<0.001	1,16	13.40	0.003	1,16	43.94	<0.001
Cortisol	1,16	98.72	<0.001	1,16	25.05	<0.001	1,16	63.56	<0.001	1,16	33.86	<0.001	1,16	54.23	<0.001

Note: significant p values are in bold.

Table 3.3 Effects of Steroid (corticosterone vs. cortisol) and Stress on glucocorticoid levels in the brain of European starlings on post-hatch day 10.

Variable	Rostral Telencephalon			Caudal Telencephalon			Dorsal Telencephalon			Diencephalon			Cerebellum			Optic Lobe		
	df	F ratio	p	df	F ratio	p	df	F ratio	p	df	F ratio	p	df	F ratio	p	df	F ratio	p
Steroid	1,20	130.45	<0.001	1,20	54.37	<0.001	1,20	65.89	0.001	1,20	24.05	<0.001	1,20	27.40	<0.001	1,20	45.53	<0.001
Stress	1,20	1.50	0.24	1,20	0.94	0.35	1,20	4.36	0.06	1,20	18.23	<0.001	1,20	4.37	0.06	1,20	2.14	0.16
Steroid x Stress	1,20	9.45	0.007	1,20	6.72	0.02	1,20	9.60	0.006	1,20	1.96	0.18	1,20	6.91	0.02	1,20	6.13	0.02

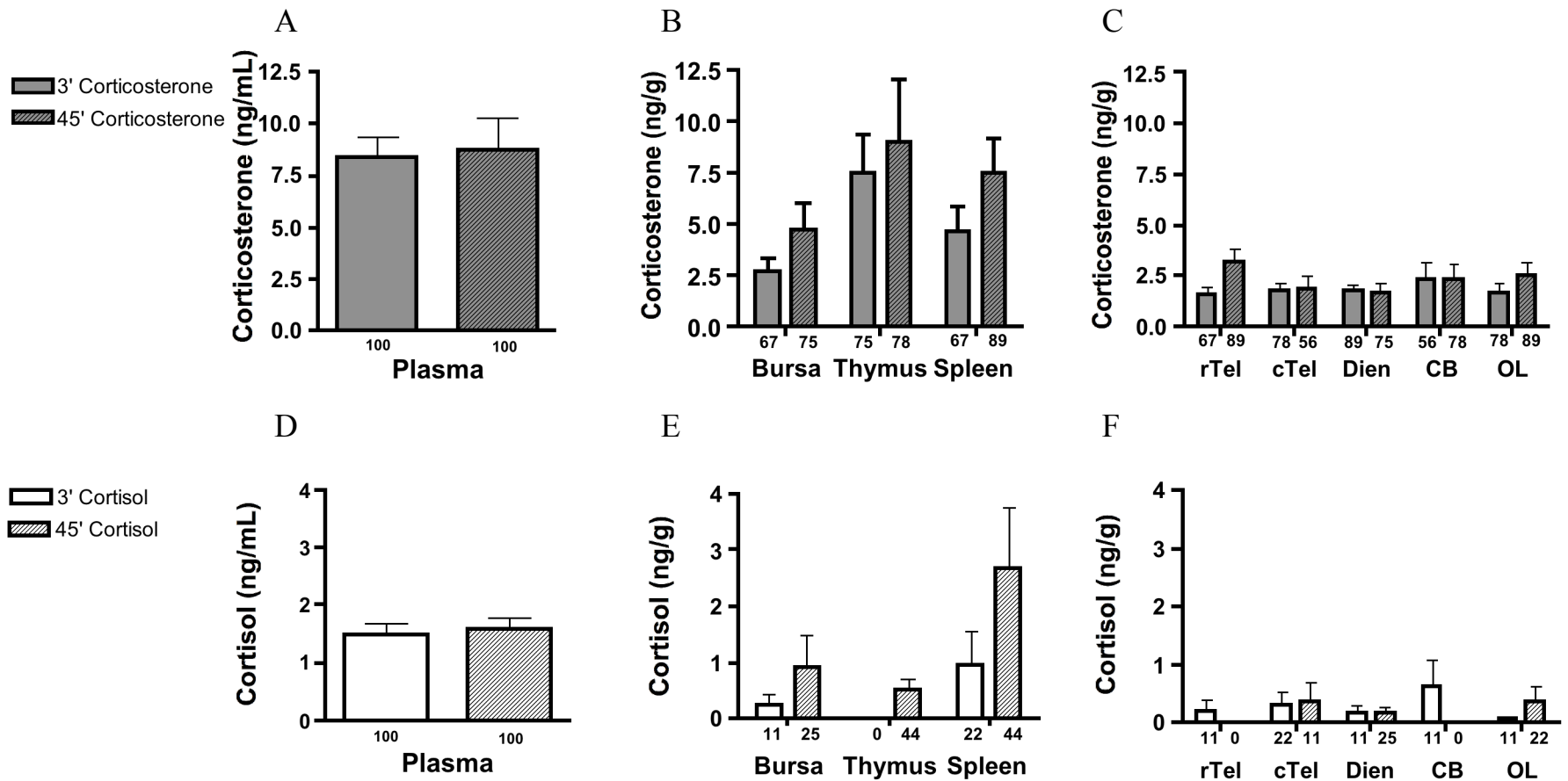
Note: significant p values are in bold.

Table 3.4 Effect of Sample (plasma vs. tissue) on glucocorticoid levels in the brain of European starlings on post-hatch day 10.

Variable	Rostral Telencephalon			Caudal Telencephalon			Dorsal Telencephalon			Diencephalon			Cerebellum			Optic Lobe		
	df	F ratio	p	df	F ratio	p	df	F ratio	p	df	F ratio	p	df	F ratio	p	df	F ratio	p
Corticosterone	1,20	58.25	<0.001	1,20	65.97	<0.001	1,20	78.74	<0.001	1,20	209.62	<0.001	1,20	164.89	<0.001	1,20	107.14	<0.001
Cortisol	1,20	0.02	0.91	1,20	0.30	0.59	1,20	0.02	0.89	1,20	0.20	0.66	1,20	0.03	0.88	1,20	0.44	0.52

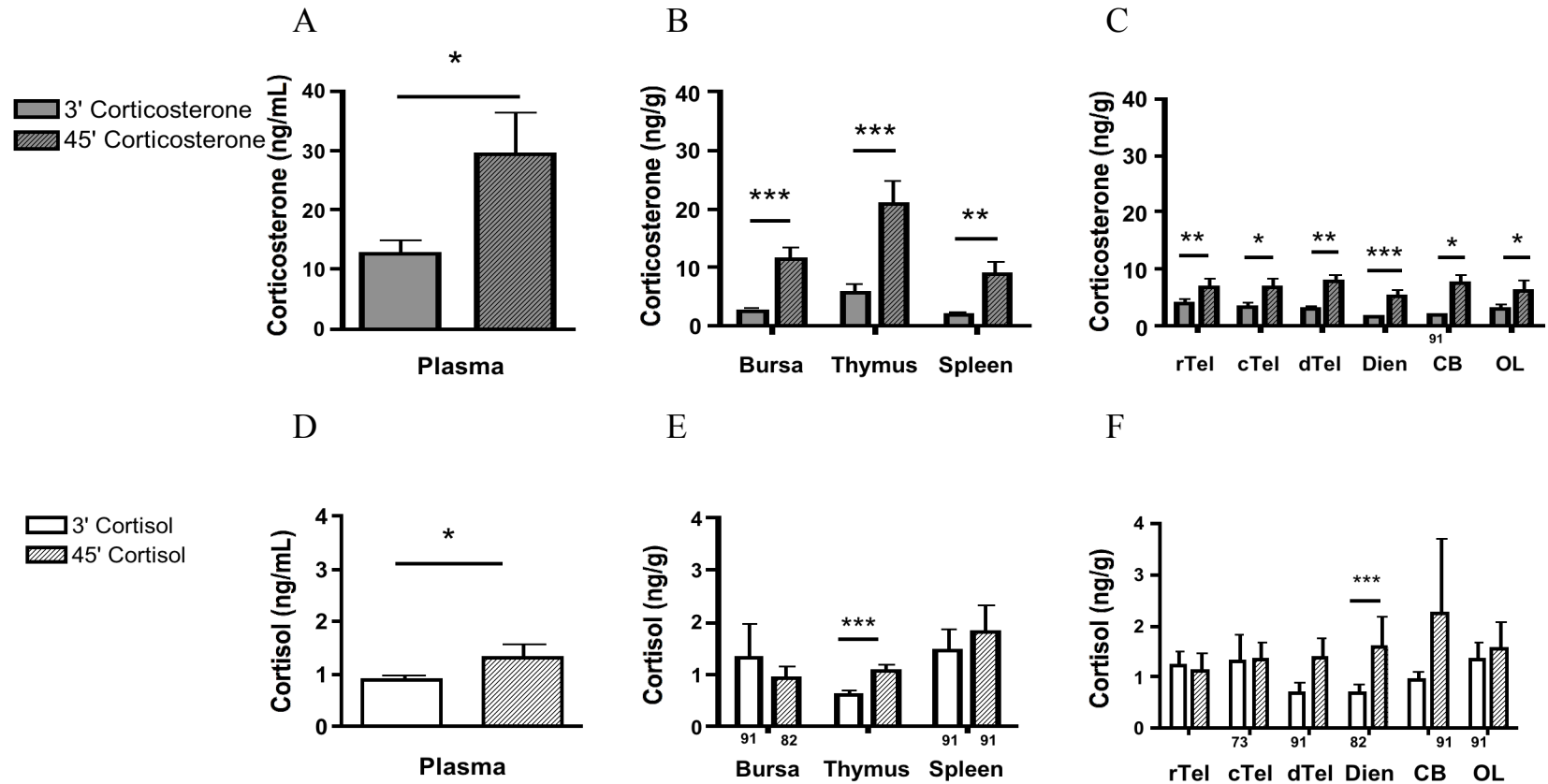
Note: significant p values are in bold.

Figure 3.1 Corticosterone and cortisol levels in European starlings on the day of hatch



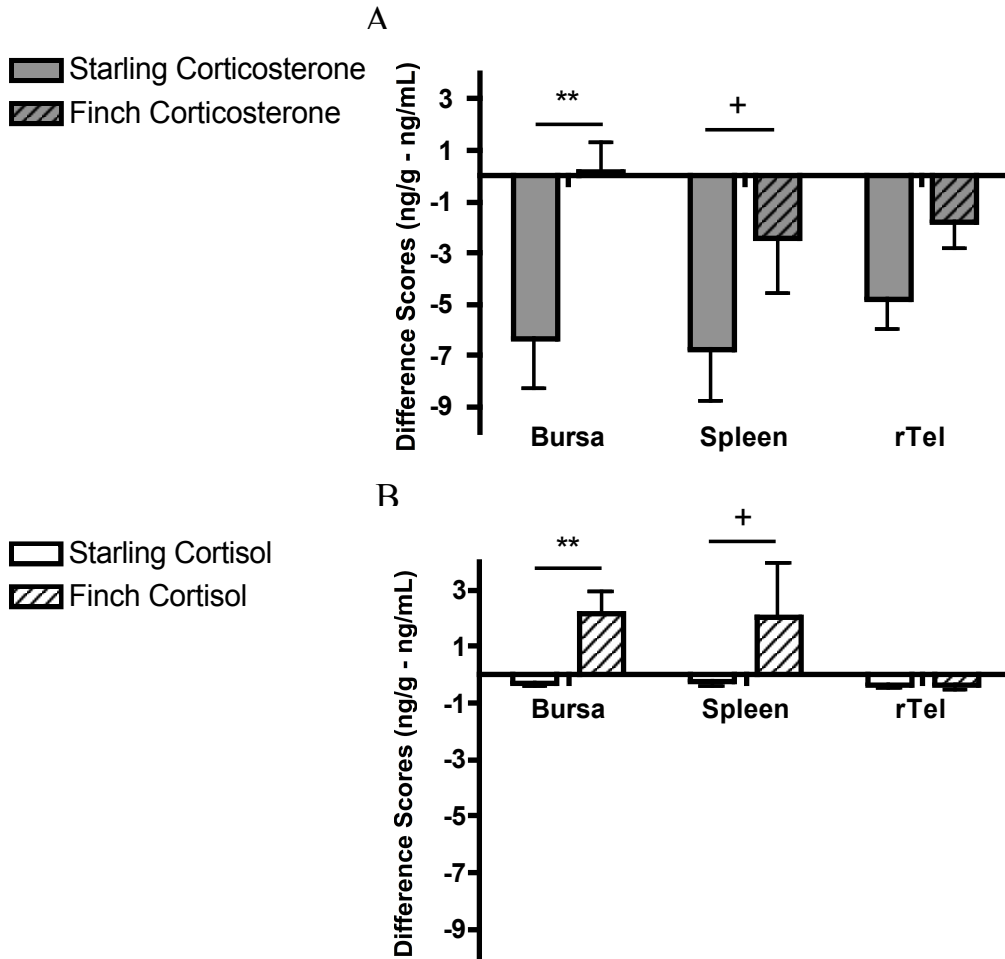
Corticosterone (A-C) and cortisol (D-F) levels in European starlings on the day of hatch in plasma (A, D), immune tissues (B, E), and brain tissue (C, F). Chicks were sampled within 3 min of disturbance (n = 9) or after 45 min of restraint stress (n = 9). rTel = rostral telencephalon; cTel = caudal telencephalon; Dien = diencephalon; CB = cerebellum; OL = optic lobes. Numbers below the bars represent the percent of detectable samples.

Figure 3.2 Corticosterone and cortisol levels in European starlings on post-hatch day 10



Corticosterone (A-C) and cortisol (D-F) levels in European starlings on post-hatch day 10 in plasma (A, D), immune tissues (B, E), and brain tissue (C, F). Chicks were sampled within 3 min of disturbance (n = 11) or after 45 min of restraint stress (n = 11). rTel = rostral telencephalon; cTel = caudal telencephalon; dTel = dorsal telencephalon; Dien = diencephalon; CB = cerebellum; OL = optic lobes. Numbers below the bars represent the percent of detectable samples. Where no number is given, 100% of the samples were detectable. * = p<0.05; ** = p<0.01; *** = p<0.001.

Figure 3.3 Corticosterone and cortisol levels in wild European starlings and captive zebra finches on post-hatch day 4



Plasma corticosterone and cortisol levels were subtracted from tissue corticosterone and cortisol levels, respectively, to create “difference scores”. All chicks were sampled within 3 min of disturbance. Starling = European starling; finch = zebra finch; rTel = rostral telencephalon. + = $p < 0.10$; ** = $p < 0.01$.

3.5 References

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4. General discussion and conclusions

One major goal of this thesis was to determine whether GCs may be locally synthesized in the immune system and/or brain by measuring endogenous corticosterone and cortisol levels in tissues. Importantly, most studies addressing this issue have used *in vitro* methods to determine the activity of steroidogenic enzymes or measured mRNA for steroidogenic enzymes, but have not measured the products of these enzymes under *in vivo* conditions. The studies described in this thesis are some of the first to measure endogenous GC levels in the immune system and brain of any species. In Chapter 2, developmental changes in local versus systemic GC levels were compared in captive zebra finch nestlings. In Chapter 3, local GC levels were measured in a wild animal, the European starling, and the effect of restraint stress on local and systemic GC levels was determined.

In Chapter two, high levels of cortisol were found in the immune system of developing zebra finches, suggesting that cortisol is locally synthesized as an immunosteroid. This finding is consistent with a study in developing chickens, which found evidence that the chicken bursa and thymus contain the enzymes necessary to synthesize cortisol *in vitro* (2). The current findings, along with past studies demonstrating activity of GC synthetic enzymes in immune tissues *in vitro* (2, 3, 19), provide strong evidence that cortisol is an immunosteroid. Additionally, our findings also suggest that although corticosterone is the predominant systemic GC in zebra finches, cortisol is the predominant GC in the immune system and is present at similar levels as corticosterone in the brain early in development. Thus the identity of the locally synthesized GC may differ from that of the systemic GC. Furthermore, cortisol levels in the immune system declined with age as systemic levels of corticosterone were increasing

In Chapter 3, in study 3.1, baseline GC levels in the immune system and brain of European starlings were low, suggesting that GCs may only be synthesized at low levels, if at all, in these tissues. After restraint stress however, P0 birds exhibited a trend for increased levels of both corticosterone and cortisol in the three immune organs studied. This trend was not observed in plasma or brain tissue, where there was no effect of restraint stress. Therefore, in P0 starlings, GCs may be synthesized in immune tissues in response to restraint. At P10, restraint increased corticosterone in plasma and all tissues studied as well as cortisol in plasma, thymus and diencephalon. This illustrates that local and systemic GC levels become more responsive to restraint stress with age in European starlings, as has been observed in other altricial species (20, 4).

In Chapter 3, in study 3.2, notable differences in local corticosterone and cortisol levels were found between European starlings and zebra finches. These differences in local GC levels could be due to differences in life history strategies of these species. Past research suggests that long-lived species that have multiple opportunities to reproduce invest a greater proportion of resources into adaptive immunity, presumably to increase the probability of long-term survival (7, 8). In contrast, short-lived species may allocate resources to rapid reproductive development and rely to a greater degree on innate immunity (7, 8). Since GCs regulate apoptosis of lymphocytes and thus play an important role in the development of adaptive immunity, local GC synthesis may be one mechanism by which investment in immune function is differently regulated between species. Alternatively, differences in GC synthesis between species may also be due to experiential factors, such as parasite exposure.

One important question raised by the findings in this thesis, is what may the function of local GC synthesis be? GCs play an important role in lymphocyte selection early in development. However, during this time systemic GC levels are often low (4, 20). It is possible that GCs synthesized locally in immune tissues may regulate lymphocyte selection. In support of this when transgenic mice that over-express glucocorticoid receptors (GR) specifically in thymocytes are adrenalectomized, GR over-expression still leads to a decrease in thymocyte number, even though systemic GCs have been removed (11). Thus Local GC synthesis in the immune system may provide adequate levels of GCs to regulate the process of lymphocyte selection during development when circulating GC levels are low. Since we found that cortisol was the predominant GC in immune tissues of zebra finches, even though corticosterone is the predominant GC in circulation, it is possible that cortisol and corticosterone differentially regulate lymphocyte apoptosis.

The studies described in this thesis highlight many novel and intriguing findings and will provide a foundation for future research in this direction. Future studies should confirm that high levels of cortisol in the immune system of zebra finches are due to the local synthesis of cortisol, rather than sequestration. This can be determined by incubating immune tissues with radiolabeled cholesterol *in vitro* and measuring the metabolites using high performance liquid chromatography (HPLC). Further, the effects of restraint stress and other stressors (e.g. immune challenge) on GC levels in tissues of zebra finches should be determined. Since local sex steroid synthesis can be rapidly regulated (12, 15), time points of restraint other than 45 min should be examined (e.g. 5 min).

The finding that the identity of the local GC may differ from the identity of the systemic GC suggests that corticosterone and cortisol may have different functions.

Implanting developing animals with either 1) cortisol, 2) corticosterone, 3) or an empty implant to examine the effect of both GCs on immune system and brain development will test this hypothesis. Interestingly, both cortisol and corticosterone administration decrease the weight of the bursa in young chickens, but cortisol causes more vacuolar spaces within the lymph follicle of the bursa (1). These data suggest that cortisol has a stronger effect on bursa histology than corticosterone

There is also evidence that ACTH and CRH are expressed in immune organs, raising the hypothesis that there is an “HPA axis homolog” in immune tissues (13). Thus future studies should use immunostaining or molecular techniques to detect CRH, CRH receptors, ACTH and ACTH receptors in immune tissues of zebra finches. If CRH and ACTH are also expressed in immune tissues, then immunosteroid synthesis may be under autonomous local regulation independent of the classical HPA axis.

It will also be important to determine if there are receptors in avian immune and brain tissue that bind cortisol in order to verify that cortisol is physiologically relevant (14). Since locally synthesized steroids can have rapid effects suggesting that they act via nongenomic mechanisms, for example by binding to membrane-bound receptors (5, 16), one hypothesis is that there is a membrane-bound receptor in immune tissue that has a higher affinity for cortisol than corticosterone (17).

Future studies should also manipulate investment into adaptive immunity to determine how this affects local GC synthesis in the immune system. Since we found evidence that local GC levels are lower in starlings, a longer-lived species that may invest more into adaptive immunity, one prediction is that increasing investment in adaptive

immunity in zebra finches, for example by administering the immunostimulant methionine (18), will decrease GC synthesis in immune tissues.

Lastly, in the present studies, GC levels in the brain tended to be low, providing little evidence for GC synthesis in the brain. However, past studies have found evidence that GCs are neurosteroids (6, 9). One explanation for this discrepancy is that GCs are synthesized only in discrete brain regions and that the dissections used in these studies were too gross to detect this regional specificity. Measuring GCs in micropunches using Palkovits punch techniques (10) will provide more detail on regional differences in GC synthesis in the brain.

In conclusion, the results presented in this thesis further support the hypothesis that GCs can be locally synthesized in extra-adrenal tissues. Importantly, systemic GC levels may not be representative of tissue GC levels. Measuring tissue GC levels can reveal important insights into how GC levels are regulated in age-specific, tissue-specific, and species-specific manners. Lastly, it is frequently thought that cortisol and corticosterone are interchangeable and a common belief is that a species has just one predominant GC, corticosterone or cortisol. Results described in this thesis challenge this notion and instead raise the novel hypothesis that cortisol and corticosterone may have unique physiological effects, particularly during development.

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