

Local glucocorticoid regulation in avian and murine lymphoid organs

by

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Abstract

Glucocorticoids are steroid hormones that circulate in the blood to coordinate organismal physiology. They have pleiotropic effects, regulating metabolic, cardiovascular, neural, and immune function. While glucocorticoids are classically thought to be secreted exclusively by the adrenal glands, evidence suggests that different organs may be able to autonomously regulate their local glucocorticoid levels via local production. Local production may be important when circulating glucocorticoids are low or absent, such as in early life of altricial young, which are unable to care for themselves. Immune (lymphoid) organs are particularly interesting candidates for tissue-specific regulation of glucocorticoid levels, as glucocorticoids are necessary for early-life immune development in altricial young. In this dissertation, I present a series of studies using birds and mice to examine whether tissue-specific regulation of glucocorticoids occurs in lymphoid organs. In brief, I report that a) glucocorticoids are locally elevated in lymphoid organs of newly-hatched altricial but not precocial birds, b) glucocorticoids are locally elevated in lymphoid organs of neonatal altricial mice, and c) lymphoid organs of both neonatal and adult mice synthesize glucocorticoids from other steroid precursors. Local glucocorticoid production in lymphoid organs may function to ensure production of functional lymphocytes, and factors that alter lymphoid glucocorticoid levels may play a role in programming the overall immune reactivity.

Preface

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List of abbreviations

| | |
|--|--|
| 3 β -HSD1 – 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4 isomerase type 1 | \times g – earth's gravitational acceleration constant |
| 3 β -HSD1 – 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4 isomerase type 6 | GC – glucocorticoid |
| 11 β -HSD1 – 11 β -hydroxysteroid dehydrogenase type 1 | GR – glucocorticoid receptor |
| 11 β -HSD2 – 11 β -hydroxysteroid dehydrogenase type 2 | HAW – Hawaiian strain of Japanese quail |
| μ g – microgram | HPA – hypothalamic pituitary adrenal |
| μ l – microliter | HPLC – high performance liquid chromatography |
| μ M – micromolar | HPA axis – hypothalamic-pituitary-adrenal axis |
| ABC – ATP-binding cassette | h, hr – hours |
| ACE – angiotensin converting enzyme | icv – intracerebroventricular |
| ACTH – adrenocorticotrophic hormone | IL-1 β – interleukin 1 β |
| ANOVA – analysis of variance | IS – internal standard |
| ATP – adenosine triphosphate | LC-MS/MS – liquid chromatography-tandem mass spectrometry |
| BCR – B cell receptor | LPS – lipopolysaccharide |
| $^{\circ}$ C – degrees Celsius | LRH-1 – liver receptor homolog-1 |
| cAMP – cyclic adenosine monophosphate | m – metres |
| CBG – corticosteroid-binding globulin | M – molar |
| cDNA – complementary DNA | MC – mineralocorticoid |
| CIHR – Canadian Institutes of Health Research | MET – metyrapone |
| CPM – counts per minute | mg – milligram |
| Cq – qPCR quantification cycle | MHC – major histocompatibility complex |
| CRH – corticotropin releasing hormone | min – minutes |
| CYP11A1 - cytochrome P450 family 11, subfamily A, polypeptide 1 | ml – milliliter |
| CYP11B1 - cytochrome P450 family 11, subfamily B, polypeptide 1 | MLN64 – StAR-related lipid transfer protein |
| CYP11B2 - cytochrome P450 family 11, subfamily B, polypeptide 2 | MPA – mobile phase A |
| CYP17A1 - cytochrome P450 family 17, subfamily A, polypeptide 1 | MPB – mobile phase B |
| DHC – 11-dehydrocorticosterone | MR – mineralocorticoid receptor |
| DNA – deoxyribonucleic acid | MRM – multiple reaction monitoring |
| DOC – 11-deoxycorticosterone | mRNA – messenger ribonucleic acid |
| DPM – decays per minute | mV – millivolts |
| E16.5 – embryonic day 16.5 | m/z – mass/charge ratio |
| EIA – enzyme immunoassay | na – not applicable |
| g – gram | nd – nondetectable |
| | NF κ B – nuclear factor κ of activated B cells |
| | ng – nanograms |
| | NR3C1 – nuclear receptor subfamily 3, group C, member 1 |

| | |
|---|---|
| NSERC – Natural Sciences and Engineering Research Council | RPMI – Roswell Park Memorial Institute medium |
| OAZ1 – ornithine decarboxylase antizyme 1 | RPS29 – ribosomal protein S29 |
| P1 – posthatch day 1 | RPL4 – ribosomal protein L4 |
| P14 – posthatch day 14 | s – seconds |
| P30 – posthatch day 30 | sc – subcutaneous |
| P42 – posthatch day 42 | sec – seconds |
| PBSg – phosphate-buffered saline containing 0.1% gelatin | SEM – standard error of the mean |
| PCR – polymerase chain reaction | SF-1 – steroidogenic factor 1 |
| pg – picograms | SD – standard deviation |
| PND1 – postnatal day 1 | SHRP – stress hyporesponsive period |
| PND5 – postnatal day 5 | SPE – solid-phase extraction |
| PND15 – postnatal day 15 | StAR – steroid acute regulatory protein |
| PND60 – postnatal day 60 | STARD3 – StAR-related lipid transfer protein |
| POMC – pro-opiomelanocortin | TCR – T cell receptor |
| PR – progesterone receptor | T _h 1 – type 1 helper T cell |
| qPCR – quantitative polymerase chain reaction | T _h 2 – type 2 helper T cell |
| RAS – renin-angiotensin system | T _h 17 – type 17 helper T cell |
| RIA – radioimmunoassay | T _{reg} – regulatory T cell |
| RIR – Rhode Island Red breed of chicken | TNF α – tumor necrosis factor α |
| RNA – ribonucleic acid | UBC – University of British Columbia |
| | UWO – University of Western Ontario |
| | VEH – vehicle |
| | WL – White Leghorn breed of chicken |

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1 Introduction

1.1 Overview

Endocrinology is concerned with understanding hormones, signaling molecules produced by specific glands of multicellular organisms that are secreted in the blood to then act on distant target tissues (Norris, 2007; Guyton and Hall, 2006). Following this definition, endocrinology has overwhelmingly focused on hormonal changes at the systemic level, in which endocrine organs secrete hormones into the circulating blood. This regulation of whole-body hormone levels functions to coordinate systemic organismal physiology.

However, many hormones known to act in an endocrine fashion, where secretion and action take place at disparate locations in the body, are now known to also act in paracrine or autocrine fashions, where they act on target cells in close proximity to the source of the hormone, or on the hormone-producing cells themselves (Schmidt et al., 2008). This has greatly expanded our understanding of the different targets that hormones can act on. More recently, a growing body of evidence has shown multiple mechanisms by which hormone-sensitive tissues may independently regulate their local hormone concentrations as well (Taves et al., 2011a, Tomlinson et al., 2004; Breuner and Orchinik, 2002; Thompson, 1995). However, the extent to which these mechanisms actually affect specific tissues is not well understood.

Glucocorticoids are a particularly interesting example to examine tissue-specific regulation. Produced by the adrenal glands, especially in response to stressors, circulating glucocorticoids have pleiotropic effects, orchestrating metabolic, cardiovascular, neural, and immune function (Sapolsky et al., 2000). Glucocorticoids act on nearly every cell of the body, and are critical effectors of development (Wada, 2008), homeostasis (Sapolsky et al., 2000), and

disease (McEwen and Wingfield, 2003). While glucocorticoids have varied effects on different cell and tissue types, these effects are widely thought to follow systemic glucocorticoid patterns. As such, measurements of the predominant adrenal glucocorticoid in the blood (e.g., cortisol in human, corticosterone in rats, mice, and birds) are widely used to understand how changes in systemic glucocorticoid levels regulate these processes (Norris, 2007; Guyton and Hall, 2006; Demas and Nelson, 2012).

Glucocorticoids are lipophilic, and as such, freely diffuse through cell membranes and into tissues. Thus, glucocorticoid levels in tissues are widely thought to parallel glucocorticoid levels in the blood. However, a variety of tissues express cellular machinery by which local glucocorticoid levels could be regulated independently of systemic glucocorticoid levels. My PhD research investigated tissue-specific regulation of glucocorticoid levels; when it occurs and how it occurs. As glucocorticoids have critical roles in early organismal development, but are often nondetectable in the blood during early life, I chose to focus on early development as a time during which tissue-specific glucocorticoid regulation would seem highly beneficial for a developing organism. My hypothesis was that in early life, when circulating glucocorticoid concentrations are minimal, immune organs would upregulate their local glucocorticoid concentrations to maintain glucocorticoid-dependent functions. My first study, in developing birds, compares blood and lymphoid organ glucocorticoids in altricial offspring, which have minimal circulating glucocorticoids, and precocial offspring, which have fully functional systemic glucocorticoid responses. My second study compares blood and lymphoid organ glucocorticoids during mouse development. My third study examines glucocorticoid production by neonatal and adult murine lymphoid organs.

1.2 Adrenal corticosteroids

Corticosteroids are steroid hormones produced in the adrenal cortex and are of two types, glucocorticoids and mineralocorticoids. Glucocorticoids, such as corticosterone and cortisol, have numerous effects and can act on nearly all cells in the body. For example, glucocorticoids regulate metabolic activity, immune function, and behavior (Sapolsky et al., 2000). Circulating glucocorticoid levels increase in response to a variety of stressors, under control of the hypothalamic-pituitary-adrenal (HPA) axis. Hypothalamic release of corticotropin-releasing hormone (CRH) triggers pituitary release of adrenocorticotrophic hormone (ACTH), which stimulates glucocorticoid production by the zona fasciculata of the adrenals. The adrenals can secrete cortisol, corticosterone or both, depending on the species.

Mineralocorticoids, such as aldosterone, promote sodium reabsorption in transporting epithelia of the kidneys, salivary glands, and large intestine. Sodium reabsorption is followed by passive reabsorption of water. Circulating aldosterone concentrations rise in response to low blood volume or sodium depletion, under control of the renin-angiotensin system (RAS). The kidneys release renin, which converts angiotensinogen to angiotensin I. Angiotensin I is then cleaved by angiotensin converting enzyme (ACE) to active angiotensin II. Angiotensin II stimulates mineralocorticoid production by the zona glomerulosa of the adrenals.

The classical corticosteroid biosynthetic pathway is shown in Figure 1.1. Traditionally, it was thought that glucocorticoids and mineralocorticoids were synthesized solely in the adrenal cortex, and research has focused overwhelmingly on measuring circulating levels of these steroids in plasma or serum. However, a growing body of evidence has demonstrated *de novo* synthesis of glucocorticoids and mineralocorticoids in other organs, such as primary lymphoid organs, intestine, skin, brain, and possibly the heart and vasculature (Davies and Mackenzie,

2003; Schmidt et al., 2008). Here, we review evidence of local *de novo* corticosteroid production, regulation of local corticosteroid production, and the potential functions of locally-produced corticosteroids.

1.3 Primary lymphoid organs

Primary lymphoid organs are the sites of T and B cell (lymphocyte) development. In mammals, both cell lineages originate from the same early precursors in the bone marrow. T cell precursors migrate to and mature in the thymus, while B cell precursors remain and mature in the bone marrow. The thymus consists of inner medullary and outer cortical epithelial cells through which immature T cells (thymocytes) migrate over the course of development (Takahama, 2006). During development, thymocyte selection ensures the ability of the T cell receptor (TCR) to recognize antigens presented by self MHC molecules (positive selection) and prevents T cell autoreactivity (negative selection). Only thymocytes expressing a TCR with intermediate affinity for antigen:MHC develop into mature T cells; the other thymocytes (96%) undergo apoptosis (Takehama, 2006). In the bone marrow, a similar process results in removal of autoreactive B cells.

Glucocorticoids can induce apoptosis of lymphocytes, and this effect is especially pronounced in immature lymphocytes. However, glucocorticoids can also inhibit TCR-mediated apoptosis and promote survival (Iwata et al., 1991). This mutual antagonism of glucocorticoid receptor (GR) signaling and TCR signaling suggested a role for glucocorticoids in thymocyte selection (Iwata et al., 1991). Since circulating glucocorticoids are very low in early postnatal life (when lymphocyte production is high), local synthesis might provide a source of glucocorticoids.

1.3.1 Evidence for local synthesis

Steroidogenic enzymes. The first demonstration of thymic steroid production was in the mouse, and using fetal thymic organ culture, Vacchio and colleagues demonstrated conversion of a cholesterol analog into pregnenolone and 11-deoxycorticosterone (Vacchio et al., 1994). Steroidogenic capability was high in thymic epithelial cells and low in thymocytes (Vacchio et al., 1994). Murine thymic epithelial cells have since been shown to have mRNA, protein, and activities of the enzymes required for *de novo* glucocorticoid synthesis (Lechner et al., 2000; Pazirandeh et al., 1999; Qiao et al., 2008; Vacchio et al., 1994) (Figure 1.1, Table 1.1). The absence of CYP17 activity results in the formation of corticosterone (Lechner et al., 2000), which is also the major adrenal glucocorticoid in mice. Thymic epithelial cells also activate GR-mediated transcription in co-cultured cells (Pazirandeh et al., 1999). In addition to thymic epithelial cells, thymocytes themselves also express StAR, CYP11A1, 3 β -HSD, CYP17, CYP21, and CYP11B1 mRNA and synthesize corticosterone (Chen et al., 2010; Qiao et al., 2008; Qiao et al., 2009). Moreover, the chicken thymus contains functional CYP11A1, 3 β -HSD, CYP21, and CYP11B1 enzymes for glucocorticoid synthesis, but the additional presence of CYP17 activity directs synthesis toward cortisol, rather than corticosterone, in contrast to the chicken adrenals (Lechner et al., 2001). All the enzymes found in the chicken thymus are also present in the bursa of Fabricius (hereafter bursa) (Lechner et al., 2001). The bursa is the site of avian B cell development, analogous to mammalian bone marrow. Together, these studies of corticosteroidogenic enzymes demonstrate that the murine and avian thymus, and also the avian bursa, contain the machinery for *de novo* glucocorticoid synthesis.

Local steroid levels. Endogenous glucocorticoid levels have not been measured in rodent thymus, but have been measured in the avian thymus and bursa (Figure 1.2, Table 1.1). The major circulating glucocorticoid in birds is corticosterone, but in zebra finch (*Taeniopygia guttata*) thymus and bursa, cortisol levels are higher than corticosterone levels. Also, cortisol (but not corticosterone) levels are higher in lymphoid tissue than in plasma, which provides evidence that cortisol is a locally-synthesized “immunosteroid” (Lechner et al., 2001; Schmidt et al., 2008; Schmidt and Soma; 2008). High local levels could also involve accumulation of circulating glucocorticoids, but the local presence of cortisol-synthetic machinery and the low circulating levels of cortisol suggest local origin. Local synthesis of cortisol, rather than corticosterone, offers an opportunity to parse the regulation and functions of lymphoid glucocorticoids from those of adrenal glucocorticoids. Further, glucocorticoid production in the avian bursa raises the possibility that similar production occurs in the mammalian bone marrow. The very high lymphoid cortisol concentrations after hatch show that local glucocorticoid synthesis can result in levels far in excess of those in the circulation.

1.3.2 Regulation

In the mouse, *in vitro* steroidogenesis by thymic epithelium is high at birth and decreases with age (Qiao et al., 2009; Vacchio et al., 1994). A similar age-related decrease is seen in zebra finch bursa; cortisol levels are high at hatch and decrease rapidly with age (Schmidt and Soma, 2008) (Figure 1.2). In the first 1-2 weeks of life, mice (Schmidt et al., 2003), zebra finches (M. D. Taves and K. K. Soma, unpublished data; Wada et al., 2009), and other altricial species undergo a period of minimal adrenal glucocorticoid production (the stress hyporesponsive period), which results in low circulating levels. A stress hyporesponsive period occurs in humans (also altricial)

(Gunnar and Cheatham, 2003). Since the stress hyporesponsive period coincides with high local synthesis in lymphoid organs, local production may serve to maintain high local glucocorticoid levels in lymphoid organs while systemic levels are low. In contrast to thymic epithelial cells, thymocyte production of glucocorticoids increases at puberty (~4 weeks in mice) (Qiao et al., 2008) and is stimulated by testosterone in males (Chen et al., 2010).

Glucocorticoid synthesis in primary lymphoid organs is regulated by HPA axis mediators (Figure 1.3A). ACTH increases *in vitro* steroid production by murine thymic epithelial cells (Vacchio et al., 1994) (Table 1.2), although a separate study found little effect on glucocorticoid response element activity (Pazirandeh et al., 1999). In thymocytes, ACTH and cAMP decrease steroidogenic enzyme expression and glucocorticoid response element activity (Qiao et al., 2009). The divergent effects of ACTH on glucocorticoid production by thymic epithelial cells and thymocytes could help to maintain local glucocorticoid levels. Pro-opiomelanocortin (POMC) mRNA is present in rat thymus (Qiao et al., 2009) and ACTH immunoreactivity has been detected in rat, bird, and human thymi (Batanero et al., 1992; Ottaviani et al., 1997). CRH mRNA is also present in rat thymus (Aird et al., 1993), and CRH immunoreactivity has been detected in rat and bird thymi (Ottaviani et al., 1998). Thus, an exciting possibility is that a local analog of the HPA axis regulates local glucocorticoid production in the thymus. In bone marrow and bursa, less is known about regulatory factors. The avian bursa contains immunoreactive ACTH (Franchini and Ottaviani, 1999), but CRH has not been reported. The bursa also contains bursal anti-steroidogenic peptide (BASP), which suppresses adrenal glucocorticoid production *in vitro* (Byrd, 1994).

1.3.3 Function

Locally-produced glucocorticoids have major effects on thymocyte development (Figure 1.3A, Table 1.3). In general, thymocyte selection is thought to be driven largely by TCR affinity for antigen presented by self MHC molecules. Low affinity (weak or absent TCR signal) results in death, intermediate affinity (moderate signal) results in positive selection and survival, and high affinity (strong signal) results in negative selection and death. The discovery of thymic glucocorticoid synthesis has suggested an alternative model (“mutual antagonism”) in which TCR signaling induces apoptotic signals in thymocytes with intermediate- or high-affinity TCR, and glucocorticoids antagonize these pro-apoptotic signals to allow survival of thymocytes with intermediate-affinity TCR (Vacchio et al., 1994). The mutual antagonism model is supported by *in vitro* observations that TCR signaling decreases glucocorticoid-induced thymocyte apoptosis (Iwata et al., 1991) and that endogenous thymic glucocorticoids decrease TCR-dependent thymocyte apoptosis (Vacchio and Ashwell, 1997; Vacchio et al., 1994). Furthermore, in thymi of fetal mice, thymocyte-specific GR reduction (via transgenic expression of antisense transcripts) reduces thymocyte numbers, suggesting that GR signaling promotes survival (King et al., 1995).

The effects of glucocorticoids on thymocytes, however, change with age. At puberty, GR underexpression (as above) increases thymocyte numbers, while overexpression decreases thymocyte numbers (Pazirandeh et al., 2002). Thus, at puberty, endogenous glucocorticoids promote thymocyte apoptosis rather than survival. GR overexpression also delays thymus growth and involution (Pazirandeh et al., 2004). The necessity of GR function for thymocyte development has also been tested in knockout mice. Mutants with partial (Purton et al., 2000) or complete (Brewer et al., 2002) abrogation of GR function retain functional thymocyte development. GR signaling is therefore not necessary for thymocyte development. Nonetheless,

in intact adult mice, inhibition of thymic glucocorticoid production by low-dose metyrapone treatment (which did not significantly affect plasma glucocorticoid levels in this study) increased thymocyte numbers, showing that locally-produced glucocorticoids have physiological effects even in the presence of functional adrenal glands (Qiao et al., 2009). Finally, T cell-specific deletion of the GR results in the production of T cells that are unable to mount a competent immune response (Mittelstadt et al., 2012).

In sum, GR signaling promotes thymocyte survival in fetal thymus and promotes apoptosis in postnatal (pubertal) thymus, but is not critical for thymocyte maturation. How glucocorticoids interact with TCR signaling is unclear, but this might involve membrane-associated receptors rather than cytosolic receptors. The unbound GR has been shown to associate at the cell membrane with TCR kinases and facilitate TCR signaling, while glucocorticoid binding causes dissociation of this complex and inhibition of TCR signaling (Lowenberg et al., 2005; Lowenberg et al., 2006).

Far less work has been done on locally-synthesized glucocorticoids and B cell development. Systemic glucocorticoid treatment depletes B lineage cells in murine bone marrow (Garvy and Fraker, 1993a) and decreases bursa size in the chicken (Norton and Wira, 1977). In the zebra finch, lymphoid cortisol may act on different receptors than adrenal corticosterone, because cortisol (but not corticosterone) shows specific binding to bursa membranes (Schmidt et al, 2010). The accessibility of the avian bursa and the ability to remove it intact make this a useful model for investigating the effects of local glucocorticoid synthesis in B cell development.

1.4 Intestine

The intestine is a critical barrier between the internal environment (within the organism) and external environment (the lumen; outside the organism). The intestinal mucosa contains the largest number of immune cells in the body and protects the epithelial surface from pathogens as well as commensal bacteria. Intestinal immune cells are concentrated in distinct lymphoid tissues (Peyer's patches, mesenteric lymph nodes, and appendix) and are also present as individual cells throughout the epithelium (Cebra, 1999). Tight regulation of immune activation is necessary to maintain intestinal homeostasis.

1.4.1 Evidence for local synthesis

Steroidogenic enzymes. The *de novo* steroidogenic capacity of the gut was first suggested in a study using *in situ* hybridization, which detected CYP11A1 and 3 β -HSD mRNA in the mouse gut during embryonic development (Keeney et al., 1995) (Table 1.1). Subsequent studies have demonstrated expression of mRNA and protein for several glucocorticoid-synthetic enzymes in murine small intestine and colon (Cima et al., 2004; Coste et al., 2007). CYP11A1 mRNA is highest in the proximal small intestine, intermediate in the middle and distal small intestine, and lowest in the colon (Cima et al., 2004). The mRNAs for steroidogenic enzymes are restricted to the proliferating epithelial cells of the crypts (Atanasov et al., 2008; Cima et al., 2004), and differentiation of immature intestinal epithelial cells into mature nonproliferating cells results in a decrease of steroidogenic enzyme mRNA (Atanasov et al., 2008). Also, CYP11A1 and CYP11B1 mRNAs are detectable in a murine epithelial cell line (Mueller et al., 2007). Murine small intestine contains CYP11A1 protein, and the activity of this and other steroidogenic enzymes is demonstrated by *ex vivo* synthesis of corticosterone (Cima et al., 2004;

Coste et al., 2007). Corticosterone synthesis is blocked by metyrapone (Cima et al., 2004; Coste et al., 2007). In humans, CYP11A1 and CYP11B2 mRNAs are present in colon biopsies (Coste et al., 2007), suggesting that the human intestine, like that of the mouse, is capable of glucocorticoid synthesis.

Local steroid levels. To our knowledge, local corticosteroid levels within the intestinal epithelium have not been measured *in vivo*.

1.4.2 Regulation

In the murine intestine, expression of specific steroidogenic enzymes increases in response to immune activation and inflammation (Fig. 1.3B). *In vivo* treatment of mice with anti-CD3 (to activate T cells) profoundly increases *ex vivo* corticosterone production by the small intestine (Cima et al., 2004) (Table 1.2). Mouse small intestine and colon constitutively express mRNA of most enzymes required for corticosterone synthesis (Cima et al., 2004; Coste et al., 2007), but CYP11A1 and CYP11B1 expression only reach high levels after T cell activation (Cima et al., 2004) or inflammation (Noti et al., 2010a; Noti et al., 2010b). The inflammation-induced increases in enzyme expression and corticosterone synthesis require the secretion of tumor necrosis factor- α (TNF α), a pro-inflammatory cytokine (Noti et al., 2010a; Noti et al., 2010b). Immune activation, possibly via TNF α induction of NF κ B (nuclear factor kappa-light-chain-enhancer of activated B cells, a pro-inflammatory transcription factor) (Noti et al., 2010a), increases intestinal expression of the transcription factor liver receptor homolog-1 (LRH-1) (Mueller et al., 2006). LRH-1 is closely related to steroidogenic factor-1 (SF-1), which regulates the expression of most steroidogenic enzymes in the adrenal (Mueller et al., 2007). LRH-1 is

important in cell cycle progression, and LRH-1 expression in the intestine is limited to proliferating crypt cells (Atanasov et al., 2008). In a murine intestinal epithelial cell line, overexpression of LRH-1 increases CYP11A1 and CYP11B1 mRNA levels and corticosterone production (Mueller et al., 2006). *In vivo*, LRH-1 haploinsufficiency prevents T cell activation-induced upregulation of CYP11A1 mRNA and corticosterone production in the small intestine, and attenuates upregulation of CYP11A1 and CYP11B1 mRNA levels in the large intestine (Mueller et al., 2006). The requirement for both TNF α and LRH-1 suggests that during immune activation, TNF α might (in addition to its pro-inflammatory effects) induce LRH-1 and subsequently increase glucocorticoid production and epithelial cell proliferation (Noti et al., 2009).

The expression of steroidogenic enzymes is differentially regulated in adrenal and intestinal cells. For example, cAMP increases CYP11A1 and CYP11B1 mRNA levels in an adrenal cell line but has the opposite effects in an intestinal epithelial cell line (Mueller et al., 2007). Also, cAMP increases adrenal corticosterone release but decreases intestinal corticosterone release in culture. Similarly, ACTH administration increases adrenal-derived serum corticosterone levels but has no effect on intestine corticosterone production (Mueller et al., 2007). Local production of ACTH in the intestine is unlikely, as POMC mRNA is nondetectable in the gut (Fink et al., 1994). In contrast to the effects of ACTH and cAMP, protein kinase activation has no effect on enzyme transcription in adrenal cells but increases CYP11B1 transcription in intestinal epithelial cells (Mueller et al., 2007). These findings show that regulatory signals have differing or opposite effects on adrenal and intestinal glucocorticoid production. Differential regulation may avoid unnecessary local production when circulating glucocorticoid levels are high.

1.4.3 Function

Excessive stimulation of T cells or macrophages results in secretion of TNF α and inflammation-mediated damage to the intestinal epithelium (Figure 1.3B). TNF α also induces expression of LRH-1, which results in increased glucocorticoid production by and increased proliferation of epithelial cells (Noti et al., 2010a; Noti et al., 2010b). Together, these processes may downregulate the inflammatory immune response and mediate repair of inflammatory damage (Table 1.3). Consistent with this hypothesis, intestinal glucocorticoids decrease damage resulting from inflammatory bowel disease in both mice and humans, and glucocorticoid-synthetic gene expression in the colon is decreased in patients with Crohn's disease and inflammatory bowel disease (Coste et al., 2007). Interestingly, endogenous glucocorticoid synthesis may regulate cell-mediated (T_h1 -polarized) but not humoral (T_h2 -polarized) immune responses, as the latter does not stimulate glucocorticoid production in mouse intestine (Noti et al., 2010a). Thus, glucocorticoid synthesis by the intestine is specifically stimulated by activation of a cell-mediated T_h1 immune response, and acts to suppress and repair the damaging effects of this response. A cell-mediated T_h17 inflammatory response also seems likely to stimulate local glucocorticoid synthesis, but this possibility remains to be tested.

1.5 Skin

The skin, like the intestine, provides a boundary between the internal and external environments and is critical as a physical and immunological barrier. The epidermis is the outermost skin layer, which consists of keratinocytes that are continuously produced. Underneath the epidermis is the dermis, which contains connective tissues, nerve endings, sweat glands, hair

follicles, and sebaceous glands. Under the dermis is the subcutaneous layer, which is composed of adipose tissue. The skin is continuously exposed to solar, thermal, mechanical, and immune stressors, and responds rapidly to varying stressors in order to maintain its physical and functional integrity. The discovery of CRH and ACTH expression in skin, along with the presence of steroid metabolizing enzymes, suggested that the skin might also synthesize glucocorticoids (Slominski and Wortsman, 2000).

1.5.1 Evidence for local synthesis

Steroidogenic enzymes. The *de novo* steroidogenic capacity of human skin was first demonstrated by the conversion of cholesterol into pregnenolone and by the expression of CYP11A1 mRNA and protein (Thiboutot et al., 2003). Human skin expresses a functional homolog of StAR, StAR-related lipid transfer protein (MLN64, or STARD3) (Slominski et al., 2004), and steroidogenic enzyme mRNA, protein, and activities needed for glucocorticoid synthesis from cholesterol

have been shown (Dumont et al., 1992; Rogoff et al., 2001; Slominski et al., 1996; Vukelic et al., 2011) (Table 1.1). Localization of glucocorticoid-synthetic enzyme mRNA and protein suggests glucocorticoid production in sebaceous cells (Thiboutot et al., 2003), keratinocytes (Vukelic et al., 2011), fibroblasts (Slominski et al., 2004) and potentially melanocytes (Slominski et al., 2005b). Cortisol appears to be the major corticosteroid product in human skin (Slominski et al., 2006).

Mouse skin expresses CYP11A1, but further metabolism from pregnenolone into other steroids has not been reported (Slominski et al., 2004). Glucocorticoid synthesis is likely to occur in rat skin, as most glucocorticoid synthetic enzymes have been detected by measurements of

mRNA (Simard et al., 1993) or activity (Slominski et al., 2000), but CYP11A1 has not specifically been shown in rat skin.

Local steroid levels. To our knowledge, local corticosteroid levels within the skin have not been measured.

1.5.2 Regulation

Human skin expresses CRH and ACTH proteins (Ito et al., 2005; Slominski et al., 2000; Slominski et al., 2005; Teofoli et al., 1999). Incubation of melanocytes with CRH increases ACTH and cAMP levels, and incubation with ACTH increases cortisol levels in conditioned media (Slominski et al., 2005a) (Figure 1.3C, Table 1.2). Remarkably, these data suggest that melanocytes may contain a miniature ‘analog’ of the HPA axis. A similar local HPA axis also regulates glucocorticoid production in human hair follicles and fibroblasts (Ito et al., 2005; Slominski et al., 2005b), complete with negative feedback of cortisol on CRH expression (Ito et al., 2005).

Mouse skin also contains CRH protein (Roloff et al., 1998). The absence of CRH mRNA in mouse skin suggests that this CRH protein originates elsewhere (Roloff et al., 1998). CRH levels in murine skin correspond with hair growth: CRH levels are high during the growth phase and low during the regression and resting phases (Roloff et al., 1998).

Glucocorticoid synthesis in skin is induced by inflammation. Insults such as tissue damage, UV radiation, or pathogens result in local production of CRH (Slominski and Wortsman, 2000; Slominski et al., 1996), which promotes inflammation. CRH-induced pro-inflammatory

cytokines IL-1 β and TNF α then increase ACTH and glucocorticoid production in the skin (Teofoli et al., 1999; Vukelic et al., 2011).

1.5.3 Function

Glucocorticoid synthesis in the skin functions as a rapid and localized stress-response system (Slominski and Wortsman, 2000) (Table 1.3). It is well-known that topical treatment with high doses of glucocorticoids is anti-inflammatory and immunosuppressive. Locally-produced glucocorticoids play a similar role, inhibiting production of pro-inflammatory signal molecules such as CRH and IL-1 β (Ito et al., 2005; Vukelic et al., 2011) (Figure 1.3C). In contrast, adrenal glucocorticoids have rapid, short-term enhancing effects on cutaneous immunity by mediating immune cell migration from circulating blood into skin tissues (Dhabhar, 2009). Localized synthesis of glucocorticoids, by downregulating production of CRH and inflammatory cytokines, aids in preventing subsequent overshoot of the inflammatory immune response and further tissue damage. The physiological functions of skin-derived glucocorticoids, especially *in vivo*, require further study.

1.6 Central nervous system

Steroids play numerous important roles in the development and function of the central nervous system. Baulieu and Robel first observed that steroids (e.g. pregnenolone, dehydroepiandrosterone) were present in the rat brain at high concentrations long after castration and adrenalectomy (Robel and Baulieu, 1994). Since then, these brain-derived steroids (“neurosteroids”) have been widely studied, but with an overwhelming focus on progestins, androgens, and estrogens (Do Rego et al., 2009). As the synthesis of sex steroids in the central

nervous system has been extensively reviewed (Compagnone and Mellon, 2000; Do Rego et al., 2009; Remage-Healey et al., 2010; Tsutsui et al., 2000), here we focus on the final steps of glucocorticoid or mineralocorticoid production.

1.6.1 Evidence for local synthesis

Steroidogenic enzymes. The rat brain expresses the mRNAs of all the steroidogenic enzymes required for *de novo* synthesis of glucocorticoids and mineralocorticoids from cholesterol (Gomez-Sanchez et al., 1997; Gomez-Sanchez et al., 1996; Kishimoto et al., 2004; MacKenzie et al., 2000; Mellon and Deschepper, 1993; Stromstedt and Waterman, 1995) (Table 1.1). CYP11B1 and CYP11B2 proteins and activities have also been detected, and activity can be blocked by specific enzyme inhibitors (Gomez-Sanchez et al., 1997; MacKenzie et al., 2000). The mouse brain contains mRNAs for most corticosteroid-synthetic enzymes, but mRNA levels for CYP11B1 and CYP11B2 are minimal (Stromstedt and Waterman, 1995). In the human brain, several regions also express enzymes for glucocorticoid and mineralocorticoid synthesis (Beyenburg et al., 2001; Yu et al., 2002). Interestingly, in both rat and human brain, CYP21 mRNA is very low or nondetectable (Mellon and Miller, 1989; Stromstedt and Waterman, 1995), but the same 21-hydroxylase function is performed by an alternate enzyme, CYP2D (CYP2D4 in the rat and CYP2D6 in the human) (Kishimoto et al., 2004). This example highlights the possibility that corticosteroid synthesis in the brain (and other organs) can differ from corticosteroid synthesis in the adrenals.

Local steroid levels. In intact rats, corticosterone and aldosterone levels are lower in brain than in plasma, but in adrenalectomized rats, aldosterone (but not corticosterone) levels are

higher in brain than in plasma (Gomez-Sanchez et al., 2005). These data suggest that aldosterone is synthesized in the rat brain. In contrast, in intact mice, corticosterone levels are similar or lower in brain compared to plasma, but in adrenalectomized mice, corticosterone levels are higher in brain than in plasma (Croft et al., 2008). This suggests that corticosterone is synthesized in the mouse brain (aldosterone was not quantified). There is also evidence for glucocorticoid synthesis in the developing and adult songbird brain. In newly-hatched zebra finches, cortisol levels are higher in caudal telencephalon than in plasma (Schmidt and Soma, 2008), and in adult song sparrows (*Melospiza melodia*), corticosterone levels can be higher in plasma from the jugular vein (exiting the brain) than in plasma from the brachial vein (Newman et al., 2008).

1.6.2 Regulation

Expression of steroidogenic enzymes in the brain during early life is tightly controlled, as sex steroids are critical in neural development (Kimoto et al., 2010). While glucocorticoids are also important in neural development, little is known regarding ontogenetic patterns of corticosteroid synthesis in brain. In the zebra finch, brain glucocorticoid levels during development are low, except in the caudal telencephalon immediately after hatch (Schmidt and Soma, 2008). Elevated glucocorticoid levels can have harmful effects during neural development, and altricial species such as finches, rodents, and humans may avoid such effects by maintaining low brain and circulating glucocorticoid levels.

In adult rats and mice, brain corticosterone production is triggered by an acute injection of alcohol (Croft et al., 2008) or by withdrawal from chronic alcohol consumption (Little et al., 2008) (Table 1.2). Alcohol withdrawal causes dramatic and region-specific increases in brain corticosterone levels, with no change in plasma corticosterone levels. In some cases,

corticosterone concentrations are much higher in brain than in plasma (Little et al., 2008). Similar effects are seen with a social stressor, social defeat (Croft et al., 2008). The effects of ethanol and social defeat could be mediated by ACTH, which increases brain CYP11B1 mRNA (Ye et al., 2008).

In the adult song sparrow, data suggest that acute restraint stimulates brain corticosterone synthesis and secretion during the molt, a life history stage in which corticosterone production by the adrenals is dramatically reduced (to allow feather growth) (Newman et al., 2008). In the zebra finch, saline perfusion was used to remove blood contamination from the brain, prior to measurement of brain steroid levels. Surprisingly, saline perfusion caused a rapid and region-specific increase in corticosterone levels in the brain, suggesting that hypoxia or ischemia could stimulate brain glucocorticoid synthesis (Taves et al., 2010).

In adult rats, the regulation of brain mineralocorticoid synthesis is well studied. Aldosterone synthesis in the rat brain is regulated by sodium intake. Low sodium intake increases expression of CYP11B2 (but not CYP11B1) mRNA in brain and in adrenals (Ye et al., 2003). However, high sodium intake or systemic angiotensin II administration does not affect CYP11B2 expression in brain (Ye et al., 2003). The lack of a response to systemic angiotensin II could be due to its limited crossing of the blood-brain barrier. Interestingly, the brain contains all the components of the renin-angiotensin system (RAS), including production of angiotensin II (MacKenzie et al., 2002). These components could thus function as a miniature analog of the classical RAS.

1.6.3 Function

Systemic glucocorticoids influence behavior and neurophysiology (Sapolsky et al., 2000). Locally-produced brain glucocorticoids could have similar, and possibly additive, effects. In birds, during the molt, when adrenal glucocorticoid production is low, local synthesis in the brain during capture and restraint might facilitate escape behavior or learning (e.g., to avoid capture in the future). Alterations in behavior are also important after social defeat (e.g., to prevent further physical aggression) (Table 1.3). In contrast, chronic elevation of brain glucocorticoid levels may contribute to cognitive and memory deficits that can result from alcohol withdrawal (Little et al., 2008).

Aldosterone acts on mineralocorticoid receptors in the brain to regulate blood pressure and salt consumption (Gomez-Sanchez et al., 2004; Sakai et al., 1986). Central production of aldosterone is involved in the development of hypertension in the Dahl salt-sensitive rat model, in which hypothalamic aldosterone synthesis is increased relative to a control rat strain (Gomez-Sanchez et al., 2010; Huang et al., 2009). Brain-derived aldosterone is critical in driving sodium-induced hypertension. Infusion of a 3β -HSD inhibitor into the lateral ventricle prevents development of systemic hypertension in the adrenal-intact Dahl salt-sensitive rat (Gomez-Sanchez et al., 2005). In addition, infusion of a CYP11B2 inhibitor into the lateral ventricle of adrenal-intact rats dramatically decreases blood pressure induced by salt consumption (Gomez-Sanchez et al., 2010; Huang et al., 2008). This is not due to an effect on adrenal aldosterone synthesis, as no decrease in blood pressure is seen with systemic administration of the same dose (Fig. 1.4). The effect of CYP11B2 inhibition is also reversible, as replacement with control vehicle results in blood pressure elevation (Gomez-Sanchez et al., 2010). These studies

demonstrate that even in the presence of adrenal aldosterone synthesis, brain-derived aldosterone is critical for the regulation of blood pressure and the development of hypertension.

Even low levels of corticosteroid synthesis in the brain could have physiological significance, as the blood-brain barrier excludes several corticosteroids. The uptake of cortisol and aldosterone from the systemic circulation into the brain is especially low due to active removal by the transporter mdr1 (p-glycoprotein) (Geerling and Loewy, 2009).

1.7 Cardiovascular system

Aldosterone plays an important role in the physiopathology of congestive heart failure, which prompted researchers to examine local synthesis of aldosterone in the heart and vasculature. The possibility of cardiovascular synthesis of aldosterone took on added importance after it was found that mineralocorticoid receptor blockade had beneficial effects in heart failure patients, even when plasma aldosterone levels were normal or low (Pitt et al., 1999).

1.7.1 Evidence for local synthesis

Steroidogenic enzymes. Cardiovascular corticosteroid production was first investigated in human blood vessels, and later in the heart itself. In human vascular cells or tissue, local synthesis was suggested by PCR detection of CYP11A1, 3 β -HSD and CYP21 mRNA (Hatakeyama et al., 1996; Kayes-Wandover and White, 2000; Young et al., 2001) (Table 1.1). However, reports are divided on whether CYP11B1 mRNA is detectable (Kayes-Wandover and White, 2000) or not (Hatakeyama et al., 1996; Young et al., 2001), and whether CYP11B2 mRNA is detectable (Hatakeyama et al., 1994; Hatakeyama et al., 1996; Kayes-Wandover and White, 2000) or not (Ahmad et al., 2004; Young et al., 2001). Similarly, in the adult human heart,

steroidogenic enzyme mRNAs have been detected (Chai et al., 2010; Kayes-Wandover and White, 2000; Young et al., 2001). Reports are again divided on whether CYP11B1 mRNA is present, but CYP11B2 mRNA is not detectable (Chai et al., 2010; Kayes-Wandover and White, 2000; Young et al., 2001) except in subjects with heart failure (Young et al., 2001).

In rats, CYP11A1, CYP11B1 and CYP11B2 mRNAs have been detected in blood vessels (Takeda et al., 1995; Takeda et al., 1994) and StAR, CYP11B1, and CYP11B2 have been detected in the heart (Casal et al., 2003; Silvestre et al., 1998). However, subsequent studies found expression of CYP11B1 and CYP11B2 mRNA to be extremely low or nondetectable in the heart of various rat strains (Gomez-Sanchez et al., 2004; Ohtani et al., 2007; Ye et al., 2005). *Ex vivo* perfused rat blood vessels convert labeled pregnenolone to labeled corticosterone (Takeda et al., 1994), and perfused blood vessels from adrenalectomized rats release corticosterone and aldosterone into the perfusate (Takeda et al., 1995). *Ex vivo* perfused rat heart also contains corticosterone and aldosterone and releases them into the perfusate (Silvestre et al., 1998). Studies in which rat heart homogenate was incubated with radiolabeled deoxycorticosterone disagree on the presence of CYP11B1 and CYP11B2 activity (Ohtani et al., 2007; Silvestre et al., 1998). In mice, the heart contains mRNA for some steroidogenic enzymes, but not mRNA for CYP11B1 and CYP11B2, arguing against corticosteroid production (Young et al., 2001). The chicken heart appears not to have steroidogenic capacity, because several steroidogenic enzyme activities are not detectable (Lechner et al., 2001).

Local steroid levels. To measure cardiac aldosterone production in humans, studies have compared blood collected from the cardiac vein or coronary sinus (draining from heart muscle) versus blood collected from the aorta. In healthy subjects, plasma aldosterone levels do not differ

between these locations, but in subjects with heart failure or hypertension, plasma aldosterone levels are higher in the cardiac vein than the aorta (Mizuno et al., 2001; Yamamoto et al., 2002). These data suggest that the heart produces aldosterone during cardiovascular disease. Other investigators have found a different pattern: plasma aldosterone levels in the coronary sinus are lower than those in the aorta in healthy subjects, while plasma aldosterone levels are similar in the coronary sinus and aorta in subjects with heart failure (Tsutamoto et al., 2000). This pattern is more difficult to interpret but suggests differential aldosterone synthesis, metabolism or uptake with heart failure.

In adrenal-intact rats, corticosterone and aldosterone levels in the heart tissue closely parallel those in plasma under varying conditions of salt intake (Gomez-Sanchez et al., 2004). In adrenalectomized rats, aldosterone (but not corticosterone) is detectable in heart tissue from 30% of subjects, but is not detectable in plasma, suggesting local production (Gomez-Sánchez et al., 2004). However, aldosterone levels in the rat heart are not increased by systemic treatment with 11-deoxycorticosterone (the precursor of aldosterone) (Gomez-Sánchez et al., 2004), indicating that precursor availability is not rate limiting. Taken together, these studies suggest that mammalian blood vessels and possibly heart produce aldosterone under certain conditions. Importantly, however, the variability in results among laboratories clearly suggests that this production is minimal and may occur only in specific contexts, subjects or anatomical locations. This issue remains controversial (Funder, 2004).

1.7.2 Regulation

Cardiovascular aldosterone production appears to be minimal under normal conditions but might increase under pathological conditions, such as heart failure. Under pathological

conditions, the regulators of adrenal aldosterone production can also be produced locally in the cardiovascular system and possibly regulate local aldosterone synthesis (Table 1.2). For example, the neonatal and adult rat hearts express the mRNA for renin and angiotensinogen (Dostal et al., 1992; Passier et al., 1996), and levels of these transcripts in the adult rat heart increase after experimental myocardial infarction (Passier et al., 1996). Angiotensin II protein and aldosterone levels also increase in the rat heart after experimental myocardial infarction (Silvestre et al., 1999). Taken together, these data raise the possibility that renin and angiotensin II of local origin regulate, at least in part, local aldosterone synthesis. Moreover, in pigs, the majority of angiotensin I and II in the heart is made locally, rather than taken up from the blood (van Kats et al., 1998). In humans, ACE activity has been detected in the heart, particularly during heart failure (Falkenhahn et al., 1995). Thus, mediators of the classic RAS are expressed locally in the cardiovascular system and may contribute to regulation of local aldosterone production (MacKenzie et al., 2002).

1.7.3 Function

Aldosterone synthesis by the heart is minimal or absent in healthy individuals. Under normal conditions, these low levels of local aldosterone synthesis are probably insufficient to affect sodium and water reabsorption by the kidney, and thus would not affect blood volume and blood pressure by this mechanism. In cases of pathophysiology, when local aldosterone synthesis might increase, chronic production of local aldosterone could paradoxically exacerbate heart problems (Table 1.3). Aldosterone acts directly upon the heart to stimulate fibrosis and left ventricular hypertrophy (MacKenzie et al., 2002), which increase the risk of heart failure. This effect is independent of any change in blood pressure. A pathological role of local aldosterone

synthesis is consistent with the observation that mineralocorticoid receptor antagonist treatment dramatically lowers human mortality from heart failure, even when circulating aldosterone levels are normal (Pitt et al., 1999). Other mechanisms can explain this finding without the need to invoke local aldosterone synthesis, such as retention of circulating, adrenal-derived aldosterone in cardiac tissue (Funder, 2004), but this possibility is unlikely (Chai et al., 2010).

1.8 Common themes

1.8.1 Locally synthesized glucocorticoids

One emerging theme is that local glucocorticoid synthesis occurs in immunologically important tissues. The thymus and bursa are critical sites of lymphocyte development, and the intestine and skin contain large numbers of immune cells. All of these are sites of exposure to antigen and lymphocyte activation. Locally-produced glucocorticoids in lymphoid organs, intestine, and skin antagonize signals that promote lymphocyte activation or proliferation, thus acting to prevent lymphocyte over-responsiveness. This role of locally-synthesized glucocorticoids is similar to a key role of circulating glucocorticoids in response to an immune challenge (Dhabhar, 2009; Sapolsky et al., 2000). The lung, another immunologically important barrier, may also synthesize glucocorticoids (Pazirandeh et al., 1999; Provost et al., 2005).

Local glucocorticoid synthesis appears to be independent of, or even in contrast to, patterns of adrenal glucocorticoid synthesis. For example, lymphoid glucocorticoid synthesis is high in early development and decreases over time, while adrenal glucocorticoid synthesis is low in early development and increases over time (Qiao et al., 2008; Schmidt and Soma, 2008; Vacchio et al., 1994). Similarly, in the intestine, ACTH suppresses local glucocorticoid synthesis, although ACTH stimulates adrenal glucocorticoid synthesis (Mueller et al., 2007). In the skin,

local glucocorticoid synthesis is regulated by a local HPA axis analog (Ito et al., 2005) and is induced by local tissue damage (Vukelic et al., 2011).

Local glucocorticoid synthesis in primary lymphoid organs, intestine and skin might have evolved as an adaptive mechanism to allow for localized action of glucocorticoids where and when they are needed, without incurring the costs of exposing all tissues to high glucocorticoid levels. Similar compartmentalization, or “Balkanization,” of steroid synthesis is seen in seasonally-breeding birds (Schmidt et al., 2008). Breeding male song sparrows (in spring) have high systemic testosterone levels, while nonbreeding males (in winter) have low systemic testosterone levels (Wingfield et al., 1994). Both breeding and nonbreeding males must aggressively defend a territory, which is critical for survival and reproduction. In nonbreeding males, local synthesis of sex steroids in the brain is upregulated to support the expression of aggression, while avoiding the costs of high systemic testosterone levels during this season (Pradhan et al., 2010; Soma, 2006).

1.8.2 Locally synthesized mineralocorticoids

Local synthesis of mineralocorticoids occurs in the brain and might occur in the heart, although cardiac production of aldosterone remains controversial. Studies in lymphoid organs and intestine have not examined aldosterone or its synthetic enzyme, CYP11B2, and this would be a useful goal for future research. Lymphoid organs contain few mineralocorticoid receptors, making aldosterone an unlikely product (Miller et al., 1990; Schmidt et al., 2008), but the possibility has not been ruled out.

In the brain and heart, local aldosterone synthesis might be regulated by local expression of upstream RAS mediators. In brain, aldosterone synthesis is increased in response to low salt

intake, in parallel with adrenal aldosterone synthesis. However, systemic treatment with angiotensin II has no effect on brain aldosterone synthesis, suggesting that brain aldosterone synthesis is independent of systemic RAS mediators and might depend on local expression of RAS mediators. Brain aldosterone synthesis plays an important role in systemic blood pressure regulation, as brain-specific inhibition of aldosterone synthesis reversibly decreases blood pressure (Gomez-Sanchez et al., 2010).

While locally-synthesized glucocorticoids have anti-inflammatory functions and serve to minimize tissue damage, local aldosterone synthesis in the brain and heart drives hypertension and heart failure, exacerbating tissue damage. Local aldosterone synthesis in healthy individuals may occur at low levels that allow beneficial effects of locally-elevated mineralocorticoid levels, while avoiding the costs of high systemic aldosterone levels (e.g., high systemic blood pressure). For the brain in particular, the blood-brain barrier only allows minimal uptake of circulating aldosterone (Geerling and Loewy, 2009), suggesting that it is important to minimize the effects of systemic aldosterone on the brain or that it is important for brain aldosterone levels to be regulated independently of systemic aldosterone levels.

1.9 Conclusions

The accumulated mass of evidence (Table 1.1) demonstrates that *de novo* corticosteroid synthesis occurs outside of the adrenal cortex. The evidence is heavily weighted towards PCR studies of steroidogenic enzyme mRNA and corticosteroid synthesis *in vitro*. However, some studies have also measured local endogenous corticosteroid levels in adrenal-intact as well as adrenalectomized subjects (Croft et al., 2008; Gomez-Sánchez et al., 2004; Gomez-Sánchez et al., 2005; Little et al., 2008; Schmidt et al., 2008; Schmidt and Soma, 2008). Together, these

different lines of evidence show that locally elevated steroid concentrations cannot simply be accounted for by the accumulation of circulating adrenal steroids. Furthermore, recent studies have used steroidogenic enzyme inhibitors *in vivo* to decrease local steroid synthesis in adrenal-intact subjects (Gomez-Sanchez et al., 2010; Huang et al., 2008; Qiao et al., 2009). These studies have convincingly demonstrated that local corticosteroid synthesis has functional consequences and is physiologically relevant. The effects of locally-synthesized steroids likely depend on high local steroid concentrations, either within an entire target organ (**Fig. 1.2**) or on a smaller scale (such as at the neuronal synapse). Thus, at level of the receptors, local corticosteroid concentrations can be far higher than those of systemic corticosteroids synthesized by distant adrenal cortices. Furthermore, most circulating glucocorticoids (90-95%) are bound with high affinity to corticosteroid-binding globulin and are unavailable to bind receptors.

In addition to the tissues discussed above, corticosteroid synthesis *de novo* from cholesterol might also occur in the lung (Pazirandeh et al., 1999; Provost and Tremblay; 2005), retina (Zmijewski et al., 2007), and kidney (Xue and Siragy, 2005). In addition to *de novo* synthesis, bones, joints, liver, muscle and fat express 11 β -HSD type I and can convert circulating inactive glucocorticoid metabolites (e.g. cortisone) into active glucocorticoids (Raza et al., 2010) (Figure 1.1). Also, tissues expressing only CYP11B1 or CYP11B2 can convert circulating precursors (e.g. 11-deoxycorticosterone) into glucocorticoids or mineralocorticoids, respectively. Overall, it is clear that the circulating systemic levels of cortisol, corticosterone and aldosterone need not (and very often do not) reflect the local levels of these steroids at crucial target tissues.

Clinically, the local administration of glucocorticoids (e.g., via inhaler, topical application, injection into joints) can be advantageous over systemic glucocorticoid administration, which has numerous side effects throughout the body. This practice mimics the

endogenous local synthesis of corticosteroids by various organs. Knowledge about these natural physiological processes may inform therapies that target corticosteroids to specific tissues or stimulate endogenous local corticosteroid synthesis at specific sites.

Compared with adrenal corticosteroids, locally-produced corticosteroids can be synthesized by different enzymes (Kishimoto et al., 2004), can differ in identity (e.g., corticosterone versus cortisol; Lechner et al., 2001, Schmidt and Soma, 2008), can bind differentially to receptors (Schmidt et al., 2010), can be differentially regulated (Mueller et al., 2007), and have greater spatial specificity. These differences allow locally-synthesized corticosteroids to complement the functions of systemic, adrenal-synthesized corticosteroids.

1.10 Objectives

In this thesis, I present three studies examining tissue-specific regulation of glucocorticoid levels in lymphoid organs.

In Chapter 2, I measured endogenous levels of glucocorticoids and their precursors over avian development, in both precocial and altricial birds, to test whether developmental strategy and corresponding systemic glucocorticoid levels are related to local glucocorticoid regulation in lymphoid organs. While precocial hatchlings had limited evidence for tissue-specific glucocorticoid regulation, altricial hatchlings had dramatically elevated lymphoid glucocorticoid concentrations. Furthermore, the major glucocorticoid in altricial hatchling lymphoid organs was cortisol, in contrast to the major adrenal glucocorticoid, corticosterone. These data suggest altricial offspring may use unique local glucocorticoids in lymphoid development, and that this may correspond with differential responsivity of immune glucocorticoid levels to environmental conditions.

In Chapter 3, I measured endogenous levels of glucocorticoids and their precursors over mouse development, to see whether local regulation was widespread across glucocorticoid-sensitive tissues. Local glucocorticoids in lymphoid and other organs were highly tissue-specific: local glucocorticoid levels could be much higher than circulating glucocorticoid levels, local glucocorticoid identities differed from circulating glucocorticoid identities, and local glucocorticoid levels changed independently of circulating glucocorticoid levels. This study suggests that glucocorticoid concentrations and therefore signaling may be largely regulated by individual tissues, especially during development. Furthermore, the major glucocorticoid in neonatal lymphoid organs was cortisol, in contrast to the major adrenal glucocorticoid in these species, corticosterone, suggesting that unique lymphoid glucocorticoid identities may occur across a range of species.

In Chapter 4, I explored the mechanism of tissue-specific glucocorticoid regulation in mouse lymphoid organs. Lymphoid organs exhibited gene expression and enzyme activity of 11 β -HSD1, and not CYP11B1, suggesting that local glucocorticoid upregulation occurs largely via regeneration of inactive glucocorticoid metabolites (i.e., dehydrocorticosterone). Furthermore, this glucocorticoid production was higher in lymphoid organs of adults, indicating that local upregulation increases with age. These data show that the bone marrow and spleen, like the thymus, can produce their own glucocorticoids, and that this glucocorticoid production may play an important role throughout life.

These studies support a growing body of data showing steroids, rather than functioning only as systemic orchestrators of physiology, can also be tightly regulated in tissue-specific manner, and may have widespread functions as paracrine or autocrine signals. This local regulation appears to be largely due to regeneration of glucocorticoid metabolites. The finding of

glucocorticoid regeneration in the bone marrow and spleen, suggests that glucocorticoids, in addition to facilitating production of functional T cells in the thymus, may also be important in production of B cells and other hematopoietic cell lineages in the bone marrow and spleen.

1.11 Tables and figures

Table 1.1: Evidence for and against local synthesis of glucocorticoids and mineralocorticoids

| | | Lymphoid | | Intestine | | Skin | | Brain | | Cardiovascular | |
|------------------------------|------------------|--------------------|----|-------------------------------|---|----------------|----|---------------------------|------------------|--------------------------------------|------------------------------|
| | | + | - | + | - | + | - | + | - | + | - |
| <i>Steroidogenic enzymes</i> | | | | | | | | | | | |
| StAR | mRNA | 10, 75, 76 | | | | | | 17, 78 | | 8, 9, 120 | |
| | Protein Activity | | | | | 99 | | 17 | | | |
| | | | | | | | | 17, 78 | | | |
| CYP11A1 | mRNA | 10, 47, 69, 75, 76 | | 3, 11, 13, 42, 57, 58, 61, 62 | | 94, 99, 106 | | 12, 17, 78 | | 9, 41, 103, 120 | 35, 47 |
| | Protein Activity | 110 | | 11 | | 99, 106 | | 12, 17 | | | |
| | | 46, 47, 110 | | | | 99, 106 | | 12, 17, 78 | | | 46, 47 |
| 3 β -HSD | mRNA | 10, 75 | | 11, 42 | | 19 | | 12, 17, 78 | | 35, 41, 103, 120 | 9 |
| | Protein Activity | 46, 47, 110 | | | | 19 | | 12, 17 | | | 46, 47 |
| CYP17 | mRNA | 75 | 47 | | | 94 | | 12, 17, 78 | 17 | 9 | 41, 47, 120 |
| | Protein Activity | 46 | 47 | | | | | 12, 17 | 17 | | 46, 47 |
| | | | | | | | | 17, 78 | | | |
| CYP21 | mRNA | 10, 69, 75 | | 11 | | 94 | | 53, 81 | 5, 101, 121 | 45, 53 | 9, 35, 41 |
| | Protein Activity | 46, 47, 110 | | | | 81, 95 | | | 45 | | 53 |
| CYP11B1 | mRNA | 10, 47, 69, 75, 76 | | 3, 11, 13, 57, 58, 61, 62 | | | | 27, 51, 54, 101, 118, 121 | 101 | 1, 9, 27, 41, 64, 91, 102, 103, 120 | 29, 34, 35, 47, 51, 117, 120 |
| | Protein Activity | 69, 110 | | | | | | 51 | | | |
| | | 46, 47 | | 11, 42 | | 38, 95–98 | | 27 | | 1, 91 | 46, 47, 64 |
| CYP11B2 | mRNA | | | | | | | 26, 51, 101, 118, 121 | 54, 101 | 1, 26, 34, 35, 41, 91, 102, 103, 120 | 1, 9, 29, 51, 64, 117, 120 |
| | Protein Activity | | | | | | 95 | 51 | 26 | 91, 102, 103 | 64 |
| <i>Local steroid levels</i> | | | | | | | | | | | |
| Glucocorticoids | | | | | | | | | | | |
| Tissue > plasma | | 85, 88 | | | | | | 14, 48, 88 | 30 | | 29 |
| Present postadrenalectomy | | | | | | | | 14 | 30 | | 29 |
| Synthesis in vitro | | 10, 69, 75, 76 | | 3, 11, 13, 57, 58, 61, 62 | | 38, 95, 97, 98 | | | 90, 91, 102, 103 | | 64 |
| Mineralocorticoids | | | | | | | | | | | |
| Tissue > plasma | | | | | | | | 30 | | | 29 |
| Present postadrenalectomy | | | | | | | 95 | 30 | | | |
| Synthesis in vitro | | | | | | | 26 | 29 | 34, 90, 91, 102 | 1, 64 | |

Nos. are references. +, detected; -, not detected. Note: this table does not account for important factors such as species, age, sex, season, etc.

Reference numbers can be found in Taves et al., 2011a (Appendix A).

Table 1.2: Regulation of local glucocorticoid and mineralocorticoid synthesis

| | Lymphoid | Intestine | Skin | Brain | Cardiovascular |
|---------------------|--|---|--|---|---|
| Stimulatory Signals | Glucocorticoids • ACTH (acting on thymic epithelial cells) • Testosterone (acting on thymocytes) | Glucocorticoids • T cell activation (T_{H1}) • Macrophage activation • TNF α • LRH-1 • Kinase activation • LPS | Glucocorticoids • UV radiation • CRH • ACTH | Glucocorticoids • Alcohol withdrawal • Social defeat • Restraint • Saline perfusion • ACTH | Mineralocorticoids • Heart failure • Angiotensin II • ACTH |
| Inhibitory Signals | • ACTH (acting on thymocytes) • cAMP (acting on thymocytes) | • ACTH • cAMP • SF-1 | • Glucocorticoids (negative feedback) • IGF-1 | Glucocorticoids • unknown | • unknown |
| References | 10, 69, 75, 76, 88, 110 | 3, 11, 13, 57, 58, 61, 62 | 38, 82, 94, 96, 97, 105, 112 | 14, 48, 52, 59, 88, 104, 118, 119 | 18, 20, 52, 67, 90, 91, 111 |

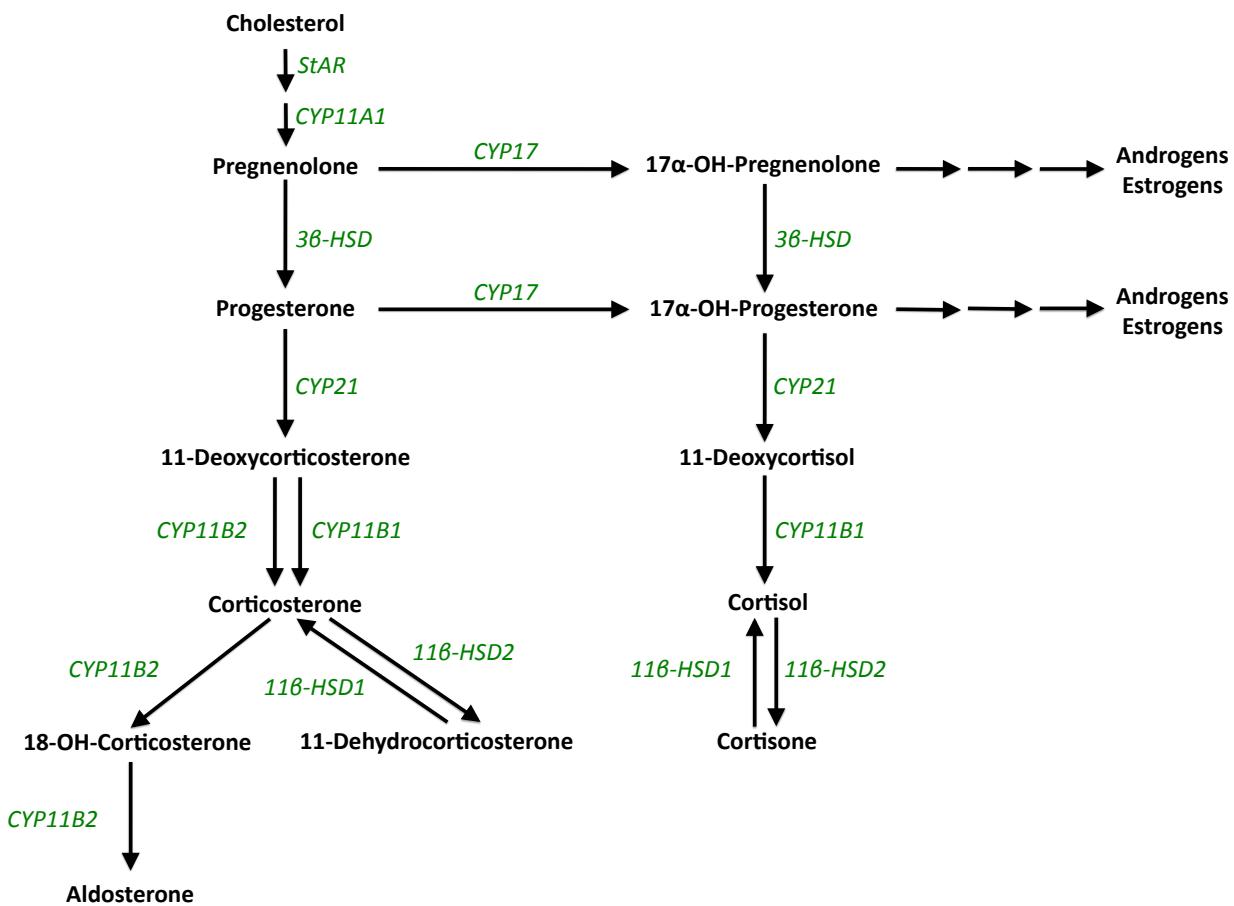
Reference numbers can be found in Taves et al., 2011a (Appendix A).

Table 1.3: Possible functions of locally synthesized glucocorticoids and mineralocorticoids

| | Lymphoid | Intestine | Skin | Brain | Cardiovascular |
|----------------|--|--|--|---|--|
| Local function | Glucocorticoids •Antagonism of T cell receptor signal •Promote thymocyte apoptosis •B cell development? | Glucocorticoids •Downregulate $T_{H}1$ immune response and inflammation •Allow tissue repair •Prevent induction of autoimmune response to commensal gut bacteria? | Glucocorticoids •Local stress response to different environmental stimuli •Regulation of inflammatory response •Regulation of skin immune response? | Glucocorticoids •Drug-seeking behavior? •Submissive behavior? •Anti-predator behavior? Mineralocorticoids •Regulation of blood pressure, systemic sodium balance | Mineralocorticoids •Collagen deposition •Regulation of blood pressure and systemic sodium balance? |
| References | 24, 44, 60, 68, 70, 86, 109, 110 | 13, 61–63 | 16, 93, 112 | 25, 28, 31, 32, 36, 37, 52, 83, 84 | 52, 71 |

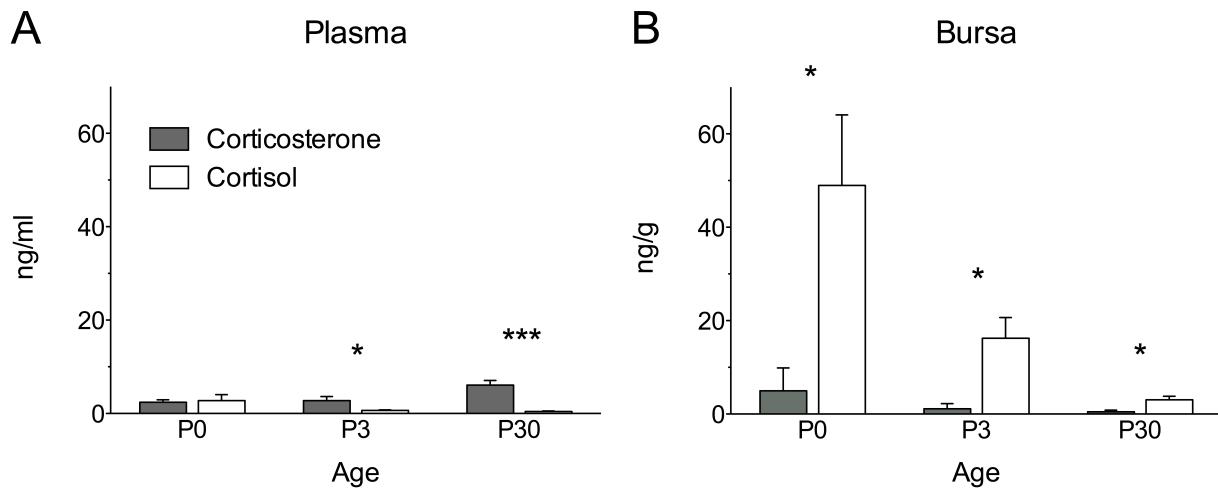
Reference numbers can be found in Taves et al., 2011a (Appendix A).

Figure 1.1 Simplified diagram of the classical corticosteroid synthetic pathway



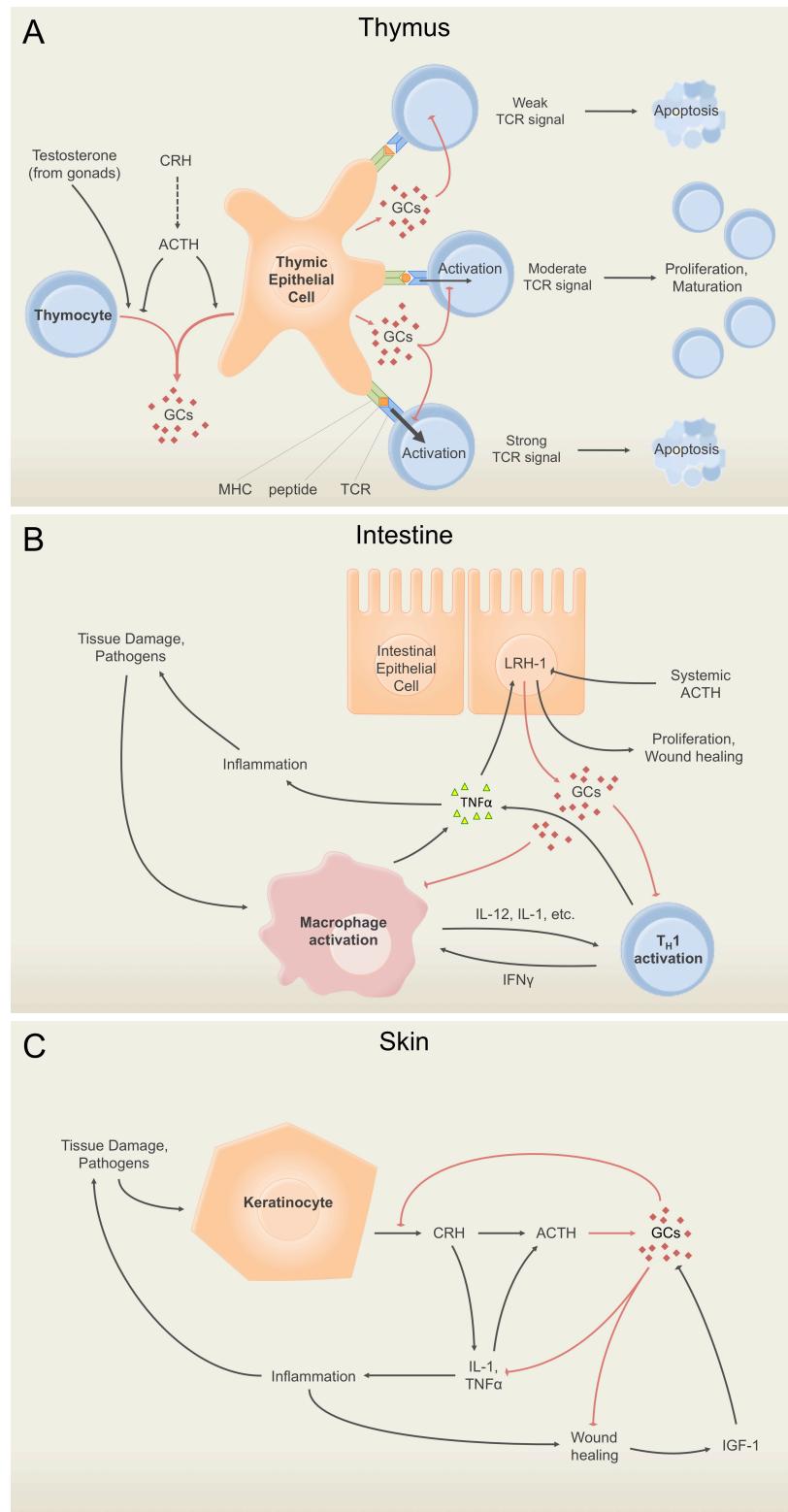
Steroid names are bolded, and steroidogenic enzyme gene names are italicized in green. *StAR* (steroidogenic acute regulatory protein) is required for translocation of cholesterol from the outer to the inner mitochondrial membrane, where **CYP11A1** (P450 side-chain cleavage or P450scc) catalyzes the conversion of cholesterol to pregnenolone. Further steroid conversions are performed by **3 β -HSD** (3β -hydroxysteroid dehydrogenase/ $\Delta 5$ - $\Delta 4$ -isomerase), **CYPs** (17α -hydroxylase/17,20-lyase or P450c17), **CYP21** (21-hydroxylase or P450c21), **CYP11B1** (11β -hydroxylase or P450c11 β), **CYP11B2** (aldosterone synthase or P450c11AS), **11 β -HSD** (11β -hydroxysteroid dehydrogenase) **types 1 and 2**.

Figure 1.2 Baseline endogenous glucocorticoid levels in plasma and lymphoid organs of developing zebra finches.



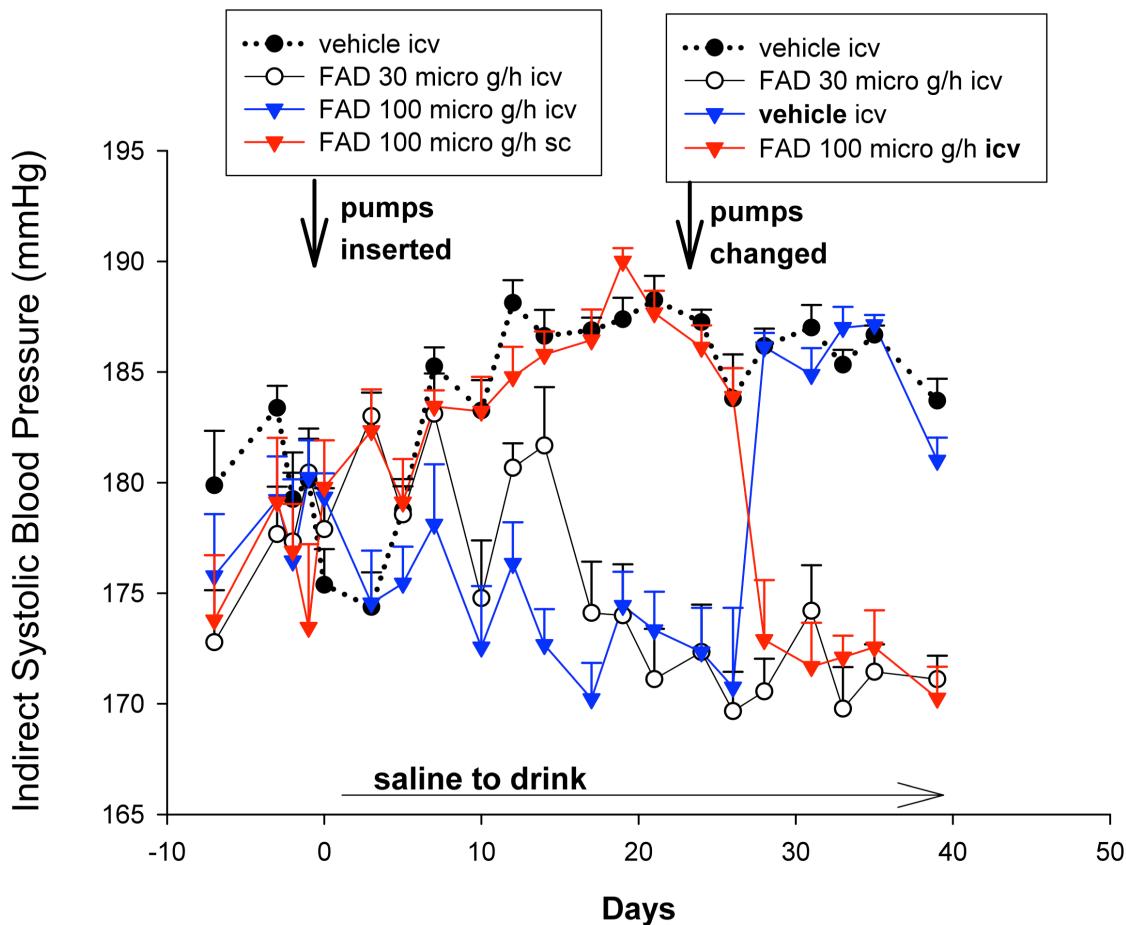
Baseline endogenous glucocorticoid levels in **A**) plasma and **B**) bursa of Fabricius (bursa) of developing zebra finches, at ages 0, 3, and 30 days posthatch. The bursa is the site of B lymphocyte development, similar to mammalian bone marrow. Adapted from Schmidt et al. (2008).

Figure 1.3 Regulation and function of local glucocorticoid synthesis in the thymus, intestine, and skin.



A) In the thymus, glucocorticoids and T cell receptor signaling each function independently as pro-apoptotic stimuli in developing thymocytes. Glucocorticoids also antagonize T cell receptor signaling, and alter the threshold of T cell receptor affinity for self peptide:MHC that results in survival and proliferation versus apoptosis. **B)** In the intestine, activated macrophages and $T_{H}1$ cells secrete the pro-inflammatory cytokine $TNF\alpha$, which upregulates the transcription factor LRH-1 in epithelial cells of the intestinal crypt. LRH-1 induces local production of glucocorticoids, which downregulate immune cell activation and resulting inflammation. **C)** Activation of keratinocytes (and other types of skin cells) results in production of CRH, which stimulates production of ACTH and pro-inflammatory cytokines such as $IL-1\beta$ and $TNF\alpha$. ACTH then stimulates local synthesis of glucocorticoids, which inhibit further production of CRH and pro-inflammatory cytokines, thus downregulating inflammation. Abbreviations: *ACTH* = *adrenocorticotrophic hormone*, *CRH* = *corticotropin-releasing hormone*, *glucocorticoids* = *glucocorticoids*, *IFN γ* = *interferon- γ* , *IGF-1* = *insulin-like growth factor 1*, *IL-1 β* = *interleukin 1 β* , *IL-12* = *interleukin 12*, *LRH-1* = *liver receptor homolog-1*, *MHC* = *major histocompatibility complex*, *TCR* = *T cell receptor*, *T_H1* = *Type I helper T cell*, *TNF α* = *tumor necrosis factor alpha*.

Figure 1.4 Effects of local versus systemic CYP11B2 inhibition on blood pressure in the Dahl salt-sensitive rat.



Effects of FAD286 (FAD, an inhibitor of CYP11B2 activity and aldosterone synthesis) on systolic blood pressure. Intracerebroventricular (icv) but not subcutaneous (sc) FAD administration decreases systolic blood pressure in hypertensive Dahl salt-sensitive rats. Cross-over treatment demonstrates the reversibility of this effect. Importantly, all subjects were adrenal-intact, and circulating aldosterone levels were similar in all groups at the conclusion of the experiment. Reprinted from Gomez-Sanchez et al. (2010).

2 Locally elevated cortisol levels in lymphoid organs of developing altricial but not precocial birds

2.1 Introduction

Animals exhibit dramatic variation along a spectrum of developmental strategies, ranging from undeveloped altricial offspring that are completely dependent on their parents, to developed and independent precocial offspring (Ricklefs, 1998). Both altricial and precocial offspring rely heavily on innate immunity and maternal antibodies for protective immunity in early life, because lymphocytes that provide antigen-specific immunity are not yet mature and functional (birds, (Davison et al., 2011; Klasing and Leshchinsky, 1999); mammals (Levy, 2007; Pilorz et al., 2005). A large proportion of lymphocytes are produced during early life (Adkins et al., 2004; Davison et al., 2011), creating a critical period during which environmental conditions can have long-term programming effects on adaptive immunity (Hodgson and Coe, 2006). The mechanisms underlying this developmental programming, however, are largely unknown.

Glucocorticoids are steroid hormones (Figure 1.1) that mediate environmental effects on immunity (Martin, 2009) and are critical for the production of functional, appropriately reactive lymphocytes (Mittelstadt et al., 2012). Circulating glucocorticoids in the blood may not be sufficient for development of competent lymphocytes, as mouse pups and songbird nestlings (like altricial neonates of other species) experience a stress hyporesponsive period (SHRP) during which circulating glucocorticoids are extremely low and unresponsive (Schmidt et al., 2003; Wada, 2008). However, murine lymphoid organs synthesize glucocorticoids (Lechner et

al., 2000; Pazirandeh et al., 1999; Vacchio et al., 1994), resulting in locally elevated glucocorticoid levels in these organs (Taves et al., 2015). Interestingly, murine lymphoid organs maintain high levels of cortisol, as well as the predominant circulating murine glucocorticoid, corticosterone (Taves et al., 2015). High cortisol levels are also seen in lymphoid organs (thymus, bursa of Fabricius, and spleen) of an altricial bird, the zebra finch (Schmidt and Soma, 2008), where the predominant circulating avian glucocorticoid is also corticosterone. Together, these findings suggest potentially distinct roles of cortisol and corticosterone in lymphoid organs of altricial young.

Precocial offspring, in contrast, have responsive circulating glucocorticoids (Brown and Spencer, 2013; Starck and Ricklefs, 1998), which may be sufficient to support normal lymphocyte production. Circulating glucocorticoids of precocial offspring, like circulating glucocorticoids of adults, are highly reactive to environmental conditions. Chronically increased or decreased glucocorticoid levels in the blood could alter lymphocyte development, resulting in lymphocytes with corresponding increased or decreased reactivity. In this way, changes in circulating glucocorticoids might be a mechanism by which environmental conditions program adaptive immunity in precocial but not altricial offspring. Alternatively, lymphoid organs of precocial chickens can synthesize cortisol *in vitro* (Lechner et al., 2001), indicating that precocial offspring may also require locally-elevated glucocorticoid levels for development of functional lymphocytes.

Here, we aimed to determine whether lymphoid glucocorticoids are locally elevated (relative to circulating glucocorticoids) during post-hatch immune development in both altricial and precocial offspring, or whether lymphoid glucocorticoid levels are locally elevated only in altricial but not precocial offspring. We measured two endogenous glucocorticoids:

corticosterone, the predominant circulating avian glucocorticoid, and cortisol, a glucocorticoid classically thought to be absent in birds, but which we have found in avian lymphoid organs (Schmidt and Soma, 2008). Locally elevated lymphoid glucocorticoid levels (in lymphoid organs relative to circulating blood) across species would suggest that maintenance of stable glucocorticoid concentrations is critical for lymphocyte development, while local levels similar to circulating levels would suggest much greater influence of environmental conditions. Furthermore, we also quantified glucocorticoid precursors, 11-deoxycorticosterone (deoxycorticosterone), 11-deoxycortisol (deoxycortisol), and progesterone. The presence of locally elevated glucocorticoids together with their precursors suggests that local synthesis from upstream precursors could play a major role in local glucocorticoid elevation (Taves et al., 2011a; Taves et al., 2015)). In addition, these steroids can independently bind to and regulate activity of steroid receptors (Gomez-Sanchez, 2014). Samples were collected from the altricial Zebra finch (*Taeniopygia guttata*), and two precocial birds, the Japanese quail (*Coturnix coturnix japonica*) and the chicken (*Gallus gallus domesticus*). The comparative study of altricial and precocial development has focused in large part on birds, making them well suited to explore the role of steroids in immune development. Furthermore, the developmental effects of glucocorticoids have been extensively investigated in these three domestic species, making them especially useful models (Schoech et al., 2011).

We examined birds as hatchlings and as juveniles, as lymphocyte production is greatest in early life (Orkin and Zon, 2008) and lymphoid organs regress with age (Davison et al., 2011; Glick, 1956). For both quail and chicken we included two strains, to look for differences both within species and across species. Because lymphocyte selection is extremely stringent to achieve immunocompetence while avoiding autoimmunity, we hypothesized that both altricial

and precocial birds would have locally elevated glucocorticoids and glucocorticoid precursors in developing lymphoid organs relative to levels in circulating blood.

2.2 Materials and methods

2.2.1 Subjects

Zebra finches, like other altricial young, are born naked, immobile, with closed eyes, and with immature tissues, and parents provide food, warmth, and protection against predators and parasites (Starck and Ricklefs, 1998; Zann and Bamford, 1996). Altricial development (perhaps in part due to the SHRP) facilitates rapid posthatch growth (Ricklefs, 1979; Wada, 2008; Wada et al., 2009). Due to immature sensory systems (Herrmann and Bischof, 1988), immature physiological responses to stimuli (Wada et al., 2009) and buffering of environmental conditions by parents (Lindström, 1999), altricial hatchlings have limited interactions with environments beyond the nest. Parents even engage in behaviors to protect the nest from parasites (Petit et al., 2002).

Zebra finches in this study were from a captive colony maintained at the Advanced Facility for Avian Research at the University of Western Ontario. Finches were housed on a 14 hr : 10 hr light:dark cycle, and had ad libitum access to grit, cuttlefish bone, water, and seed (11% protein, 6% lipid; Living World premium finch seed). Breeding pairs were given a nest box, and received daily supplements of hardboiled eggs, cornmeal, and bread. Hatchling finches were collected from nest boxes on the day of hatch (P0, or posthatch day 0), and juveniles (still housed with parents) were collected at P30, which is approximately one-third of the age of sexual maturity (gonads mature at approximately P90).

Japanese quail and chickens have relatively precocial young, which are born feathered, mobile, with open eyes, and able to actively forage for themselves ((McNabb and McNabb, 1977; Nichelmann and Tzschenke, 2002; Ottinger, 2001). These functional physiological systems come at the cost of reduced post-hatch growth rates (Ricklefs, 1979). Precocial offspring experience little or no post-hatch SHRP; they have a functioning glucocorticoid response and they are responsive to environmental stimuli (Brown and Spencer, 2013; Freeman and Manning, 1984). While they may still be led to food, brooded, and protected by their parents, precocial offspring are immediately exposed to environmental conditions beyond the nest that immediately impact their survival.

Japanese quail in this study were from pure lines maintained in a UBC animal facility at the Agassiz Pacific Agri-Food Research Centre (Silversides et al., 2013). We looked at two strains of Japanese quail: the first strain (UBC line) is a long-domesticated line closed in 1968, while the second strain was derived from feral birds captured in Hawaii in 1981 (HAW line). Quail were housed on a 12 hr : 12 hr light:dark cycle, and received a nutritionally complete diet containing 2900 kcal/kg metabolizable energy and 24% crude protein and water to allow for ad libitum consumption. Hatchling (P0) quail were collected from a 37 °C incubator, and group-housed juveniles were kept in a 33 °C brooder and collected P14, which is approximately one-third of the age of sexual maturity.

Chickens in this study were from pure lines maintained at the same facility at the Pacific Agri-Food Research Centre (Silversides et al., 2007). We looked at two breeds of chickens both bred for egg laying, the White Leghorn and the Rhode Island Red. These breeds have been previously used to investigate bursa of Fabricius (hereafter bursa) development; the bursa is larger at hatch and grows faster in the White Leghorn than the Rhode Island Red (Glick, 1956).

Chickens were housed on a 12 hr : 12 hr light:dark cycle, and received a nutritionally complete diet containing 2800 kcal/kg and 18.5% crude protein and water to allow for ad libitum consumption. Hatchling (P0) chicks were collected from a 37 °C incubator, and group-housed juveniles were kept in a 33 °C brooder and collected at P42, which is approximately one-third of the age of sexual maturity.

For all three species, no subject was ever housed in isolation, and no subjects received antibiotics or vaccines. Egg incubation periods for these species are 12-15 d (zebra finch), 17-18 d (Japanese quail), and 21 d (chicken). Sex was determined by visual inspection of the gonads except for hatchling finches, in which case sex was determined using PCR as previously described (Soderstrom et al., 2007). The number of subjects collected for each species, strain, and sex is given in Table 2.1. Protocols followed approved institutional guidelines (UBC protocol A09-0395, UWO protocol 2007-089, and Agassiz Research Centre protocol P1101), and were in compliance with regulations established by the Canadian Council on Animal Care.

2.2.2 Tissue collection

Finch hatchling blood samples were collected by cardiac puncture within 3 min of researchers approaching the cage, and blood was stored on wet ice. Brachial blood samples from P30 finches were also collected within 3 min, into two tubes, one kept as whole blood and the other centrifuged for separation of plasma. Due to small sample volume, only whole blood was collected from P0 finches to optimize the chance of steroid detection. Immediately after blood collection, subjects were sacrificed by rapid decapitation and tissues (thymus, bursa of Fabricius, and spleen) were dissected and frozen on dry ice. All samples (plasma, whole blood, and lymphoid organs) were stored at -80 °C.

For quail and chickens, subjects were sacrificed by rapid decapitation, trunk blood was collected into two tubes within 3 min of researchers approaching the cage, and blood was stored on wet ice. One tube was kept as whole blood, and the other centrifuged for separation of plasma. Immediately after blood collection, tissues (thymus, bursa, and spleen) were dissected and frozen on dry ice. All samples were stored at -80 °C.

2.2.3 Steroid extraction

Steroids were extracted from all samples using solid-phase extraction (SPE) with C₁₈ cartridges (Newman et al., 2008; Taves et al., 2010; 2015). Briefly, samples were measured to the closest µl (plasma, blood) or the closest 0.1 mg (thymus, bursa, spleen), and transferred into a chilled 5-ml tube containing five 2.3 mm ceramic beads. The maximum tissue sample size was 250 µl or mg; larger tissues were bisected until a portion under this size was obtained. All samples were then diluted with 19 volumes of 84% methanol and homogenized using an Omni Bead Ruptor. Homogenates were incubated overnight at 4 °C, and the following day supernatants (up to 1 ml) were diluted with 10 ml water. Cartridges (Agilent #12113045) were primed with 3 ml methanol, equilibrated with 10 ml water, and then samples were loaded. Samples were washed with 10 ml 40% methanol, and steroids eluted with 5 ml 90% methanol. All reagents were HPLC-grade. Eluates were dried in a vacuum centrifuge (ThermoElectron SPD111V) at 60 °C.

2.2.4 Steroid separation

Steroids were then separated using reverse-phase high performance liquid chromatography (HPLC, Gilson 322), as previously described (Taves et al., 2015). Briefly, dried steroid residues were resuspended in Solvent A (30% acetonitrile, 0.01% formic acid), run through a Waters SymmetryShield C₁₈ column (4.6 × 250 mm), and eluted at a flow rate of 1.0 ml/min over 45 min.

Elution began with 100% Solvent A, with a linear increase to 100% Solvent B (100% acetonitrile, 0.01% formic acid) from 20 to 45 min. This run time resulted in clean separation of our steroids of interest, which were collected in 3-min fractions. Other steroids did not co-elute with our steroids of interest, with the exception of cortisone, which co-eluted in the cortisol fraction (Taves et al., 2015). It is unlikely that this affected our cortisol measurements, as we measured cortisol with an antibody that has minimal crossreactivity with cortisone (0.13%, Table 2.3), and as this protocol has been validated for identification of cortisol using liquid chromatography-tandem mass spectrometry (Taves et al., 2015).

2.2.5 Steroid immunoassays

Steroids (Figure 1.1, Table 2.2) were measured in duplicate using specific and sensitive immunoassays, as previously described (Taves et al., 2014). Briefly, progesterone, corticosterone, and cortisol were quantified using commercial immunoassay kits (Table 2.3), and deoxycorticosterone and deoxycortisol were quantified by radioimmunoassay (RIA) using commercially available antibodies (Table 2.3), with charcoal-dextran separation of antibody-bound and free steroids prior to counting in a liquid scintillation counter. For each steroid, recovery was determined by spiking known steroid amounts into tissue samples, and comparing these with unspiked samples from the same tissue pools (Taves et al., 2011b).

2.2.6 Statistical analysis

Nondetectable steroid samples (those below the lowest standard on the standard curve or those lower than the average water blank + two standard deviations, whichever was greater) were set to zero. Log-transformed data were analyzed using linear mixed-effects models in R (R Core

Team, 2005) and Prism 5, with tissue type (e.g. whole blood, bursa) as a within-subjects factor and with sex, age, and strain as between-subjects factors. Planned comparisons were conducted using paired t-tests. Significance was set at $\alpha = 0.05$, and data are shown as mean \pm SEM.

2.3 Results

2.3.1 Lymphoid organ steroid concentrations

We examined subjects at two developmental timepoints, 1) as hatchlings and 2) as juveniles at one third of the age at sexual maturity. Relative body masses are given in Figure 2.5 and Table 2.4. To look for evidence of tissue-specific glucocorticoid regulation, we compared concentrations of steroids in lymphoid organs with those in circulating blood. Locally elevated glucocorticoids, especially together with locally elevated precursors, are consistent with local glucocorticoid synthesis (Taves et al., 2011a).

2.3.1.1 Zebra finch steroids

In the hatchling zebra finch, all steroids were at low concentrations in circulating blood. In contrast, the hatchling bursa and spleen had highly elevated cortisol levels, with cortisol levels 30- and 35-fold higher, respectively, than blood cortisol levels. In contrast, corticosterone, the expected avian glucocorticoid, was not locally elevated (Figure 2.2d, e, Table 2.5). Within lymphoid organs, cortisol was the predominant glucocorticoid, with concentrations nearly an order of magnitude higher than corticosterone concentrations, whereas cortisol and corticosterone concentrations were similar in the blood (Table 2.6). Hatchling lymphoid organs also had locally elevated progesterone and deoxycorticosterone (Figure 2.2a, b, Table 2.5). The

hatchling spleen had near-identical patterns to the hatchling bursa, with locally elevated cortisol but not corticosterone, and cortisol as the predominant glucocorticoid (Figure 2.2d, e, Table 2.5, Table 2.6). Progesterone and deoxycorticosterone were also similarly elevated (Figure 2.2a, b, Table 2.5). Patterns were similar in female and male hatchlings. Thymus could not be collected from hatchling finches, due to its small size.

In juvenile zebra finches, all steroids remained low in circulating blood. As in hatchlings, lymphoid organs (thymus and bursa) had locally elevated cortisol levels, with cortisol levels approximately 4-fold higher than blood cortisol levels. Corticosterone was not locally elevated (Figure 2.2d, e, Table 2.5). In thymus and spleen, cortisol concentrations were between 4- and 5-fold higher than corticosterone concentrations, while in blood cortisol concentrations were less than half of corticosterone concentrations (Table 2.6). Juvenile thymus also had locally elevated progesterone and deoxycortisol (Figure 2.2a, c; Table 2.5). Patterns were similar between female and male juveniles.

2.3.1.2 Japanese quail steroid concentrations

In hatchling Japanese quail, corticosterone was present at higher concentrations (10.5 ± 1.2 ng/ml) than other steroids in circulating blood, and steroid levels were similar between UBC and HAW hatchlings. Hatchling lymphoid organs did not have elevated levels of either cortisol or corticosterone (Figure 2.3d, e), and cortisol concentrations were similar to or lower than corticosterone concentrations in all tissues (Table 2.6). However, all hatchling lymphoid organs had locally elevated levels of the glucocorticoid precursors: progesterone, deoxycorticosterone, and deoxycortisol (Figure 2.3a-c, Table 2.5). These were especially high in the spleen.

In juvenile quail, circulating steroid patterns were similar in UBC and Hawaiian strains. However, there were strain-specific patterns in local glucocorticoid levels. Corticosterone was locally elevated in all lymphoid organs of UBC but not HAW quail juveniles (Table 2.5), while cortisol was similarly elevated in spleen regardless of strain (Figure 2.3e). Corticosterone was higher than cortisol, and the ratio of these steroids was the same across compartments. Juveniles however had limited, and strain-specific, elevation of glucocorticoids precursors, with elevated deoxycorticosterone in the bursa (UBC only) and spleen (HAW only), and deoxycortisol in the bursa of both strains (Figure 2.3b, c, Table 2.5). Progesterone was not elevated in any juvenile tissue (Figure 2.3a).

2.3.1.3 Chicken steroid concentrations

In hatchling chickens, corticosterone was present at higher concentrations (5.7 ± 1.2 ng/ml) than other steroids in the blood, and circulating steroid levels were similar between White Leghorn and Rhode Island Red strains. Hatchling lymphoid organs did not have locally elevated levels of cortisol or corticosterone (Figure 2.4d, e), and corticosterone was clearly the predominant glucocorticoid in hatchling lymphoid organs (Table 2.6). Lymphoid deoxycorticosterone and deoxycortisol were also not locally elevated (Figure 2.4b, c). Progesterone alone was locally elevated in all hatchling lymphoid organs (Figure 2.4a).

In juvenile chickens, the bursa and spleen had locally elevated corticosterone, with levels slightly higher than levels in the blood (Table 2.5), and higher than levels of cortisol (Table 2.6). The thymus, in contrast, had locally elevated deoxycortisol (Figure 2.4c). Progesterone had sex-specific patterns, with locally elevated concentrations in juvenile male but not female chickens

(Table 2.5). Interestingly, relative size of lymphoid organs was larger in hatchling and juvenile chickens than in zebra finches and quail (Figure 2.5b, c).

2.4 Discussion

Here, we compared steroid profiles across altricial and precocial avian development, and found evidence for tissue-specific steroid regulation in lymphoid organs of different avian species. Circulating glucocorticoids were similar in zebra finch hatchlings and juveniles, while circulating glucocorticoids were higher in precocial quail and chicken hatchlings than in juveniles. In contrast, lymphoid organs of altricial finches had local elevation of glucocorticoids and glucocorticoid precursors in lymphoid organs (compared to levels in blood), while lymphoid organs of precocial quail and chickens only had locally elevated glucocorticoid precursors.

2.4.1 The predominant lymphoid glucocorticoid differs in altricial and precocial birds

Our data provide strong evidence for tissue-specific glucocorticoid regulation in lymphoid organs of avian species and confirms the presence of cortisol in birds. Previous measurements of cortisol in zebra finches used immunoassay after SPE (Schmidt et al., 2009; Schmidt and Soma, 2008), and here we obtained very similar cortisol measurements after additional HPLC cortisol separation. Notably, while the major circulating glucocorticoid in birds is corticosterone, the predominant lymphoid glucocorticoid differed in altricial and precocial species: cortisol was higher in the zebra finch, while corticosterone was higher in the Japanese quail and chicken. In zebra finches, the presence of distinct systemic and lymphoid glucocorticoids suggests that they could have different mechanisms of action. Corticosterone binds to both the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) in zebra

finch thymus and bursa cytosol, while cortisol binds only to GR (Schmidt et al., 2010). As MR and GR have distinct and sometimes antagonistic activities (Chantong et al., 2012; Ehrchen et al., 2007; Usher et al., 2010), variations in cortisol and corticosterone could result in fundamentally different cellular responses. Furthermore, immune cells can contain membrane-associated GRs (Bartholome et al., 2004; Vernocchi et al., 2013), and cortisol but not corticosterone binds to GR-like membrane site in the finch bursa (Schmidt et al., 2010). Thus, lymphoid cells may distinguish between locally-derived cortisol and systemically-derived corticosterone. Indeed, cortisol has much stronger pro-apoptotic effects on chicken lymphocytes (Compton et al., 1990).

Cortisol and corticosterone signaling may both occur in quail lymphoid organs, which may have both glucocorticoids. In the chicken, however, corticosterone levels in lymphoid organs are much higher than cortisol, which is present at very low levels, and the use of distinct signaling pathways appears less likely. This finding of minimal concentrations of cortisol *in vivo* contrasts findings of another study also using female and male juvenile White Leghorn chickens, which found that lymphoid organs produced cortisol *in vitro* (Lechner et al., 2001). The reason for this difference is unclear, but endogenous glucocorticoid production may be altered by various environmental factors, such as diet or pathogen exposure.

In contrast to glucocorticoids, progesterone was locally elevated in hatchling lymphoid organs of all three species. Progesterone may function as a precursor for local glucocorticoid synthesis in the finch and quail, but was also elevated in lymphoid organs of the chicken, where there appears to be little glucocorticoid production. Progesterone could function as a distinct signal, binding progesterone receptors in avian and mammalian lymphoid organs (Pasanen et al., 1998; Pearce et al., 1983; Tibbetts et al., 1999). Local progesterone in neonatal birds and mice (Taves et al., 2015) indicates a conserved function in lymphoid development. In the periphery,

progesterone promotes lymphocyte differentiation toward regulatory (Lee et al., 2012) and humoral (Hughes et al., 2013; Miyaura and Iwata, 2002) phenotypes, facilitating tolerance and preventing autoimmunity. Progesterone also affects lymphocyte development, regulating production of different lymphocyte subsets (Leposavić et al., 2014) and probably attenuating cell-mediated immunity (Perišić et al., 2013). Since lymphocyte heterogeneity and tolerance are fundamental to immunity, locally elevated lymphoid progesterone levels may facilitate this across vertebrates. Furthermore, it may be the balance between glucocorticoids and progesterone that regulates immune reactivity and tolerance.

2.4.2 Evidence for local glucocorticoid synthesis

Adrenal glucocorticoids circulate through the blood and diffuse into various tissues, and are thus expected to be present at similar concentrations in blood and other tissues. In contrast, the presence of locally elevated glucocorticoid levels in specific tissues (versus circulating blood) demonstrates tissue-specific regulation (Taves et al., 2011b; Schmidt et al., 2008). Furthermore, when glucocorticoid precursors are also locally elevated, this could be consistent with local glucocorticoid synthesis, rather than glucocorticoid uptake from the blood (Taves et al., 2011a; 2015). Our findings indicate that zebra finches, Japanese quail, and chickens may fall along a gradient of local versus systemic glucocorticoid regulation.

In the zebra finch hatchling, progesterone, deoxycorticosterone, and cortisol were all locally elevated in lymphoid organs, suggesting that lymphoid cortisol is synthesized from upstream precursors. While local elevation of deoxycortisol was nonsignificant, deoxycortisol levels across tissues paralleled cortisol patterns, further supporting this interpretation. The

juvenile thymus (but not bursa or spleen) also had elevated progesterone, deoxycortisol, and cortisol, indicating that local glucocorticoid synthesis, while reduced, continues at this age.

In the Japanese quail, lymphoid corticosterone may be largely derived from the blood, as circulating and lymphoid corticosterone levels are similar. However, the precursors progesterone, deoxycorticosterone, and deoxycortisol were locally elevated in lymphoid organs, consistent with local synthesis – possibly of cortisol, as deoxycortisol and cortisol patterns are similar. It is possible that local synthesis does occur, but at a relatively low level that is masked by circulating glucocorticoids. Alternatively, if Cyp11b1 (Figure 1.1) is not expressed, this could mean that deoxycorticosterone and deoxycortisol are end products of local steroid synthesis and bind to steroid receptors.

In the chicken hatchling, only progesterone was locally elevated in lymphoid organs, suggesting that lymphoid corticosterone is largely derived from the blood. Interestingly, this also suggests that progesterone on its own may be an important local signal; this might also be the case in the quail and finch. In juveniles, lymphoid organs had elevated corticosterone but not upstream precursors. Our data appear to contrast previous work in which juvenile White Leghorn lymphoid organ cultures demonstrated the full range of enzyme activities needed to produce glucocorticoids (Lechner et al., 2001). Furthermore, Lechner et al. found that chicken lymphoid organs preferentially synthesized cortisol, rather than corticosterone. Our data are not strongly suggestive of local glucocorticoid synthesis, especially of cortisol, but it may be that local cortisol synthesis occurs at a low level that we were unable to detect, or differs because of other unknown factors differing between our studies.

2.4.3 Across species, lymphoid glucocorticoid levels decrease with age

In all three species we examined, lymphoid glucocorticoid (and progesterone) levels were higher in hatchlings than in juveniles. This age-related decrease corresponds with previous findings in developing zebra finches (Schmidt et al., 2009; Schmidt and Soma, 2008) and mice (Vacchio et al., 1994; Taves et al., 2015). This age-related decrease also corresponds with lymphocyte production, which is highest in early life and decreases with age as the lymphoid organs regress (Davison et al., 2011). Zebra finch glucocorticoid patterns in particular are extremely similar to those of developing mice (Taves et al., 2014), suggesting a similar glucocorticoid role in selection of appropriately reactive lymphocytes. As lymphocyte selection is critical for adaptive immunity, and glucocorticoid hormones (Denver, 2009) and adaptive immunity (Cooper and Alder, 2006) have similar functions across vertebrates, a similar glucocorticoid function in this process seems quite plausible. While the timeline of lymphoid organ colonization and lymphocyte production likely differs somewhat between the species in this study, generation of a diverse lymphocyte repertoire (and therefore selection of these lymphocytes) almost certainly continues posthatch in each of these species (Klasing and Leshchinsky, 1999).

2.4.4 Lymphoid glucocorticoids may differentially regulate immunity across species

Differential regulation of glucocorticoids across bird species could result in differential plasticity of the developing immune system. In altricial finch hatchlings, where immune glucocorticoids appear to be locally synthesized, programming of immune reactivity likely follows local glucocorticoid patterns, as circulating glucocorticoids are low and unresponsive (Wada et al., 2009). In precocial quail and chicken offspring, however, lymphoid glucocorticoid

levels are similar to circulating glucocorticoids, and environmentally-induced changes in circulating glucocorticoids (due to factors such as predation, food shortage, or high pathogen load) could potently affect immune development. Thus, our data raise the possibility that the posthatch environment may impact the development of immune reactivity to a greater extent in precocial than in altricial hatchlings.

However, the differences in lymphoid glucocorticoids could be due to factors other than the mode of development. For example, zebra finches, Japanese quail, and chickens have been domesticated for dramatically different lengths of time (zebra finches, ~200 years (Griffith and Buchanan, 2010); Japanese quail, ~1000 years (Mills et al., 1997); chickens, ~8000 years (Fumihito et al., 1996; West and Zhou, 1988), and the disparate profiles of lymphoid glucocorticoids in developing finches, quail, and chickens may be the result of domestication, and its accompanying selection for resistance to specific pathogens rather than general resistance to all pathogens (Bishop et al., 2010; Chaves et al., 2010; Homberger et al., 2013). Species differences in lymphoid glucocorticoids could also stem from phylogenetic effects unrelated to developmental mode, and examination of a greater number of altricial and precocial species would be useful to investigate this.

2.5 Conclusions

Our data show that lymphoid glucocorticoids are differentially regulated in altricial and precocial birds, with strong evidence for local cortisol synthesis in the altricial finch, and systemic regulation of lymphoid corticosterone in the quail and chicken. This differential regulation may be an important determinant of immune reactivity, and therefore susceptibility to pathogens and parasites throughout life. Furthermore, as progesterone levels are locally elevated

in lymphoid organs of all hatchlings, this steroid may play a fundamental, heretofore unrecognized role in lymphoid development. This contrasts the traditional view of steroids as immunosuppressive, instead indicating multiple roles in the development of a functional immune system.

2.6 Tables and figures

Table 2.1: Number of subjects of each species, strain, and sex.

| Species | Strain | Sex | Number | |
|----------------|------------------|--------|----------|----------|
| | | | Hatching | Juvenile |
| Zebra finch | na | Female | 6 | 3 |
| | | Male | 10 | 5 |
| Japanese quail | UBC | Female | 8 | 5 |
| | | Male | 0 | 4 |
| | Hawaiian | Female | 4 | 5 |
| | | Male | 4 | 4 |
| Chicken | White Leghorn | Female | 3 | 3 |
| | | Male | 6 | 5 |
| | | Female | 5 | 2 |
| | Rhode Island Red | Male | 4 | 6 |

Note: For zebra finch hatchlings only, tissues from two subjects were pooled, resulting in the number of analyzed samples being half of the number of subjects collected (i.e., 3 hatching female and 5 hatching male samples [pools] for each tissue). na = not applicable.

Table 2.2: HPLC steroid separation

| Steroid | Retention peak (min) |
|-------------------------------------|----------------------|
| 18-Hydroxycorticosterone | 7.25 |
| Aldosterone | 7.79 |
| Cortisol | 10.58 |
| Cortisone | 11.22 |
| Dehydrocorticosterone | 15.56 |
| 18-Hydroxy-11-Deoxycorticosterone | 18.00 |
| Corticosterone | 19.26 |
| Tetrahydrocorticosterone | 22.76 |
| Tetrahydro-11-Dehydrocorticosterone | 22.78 |
| Deoxycortisol | 24.44 |
| Deoxycorticosterone | 31.33 |
| 17 α -Hydroxyprogesterone | 33.73 |
| Tetrahydrodeoxycorticosterone | 34.43 |
| Allotetrahydrodeoxycorticosterone | 35.75 |
| Progesterone | 37.81 |

Note: Steroids were separated using a Waters SymmetryShield C₁₈ column (4.6 × 250 mm) and eluted at a flow rate of 1.0 ml/min. Elution began with 100% Solvent A (30% acetonitrile, 0.01% formic acid), with a linear increase to 100% Solvent B (100% acetonitrile, 0.01% formic acid) from 20 to 45 min (see Figure 2.1). Retention times were determined using UV detection of unlabeled steroids and collection of radiolabeled steroids in 0.5 min fractions. Steroids in bold were collected in 3 min fractions, beginning at -1.5 min and ending at +1.5 min from the retention peak.

Table 2.3: Immunoassay specifications.

| Steroid | Assay / Antibody | Detection limit | Reference |
|---------------------|---------------------------------------|-----------------|---------------------------|
| Progesterone | MP Biomedicals 07170105 | 1.95 pg/tube | Taves et al., 2015 |
| Deoxycorticosterone | Novus Biologicals NB100-62098 | 1.95 pg/tube | Taves et al., 2015 |
| Corticosterone | MP Biomedicals 07120103 | 1.56 pg/tube | Schmidt and Soma, 2008 |
| Deoxycortisol | American Research Products 13-2219 | 1.95 pg/tube | Close et al., 2010 |
| Cortisol | Salimetrics 1-3002 | 3.00 pg/well | Schmidt and Soma, 2008 |

Table 2.4: Mean hatchling and juvenile body masses.

| Species | Strain | Sex | Body mass, g | | | Ref. |
|----------------|------------------|--------|-------------------------|---------------------|------------|---------------------------|
| | | | Hatchling | Juvenile | Adult | |
| Zebra finch | na | Female | 0.85 ± 0.03 (6) | 14.43 ± 0.54 (3) | 14.0 | Kriengwatana et al., 2014 |
| | | Male | 0.86 ± 0.04 (10) | 15.22 ± 0.36 (5) | | |
| Japanese quail | UBC | Female | 6.43 ± 0.15 (8) | 51.62 ± 0.70 (5) | 96.6 | Dunning, 2007 |
| | | Male | na (0) | 49.08 ± 3.04 (4) | 93.0 | |
| | Hawaiian (HAW) | Female | 5.48 ± 0.10 (4) | 39.25 ± 0.98 (5) | 96.6 | |
| | | Male | 5.97 ± 0.37 (4) | 37.03 ± 1.92 (4) | 93.0 | |
| Chicken | White Leghorn | Female | 42.55 ± 3.35 (3) | 881.60 ± 43.60 (3) | 1516 ± 169 | Silversides et al., 2007 |
| | | Male | 41.24 ± 2.22 (6) | 1055.38 ± 19.28 (5) | 2063 ± 183 | |
| | Rhode Island Red | Female | 43.45 ± 1.01 (5) | 996.30 ± 71.7 (2) | 2045 ± 238 | |
| | | Male | 41.93 ± 2.11 (4) | 1140.50 ± 55.99 (6) | 2737 ± 246 | |

Note: Mean body mass values are in grams (\pm SEM), with significant sex differences in bold.

The numbers of subjects are given in parentheses. For zebra finch hatchlings only, tissues from two subjects were pooled, resulting in the number of analyzed samples being half the number of subjects collected (i.e., 3 hatchling female and 5 hatchling male samples for each tissue). Adult body masses are shown as reported elsewhere (see references). No male hatchling UBC Japanese quail were collected. na = not applicable.

Table 2.5: Relative steroid concentrations in lymphoid organs compared to blood. Ratios are expressed as a fraction (organ steroid concentrations / blood steroid concentration).

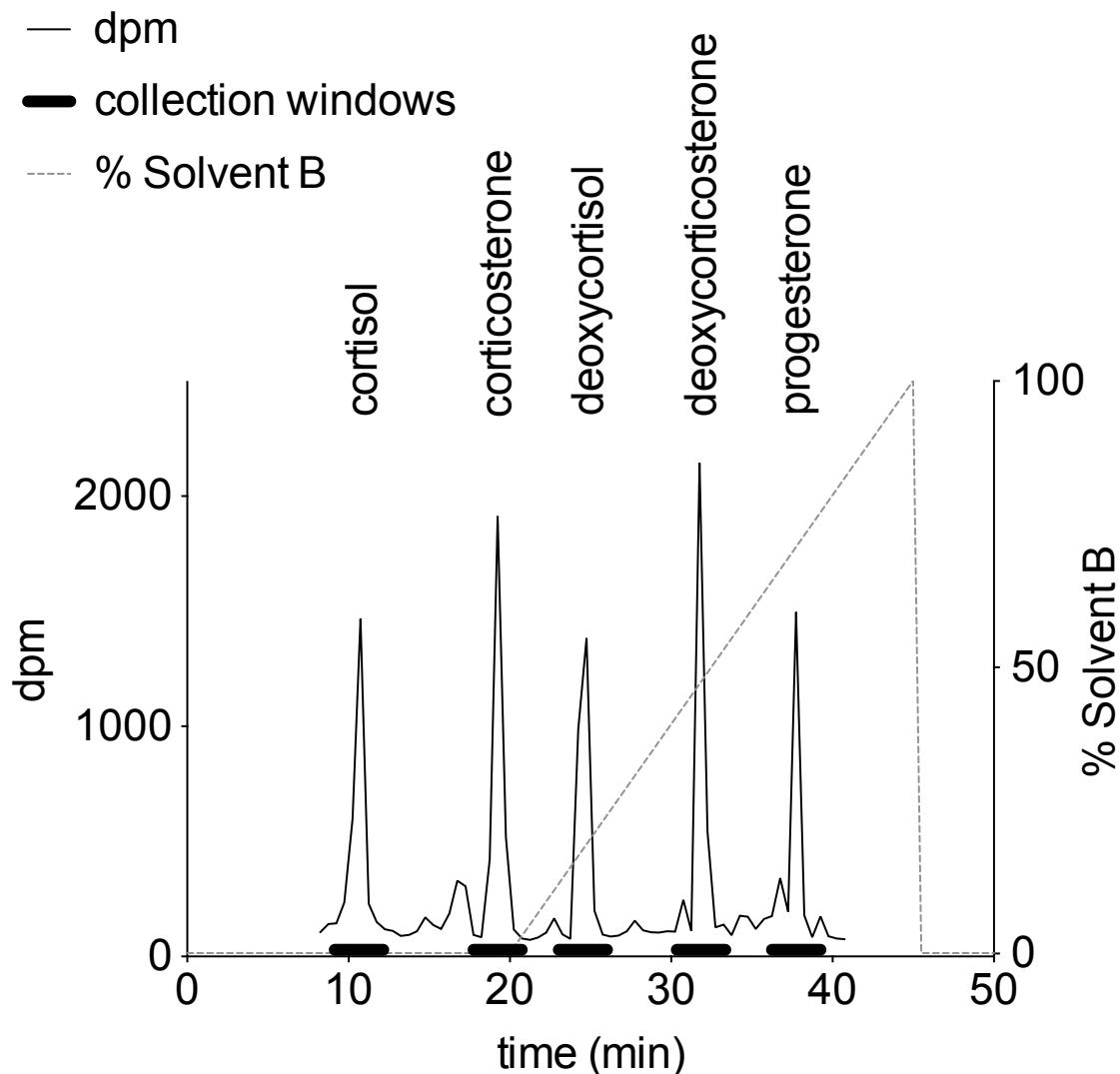
| Species | Strain | Sex | Steroid | Hatching | | | Juvenile | | |
|----------------|------------|-----|---------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| | | | | Thymus | Bursa | Spleen | Thymus | Bursa | Spleen |
| Zebra finch | | | Progesterone | na | 19 ± 5 | 16 ± 5 | 2.2 ± 0.6 | 1.2 ± 0.2 | 1.7 ± 0.7 |
| | | | Deoxycorticosterone | na | 51 ± 20 | 28 ± 13 | 2.3 ± 1.0 | 3.4 ± 1.7 | 1.0 ± 0.4 |
| | | | Corticosterone | na | 0.6 ± 0.6 | 0.9 ± 0.5 | 0.5 ± 0.2 | 0.7 ± 0.3 | 0.6 ± 0.4 |
| | | | Deoxycortisol | na | 5.1 ± 3.0 | 10 ± 6 | 2.7 ± 0.9 | 0.8 ± 0.3 | 0.7 ± 0.3 |
| | | | Cortisol | na | 30 ± 9 | 35 ± 9 | 3.9 ± 1.2 | 4.3 ± 1.9 | 3.2 ± 1.7 |
| Japanese quail | UBC HAW | | Progesterone | 8.9 ± 4.0 | 21 ± 12 | 49 ± 24 | 1.1 ± 0.3 | 11 ± 10 | 5.0 ± 3.4 |
| | | | Deoxycorticosterone | 5.2 ± 1.8 | 7.4 ± 2.9 | 12 ± 5 | 1.1 ± 0.2 | 1.5 ± 0.5 | 2.7 ± 1.5 |
| | | | Corticosterone | 0.5 ± 0.1 | 0.7 ± 0.2 | 0.8 ± 0.2 | 0.8 ± 0.1 | 1.0 ± 0.4 | 3.2 ± 1.0 |
| | | | Deoxycortisol | 17 ± 7 | 13 ± 4 | 29 ± 10 | 0.8 ± 0.1 | 1.5 ± 0.4 | 1.2 ± 0.3 |
| | | | Cortisol | 0.8 ± 0.2 | 1.2 ± 0.3 | 2.2 ± 1.4 | 0.9 ± 0.1 | 1.2 ± 0.3 | 2.7 ± 1.1 |
| | F M | | Progesterone | 1.7 ± 0.2 | 3.6 ± 0.3 | 4.7 ± 0.5 | 0.8 ± 0.8 | 1.8 ± 1.8 | 0.7 ± 0.7 |
| | | | Deoxycorticosterone | 0.7 ± 0.2 | 1.0 ± 0.3 | 1.0 ± 0.6 | 1.3 ± 0.1 | 2.6 ± 0.3 | 1.2 ± 0.1 |
| | | | Corticosterone | 0.8 ± 0.1 | 0.9 ± 0.2 | 1.0 ± 0.2 | 1.1 ± 0.2 | 1.8 ± 0.4 | 2.2 ± 0.5 |
| | | | Deoxycortisol | 0.9 ± 0.1 | 1.3 ± 0.3 | 1.6 ± 0.4 | 1.2 ± 0.2 | 1.1 ± 0.2 | 1.0 ± 0.1 |
| | | | Cortisol | 0.8 ± 0.1 | 0.9 ± 0.1 | 1.2 ± 0.3 | 0.9 ± 0.1 | 0.8 ± 0.1 | 0.9 ± 0.1 |

Bolded values indicate tissue steroid concentrations significantly higher than blood steroid concentrations (e.g., values significantly greater than 1; $p < 0.05$). Strain or sex are only listed separately when steroid ratios differ between strains or between females and males. Cases where tissue steroids were detectable and blood steroids were nondetectable were not included when calculating means. na = not applicable.

Table 2.6: Relative cortisol:corticosterone concentrations in blood and lymphoid organs of hatchling and juvenile finches, quail, and chickens.

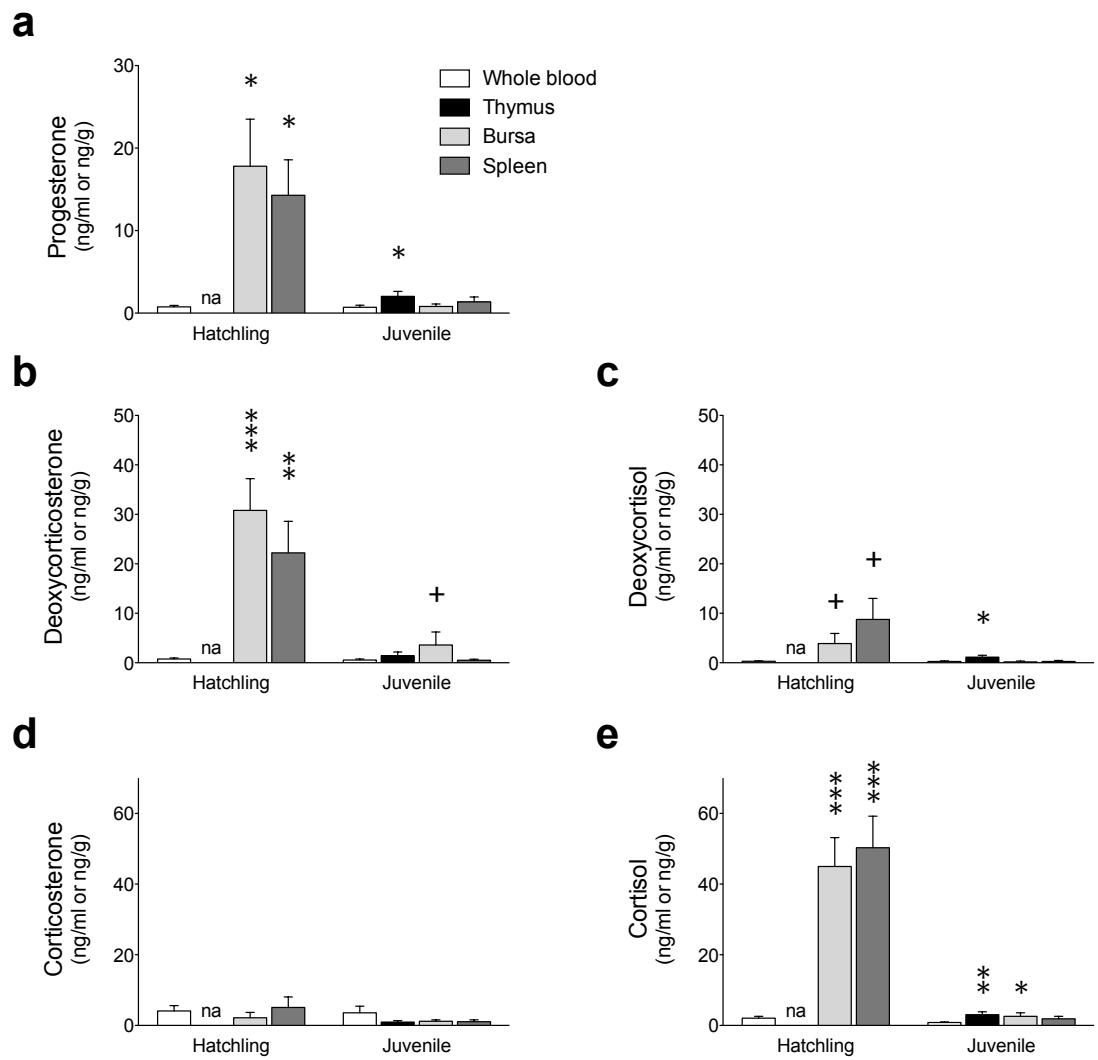
| Species | Hatchling | | | | Juvenile | | | |
|----------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| | Blood | Thymus | Bursa | Spleen | Blood | Thymus | Bursa | Spleen |
| Zebra finch | 1.2 ± 0.4 | n.a. | 9.2 ± 0.6 | 8.8 ± 1.1 | 0.4 ± 0.1 | 4.8 ± 1.5 | 3.2 ± 1.5 | 4.6 ± 1.6 |
| Japanese quail | 0.1 ± 0.1 | 1.1 ± 0.8 | 0.5 ± 0.2 | 1.0 ± 0.6 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 2.6 ± 1.5 |
| Chicken | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.5 ± 0.3 | 0.7 ± 0.4 | 0.7 ± 0.3 | 0.4 ± 0.3 | 0.3 ± 0.1 |

Figure 2.1 Steroid separation using reverse-phase HPLC.



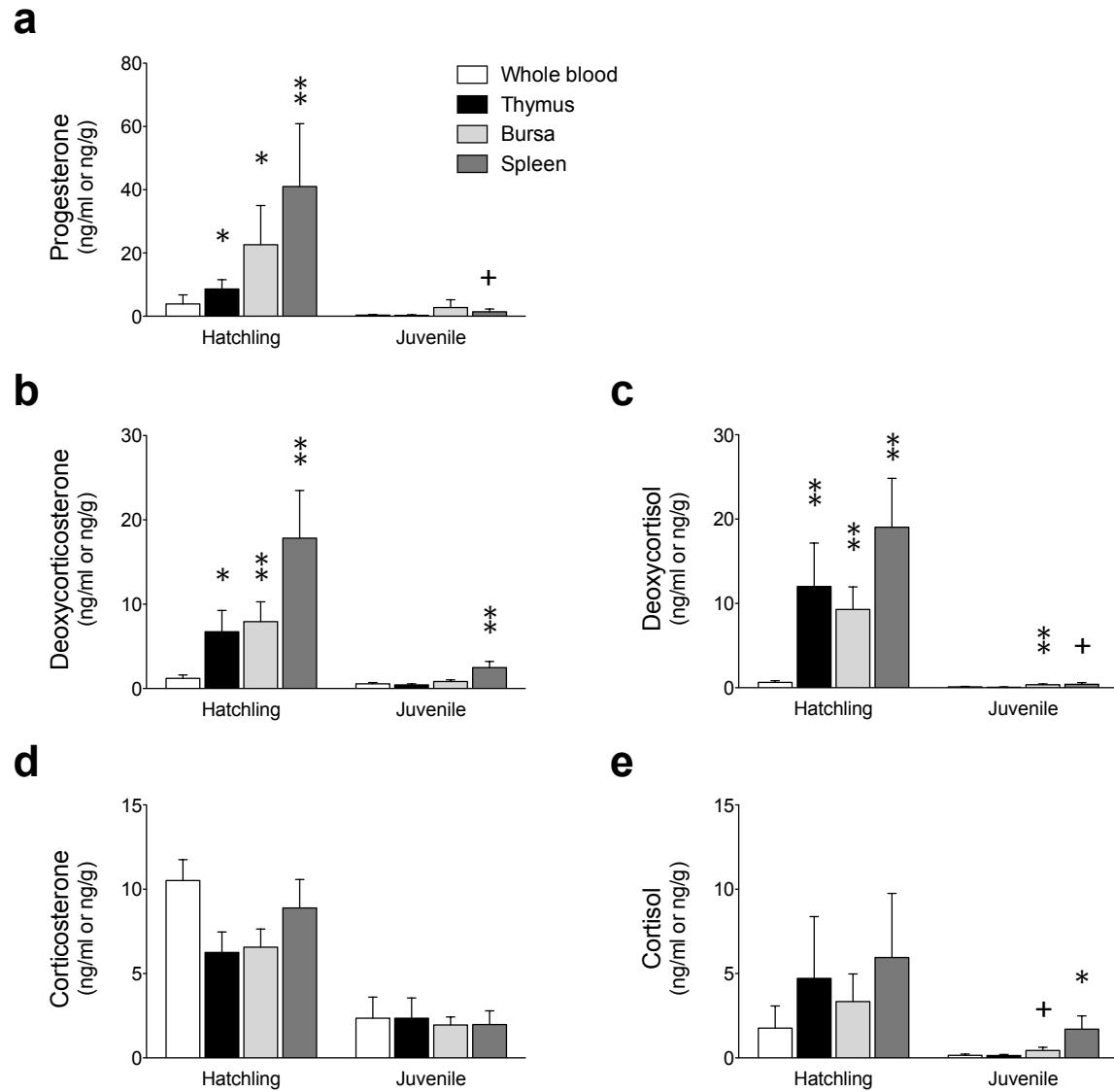
HPLC separation of cortisol, corticosterone, deoxycortisol, deoxycorticosterone, and progesterone (in order of increasing retention time). Retention times were determined by collection of ^3H -labeled steroids in 0.5 min fractions and counting in a liquid scintillation counter.

Figure 2.2 Steroid concentrations in blood and lymphoid organs of hatchling and juvenile zebra finches.



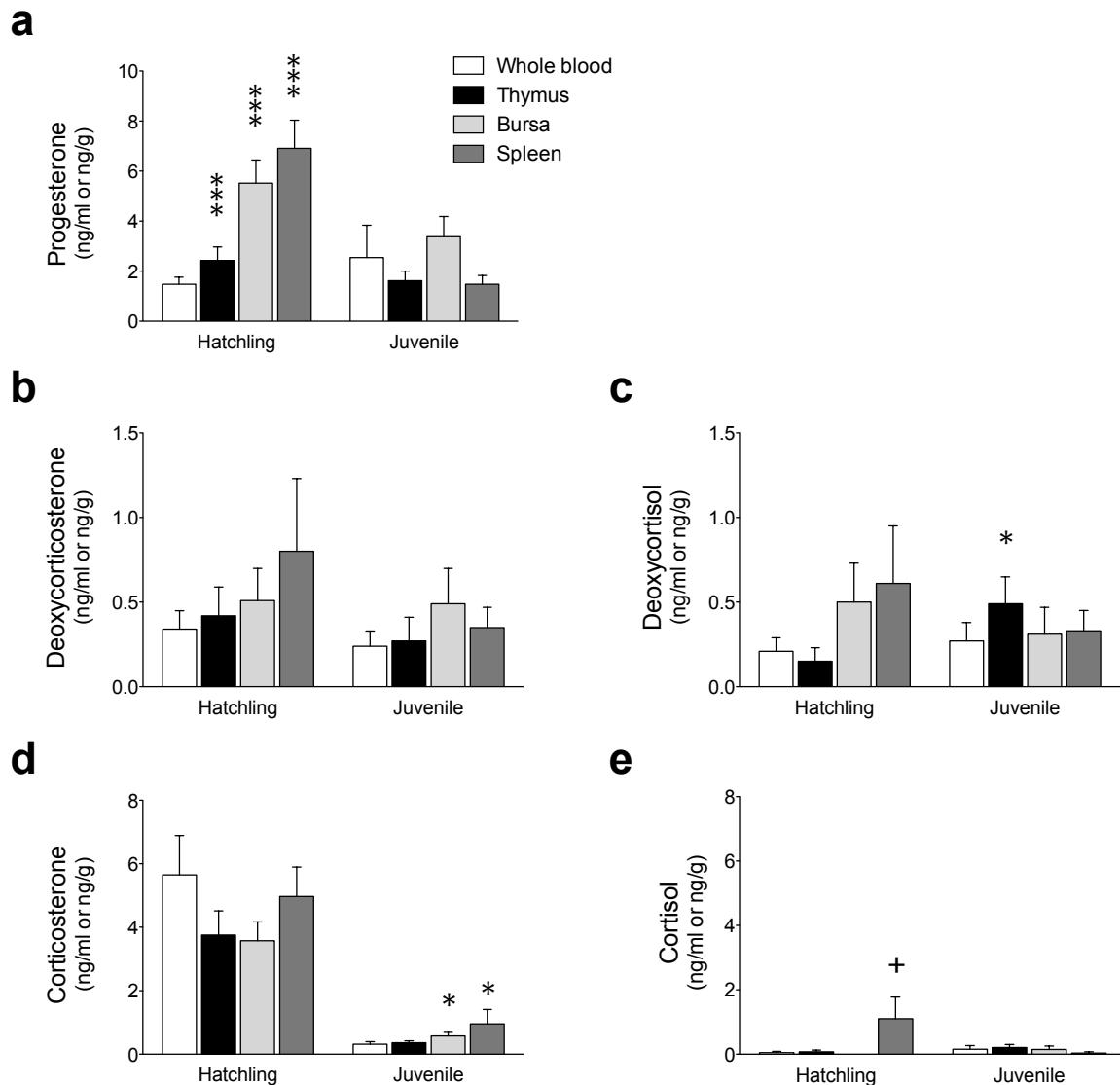
a) Progesterone, b) deoxycorticosterone, c) deoxycortisol, d) corticosterone, and e) cortisol concentrations in whole blood (ng/ml), thymus, bursa of Fabricius, and spleen (ng/g) of zebra finch hatchlings (posthatch day 0) and juveniles (posthatch day 30). Thymus was not collected from hatchlings (na = not applicable). Statistically significant differences between tissue steroid levels and blood steroid levels are indicated as follows, + p < 0.10, * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 2.3 Steroid levels in blood and lymphoid organs of hatchling and juvenile Japanese quail.



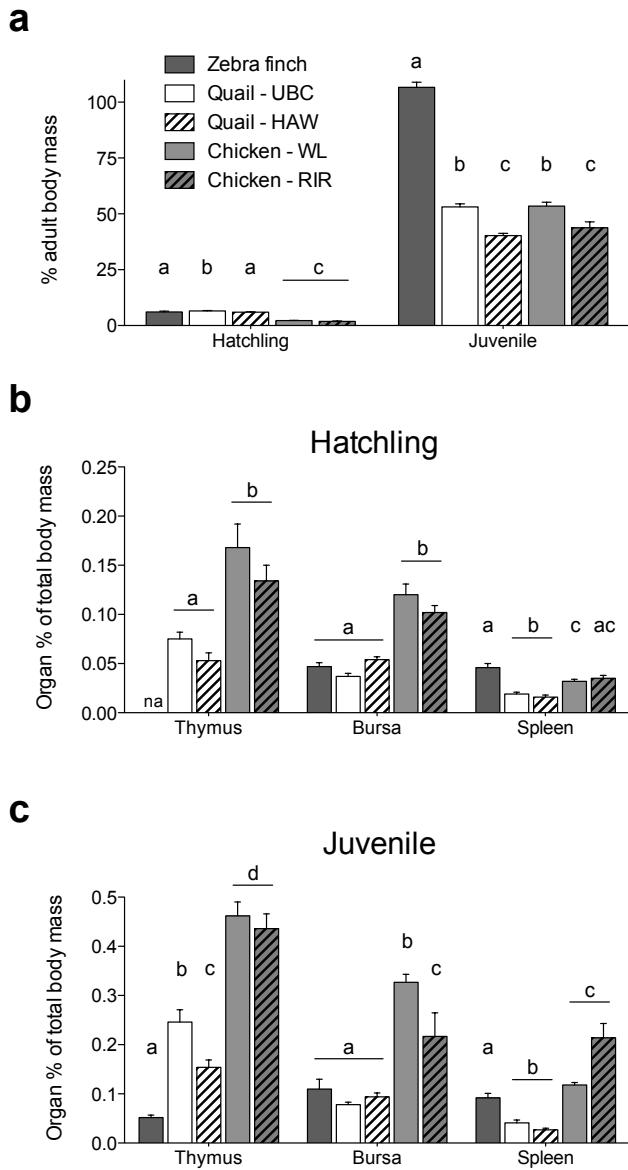
a) Progesterone, b) deoxycorticosterone, c) deoxycortisol, d) corticosterone, and e) cortisol concentrations in whole blood (ng/ml), thymus, bursa of Fabricius, and spleen (ng/g) of Japanese quail hatchlings (posthatch day 0) and juveniles (posthatch day 14). Statistically significant differences between tissue steroid levels and blood steroid levels are indicated as follows, + p < 0.10, * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 2.4 Steroid concentrations in blood and lymphoid organs of hatchling and juvenile chickens.



a) Progesterone, b) deoxycorticosterone, c) deoxycortisol, d) corticosterone, and e) cortisol concentrations in whole blood (ng/ml), thymus, bursa of Fabricius, and spleen (ng/g) of chicken hatchlings (posthatch day 0) and juveniles (posthatch day 42). Statistically significant differences between tissue steroid levels and blood steroid levels are indicated as follows, + p < 0.10, * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 2.5 Relative body and organ sizes of hatchling and juvenile zebra finches, Japanese quail, and chickens.



letters indicate significant differences at $p < 0.05$.

a) Hatchling and juvenile body
masses are shown as a percent of expected adult body mass (adult body masses published elsewhere, see Table 2.4). **b, c) Relative** thymus, bursa, and spleen masses of hatchlings (b) and juveniles (c) are given as a percent of total body mass. Hatchlings were collected on the day of hatch (posthatch day 0, or P0), while juveniles were collected at P30 (zebra finches), P14 (UBC and Hawaiian strains of Japanese quail), and P42 (White Leghorn and Rhode Island Red strains of chicken). Data are presented as \pm SEM, and different

3 Steroid profiling reveals widespread local regulation of glucocorticoid levels during mouse development

3.1 Introduction

Traditionally, endocrinology has focused on hormonal changes at the systemic level, in which endocrine organs secrete hormones into the circulating blood (Norris, 2007; Guyton and Hall, 2006). This results in regulation of whole-body hormone levels and functions to coordinate organismal physiology. More recently, a growing body of evidence has shown multiple mechanisms by which hormone-sensitive tissues may independently regulate their local hormone concentrations (Taves et al., 2011a; Seckl and Holmes, 2007; Breuner and Orchinik, 2002). However, the extent to which these mechanisms differentially affect specific tissues is not well understood.

Glucocorticoids are a particularly interesting example to examine tissue-specific regulation. Under control of the hypothalamic-pituitary-adrenal (HPA) axis, circulating glucocorticoids have pleiotropic effects, orchestrating immune, cardiovascular, metabolic, and neural function (Sapolsky et al., 2000). Glucocorticoids act on nearly every cell of the body, and are critical effectors of development (Wada, 2008), homeostasis (Sapolsky et al., 2000; Wada, 2008), and disease (Seckl and Holmes, 2007; Walker, 2007; Vegiopoulos and Herzog, 2007). While glucocorticoids have varied effects on different cell and tissue types, these effects are widely thought to follow systemic glucocorticoid patterns. As such, measurements of the predominant adrenal glucocorticoid in the blood (e.g. cortisol in humans, corticosterone in rats and mice) are widely used to understand how changes in systemic glucocorticoid levels regulate

these processes (Norris, 2007; Guyton and Hall, 2006; Demas and Nelson, 2012; Makin and Gower, 2010).

Although glucocorticoid levels in tissues are thought to parallel glucocorticoid levels in the blood, a variety of tissues express cellular machinery by which local glucocorticoid levels could be regulated independently of systemic glucocorticoid levels. Glucocorticoid synthesis *de novo* from cholesterol may occur in extra-adrenal tissues, such as the thymus (Taves et al., 2011a). Cultured murine thymus converts cholesterol into corticosterone, the predominant murine glucocorticoid (Vacchio et al., 1994; Lechner et al., 2000), and this activity was especially high in thymi of young mice (Vacchio et al., 1994). Similarly, glucocorticoid-synthetic activity was found in cultured chicken thymus and other lymphoid organs (Lechner et al., 2001). Interestingly, while corticosterone is the predominant circulating avian glucocorticoid, as in mice and rats, lymphoid organs of chickens synthesized cortisol, suggesting the possibility of distinct adrenal and extra-adrenal glucocorticoids (Lechner et al., 2001). We measured glucocorticoid levels in songbirds and indeed found high cortisol (but not corticosterone) levels in developing lymphoid organs and low levels of both glucocorticoids in blood (Schmidt and Soma, 2008). We also found high local levels of upstream precursors, coinciding with high local levels of cortisol (see Chapter 2 above). Taken together, these data suggest that tissue-specific regulation of glucocorticoids occurs *in vivo* and that the identities of systemic and local glucocorticoids can differ. However, in mice, little is known regarding the local levels of glucocorticoids *in vivo* and their identities.

Here, we quantify endogenous glucocorticoids in a variety of glucocorticoid-sensitive tissues to look for local glucocorticoid regulation *in vivo* over murine development. In addition to measuring the predominant murine glucocorticoid, corticosterone, we also quantify, cortisol

and the precursors 11-deoxycorticosterone (deoxycorticosterone), 11-deoxycortisol (deoxycortisol), and progesterone, which can also bind mineralocorticoid and glucocorticoid receptors (MR and GR) (Vinson, 2011; Close et al., 2010; Duval et al., 1984; Gomez-Sanchez and Gomez-Sanchez, 2014). We also measured gene expression of steroidogenic enzymes using qPCR. Early development is especially well suited for examining local glucocorticoid regulation. In altricial species, whose offspring are completely dependent on their parents, the adrenal glands undergo a period of quiescence in early neonatal life, resulting in low or nondetectable glucocorticoid levels in blood (the stress hyporesponsive period, SHRP) (Meaney et al., 1985). The reduction in adrenal glucocorticoids is thought to promote neural development and body growth (Wada, 2008; Lupien et al., 2009), but might deprive other organs of glucocorticoids where they are beneficial, such as the thymus (Mittelstadt et al., 2012), heart (Rog-Zielinska et al., 2013), and liver (Liggins, 1994). Thus, these and other developing organs are good candidates for examining locally elevated glucocorticoid levels, especially during the SHRP.

3.2 Materials and methods

3.2.1 Subjects

Samples were collected from C57BL/6 mice at embryonic day 16.5 (E16.5) and postnatal days (PND) 1, 5, 15, and 60 ($n = 8, 14, 12, 12$, and 13 , respectively), with PND0 defined as the first day pups were present in the cage. PND5 was specifically selected because this age is well within the SHRP, when the predominant murine adrenal glucocorticoid, corticosterone, is extremely low in circulating blood (Schmidt et al., 2003). Mice were housed in a specific pathogen-free colony in the Wesbrook Animal Unit at the University of British Columbia, with corn cob bedding, under a 12:12 light:dark cycle, with free access to water and food (LabDiet

5021 for breeding parents, and LabDiet 5010 after weaning at PND20). Samples were collected in the morning (from 0900 to 1100 h) to reduce diel variation in steroid levels. Protocols were approved by the UBC Animal Care Committee (A07-0417) and were in compliance with regulations established by the Canadian Council on Animal Care.

3.2.2 Tissue collection

Postnatal mice were deeply anesthetized with isoflurane delivered in oxygen (<1 min) and euthanized by decapitation. Trunk blood was collected into two heparinized tubes and immediately placed on wet ice. Blood collection was completed within 3 min of initial disturbance (2.42 ± 0.05 min) to avoid any rise in circulating glucocorticoid levels. Spleen, liver, thymus, and brain were dissected and immediately frozen on dry ice. The heart was briefly blotted to remove blood prior to freezing on dry ice. Femurs were isolated, ends cut off, and also frozen on dry ice. For collection of embryonic tissues, the pregnant dam was deeply anesthetized with isoflurane and euthanized, after which embryos were euthanized by decapitation and chilled on wet ice as tissues were collected. Spleen and bone marrow could not be obtained from embryos. Genomic DNA was extracted from tail clips and sex determined as previously described (Clapcote and Roder, 2005).

3.2.3 Steroid extraction

Steroids were extracted from all samples (plasma, whole blood, thymus, bone marrow, heart, liver, spleen, and brain) using solid phase extraction (SPE) with C₁₈ columns as previously described (Newman et al., 2008; Taves et al., 2010). Organs were weighed, homogenized in 84% methanol with a bead homogenizer (Omni Bead Ruptor), and supernatants were loaded onto SPE

columns (Agilent #12113045). For PND1 mice, whole femurs containing marrow were weighed and homogenized, while marrow from older mice was flushed from femurs with ice-cold water before being weighed and homogenized. Samples were washed with 10 ml of 40% methanol to remove conjugated (glucuronidated and sulfated) steroids and interfering substances (Taves et al., 2011b), and unconjugated steroids were eluted with 5 ml of 90% methanol and dried at 60°C in a vacuum centrifuge (ThermoElectron SPD111V).

3.2.4 Steroids separation

Samples were further processed using reversed-phase HPLC (Gilson 322) with a Waters SymmetryShield C₁₈ column (4.6 × 250 mm, 5 µm silica particles) kept at 40°C with a column heater (Torrey Pines #CO20), using 30% acetonitrile, 0.01% formic acid in water as mobile phase A (MPA) and 100% acetonitrile, 0.01% formic acid as mobile phase (MPB). Dried steroid residues were resuspended in 475 µl MPA, centrifuged at 16,000 g for 5 min, and supernatants transferred to HPLC vials. 400 µl of each sample was loaded onto the HPLC column using an autoinjector (Gilson 234). The gradient profile started at 0% MPB for 20 min, ramped to 100% MPB over 25 min, returned to starting conditions in 0.5 min, and held for 15 min to re-equilibrate the column. The total run time was 60.5 min. Samples were eluted at a flow rate of 1.0 ml/min, and steroid fractions were obtained using 3-min collection windows (Figure 2.1, Table 2.2) with a fraction collector (Gilson FC 204). We established elution times of other steroids to determine if they co-eluted with our steroids of interest (Table 2.2). Cortisone co-eluted with cortisol, but this is highly unlikely to affect our cortisol measurements, due to minimal cross-reactivity of cortisone with our anti-cortisol antibody (0.13%, Table 3.1). Fractions were dried at 60°C in a vacuum centrifuge.

3.2.5 Immunoassays

Steroids were measured in duplicate using specific and sensitive immunoassays. Assay details and specificities are given in Table 3.1. Corticosterone was quantified using a radioimmunoassay following the manufacturer's instructions. The lowest standard was further diluted to allow detection of lower corticosterone quantities (Schmidt and Soma, 2008). Cortisol quantification was performed by enzyme immunoassay following the manufacturer's instructions (Schmidt and Soma, 2008). Deoxycorticosterone and deoxycortisol were each quantified using specific antibodies, steroid standards, and tritiated steroids with charcoal-dextran separation of free and bound steroids (Close et al., 2010). Briefly, 100 µl of resuspended sample (with 2.5% ethanol to aid in resuspension) was added to 100 µl of assay buffer containing tracer (~6000 cpm) and antibody (anti-deoxycorticosterone, 1:14,000 final dilution; anti-deoxycortisol, 1:3333 final dilution). Tubes were incubated overnight at 4°C after which 500 µl of dextran-coated charcoal was added. Tubes were incubated for 15 min on ice and centrifuged at 1455 g for 12 min at 4°C to pellet charcoal and charcoal-bound free steroids. Supernatants were decanted, mixed with 5 ml of scintillant, and counted for 5 min in a scintillation counter. Progesterone was quantified using a double-antibody ^{125}I RIA that we modified to increase sensitivity. Sixty microlitres of tracer was added to 390 µl of resuspended sample (with 1% ethanol to aid resuspension), followed by 250 µl of primary antibody. Samples were incubated for 1 hr in a 37°C water bath. Five hundred microlitres of precipitant (secondary antibody) was added, and tubes centrifuged at 1000 g for 20 min at 4°C. Supernatants were decanted, and pellet radioactivity counted for 1 min in a gamma counter. Steroid recovery or our extraction and

separation protocol was estimated by spiking tissue homogenates with known amounts of steroids and comparing these samples with unspiked samples from the same pools.

3.2.6 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Although we used a combination of SPE, HPLC separation with a long run time, and specific immunoassays, LC-MS/MS is useful for definitive steroid identification. We used a non-quantitative method with pooled samples (one pool of each tissue type, at each age) to optimize steroid detection. We spiked pools with deuterated internal standards (d4-cortisol, d8-corticosterone), and extracted and separated steroids with SPE and HPLC as described above. Dried HPLC fractions were resuspended in 150 µl of 22% Optima-grade methanol and transferred into a 96-well autoinjector sample plate. 50 µL of resuspended sample was injected into a Shimadzu Prominence LC20AC and separated on a Phenomenex Gemini NX-C₁₈ column (100 × 2.1 mm, 3.5 µm) in a 55°C column oven using 2 mM ammonium acetate in water as mobile phase A (MPA), and 2 mM ammonium acetate in methanol as mobile phase B (MPB). The gradient profile started at 20% MPB for 1 minute, ramped to 70% MPB for 4 min, held for 1 minute, ramped to 90% MPB for 0.5 min, held for 1 minute, and returned to starting conditions in 0.1 minute and held for an additional 2.4 min to re-equilibrate the column. The total run time was 10 min. Steroids were detected with multiple reaction monitoring (MRM), with two MRM transitions each for corticosterone (m/z 347.4 → 121.0, m/z 347.4 → 97.1) and cortisol (m/z 363.4 → 121.1, m/z 363.4 → 97.1) and 1 MRM transition for each internal standard (corticosterone-d8 m/z 355.4 → 125.2, cortisol-d4 367.2 → 121.2), acquired on an AB SCIEX 5500Qtrap triple quadrupole tandem mass spectrometer in positive electrospray ionization mode. None of the endogenous steroids tested interfered with the LC-MS/MS cortisol assay (Table 3.2).

Product ion spectra for cortisol in a representative standard and sample were obtained by acquiring product ion scans on the cortisol parent (Q1) mass of 363.4, using the same liquid chromatography parameters as described for the MRM method.

3.2.7 Real-time quantitative PCR (qPCR)

Tissue samples from PND5 and PND15 mice ($n = 3$ mice at each age) were collected and snap-frozen on dry ice as described above. Samples were then homogenized in ice-cold isola-
RNA lysis reagent (5 Prime #2302700), and RNA was extracted with chloroform, precipitated with isopropanol, and washed with 75% ethanol. Next, cDNA was synthesized from 4 μ g of total RNA using a Maxima First Strand synthesis kit (Thermo #K1641) according to the manufacturer's instructions. Then qPCR for gene expression of steroidogenic enzymes was performed using SYBR Green assays (using SsoFast EvaGreen supermix, Bio-Rad #172-5201) with previously published primers (Qiao et al., 2008) (Table 3.3). Assays were run at 95°C for 3 min, followed by 50 cycles of 95°C for 10 sec, 60°C for 15 sec on a Bio-Rad CFX96 real-time PCR system. Product specificity was examined by dissociation curve analysis and gel electrophoresis. For two genes, Cyp21a1 and Cyp11b1, SYBR Green assays gave non-specific results, so we used instead previously validated 5' nuclease probe-based assays (using Brilliant III Ultra-Fast QPCR master mix, Agilent #600880) to quantify expression of these genes (Table 3.4). These assays were run at 95°C for 3 min, followed by 45 cycles of 95°C for 5 sec, 60°C for 10 sec. The ribosomal gene *Rps29* was used as a reference gene to normalize sample loading (de Jonge et al., 2007), and expression of steroidogenic enzymes was expressed relative to PND5 thymus, as the developing thymus is known to express mRNA, protein, and activity of the full suite of glucocorticoid-synthetic enzymes (Vacchio et al., 1994; Lechner et al., 2000; Qiao et al.,

2008; Pazirandeh et al., 1999). Negative controls (no RNA, no reverse transcription) were always nondetectable.

3.2.8 Statistical analysis

For immunoassays, nondetectable samples (below the lowest standard on the standard curve and lower than the average water blank + two SD) were set to zero. Log-transformed data were analyzed with linear mixed-effects models, using R (R Core Team, 2005) and Prism 5. The E16.5 subjects were analyzed separately, as several tissues could not be obtained at this age. Concentrations of all steroids, each analyzed separately, varied with tissue type ($p < 0.0001$) and showed age \times tissue interactions ($p < 0.0001$). The relationships between tissue and circulating steroid levels were similar in females and males, so sexes were pooled for further analyses. At each age we compared organ steroid levels with whole blood (hereafter “blood”) steroid levels, as blood is more reflective of circulating steroid levels than plasma (Taves et al., 2011b; Hiramatsu and Nisula, 1987). However, plasma steroids were also quantified to allow comparison with previously published data (Figure 3.1), and observed patterns were similar whether organs were compared with plasma or blood. We conducted planned pairwise comparisons, using paired t-tests or Wilcoxon tests as appropriate to compare steroid levels in different tissues with steroid levels in blood. As we were looking specifically for tissue-specific local elevation of steroid concentrations, tests were one-directional. Within a given tissue sample, concentrations of other steroids relative to corticosterone were also calculated, as this could relate to precursor conversion to active glucocorticoids or the relative ligand availability of two different glucocorticoids. Gene expression of steroidogenic enzymes was compared between PND5 and PND15 mice using unpaired two-directional t-tests. Significance set at $\alpha = 0.05$.

3.3 Results

3.3.1 Tissue and blood steroid levels

To assess the evidence for or against tissue-specific steroid regulation, we measured steroids (Figure 1.1, Table 2.2) in organs of interest, and compared these tissue steroid levels with corresponding steroid levels in circulating blood. When steroid concentrations in an organ are higher than concentrations in blood, this indicates that the organ is actively increasing its local steroid content.

Corticosterone and cortisol are locally elevated in the embryonic and neonatal thymus.

Corticosterone, the major circulating glucocorticoid in mice, was present at higher concentrations in the thymus than in circulating blood of embryos and neonates to PND5 (Figure 3.2C). Corticosterone in blood was only detectable in 25% of subjects at PND5 (Table 3.5), consistent with this age being during the SHRP. At later ages, corticosterone levels were similar in thymus and blood. Cortisol, a glucocorticoid that is not (or minimally produced by mouse adrenals (Norris, 2007; van Weerden et al., 1992; Touitou et al., 1990), was locally elevated in the thymus of embryonic and PND1 mice (Figure 3.2E). Cortisol levels in the embryonic thymus were more than 30-fold higher than levels in blood, and decreased thereafter with age. At PND5, cortisol concentrations were 3.67-fold greater than corticosterone concentrations in the thymus (Table 3.6). Consistent with minimal cortisol secretion by the adrenals, circulating cortisol was non-detectable in the majority of subjects (Table 3.5). Both of the glucocorticoid precursors, deoxycorticosterone and deoxycortisol, followed similar local patterns as their respective downstream glucocorticoids. Uniquely, progesterone was locally elevated in the thymus into adulthood (Figure 3.2A, B, D).

Corticosterone and cortisol are locally elevated in neonatal bone marrow.

Corticosterone and cortisol were both locally elevated in the bone marrow at PND5 (Figure 3.2H, J). Cortisol levels in bone marrow were approximately 1000-fold higher than cortisol levels in blood, and more than 35-fold higher than corticosterone levels in bone marrow (Table 3.6), suggesting that at this age, cortisol might be considered the predominant glucocorticoid in bone marrow. The glucocorticoid precursors deoxycorticosterone and deoxycortisol were both elevated in PND1 and PND5 bone marrow (Figure 3.2G, I), while progesterone again remained locally elevated into adulthood (Figure 3.2F), as in the thymus.

Deoxycorticosterone is locally elevated in the developing and adult heart.

Deoxycorticosterone was locally elevated in the heart at all ages except PND5, when there was a non-significant trend for local elevation ($p=0.057$) (Figure 3.2L). Mean deoxycorticosterone levels in the heart were always more than double the levels in blood. Its precursor, progesterone, had a nearly identical pattern of local elevation in the heart (Figure 2K). Cortisol and deoxycortisol were elevated at PND5 (Figure 3.2N, O), while corticosterone was locally elevated at PND1 and PND5 (Figure 3.2M).

Corticosterone is locally elevated in the liver, spleen, and brain. Corticosterone levels were locally elevated in the liver of postnatal and adult mice (Figure 3.3C), while its precursors deoxycorticosterone and progesterone were locally elevated in the developing but not adult liver (Figure 3.3A, B). Corticosterone was also elevated in the spleen at PND1 and PND5 (Figure 3.3H), and in the brain at PND5 (Figure 3.3M). Cortisol and deoxycortisol were not significantly locally elevated in liver (Figure 3.3D, E), spleen (Figure 3.3I, J), or brain (Figure 3.3N, O) at any age, and cortisol and deoxycortisol concentrations were always much lower than those of corticosterone (Table 3.6).

3.3.2 LC-MS/MS detection of cortisol

As cortisol is an unexpected steroid in the mouse, we used non-quantitative LC-MS/MS to confirm the presence of both cortisol and corticosterone as endogenous steroids in mouse tissues. We pooled tissue from several developing mice and several adult mice, and after SPE and HPLC separation, used LC-MS/MS to detect corticosterone and cortisol. Ion chromatograms of MRM specific to corticosterone or cortisol showed peaks at identical retention times to spiked corticosterone or cortisol, respectively, and exhibited the same positive fragmentation ion mass spectra as spiked standards. Specifically, corticosterone was present in all tissue types in both developing and adult mice, as expected (data not shown). We also confirmed the presence of cortisol in the developing whole femur (including bone marrow) and brain, and the adult thymus and heart (representative product ion scans from prepared standards or thymic extracts obtained at the elution time of cortisol shown in Figure 3.4A, B; MRM detection shown in Figure 3.4C, D and Figure 3.5). However, cortisol was not detected in plasma, whole blood, liver, or spleen (3.5), consistent with immunoassay findings.

3.3.3 Gene expression of steroidogenic enzymes

To test whether the enzymes needed for glucocorticoid synthesis are present in these tissues, we next looked at gene expression of these enzymes in mouse tissues during and after the SHRP (in PND5 neonates and PND15 juveniles, respectively). We found that transcripts for the upstream glucocorticoid-synthetic enzymes *Star*, *Cyp11a1*, *Hsd3b6*, *Cyp21a1*, and *Cyp11b1* were widely expressed across tissues, in both PND5 and PND15 mice (Figure 3.6A-F). *Hsd3b1* expression was also detected, although at very low levels (usually detectable after 40 qPCR

cycles for tissues other than thymus and liver). Negative controls were all nondetectable. *Cyp21a1* and *Cyp11b1* expression were lower at PND5 than PND15 in the bone marrow. Importantly, transcripts of *Cyp17a1*, which is necessary for the production of cortisol, were widely expressed in mice (Figure 3.6G). In the thymus, *Cyp17a1* transcripts were present at similar levels in PND5 and PND15 mice, while expression decreased with age in bone marrow and possibly heart (Figure 3.6G). Liver expression of *Hsd3b1*, *Hsd3b6*, and *Cyp21a1* was very high (Figure 3.6C-E). Gene expression varied in the brain; *Star* expression decreased with age, while *Cyp11a1* and *Cyp11b1* increased with age. Overall, we found the full suite of steroidogenic enzyme genes to be expressed in each of the tissues examined.

In tissues where glucocorticoids were locally elevated in the absence of elevated glucocorticoid precursors, *Hsd11b1* regeneration of inactive glucocorticoid metabolites (Figure 1.1) could be a major contributor to endogenous glucocorticoid levels. Thus, we also examined *Hsd11b1* gene expression, and found that transcript levels were very high in the liver, and increased with age in the thymus, brain, and potentially heart (Figure 3.6H).

3.4 Discussion

Here we present evidence that different tissues are able to regulate their local glucocorticoid levels *in vivo*, independent of glucocorticoid patterns in circulating blood. Specifically, we quantified endogenous glucocorticoids and precursors over multiple developmental timepoints and in multiple organs where glucocorticoids have important actions. Strikingly, each organ had a unique pattern of elevated glucocorticoid (and glucocorticoid precursor) concentrations over development, and several organs had elevated levels of glucocorticoids that are unexpected in mice (e.g., deoxycortisol, cortisol). These results

demonstrate that local regulation allows organisms to maintain high steroid concentrations at specific locations where they are beneficial. Local regulation also allows steroid levels in different locations to follow distinct age-related patterns. Finally, local regulation allows tissues to utilize different steroid ligands from those in systemic circulation (e.g. cortisol in thymus, bone marrow, and heart, deoxycorticosterone in heart versus corticosterone in blood). These distinct local steroids may have different mechanisms of action and effects than systemic steroids.

Local glucocorticoid regulation is widespread across organs, and may even be the norm rather than the exception. Such autonomous regulation allows the organism to limit high glucocorticoid concentrations to where they are needed, while keeping systemic glucocorticoid concentrations low to avoid the detrimental effects of chronic glucocorticoid exposure. This strategy may be beneficial in reducing glucocorticoid-induced “wear and tear,” or allostatic load (McEwen and Wingfield, 2003). Furthermore, it explains how the distinct glucocorticoid requirements of individual organs (such as the thymus, heart, and liver) can be met when circulating glucocorticoids are minimal, such as during the SHR, or variable, as they are in later development and in adulthood.

Local regulation could occur via different mechanisms. Previous work has shown that several organs have the enzyme activities needed for synthesis of glucocorticoids from cholesterol or upstream steroids (thymus, (Vacchio et al., 1994; Pazirandeh et al., 1999; Lechner et al., 2000; Lechner et al., 2001); heart, (Silvestre et al., 1998; Silvestre et al., 1999) but see (Funder, 2004); brain, (Gomez-Sanchez et al., 1997; Higo et al., 2010)). Additionally, studies have shown widespread activity of 11 β -hydroxysteroid dehydrogenase type 1 (coded by the *Hsd11b1* gene), which converts inactive metabolites (11-dehydrocorticosterone, cortisone) back into active glucocorticoids (corticosterone, cortisone) (Tomlinson et al., 2004). Glucocorticoids

can also be preferentially imported into (or out of) cells and tissues by ATP-binding cassette (ABC) transporters (Karssen et al., 2001), potentially resulting in local accumulation of specific glucocorticoids that are present in circulating blood. Finally, glucocorticoids may be sequestered by high-affinity binding to membrane and cytoplasmic proteins, such as corticosteroid-binding globulin (Breuner et al., 2002; Sivukhina et al., 2013) and glutathione S-transferase (Homma et al., 1985; Rowe et al., 1997). Such proteins could affect access of glucocorticoids to their cognate receptors. Differential expression of receptors might also affect tissue glucocorticoid distributions.

Multiple mechanisms likely contribute to our observations. Glucocorticoid synthesis from upstream precursors might be the most important during the SHRP, as circulating glucocorticoids are minimal. Our detection of widespread gene expression of glucocorticoid-synthetic enzymes is consistent with this possibility, and in the thymus, heart, and brain, these gene expression data are consistent with previous demonstrations of enzyme activity in these tissues (Vacchio et al., 1994; Lechner et al., 2000; Silvestre et al., 1998; Gomez-Sanchez et al., 1997). In many cases, we also found that glucocorticoid precursors had similar patterns of elevation as their downstream glucocorticoids, which is also consistent with local synthesis. Outside of the SHRP, and especially in adulthood, regeneration of circulating glucocorticoid metabolites and binding of circulating glucocorticoids to high-affinity intracellular proteins likely play increased roles. This is consistent with increased expression of *Hsd11b1* in certain tissues after the SHRP and with the absence of elevated glucocorticoid precursors, such as in the adult liver.

In the thymus, corticosterone, cortisol, and their precursors were locally elevated in the embryo and neonate. The thymus produces T cells, a critical arm of adaptive immunity, and the

majority of T cells are produced during embryonic and early postnatal development, coincident with locally elevated thymic glucocorticoid levels. Glucocorticoids promote T cell immunocompetence, ensuring development of a functional immune system (Mittelstadt et al., 2012), and local glucocorticoid synthesis may ensure this when circulating glucocorticoids are low (Vacchio et al., 1994; Lechner et al., 2000; Pazirandeh et al., 1999). The presence of cortisol and *Cyp17a1* mRNA in the thymus is intriguing, as cortisol is often considered to be minimal or absent in the mouse (Norris et al., 2007; van Weerden et al., 1992; Touitou et al., 1990). While the embryonic adrenal expresses *Cyp17a1* transcripts before birth (Keeney et al., 1995) and may produce cortisol in early life (Varon et al., 1964), circulating cortisol was usually nondetectable in this study. As murine T cell activation is regulated more potently by cortisol than corticosterone (Gillis et al., 1979; Iwata et al., 1991), local and systemic glucocorticoids could differentially regulate T cells, especially at PND5 when thymic cortisol nearly four times higher than corticosterone. Interestingly, a highly similar pattern is seen in altricial songbirds, where corticosterone is the major circulating glucocorticoid but thymus has higher cortisol levels (Schmidt and Soma, 2008). Progesterone, in contrast, remained elevated in the adult mouse thymus; progesterone may regulate T cell differentiation (Hughes et al., 2013; Lee et al., 2012) or thymic involution (Tibbetts et al., 1999).

Similar to the thymus, bone marrow corticosterone and cortisol levels were locally elevated in early postnatal development, with cortisol and glucocorticoid precursor levels far higher than those in blood. Glucocorticoids were quite high in the bone marrow during the SHRP, when they were usually nondetectable in the blood. These data, together with gene expression of the full suite of glucocorticoid-synthetic enzymes, provide the first indication of local steroid synthesis in the bone marrow. Bone marrow hematopoiesis, which peaks in the neonate and

decreases thereafter (Orkin and Zon, 2008), parallels the neonatal peak and drop in local glucocorticoid levels and glucocorticoid-synthetic enzymes with age. Immature B cells express especially high levels of the glucocorticoid receptor (Gruver-Yates et al., 2013) and are more sensitive to cortisol than corticosterone (Garvy et al., 1993b), suggesting a role for local glucocorticoids (especially cortisol) in B cell development. Local elevation of cortisol is also seen in the corresponding avian site of B cell development, the bursa of Fabricius, contrasting with adrenal production of corticosterone (Lechner et al., 2001; Schmidt and Soma, 2008). Furthermore, cortisol (but not corticosterone) specifically binds to membrane glucocorticoid receptors in the bursa of Fabricius (Schmidt et al., 2010). Membrane glucocorticoid receptors are also present in mammalian B cells (Bartholome et al., 2004) and could be a mechanism for cortisol-specific signaling. Locally regulated glucocorticoids could also promote production of innate immune cells (Trottier et al., 2008) and erythrocytes (Bauer et al., 1999).

Heart levels of deoxycorticosterone and progesterone were locally elevated in development and adulthood, while corticosterone and cortisol were also elevated in the neonatal heart. The timing of cortisol, corticosterone, and *Cyp11a1* mRNA elevation correspond with the requirement of glucocorticoids for murine cardiac maturation (Rog-Zielinska et al., 2013). Clinical findings (Pitt et al., 1999) have motivated a search for local mineralocorticoid synthesis in the adult heart, and aldosterone, which is downstream of corticosterone, is the major mineralocorticoid in rodents and humans. Some studies have found aldosterone-synthetic activity in heart tissue (e.g., Silvestre et al., 1998), while others have only found expression of deoxycorticosterone-synthetic enzymes (e.g., Young et al., 2001; Kayes-Wandover and White, 2000; Ye et al., 2005). Further, aldosterone levels are extremely low or absent in the rat heart after adrenalectomy (Gomez-Sanchez et al., 2004). While often dismissed as an inactive

precursor, deoxycorticosterone functions as a mineralocorticoid (Vinson et al., 2011), and our results suggest that locally-elevated deoxycorticosterone could bind to mineralocorticoid receptors in the heart to regulate cardiac remodeling in heart failure.

The liver had elevated corticosterone and precursor levels during development, consistent with expression of upstream steroidogenic enzymes. Corticosterone, however, remained elevated in the adult liver, potentially due to production by *Hsd11b1* (Jamieson et al., 1995), binding to corticosteroid-binding globulin (Breuner and Orchinik, 2002), and binding to glutathione S-transferase (Homma et al., 1985), all of which are present at high levels in the liver. Locally elevated corticosterone in the liver and also spleen coincides with and could promote erythropoiesis (Hattangadi et al., 2011) and clearance of toxic free hemoglobin (Schaer et al., 2002).

Brain levels of deoxycorticosterone and progesterone were elevated in the embryo, and corticosterone was elevated at PND5. Elevated corticosterone and high *Cyp11b1* expression during the SHRP was surprising, as glucocorticoids generally impede neural growth, and the SHRP is believed to minimize circulating glucocorticoids to facilitate brain development (Wada, 2008; Lupien et al., 2009). However, brain regulation of glucocorticoids is heterogeneous and likely occurs at some locations while levels are low elsewhere (Gomez-Sanchez et al., 1997; Higo et al., 2011). Future steroid measurements, in brain and other tissues, would benefit from the use of techniques with greater spatial resolution (Taves et al., 2011b).

3.5 Conclusions

Taken together, these data show that tissue-specific regulation of local glucocorticoid levels occurs widely in the developing mouse. Local regulation may involve widespread expression of

steroid metabolic enzymes, and further work should test for enzyme activity in tissues such as bone marrow where this has not been shown. This heterogeneity of endogenous glucocorticoid levels is likely important to understanding glucocorticoid functions in health and disease. For example, it is often thought that low systemic glucocorticoids are beneficial for some organs, such as the thymus, which atrophies in response to chronic elevation of adrenal glucocorticoids. However, the presence of locally elevated glucocorticoids in multiple developing organs of the mouse suggests that physiological (intermediate) levels of glucocorticoids, including different glucocorticoids than those in circulating blood, may have diverse and unsuspected roles in a variety of organs (Schmidt et al., 2008). This is clearly illustrated by the thymus, which requires glucocorticoids for production of immunocompetent lymphocytes (Mittelstadt et al., 2012). Furthermore, while the measurement of one circulating glucocorticoid (corticosterone or cortisol) is widely employed (Norris, 2007; Guyton and Hall, 2005; Demas and Nelson, 2012), such single measurements are often misleading with respect to glucocorticoid levels in different organs. Instead, different organs display a remarkable variety of glucocorticoid patterns, and glucocorticoid functions in different tissues should therefore be evaluated in the context of local, not just systemic, glucocorticoid levels.

3.6 Tables and figures

Table 3.1: Immunoassay specifications

| Steroid | Progesterone | Deoxycorticosterone | Corticosterone | Deoxycortisol | Cortisol |
|--|--------------------|---------------------|---------------------|----------------------------|---------------------|
| Manufacturer | MP Biomedicals | Novus Biologicals | MP Biomedicals | American Research Products | Salimetrics |
| Assay/Antibody cat. # | 07170105 | NB100-62098 | 07120103 | 13-2219 | 1-3002 |
| Poly/Monoclonal | Rabbit, polyclonal | Sheep, polyclonal | Rabbit, polyclonal | Sheep, polyclonal | Mouse, monoclonal |
| Dilution | see methods | 1:14,000 | as per manufacturer | 1:3333 | as per manufacturer |
| Modification / Validation | see methods | see methods | see methods | see methods | see methods |
| Standard curve range (pg/well or pg/tube) | 1.95 - 1000 | 1.95 - 500 | 1.56 - 250 | 1.95 - 500 | 3.00 - 750 |
| Cross-reactivity (%) | | | | | |
| Progesterone | 100 | na | 0.02 | 2.0 | <0.02 |
| Deoxycorticosterone | 3.80 | 100 | 0.34 | 7.0 | 2.24 |
| Corticosterone | 0.70 | na | 100 | 0.5 | 0.20 |
| Dehydrocorticosterone | na | na | 0.50 | na | 2.03 |
| Aldosterone | <0.01 | na | 0.03 | <0.01 | <0.01 |
| 17 α -OH-progesterone | 0.67 | na | <0.01 | 7.0 | <0.01 |
| Deoxycortisol | <0.01 | <1.0 | <0.30 | 100 | 0.16 |
| Cortisol | <0.01 | 2.0 | 0.05 | 0.5 | 100 |
| Cortisone | na | na | <0.30 | <0.01 | 0.13 |
| Estimate of steroid recovery (%) | | | | | |
| Whole blood | 90.5 | 94.1 | 84.9 | 66.9 | 81.1 |
| Thymus | 77.0 | 95.7 | 66.0 | 32.8 | 42.8 |
| Bone marrow | 77.3 | 88.7 | 70.9 | 61.8 | 66.9 |
| Heart | 46.3 | 64.1 | 74.0 | 37.3 | 59.5 |
| Liver | 64.4 | 95.7 | 77.3 | 57.3 | 70.1 |
| Spleen | 68.9 | 89.8 | 82.1 | 63.6 | 80.7 |
| Brain | 76.0 | 86.7 | 70.4 | 52.4 | 34.3 |
| Plasma | 71.0 | 90.6 | 78.3 | 49.8 | 61.0 |

Note: Antibody cross-reactivities were previously determined (Schmidt et al., 2008) or reported by the manufacturer. na = not assessed.

Table 3.2: Steroids tested for interference with LC-MS/MS cortisol assay.

| Steroid | Amount tested (nM) | Interference |
|-----------------------------------|--------------------|--------------|
| Progesterone | 15.90 | none |
| 17 α -OH-Progesterone | 15.13 | none |
| Deoxycorticosterone | 4.24 | none |
| 18-Hydroxy-11-Deoxycorticosterone | 2.89 | none |
| Deoxycortisol | 99.00 | none |
| 21-Deoxycortisol | 9.00 | none |
| Corticosterone | 7.25 | none |
| 18-Hydroxycorticosterone | 6.35 | none |
| Cortisone | 277.44 | none |
| Aldosterone | 5.21 | none |
| Dehydroepiandrosterone | 17.25 | none |
| Dehydroepiandrosterone sulfate | 3390.00 | none |
| Androstenedione | 4.38 | none |
| Testosterone | 350.00 | none |
| 17 β -Estradiol | 18.25 | none |
| 25-Hydroxyvitamin D3 | 62.25 | none |

Table 3.3: SYBR green qPCR assays

| Gene | Forward (5'-3') | Reverse (5'-3') | Exons |
|---------|-------------------------|--------------------------|-------|
| Star | TCACTTGGCTGCTCAGTATTGAC | GCGATAGGACCTGGTTGATGA | 6-7 |
| Cyp11a1 | GACCTGGAAGGACCATGCA | TGGGTGTACTCATCAGCTTATTGA | 4-5 |
| Hsd3b1 | CTTTTCAGCCACCACCATCT | GGTCTGTCCTTCCCAGTGAT | 1-2 |
| Hsd3b6 | AGACCAGAAACCAGGGAGCAA | TCTCCTTCCAACACTGTCACCTT | 2-3 |
| Cyp17a1 | GTGGACATATTCCCGTGGTT | TCAAACATTCAACCAGTGTFFFF | 3-4 |
| Hsd11b1 | TGGTGCTCTCCTGGCCTACT | CTGGCCCCAGTGACAATCA | 1-2 |
| Rps29 | ACGGTCTGATCCGCAAATAC | CATGATCGGTTCCACTTGGT | 2-3 |

Table 3.4: 5' nuclease probe qPCR assays.

| Gene | Forward (5'-3') | Reverse (5'-3') | Probe (5'-3') | Exons |
|---------|-----------------------|-------------------------|--------------------------|-------|
| Cyp21a1 | GGTTCCAGGAAGCGATCTG | CAACTAGGGCTAGCAGCATC | TCATCATCCCCAACATCCAAGGCG | 8-10 |
| Cyp11b1 | GCCAGCTCAAAGAAAGTCAT | CAGGCTAACTCAATGGAACCTCA | ACTGACACGACATCAACCCCCTG | 5-6 |
| Rps29 | TCCATTCAAGGTCGCTTAGTC | CGGTCTGATCCGCAAATACG | AAGCCTATGTCCTTCGCGTACTGC | 1-3 |

Note: Pre-designed 5' nuclease probe qPCR assays were purchased from Integrated DNA Technologies (cat. # Mm.PT.58.29960109, Mm.PT.58.41798181, Mm.PT.58.32879049).

Table 3.5: Percent of mice with detectable steroid levels in circulating whole blood.

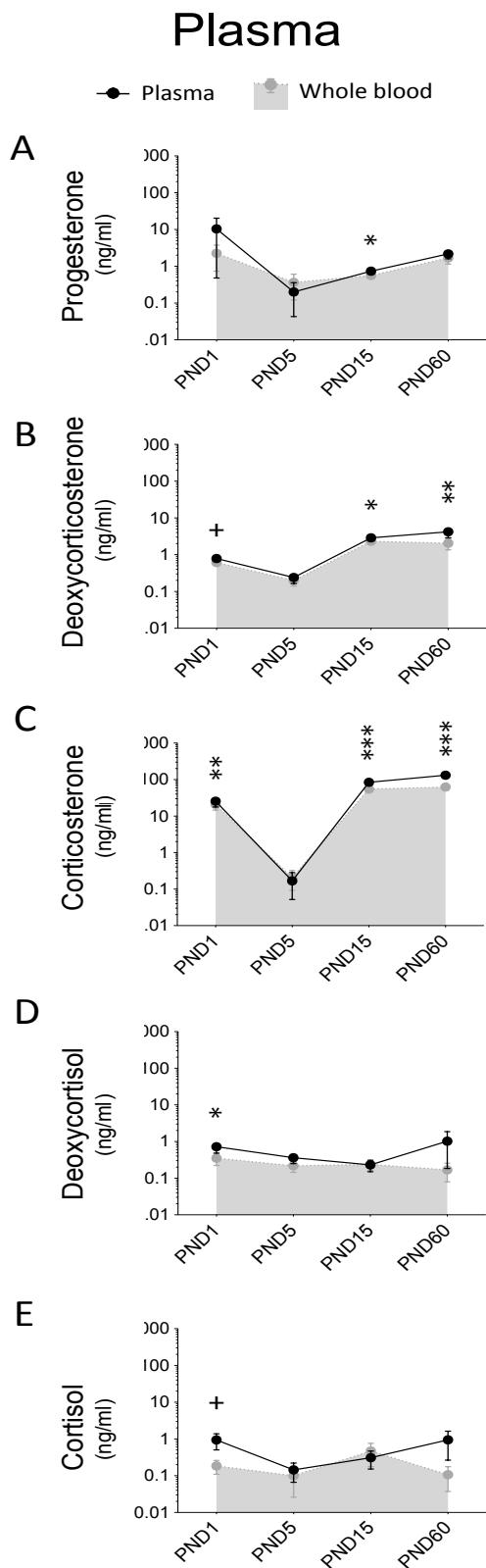
| Steroid | E16.5 | PND1 | PND5 | PND15 | PND60 |
|---------------------|-------|------|------|-------|-------|
| Progesterone | 100 | 79 | 75 | 75 | 77 |
| Deoxycorticosterone | 100 | 86 | 67 | 100 | 69 |
| Corticosterone | 100 | 100 | 25 | 100 | 100 |
| Deoxycortisol | 100 | 50 | 50 | 67 | 33 |
| Cortisol | 38 | 36 | 17 | 42 | 23 |

Table 3.6: Relative steroid concentrations across ages and tissues.

| Ratio | Age | Blood | Thymus | Bone marrow | Heart | Liver | Spleen | Brain |
|--|-------|-------|--------|-------------|-------|-------|--------|-------|
| Progesterone / Corticosterone (%) | E16.5 | 3 | 13 | na | 39 | 23 | na | 45 |
| | PND1 | 15 | 13 | 32 | 29 | 24 | 67 | 7 |
| | PND5 | (176) | (38) | (941) | (63) | 66 | (49) | (40) |
| | PND15 | 1 | 3 | 18 | 5 | 2 | 4 | 1 |
| | PND60 | 2 | 8 | 19 | 10 | 2 | 8 | 2 |
| Deoxycorticosterone / Corticosterone (%) | E16.5 | 5 | 10 | na | 27 | 7 | na | 26 |
| | PND1 | 9 | 14 | 68 | 17 | 6 | 37 | 11 |
| | PND5 | (97) | (128) | (1033) | (37) | 20 | (25) | (18) |
| | PND15 | 4 | 7 | 24 | 13 | 2 | 7 | 5 |
| | PND60 | 3 | 4 | 5 | 7 | 1 | 4 | 4 |
| Deoxycortisol / Corticosterone (%) | E16.5 | 2 | 13 | na | 14 | 1 | na | 3 |
| | PND1 | 6 | 43 | 56 | 19 | 1 | 26 | 4 |
| | PND5 | (106) | 59 | (1146) | (53) | 5 | 24 | (18) |
| | PND15 | 1 | 2 | 83 | 2 | 0 | 1 | 0 |
| | PND60 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| Cortisol / Corticosterone (%) | E16.5 | 1 | 21 | na | 19 | 1 | na | 4 |
| | PND1 | 3 | 13 | 23 | 7 | 1 | 25 | 6 |
| | PND5 | 64 | 367 | (3556) | (42) | 12 | 24 | (18) |
| | PND15 | 2 | 3 | 6 | 1 | 0 | 0 | 0 |
| | PND60 | 0 | 1 | 12 | 2 | 0 | 9 | 0 |

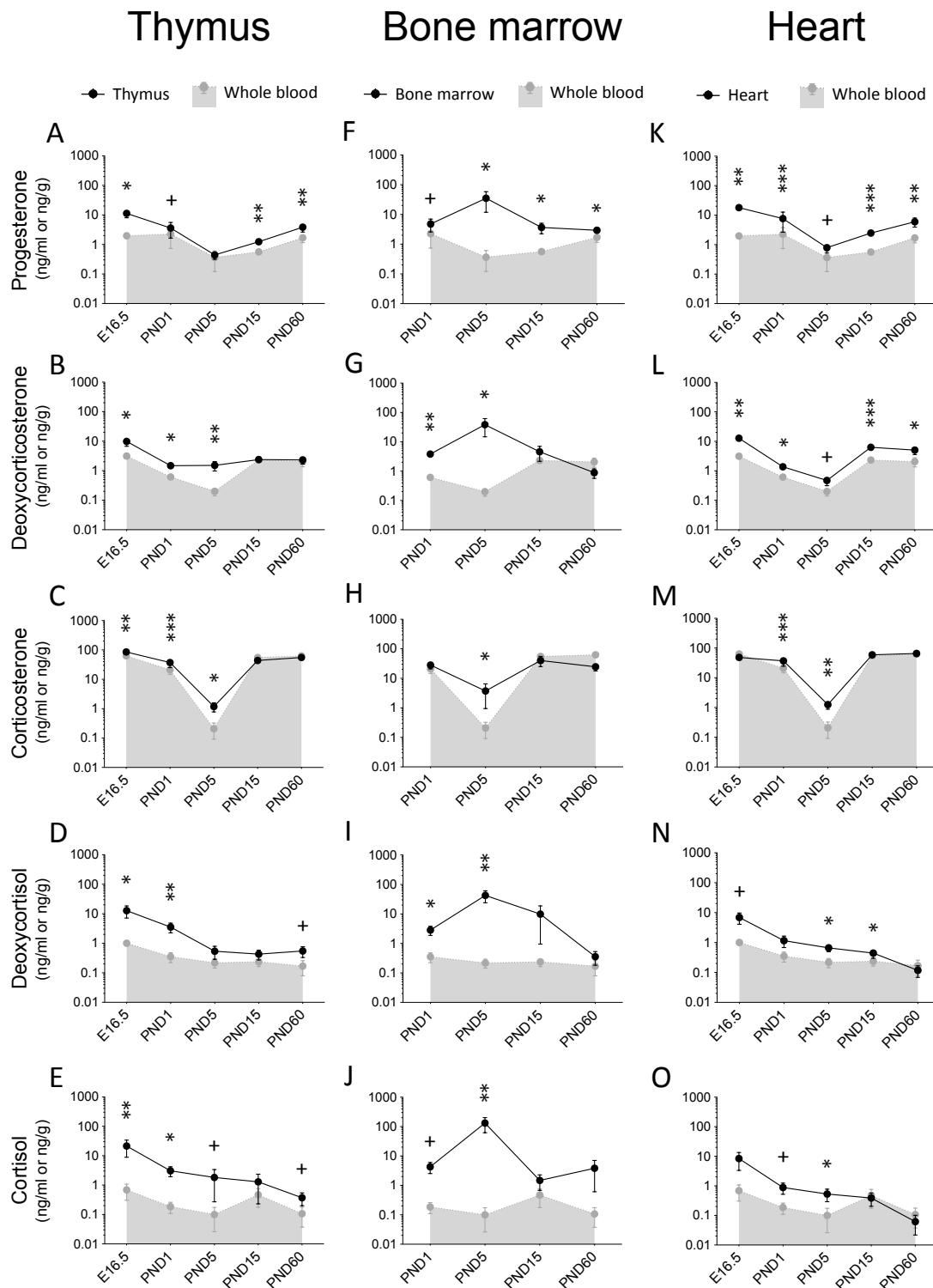
Note: Relative levels of progesterone, deoxycorticosterone, deoxycortisol, and cortisol compared to corticosterone (the major systemic glucocorticoid in the mouse), given as percent values. Each of these steroids regulates activity of the same nuclear receptors as corticosterone, thus relative concentrations are important for interpreting regulation of signaling. At PND5 (during the SHR) corticosterone was often nondetectable, resulting in non-calculable ratios. For these, estimates based on mean tissue concentrations (Figures 2 and 3) are given in parentheses. na = not assessed (tissues were not obtained).

Figure 3.1 Steroid concentrations in plasma and whole blood of developing mice



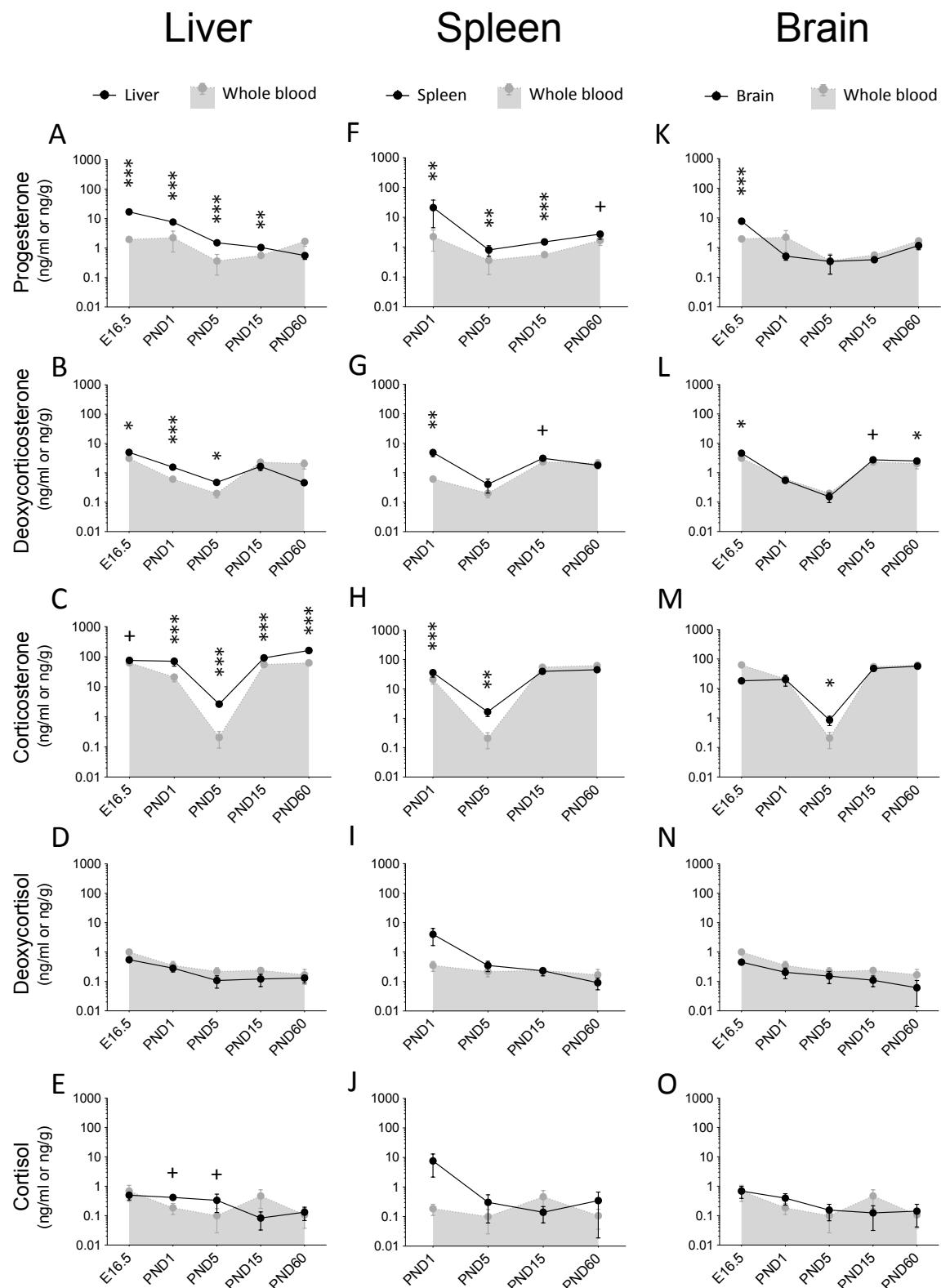
Steroid concentrations in plasma (black circles) relative to whole blood (shaded circles, shaded region) of mice at postnatal day (PND) 1, 5, 15, and 60, as measured by immunoassay. Data are presented as means \pm SEM, and plasma steroid concentrations greater than whole blood steroid concentrations are indicated at each age as follows, + $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Plasma was not obtained from embryos.

Figure 3.2 Steroid concentrations in thymus, bone marrow, and heart of developing mice.



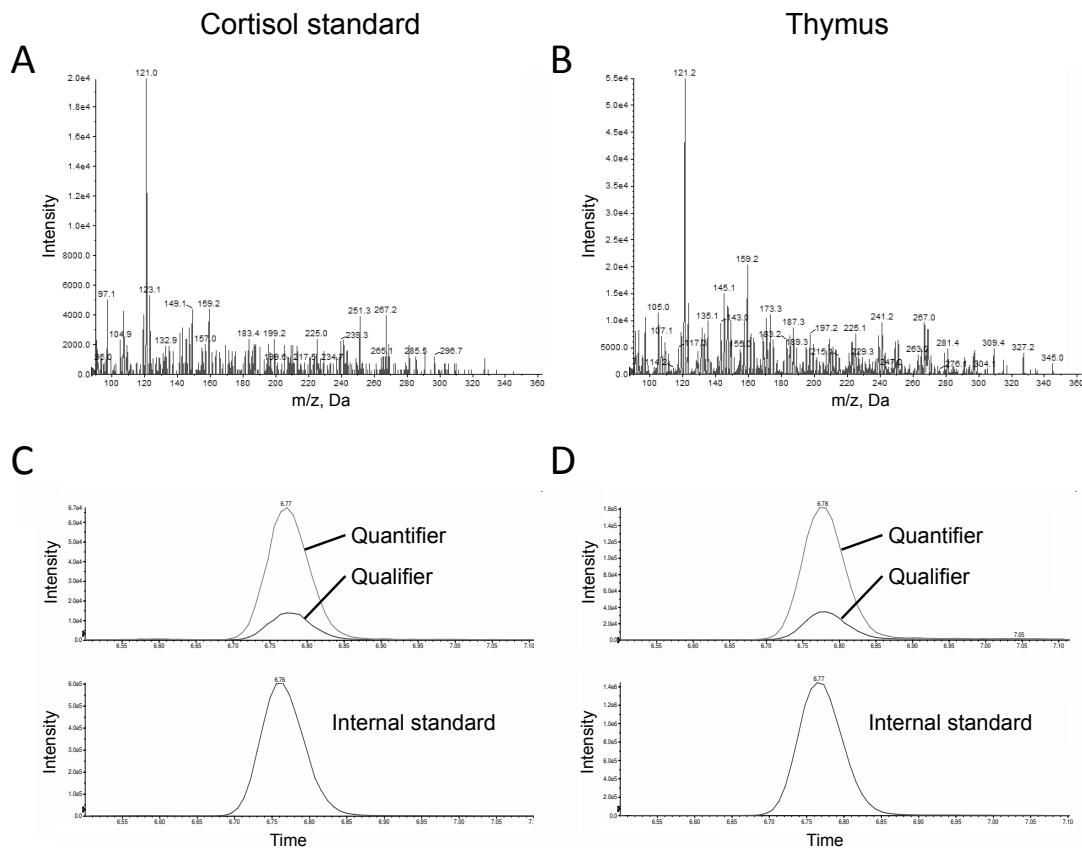
Steroid concentrations in thymus, bone marrow, and heart (black circles, nanograms per gram) relative to those in whole blood (shaded circles, shaded region, nanograms per milliliter) of mice at E16.5 and PND1, PND5, PND15, and PND60. Data are presented as means \pm SEM, and tissue steroid concentrations greater than blood steroid concentrations are indicated at each age as follows, + $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Bone marrow was not obtained from embryos. Furthermore, we were unable to flush marrow from femurs at PND1, and we thus quantified steroids in whole femur, including solid bone. Steroid concentrations in PND1 bone marrow may thus be higher than shown here.

Figure 3.3 Steroid concentrations in liver, spleen, and brain of developing mice.



Steroid concentrations in liver, spleen, and brain (black circles, nanograms per gram) relative to those in whole blood (shaded circles, shaded region, nanograms per milliliter) of mice at E16.5 and PND1, PND5, PND15, and PND60. Data are presented as means \pm SEM, and tissue steroid concentrations greater than blood steroid concentrations are indicated at each age as follows, + p ≤ 0.10 , * p ≤ 0.05 , ** p ≤ 0.01 , *** p ≤ 0.001 . Spleens were not obtained from embryos.

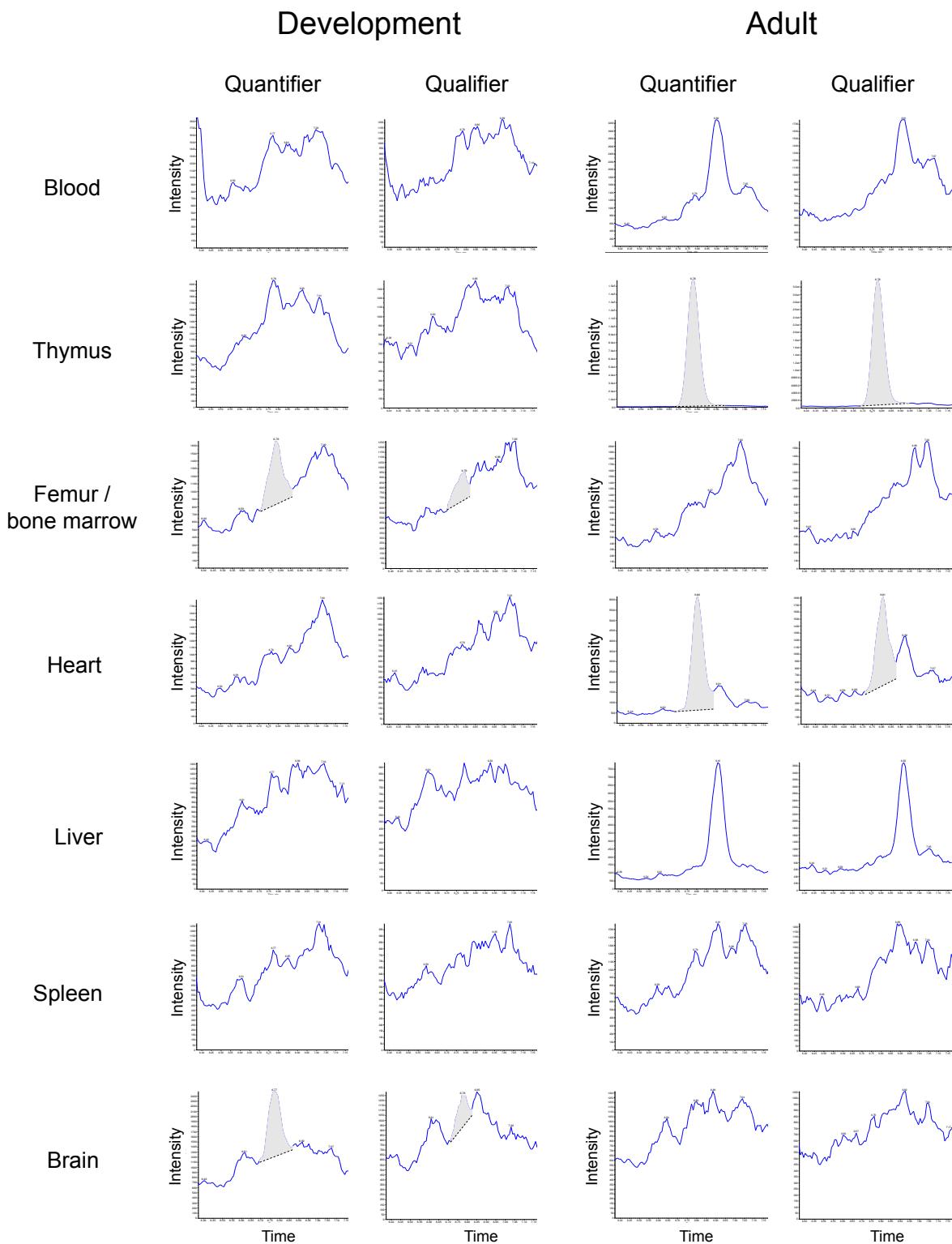
Figure 3.4 Detection of cortisol in mouse thymus by LC-MS/MS.



The fragmentation pattern of the cortisol standard (A) matches that from pooled adult thymus (B).

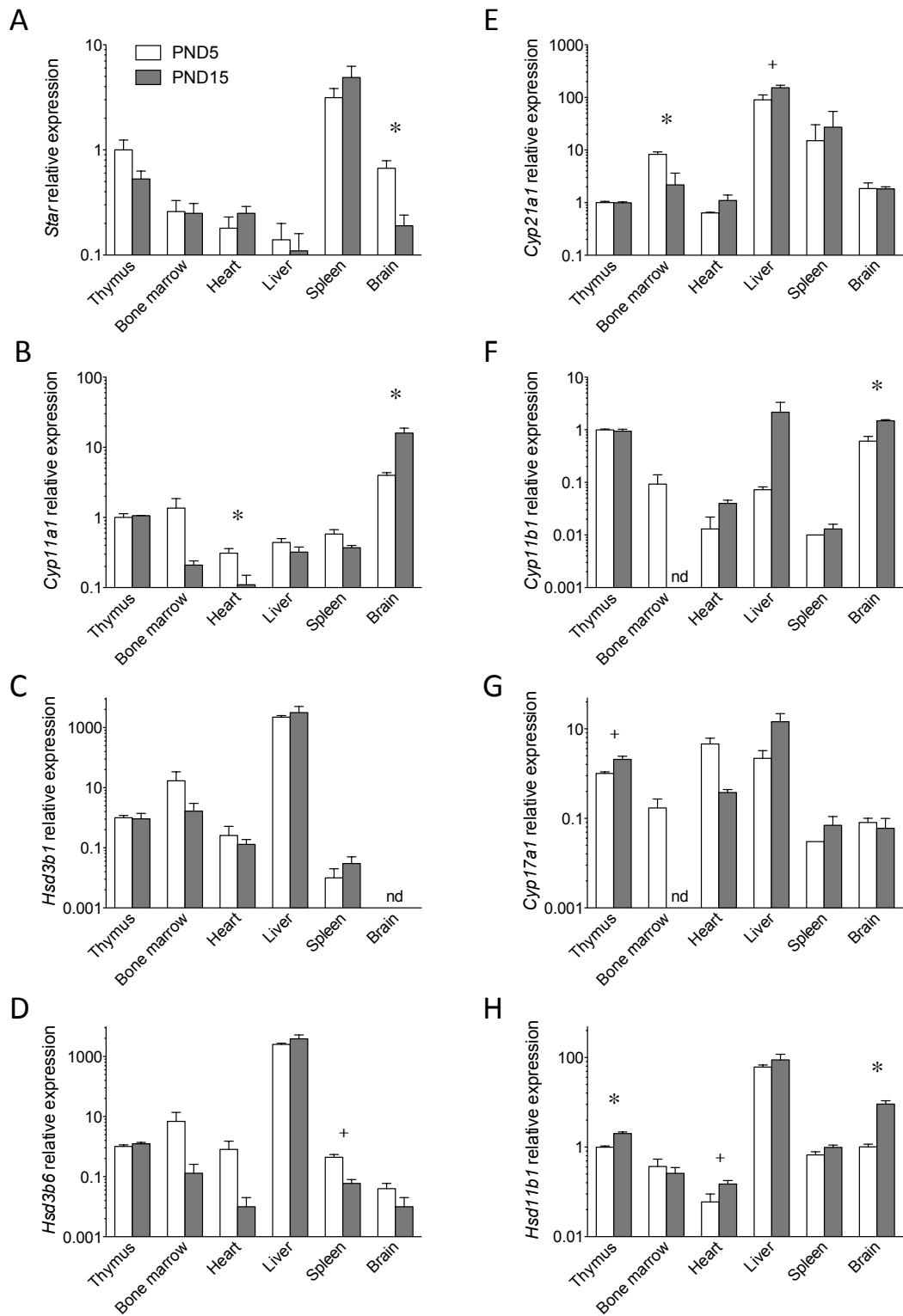
C and D, Cortisol was also detected with MRM, with 2 MRM transitions for cortisol (quantifier m/z 363.4 → 121.1 and qualifier m/z 363.4 → 97.1) (top panels) and one MRM transition for the cortisol-d₄ internal standard (m/z 367.2 → 121.4) (bottom panels). Cortisol was also detected using LC-MS/MS in developing femur (containing bone marrow), developing brain, and adult heart (see below), but cortisol was not detected in plasma, whole blood, liver, or spleen.

Figure 3.5 Cortisol detection across mouse tissues by LC-MS/MS.



Liquid chromatograph-tandem mass spectrometry (LC-MS/MS) multiple reaction monitoring (MRM) detection of cortisol quantifier (m/z 363.4 → 121.1) and qualifier (m/z 363.4 → 97.1) product ions in blood and tissues of developing (PND5 and PND15, left) and adult (PND60, right) mice. Whole femur was used from developing mice, while bone marrow was isolated from adult mice. The assay limit of quantification was 0.018 ng/ml. Grey shaded regions represent cortisol.

Figure 3.6 Gene expression of steroid synthetic enzymes across mouse development.



Gene expression of steroid synthetic enzymes Star (A), Cyp11a1 (B), Hsd3b1 (C), Hsd3b6 (D), Cyp21a1 (E), Cyp11b1 (F), Cyp17a1 (G), and Hsd11b1 (H) in thymus, bone marrow, heart, liver, spleen, and brain. Relative expression was corrected using Rps29 as a reference gene and is presented as relative abundance compared to that of PND5 thymus, in which these transcripts (Qiao et al., 2008; Pazirandeh et al., 1999) and their corresponding enzymatic activities (Vacchio et al., 1994; Lechner et al., 2000) have been shown. Data are presented as means \pm SEM, and differences in transcript levels between PND5 and PND15 tissues are indicated for each tissue as follows, + p \leq 0.10, * p \leq 0.05, nd = nondetectable.

4 Glucocorticoid regeneration by 11 β -HSD type 1 in bone marrow, spleen, and thymus of neonatal and adult mice

4.1 Introduction

Glucocorticoids are steroid hormones synthesized by the adrenal glands that circulate through the blood to coordinate systemic physiological function. Glucocorticoids have critical functions in lymphoid organs, regulating development, differentiation, and function of various hematopoietic cells (i.e., lymphocytes, Mittelstadt et al., 2012; macrophages, Schaer et al., 2002, Tuckermann et al., 2007; neutrophils, Tuckermann et al., 2007; erythrocytes, Bauer et al., 1999). An especially well-studied example involves the thymus, in which glucocorticoid signaling is necessary for production of immunocompetent T lymphocytes (Mittelstadt et al., 2012) and functional adaptive immunity. Due to the critical functions of glucocorticoids in lymphoid organs, therefore, it is important to understand how glucocorticoid concentrations are regulated in these organs.

As circulating glucocorticoids act on tissues throughout the body, it is often assumed that glucocorticoid concentrations in different tissues parallel concentrations in the blood. However, some tissues appear to regulate their local glucocorticoid levels independent of systemic glucocorticoid levels. Specifically, endogenous glucocorticoid concentrations in murine lymphoid organs (bone marrow, spleen, and thymus) are locally elevated *in vivo* compared to concentrations in circulating blood, especially during early development (Taves et al., 2015). This tissue-specific elevation could occur via local production of glucocorticoids, either by 1)

synthesis from upstream precursors (Taves et al., 2011a), or 2) regeneration of glucocorticoid metabolites (Tomlinson et al., 2004).

Here, we investigated whether murine bone marrow and spleen, like the thymus, have the capacity to independently produce glucocorticoids. Specifically, we tested for metyrapone-blockable glucocorticoid production (“production” referring to both the generation of glucocorticoids from precursors and from metabolites), gene expression of steroidogenic enzymes, and conversion of steroid substrates into active glucocorticoids. We examined lymphoid organs from 5-day old neonatal mice, which are altricial and have very low circulating glucocorticoid levels, and adults, which have high circulating glucocorticoid levels (Taves et al., 2015), to further test whether the magnitude or mechanism of glucocorticoid production changes with age. Due to the presence of locally elevated glucocorticoid precursor concentrations in neonatal lymphoid organs (Taves et al., 2015), we hypothesized that neonatal lymphoid organs would produce glucocorticoids via synthesis (“synthesis” referring to CYP11B1 conversion of precursors into glucocorticoids). As local elevation of precursors is less clear in adult lymphoid organs (Taves et al., 2015), we hypothesized that glucocorticoid synthesis from precursors would be reduced, and regeneration (“regeneration” referring to 11 β -HSD1 conversion of glucocorticoid metabolites into glucocorticoids) would be increased, in adults.

4.2 Materials and methods

4.2.1 Subjects

Female and male C57BL/6 mice were bred and housed at the Centre for Disease Modeling, a specific pathogen-free facility at the University of British Columbia. Mice were group housed with corn cob bedding, under a 12:12 light:dark cycle, with *ad libitum* water and

food (Teklad, diet 2018 for adults, diet 2019 for breeders). All protocols were approved by the UBC Animal Care Committee (A12-0119).

4.2.2 Tissue collection

Neonates (5 days old, with day 0 being the morning pups were first present) or adults (2 – 3 months old) were deeply anesthetized with isoflurane in oxygen (<2 min) and euthanized by rapid decapitation. Decapitation and blood collection was completed in less than 3 min after initial disturbance. Immediately after euthanasia, spleen, thymus, and femurs were collected and cleaned of fat and connective tissue. Tissue samples were collected in the morning (between 0800 and 1200 h), to reduce diel variation in hormone levels. Tissues were collected onto wet ice (for tissue culture experiments), or onto dry ice and stored at -80°C (for gene expression experiments).

4.2.3 Tissue culture

Subjects were anesthetized and euthanized as described above, and the thymus, spleen, and femurs were removed and cleaned of fat and connective tissue. Bone marrow was flushed from femurs, and thymus and spleen were minced in culture media consisting of RPMI 1640 (Hyclone SH3009601) supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone SH3006802), 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol, and 12 µM L-glutamine. All three tissue types were dispersed with a pipette, and incubated for 24 h in 5% CO₂ at 37°C in 4 ml medium containing vehicle (0.2% ethanol), 200 µg/ml metyrapone (Cima et al., 2004; Tocris), or 10⁶ M (Vukelic et al., 2011) steroid substrate (deoxycorticosterone or dehydrocorticosterone, Steraloids) (Figure 1.1). For each adult subject,

tissues were split in half, one half treated with vehicle and the other half treated with metyrapone or steroid substrate, allowing for within-subject analysis. Tissues from neonates were not split due to the small size. After 24 hours, adherent cells were loosened with a pipet and cells and supernatant were transferred into a conical tube. In some experiments, 1 ml of methanol was immediately added and samples were frozen at -80°C, while in other experiments, samples were spun for 6 min at 500 × g and 1 ml of methanol added to the supernatant before being frozen at -80°C.

4.2.4 Steroid extraction

Steroids were extracted from cultured samples and medium using solid phase extraction (SPE) with C₁₈ columns as previously described (Newman et al., 2008a; Taves et al., 2010). Conditioned media samples were collected, 1 ml of methanol added, and then stored at -20°C. Samples were thawed, diluted with 5 ml of water and loaded onto SPE columns (Agilent, cat. 12113045). Prior to sample loading, SPE columns were primed with 3 ml methanol and equilibrated with 10 ml water. After sample loading, samples were washed with 10 ml 40% methanol to remove interfering substances (Brummelte et al., 2010; Taves et al., 2011b). Unconjugated steroids were eluted with 5 ml 90% methanol and dried in a vacuum centrifuge (ThermoElectron SPD111V).

4.2.5 Steroid separation

When tissues were cultured in the presence of exogenous steroid substrate (corticosterone precursor or metabolite), extracted steroid residues were further processed using reversed-phase HPLC to avoid any cross-reactivity (Gilson 322 pump, Gilson FC 204 fraction collector), based

on a previously published protocol (Taves et al., 2015). A Waters SymmetryShield C₁₈ column (4.6 × 250 mm, 5 µl silica particles) kept at 40°C was used for steroid separation. The mobile phases were 0.01% formic acid in water (Solvent A) and 0.01% formic acid in acetonitrile (Solvent B). Following SPE, steroid residues were resuspended in 45 µl 20% acetonitrile, 0.01% formic acid in water, centrifuged at 16,000 g for 5 min, and supernatants transferred to HPLC vials. From each sample, 16 µl was loaded onto the HPLC column and eluted at a flow rate of 1.0 ml/min. From 0 to 15 min the concentration of Solvent B increased from 20% to 45%, and from 15 to 19 min the concentration of Solvent B increased from 45% to 100%. The Solvent B concentration was maintained at 100% until 25 min, when it was decreased back to 20% and the column was equilibrated until 35 min. The gradients and steroid elution times are given in Figure 4.1. The corticosterone fraction was collected in a 0.45 min fraction, which was dried in a vacuum centrifuge. To further ensure that there was no sample carry-over, we used a stringent cleaning protocol between samples, which included washing the column with 100% Solvent B for 15 min, washing the fraction collector with 100% Solvent B for 4 min, and washing the injection needle, syringe, and sample loop with 50% Solvent B.

4.2.6 Immunoassays

Previously, we have found locally elevated levels of corticosterone and cortisol in lymphoid organs of neonatal C57BL/6 mice (Taves et al., 2015), but in this study we detected little or no cortisol in any tissue (either in freshly collected and frozen tissue samples or in cultured tissue samples; data not shown). Thus, we focused on corticosterone for the rest of this study. Corticosterone was quantified after SPE or after SPE+HPLC using a specific and sensitive radioimmunoassay kit (MP Biomedicals), as previously described (Taves et al., 2015).

4.2.7 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Even though our HPLC separation resulted in clear separation of corticosterone from major precursors and metabolites, we wanted to ensure that our measurements were not affected by any crossreactivity to unknown co-eluting steroids. Therefore, we quantified corticosterone in our HPLC fractions using LC-MS/MS, as previously described (Taves et al., 2015). Dried HPLC fractions were resuspended in 22% Optima-grade methanol, transferred into a 96-well autoinjector sample plate, and 50 µl of resuspended sample injected into a Shimadzu Prominence LC20AC system. Separation was performed using a Phenomenex Gemini NX-C18 column in a 55°C column oven. The gradient profile was the same as previously described (Taves et al., 2015), and corticosterone was detected with multiple reaction monitoring (MRM), with two MRM transitions (quantifier m/z 347.4 → 121.0 and qualifier m/z 347.4 → 97.1).

4.2.8 Real-time quantitative PCR (qPCR)

Tissue samples were collected and snap frozen on dry ice as described above. Samples were then homogenized in ice-cold isol-RNA lysis reagent (5 prime 2302700), RNA extracted with chloroform, precipitated with isopropanol, and washed with 75% ethanol. Complementary DNA was then synthesized from 5 µg of total RNA with a Maxima first strand synthesis kit (Thermo K1641), and qPCR for gene expression of steroidogenic enzymes was performed in triplicate with 5' nuclease probe-based assays (Integrated DNA Technologies, details in Table 4.1). Quantitative PCR was performed with Brilliant III QPCR mastermix (Agilent, 600880), run at 95°C for 3 min, followed by 45 cycles of 95°C for 5 seconds and 60°C for 10 seconds. All

detectable samples had Cq (quantification cycle) values less than 40 cycles. The Rps29, Rpl4, and Oaz1 genes were used as reference genes (de Jonge et al., 2007), with the geometric mean of these three Cq values used as the reference value (Vandesompele et al., 2002). Gene expression of steroidogenic enzymes was expressed relative to that of adult thymus Cyp11b1, whose activity has previously been reported (Lechner et al., 2000; Qiao et al., 2008). Negative controls (no RNA or no reverse transcription) were always nondetectable.

4.2.9 Statistical analysis

Nondetectable steroid sample values (those below the lowest standard on the standard curve or those lower than the average water blank + two standard deviations, whichever was greater) were set to zero. Log-transformed data were analyzed using linear mixed-effects models in R (R Core Team, 2005) and Prism 5. Planned comparisons were conducted using paired or unpaired t-tests or Wilcoxon tests as appropriate. Significance was set at $\alpha = 0.05$, and data are shown as mean \pm SEM. Data from females and males were pooled, because no sex differences were detected.

4.3 Results

4.3.1 Glucocorticoid production by bone marrow, spleen, and thymus increases with age

To test whether lymphoid organs produce glucocorticoids, we incubated minced tissues in the presence or absence of metyrapone, which inhibits glucocorticoid synthesis by CYP11B1, regeneration by 11 β -HSD1, and inactivation by 11 β -HSD2. We then quantified corticosterone in extracted culture medium and tissue. We found that in the presence of metyrapone, conditioned

media from bone marrow, spleen, and thymus all had dramatically reduced concentrations of corticosterone (Figure 4.2A, B), demonstrating glucocorticoid production in each of these tissues, which could be blocked by inhibition of glucocorticoid-producing enzymes. Surprisingly, the magnitude of metyrapone-blockable corticosterone, even after correction for tissue mass, was much higher in adults than in neonates, across all tissues (Figure 4.2C). This suggests that lymphoid glucocorticoid production occurs throughout life, and actually increases in adults. As these experiments were performed with steroid-free culture medium, these experiments also show that glucocorticoid production occurs in the presence of an endogenous substrate present in these tissues.

4.3.2 Lymphoid organs express high levels of *Hsd11b1* but little or no *Cyp11b1* mRNA

To further explore the potential for lymphoid organ conversion of steroid substrates into active glucocorticoids, we quantified gene expression of glucocorticoid-producing enzymes using specific, probe-based qPCR assays. The enzyme CYP11B1 (P450c11B1) converts the upstream precursor, deoxycorticosterone, into corticosterone, while the enzyme 11 β -HSD1 (11 β -hydroxysteroid dehydrogenase type 1) converts the inactive metabolite, dehydrocorticosterone, into active corticosterone (Norris, 2007; Tomlinson et al., 2004). The enzyme 11 β -HSD2 catalyzes the reverse reaction, converting active corticosterone into inactive dehydrocorticosterone (Tomlinson et al., 2004).

Across lymphoid organs, patterns were very similar. In neonates, gene expression of 11 β -HSD1 (coded by the *Hsd11b1* gene) was approximately 1000-fold higher than gene expression of CYP11B1, which was often nondetectable. Gene expression of 11 β -HSD2 (*Hsd11b2*) was intermediate (Figure 4.3A-C). In adults, expression of CYP11B1 was nondetectable, 11 β -HSD1

was higher, and 11 β -HSD2 was lower, relative to levels in neonates (Figure 4.3A-C). Negative controls (no template or no reverse transcriptase) were always nondetectable. These data suggest that in neonatal lymphoid organs, metyrapone-blockable glucocorticoid production may occur largely via regeneration from dehydrocorticosterone, rather than synthesis from deoxycorticosterone. Furthermore, the increased metyrapone-blockable glucocorticoid production in adults (Figure 4.2C) is associated with increased expression of 11 β -HSD1 and reduced expression of 11 β -HSD2 (Figure 4.3A-C).

We further assayed gene expression of all of the steroidogenic enzymes upstream of CYP11B1. In neonatal tissues, we detected mRNA for all of the enzymes needed for the conversion of cholesterol, the precursor of all steroids, into deoxycorticosterone (Figure 4.4A-C). As CYP11B1 gene expression was lower than that for all other enzymes, low levels of CYP11B1 might be the limiting step in lymphoid glucocorticoid synthesis. In adult lymphoid organs, gene expression of upstream enzymes was mostly reduced, although StAR and CYP21A1 expression increased in the spleen. Expression of 3 β -HSD6 (coded by *Hsd3b6*) was nondetectable in adult bone marrow and spleen, similar to expression of CYP11B1. These data suggest that glucocorticoid synthesis from precursors could occur in neonates, although gene expression of 11 β -HSD1 was generally much higher than that of upstream glucocorticoid-synthetic enzymes. In adults, the absence of 3 β -HSD6, 3 β -HSD1 (coded by *Hsd3b1*, which was nondetectable in all tissues at both ages), and CYP11B1 suggests that regeneration is the major pathway for production of glucocorticoids, and synthesis from precursors is unlikely.

4.3.3 Lymphoid organs convert the metabolite dehydrocorticosterone but not the precursor deoxycorticosterone into corticosterone

To test whether our gene expression data corresponded with actual CYP11B1 or 11 β -HSD1 enzyme activity, we next incubated tissues in medium supplemented with exogenous deoxycorticosterone (which is converted to corticosterone by CYP11B1) or exogenous dehydrocorticosterone (which is converted to corticosterone by 11 β -HSD1). Conditioned media was extracted and steroids were separated with HPLC to avoid potential antibody cross-reactivity.

Consistent with our gene expression results, incubation of neonatal tissues with deoxycorticosterone resulted in moderate (bone marrow) or nondetectable (spleen, thymus) increases in corticosterone levels, while incubation with dehydrocorticosterone resulted in dramatic increases in corticosterone levels (Figure 4.5A). A similar pattern was observed in adult tissues, where incubation with deoxycorticosterone had little effect on corticosterone levels but incubation with dehydrocorticosterone resulted in large increases in corticosterone levels (Figure 4.5B). The corticosterone increase in the presence of dehydrocorticosterone was much higher with adult tissues than with neonatal tissues, and when corrected for tissue mass, adult bone marrow and thymus had higher corticosterone-producing activity than neonatal bone marrow and thymus (Figure 4.5C).

Medium containing either deoxycorticosterone or dehydrocorticosterone, incubated without tissue and processed in parallel with tissue-containing medium, contained little or no detectable corticosterone, indicating that deoxycorticosterone and dehydrocorticosterone did not contaminate the corticosterone HPLC fraction or spontaneously undergo conversion into corticosterone in the absence of tissue. Deoxycorticosterone and dehydrocorticosterone conversion to corticosterone could be inhibited by coincubation with metyrapone (Figure 4.6A,

B), showing that our original finding of metyrapone-blockable glucocorticoid production is consistent with inhibition of 11β -HSD1 conversion of dehydrocorticosterone into corticosterone. Dehydrocorticosterone conversion to corticosterone was blocked by boiling tissue before incubation (Figure 4.6C), showing that conversion requires intact tissue and enzyme function. Furthermore, the minimal conversion of deoxycorticosterone into corticosterone was not due to an artifact of our tissue culture protocol, as adrenal corticosterone production was increased in the presence of deoxycorticosterone (Figure 4.6D).

4.4 Discussion

Here, we show that murine bone marrow, spleen, and thymus are each able to produce glucocorticoids from endogenous substrates, as shown by inhibition of glucocorticoid production with metyrapone. High gene expression of 11β -HSD1 relative to CYP11B1, together with greater conversion of the metabolite dehydrocorticosterone, compared to the precursor deoxycorticosterone, into active corticosterone, indicate that the major contributor to tissue-specific production of corticosterone is via glucocorticoid regeneration by 11β -HSD1, rather than synthesis by CYP11B1. Also unexpectedly, we found that instead of decreasing with age, lymphoid corticosterone production was higher in adult than in neonate lymphoid organs, while similar in females and males. These findings are highly relevant to understanding the wide range of glucocorticoid functions in development and function of lymphocytes and other hematopoietic cells.

4.4.1 Bone marrow, spleen, and thymus produce glucocorticoids

Glucocorticoids potently regulate the activity of lymphocytes (Ashwell et al., 2000) and other immune cells (Tuckermann et al., 2007), and this glucocorticoid activity is generally assumed to follow systemic patterns of glucocorticoid synthesis by the adrenal glands. However, we previously found that endogenous glucocorticoid concentrations were locally elevated in murine lymphoid organs, especially in early life when circulating adrenal-derived glucocorticoid concentrations were low (Taves et al., 2015). Here, we found that the bone marrow and spleen are able to locally produce glucocorticoids, and that this activity is blocked by inhibition of steroidogenic enzymes with metyrapone. Glucocorticoid production in bone marrow and spleen was highly similar to glucocorticoid production in the thymus, which has also been shown previously (Lechner et al., 2000; Qiao et al., 2008). These findings show that lymphoid organs can have local control of glucocorticoid signaling, rather than merely functioning as passive recipients of circulating, adrenal-derived glucocorticoids. Unexpectedly, lymphoid glucocorticoid production increased with age, suggesting that local glucocorticoid upregulation is not only important when systemic glucocorticoid levels are minimal or absent (Schmidt et al., 2008). Local glucocorticoid production may instead function to regulate the development and function of lymphocytes and other hematopoietic cells throughout life.

The role of locally-regenerated glucocorticoids in the bone marrow and spleen is unclear. In the thymus, glucocorticoids antagonize signaling through the T cell receptor (Iwata et al., 1991; Van Laethem et al., 2001b), and ensure the survival and export of functional, competent T cells (Mittelstadt et al., 2012). Glucocorticoids also antagonize T cell receptor signaling in mature T cells, and in bone marrow and spleen may regulate T cell survival and expansion (Jamieson and Yamamoto, 2000), and potentially T cell-mediated immune responses.

Analogous to their role in the thymus, glucocorticoids in bone marrow and spleen might also function to antagonize signaling through the B cell antigen receptor, ensuring the production of an appropriately reactive B cell repertoire. Consistent with this, immature B cell subsets in the bone marrow and spleen have higher expression of the glucocorticoid receptor (GR) and greater sensitivity to glucocorticoids than do mature B cells in the same tissues (Gruver-Yates et al., 2013). Furthermore, the avian bursa of Fabricius, which functions specifically in the production of B cells (Glick et al., 1956), is also capable of producing its own glucocorticoids (Lechner et al., 2001). Together, these data indicate that B cells are probably a major target of locally-produced glucocorticoids.

In addition to regulating development within a certain hematopoietic lineage, glucocorticoids also alter the relative proportions of different hematopoietic lineages present within murine bone marrow (Trottier et al., 2008). Local activity of 11β -HSD1 may thus be a regulator both of the developmental trajectories of hematopoietic cells, and of the reactivity of the resulting immune cells.

4.4.2 Glucocorticoid regeneration versus synthesis

We have previously found that upstream glucocorticoid precursors are locally elevated in parallel with glucocorticoids in developing bone marrow, spleen, and thymus (Taves et al., 2015). Additionally, in the thymus, other studies have also found expression of upstream glucocorticoid-synthetic enzymes (Vacchio et al., 1994; Pazirandeh et al., 1999; Lechner et al., 2000; Qiao et al., 2008). Thus, we expected that glucocorticoid production would occur via conversion of upstream precursors, via deoxycorticosterone, into corticosterone (Figure 1.1). However, while generally used as an inhibitor of CYP11B1, metyrapone is also an inhibitor of

the 11 β -HSD enzymes (Sampath-Kumar et al., 1997; Hostettler et al., 2012). Our highly specific probe-based qPCR assays found that gene expression of 11 β -HSD1 was approximately 1000-fold higher than expression of CYP11B1. Moreover, expression of CYP11B1 was nondetectable in adult bone marrow and spleen. This large difference in gene expression indicates that metyrapone-blockable glucocorticoid production is due primarily to the presence of 11 β -HSD1 rather than CYP11B1. Correspondingly, dehydrocorticosterone was converted into corticosterone much more rapidly and consistently across tissues compared to deoxycorticosterone. Thus, our data show that 11 β -HSD1 activity is the major contributor to lymphoid glucocorticoid production.

In the bone marrow and spleen, our finding of predominant 11 β -HSD1 activity is consistent with previous findings of 11 β -HSD1 (in osteoblasts, Cooper et al., 2002; in spleen homogenates, Tomlinson et al., 2004; in spleen-derived lymphocytes, Zhang et al., 2005). However, our results appear to conflict with existing work in the murine thymus. While several studies have examined thymic glucocorticoid synthesis, only one has tested for activity of CYP11B1, looking at mitochondrial conversion of deoxycortisol to cortisol (Lechner et al., 2000). Other studies have focused on metyrapone inhibition of glucocorticoid production (Qiao et al., 2008) or signaling (Vacchio et al., 1994; Pazirandeh et al., 1999), and those observations might be due to inhibition of 11 β -HSD1 as shown here, rather than inhibition of CYP11B1. Indeed, thymus homogenates have been clearly shown to have 11 β -HSD1 activity, which increased with age as seen here (Nuotio-Antar et al., 2006).

The pathway utilized for glucocorticoid production is important for understanding how local glucocorticoid levels are regulated. In rats, dehydrocorticosterone is present in circulating blood of both neonates and adults (Tagawa et al., 2007), and circulating dehydrocorticosterone

levels can remain relatively stable after acute or chronic stress (Obut et al., 2009). This suggests that circulating dehydrocorticosterone may provide a reliably available substrate reservoir for local glucocorticoid production, and one that is less energetically expensive than *de novo* glucocorticoid production from cholesterol. Spleen and thymus 11 β -HSD1 activities are also unchanged after chronic stress (Jellinck et al., 1997), suggesting that glucocorticoid regeneration may function to constitutively maintain elevated glucocorticoid signaling in these tissues, rather than responding rapidly to environmental stimuli. Constitutive activity of 11 β -HSD1 may also mean that therapeutic glucocorticoids that require regeneration into their active products (e.g., cortisone, prednisone) may preferentially target 11 β -HSD1-expressing lymphoid organs.

4.4.3 Corticosterone versus cortisol in lymphoid organs

Our previous work quantifying endogenous lymphoid glucocorticoids found that lymphoid organs had locally-elevated levels of corticosterone, the major adrenal glucocorticoid in mice (Taves et al., 2015). However, that study also found locally-elevated levels of another glucocorticoid, cortisol, which is generally thought to be absent in mice. In the current study, we quantified endogenous cortisol concentrations in blood and lymphoid organs and cortisol production *in vitro*, and found levels to be extremely low or nondetectable, although endogenous corticosterone levels were similar in both studies. This cortisol difference may be due to these two studies being conducted in different animal facilities, including various differences in environmental conditions, such as diet, excluded microbiota, or light cycle (Table 4.2, Appendix B, C). Major alterations in diet (e.g., high fat diet (Tannenbaum et al., 1997; Boullu-Ciocca et al., 2008)) or gut microbiome (e.g. germ-free mice (Sudo et al., 2004)) are known to alter corticosterone production, and while unexplored, might also alter expression of CYP17 and

cortisol production. However, it is unclear whether more subtle differences in diet (such as the source of lipids) or microbiome composition alter glucocorticoid production. Alternatively, the presence of steroids in the diet (such as 17 α -hydroxyprogesterone, deoxycortisol or cortisone) might provide substrates which are endogenously converted into cortisol, alongside the endogenous glucocorticoid, corticosterone.

The mechanism underlying local elevation of glucocorticoid precursors (progesterone, deoxycorticosterone) in our previous study (Taves et al., 2015) is also unclear, but the present findings suggest that these precursors may not be used as corticosterone precursors, even though upstream steroidogenic enzyme genes are expressed. Instead, these locally elevated “precursors” might themselves function as signal molecules and bind lymphoid steroid receptors. Indeed, this has already been shown for progesterone (Tibbetts et al., 1999).

4.5 Conclusions

Here, we demonstrated local glucocorticoid production in lymphoid organs of developing and adult mice, and found that this production occurs primarily via corticosterone regeneration from dehydrocorticosterone, rather than corticosterone synthesis from deoxycorticosterone. Our findings indicate that glucocorticoid regeneration occurs across lymphoid organs, and increases over development. Together with previous work on the local elevation of endogenous glucocorticoids (Taves et al., 2015), these results indicate that the lymphoid glucocorticoid milieu is locally controlled by expression of glucocorticoid metabolic enzymes (especially 11 β -HSD1 and 11 β -HSD2). This local control of glucocorticoid levels is likely instrumental in the production of appropriately reactive T cells (Mittelstadt et al., 2012), and may be similarly critical in development of B cells (Lechner et al., 2001; Schmidt et al., 2010; Gruver-Yates et al.,

2013; Taves et al., 2011a). Lymphoid 11 β -HSD1 may also more broadly regulate the development and relative output of multiple hematopoietic lineages. Targeted inhibition of 11 β -HSD enzymes may offer a tool for clinical modification of immune development and cell output, and resulting alteration of immune reactivity.

4.6 Tables and figures

Table 4.1: qPCR primers and probes

| Gene | Forward (5'-3') | Reverse (5'-3') | Probe (5'-3') | Exons |
|---------|--------------------------|------------------------|-----------------------------|-------|
| Star | tatctgggtctgcgaatagga | tccctccaagactaaactcact | agecaccccttcaggtcaatactg | 6-7 |
| Cyp11a1 | gccttaagtcccaggtagaagttc | tgcactcctcagaactaagacc | tgaaaatcacatcccaggcagctg | 4-5 |
| Hsd3b1 | gtctgtcctccaggtagtttgc | cctgatcttcagccacca | tttgctctcagttgtgaccattcct | 1-2 |
| Hsd3b6 | cactgtcacctgtatgttgtatc | gatgcaggagaagatctggag | tgctccctggttctggctgaag | 2-3 |
| Cyp21a1 | ggttccaggaaagcgatctg | caactaggcttagcagcatc | tcatcatccccaaacatccaaggcg | 8-10 |
| Cyp11b1 | gccagctcaaagaaagtcat | caggctaactcaatggactca | actgacacgcacatcaaccccttg | 5-6 |
| Hsd11b1 | atgtccaggccat | gaggaaggctccagaaggta | ccatagtgccagcaatgttagtgagca | 3-5 |
| Hsd11b2 | cgtgaagccatggcat | ggctggatcgcggtgc | cggttgtgacactgggttggcaa | 1-2 |
| Cyp17a1 | cctttcccttggccgacaa | cagagaagtgcgtgtgaaga | ctagagtcaccatctggggccga | 1-2 |
| Rps29 | tccattcaaggctcgcttagtc | cggctgtatccgcaatacg | aagectatgccttcgcgtactgc | 2-3 |
| Rpl4 | cttgccagcttcattctcg | tggtggttgaagataaggttga | ctgaacagcccttggcttttgta | 5-6 |
| Oaz1 | gccaatgaacgagatcactt | gctgttaagatggtcagggtga | atttgagccaggggagggtgacac | 5-5 |

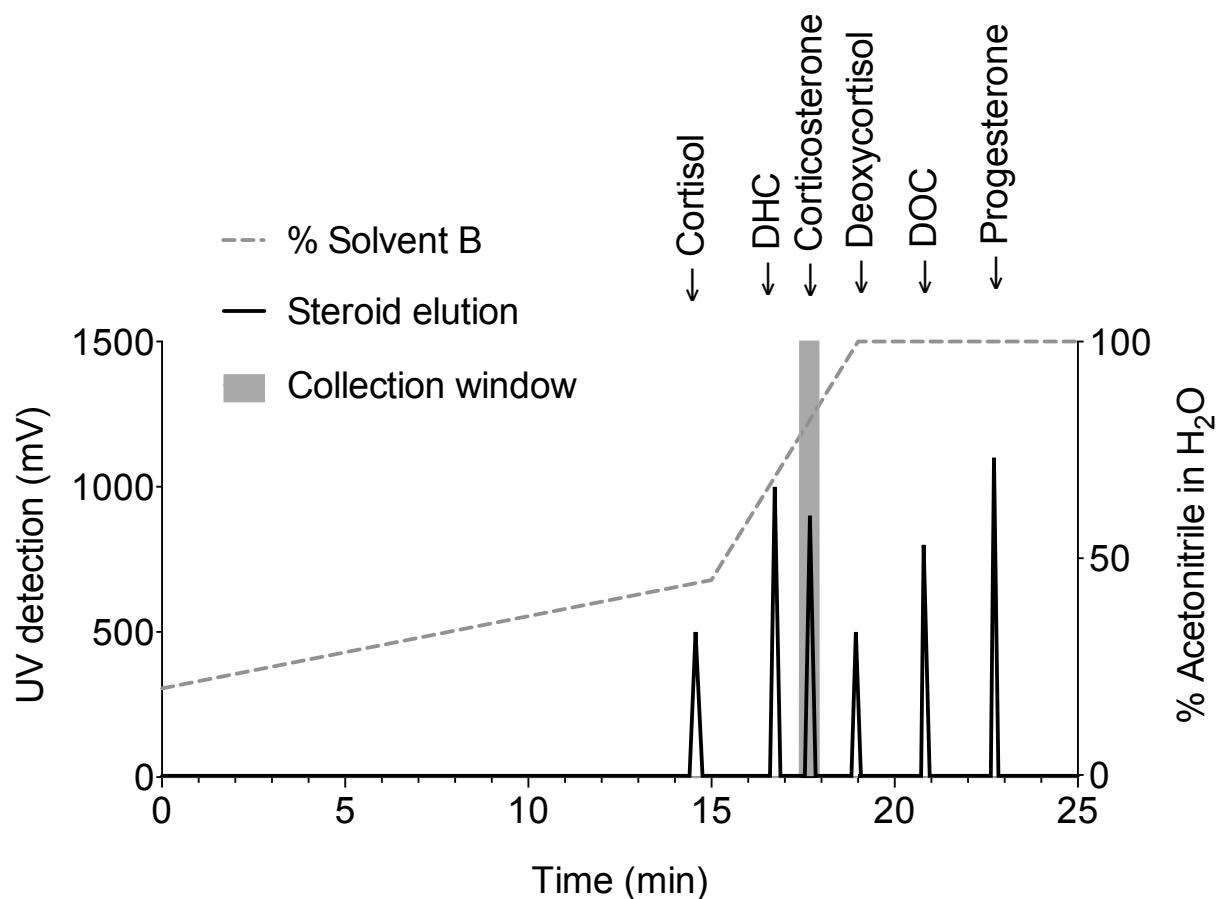
Note: 5' nuclease probe qPCR assays were purchased from Integrated DNA Technologies (cat. # Mm.PT.58.8323852, Mm.PT.58.29648776, Mm.PT.58.43238915, Mm.PT.58.5793064, Mm.PT.58.29960109, Mm.PT.58.41798181, Mm.PT.58.30853261, Mm.PT.58.13323852, Mm.PT.58.10555317, Mm.PT.58.21577577, Mm.PT.58.17609218, Mm.PT.58.33130152.g.

Table 4.2: Overview of Chapter 3 and Chapter 4 mouse housing conditions

| | Chapter 3 (Wesbrook Animal Unit) | Chapter 4 (Centre for Disease Modeling) |
|--------------------|---|--|
| Mouse strain | C57BL/6 | C57BL/6 (rederived) |
| Sex | Female & male | Female & male |
| Ages | PND5 & PND60 | PND5 & ~PND60 |
| Breeding strategy | Mating pair | Mating pair |
| Diet (standard) † | Labdiet 5010 (Appendix B) | Teklad 2918 (Appendix B) |
| Diet (breeding) † | Labdiet 5021 (Appendix B) | Teklad 2919 (Appendix B) |
| Cage bedding | Corncob | Corncob |
| Individuals/cage | 2-5 | 2-5 |
| Light:dark cycle † | 12:12 | 14:10 |
| SPF barrier † | (Appendix C) | (Appendix C) |
| Disinfectant † | Virkon | Virox |
| Enrichment | Nestlets, igloo | Nestlets, igloo |

† Conditions differ between facilities.

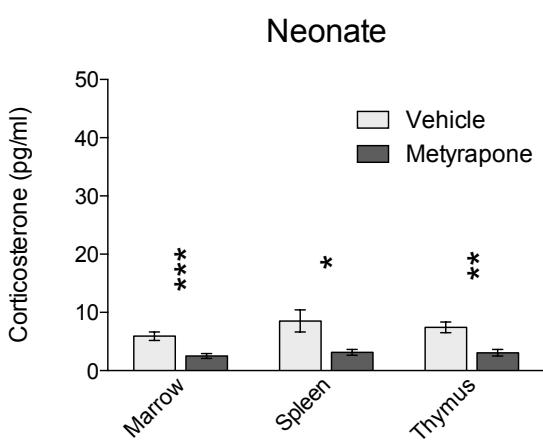
Figure 4.1 HPLC steroid separation



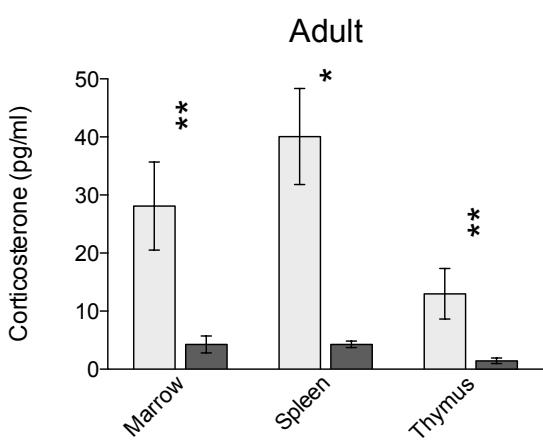
HPLC separation of glucocorticoids. Retention times were determined by UV detection (shown above) and corticosterone elution time was confirmed by collection of ³H-labeled corticosterone in 0.1 min fractions and counting in a liquid scintillation counter (data not shown). DHC = dehydrocorticosterone, DOC = deoxycorticosterone.

Figure 4.2 Glucocorticoids are produced by bone marrow, spleen, and thymus of neonatal and adult mice.

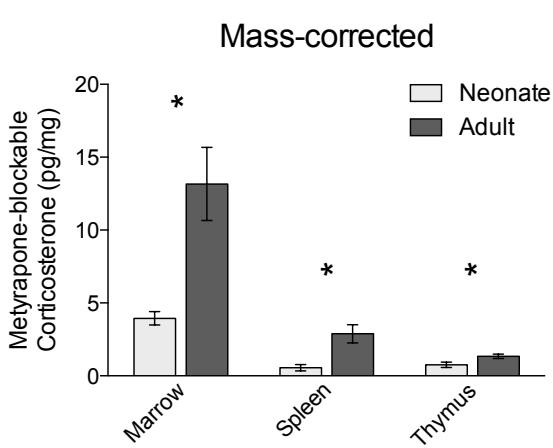
A



B

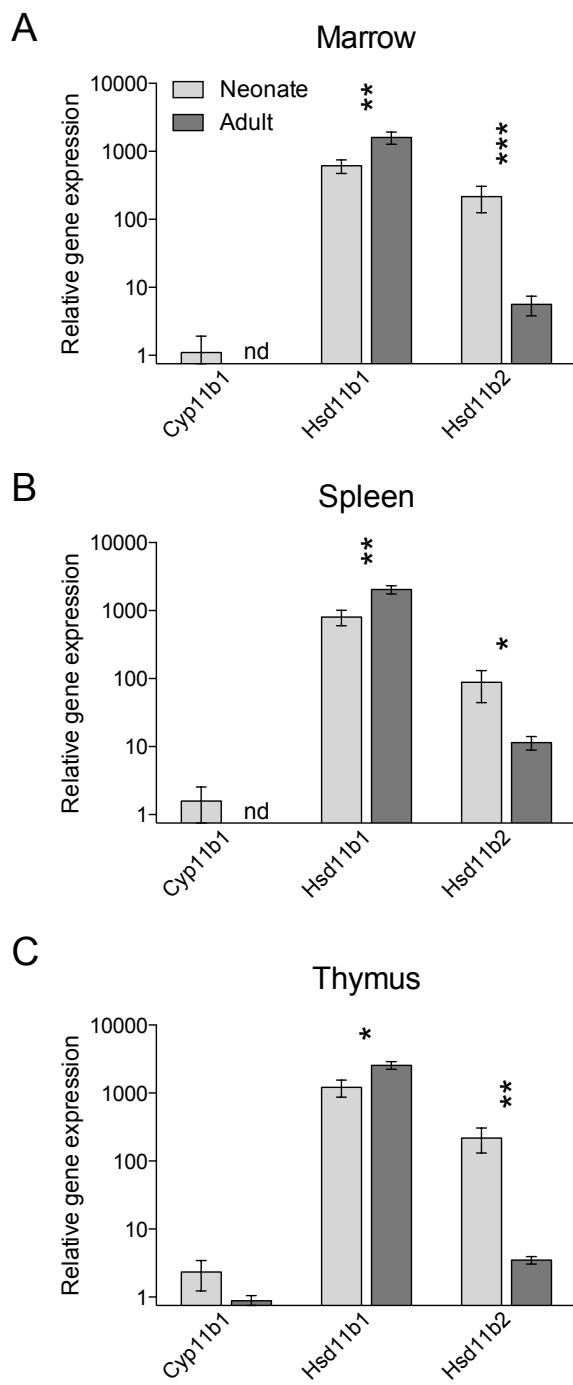


C



Glucocorticoid production by bone marrow, spleen, and thymus of (A) neonatal and (B) adult mice cultured *ex vivo* with vehicle or metyrapone, which inhibits glucocorticoid production by CYP11B1 and 11 β -HSD type 1. No exogenous substrate was added. Tissue and conditioned medium was extracted using SPE and corticosterone was quantified with a sensitive and specific RIA. (C) Metyrapone-blockable glucocorticoid production was estimated by subtracting corticosterone levels in metyrapone-incubated samples from corticosterone levels in paired vehicle-incubated samples, and correcting for average tissue mass in each sample. All data are presented as means \pm SEM, and treatment or age differences are indicated as follows, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Sample sizes are 7 for neonate samples and 5, 5, and 4 for adult bone marrow, spleen, and thymus, respectively.

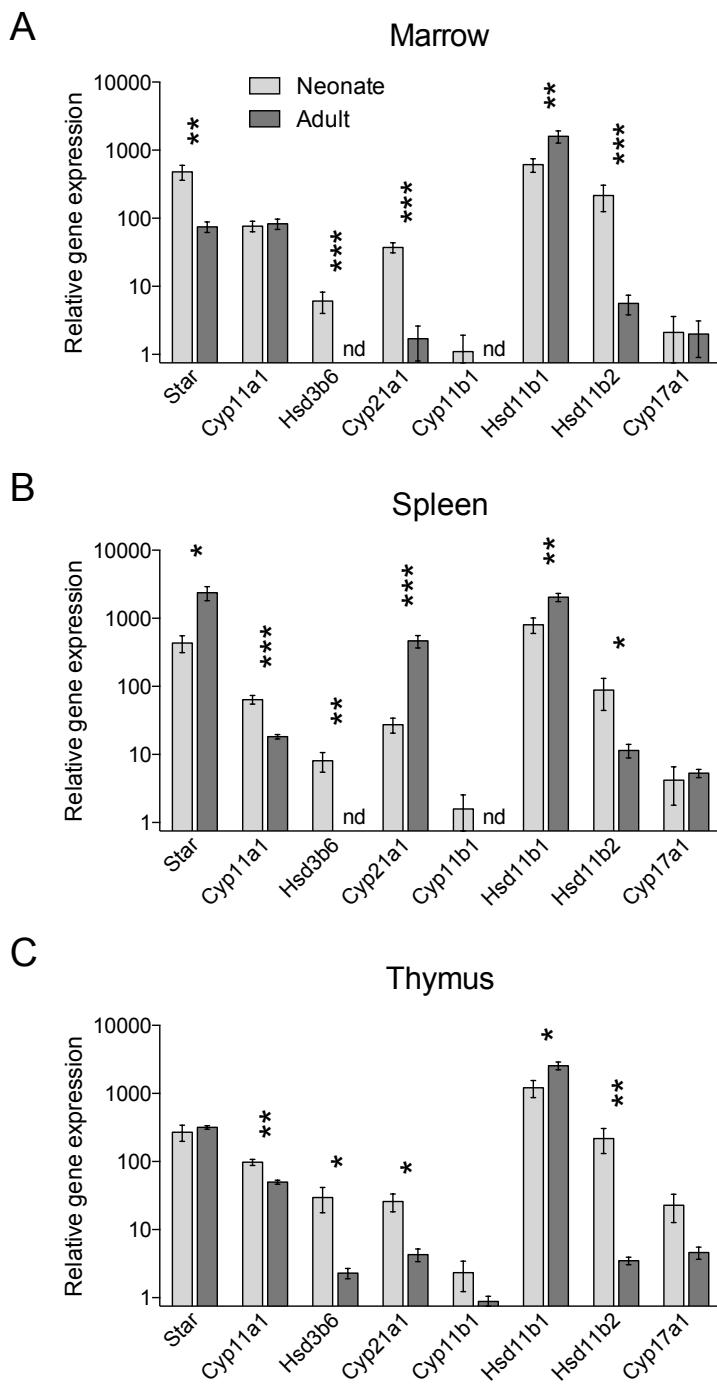
Figure 4.3 Lymphoid organ expression *Hsd11b1* is much higher than *Cyp11b1* and increases with age



Relative gene expression of *Cyp11b1*, *Hsd11b1*, and *Hsd11b2*, which code for the enzymes CYP11B1, 11 β -HSD1, and 11 β -HSD2 which are responsible for glucocorticoid synthesis, regeneration, and inactivation, respectively.

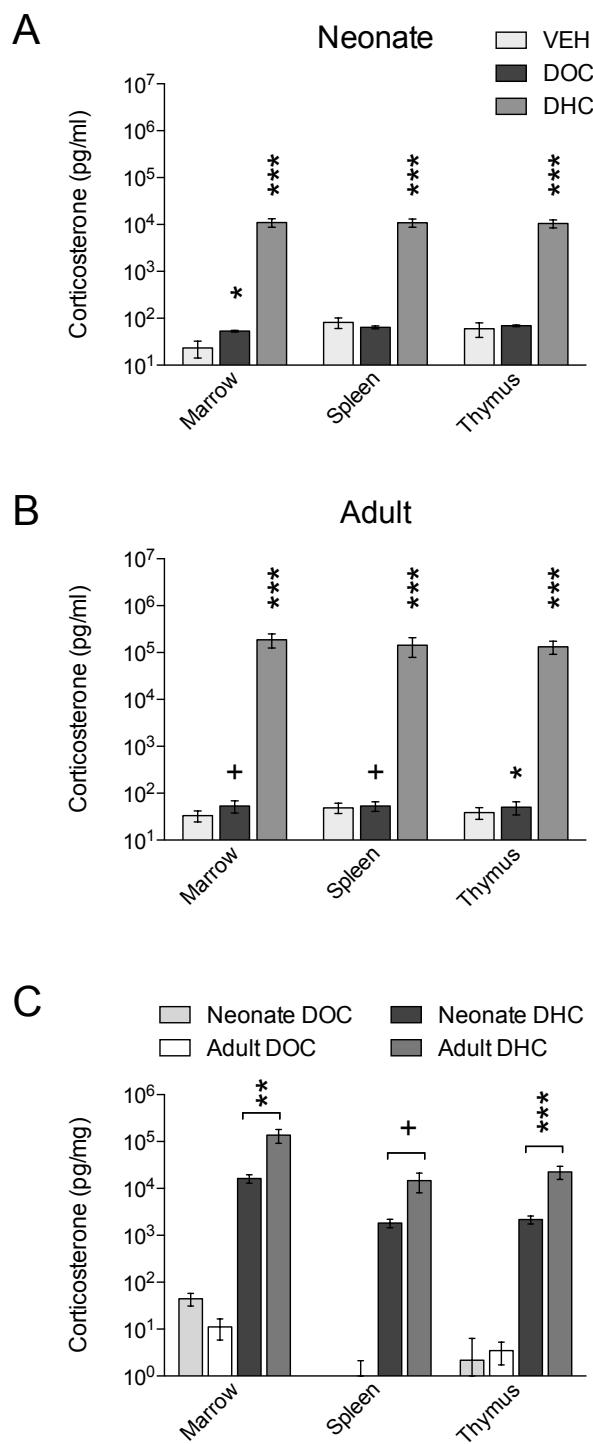
Relative expression was quantified in neonatal and adult (A) bone marrow, (B) spleen, and (C) thymus, using highly specific 5' nuclease probe-based qPCR assays. Expression was corrected using the reference genes *Rps29*, *Rpl4*, and *Oaz1*, and expressed relative to adult thymus *Cyp11b1* (note the log scale for the y-axis). In some cases, *Cyp11b1* expression was not detectable (nd = nondetectable). Data are presented as means \pm SEM, and age-related differences in expression of a particular gene are indicated as follows, * p ≤ 0.05 , ** p ≤ 0.01 , *** p ≤ 0.001 . Sample sizes are 9 and 8 for neonate and adult samples, respectively.

Figure 4.4 Gene expression of steroidogenic enzymes in murine lymphoid organs



Relative gene expression of steroidogenic enzymes in neonatal and adult (A) bone marrow, (B) spleen, and (C) thymus, as determined using highly specific 5' nuclease probe-based qPCR assays. Expression was corrected using the reference genes *Rps29*, *Rpl4*, and *Oaz1*, and expressed relative to adult thymus *Cyp11b1* (note the log scale for the y-axis). In some cases, gene expression was not detectable (nd = nondetectable). Data are presented as means \pm SEM, and age-related differences in expression of a particular gene are indicated as follows, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Sample sizes are 9 and 8 for neonate and adult samples, respectively.

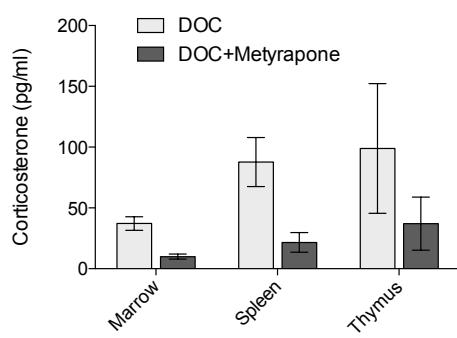
Figure 4.5 Lymphoid organs primarily convert dehydrocorticosterone, rather than deoxycorticosterone, into corticosterone



Corticosterone production by bone marrow, spleen, and thymus of (A) neonatal and (B) adult mice cultured *ex vivo* with vehicle (VEH), the precursor deoxycorticosterone (DOC), or the metabolite dehydrocorticosterone (DHC). Conditioned medium was extracted using SPE, corticosterone isolated using HPLC, and quantified via radioimmunoassay. DOC and DHC were not converted to corticosterone in tissue-free cultured medium processed in parallel with tissue samples. Asterisks indicate corticosterone levels higher than in corresponding vehicle-treated samples. (C) DOC and DHC conversion to corticosterone in neonatal and adult lymphoid organs, corrected for average tissue mass in each sample. All data are presented as means \pm SEM, with significance (difference from vehicle samples) indicated as follows, + $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Sample sizes are 5 – 6 for neonatal samples and 6 for adult samples.

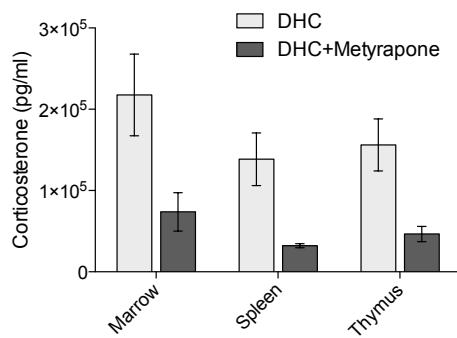
Figure 4.6 Dehydrocorticosterone conversion to corticosterone is inhibited by metyrapone or enzyme denaturing

A

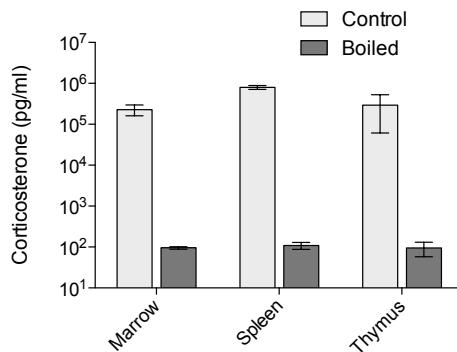


Corticosterone production in the presence of (A) DOC or (B) DHC was inhibited by co-incubation with metyrapone. (C) DHC conversion to corticosterone was also prevented by boiling tissue samples before incubation. (D) Adrenal corticosterone production was increased in the presence of DOC. For all experiments, conditioned medium was extracted using SPE, corticosterone isolated using HPLC, and quantified via radioimmunoassay. Data are presented as means \pm SEM, and all sample sizes are 3.

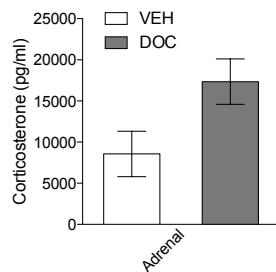
B



C



D



5 General discussion

Glucocorticoids are best known as stress hormones that function to maintain systemic organismal homeostasis. However, there is an expanding literature describing organ- and cell-specific regulation of glucocorticoid signaling, through mechanisms including local synthesis (Taves et al., 2011a), inactivation and regeneration (Tomlinson et al., 2004), uptake or exclusion from target cells (KarsSEN et al., 2001), and expression of different receptor subtypes (Yudt et al., 2003; Bartholome et al., 2004).

In this thesis, I have presented three studies examining the hypothesis that lymphoid organs locally upregulate their glucocorticoid concentrations, and that this local regulation changes over development. I found that 1) endogenous glucocorticoids and their precursors are locally elevated in lymphoid organs of altricial but not precocial bird hatchlings, 2) endogenous glucocorticoids and their precursors are locally elevated in lymphoid organs of developing but not adult mice, and 3) that murine lymphoid organs locally produce glucocorticoids via regeneration of metabolites, rather than synthesis from upstream precursors. Together, these studies show that lymphoid organs independently regulate their local steroid levels, that this local regulation is conserved across altricial birds and mammals, and that glucocorticoid regeneration is the preferential pathway of local glucocorticoid production in lymphoid organs. This demonstration that the immune system autonomously regulates local glucocorticoid levels is important in understanding the actions of glucocorticoids, both endogenous and therapeutic, on immune development and function.

5.1 Major findings – local glucocorticoid regulation in lymphoid organs

5.1.1 Locally-elevated glucocorticoid levels in lymphoid organs during early altricial life

Tissue-specific glucocorticoid production has been examined in a variety of organs, but prior work has largely focused on glucocorticoid synthesis *in vitro*. We measured endogenous steroid concentrations in lymphoid organs (Chapters 2, 3), and found that lymphoid glucocorticoid concentrations were often much higher than circulating glucocorticoid concentrations. In birds, this pattern was clear in altricial but not precocial species, suggesting that local glucocorticoid production is especially important when circulating glucocorticoids are minimal or absent. In mice, which are also altricial, this was the first *in vivo* evidence for lymphoid glucocorticoid production, supporting previous *in vitro* studies. Local upregulation of glucocorticoid concentrations, and glucocorticoid precursor concentrations, thus does not appear to be an artifact of tissue culture systems, but a pattern that also occurs endogenously. Furthermore, as patterns were highly similar in newly-hatched altricial birds and newborn altricial mice, lymphoid glucocorticoid regulation appears to be conserved across a range of animal species, and consistent across females and males.

5.1.2 Different glucocorticoid identities present in lymphoid organs

Another pattern we observed when measuring endogenous glucocorticoids in lymphoid organs (Chapters 2, 3) was that local glucocorticoid identities were unique from glucocorticoid identities in the blood. In both birds and mice, corticosterone is the major adrenal-derived glucocorticoid in the blood, but we found that lymphoid organs of altricial birds and mice had locally-elevated levels of cortisol, a distinct glucocorticoid which is generally thought to be absent in these species. The presence of a distinct local glucocorticoid raises the possibility that

there might be differential signaling between the unique local glucocorticoid (i.e. cortisol) and the major circulating glucocorticoid (i.e. corticosterone). While these glucocorticoids are generally thought to be interchangeable, some studies suggest different signaling pathways (Schmidt et al., 2010) and downstream effects (Gillis et al., 1979; Iwata et al., 1991) of these two glucocorticoids. Our data certainly suggest that cortisol signaling may allow lymphoid organs to distinguish between glucocorticoids of local and systemic origin.

In addition to the unexpected presence of cortisol, we also found locally-elevated levels of the steroids deoxycorticosterone and progesterone. Deoxycorticosterone and progesterone could function as precursors for glucocorticoid synthesis, and could bind steroid receptors and have important effects in lymphoid organs. As there are limited data on progesterone and deoxycorticosterone effects on lymphoid development, this is an area that may prove especially useful to investigate.

5.1.3 Local glucocorticoid levels follow different patterns than systemic glucocorticoid levels

Finally, quantification of endogenous lymphoid glucocorticoid levels demonstrated that local glucocorticoid concentrations follow organ-specific patterns, rather than simply paralleling age-related changes in systemic, circulating glucocorticoids (Chapters 2, 3). For example, while systemic glucocorticoid levels are low and unresponsive in early altricial development (Wada, 2008; Wada et al., 2009; Schmidt et al., 2003), this was the period during which lymphoid glucocorticoid concentrations were most clearly elevated relative to circulating concentrations. In this way, local regulation of glucocorticoids might function as a way for lymphoid organs to maintain optimal local glucocorticoid levels, rather than simply having local glucocorticoid

levels fluctuate with changes in systemic glucocorticoid levels. Depending on the mechanisms of local glucocorticoid upregulation, local glucocorticoid levels might be maintained completely independently of systemic glucocorticoid levels, or might be dependent on or responsive to systemic glucocorticoid levels.

5.1.4 Similar patterns of local regulation in altricial birds and mammals

The studies in Chapter 2 and Chapter 3 found remarkably similar patterns in developing zebra finches and developing mice, both of which are altricial species. Both had highly elevated glucocorticoids in lymphoid organs, especially in early life, and both species had high local levels of cortisol, despite having adrenal synthesis of corticosterone. These striking similarities strongly suggest that glucocorticoids have highly conserved functions in lymphoid development. The avian and mammalian thymus function solely for the production of functional T cells, thus glucocorticoids are almost certainly important in avian T cell development, as they are in mammalian T cell development (Mittelstadt et al., 2012). The function of glucocorticoids in mammalian bone marrow is less clear, as the bone marrow functions in development and homeostasis of a variety of cell types. However, the presence of elevated glucocorticoids in the altricial avian bursa, which functions solely for the production of functional B cells, indicates that glucocorticoids are likely important for B cell development as well. Both glucocorticoids and lymphocytes have conserved functions across all except perhaps the earliest vertebrate lineages (Close et al., 2010; Pancer and Cooper, 2006), thus glucocorticoid signaling could be a feature of lymphocyte development across vertebrates.

5.1.5 Mechanisms of local glucocorticoid regulation

Our final study demonstrated that a major mechanism underlying lymphoid glucocorticoid elevation was regeneration of dehydrocorticosterone, rather than synthesis from deoxycorticosterone and other upstream precursors (Chapter 4). While studies have individually examined glucocorticoid synthesis (primarily in thymus, Lechner et al., 2000; 2001) or glucocorticoid regeneration (in thymus, Nuotio-Antar et al., 2006; osteoblasts, Cooper et al., 2002), the relative contribution of these different pathways to local glucocorticoid production has not been tested. Our finding that regeneration of dehydrocorticosterone is the major contributor to glucocorticoid production has a range of implications, both for endogenous regulation of lymphoid development and function, and for pharmacological glucocorticoid effects on immune function. Dehydrocorticosterone appears to be present in the circulating blood during early development (Tagawa et al., 2007), and is highly bioavailable for uptake. This means that lymphoid glucocorticoid levels are not completely under autonomous regulation, but are instead affected by systemic DHC levels, which are primarily regulated by adrenal glucocorticoid synthesis, kidney glucocorticoid inactivation, and liver glucocorticoid regeneration. Adrenal glucocorticoid synthesis is especially responsive to environmental conditions and stimuli, and these may thus also affect lymphoid glucocorticoid levels. Furthermore, as many widely prescribed glucocorticoids are administered as inactive glucocorticoid metabolites (i.e., cortisone, prednisone) which must be regenerated by 11β -HSD1 into active glucocorticoids (i.e., cortisol, prednisolone), this indicates that these drugs may have disproportionately high activity in these (and other 11β -HSD1-expressing) tissues. While glucocorticoids are often prescribed specifically for their immunosuppressive effects, consistently increased glucocorticoid signaling in lymphoid

organs could have undesirable actions, such as selection for developing T cells with increased reactivity (Mittelstadt et al., 2012; Van Laethem et al., 2001b).

5.2 Additional contributions and future directions

5.2.1 Unique steroids and their actions

In addition to finding that cortisol can be present in lymphoid organs of corticosterone-dominant species, we also found locally elevated levels of progesterone and deoxycorticosterone. These steroids can bind the glucocorticoid receptor (GR), although with lower affinity than cortisol and corticosterone, but are also ligands for the progesterone receptor (PR) and mineralocorticoid receptor (MR). It is likely that activation of each of these different receptors has different downstream effects, and the relative concentrations of different steroids and their activation (or inhibition) of different steroid receptors is likely to be important for understanding overall steroid functions in these organs. The actions of deoxycorticosterone have received a surprisingly little amount of investigation (Vinson, 2011), despite being a steroid receptor ligand (Gomez-Sanchez, 2014) and being present in appreciable concentrations in circulating blood (Chapter 2, 3). Furthermore, while the studies in this thesis focused only on a subset of C-21 steroids (progestins and corticosteroids), sex steroids are known to regulate lymphoid development (Chen et al., 2010) and local regulation (production or inactivation) of androgens and estrogens would also be relevant to explore. To this end, the development of LC-MS/MS assays that simultaneously quantify a large number of steroids in single samples may be a useful next step for investigating the role of a much greater number of steroid hormones and unraveling lymphoid steroid metabolism. Furthermore, preliminary work has begun to look at the expression of steroid receptors in different immune cells (Berki et al., 2002; Tuckermann et al., 2007; Butts

et al., 2011; Gruver-Yates et al., 2013), but the expression of different steroid receptors and their signaling in the wide variety of hematopoietic and lymphoid cell types has largely been unexplored.

5.2.2 Glucocorticoid functions in bone marrow and spleen

While the potential functions of locally-enriched glucocorticoid levels in the thymus have been studied for more than twenty years (Vacchio et al., 1994), the functions of glucocorticoid production in the bone marrow and spleen are unclear. Glucocorticoid antagonism of T cell receptor signaling may regulate homeostatic survival and expansion of mature T cells (Jamieson and Yamamoto, 2000). Based on work in developing T cells (Van Laethem et al., 2001; Mittelstadt et al., 2012), glucocorticoids in bone marrow and spleen may also play an important role in B cell development, consistent with relatively high GR expression in developing B cells (Gruver-Yates et al., 2013). However, other hematopoietic lineages also express GR and are responsive to glucocorticoids (Bauer et al., 1999; Tuckermann et al., 2007), and it is likely that glucocorticoids in the bone marrow and spleen have a variety of effects on different cell types. One of the best tools to test different functions may be the use of mutant mouse models, specifically conditional GR knockout mouse models in which specific cell lineages are deficient for the GR. Use of such models has been instrumental in testing the role of the GR in thymocytes (Mittelstadt et al., 2012), and would be equally useful for testing glucocorticoid functions in B cells and other blood cells. To complement these studies, tissue-specific targeting of the *Hsd11b1* gene could then abrogate local glucocorticoid regeneration, and demonstrate the organ-level effect of a reduction in local glucocorticoid upregulation by 11 β -HSD1.

5.2.3 Environmental regulation of local glucocorticoid production and effects on immunity

In Chapter 2, we found that lymphoid organs of hatchling zebra finches had high levels of cortisol. However, we found that lymphoid organs of hatchling zebra finches in a colony infected with *Mycobacterium avium* had locally-elevated corticosterone, but little or no cortisol (Taves and Soma, unpublished data). Furthermore, we found that lymphoid cortisol levels in hatchlings were lower if parents received hard-boiled egg diet supplements, compared with those whose parents not receiving this supplement. These preliminary data suggest that parental diet may potently affect the identity and quantity of glucocorticoids present in offspring lymphoid organs.

Along similar lines, in Chapter 3 we found that murine lymphoid organs had high levels of cortisol; but in Chapter 4, little or no cortisol was detected. We were unable to determine the cause of this difference, but as these studies were conducted in different facilities (Chapter 3, Wesbrook Animal Unit, UBC; Chapter 4, Centre for Disease Modeling, UBC), it suggests that local glucocorticoid identity and quantity of murine neonatal lymphoid organs may also be potently affected by the parental environment. While bedding and housing were similar between facilities, diet was one obvious difference. These preliminary data suggests that different diets, and potentially the resulting difference in composition of the microbiome, may alter murine lymphoid glucocorticoids.

In both birds and mammals, it may be that diet, and other environmental factors, play an important role in lymphoid steroid levels and immune development. It would be useful to investigate the effects of environmental conditions (experienced by parents or offspring) on lymphoid glucocorticoids, and the effect of these on downstream immune function. It may be that by altering glucocorticoids (systemically- and/or locally-produced), environmental stimuli

have a role in long-term “programming” of immunity, especially during early development. Future studies would benefit from capitalizing on the strengths of both avian and rodent models. In particular, birds have the unique benefit of an organ dedicated to B cell development, the bursa. Birds also exhibit a diverse range of life histories and developmental strategies, which can be easily studied in captivity and in the wild. On the other hand, mice have been the primary model for immunology, and have the best characterized lymphocyte development and function. Furthermore, the availability of mouse strains with a range of targeted mutations makes it possible to determine the specific roles and interactions of different gene products in lymphoid function.

5.2.4 Glucocorticoid production in the heart

In Chapter 2, we found that endogenous levels of cortisol and deoxycorticosterone were locally elevated in the heart, as well as lymphoid organs, in early mouse development. A large amount of previous work has looked for production of mineralocorticoids, focusing on the synthesis of aldosterone and its requirement for activity of CYP11B2 (Taves et al., 2011a). However, the glucocorticoid precursor deoxycorticosterone also binds the MR, and it may be that deoxycorticosterone is at least in part involved in the effects attributed to locally-produced aldosterone, especially since aldosterone synthesis does not appear to occur in healthy cardiac tissue (Funder, 2004; Taves et al., 2011a). This, and effects of deoxycorticosterone in other tissues, merit further investigation (Vinson, 2011).

5.3 Conclusions

This dissertation presents the first studies to measure endogenous lymphoid glucocorticoids in precocial and altricial avian species, and endogenous lymphoid glucocorticoids in altricial mammals. It also presents the first comparison of glucocorticoid synthesis and regeneration in lymphoid organs, and shows that that regeneration may be the predominant mechanism of local glucocorticoid production. These results demonstrate the importance of tissue-specific regulation of glucocorticoids when examining the actions of glucocorticoids in lymphoid organs, and indicate that local glucocorticoid regulation, in addition to controlling development of T cells in the thymus, may also control development and homeostasis of a range of hematopoietic cell lineages in the bone marrow and spleen. More broadly, this work contributes to a growing body of evidence suggesting that glucocorticoids function not only as systemic hormones, but also as local signals in a range of different tissues.

References

- Adkins B, Leclerc C, Marshall-Clarke S. Neonatal adaptive immunity comes of age. *Nat Rev Immunol* 4: 553-564, 2004.
- Ahmad N, Romero DG, Gomez-Sanchez EP, Gomez-Sanchez CE. Do human vascular endothelial cells produce aldosterone? *Endocrinology* 145: 3626-3629, 2004.
- Aird F, Clevenger CV, Prystowsky MB, Redei EVA. Corticotropin-releasing factor mRNA in rat thymus and spleen. *Proc Natl Acad Sci USA* 90: 7104-7108, 1993.
- Ashwell JD, Lu FWM, Vacchio MS. Glucocorticoids in T cell development and function. *Ann Rev Immunol* 18: 309-345, 2000.
- Atanasov AG, Leiser D, Roesselet C, Noti M, Corazza N, Schoonjans K, Brunner T. Cell cycle-dependent regulation of extra-adrenal glucocorticoid synthesis in murine intestinal epithelial cells. *FASEB J* 22: 4117-4125, 2008.
- Bartholome B, Spies CM, Gaber T, Schuchmann S, Berki T, Kunkel D, Bienert M, Radbruch A, Burmester G-R, Lauster R, Scheffold A, Buttgereit F. Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis. *FASEB J* 18: 70-80, 2004.
- Batanero E, de Leeuw FE, Jansen GH, van Wichen DF, Huber J, Schuurman HJ. The neural and neuro-endocrine component of the human thymus. II. Hormone immunoreactivity. *Brain Behav Immun* 6: 249-264, 1992.
- Bauer A, Tronche F, Wessely O, Kellendonk C, Reichardt HM, Steinlein P, Schutz G, Beug H. The glucocorticoid receptor is required for stress erythropoiesis. *Genes Dev* 13: 2996-3002, 1999.
- Berki T, Palinkas L, Boldizsar F, Nemeth P. Glucocorticoid (GC) sensitivity and glucocorticoid receptor expression differ in thymocyte populations. *Int Immunol* 14: 463-469, 2002.
- Beyenburg S, Watzka M, Clusmann H, Blumcke I, Bidlingmaier F, Elger CE, Stoffel-Wagner B. Messenger RNA of steroid 21-hydroxylase (CYP21) is expressed in the human hippocampus. *Neurosci Lett* 308: 111-114, 2001.
- Bishop SC, Axford RF, Nicholas FW, Owen JB. *Breeding for Disease Resistance in Farm Animals*. 3rd Ed. CABI: Wallingford. 2010.
- Boullu-Ciocca S, Achard V, Tassistro V, Dutour A, Grino M. Postnatal programming of glucocorticoid metabolism in rats modulates high-fat diet-induced regulation of visceral adipose tissue glucocorticoid exposure and sensitivity and adiponectin and proinflammatory adipokines gene expression in adulthood. *Diabetes* 57: 669-677, 2008.

- Breuner CW, Orchinik M. Plasma binding proteins as mediators of corticosteroid action in vertebrates. *J Endocrinol* 175: 99-112, 2002.
- Brewer JA, Kanagawa O, Sleckman BP, Muglia LJ. Thymocyte apoptosis induced by T cell activation is mediated by glucocorticoids in vivo. *J Immunol* 169: 1837–1843, 2002.
- Brown G, Spencer K. Steroid hormones, stress and the adolescent brain: a comparative perspective. *Neuroscience* 249: 115-128, 2013.
- Butts CL, Jones YL, Lim JK, Salter CE, Belyavskaya E, Sternberg EM. Tissue expression of steroid hormone receptors is associated with differential immune responsiveness. *Brain Behav Immun* 25: 1000-1007, 2011.
- Byrd J. The effect of the humoral immune system-derived bursal anti-steroidogenic peptide (BASP) on corticosteroid biosynthesis in avian, porcine and canine adrenal cortical cells. *Comp Biochem Physiol* 108: 221–227, 1994.
- Casal AJ, Silvestre J-S, Delcayre C, Capponi AM. Expression and Modulation of Steroidogenic Acute Regulatory Protein Messenger Ribonucleic Acid in Rat Cardiocytes and after Myocardial Infarction. *Endocrinology* 144: 1861–1868, 2003.
- Cebra JJ. Influences of microbiota on intestinal immune system development. *Am J Clin Nutr* 69: 1046S-1051S, 1999.
- Chai W, Hofland J, Jansen PM, Garrelds IM, de Vries R, van den Bogaerd AJ, Feelders RA, de Jong FH, Danser AHJ. Steroidogenesis vs. steroid uptake in the heart: do corticosteroids mediate effects via cardiac mineralocorticoid receptors? *J Hypertens* 28: 1044-1053, 2010.
- Chantong B, Kratschmar DV, Nashev LG, Balazs Z, Odermatt A. Mineralocorticoid and glucocorticoid receptors differentially regulate NF-kappaB activity and pro-inflammatory cytokine production in murine BV-2 microglial cells. *J Neuroinflammation* 9:260, 2012.
- Chaves, LD, Faile GM, Krueth SB, Hendrickson JA, Reed KM. Haplotype variation, recombination, and gene conversion within the turkey MHC-B locus. *Immunogenetics* 62:465-477, 2010.
- Chen Y, Qiao S, Tuckermann J, Okret S, Jondal M. Thymus-derived glucocorticoids mediate androgen effects on thymocyte homeostasis. *FASEB J* 24: 5043-5051, 2010.
- Cima I, Corazza N, Dick B, Fuhrer A, Herren S, Jakob S, Ayuni E, Mueller C, Brunner T. Intestinal epithelial cells synthesize glucocorticoids and regulate T cell activation. *J Exp Med* 200: 1635–1646, 2004.

- Clapcote SJ, Roder JC. Simplex PCR assay for sex determination in mice. *BioTechniques* 38: 702-706, 2005.
- Close DA, Yun S-S, McCormick SD, Wildbill AJ, Li W. 11-Deoxycortisol is a corticosteroid hormone in the lamprey. *Proc Natl Acad Sci USA* 107:13942-13947, 2010.
- Compagnone NA, Mellon SH. Neurosteroids: biosynthesis and function of these novel neuromodulators. *Front Neuroendocrinol* 56: 1-56, 2000.
- Compton MM, Gibbs PS, Swicegood LR. Glucocorticoid-mediated activation of DNA degradation in avian lymphocytes. *Gen Comp Endocrinol* 80: 68-79, 1990.
- Cooper MD, Alder MN. The evolution of adaptive immune systems. *Cell* 124:815-822, 2006.
- Cooper MS, Rabbitt EH, Goddard PE, Bartlett WA, Hewison M, Stewart PM. *J Bone Miner Res* 17: 979-986, 2002.
- Coste A, Dubuquoy L, Barnouin R, Annicotte J-S, Magnier B, Notti M, Corazza N, Antal MC, Metzger D, Desreumaux P, Brunner T, Auwerx J, Schoonjans K. LRH-1-mediated glucocorticoid synthesis in enterocytes protects against inflammatory bowel disease. *Proc Natl Acad Sci USA* 104: 13098–13103, 2007.
- Croft AP, O'Callaghan MJ, Shaw SG, Connolly G, Jacquot C, Little HJ. Effects of minor laboratory procedures, adrenalectomy, social defeat or acute alcohol on regional brain concentrations of corticosterone. *Brain Res* 1238: 12–22, 2008.
- Davies E, Mackenzie SM. Extra-adrenal production of corticosteroids. *Clin Exp Pharmacol Physiol* 30: 437–445, 2003.
- Davison, F, Kaspers B, Schat KA, Kaiser P. *Avian Immunology*. Academic Press: San Diego. 2011.
- de Jonge HJM, Fehrmann RSN, de Bont ESJM, Hofstra RMW, Gerbens F, Kamps WA, de Vries EGE, van der Zee AGJ, te Meerman GJ, ter Elst A. Evidence based selection of housekeeping genes. *PLoS ONE* 2:e898, 2007.
- Demas GE, Nelson RJ. *Ecoimmunology*. New York: Oxford, 2012.
- Denver RJ. Structural and functional evolution of vertebrate neuroendocrine stress systems. *Annals of the New York Academy of Sciences* 1163:1-16, 2009.
- Dhabhar FS. Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology. *Neuroimmunomodulation* 16: 300–317, 2009.
- Do Rego JL, Seong JY, Burel D, Leprince J, Luu-The V, Tsutsui K, Tonon M-C, Pelletier G, Vaudry H. Neurosteroid biosynthesis: enzymatic pathways and neuroendocrine

regulation by neurotransmitters and neuropeptides. *Front Neuroendocrinol* 30: 259–301, 2009.

Dostal DE, Rothblum KN, Chernin MI, Cooper GR, Baker KM. Intracardiac detection of angiotensinogen and renin: a localized renin-angiotensin system in neonatal rat heart. *Am J Physiol Cell Physiol* 263: C838–C850, 1992.

Dumont M, Luu-The V, Dupont E, Pelletier G, Labrie F. Characterization, expression, and immunohistochemical localization of 3beta-hydroxysteroid dehydrogenas/ Δ 5- Δ 4 isomerase in human skin. *J Invest Dermatol* 99: 415–421, 1992.

Dunning JB. *Handbook of Avian Body Masses*, 2nd Ed. Boca Raton: Taylor & Francis, 2008.

Duval D, Durant S, Homo-Delarche F. Effect of antiglucocorticoids on dexamethasone-induced inhibition of uridine incorporation and cell lysis in isolated mouse thymocytes. *J Steroid Biochem* 20:283-287, 1984.

Ehrchen, J, Steinmüller L, Barczyk K, Tenbrock K, Nacken W, Eisenacher M, Nordhues U, Sorg C, Sunderkötter C, Roth J. Glucocorticoids induce differentiation of a specifically activated, anti-inflammatory subtype of human monocytes. *Blood* 109:1265-1274, 2007.

Falkenhahn M, Franke F, Bohle RM, Zhu YC, Stauss HM, Bachmann S, Danilov S, Unger T. Cellular distribution of angiotensin-converting enzyme after myocardial infarction. *Hypertension* 25: 219–226, 1995.

Fink T, Schafer MKH, di S, P, Friess H, Buchler M, Weihe E. Differential opioid gene expression in neurons and neuroendocrine cells of rat and human gastrointestinal tract. *Regul Pept* 53: S149–S150, 1994.

Franchini A, Ottaviani E. Immunoreactive POMC-derived peptides and cytokines in the chicken thymus and bursa of Fabricius microenvironments: age-related changes. *J Neuroendocrinol* 11: 685–692, 1999.

Freeman, B, Manning A. Re-establishment of the stress response in Gallus domesticus after hatching. *Comparative Biochemistry and Physiology Part A: Physiology* 78:267-270, 1984.

Fumihibo, A, Miyake T, Takada M, Shingu R, Endo T, Gojobori T, Kondo N, Ohno S. Monophyletic origin and unique dispersal patterns of domestic fowls. *Proceedings of the National Academy of Sciences* 93:6792-6795, 1996.

Funder JW. Cardiac synthesis of aldosterone: going, going, gone.? *Endocrinology* 145: 4793–4795, 2004.

Garvy BA, King LE, Telford WG, Morford LA, Fraker PJ. Chronic elevation of plasma corticosterone causes reductions in the number of cycling cells of the B lineage in murine bone marrow and induces apoptosis. *Immunology* 80: 587–592, 1993a.

Garvy BA, Telford WG, King LE, Fraker PJ. Glucocorticoids and irradiation-induced apoptosis in normal murine bone marrow B-lineage lymphocytes as determined by flow cytometry. *Immunology* 79:270-277, 1993b.

Geerling JC, Loewy AD. Aldosterone in the brain. *Am J Physiol Renal Physiol* 297: F559-F576, 2009.

Gillis S, Crabtree GR, Smith KA. Glucocorticoid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. *J Immunol* 123:1624-1631, 1979.

Gilmour JS, Coutinho AE, Cailhier J-F, Man TY, Clay M, Thomas G, Harris HJ, Mullins JJ, Seckl JR, Savill JS, Chapman KE. Local amplification of glucocorticoids by 11 β -hydroxysteroid dehydrogenase type 1 promotes macrophage phagocytosis of apoptotic leukocytes. *J Immunol* 176: 7605-7611, 2006.

Glick B. Normal growth of the bursa of Fabricius in chickens. *Poult Sci* 35:843-851, 1956.

Glick B, Chang TS, Jaap RG. The bursa of Fabricius and antibody production. *Poult Sci* 35: 224-225, 1956.

Gomez-Sanchez E, Gomez-Sanchez CE. The multifaceted mineralocorticoid receptor. *Compr Physiol* 4:965-994, 2014.

Gomez-Sanchez CE, Zhou MY, Cozza EN, Morita H, Foecking MF, Gomez-Sanchez EP. Aldosterone biosynthesis in the rat brain. *Endocrinology* 138: 3369–3373, 1997.

Gomez-Sanchez CE, Zhou MY, Cozza EN, Morita H, Eddleman FC, Gomez-Sanchez EP. Corticosteroid synthesis in the central nervous system. *Endocr Res* 22: 463–470, 1996.

Gomez-Sanchez EP. Brain mineralocorticoid receptors: orchestrators of hypertension and end-organ disease. *Curr Opin Nephrol Hypertens* 13: 191–196, 2004.

Gomez-Sanchez EP, Ahmad N, Romero DG, Gomez-Sanchez CE. Origin of aldosterone in the rat heart. *Endocrinology* 145: 4796–4802, 2004.

Gomez-Sanchez EP, Ahmad N, Romero DG, Gomez-Sanchez CE. Is aldosterone synthesized within the rat brain? *Am J Physiol Endocrinol Metab* 288: E342–E346, 2005.

Gomez-Sanchez EP, Gomez-Sanchez CM, Plonczynski M, Gomez-Sanchez CE. Aldosterone synthesis in the brain contributes to Dahl salt-sensitive rat hypertension. *Exp Physiol* 95: 120–130, 2010.

Gomez-Sanchez EP, Samuel J, Vergara G, Ahmad N. Effect of 3beta-hydroxysteroid dehydrogenase inhibition by trilostane on blood pressure in the Dahl salt-sensitive rat. *Am J Physiol Regul Integr Comp Physiol* 288: R389–R393, 2005.

Griffith SC, Buchanan KL. The zebra finch: the ultimate Australian supermodel. *Emu* 110: v-xii, 2010.

Gruver-Yates AL, Quinn MA, Cidlowski JA. Analysis of glucocorticoid receptors and their apoptotic response to dexamethasone in male murine B cells during development. *Endocrinology* 155: 463-474, 2013.

Gunnar MR, Cheatham CL. Brain and behavior interface: stress and the developing brain. *Infant Ment Health J* 24: 195– 211, 2003.

Guyton AC, Hall JE. *Textbook of Medical Physiology* 11th Ed. Philadelphia: Elsevier, 2006.

Hatakeyama H, Miyamori I, Fujita T, Takeda Y, Takeda R, Yamamoto H. Vascular aldosterone: biosynthesis and a link to angiotensin II-induced hypertrophy of vascular smooth muscle cells. *J Biol Chem* 269: 24316–24320, 1994.

Hatakeyama H, Miyamori I, Takeda Y, Yamamoto H, Mabuchi H. The expression of steroidogenic enzyme genes in human vascular cells. *Biochem Mol Biol Int* 40: 639–645, 1996.

Hattangadi SM, Wong P, Zhang L, Flygare J, Lodish HF. From stem cell to red cell: regulation of erythropoiesis at multiple levels by multiple proteins, RNAs, and chromatin modifications. *Blood* 118: 6258-6268, 2011

Herrmann K, Bischof HJ. Development of neurons in the ectostriatum of normal and monocularly deprived zebra finches: a quantitative Golgi study. *J Comp Neurol* 277:141-154, 1988.

Higo S, Hojo Y, Ishii H, Komatsuzaki Y, Ooishi Y, Murakami G, Mukai H, Yamazaki T, Nakahara D, Barron A, Kimoto T, Kawato S. Endogenous synthesis of corticosteroids in the hippocampus. *PLoS ONE* 6:e21631, 2011.

Hiramatsu R, Nisula BC. Erythrocyte-associated cortisol - measurement, kinetics of dissociation, and potential physiological significance. *J Clin Endocrinol Metab* 64: 1224-1232, 1987.

Hodgson DM, Coe CL. *Perinatal Programming: Early Life Determinants of Adult Health and Disease*. Taylor & Francis: Andover. 2006.

Holmes MC, Carter RN, Noble J, Chitnis S, Dutia A, Paterson JM, Mullins JJ, Seckl JR, Yau JLW. 11beta-Hydroxysteroid dehydrogenase type 1 expression is increased in the aged

mouse hippocampus and parietal cortex and causes memory impairments. *J Neurosci* 30:6916-6920, 2010.

Homberger B, Jenni-Eiermann S, Roulin A, Jenni L. The impact of pre- and post-natal contexts on immunity, glucocorticoids and oxidative stress resistance in wild and domesticated grey partridges. *Funct Ecol* 27: 1042-1054, 2013.

Homma H, Listowsky I. Identification of Yb-glutathione-S-transferase as a major rat liver protein labeled with dexamethasone 21-methanesulfonate. *Proc Natl Acad Sci USA* 82: 7165-7169, 1985.

Hostettler N, Bianchi P, Gennari-Moser C, Kassahn D, Schoonjans K, Corazza N, Brunner T. Local glucocorticoid production in the mouse lung is induced by immune cell stimulation. *Allergy* 67: 227-234, 2012.

Huang BS, White RA, Ahmad M, Jeng AY, Leenen FHH. Central infusion of aldosterone synthase inhibitor prevents sympathetic hyperactivity and hypertension by central Na⁺ in Wistar rats. *Am J Physiol Regul Integr Comp Physiol* 295: R166-R172, 2008.

Huang BS, White RA, Jeng AY, Leenen FHH. Role of central nervous system aldosterone synthase and mineralocorticoid receptors in salt-induced hypertension in Dahl salt-sensitive rats. *Am J Physiol Regul Integr Comp Physiol* 296: R994-R1000, 2009.

Hughes GC, Clark EA, Wong AH. The intracellular progesterone receptor regulates CD4+ T cells and T cell-dependent antibody responses. *J Leukoc Biol* 369-375, 2013.

Ito N, Ito T, Kromminga A, Bettermann A, Takigawa M, Kees F, Straub RH, Paus R. Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol. *FASEB J* 19: 1332-1334, 2005.

Iwata M, Hanaoka S, Sato K. Rescue of thymocytes and T cell hybridomas from glucocorticoid-induced apoptosis by stimulation via the T cell receptor/CD3 complex: a possible in vitro model for positive selection of the T cell repertoire. *Eur J Immunol* 21: 643-648, 1991.

Jamieson PM, Chapman KE, Edwards CR, Seckl JR. 11 beta-hydroxysteroid dehydrogenase is an exclusive 11 beta-reductase in primary cultures of rat hepatocytes: effect of physicochemical and hormonal manipulations. *Endocrinology* 136:4754-4761, 1995.

Jamieson CAM, Yamamoto KR. Crosstalk pathway for inhibition of glucocorticoid-induced apoptosis by T cell receptor signaling. *Proc Natl Acad Sci USA* 97: 7319-7324, 2000.

Jellinck PH, Dhabhar FS, Sakai RR, McEwen BS. Long-term corticosteroid treatment but not chronic stress affects 11 β -hydroxysteroid dehydrogenase type I activity in rat brain and peripheral tissues. *J Steroid Biochem Mol Biol* 60: 319-323, 1997.

Karssen AM, Meijer OC, van der Sandt ICJ, Lucassen PJ, de Lange ECM, de Boer AG, de Kloet ER. Multidrug resistance P-glycoprotein hampers the access of cortisol by not of corticosterone to mouse and human brain. *Endocrinology* 142: 2686-2694, 2001.

Kayes-Wandover KM, White PC. Steroidogenic enzyme gene expression in the human heart. *J Clin Endocrinol Metab* 85: 2519–2525, 2000.

Keeney DS, Ikeda Y, Waterman MR, Parker KL. Cholesterol side-chain cleavage cytochrome P450 gene expression in the primitive gut of the mouse embryo does not require steroidogenic factor 1. *Mol Endocrinol* 9: 1091–1098, 1995.

Keeney DS, Jenkins CM, Waterman MR. Developmentally regulated expression of adrenal 17alpha-hydroxylase cytochrome P450 in the mouse embryo. *Endocrinology* 136:4872-4879, 1995.

Kimoto T, Ishii H, Higo S, Hojo Y, Kawato S. Semicomprehensive analysis of the postnatal age-related changes in the mRNA expression of sex steroidogenic enzymes and sex steroid receptors in the male rat hippocampus. *Endocrinology* 151: 5795-5806, 2010.

King LB, Vacchio MS, Dixon K, Hunziker R, Margulies DH, Ashwell JD. A targeted glucocorticoid receptor antisense transgene increases thymocyte apoptosis and alters thymocyte development. *Immunity* 3: 647–656, 1995.

Kishimoto W, Hiroi T, Shiraishi M, Osada M, Imaoka S, Kominami S, Igarashi T, Funae Y. Cytochrome P450 2D catalyze steroid 21-hydroxylation in the brain. *Endocrinology* 145: 699–705, 2004.

Klasing KC, Leshchinsky TV. Functions, costs, and benefits of the immune system during development and growth. *Ostrich* 2817-2832, 1999.

Lechner O, Dietrich H, Wiegers GJ, Vacchio MS, Wick G. Glucocorticoid production in the chicken bursa and thymus. *Int Immunol* 13: 769–776, 2001.

Lechner O, Wiegers GJ, Oliveira-Dos-Santos AJ, Dietrich H, Recheis H, Waterman M, Boyd R, Wick G. Glucocorticoid production in the murine thymus. *Eur J Immunol* 30: 337–346, 2000.

Lee JH, Lydon JP, Kim CH. Progesterone suppresses the mTOR pathway and promotes generation of induced regulatory T cells with increased stability. *Eur J Immunol* 2683-2696, 2012.

Leposavić G, Nanut MP, Pilipović I, Kosec D, Arsenović-Ranin N, Stojić-Vukanić Z, Djikić J, Nacka-Aleksić M. Reshaping of T-lymphocyte compartment in adult prepubertal ovariectomised rats: A putative role for progesterone deficiency. *Immunobiology* 219: 118-130, 2014.

- Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat Rev Immunol* 7: 379-390, 2007.
- Liggins GC. The role of cortisol in preparing the fetus for birth. *Reprod Fertil Dev* 6: 141-150, 1994.
- Lindström J. Early development and fitness in birds and mammals. *Trends Ecol Evol* 14: 343-348, 1999.
- Little HJ, Croft AP, O'Callaghan MJ, Brooks SP, Wang G, Shaw SG. Selective increases in regional brain glucocorticoid: a novel effect of chronic alcohol. *Neuroscience* 156: 1017-1027, 2008.
- Lowenberg M, Tuynman J, Bilderbeek J, Gaber T, Buttgereit F, van Deventer S, Peppelenbosch M, Hommes D. Rapid immunosuppressive effects of glucocorticoids mediated through Lck and Fyn. *Blood* 106: 1703-1710, 2005.
- Lowenberg M, Verhaar AP, Bilderbeek J, van Marle J, Buttgereit F, Peppelenbosch MP, van Deventer SJ, Hommes DW. Glucocorticoids cause rapid dissociation of a T-cell-receptor-associated protein complex containing LCK and FYN. *EMBO Rep* 7: 1023-1029, 2006.
- Lupien SJ, McEwen BS, Gunnar MR, Heim C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat Rev Neurosci* 10: 434-445, 2009.
- MacKenzie SM, Clark CJ, Fraser R, Gomez-Sanchez CE, Connell JM, Davies E. Expression of 11beta-hydroxylase and aldosterone synthase genes in the rat brain. *J Mol Endocrinol* 24: 321-328, 2000.
- MacKenzie SM, Fraser R, Connell JMC, Davies E. Local renin-angiotensin systems and their interactions with extra-adrenal corticosteroid production. *J Renin Angiotensin Aldosterone Syst* 3: 214-221, 2002.
- Makin HLJ, Gower DB. *Steroid Analysis*. New York: Springer, 2010.
- Martin LB. Stress and immunity in wild vertebrates: Timing is everything. *Gen Comp Endocrinol* 163: 70-76, 2009.
- McEwen BS, Wingfield JC. The concept of allostasis in biology and biomedicine. *Horm Behav* 43: 2-15, 2003.
- McNabb FA, McNabb RA. Thyroid development in precocial and altricial avian embryos. *Auk* 736-742, 1977.
- Meaney MJ, Sapolsky RM, McEwen BS. The development of the glucocorticoid receptor system in the rat limbic brain. I. Ontogeny and autoregulation. *Dev Brain Res* 18: 159-164, 1985.

- Mellon SH, Miller WL. Extraadrenal steroid 21-hydroxylation is not mediated by P450c21. *J Clin Invest* 84: 1497–1502, 1989.
- Mellon SH, Deschepper CF. Neurosteroid biosynthesis: genes for adrenal steroidogenic enzymes are expressed in the brain. *Brain Res* 629: 283–292, 1993.
- Miller AH, Spencer RL, Stein M, McEwen BS. Adrenal steroid receptor binding in spleen and thymus after stress or dexamethasone. *Am J Physiol Endocrinol Metab* 259: E405–E412, 1990.
- Mills AD, Crawford LL, Domjan M, Faure JM. The behavior of the Japanese or domestic quail *Coturnix japonica*. *Neurosci Biobehav Rev* 21:261-281, 1997.
- Mittelstadt PR, Monteiro JP, Ashwell JD. Thymocyte responsiveness to endogenous glucocorticoids is required for immunological fitness. *J Clin Invest* 122: 2384-2394, 2012.
- Miyaura H, Iwata M. Direct and indirect inhibition of Th1 development by progesterone and glucocorticoids. *J Immunol* 168: 1087-1094, 2002.
- Mizuno Y, Yoshimura M, Yasue H, Sakamoto T, Ogawa H, Kugiyama K, Harada E, Nakayama M, Nakamura S, Ito T, Shimasaki Y, Saito Y, Nakao K. Aldosterone production is activated in failing ventricle in humans. *Circulation* 103: 72–77, 2001.
- Mueller M, Atanasov A, Cima I, Corazza N, Schoonjans K, Brunner T. Differential regulation of glucocorticoid synthesis in murine intestinal epithelial versus adrenocortical cell lines. *Endocrinology* 148: 1445–1453, 2007.
- Mueller M, Cima I, Noti M, Fuhrer A, Jakob S, Dubuquoy L, Schoonjans K, Brunner T. The nuclear receptor LRH-1 critically regulates extra-adrenal glucocorticoid synthesis in the intestine. *J Exp Med* 203: 2057–2062, 2006.
- Newman AEM, Chin EH, Schmidt KL, Bond L, Wynne-Edwards KE, Soma KK. Analysis of steroids in songbird plasma and brain by coupling solid phase extraction to radioimmunoassay. *Gen Comp Endocrinol* 155: 503-510, 2008.
- Newman AEM, Pradhan DS, Soma KK. Dehydroepiandrosterone and corticosterone are regulated by season and acute stress in a wild songbird: jugular versus brachial plasma. *Endocrinology* 149: 2537–2545, 2008.
- Nichelmann M, Tzschenk B. Ontogeny of thermoregulation in precocial birds. *Comp Biochem Physiol A* 131:751-763, 2002.
- Norris DO. *Vertebrate Endocrinology* 4th Ed. Burlington: Elsevier, 2007.
- Norton JM, Wira CR. Dose-related effects of the sex hormones and cortisol on the growth of the bursa of fabricius in chick embryos. *J Steroid Biochem* 8: 985–987, 1977.

- Noti M, Corazza N, Mueller C, Berger B, Brunner T. TNF suppresses acute intestinal inflammation by inducing local glucocorticoid synthesis. *J Exp Med* 207: 1057–1066, 2010.
- Noti M, Corazza N, Tuffin G, Schoonjans K, Brunner T. Lipopolysaccharide induces intestinal glucocorticoid synthesis in a TNF α -dependent manner. *FASEB J* 24: 1340–1346, 2010.
- Noti M, Sidler D, Brunner T. Extra-adrenal glucocorticoid synthesis in the intestinal epithelium: more than a drop in the ocean? *Semin Immunopathol* 31: 237–248, 2009.
- Nuotio-Antar AM, Hasty AH, Kovacs WJ. Quantitation and cellular localization of 11 β -HSD1 expression in murine thymus. *J Steroid Biochem Mol Biol* 99: 93-99, 2006.
- Obut TA, Ovsyukova MV, Dement'eva TY, Cherkasova OP, Saryg SK, Grigor'eva TA. Effects of dehydroepiandrosterone sulfate on the conversion of corticosterone into 11-dehydrocorticosterone in stress: a regulatory scheme. *Neurosci Behav Physiol* 39: 695–699, 2009.
- Ogawa T, Kitagawa M, Hirokawa K. Age-related changes of human bone marrow: a histometric estimation of proliferative cells, apoptotic cells, T cells, B cells and macrophages. *Mech Ageing Develop* 117: 57-68, 2000.
- Ohtani T, Ohta M, Yamamoto K, Mano T, Sakata Y, Nishio M, Takeda Y, Yoshida J, Miwa T, Okamoto M, Masuyama T, Nonaka Y, Hori M. Elevated cardiac tissue level of aldosterone and mineralocorticoid receptor in diastolic heart failure: beneficial effects of mineralocorticoid receptor blocker. *Am J Physiol Regul Integr Comp Physiol* 292: R946-R954, 2007.
- Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* 132: 631–644, 2008.
- Ottaviani E, Franchini A, Franceschi C. Evolution of neuroendocrine thymus: studies on POMC-derived peptides, cytokines and apoptosis in lower and higher vertebrates. *J Neuroimmunol* 72: 67–74, 1997.
- Ottaviani E, Franchini A, Franceschi C. Presence of immunoreactive corticotropin-releasing hormone and cortisol molecules in invertebrate haemocytes and lower and higher vertebrate thymus. *Histochem J* 30: 61–67, 1998.
- Ottinger MA. Quail and other short-lived birds. *Exp Gerontol* 36: 859-868, 2001.
- Pancer Z, Cooper MD. The evolution of adaptive immunity. *Ann Rev Immunol* 24: 497-518, 2006.

- Pasanen S, Ylikomi T, Palojoki E, Syvala H, Pelto-Huikko M, Tuohimaa P. Progesterone receptor in chicken bursa of Fabricius and thymus: evidence for expression in B-lymphocytes. *Mol Cell Endocrinol* 141: 119-128, 1998.
- Passier RC, Smits JF, Verluyten MJ, Daemen MJ. Expression and localization of renin and angiotensinogen in rat heart after myocardial infarction. *Am J Physiol Heart Circ Physiol* 271: H1040-H1048, 1996.
- Pazirandeh A, Jondal M, Okret S. Glucocorticoids delay age-associated thymic involution through directly affecting the thymocytes. *Endocrinology* 145: 2392–2401, 2004.
- Pazirandeh A, Xue Y, Rafter I, Sjovall J, Jondal M, Okret S. Paracrine glucocorticoid activity produced by mouse thymic epithelial cells. *FASEB J* 13: 893–901, 1999.
- Pazirandeh A, Xue Y, Prestegaard T, Jondal M, Okret S. Effects of altered glucocorticoid sensitivity in the T cell lineage on thymocyte and T cell homeostasis. *FASEB J* 16: 727–729, 2002.
- Pearce P, Khalid B, Funder J. Progesterone receptors in rat thymus. *Endocrinology* 113:1287-1291, 1983.
- Perišić M, Stojić-Vukanić Z, Pilipović I, Kosec D, Nacka-Aleksić M, Đikić J, Arsenović-Ranin N, Leposavić G. Role of ovarian hormones in T-cell homeostasis: From the thymus to the periphery. *Immunobiology*. 218: 353-367, 2013.
- Petit C, Hossaert-McKey M, Perret P, Blondel J, Lambrechts MM. Blue tits use selected plants and olfaction to maintain an aromatic environment for nestlings. *Ecol Lett* 5: 585-589, 2002.
- Pilorz V, Jäckel M, Knudsen K, Trillmich F. The cost of a specific immune response in young guinea pigs. *Physiol Behav* 85: 205-211, 2005.
- Pitt B, Zannad F, Remme WJ, Cody R, Castaigne a, Perez a, Palensky J, Witten J. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *N Engl J Med* 341: 709–717, 1999.
- Pradhan DS, Newman AEM, Wacker DW, Wingfield JC, Schlinger BA, Soma KK. Aggressive interactions rapidly increase androgen synthesis in the brain during the non-breeding season. *Horm Behav* 57: 381–389, 2010.
- Provost PR, Tremblay Y. Genes involved in the adrenal pathway of glucocorticoid synthesis are transiently expressed in the developing lung. *Endocrinology* 146: 2239–2245, 2005.

- Purton JF, Boyd RL, Cole TJ, Godfrey DI. Intrathymic T-cell development and selection proceeds normally in the absence of glucocorticoid receptor signaling. *Immunity* 13: 179–186, 2000.
- Qiao S, Chen L, Okret S, Jondal M. Age-related synthesis of glucocorticoids in thymocytes. *Exp Cell Res* 314: 3027–3035, 2008.
- Qiao S, Okret S, Jondal M. Thymocyte-synthesized glucocorticoids play a role in thymocyte homeostasis and are down-regulated by adrenocorticotrophic hormone. *Endocrinology* 150: 4163–4169, 2009.
- Raza K, Hardy R, Cooper MS. The 11beta-hydroxysteroid dehydrogenase enzymes--arbiters of the effects of glucocorticoids in synovium and bone. *Rheumatology* 49: 2016–2023, 2010.
- Remage-Healey L, London SE, Schlinger BA. Birdsong and the neural production of steroids. *J Chem Neuroanat* 39: 72–81, 2010.
- Revskoy S, Halasz I, Redei E. Corticotropin-releasing hormone and proopiomelanocortin gene expression is altered selectively in the male rat fetal thymus by maternal alcohol consumption. *Endocrinology* 138: 389-396, 1997.
- Ricklefs RE. Patterns of growth in birds. V. A comparative study of development in the starling, common tern, and Japanese quail. *Auk* 96: 10-30, 1979.
- Robel P, Baulieu E-E. Neurosteroids: biosynthesis and function. *Trends Endocrinol Metab* 5: 1–8, 1994.
- Rog-Zielinska EA, Thomson A, Kenyon CJ, Brownstein DG, Moran CM, Szumska D, Michailidou Z, Richardson J, Owen E, Watt A, Morrison H, Forrester LM, Bhattacharya S, Holmes MC, Chapman KE. Glucocorticoid receptor is required for foetal heart maturation. *Hum Mol Genet* 22: 3269-3282, 2013.
- Rogoff D, Gomez-Sanchez CE, Foecking MF, Wortsman J, Slominski A. Steroidogenesis in the human skin: 21-hydroxylation in cultured keratinocytes. *J Steroid Biochem Mol Biol* 78: 77–81, 2001.
- Roloff B, Fechner K, Slominski A, Furkert J, Botchkarev VA, Bulfone-Paus S, Zipper J, Krause E, Paus R. Hair cycle-dependent expression of corticotropin-releasing factor (CRF) and CRF receptors in murine skin. *FASEB J* 12: 287–297, 1998.
- Rowe JD, Nieves E, Listowsky I. Subunit diversity and tissue distribution of human glutathione S-transferases: interpretations based on electrospray ionization-MS and peptide sequence-specific antisera. *Biochem J* 325:481-486, 1997.

- Rupprecht R., Reul JM, van Steensel B, Spengler D, Söder M, Berning B, Holsboer F, Damm K. Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. *Eur J Pharmacol* 247:145-154, 1993.
- Sakai RR, Nicolaidis S, Epstein AN. Salt appetite is suppressed by interference with angiotensin II and aldosterone. *Am J Physiol Regul Integr Comp Physiol* 251: R762–R768, 1986.
- Sampath-Kumar R, Yu M, Khalil MW, Yang K. Metyrapone is a competitive inhibitor of 11 β -hydroxysteroid dehydrogenase type 1 reductase. *J Steroid Biochem Mol Biol* 62: 195-199, 1997.
- Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev* 21: 55–89, 2000.
- Schaer DJ, Boretti FS, Schoedon G, Schaffner A. Induction of the CD163-dependent haemoglobin uptake by macrophages as a novel anti-inflammatory action of glucocorticoids. *Br J Haematol* 119:239-243, 2002.
- 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. *Am J Physiol Regul Integr Comp Physiol* 297: R42–R51, 2009.
- Schmidt KL, Malisch JL, Breuner CW, Soma KK. Corticosterone and cortisol binding sites in plasma, immune organs and brain of developing zebra finches: intracellular and membrane-associated receptors. *Brain Behav Immun* 24: 908–918, 2010.
- 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.
- Schmidt KL, Soma KK. Cortisol and corticosterone in the songbird immune and nervous systems: local vs. systemic levels during development. *Am J Physiol Regul Integr Comp Physiol* 295: R103–R110, 2008.
- Schmidt M, Enthoven L, van der Mark M, Levine S, de Kloet ER, Oitzl MS. The postnatal development of the hypothalamic-pituitary-adrenal axis in the mouse. *Int J Dev Neurosci* 21: 125–132, 2003.
- Seckl JR, Holmes MC. Mechanisms of disease: glucocorticoids, their placental metabolism and fetal "programming" of adult pathophysiology. *Nat Clin Pract Endocrinol Metab* 3: 479-488, 2007.

Sheppard KE, Autelitano DJ. 11 β -Hydroxysteroid dehydrogenase 1 transforms 11-dehydrocorticosterone into transcriptionally active glucocorticoid in neonatal rat heart. *Endocrinology* 143: 198-204, 2002.

Silversides FG, Robertson MC, Liu J. Cryoconservation of avian gonads in Canada. *Poult Sci* 92: 2613-2617, 2013.

Silversides FG, Shaver D, Song Y. Pure line laying chickens at the Agassiz Research Centre. *Animal Genetic Resources Information*. Cambridge Univ Press, 79-85, 2007.

Silvestre JS, Robert V, Heymes C, Aupetit-Faisant B, Mouas C, Moalic JM, Swynghedauw B, Delcayre C. Myocardial production of aldosterone and corticosterone in the rat. *J Biol Chem* 273: 4883-4891, 1998.

Silvestre J-S, Heymes C, Oubenaissa A, Robert V, Aupetit-Faisant B, Carayon A, Swynghedauw B, Delcayre C. Activation of cardiac aldosterone production in rat myocardial infarction: effect of angiotensin II receptor blockade and role in cardiac fibrosis. *Circulation* 99: 2694-2701, 1999.

Simard J, Couet J, Durocher F, Labrie Y, Sanchez R, Breton N, Turgeon C, Labrie F. Structure and tissue-specific expression of a novel member of the rat 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4 isomerase (3 β -HSD) family. *J Biol Chem* 268: 19659-19668, 1993.

Sivukhina E, Helbling J-C, Minni AM, Schafer HH, Pallet V, Jirikowski GF, Moisan M-P. Intrinsic expression of transcartin in neural cells of the mouse brain: a histochemical and molecular study. *J Exp Biol* 216: 245-252, 2013.

Slominski A, Wortsman J. Neuroendocrinology of the skin. *Endocr Rev* 21: 457-487, 2000.

Slominski A, Ermak G, Mihm M. ACTH receptor, CYP11A1, CYP17 and CYP21A2 genes are expressed in the skin. *J Clin Endocrinol Metab* 81: 2746-2749, 1996.

Slominski A, Gomez-Sanchez CE, Foecking MF, Wortsman J. Active steroidogenesis in the normal rat skin. *Biochim Biophys Acta* 1474: 1-4, 2000.

Slominski A, Zbytek B, Semak I, Sweatman T, Wortsman J. CRH stimulates POMC activity and corticosterone production in dermal fibroblasts. *J Neuroimmunol* 162: 97-102, 2005.

Slominski A, Zbytek B, Szczesniewski A, Semak I, Kaminski J, Sweatman T, Wortsman J. CRH stimulation of corticosteroids production in melanocytes is mediated by ACTH. *Am J Physiol Endocrinol Metab* 288: E701-E706, 2005.

Slominski A, Zbytek B, Szczesniewski A, Wortsman J. Cultured human dermal fibroblasts do produce cortisol. *J Invest Dermatol* 126: 1177-1178, 2006.

- Slominski A, Zjawiony J, Wortsman J, Semak I, Stewart J, Pisarchik A, Sweatman T, Marcos J, Dunbar C, Tuckey RC. A novel pathway for sequential transformation of 7-dehydrocholesterol and the expression of the P450scc system in mammalian skin. *Eur J Biochem* 271: 4178–4188, 2004.
- Soderstrom, K., W. Qin, and M.H. Leggett. A minimally invasive procedure for sexing young zebra finches. *J Neurosci Meth* 164: 116-119, 2007.
- Soma KK. Testosterone and aggression: Berthold, birds and beyond. *J Neuroendocrinol* 18: 543–551, 2006.
- Starck JM, Ricklefs RE. *Avian Growth and Development: Evolution Within the Altricial-Precocial Spectrum*. Oxford: New York. 1998.
- Stromstedt M, Waterman MR. Messenger RNAs encoding steroidogenic enzymes are expressed in rodent brain. *Mol Brain Res* 34: 75–88, 1995.
- Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu X-N, Kubo C, Koga Y. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J Physiol* 558: 263-275.
- Takahama Y. Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol* 6: 127-135.
- Tagawa N, Ikariko N, Fukumura K, Kobayashi Y. Development of an enzyme-linked immunosorbent assay for serum 11-dehydrocorticosterone in rat and mouse. *Biol Pharm Bull* 30: 403-407, 2007.
- Takeda R, Hatakeyama H, Takeda Y, Iki K, Miyamori I, Sheng WP, Yamamoto H, Blair IA. Aldosterone biosynthesis and action in vascular cells. *Steroids* 60: 120–124, 1995.
- Takeda Y, Miyamori I, Yoneda T, Iki K, Hatakeyama H, Blair IA, Hsieh FY, Takeda R. Synthesis of corticosterone in the vascular wall. *Endocrinology* 135: 2283–2286, 1994.
- Tannenbaum BM, Brindley DN, Tannenbaum GS, Dallman MF, McArthur MD, Meaney MJ. High-fat feeding alters both basal and stress-induced hypothalamic-pituitary-adrenal activity in the rat. *Am J Physiol Endocrinol Metab* 273: E1168-E1177, 1997.
- Taves MD, Gomez-Sanchez CE, Soma KK. Extra-adrenal glucocorticoids and mineralocorticoids: evidence for local synthesis, regulation, and function. *Am J Physiol Endocrinol Metab* 301: E11-E24, 2011a.
- Taves MD, Ma C, Heimovics SA, Saldanha CJ, Soma KK. Measurement of steroid concentrations in brain tissue: methodological considerations. *Front Endocrinol* 2:39, 2011b.

Taves MD, Plumb AW, Sandkam BA, Ma C, Van Der Gugten JG, Holmes DT, Close DA, Abraham NA, Soma KK. Steroid profiling reveals widespread local regulation of glucocorticoid levels during mouse development. *Endocrinology* 156: 511-522, 2015.

Taves MD, Schmidt KL, Ruhr IM, Kapusta K, Prior NH, Soma KK. Steroid concentrations in plasma, whole blood and brain: effects of saline perfusion to remove blood contamination from brain. *PLoS ONE* 5: e15727, 2010.

R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, 2005.

Teofoli P, Frezzolini a, Puddu P, De Pita O, Mauviel a, Lotti T. The role of proopiomelanocortin-derived peptides in skin fibroblast and mast cell functions. *Ann N Y Acad Sci* 885: 268–276, 1999.

Thiboutot D, Jabara S, McAllister JM, Sivarajah A, Gilliland K, Cong Z, Clawson G. Human skin is a steroidogenic tissue: steroidogenic enzymes and cofactors are expressed in epidermis, normal sebocytes, and an immortalized sebocyte cell line (SEB-1). *J Invest Dermatol* 120: 905–914, 2003.

Thompson EB. Membrane transporters of steroid hormones. *Curr Biol* 5: 730-732, 1995.

Tibbetts, T, DeMayo F, Rich S, Conneely OM, O'Malley BW. Progesterone receptors in the thymus are required for thymic involution during pregnancy and for normal fertility. *Proc Natl Acad Sci USA* 96: 12021-12026, 1999.

Tomlinson JW, Walker EA, Bujalska IJ, Draper N, Lavery GG, Cooper MS, Hewison M, Stewart PM. 11 β -hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response. *Endocr Rev* 25: 831-866, 2004.

Touitou Y, Auzeby A, Bogdan A. Cortisol and cortisone production in rat and mouse adrenal incubations. *J Steroid Biochem Mol Biol* 37: 279-284, 1990.

Trottier MD, Newsted MM, King LE, Fraker PJ. Natural glucocorticoids induce expansion of all developmental stages of murine bone marrow granulocytes without inhibiting function. *Proc Natl Acad Sci USA* 105:2028-2033, 2008.

Tsutamoto T, Wada A, Maeda K, Mabuchi N, Hayashi M, Tsutsui T, Ohnishi M, Sawaki M, Fujii M, Matsumoto T, Horie H, Sugimoto Y, Kinoshita M. Spironolactone inhibits the transcardiac extraction of aldosterone in patients with congestive heart failure. *J Am Coll Cardiol* 36: 838–844, 2000.

Tsutsui K, Ukena K, Usui M, Sakamoto H, Takase M. Novel brain function: biosynthesis and actions of neurosteroids in neurons. *Neurosci Res* 36: 261–273, 2000.

- Tuckermann JP, Kleiman A, Moriggi R, Spanbroek R, Neumann A, Illing A, Clausen BE, Stride B, Forster I, Habenicht AJR, Reichardt HM, Tronche F, Schmid W, Schutz G. Macrophages and neutrophils are the targets for immune suppression by glucocorticoids in contact allergy. *J Clin Invest* 117: 1381-1390, 2007.
- Usher MG, Duan SZ, Ivaschenko CY, Frieler RA, Berger S, Schütz G, Lumeng CN, Mortensen RM. Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice. *J Clin Invest* 120: 3350-3364, 2010.
- Vacchio MS, Ashwell JD. Thymus-derived glucocorticoids regulate antigen-specific positive selection. *J Exp Med* 185: 2033-2038, 1997.
- Vacchio MS, Papadopoulos V, Ashwell JD. Steroid production in the thymus: implications for thymocyte selection. *J Exp Med* 179: 1835-1846, 1994.
- van Kats JP, Danser AH, van Meegen JR, Sassen LM, Verdouw PD, Schalekamp MA. Angiotensin production by the heart: a quantitative study in pigs with the use of radiolabeled angiotensin infusions. *Circulation* 98: 73-81, 1998.
- Van Laethem F, Baus E, Andris F, Urbain J, Leo O. A novel aspect of the anti-inflammatory actions of glucocorticoids: inhibition of proximal steps of signaling cascades in lymphocytes. *Cell Mol Life Sci* 58: 1599-1606. 2001a.
- Van Laethem F, Baus E, Smyth LA, Andris F, Bex F, Urbain J, Kioussis D, Leo O. Glucocorticoids attenuate T cell receptor signaling. *J Exp Med* 193: 803-814. 2001b.
- Van Weerden W, Bierings H, Van Steenbrugge G, De Jong F, Schröder F. Adrenal glands of mouse and rat do not synthesize androgens. *Life Sci* 50: 857-861, 1992.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3: research0034-34.11, 2002.
- Varon HH, Touchstone JC. 17-Hydroxycorticoids in mouse adrenal incubates. *Acta Endocrinol* 46: 65-70, 1964.
- Vegiopoulos A, Herzig S. Glucocorticoids, metabolism and metabolic diseases. *Mol Cell Endocrinol* 275:43-61, 2007.
- Vernocchi S, Battello N, Schmitz S, Revets D, Billing AM, Turner JD, Muller CP. Membrane Glucocorticoid Receptor activation induces proteomic changes aligning with classical glucocorticoid effects. *Mol Cell Proteomics* 12, 1764-1779, 2013.
- Vinson GP. The mislabelling of deoxycorticosterone: making sense of corticosteroid structure and function. *J Endocrinol* 211: 3-16, 2011.

- Vukelic S, Stojadinovic O, Pastar I, Rabach M, Krzyzanowska A, Lebrun E, Davis SC, Resnik S, Brem H, Tomic-Canic M. Cortisol synthesis in epidermis is induced by IL-1 and tissue injury. *J Biol Chem* 286: 10265-10275, 2011.
- Wada H. Glucocorticoids: Mediators of vertebrate ontogenetic transitions. *Gen Comp Endocrinol*, 441-453, 2008.
- Wada H, Salvante KG, Wagner E, Williams TD, Breuner CW. Ontogeny and individual variation in the adrenocortical response of zebra finch (*Taeniopygia guttata*) nestlings. *Physiol Biochem Zool* 82: 325–331, 2009.
- Walker BR. Glucocorticoids and cardiovascular disease. *Eur J Endocrinol* 157:545-559, 2007.
- West B, Zhou B-X. Did chickens go north? New evidence for domestication. *J Archaeol Sci* 15:515-533, 1988.
- Wingfield JC, Hahn TP. Testosterone and territorial behaviour in sedentary and migratory sparrows. *Anim Behav* 47: 77–89, 1994.
- Xue C, Siragy HM. Local renal aldosterone system and its regulation by salt, diabetes, and angiotensin II type 1 receptor. *Hypertension* 46: 584–590, 2005.
- Yamamoto N, Yasue H, Mizuno Y, Yoshimura M, Fujii H, Nakayama M, Harada E, Nakamura S, Ito T, Ogawa H. Aldosterone is produced from ventricles in patients with essential hypertension. *Hypertension* 39: 958–962, 2002.
- Ye P, Kenyon CJ, MacKenzie SM, Jong AS, Miller C, Gray GA, Wallace A, Ryding AS, Mullins JJ, McBride MW, Graham D, Fraser R, Connell JMC, Davies E. The aldosterone synthase (CYP11B2) and 11beta-hydroxylase (CYP11B1) genes are not expressed in the rat heart. *Endocrinology* 146: 5287–5293, 2005.
- Ye P, Kenyon CJ, MacKenzie SM, Seckl JR, Fraser R, Connell JMC, Davies E. Regulation of aldosterone synthase gene expression in the rat adrenal gland and central nervous system by sodium and angiotensin II. *Endocrinology* 144: 3321–3328, 2003.
- Ye P, Kenyon CJ, Mackenzie SM, Nichol K, Seckl JR, Fraser R, Connell JMC, Davies E. Effects of ACTH, dexamethasone, and adrenalectomy on 11beta-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) gene expression in the rat central nervous system. *J Endocrinol* 196: 305–311, 2008.
- Young MJ, Clyne CD, Cole TJ, Funder JW. Cardiac steroidogenesis in the normal and failing heart. *J Clin Endocrinol Metab* 86: 5121–5126, 2001.
- Yu L, Romero DG, Gomez-Sanchez CE, Gomez-Sanchez EP. Steroidogenic enzyme gene expression in the human brain. *Mol Cell Endocrinol* 190: 9–17, 2002.

Yudt MR, Jewell CM, Bienstock RJ, Cidlowski JA. Molecular origins for the dominant negative function of human glucocorticoid receptor beta. *Mol Cell Biol* 23: 4319-4330, 2003.

Zann RA. *The Zebra Finch: A Synthesis of Field and Laboratory Studies*. Oxford: New York. 1996.

Zhang TY, Ding X, Daynes RA. The expression of 11 β -hydroxysteroid dehydrogenase type 1 by lymphocytes provides a novel means for intracrine regulation of glucocorticoid activities. *J Immunol* 174: 879-889, 2005.

Zmijewski MA, Sharma RK, Slominski A. Expression of molecular equivalent of hypothalamic-pituitary-adrenal axis in adult retinal pigment epithelium. *J Endocrinol* 193: 157-169, 2007.

Appendices

Appendix A: Taves et al., 2011a references (for Tables 1.1, 1.2, 1.3)

1. Ahmad N, Romero DG, Gomez-Sanchez EP, Gomez-Sanchez CE. *Endocrinology* 145: 3626–3629, 2004.
2. Aird F, Clevenger CV, Prystowsky MB, Redei EVA. *Proc Natl Acad Sci USA* 90: 7104–7108, 1993.
3. Atanasov AG, Leiser D, Roesselet C, et al. *FASEB J* 22: 4117–4125, 2008.
4. Batanero E, de Leeuw FE, Jansen GH, et al. *Brain Behav Immun* 6: 249–264, 1992.
5. Beyenburg S, Watzka M, Clusmann H, et al. *Neurosci Lett* 308: 111–114, 2001.
6. Brewer JA, Kanagawa O, Sleckman BP, Muglia LJ. *J Immunol* 169: 1837–1843, 2002.
7. Byrd J. *Comp Biochem Physiol* 108: 221–227, 1994.
8. Casal AJ, Silvestre J-S, Delcayre C, Capponi AM. *Endocrinology* 144: 1861–1868, 2003.
9. Chai W, Hofland J, Jansen PM, et al. *J Hypertens* 28: 1044–1053, 2010.
10. Chen Y, Qiao S, Tuckermann J, Okret S, Jondal M. *FASEB J* 24: 5043–5051, 2010.
11. Cima I, Corazza N, Dick B, et al. *J Exp Med* 200: 1635–1646, 2004.
12. Compagnone NA, Mellon SH. *Front Neuroendocrinol* 56: 1–56, 2000.
13. Coste A, Dubuquoy L, Barnouin R, et al. *Proc Natl Acad Sci USA* 104: 13098–13103, 2007.
14. Croft AP, O'Callaghan MJ, Shaw SG, et al. *Brain Res* 1238: 12–22, 2008.
15. Davies E, Mackenzie SM. *Clin Exp Pharmacol Physiol* 30: 437–445, 2003.
16. Dhabhar FS. *Neuroimmunomodulation* 16: 300–317, 2009.
17. Do Rego JL, Seong JY, Burel D, et al. *Front Neuroendocrinol* 30: 259–301, 2009.
18. Dostal DE, Rothblum KN, Chernin MI, et al. *Am J Physiol Cell Physiol* 263: C838–C850, 1992.
19. Dumont M, Luu-The V, Dupont E, Pelletier G, Labrie F. *J Invest Dermatol* 99: 415–421, 1992.
20. Falkenhahn M, Franke F, Bohle RM, et al. *Hypertension* 25: 219–226, 1995.
21. Fink T, Schafer MKH, di S, P, Frieß H, Buchler M, Weihe E. *Regul Pept* 53: S149–S150, 1994.
22. Franchini A, Ottaviani E. *J Neuroendocrinol* 11: 685–692, 1999.
23. Funder JW. *Endocrinology* 145: 4793–4795, 2004.
24. Garvy BA, King LE, Telford WG, Morford LA, Fraker PJ. *Immunology* 80: 587–592, 1993.
25. Geerling JC, Loewy AD. *Am J Physiol Renal Physiol* 297: F559–F576, 2009.
26. Gomez-Sanchez CE, Zhou MY, Cozza EN, et al. *Endocrinology* 138: 3369–3373, 1997.
27. Gomez-Sanchez CE, Zhou MY, Cozza EN, et al. *Endocr Res* 22: 463–470, 1996.
28. Gomez-Sanchez EP. *Curr Opin Nephrol Hypertens* 13: 191–196, 2004.
29. Gomez-Sanchez EP, Ahmad N, Romero DG, Gomez-Sanchez CE. *Endocrinology* 145: 4796–4802, 2004.
30. Gomez-Sanchez EP, Ahmad N, Romero DG, Gomez-Sanchez CE. *Am J Physiol Endocrinol Metab* 288: E342–E346, 2005.
31. Gomez-Sanchez EP, Gomez-Sanchez CM, Plonczynski M, Gomez-Sanchez CE. *Exp Physiol* 95: 120–130, 2010.
32. Gomez-Sanchez EP, Samuel J, Vergara G, Ahmad N. *Am J Physiol Regul Integr Comp Physiol* 288: R389–R393, 2005.
33. Gunnar MR, Cheatham CL. *Infant Ment Health J* 24: 195–211, 2003.
34. Hatakeyama H, Miyamori I, Fujita T, et al. *J Biol Chem* 269: 24316–24320, 1994.
35. Hatakeyama H, Miyamori I, Takeda Y, et al. *Biochem Mol Biol Int* 40: 639–645, 1996.
36. Huang BS, White RA, Ahmad M, et al. *Am J Physiol Regul Integr Comp Physiol* 295: R166–R172, 2008.
37. Huang BS, White RA, Jeng AY, Leenen FHH. *Am J Physiol Regul Integr Comp Physiol* 296: R994–R1000, 2009.
38. Ito N, Ito T, Kromminga A, et al. *FASEB J* 19: 1332–1334, 2005.
39. Iwata M, Hanaoka S, Sato K. *Eur J Immunol* 21: 643–648, 1991.
40. Janeway CA, Travers P, Walport M, Shlomchik MJ. *Immunobiology*. New York: Garland, 2005.
41. Kayes-Wandover KM, White PC. *J Clin Endocrinol Metab* 85: 2519–2525, 2000.
42. Keeney DS, Ikeda Y, Waterman MR, Parker KL. *Mol Endocrinol* 9: 1091–1098, 1995.
43. Kimoto T, Ishii H, Higo S, Hojo Y, Kawato S. *Endocrinology* 151: 5795–5806, 2010.
44. King LB, Vacchio MS, Dixon K, et al. *Immunity* 3: 647–656, 1995.
45. Kishimoto W, Hiroi T, Shiraishi M, et al. *Endocrinology* 145: 699–705, 2004.
46. Lechner O, Dietrich H, Wiegers GJ, Vacchio MS, Wick G. *Int Immunol* 13: 769–776, 2001.
47. Lechner O, Wiegers GJ, Oliveira-Dos-Santos AJ, et al. *Eur J Immunol* 30: 337–346, 2000.
48. Little HJ, Croft aP, O'Callaghan MJ, Brooks SP, Wang G, Shaw SG. *Neuroscience* 156: 1017–1027, 2008.
49. Lowenberg M, Tuynman J, Bilderbeek J, et al. *Blood* 106: 1703–1710, 2005.
50. Lowenberg M, Verhaar AP, Bilderbeek J, et al. *EMBO Rep* 7: 1023–1029, 2006.
51. MacKenzie SM, Clark CJ, Fraser R, et al. *J Mol Endocrinol* 24: 321–328, 2000.
52. MacKenzie SM, Fraser R, Connell JMC, Davies E. *J Renin Angiotensin Aldosterone Syst* 3: 214–221, 2002.
53. Mellon SH, Miller WL. *J Clin Invest* 84: 1497–1502, 1999.
54. Mellon SH, Deschepper CF. *Brain Res* 629: 283–292, 1993.
55. Miller AH, Spencer RL, Stein M, McEwen BS. *Am J Physiol Endocrinol Metab* 259: E405–E412, 1990.
56. Mizuno Y, Yoshimura M, Yasue H, et al. *Circulation* 103: 72–77, 2001.
57. Mueller M, Atanasov A, Cima I, et al. *Endocrinology* 148: 1445–1453, 2007.
58. Mueller M, Cima I, Noti M, et al. *J Exp Med* 203: 2057–2062, 2006.

59. Newman AEM, Pradhan DS, Soma KK. *Endocrinology* 149: 2537–2545, 2008.
60. Norton JM, Wira CR. *J Steroid Biochem* 8: 985–987, 1977.
61. Noti M, Corazza N, Mueller C, Berger B, Brunner T. *J Exp Med* 207: 1057–1066, 2010.
62. Noti M, Corazza N, Tuffin G, Schoonjans K, Brunner T. *FASEB J* 24: 1340–1346, 2010.
63. Noti M, Sidler D, Brunner T. *Semin Immunopathol* 31: 237–248, 2009.
64. Ohtani T, Ohta M, Yamamoto K, et al. *Am J Physiol Regul Integr Comp Physiol* 292: R946–R954, 2007.
65. Ottaviani E, Franchini A, Franceschi C. *J Neuroimmunol* 72: 67–74, 1997.
66. Ottaviani E, Franchini A, Franceschi C. *Histochem J* 30: 61–67, 1998.
67. Passier RC, Smits JF, Verluyten MJ, Daemen MJ. *Am J Physiol Heart Circ Physiol* 271: H1040–H1048, 1996.
68. Pazirandeh A, Jondal M, Okret S. *Endocrinology* 145: 2392–2401, 2004.
69. Pazirandeh A, Xue Y, Rafter I, Sjovall J, Jondal M, Okret S. *FASEB J* 13: 893–901, 1999.
70. Pazirandeh A, Xue Y, Prestegaard T, Jondal M, Okret S. *FASEB J* 16: 727–729, 2002.
71. Pitt B, Zannad F, Remme WJ, et al. *N Engl J Med* 341: 709–717, 1999.
72. Pradhan DS, Newman AEM, Wacker DW, et al. *Horm Behav* 57: 381–389, 2010.
73. Provost PR, Tremblay Y. *Endocrinology* 146: 2239–2245, 2005.
74. Purton JF, Boyd RL, Cole TJ, Godfrey DI. *Immunity* 13: 179–186, 2000.
75. Qiao S, Chen L, Okret S, Jondal M. *Exp Cell Res* 314: 3027–3035, 2008.
76. Qiao S, Okret S, Jondal M. *Endocrinology* 150: 4163–4169, 2009.
77. Raza K, Hardy R, Cooper MS. *Rheumatology* 49: 2016–2023, 2010.
78. Remage-Healey L, London SE, Schlinger BA. *J Chem Neuroanat* 39: 72–81, 2010.
79. Revskoy S, Halasz I, Redei E. *Endocrinology* 138: 389–396, 1997.
80. Robel P, Baulieu E-E. *Trends Endocrinol Metab* 5: 1–8, 1994.
81. Rogoff D, Gomez-Sanchez CE, Foecking MF, et al. *J Steroid Biochem Mol Biol* 78: 77–81, 2001.
82. Roloff B, Fechner K, Slominski A, et al. *FASEB J* 12: 287–297, 1998.
83. Sakai RR, Nicolaidis S, Epstein AN. *Am J Physiol Regul Integr Comp Physiol* 251: R762–R768, 1986.
84. Sapolsky RM, Romero LM, Munck AU. *Endocr Rev* 21: 55–89, 2000.
85. Schmidt KL, Chin EH, Shah AH, Soma KK. *Am J Physiol Regul Integr Comp Physiol* 297: R42–R51, 2009.
86. Schmidt KL, Malisch JL, Breuner CW, Soma KK. *Brain Behav Immun* 24: 908–918, 2010.
87. Schmidt KL, Pradhan DS, Shah AH, et al. *Gen Comp Endocrinol* 157: 266–274, 2008.
88. Schmidt KL, Soma KK. *Am J Physiol Regul Integr Comp Physiol* 295: R103–R110, 2008.
89. Schmidt M, Enthoven L, van der Mark M, et al. *Int J Dev Neurosci* 21: 125–132, 2003.
90. Silvestre JS, Robert V, Heymes C, et al. *J Biol Chem* 273: 4883–4891, 1998.
91. Silvestre J-S, Heymes C, Oubenaissa A, et al. *Circulation* 99: 2694–2701, 1999.
92. Simard J, Couet J, Durocher F, et al. *J Biol Chem* 268: 19659–19668, 1993.
93. Slominski A, Wortsman J. *Endocr Rev* 21: 457–487, 2000.
94. Slominski A, Ermak G, Mihm M. *J Clin Endocrinol Metab* 81: 2746–2749, 1996.
95. Slominski A, Gomez-Sanchez CE, Foecking MF, Wortsman J. *Biochim Biophys Acta* 1474: 1–4, 2000.
96. Slominski A, Zbytek B, Semak I, Sweatman T, Wortsman J. *J Neuroimmunol* 162: 97–102, 2005.
97. Slominski A, Zbytek B, Szczesniewski A, et al. *Am J Physiol Endocrinol Metab* 288: E701–E706, 2005.
98. Slominski A, Zbytek B, Szczesniewski A, Wortsman J. *J Invest Dermatol* 126: 1177–1178, 2006.
99. Slominski A, Zjawiony J, Wortsman J, et al. *Eur J Biochem* 271: 4178–4188, 2004.
100. Soma KK. *J Neuroendocrinol* 18: 543–551, 2006.
101. Stromstedt M, Waterman MR. *Mol Brain Res* 34: 75–88, 1995.
102. Takeda R, Hatakeyama H, Takeda Y, et al. *Steroids* 60: 120–124, 1995.
103. Takeda Y, Miyamori I, Yoneda T, et al. *Endocrinology* 135: 2283–2286, 1994.
104. Taves MD, Schmidt KL, Ruhr IM, et al. *PLoS ONE* 5: e15727, 2010.
105. Teofoli P, Frezzolini a, Puddu P, et al. *Ann NY Acad Sci* 885: 268–276, 1999.
106. Thiboutot D, Jabara S, McAllister JM, et al. *J Invest Dermatol* 120: 905–914, 2003.
107. Tsutamoto T, Wada A, Maeda K, et al. *J Am Coll Cardiol* 36: 838–844, 2000.
108. Tsutsui K, Ukena K, Usui M, Sakamoto H, Takase M. *Neurosci Res* 36: 261–273, 2000.
109. Vaccchio MS, Ashwell JD. *J Exp Med* 185: 2033–2038, 1997.
110. Vaccchio MS, Papadopoulos V, Ashwell JD. *J Exp Med* 179: 1835–1846, 1994.
111. van Kats JP, Danser AH, van Meegen JR, et al. *Circulation* 98: 73–81, 1998.
112. Vukelic S, Stojadinovic O, Pastar I, et al. *J Biol Chem* 286: 10265–10275, 2011.
113. Wada H, Salvante KG, Wagner E, et al. *Physiol Biochem Zool* 82: 325–331, 2009.
114. Wingfield JC, Hahn TP. *Anim Behav* 47: 77–89, 1994.
115. Xue C, Siragy HM. *Hypertension* 46: 584–590, 2005.
116. Yamamoto N, Yasue H, Mizuno Y, et al. *Hypertension* 39: 958–962, 2002.
117. Ye P, Kenyon CJ, MacKenzie SM, et al. *Endocrinology* 146: 5287–5293, 2005.
118. Ye P, Kenyon CJ, MacKenzie SM, et al. *Endocrinology* 144: 3321–3328, 2003.
119. Ye P, Kenyon CJ, MacKenzie SM, et al. *J Endocrinol* 196: 305–311, 2008.
120. Young MJ, Clyne CD, Cole TJ, Funder JW. *J Clin Endocrinol Metab* 86: 5121–5126, 2001.
121. Yu L, Romero DG, Gomez-Sanchez CE, Gomez-Sanchez EP. *Mol Cell Endocrinol* 190: 9–17, 2002.
122. Zmijewski MA, Sharma RK, Slominski A. *J Endocrinol* 193: 157–169, 2007.

Appendix B: Chapter 3 versus Chapter 4 mouse diets

| Study | Chapter 3 | | Chapter 4 | |
|-----------------------------------|-----------------------------------|------------------------------------|------------------------------------|------------------------------------|
| Animal Facility | Wesbrook Animal Unit | | Centre for Disease Modeling | |
| Type of diet | Standard diet | Breeding diet | Standard diet | Breeding diet |
| Vendor & cat # | Labdiet 5010 | Labdiet 5021 | Teklad 2918 | Teklad 2919 |
| Energy density (kcal/g) | 3.1 | 3.4 | 3.1 | 3.3 |
| Protein calories (%) | 29 | 24 | 24 | 23 |
| Fat calories (%) | 13 | 22 | 18 | 22 |
| Carbohydrates cal (%) | 59 | 55 | 58 | 55 |
| Ingredients | | | | |
| Ground corn | Ground corn | Ground wheat | Ground wheat | Ground wheat |
| Dehulled soybean meal | Wheat middlings | Ground corn | Ground corn | Ground corn |
| Wheat middlings | Dehulled soybean meal | Wheat middlings | Corn gluten meal | Corn gluten meal |
| Fish meal | Wheat germ | Dehulled soybean meal | Wheat middlings | Wheat middlings |
| Ground wheat | Fish meal | Corn gluten meal | Soybean oil | Soybean oil |
| Wheat germ | Ground wheat | Soybean oil | Calcium carbonate | Calcium carbonate |
| Brewers dried yeast | Porcine animal fat | Calcium carbonate | Dicalcium phosphate | Dicalcium phosphate |
| Ground oats | Brewers dried yeast | Dicalcium phosphate | Brewers dried yeast | Brewers dried yeast |
| Dehydrated alfalfa meal | Soybean oil | Brewers dried yeast | L-lysine | L-lysine |
| Calcium carbonate | Calcium carbonate | Iodized salt | Iodized salt | Iodized salt |
| Porcine animal fat | Salt | L-lysine | Magnesium oxide | Magnesium oxide |
| Ground soybean hulls | Dried beet pulp | DL-methionine | Choline chloride | Choline chloride |
| Soybean oil | Ground oats | Choline chloride | DL-methionine | DL-methionine |
| Salt | Choline chloride | Kaolin | Calcium propionate | Calcium propionate |
| Dried beet pulp | Pyridoxine hydrochloride | Magnesium oxide | L-tryptophan | L-tryptophan |
| Pyridoxine hydrochloride | Menadione dimethylpyrimidinol bis | Vitamin E acetate | Vitamin E acetate | Vitamin E acetate |
| Choline chloride | Thiamin mononitrate | Menadione sodium bisulfite complex | Menadione sodium bisulfite complex | Menadione sodium bisulfite complex |
| Menadione dimethylpyrimidinol bis | DL-methionine | Manganous oxide | Manganous oxide | Manganous oxide |
| Thiamin mononitrate | Vitamin A acetate | Ferrous sulfate | Ferrous sulfate | Ferrous sulfate |
| Vitamin A acetate | Silicon dioxide | Zinc oxide | Zinc oxide | Zinc oxide |
| Cholecalciferol | Cholecalciferol | Niacin | Niacin | Niacin |
| Silicon dioxide | Folic acid | Calcium pantothenate | Calcium pantothenate | Calcium pantothenate |
| Dicalcium phosphate | dl-alpha tocopheryl acetate | Copper sulfate | Copper sulfate | Copper sulfate |
| Monocalcium phosphate | Biotin | Pyridoxine hydrochloride | Pyridoxine hydrochloride | Pyridoxine hydrochloride |
| Folic acid | Calcium pentathenate | Riboflavin | Riboflavin | Riboflavin |
| DL-methionine | Riboflavin | Thiamin mononitrate | Thiamin mononitrate | Thiamin mononitrate |
| Biotin | Nicotinic acid | Vitamin A acetate | Vitamin A acetate | Vitamin A acetate |
| dl-alpha tocopheryl acetate | Vitamin B12 supplement | Calcium iodate | Calcium iodate | Calcium iodate |
| Calcium pentathenate | Manganous oxide | Vitamin B12 supplement | Vitamin B12 supplement | Vitamin B12 supplement |
| Riboflavin | Zinc oxide | Folic acid | Folic acid | Folic acid |
| Nicotinic acid | Ferrous carbonate | Biotin | Biotin | Biotin |
| Vitamin B12 supplement | Copper sulfate | Vitamin D3 supplement | Vitamin D3 supplement | Vitamin D3 supplement |
| Manganous oxide | Zinc sulfate | Cobalt carbonate | Cobalt carbonate | Cobalt carbonate |
| Zinc oxide | Calcium iodate | | | |
| Ferrous carbonate | Cobalt carbonate | | | |
| Copper sulfate | Sodium selenite | | | |
| Zinc sulfate | | | | |
| Calcium iodate | | | | |
| Cobalt carbonate | | | | |

Appendix C: Chapter 3 versus Chapter 4 animal facility excluded pathogens

| Pathogen | Chapter 3 | Chapter 4 |
|--|----------------------|-----------------------------|
| | Wesbrook Animal Unit | Centre for Disease Modeling |
| Viruses | | |
| Mouse hepatitis virus (MHV) | excluded | excluded |
| Mouse minute virus (MMV) | excluded | excluded |
| Mouse parvovirus (MPV) | excluded | excluded |
| Theiler's murine encephomyelitis virus (TMEV) | excluded | excluded |
| Rotavirus epizootic diarrhea of infant mice (EDIM) | excluded | excluded |
| Sendai virus | excluded | excluded |
| Pneumonia virus of mice | excluded | excluded |
| Lymphocytic choriomeningitis virus (LCMV) | excluded | excluded |
| Ectromelia virus | excluded | excluded |
| Mouse adenovirus I/II | excluded | excluded |
| Polyoma virus | excluded | excluded |
| Reo3 virus | excluded | |
| Reovirus 2 | | excluded |
| Mouse thymic virus (MTLV) | | excluded |
| Mouse norovirus (MNV) | | excluded |
| Mouse encephalomyelitis virus | | excluded |
| K virus | | excluded |
| Lactic dehydrogenase elevating virus (LDEV) | | excluded |
| Hantaan virus | | excluded |
| Mouse cytomegalovirus (MCMV) | | excluded |
| Bacteria | | |
| Mycoplasma pumonis | excluded | excluded |
| Helicobacter spp. | | excluded |
| CAR bacillus | | excluded |
| Clostridium piliforme (Tyzzer's disease) | | excluded |
| Bordetella bronchiseptica | | excluded |
| Citrobacter rodentium | | excluded |
| Corynebacterium bovis | | excluded |
| Corynebacterium kutscheri | | excluded |
| Pasteurella pneumotropica | | excluded |
| Salmonella spp. | | excluded |
| Streptococcus pneumoniae | | excluded |
| Streptobacillus moniliformis | | excluded |
| Pseudomonas aeruginosa | | excluded |
| B-hemolytic Streptococcus | | excluded |
| Klebsiella pneumoniae | | excluded |
| Parasites | | |
| Pinworm | excluded | excluded |
| Fur mites | excluded | excluded |
| Encephalitozoon cuniculi | | excluded |
| Pneumocystis spp. | | excluded |