A DUALITY IN MAMMALIAN GLUCOCORTICOID SIGNALING

Evidence against the dominant glucocorticoid/ interchangeability assumption

by

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ABSTRACT

I tested a prevalent assumption in glucocorticoid research that states that each species has a dominant glucocorticoid, and cortisol and corticosterone are interchangeable steroids. A comprehensive analysis of historical and current data failed to support this assumption and revealed evidence of drift away from exploration of cortisol and corticosterone as dual, important adrenal products to the exclusive quantification of one, dominant glucocorticoid. Originating approximately 30 years ago, the dominant glucocorticoid/ interchangeability assumption is now portrayed in textbook images used to represent adrenal steroid biosynthesis and is widespread throughout empirical research. Less than 1% of over 50,000 published papers relating to the glucocorticoids have considered the potential for independence in glucocorticoid signaling by quantifying both cortisol and corticosterone within a sample. A dispersed literature shows independent regulation of cortisol and corticosterone, extensive inter-species variation in glucocorticoid concentrations and cortisol: corticosterone ratios and adrenal synthesis of the non-dominant glucocorticoid during early development. We hypothesize that there is a functional duality in glucocorticoid signaling and use mass spectrometry to explore the glucocorticoid profile of the full-term human (n = 125) and guinea pig (n = 28) fetus (both cortisol-dominant species). The sample preparation method yielded poor steroid recoveries (~ 4-28%), which made quantification by mass spectrometry challenging, but in both species corticosterone concentrations were significantly higher in fetal blood compared to umbilical venous or umbilical mixed blood ($p < 0.0001$), suggesting fetal corticosterone enrichment. Within an individual, cortisol was not an accurate predictor of corticosterone for either species (human, $r = 0.001$, $p > 0.05$; guinea pig, $r = 0.14$, $p > 0.05$) and our data suggests independent glucocorticoid responses; in humans, cortisol

was significantly higher in vaginal deliveries relative to elective Caesarian sections ($p < 0.0001$) but corticosterone was unaffected. Guinea pig fetal corticosterone was not affected by daily maternal stress during gestation but cortisol was significantly lower in stressed fetuses ($p < 0.05$). While these preliminary data require further investigation, we conclude that fetuses from the human and guinea pig actively secrete the non-dominant glucocorticoid in late gestation and suggest that there is a functional duality in glucocorticoid signaling.

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CHAPTER 1: General introduction

Cortisol and corticosterone, collectively known as the glucocorticoids (GCs), are steroid hormones produced in the cortex of the adrenal gland (in mammals) or in the adrenocortical tissue (in lower vertebrates). The GCs have been under investigation since the 1950s and are now known to exert a wide variety of genomic and non-genomic actions (Lösel & Wehling 2003). These steroids are critical for survival and a list of GC actions include (but is certainly not limited to) glucose synthesis (Exton *et al.* 1972), cellular maturation and differentiation (Piemonti *et al.* 1999), fat breakdown (Exton *et al.* 1972), circadian rhythm regulation (Veldhuis *et al.* 1989), energy metabolism (McMahon *et al.* 1988), immune response (Besedovsky *et al.* 1975) and neural excitability (Abraham *et al.* 1996). The GCs are best known for their role in the stress response. Upon exposure to a physical or psychological stressor, the GCs act to modulate an organism's response to the stressor and help the body to regain homeostasis soon after. The GCs can also alter an organism's response to a subsequent stressor and help an individual cope with chronic stress (Sapolsky *et al.* 2000). Overexposure to GCs or irregular GC secretion can make an organism more susceptible to disease (Corcoran *et al.* 2003) and can lead to a number of maladies (Addison 1855; Cushing 1932; Woolley *et al.* 1990, Allen 1996).

Synthesis of cortisol and corticosterone is controlled by way of the hypothalamic-pituitary-adrenal (HPA) axis. Corticotropin-releasing hormone (CRH) gets released from the hypothalamus, travels to the pituitary gland and binds to anterior pituitary receptors to trigger the release of adrenocorticotropic hormone (ACTH) into systemic circulation. The binding of ACTH to the adrenal cortical receptors then initiates the synthesis and secretion of cortisol and/ or corticosterone.

Both cortisol and corticosterone are produced by most (if not all) vertebrate species but in most vertebrate species, one of the two GCs is produced in a higher concentration and this GC is commonly referred to as the 'dominant', 'principal' or 'major' glucocorticoid. Cortisol is the dominant GC in the majority of mammals whereas corticosterone is the dominant GC in lizards and birds.

Historical comparisons of GC secretion and function among cortisol- and corticosterone-dominant species have noted that the two hormones showed similar responses to various experimental manipulations and often changed concurrently. This led to the idea that the dominant GC would provide a measure of total GC activity when designing and interpreting empirical studies. The GCs were considered interchangeable. Measuring only the dominant GC has since been a universally accepted practice in glucocorticoid research. However, this dominant GC/ interchangeability assumption has never been explicitly tested and there is little empirical evidence to support it. The following thesis presents a comprehensive review of the literature, and an empirical study in two mammalian species, which collectively suggest that the GCs are independently regulated.

CHAPTER 2: Background and literature review

2.1. Introduction

Cortisol and corticosterone are steroid hormones that are assumed to be physiologically interchangeable such that some species secrete corticosterone, whereas other species secrete cortisol, but either cortisol or corticosterone can manage all of the same physiological processes. Although most (if not all) vertebrate species secrete a combination of both GCs, the dominant GC is regularly used as a proxy for endogenous GC activity. For example, researchers studying reptiles would, as a matter of course, report corticosterone values (French *et al.* 2008), as would avian researchers (Bonier *et al.* 2007). On the other hand, human studies (Buchanan *et al.* 2009), like all studies with non-human primates (Ziegler *et al.* 1996), report cortisol. Even within laboratory rodents, there are differences, with rats and mice reputed to be unable to synthesize cortisol (Mathieu *et al.* 2002), and hamsters (*Mesocricetus*) reputed to secrete predominantly cortisol (Dunlap & Grizzle, 1984; Ronchi *et al.* 1998; Mathieu *et al.* 2002), so that initial research on dwarf hamsters (*Phodopus*) explicitly compared the relative concentrations of corticosterone and cortisol, to establish the 'dominant' glucocorticoid for that species, before proceeding to measure cortisol (Reburn & Wynne-Edwards, 1999). When the intent of the study is to use an animal to model aspects of human responses to stress, or the role of stress in the etiology of human disease, this assumption often becomes explicit with a 'reminder' that the study of corticosterone in rats is functionally equivalent to the study of cortisol in humans. As a typical example, Khaksari et al. (2007) start their abstract with "Previous studies indicated that levels of glucocorticoids (cortisol in humans; corticosterone in rats) induce impairment of long-term memory…".

Foremost among the *a priori* reasons why this 'either cortisol or corticosterone' assumption is unlikely to be valid is the plausibility of any hypothesis that requires natural selection to render either cortisol or corticosterone redundant, and remove it from biological signaling pathways. The steroid biosynthetic pathway is ancient, and the homology of glucocorticoid receptors has been stretched back to include our common ancestor with the octopus, as well as with cartilaginous fishes (Carroll *et al.* 2008). In addition, corticosterone is the substrate for the synthesis of aldosterone, and is therefore going to be synthesized in the course of aldosterone synthesis, even in species with cortisol 'dominance'. Both cortisol and corticosterone also bind to both glucocorticoid and mineralocorticoid receptors (Sutanto & de Kloet 1987), so each has the potential to stimulate or antagonize receptor-mediated actions of the other.

Of course, this assumption of either/or exclusivity is not unique to cortisol and corticosterone. Until recently, endocrinologists would never have measured estrogens in samples from men unless they were growing unwanted breast tissue or had presented with breast cancer. Now, the early stages of comparisons across species have revealed high levels of natural estrogens in stallions (Claus *et al.* 1992), alligators (Guillette *et al.* 1999), and dwarf hamsters (Schum & Wynne-Edwards 2005), as well as changes in estrogen concentration associated with fatherhood and pair-bonding in men (Berg & Wynne-Edwards 2001). The same is true for testosterone in women. Once associated with unwanted facial hair, there is now an extensive literature on effects on pubertal development (Bond *et al.* 2006), libido (van Anders *et al.* 2007), and natural ability to mentally rotate three-dimensional objects (Alexander & Son 2007). Thus, the assumption that any species would reject the opportunity to signal through corticosterone in favor of signaling through cortisol (or *vice versa*) is as unlikely to be supported as the old concept

of sex-specificity in sex steroid actions has proven to be.

2.2. Pervasiveness of the dominant glucocorticoid assumption

Using the 'Web of Science' online database to perform a search for articles with either cortisol or corticosterone yields an extraordinary 53,000 published documents. Of those, only 3% remain if the search is repeated with 'or' replaced by 'and'. In 2007, the total number of articles was 2,825 and 70 (2.5%) remained. However, there were a lot of different reasons for both cortisol and corticosterone to be keywords in a search, very few of which related to simultaneous quantification of cortisol and corticosterone in the same biological sample. An in depth examination of these 70 papers revealed that only 3 (0.1%) were in fact, empirical studies that measured both glucocorticoids within the same sample – and they were mass spectrometry studies exploring the broad hormonal signatures of adrenal carcinomas. The other 67 publications were reviews that covered different species, or basic research that inferred the implications of research in rats and mice for their validity as models for human health. Thus, around 1 in 1000 published papers with cortisol or corticosterone as a keyword actually consider the possibility that it is important to measure more than one of them, and those papers are not concerned with the stress response. Based on this extraordinary prevalence, the 'either/or' assumption is pervasive. Indeed, the dominant glucocorticoid hypothesis is so pervasive that, although, the golden hamster clearly secretes approximately equal amounts of cortisol and corticosterone (Albers *et al.* 1985; Ottenweller *et al.* 1985; Huhman *et al.* 1990; Kollack-Walker *et al.* 1997), it is still referred to as cortisol-dominant (Dunlap & Grizzle, 1984; Ronchi *et al.* 1998; Wommack *et al.* 2004).

2.3. Historical comparative approaches

Thomas Addison (1855) was the first to show that the adrenal glands were physiologically important and, within a year, Brown-Séquard (1856) established that dogs would not survive adrenalectomy. Over 70 years passed before a purified extract of the adrenal cortex was used to prolong the life of adrenalectomized cats (Hartman *et al.* 1927; Swingle & Pfiffner 1931), which led to synthesis (Reichstein 1936), isolation (Mason *et al.* 1937, Mason *et al.* 1938; Steiger & Reichstein 1938) and the pharmaceutical use of glucocorticoids (cortisol) as anti-inflammatory treatment for rheumatoid arthritis (Hench *et al.* 1949).

Soon, chromatographic methods uncovered considerable diversity from species to species in the ratio of Compound F (cortisol) to Compound B (corticosterone) with the rat showing a two-fold bias towards corticosterone (ratio =0.5) and the rhesus macaque a 20 fold bias towards cortisol (ratio = 20) (Bush 1953). The author concluded that: "Compounds F and B have essentially the same function in these species during 'stress', but are secreted in a ratio determined by the biochemical properties of the adrenal cortex." Although the ratio of cortisol to corticosterone (F: B ratio) was not easily predicted by mode of life or dietary habits, it was possible to classify species as 'F' or 'B' secretors. Over the next 25 years, commonalities between the physiological secretion patterns of cortisol and corticosterone in 'F' and 'B' secretors accumulated with evidence that both responded to ACTH stimulation with a surge and subsequent recovery (Peterson 1957; Gwazdauskas *et al.* 1972), and both had an endogenous daily rhythm with a peak after awakening (Peterson 1957; Turner 1984). Gradually, the unanswered question about why some species secreted more cortisol and others more corticosterone gave way to the valuable insight that the dominant glucocorticoid was a useful proxy measure for an

individual's response to stress. From that point, it was only a short step to ignoring the non-dominant glucocorticoid completely, although neither interchangeability, nor exclusivity, had been conclusions from the research.

2.4. The dogma of textbooks

In an opportunistic survey of textbooks available in the Queen's University library system (N=34) three expected trends were confirmed. First, the textbooks depicting the adrenal steroid biosynthesis pathway in the Medical library overwhelmingly ignored corticosterone (10/11 = 91%) except as a substrate for aldosterone synthesis, whereas 74% of the 23 textbooks in the Engineering & Science library presented an illustration representing, and highlighting, cortisol and corticosterone as separate, but equal (Figure 2.1). This was an anthropocentric perspective, as 15 of 20 textbooks with a stated human focus failed to label or highlight corticosterone. There was also some evidence for historical change in this schematic representation with 6 of 7 textbooks published prior to 1980, including one from the medical library, balancing cortisol and corticosterone equally (Table 2.1).

2.5. 'Rats and mice lack the enzyme to synthesize cortisol'

Textbooks are not the only source of unchallenged dogma. Researchers that report corticosterone concentrations in rat serum or plasma often state that rats and mice are unable to synthesize cortisol due to a lack of 17α -hydroxylase activity in the adrenal gland. One paper, with a lifetime citation record of 80 (van Weerden *et al.* 1992) is most often cited to support this type of statement. However, in that study, the absence of the enzyme was inferred from low plasma levels of cortisol, 17-hydroxyprogesterone and

androstenedione that all require 17α -hydroxylase in their biosynthesis pathway. Other cited references for the lack of this enzyme in the adrenal cells of rats (Namiki *et al.* 1988; Fevold *et al.* 1989) and mice (Youngblood & Payne 1992), have worked with testicular rather than adrenal cortical cells. On the other hand, 17α-hydroxylase activity has been identified in rat and mouse adrenals *in vitro* (Touitou *et al.* 1990), and the adrenal glands of late gestation mouse embryos express this enzyme (Keeney *et al.* 1995). Several studies have also reported cortisol concentrations in the plasma of rats (Milanés *et al.* 1991; Pignatelli *et al.* 2006; Zhao *et al.* 2007; Mirunalini & Subramanian 2008) and mice (Knight *et al.* 2007). Thus, even if some strains of rat do not synthesize cortisol, it is likely that ancestral wild rats do synthesize cortisol in the adrenal cortex, at least at some points in the lifespan (Amirat *et al.* 1980).

2.6. The dominant glucocorticoid assumption

Thus, there are two distinct inferences that have been collectively drawn from the historical research and constitute the dominant glucocorticoid assumption: 1) the dominant glucocorticoid is the only important signal and 2) cortisol and corticosterone are functionally interchangeable, depending on the species. These two inferences are linked because evidence of a dominant glucocorticoid led to a focus on commonalities across species, which led to the concept of interchangeability, and the supposition of interchangeability then fueled the assumption that only the dominant glucocorticoid was important. However, the original research claims only that both glucocorticoids are responsive to an imposed stressor, have a similar circadian rhythm, and vary widely in dominance across species with no obvious pattern.

2.7. Cortisol and corticosterone dominance across species

Table 2.2 summarizes a literature review of studies that measured both cortisol and corticosterone in plasma or serum from the same individuals. The original intent was to include the results of fecal glucocorticoid metabolite studies as there are well-established links between adrenal activity and fecal GC metabolites across many species (mice (Good *et al.* 2003), voles (Harper & Austad 2000), ground squirrels (Mateo & Cavigelli 2005), cats (Graham & Brown 1996), sea lions (Mashburn & Atkinson 2004), cows (Morrow *et al.* 2002) and primates (Boinski *et al.* 1999)). However, except for one report that distinguished cortisol and corticosterone metabolites (Young *et al.* 2004), fecal metabolite studies assumed that the fecal metabolites represented the sum of all glucocorticoids, and validated their approach through positive association with circulating levels of the dominant glucocorticoid.

The key feature of Table 2.2 is the species-to-species diversity. The ratio of cortisol to corticosterone is not shared, the sum of cortisol plus corticosterone is not conserved, and different researchers get different values. This is not surprising since immune-based assays differ from lab to lab and the glucocorticoids are known for their changes in concentration in response to stress, which includes handling and anesthesia. However, Table 2.2 does not represent a systematic survey of the mammals, or even a random survey of the 5,400 recognized mammalian species (Wilson & Reeder 2005). It also does not reflect the number of studies that failed to measure a second glucocorticoid because those would often not be published. Nevertheless, it is clear from these 46 species that there are species with cortisol dominance (N=35), species with corticosterone dominance (N=6), and species where the ratio was close to 1 (N=4). Those four were the golden hamster (Kollack-Walker *et al.* 1997), domestic cat (Henkin *et al.* 1968), white

rhinoceros (Turner *et al.* 2002) and ferret (Rosenthal *et al.* 1993), which are not close relatives. The koala, on the other hand, was reported as cortisol dominant based on a sample from a free-living individual (Weiss & Richards 1970) and corticosterone dominant based on a group of 7 captive individuals (Oddie *et al.* 1976).

2.8. Potential mechanisms for duality in signaling

2.8.1. Receptor evolution. The phylogeny of the glucocorticoid and mineralocorticoid receptors has recently been deduced over a 600 million year evolutionary history (elasmobranchs and octopus; Carroll *et al.* 2008, Bridgham *et al.* 2008) focused upon interactions of the ancestral receptors with response elements in the DNA sequence before their interaction with the steroid ligand, pre-adaptation of the structural binding site of the receptor prior to the evolution of the enzyme pathway to synthesize the modern ligand, and subsequent differentiation in affinity and specificity (Bridgham *et al.* 2006; Ortlund *et al.* 2007). In these studies, multiple glucocorticoids have high and equal affinity for ancestral receptors and high cross-reactivity to the non-dominant glucocorticoid is seen at the mineralocorticoid receptor (Ortlund *et al.* 2007).

2.8.2. Mineralocorticoid versus glucocorticoid receptor binding affinity. Both cortisol and corticosterone bind to mineralocorticoid as well as glucocorticoid receptors. Both types of receptors are expressed in diverse tissues, with mineralocorticoid receptors found in the cell nucleus in the presence or absence of a steroid whereas the glucocorticoid receptors are tethered in the cytoplasm until steroid binding occurs and the steroid-receptor complex can be translocated to the nucleus. The receptors share 57% homology in the steroid binding domain and 94% homology in the DNA binding domain (Stewart

2007), and thus both receptor types bind cortisol and corticosterone as well as aldosterone and synthetic glucocorticoids such as dexamethasone and betamethasone.

Notably, the binding affinity of the mineralocorticoid receptor for either cortisol or corticosterone is ten-fold higher than the affinity of the glucocorticoid receptor for either cortisol or corticosterone (Reul & de Kloet 1985). This preferential binding to the mineralocorticoid receptor has been replicated in the brains of hamsters and rats (Sutanto & de Kloet 1987), pigs (Perreau *et al.* 1999), sheep (Richards & Keller-Wood 2003), non-human primates (Brooke *et al.* 1994), and humans (Rupprecht *et al.* 1993). In fact, glucocorticoids are also now emerging as important modulators of mineralocorticoid receptors in diverse tissues and contexts where aldosterone had been assumed to be the only available ligand (Odermatt & Atanasov 2009). Pure pharmacological antagonists of the glucocorticoid receptor have also proven elusive, with candidate agents tending to interact with other steroid receptors, in diverse tissues, with broad affinity rather than specificity (Pecci *et al.* 2009).

2.8.3. Competitive binding of cortisol and corticosterone on the same receptors. Few studies have looked at differential binding of cortisol and corticosterone to the same receptors. In one early study, there was a correlation with the dominant glucocorticoid because lung receptors in cortisol-dominant humans and guinea pigs bound cortisol with higher affinity than corticosterone whereas corticosterone-dominant rat and mouse pulmonary receptors bound cortisol with higher affinity (Giannopoulos & Keichline 1981). However, in three cortisol-dominant species the glucocorticoid receptor had similar binding affinity for each whereas the mineralocorticoid receptor preferred cortisol in the golden hamster, corticosterone in the dog, and corticosterone in the rat (Sutanto &

de Kloet 1987; Reul *et al.* 1990). Of course, the assumption that the golden hamster was a cortisol-dominant species was also flawed. Other than these studies, and the evolutionary reconstructions, receptor binding is studied within the framework of the dominant glucocorticoid assumption, and the non-dominant glucocorticoid is not used as a ligand.

2.8.4. Implications of different receptor isoforms. Receptors also have different isoforms that are likely to have differential binding affinities for cortisol and corticosterone. Two isoforms of the human glucocorticoid receptor (hGRα and hGRβ) are known (Hollenberg *et al.* 1985), are ubiquitously expressed (Bamberger *et al.* 1995; Oakley *et al.* 1996; Oakley *et al.* 1997) and have similar cellular localization (deCastro *et al.* 1996). At first (Hollenberg *et al.* 1985; Giguère *et al.* 1986), evidence suggested that GRβ was unable to bind natural or synthetic glucocorticoids and was transcriptionally inactive but this has recently been challenged (Lewis-Tuffin *et al.* 2007; Kino *et al.* 2009). Even without considering any functional role for the non-dominant glucocorticoid, there is considerable complexity in glucocorticoid receptor function with independent actions, sub-type interactions, and heterodimerization implicated in pathological glucocorticoid resistance as well as normal cellular signaling (Hecht *et al.* 1997; Otto *et al.* 1997; Hamid *et al.* 1999; Chikanza 2002; Hauk *et al.* 2002). The studies have, of necessity, also focused on a small set of animal models that cannot yet be used to generalize to signaling pathways in other species.

2.9. Evidence for independent regulation of cortisol and corticosterone

Although scattered in the literature, there is evidence that cortisol and corticosterone are

independently regulated within individuals. Thus, within an individual they are not interchangeable in spite of the possibility that cortisol and corticosterone have homologous roles in 'F' and 'B' secretors. However, more systematically collected data are clearly needed. All of these studies use different methods and could be profoundly altering the secretion of cortisol relative to corticosterone as a result of those methods. For example, post surgical cortisol/ corticosterone ratio in guinea pigs was 204 when measured by indwelling cannula and 30 when measured by cardiac puncture (Malinowska & Nathanielsz 1974).

2.9.1. Circadian and seasonal variation. In spite of the early focus on similarities of circadian secretion for cortisol and corticosterone across species (Dixit & Buckley 1967; Akerstedt & Levi 1978) there is mixed support for the hypothesis that the two glucocorticoids change in tandem within individuals. Studies in big-horn sheep (Turner 1984) and humans (Peterson 1957) found parallel patterns whereas pigs showed independent cortisol and corticosterone rhythms (Bottoms *et al.* 1972) and the golden hamster has a bias towards corticosterone in the early hours of the light phase and cortisol thereafter (Albers *et al.* 1985). In contrast, however, other researchers describe cortisol dominance throughout the day with a corticosterone increase in the evening hours (Sutanto & de Kloet 1987). Both glucocorticoids were tracked throughout the year in sand rats, a rodent with cortisol dominance, and patterns were similar with the exception of a decrease of cortisol in December and January that was not seen for corticosterone (Amirat *et al.* 1980).

2.9.2. Responses to ACTH stimulation and imposed stress. As seen in the circadian

comparisons, results are often contradictory about whether comparative results for cortisol and corticosterone in the same samples are similar, or divergent. In ferrets, which do not have a dominant glucocorticoid, cortisol and corticosterone increase together in response to ACTH stimulation (Rosenthal *et al.* 1993). However, cortisol-dominant humans had a stronger corticosterone reaction (Nishida *et al.* 1977) whereas cortisol-dominant rusa deer had equal responses (van Mourik *et al.* 1985), and results in cows were completely opposite in two studies (Venkataseshu & Estergreen 1969; Gwazdauskas *et al.* 1972). Similarly, in the golden-mantled ground squirrel, cortisol increased more drastically than corticosterone in response to stress induced by capture (Romero *et al.* 2008).

In response to chronic, rather than acute, ACTH administration, corticosterone-dominant rabbits switch to become cortisol-dominant (Kass *et al.* 1954; Ganjam *et al.* 1972) or at least increase the cortisol/ corticosterone ratio (Krum & Glenn 1965). This is possibly a physiological trait unique to rabbits, as they also switch from cortisol-dominance to corticosterone-dominance as they develop (Mulay *et al.* 1973). However, fetal secretion of the non-dominant glucocorticoid might be the rule, rather than the exception.

2.9.3. Pregnancy and fetal secretion of the non-dominant glucocorticoid. In general, both cortisol and corticosterone, when both have been measured, tend to increase across pregnancy. This is true for humans (Wintour *et al.* 1978) and for the little brown bat (Reeder *et al.* 2004) although there is also evidence that cortisol and corticosterone diverge after the birth with species as different as the variable flying fox (Reeder *et al.* 2004), the degu (Kenagy *et al.* 1999), and the chipmunk (Kenagy & Place 2000).

However, it is likely that these patterns are explained by contributions from the fetal adrenal. As early as 1971, significant newborn corticosterone synthesis (through urinary analysis of metabolites of injected glucocorticoid isotopes including corticosterone) was established (Hall *et al.* 1971). Comparison of pregnancies with a normal or an anencephalic fetus confirmed that corticosterone of late pregnancy was of fetal origin (Oakey *et al.* 1977). Corticosterone remained in fetal peripheral circulation after the birth but was slowly replaced by newborn cortisol (Sippell *et al.* 1978). A decade later, longitudinal samples from pregnant women showed a gradual doubling of corticosterone across weeks 10-38 of pregnancy and an increase of 250% between week 38 and admission for delivery (Dörr *et al.* 1989).

However, the dominant glucocorticoid assumption was strong, and this corticosterone was not considered biologically important relative to the maturation of the fetal cortisol system. Instead, the tone reflected an influential Nature paper in 1980 (Fencl *et al.* 1980), which assumed that cortisol was the critical fetal response to initiate labour but argued that maternal cortisol secretion obscured this effect. That paper highlighted fetal production of corticosterone in a pregnancy without maternal adrenals, but then argued that fetal corticosterone was a proxy for the obscured cortisol response indicated by concurrent developmental hypertrophy and differentiation of the adrenal cortex. Since that time, corticosterone has not been quantified in the mother or fetus in human research.

2.10. Conclusion

There is a measurement gap in our understanding of the potential for independent biological signaling by cortisol and corticosterone within an individual. Glucocorticoid research, from the level of the gene to the level of domestication processes, has accepted

the dominant glucocorticoid assumption and generalized it as a rule, focusing all measurement on the 'expected' glucocorticoid. Recent lessons about estrogen effects in males and testosterone effects in females, as well as emerging evidence that less-studied steroids such as dihydrotestosterone and dehydroepiandrosterone are important in disease etiology, and clear evidence that steroids of abuse have wide-ranging effects, all urge caution before making any assumption about the absence of biological effects of steroids. Duality in glucocorticoid signaling could be restricted to one or more specific windows in development when, for example, efficient maternal-fetal bidirectional communication about stress status could be adaptive for both. It could also be ubiquitous, and undetected, because research has tended to focus on males and has not compared cortisol and corticosterone secretion patterns within an individual over critical biological transitions such as pair-bond formation, territory establishment, or social defeat. However, it is not plausible that natural selection rendered either cortisol or corticosterone redundant, or non-functional, in different vertebrate phylogenies.

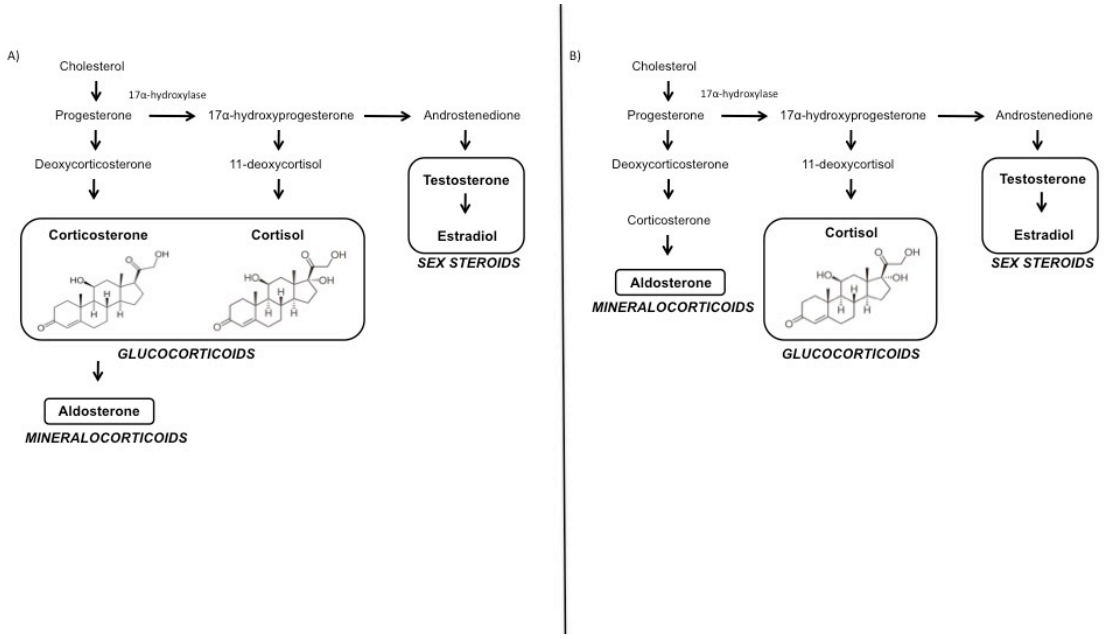

Figure 2.1. Textbooks that contain a diagram of the adrenal steroidogenesis pathway represent the glucocorticoids in one of two ways; A) cortisol and corticosterone are presented as equally important, balanced end products of the synthesis pathway or B) cortisol is presented as the only glucocorticoid while corticosterone is left unmentioned and is buried along the pathway to aldosterone.

Table 2.1. Classification of textbooks in the Queen's University library system

TEXTBOOK TITLE	AUTHOR(S)/ EDITOR(S)	PUBLICATION YEAR	SOURCE LIBRARY	COMPARATIVE/ HUMAN	BALANCED REPRESENTATION?
Animal Physiology	Scheer	1963	Science	Comparative	Yes
Animal Physiology (2nd ed.)	Hill & Wyse	1989	Medical	Comparative	No
Basic Medical Endocrinology (4th ed.)	Goodman	2009	Medical	Human	No
Biochemistry, Genetics & Embryology	Vargas, Caughey, Tan, Li	2004	Medical	Human	No
Biochemistry of Steroid Hormones	Makin	1975	Science	Human	Yes
Clinical Endocrine Physiology	Hedge, Colby, Goodman	1987	Medical	Human	No
Comparative Animal Physiology	Withers	1992	Medical	Comparative	Yes
Comparative Endocrinology	Gorbman	1958	Science	Comparative	Yes
Comparative Vertebrate Endocrinology (2nd ed.)	Bentley	1982	Science	Comparative	Yes
Endocrine Physiology	Balint Kacsoh	2000	Medical	Human	Yes
Endocrinology (2nd ed.)	Hadley	1988	Science	Comparative	Yes
Essential Endocrinology (3rd ed.)	Brook & Marshall	1996	Medical	Human	No
General Endocrinology	Turner	1966	Science	Comparative	No
Hormones and Evolution	Barrington	1964	Science	Comparative	Yes
Hormones and Their Actions (part 1)	Cooke, King, van der Molen	1988	Medical	Comparative	No
Hormones (from molecules to disease)	Baulieu & Kelly	1990	Medical	Human	No
Human Biochemistry (10th ed.)	Orten & Neuhaus	1982	Medical	Human	No

Introduction to Behavioral Endocrinology	Nelson	1995	Medical	Comparative	Yes
Introduction to Neuroendocrinology	Brown	1994	Medical	Human	Yes
Mammalian Neuroendocrinology	Bennett & Whitehead	1983	Medical	Comparative	Yes
Maternal-Fetal Endocrinology (2nd ed.)	Tulchinsky & Little	1994	Medical	Human	No
Metabolic and Endocrine Physiology	Tepperman	1962	Science	Human	Yes
Molecular Endocrinology- Basic concepts and clinical correlations	Weintraub	1995	Medical	Human	No
Pediatric Endocrinology (3rd ed.)	Sperling	2008	Medical	Human	No
Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases (2nd ed.)	Blau, Duran, Blaskovics, Gibson	2003	Medical	Human	No
Principles of Animal Physiology (2nd ed.)	Moyes & Schulte	2008	Comparative	Science	Yes
Principles of Endocrine Pharmacology	Thomas & Keenan	1986	Human	Medical	No
Principles & Practice of Endocrinology and Metabolism	Becker	1990	Human	Medical	Yes
Textbook of Biochemistry with Clinical Correlations (6th ed.)	Devlin	2006	Medical	Human	No
Textbook of Comparative Endocrinology	Gorbman & Bern	1962	Science	Comparative	Yes

Textbook of Endocrine Physiology (3rd ed.)	Griffin & Ojeda	1996	Medical	Human	No
Vertebrate Endocrinology (3rd ed.)	Norris	1997	Science	Comparative	Yes
William's Textbook of Endocrinology (11th ed.)	Kronenberg, Melmed, Polonsky, Larsen	2008	Medical	Human	No
Yen & Jaffe's Reproductive Endocrinology	Yen & Jaffe	2004	Medical	Human	No

Table 2.2. Plasma or serum glucocorticoid concentrations in mammalian species that have had cortisol and corticosterone measured in the same sample.

Order	Species	Sex	Cortisol (ng/ml)	Corticosterone (ng/ml)	Dominant	Notes	Reference
Artiodactyla	*Bos taurus* (cow)	Female	30-120	20-40	Cortisol	Lactating, non-pregnant	Venkataseshu & Estergreen 1969
		Female	1-10	0.5-1.5	Cortisol	Lactating, non-pregnant	Gwazdauskas *et al* 1972
		Male	10-35	2-8	Cortisol	Agitated; difficult to bleed	Rhynes & Ewing 1973
	Cervus rusa timorensis (rusa deer)	Male	5-15	1-5	Cortisol	Baseline samples	van Mourik *et al* 1985
	Ovis canadensis cremnobates (desert big-horn sheep)	Both	20-40	0.5-2.5	Cortisol	Captive, bled using indwelling cannula	Turner 1984
		Both	40-60	0.5-2.5	Cortisol	Free-ranging, bled using indwelling cannula	
	Ovis aries (sheep)	Female	< 500	20-50	Cortisol	Adrenal venous outflow (n = 1)	Bush & Ferguson 1953
	Sus scrofa domestica (pig)	Female	5-15	1-4	Cortisol	Measured using indwelling cannula; circadian variation	Akerstedt & Levi 1978
Carnivora	*Eumetopias jubatus* (Stellar sea lion)	Both	50-100	0-100 ng/g (fecal)	Cortisol	Captive, trained to permit blood sampling	Mashburn & Atkinson 2004

	Halichoerus grypus (gray seal)	Male	330-354	46-82	Cortisol	Free-ranging, shot, January (possibly breeding) (*n* = 2)	Sangalang & Freeman 1976
	Felis catus (cat)	Both	10-20	8-12	Both	Bled using indwelling cannula	Henkin *et al* 1968
	Canis familiaris dingo (dingo)	Both	0-40	10-55	Cortisol	Captive	Oddie *et al* 1976
	Mustela putorius furo (ferret)	Both	1-10	0.5-16	Both	Spayed/ neutered; bled while manually restrained	Rosenthal *et al* 1993
Cetacea	*Tursiops truncates* (bottlenose dolphin)	Both	10-60	2-18	Cortisol	Free-ranging, captured	Ortiz & Worthy 2000
		Both	10-41			Free-ranging, captured	St Aubin *et al* 1996
		Both	5-40			Semidomesticated	St Aubin *et al* 1996
Chiroptera	*Myotis lucifugus* (little brown bat)	Male	100-300	5-15	Cortisol	Measured through active period (May-Sept)	Reeder *et al* 2004
		Female	100-600	5-20	Cortisol	Throughout pregnancy & lactation	

	Pteropus hypomelanus (variable flying fox)	Male Female Both	700-1200 500-1000 1000-1500	100-300 100-300 400-700	Cortisol Cortisol Cortisol	Captive, measured in March (after mating) Captive, measured in March (during early pregnancy) Captive, measured in August (non-breeding animals) (sample time ~15min)	Reeder *et al* 2004 Widmaier & Kunz 1993
	Pteropus vampyrus (large flying fox)	Both	500-700	50-150	Cortisol	Captive, measured in August (non-breeding) (sample time ~15min)	Widmaier & Kunz 1993
Didelphimorphia	*Didelphis virginiana* (Virginia opossum)	Both	400-800 ng/min	75-125 ng/min	Cortisol	Adrenal venous outflow; surgically stressed	Johnston *et al* 1967
Lagomorpha	*Oryctolagus cuniculus* (New Zealand white rabbit)	Male	2-15	10-50	Corticosterone	Baseline (<5min)	Szeto *et al* 2004
Marsupialia	*Phascolarctos cinereus* (koala)	Male Male	63 10-20 (Not detected in 7/9 animals)	5 1-3	Cortisol Corticosterone	Free-ranging; anesthetized (n = 1) Captive (n = 7)	Weiss & Richards 1970 Oddie *et al* 1976
	Dasyurus geoffroyii (Western native cat)	Both	10-25	0.5-3	Cortisol	Captive	Oddie *et al* 1976

CHAPTER 3: Non-dominant glucocorticoid secretion by the term fetus is weakly related to dominant glucocorticoid secretion; a comparative study with the human and guinea pig

3.1. Introduction

There are two glucocorticoids (GCs) secreted by the mammalian adrenal cortex: cortisol and corticosterone. Some species secrete cortisol predominantly, whereas others secrete corticosterone, and it has long been assumed that the dominant GC is the only important endocrine signal. However, a recent review (Hancock & Wynne-Edwards, Chapter 2) has illustrated that all mammals examined secrete both GCs and there is little evidence to support the dominant GC assumption. The GCs do not always change in tandem, cortisol and corticosterone often exhibit independent responses and divergent patterns of secretion, and the dominant GC exhibits a wide variation across individuals of the same species (Guimont & Wynne-Edwards 2006).

For the last 30 years, common practice in steroid research has been to use the dominant GC as a representative measure of endogenous GC activity because it was assumed that the GCs were interchangeable. This drift away from exploring cortisol and corticosterone as dual, important GCs has led to a large measurement gap surrounding non-dominant GC signaling. This disparity is likely due, in part, to limitations with existing measurement techniques because antibody-based methods lack the specificity necessary for the accurate measurement of two structurally similar compounds that vary up to 10-fold (and even 100-fold) in concentration. However, mass spectrometry is an emerging technology that affords superior sensitivity and precision to allow for reliable measurement of both GCs within many biological samples.

The literature review by Hancock & Wynne-Edwards identified a number of examples of adrenal synthesis of the non-dominant GC during fetal development. Fetal rabbits are cortisol-dominant and then switch to a corticosterone-dominant profile in adulthood (Obenberger *et al.* 1971) and the human fetus increases non-dominant GC secretion, but not dominant, in response to a stressor at mid-gestation (Partsch *et al.* 1991). Fetal lambs increase corticosterone output throughout the course of gestation to surpass maternal non-dominant GC levels (Alexander *et al.* 1968) and mouse embryos display a late gestational peak in adrenal 17α-hydroxylase activity (the enzyme needed for cortisol production) (Knight *et al.* 2007). The biological relevance of non-dominant GC signaling has never been considered in relation to fetal maturation but the functional significance of non-dominant GC secretion during the prenatal period is an important issue. Antenatal GC therapy is a widely used, valuable component of obstetric care but can lead to a variety of negative effects on the fetus (Murphy 2008). The mechanisms behind these effects are unknown and no current studies consider the non-dominant GC signal to be a relevant signal.

With this study, mass spectrometry is used to quantify the GC concentrations of the full-term human and guinea pig fetus. Both of these species are cortisol-dominant and thus the hypothesis predicts that; 1) the term fetus secretes the non-dominant GC (in this case, corticosterone), as well as the dominant GC (cortisol), 2) maternal stress results in cortisol enhancement in venous circulation whereas fetal distress results in corticosterone enhancement in arterial circulation, and 3) corticosterone and cortisol responses are independent.

3.2. Methods

3.2.1. Human

From Dr. Heather Edwards, OB/GYN at Rockyview General Hospital (Calgary, Alberta), we obtained archival matched blood samples from the umbilical artery and umbilical vein of 125 singleton births collected in 2008. These samples are routinely collected at delivery to assess the newborn condition by analysis of pH, blood gases and fetal acid-base levels (known as base excess). Collectively, these measures provide information about presence or absence of acidosis and whether the cause is respiratory or metabolic. Worsening acidosis (i.e. lower base excess values) is associated with higher risk of respiratory distress and/ or central neurologic injury so base excess measurements allow for more planned, prompt treatments. Umbilical venous blood represents a steroid mixture that originated in maternal circulation and is on route to fetal circulation after passing through the placenta. Arterial blood from the cord carries blood derived from the fetus that is travelling to the placenta. Correct umbilical vessel assignment was identified with analysis of blood gases (venous blood is more oxygenated than arterial blood). Whole blood samples were centrifuged and serum was separated and stored at -20C until hormone analysis.

It has been established that delivery by elective C-section is less stressful for the fetus than normal vaginal delivery and emergency C-section following labour is implemented only in cases where "acute fetal compromise is suspected and vaginal delivery is not imminent" (Society of Obstetricians and Gynecologists of Canada Clinical Practice Guideline #89, 2000). Thus, samples were classified according to mode of delivery as follows; elective Caesarian section without labour (n = 41), spontaneous vaginal delivery (n = 53), and emergency Caesarian section following labour (n = 31).

3.2.2. Guinea pig

Guinea pig serum was obtained through collaboration with Drs. James Brien and James Reynolds at Queen's University (Kingston, Ontario). Animals were nulliparous females of the Dunkin-Hartley strain (Charles River Canada Inc., St Constant, Quebec) who were mated in the Brien-Reynolds lab and randomly assigned to one of six treatment groups in a study designed to assess offspring effects of chronic ethanol exposure *in utero*. Pregnant animals that received sucrose were each yoked to an ethanol-treated animal and received the amount of food that was consumed by the ethanol-treated animal on the previous day. As ethanol treatment is known to affect glucocorticoids (Iqbal *et al.* 2005; Weinberg *et al.* 2008), we did not use animals from the two ethanol-treated groups. Our animals were divided as follows; 1) *ad libitum* feeding (n = 3), 2) vehicle (sodium bicarbonate) + folic acid (2mg folic acid/kg body weight) + *ad lib* feeding (n = 4), 3) vehicle + isocaloric-sucrose (42% weight/volume, prepared in tap water) + paired food amount (n = 3), 4) folic acid (2mg folic acid/kg body weight) + isocaloric-sucrose + paired food amount (n = 2). Water was available to all animals *ad libitum* throughout the experiment.

All females were handled and weighed daily at 0830h (beginning on day 2 of pregnancy). Treated animals were administered the first dose (either vehicle or folic acid) at 1030h and the second dose (sucrose or folic acid) at 1230h. Solutions were dispensed deep into the oral cavity to induce swallowing.

On gestational day 65 (term is approximately 68 days in this species), guinea pigs were anesthetized with a subcutaneous injection of ketamine (50 mg/kg body weight) and xylazine (5 mg/kg body weight) and euthanized by decapitation between 1300h and 1500h. Maternal trunk blood was collected and fetuses were delivered individually by C-section. Each fetus was removed from the uterus with placenta intact, placed on a heating

pad and membranes cleared to facilitate breathing. Placenta was separated from each pup and since the cord is too small to permit separate arterial and venous sampling, mixed umbilical cord blood was collected. Each pup was then decapitated and trunk blood collected. Following collection, whole blood samples were centrifuged and serum stored at -20C until hormone analysis.

3.2.3. Hormone extraction

Prior to hormone analysis, all human and guinea pig serum samples were extracted by a solid phase extraction protocol established and validated in this laboratory (Love *et al.* 2005; 2008). This procedure is used to remove impurities (such as fats and proteins) from a serum sample and isolate the steroids of interest. Non-endcapped 6mL columns with 500mg C_{18} packing material (Chromatographic Specialties, Inc., Brockville, ON) were used for extractions. C_{18} packing begins with raw silica material and uses hydrophobic alkyl functional groups to modify the hydrophilic silanol groups (by reactions with the corresponding silanes). The resulting material retains non-polar analytes (such as the glucocorticoids) well. Non-endcapped refers to the fact that there are many residual silanol groups that can provide secondary interactions with analytes. The columns are attached to a 24-place vacuum manifold (United Chemical Technologies) and primed with 3mL non-polar HPLC-grade methanol (Fisher Scientific, cat. no. A4524) to wet the surface of the sorbent and penetrate the bonded alkyl phases. The silica surface is then wetted with 10mL Millipore® water. 50µL serum are brought up into 1mL Millipore water to allow a sufficient sample volume to load the columns and maintain proper wetting of the packing material. The entire sample volume is added to column at a flow rate of 1ml/ minute and van der Waals forces cause the retention of the non-polar

glucocorticoids by the non-polar packing material. Columns are then washed with 10mL Millipore water to remove weakly retained, unwanted compounds. Glucocorticoids are eluted into 7mL borosilicate glass vials (Fisher Scientific, cat. no. 03-337-26) with 5mL of 90% methanol. A 90% methanol solution has high enough polarity to disrupt the retention of the glucocorticoids but not enough to permit the washing of remaining unwanted compounds. Samples are dried under vacuum (10 mmHg).

The guinea pig samples were extracted over a five-day period and each extraction run contained one pooled serum sample (serum combined from 15 adult female guinea pigs) and four standards of known concentrations (40 ng/ml cortisol, 400 ng/ml cortisol, 50 ng/ml corticosterone, 500 ng/ml corticosterone; Diagnostics Systems Laboratories, Inc. cat. no. 10-2004, 10-2007, 80103, 80105, respectively). Multiple standards were used to cover a wide range of concentrations, as expected concentrations in these samples were unknown. All standards contained the steroid in a protein-based buffer (bovine serum albumin) and were used to assess steroid recovery following extraction and measure variability between extraction runs. Matched mom-cord-pup triplets were always extracted in the same run.

The human serum samples were extracted over a twelve-day period and each run contained two standards (400 ng/ml cortisol and 50 ng/ml corticosterone, as above), chosen based on expected glucocorticoid concentrations. All matched arterial-venous samples were extracted in the same run.

3.2.4. Hormone quantitation

All samples were sub-contracted to Vogon Laboratory Services Ltd. (Calgary, AB) for cortisol and corticosterone quantification by liquid chromatography coupled to mass

spectrometry (LC-MS/MS). LC separates the sample into a series of components before introduction into the MS for sequential analysis by molecular mass. Dried samples were reconstituted in 1mL of 25% methanol in 0.1% formic acid and liquid chromatography was performed using the Agilent 1200 series binary pump. The sample was dissolved in 5mM ammonium formate in 0.1% formic acid followed by 5mM ammonium formate in acetonitrile with 0.1% formic acid (gradient 40-100%B) and pumped through the Zorbax Eclipse Plus C_{18} RRHT column (2.1 x 100 mm, 1.8μ) at a flow rate of 0.4 ml/min and a temperature of 50^{o}C. The retention time of each glucocorticoid is different so they become separated and enter the mass spec individually for analysis.

Mass spectrometry was performed on the Agilent 6410B triple quadrupole LC-MC/MC using five calibrators to set the scale of expected mass-to-charge ratios (25% methanol; 0.01, 0.1, 1, 10, 100 ng/ml, prepared by Vogon Labs). At a voltage of 4000 V, the glucocorticoids are pushed through a capillary tip and disperse into an aerosol of droplets with a high positive charge. Nitrogen (nebulizer 45 psi, flow rate 12L/min, 350^{o}C) then evaporates the solvent and the charged droplets release sample ions. These charged sample ions enter the analyzer of the mass spec and are separated into fragments according to their mass-to-charge ratios for subsequent analysis of specific mass-to-charge ratios. The ion current is monitored and amplified and the signal is transmitted to the data system where it becomes recorded in the form of mass spectra. From this, we can evaluate the number of components in the sample and the molecular mass and abundance of each.

3.3. Results

3.3.1. Technical challenges

We encountered several issues with both sample preparation and mass spectrometry methods (see Appendix 1 for full details). The solid phase extraction procedure yielded very low steroid recoveries for the human serum (corticosterone = 16.7%, cortisol = 4.1%) and guinea pig serum (corticosterone = 28.4%, cortisol = 8.1%) such that many of our samples fell below the mass spec limit of quantitation (LOQ) for corticosterone. The LOQ was reported to be 0.05 ng/ml for the human samples and 0.1 ng/ml for the guinea pig serum. However, an analysis of seven guinea pig samples above the LOQ revealed no repeatability across the range of these samples ($r^2 = 0.03$, $p = 0.71$) so the reported LOQ was not supported by the empirical test. As a result, exceptional caution was needed in the interpretation of the corticosterone results.

3.3.2. Hypothesis 1: The term fetus secretes corticosterone, as well as cortisol

Fetal secretion of corticosterone should result in higher concentrations of corticosterone in the arterial, than in the venous, paired samples. Of the human venous samples, 71% ($n = 89$) contained corticosterone concentrations above the reported LOQ (mean ± SEM = 1.0 ± 0.15 ng/ml) whereas 91% ($n = 114$) of the arterial samples contained quantifiable corticosterone (mean = 2.15 ± 0.19 ng/ml; $\chi 2$ for probability of detection (125) = 12.4, $p < 0.001$). Of the samples that were above the LOQ, concentration of corticosterone was also higher in the arterial sample ($n = 87$ matched pairs, $t_{86} = 8.88$, $p < 0.0001$; Figure 3.1). Cortisol was readily quantifiable in all human venous (mean = 13.5 ± 1.5 ng/ml) and arterial (mean = 19.5 ± 1.6 ng/ml) samples with the concentration of cortisol significantly

higher in the arterial sample relative to the venous sample (matched pairs, $t_{124} = 10.4$, $p < 0.0001$; Figure 3.1). Thus, both corticosterone and cortisol were enriched in the post-fetal arterial samples.

For guinea pig, 28 matched fetal and mixed cord serum samples from 12 pregnancies (1-3 fetuses per dam) were collected. Corticosterone was above the LOQ in 27/28 fetal samples (mean ± SEM = 2.45 ± 0.35 ng/ml) and 22/28 cord samples (mean = 0.70 ± 0.11 ng/ml). When both samples were above the LOQ, corticosterone in fetal samples was significantly higher than in mixed cord samples ($n = 22$ matched pairs, $t_{21} = 4.71$, $p < 0.0001$; Figure 3.2). Cortisol was well above the LOQ in all 28 fetal and cord samples (mean = 729 ± 49.8 ng/ml; 397 ± 35.7 ng/ml, respectively) with concentrations higher in the fetus (matched pairs, $t_{27} = 9.68$, $p < 0.0001$; Figure 3.2). Thus, guinea pig samples also supported the hypothesis of corticosterone enrichment by the fetus.

3.3.3. Hypothesis 2: Maternal stress results in cortisol enhancement in venous circulation whereas fetal distress results in corticosterone enhancement in arterial circulation

Cortisol concentration in the umbilical vein has been reduced by passage through the placenta, which actively converts cortisol to cortisone (Krozowski *et al.* 1995). Nevertheless, maternal stress should result in an increase in cortisol, of maternal origin, reaching the fetus (spontaneous vaginal delivery > emergency C-section > planned C-section). As predicted, venous cortisol was elevated in spontaneous vaginal deliveries (full labor) relative to elective C-section (no labor), with emergency C-sections (labor

followed by surgical anesthesia) intermediate ($F_{2, 122} = 6.15$, $p < 0.01$ followed by post hoc Tukey test; Figure 3.3). No change in venous corticosterone was seen ($p = 0.20$).

Similarly, if the fetal adrenal secretes corticosterone in response to stress then fetal stress (emergency C-section > spontaneous vaginal delivery > planned C-section) was expected to enhance fetal corticosterone production, as measured by an elevation in arterial concentration. However, there was no evidence of changes in corticosterone concentration across the groups ($F_{2,112} = 2.19$, $p = 0.12$), although arterial cortisol followed the maternal pattern established in the venous samples ($F_{2,122} = 10.4$, $p < 0.0001$; Figure 3.4). This was not surprising, as the positive arterio-venous association for cortisol ($r^2 = 0.88$, $p < 0.0001$; Figure 3.5) was strong.

There was also an arterio-venous association for corticosterone ($r^2 = 0.53$, $p < 0.0001$; Figure 3.6). Likewise, within a guinea pig, maternal GCs were positively related to fetal GCs (corticosterone, $r^2 = 0.73$, $p < 0.001$, Figure 3.7; cortisol, $r^2 = 0.46$, $p < 0.05$, Figure 3.8).

3.3.4. Hypothesis 3: Corticosterone and cortisol responses are independent

There was no evidence that high cortisol was associated with high corticosterone within human umbilical venous ($r^2 = 0.002$, $p = 0.65$, Figure 3.9) or arterial samples ($r^2 = 0.001$, $p = 0.64$, Figure 3.10). Likewise, in the guinea pig, there was no evidence of an association between cortisol and corticosterone in maternal ($r^2 = 0.14$, $p = 0.23$; Figure 3.11) or fetal ($r^2 = 0.07$, $p = 0.17$) serum (Figure 3.12).

3.4. Discussion

The absence of repeatability for corticosterone quantification, in combination with the poor extraction efficiency, severely constrains the interpretation of these results. Nevertheless, the results suggest that these experiments are worth repeating because both the human and guinea pig term fetus secretes measurable amounts of the non-dominant GC, which is not typically measured in these species. Our data also suggest that endogenous concentrations of cortisol and corticosterone within an individual are unrelated and the two GCs do not show identical responses to mode of delivery in humans. While the dominant GC cannot predict the non-dominant GC within a mother or within a fetus, maternal or umbilical venous GCs can predict fetal GCs at parturition. In the human, umbilical venous GCs, which have passed through placental metabolism, can predict GCs in fetal circulation. In the guinea pig, maternal corticosterone can predict fetal corticosterone and the same is true for maternal and fetal cortisol.

The steroid biosynthetic pathway is ancient, and the homology of glucocorticoid receptors has been stretched back to include our common ancestor with the octopus, as well as with the cartilaginous fishes (Carroll *et al.* 2008). The mammalian endocrine system has been evolving for approximately 200 million years and is highly conserved across species. Accordingly, if our hypothesis is correct and non-dominant glucocorticoid signaling is important for humans in early development, it seems likely that the same would be true for all placental mammals. Indeed, our results suggest that this is the case for the guinea pig and indicate that multiple GCs should be quantified in all species in the early developmental period.

The permanent effects of prenatal stress have been well documented in many species (rats, McCormick *et al.* 1995; guinea pigs, Dean *et al.* 2001; foxes, Braastad *et al.*

1998; sheep, Roussel *et al.* 2004; rhesus monkeys, Clarke *et al.* 1994; humans, Buitelaar *et al.* 2003) but the mechanisms regulating alteration of the fetal phenotype are unknown. Few studies have considered the potential importance of the non-dominant GC signal but it is possible that a separation of GC signals could be one of the many mechanisms driving these epigenetic effects. There are multiple, plausible and demonstrated mechanisms through which corticosterone could carry a hormone signal that is independent from cortisol (i.e. competitive binding affinity to the same receptors (Sutanto & de Kloet 1987; Reul *et al.* 1990)). The guinea pig is a useful animal model that is amenable to experimental manipulations for establishing causal relationships in future research and mass spectrometry is the technology that will allow accurate determination of GC concentrations.

As mentioned previously, antenatal administration of GCs is often beneficial in obstetric care but the exact mechanisms behind the GC action are still unknown. Since antenatal GC administration has also been shown to cause a variety of negative effects on the fetus (Murphy 2008), a comprehensive examination of human GC secretions during the prenatal period is important. Future research could identify the mechanisms regulating both positive and negative fetal effects and could potentially lead to more targeted, personalized courses of GC therapy.

Although there were various technical challenges with our extraction and quantification methods, the data supports our hypotheses and suggests that these experiments should be repeated. Both the human and guinea pig full-term fetus secretes the non-dominant GC. Most current research ignores the non-dominant GC but there is reason to believe that the non-dominant GC is a functional endocrine signal, independent of the dominant GC.

CHAPTER 4: General discussion

There is a general bias in endocrinology research whereby researchers measure only the dominant glucocorticoid when studying the stress steroids. This bias is mainly due to an assumption that the dominant GC is the important endogenous signal because the GCs are functionally interchangeable. We have traced the emergence of the dominant GC/ interchangeability assumption from historical research and provide evidence suggesting that this assumption is pervasive in current textbooks and empirical research and has been biasing the field for about 30 years.

The literature review in Chapter 2 compiled mammalian studies that have measured both GCs. This review illustrated the paucity of evidence to support the dominant GC/ interchangeability assumption and also provided sufficient data to suggest that the GCs are independently regulated within an individual. At present, there are extensive knowledge gaps related not only to the endogenous function(s) of the GCs, but also to the general understanding of GC synthesis and secretion rates within and across species. The GC profile has been explicitly tested in only 46 mammalian species (i.e. 1% of all extant mammals) and of these 46 species, all have been assigned a dominant GC for research purposes. However, several species do not actually exhibit preferential secretion of one GC over the other and the cortisol: corticosterone ratio variability is quite large with up to a 100-fold difference across species. Likewise, the range of GC concentrations varies at least 100-fold within the Mammalia class and the distribution of ratio and/ or concentration differences does not appear to be related to phylogeny.

Although often in small amounts, non-dominant GC secretion is a characteristic of all mammalian species examined and seems to occur throughout the lifespan but several

studies have shown a divergence in non-dominant GC secretion patterns during the fetal period. Using mass spectrometry, Chapter 3 characterized the GC concentration of the full-term fetus in two species and confirmed that the non-dominant GC is an active secretion of the maturing fetal adrenal gland in the human and guinea pig. Though hampered by problems with hormone quantitation, the data provide evidence against the dominant GC/ interchangeability assumption by revealing no predictable relationship between cortisol and corticosterone within an individual and variable secretion patterns in relation to mode of delivery in humans.

Collectively, our research has established that, in the field of endocrinology, there is an inherent dominant GC/ interchangeability bias that is unwarranted. Our current glucocorticoid data from two mammalian species has provided further reason to reject this assumption in favour of a hypothesis that states that there is a duality in glucocorticoid signaling.

Independence in glucocorticoid signaling could be ubiquitous and critical in several biological transitions such as adrenarche, puberty, pair-bond formation, territory establishment, and parenthood. Alternatively, a functional glucocorticoid independence could be restricted to one or more specific windows in development. For example, during the early developmental period, efficient maternal-fetal bidirectional communication about stress status could be adaptive for both parties. Maternal stress during pregnancy has been shown to alter the physiological stress response and cognitive development in the offspring (Francis *et al.* 1999; Welberg *et al.* 2000; Osadchuk *et al.* 2001; Griffin *et al.* 2003; Maccari *et al.* 2003) and it is plausible that this modification of phenotype is a fetal adaptation that results in a phenotype that is better equipped for the surrounding environment in which it will live. Indeed, several studies suggest that this is the case

(Meylan & Clobert 2005; Love *et al.* 2008). On the other hand, offspring effects of maternal stress could be adaptive for the mother. Maternal GCs could alter offspring phenotype such that offspring need is matched to maternal ability and thus, the burden of parental care is lowered in harsh environments (Hayward & Wingfield 2004; Love *et al.* 2005).

Whatever the reason for epigenetic modification of offspring phenotype by maternal GCs, the separation of stress signals is a plausible mechanism that could be mediating these effects. If the fetal adrenal, in a cortisol-dominant species such as humans, secreted corticosterone *in utero*, then that signal could potentially pass the placenta, act on maternal receptors, and signal fetal distress to the mother. Likewise, fetal synthesis of the non-dominant corticosterone might allow the fetus to use differential receptor affinity for maternal cortisol to sense maternal stress.

At present, any duality in glucocorticoid function will remain undetected because research does not compare cortisol and corticosterone secretion patterns within an individual. Based on our research, future work in this field should perform a comprehensive assessment of glucocorticoid synthesis and secretion at all stages of the lifespan, so that precise glucocorticoid function(s) can eventually be understood.

SUMMARY

1) The dominant glucocorticoid/ interchangeability assumption is pervasive in textbooks and empirical research and has been biasing the field of endocrinology for approximately 30 years
2) There is little evidence to support the dominant glucocorticoid/ interchangeability assumption but instead, there is a dispersed literature that suggests independence, rather than interchangeability, in glucocorticoid secretion
3) All mammalian species examined secrete both glucocorticoids and the diversity in glucocorticoid concentrations and cortisol: corticosterone ratio varies up to 100-fold across mammalian species
4) The full-term human fetus and guinea pig fetus secretes the non-dominant glucocorticoid
5) Preliminary analyses suggest that the glucocorticoids might be independently regulated and the dominant glucocorticoid is not an accurate proxy of non-dominant glucocorticoid activity

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APPENDIX 1

According to our hypothesis, we expected all samples to contain detectable quantities of corticosterone. However, 47 of the 250 human samples (19%) had non-detectable corticosterone following mass spec and similarly, 7 of the 68 (10%) guinea pig samples had corticosterone levels too low for detection.

An examination of the procedures used for sample preparation and hormone quantification revealed substantial issues. The main problem was the solid phase extraction protocol used for sample preparation. The procedure was inadequate and the clean up was poor. Extracted samples contained numerous interfering compounds and the glucocorticoids were removed during the procedure. There were also concerns with the mass spectrometry data as revealed by a comparison of analyses performed by two separate mass spectrometry teams.

1. Sample preparation and quality control

During each extraction run, there were several standards of known concentrations that were subject to the same extraction procedure as the serum samples. These standards were used for quality control to assess the performance of the extraction procedure as well as the consistency across runs. An analysis of the standards by mass spectrometry showed that GC recoveries were extremely poor following the extraction of the human samples (corticosterone = 16.7%, cortisol = 4.1%) and the guinea pig samples (corticosterone = 28.4%, cortisol = 8.1%).

The improved recoveries of standards in the guinea pig extractions are puzzling since the procedure (including same reagents and same column lot numbers) was identical

for the human and guinea pig samples. The standards in each extraction run were taken directly from the same stock solutions so variation across lot number is not a concern. The variability across columns has previously been analyzed in hamster serum samples extracted with this procedure. Analysis of corticosterone with an enzyme-immunoassay revealed a coefficient of variation less than 5% from column-to-column. Variability across the 5 guinea pig extraction runs was approximately 6% but was much higher (36%) across the 12 human extractions. However, this increased level of variability does not explain the lower recovery rates.

The solid phase extraction procedure used was initially developed and validated in the Wynne-Edwards lab for the extraction of estradiol from bird yolk and plasma (Williams *et al* 2005). The procedure has also been validated for the extraction of corticosterone from bird yolk samples (Love *et al* 2005) but not for extraction of glucocorticoids from serum. The mass spec analysis revealed many interfering compounds in our extracted samples and it is clear that the SPE protocol needs to be refined for glucocorticoid extraction from serum.

There are several stages at which the SPE procedure could be modified but only one step must be altered in each trial to optimize the procedure. Because the extracted samples contained many compounds that should have been removed during the extraction procedure, an obvious first step to improve this protocol might be protein precipitation of the serum sample prior to loading onto the column. The specific compounds that caused interference in the mass spec analysis are unknown but blood contains high levels of proteins that could be the problem. Protein precipitation would remove a lot of the contaminants in the serum.

With such a small sample volume (50μl) added to the column, using a smaller column with less C_{18} packing material might improve the retention of the glucocorticoids. Endcapped columns would decrease the number of silanol groups (by reacting the bonded silica with trimethyl silane) and thus decrease any secondary polar interactions that might interfere with steroid extraction.

Perhaps the lower recovery rates of cortisol, compared to corticosterone, are due to a slight increase in polarity for cortisol. This could be important during the sample loading phase when a polarity increase will decrease the van der Waals forces that cause compound retention. A slightly higher polarity could also be important during the interference washing stage, when 10 mL of water is passed through the columns to wash away weakly retained compounds. If the forces retaining cortisol are slightly weaker than those for corticosterone, it is possible that some of the cortisol gets washed away in this phase.

The loading rate of the samples onto the columns might need to be decreased. If the sample loading is too fast, breakthrough of the sample can occur and this would cause a dramatic decrease in steroid recovery rates. However, by most standards, our loading rate is slower than should be required so it is more probable that an increase in flow rate might prove useful.

It is possible that the columns were not dry enough prior to the elution stage and this led to a dilution of the 90% methanol used for the elution. A dilution would decrease the ability of the solvent to disrupt the van der Waals forces that keep the glucocorticoids bound to the packing material. Thus, an increase in the drying time between the interference washing and the elution stage might be beneficial for removing the GCs from the column.

These are just a few changes that might improve the current protocol but there are endless adjustments and variations that can be made to optimize the extraction efficiency. Future work will vary one aspect of the protocol in a series of extraction runs until a method is in place that yields recovery rates above 90% for both glucocorticoids in all extractions.

2. Quantification by mass spectrometry

All human and guinea pig serum samples were subcontracted to Vogon Laboratory Services Ltd. (Calgary, AB) for analysis by mass spectrometry. At the time that samples were shipped to Vogon for analysis, Dr. Wynne-Edwards was shopping for a mass spectrometer for her own lab at the University of Calgary and had two companies bidding for the purchase (Agilent and MDS Sciex). The individual responsible for the analysis of our samples at Vogon Labs was simultaneously employed to showcase the advantages of the Agilent machine. It was the analysis of two identical samples by both companies that demonstrated the concerns with data presentation by Vogon Labs but unfortunately, these concerns were not recognized until after the analyses were complete.

Limit of quantitation (LOQ) is an important value as it provides a measure of instrument performance and dictates the concentration at which the mass spec can no longer determine concentration accurately (due to interfering noise). Agilent and MDS Sciex were given identical samples of SPE extracted human saliva and robin yolk and were each asked to detect cortisol and corticosterone. When results were compared, MDS Sciex identified 0.5 ng/ml as the LOQ for both glucocorticoids in these samples whereas Agilent specified a much lower LOQ of 0.1 ng/ml for both. On the surface, it would seem that the Agilent mass spec is more sensitive and can provide more accurate measurements

of glucocorticoids at concentrations between 0.1-0.5 ng/ml. However, upon further inspection, the discrepancy in LOQ can be explained by the method used to define the LOQ.

The signal-to-noise ratio (S:N), or the ratio of a peak's height to the variability in the background signal, is often used to identify the LOQ and the LOQ is defined as the concentration that produces a peak with a S:N ratio of 10 or higher. This should be assessed using several injections of multiple concentrations of the analyte in question (usually 5 injections at each of 5 concentrations) as a single S:N ratio from a single injection does not always provide an accurate assessment. MDS Sciex used 4 injections at multiple concentrations for both glucocorticoids and determined that there was too much interference below 0.5 ng/ml for quantification to be reliable. Vogon Labs boasted a higher sensitivity and lower LOQ (0.1 ng/ml) on the Agilent mass spec but used only one injection of each concentration to assess the LOQ.

An additional tool that is sometimes used for improving the presentation of mass spec data is smoothing of the peaks in the chromatogram. Smoothing has no effect on the actual LOQ or instrument accuracy but improves the appearance and the S:N ratio of a peak and can be used to conceal underlying problems with interference. As shown in Appendix Figure 1.1, Vogon Labs used a smoothing technique to improve the presentation of the data obtained on the Agilent instrument, which provides further reason to be cautious of the human and guinea pig data.

3. LOQ as a criterion for data exclusion

Of the 61 guinea pig samples that had detectable amounts of corticosterone, 51% (n = 31) had concentrations that fell at or below the limit of detection. The definition of the LOQ

as 0.1 ng/ml for the guinea pig samples is questionable as it was based only on a single injection at each concentration. In fact, seven samples that were each above the LOQ were subject to a second run through the mass spec and a regression analysis revealed absolutely no relationship between the two (the first value could explain only 3% of the variance in the second value; Appendix Figure 1.2). The poor repeatability for corticosterone values above the LOQ makes it clear that the LOQ is not accurate in these samples. In this case, we decided to include all samples in the statistical analyses because LOQ is not a useful tool for excluding data.

Of the 203 human cord samples that had measureable corticosterone, only 3 fell at or below the LOQ. The LOQ for the human serum samples was reported to be 0.05 ng/ml but there were no samples analyzed twice so the reliability of this measurement is unknown. Based on the poor repeatability of the guinea pig samples and a reportedly lower LOQ in the human samples, we included these three data points in all analyses.