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AN INTEGRATED EVALUATION OF COSTS AND BENEFITS OF CORTICOSTERONE SECRETION THROUGH DEVELOPMENT

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AN INTEGRATED EVALUATION OF COSTS AND BENEFITS OF CORTICOSTERONE SECRETION THROUGH DEVELOPMENT

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Dedication

This dissertation is dedicated to my families in Japan and the United States, especially to my parents and Guillaume Salze for their amazing love and support.

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AN INTEGRATED EVALUATION OF COSTS AND BENEFITS OF CORTICOSTERONE SECRETION THROUGH DEVELOPMENT

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Glucocorticoids (GCs) play critical roles during development: transient increases in GCs facilitate anticipatory physiological changes and trigger ontogenetic transitions such as promoting fetal/embryonic organ maturation and initiating birth/hatching. In contrast, chronically elevated GCs can be detrimental to growth, cognition, and survival. Thus, animals going through substantial growth may have higher corticosteroid binding globulin (CBG) levels, or enhance negative feedback/tonic inhibition on the hypothalamic-pituitary-adrenal (HPA) axis to keep GCs levels low. Here I investigated these hypotheses using altricial white-crowned sparrow nestlings. I examined 1) the ontogeny of the corticosterone (CORT) response (both total and free hormone levels), 2) changes in corticosteroid receptor levels in brain with age, and 3) effects of acute and extended elevation of CORT on behavior and growth.

In response to acute stress, nestlings showed a low HPA reactivity in total CORT during the first 1/3 of the nestling period. When free CORT is considered, this hyporesponsive period was extended to 2/3 of the nestling period, suggesting CBG is one

of the mechanisms to keep free CORT low. These periods coincided with rapid mass gain and acquiring thermoregulatory ability. The low reactivity was partly due to a dampened sensitivity at pituitary level or higher as all stages of nestlings responded to adrenocorticotropic hormone challenges; however it was not due to an enhanced negative feedback/tonic inhibition on hypothalamus or hippocampus. When CORT levels were artificially elevated, I only observed detrimental effects on begging behavior and growth. These series of data elucidated the ontogeny of the HPA axis in altricial nestlings regarding CORT, binding globulin, and receptor levels. In addition, I found that measured effects of exogenous CORT are primarily costly and highly age-specific.

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INTRODUCTION

Numerous physical and physiological mechanisms are critical in transitional events such as birth, puberty, senescence, as well as seasonal breeding cycles. In adult vertebrates, glucocorticoids (GCs: corticosterone (CORT) and cortisol) are implicated as one of the hormones to trigger transitions within breeding cycles. For instance in birds, CORT triggers transitions from regular to emergency life history stages, such as breeding to irruptive migration (reviewed in Wingfield and Kitaysky, 2002). Similarly, CORT facilitates finer transitions between behavioral stages across taxa, such as from courtship to flight (reviewed in Orchinik, 1998). However, the effects of GCs on life history or behavioral stages are not universal: they are context-dependent due to receptor characteristics and interactions with other neuropeptides (Orchinik, 1998).

Elevations of GCs also coincide with ontogenetic life history transitions. It is well established that fetal cortisol triggers parturition in certain mammals (see below). But GCs may trigger or modify other physiological changes necessary for ontogenetic processes as well. In this review, I will 1) discuss the ontogeny of the Hypothalamic-Pituitary-Adrenal/Interrenal (HPA/HPI) axes in relation to ontogenetic events, 2) explore the direct or permissive actions of GCs in diverse ontogenetic transitions across vertebrate taxa including hatching/birth, fledging/dispersal, metamorphosis, and smoltification, and 3) bring to light common elements of GCs during such transitions.

Some aspects of this review are extensively studied and well reviewed elsewhere. The aim of this review is not to provide details on each of the subjects, rather to bring a comparative view on the role of GCs in different ontogenetic transitions. Here I will focus mainly on the role of GCs but it is important to keep in mind that GCs interact with other hormones during

ontogeny, e.g., thyroid hormones, prolactin, and growth hormone. We should not overlook the importance of those hormones or interactions between them.

COSTS AND BENEFITS OF GC SECRETION DURING DEVELOPMENT

Young may face a trade-off between the benefits and costs of GCs (e.g. Kitaysky et al., 2003). GCs stimulate begging behavior in birds (Kitaysky and Wingfield, 2001) (but also see Rubolini et al., 2005), food intake (Kitaysky et al., 2003), and locomotor activity in young (Crespi and Denver, 2004; Freire et al., 2006). GCs also play an important role in metabolism, mobilizing glucose when needed (reviewed in Sapolsky et al., 2000). At the same time, they impair growth (Glennemeier and Denver, 2002c; Hayward and Wingfield, 2004; Janczak et al., 2006; Mashaly, 1991; Meylan and Clobert, 2005; Morici et al., 1997; Saino et al., 2005; Spencer et al., 2003; Wan et al., 2005), cognition (Kitaysky et al., 2003) (but also see Catalani et al., 2000), and immune function (Morici et al., 1997; Rubolini et al., 2005) and can cause neuronal death (Howard and Benjamins, 1975) and high mortality (Eriksen et al., 2006; Janczak et al., 2006; Mashaly, 1991; Saino et al., 2005) (but also see Meylan and Clobert, 2005). GCs may also alter sensitivity to stress later in life (Catalani et al., 2000; Hayward and Wingfield, 2004). Thus the timing of HPA/HPI axes development is critical; it would appear important to strictly regulate the use of GCs during certain period of development, so as to avoid the costs associated with elevated levels.

ONTOGENY OF THE STRESS RESPONSE

Mammals

Ontogeny of the HPA axis is well documented in mammals, particularly in rodents. Total (bound and unbound to binding proteins) baseline CORT of the rat fetus increases just prior to birth, reaching adult-like levels (Henning, 1978; Hiroshige and Sato, 1971; Martin et al.,

1977; Meaney et al., 1985a; Tinnikov, 1993; van Baelen et al., 1977) (Fig. 1.1a). This hypersecretion of CORT is independent of maternal CORT secretion and is accompanied by decrease in fetal corticosteroid binding globulin (CBG), allowing free (unbound) CORT to elevate even further. Total CORT then declines sharply to very low levels in the first couple of days after birth and stays low until the 2nd week of life. The levels then increase gradually to adult levels by ~15 days.

The development of the HPA axis appears to depend on the developmental maturity at birth. Altricial mammals show a brief period of HPA axis quiescence, called the stress hyporesponsive period (SHRP) during the first days of life (first described as non-responsive period by Schapiro, 1962, reviewed in Sapolsky and Meaney, 1986; Walker, 2001; and Vazquez, 1998). The SHRP is thought to be beneficial, especially for altricial species, considering the negative effect of CORT on growth. Rat fetuses and neonates can respond to various stressors (e.g. leg fracture, maternal ether inhalation, and histamine injection) by secreting CORT and ACTH in late gestation and soon after birth (Cohen et al., 1983; Cote and Yasumura, 1975; Milkovic and Milkovic, 1963; Milkovic et al., 1973). However, the responsiveness declines shortly after birth and various stressors (e.g. ether, shock, heat, histamine injection) elicit no or blunted adrenocortical response until the 2nd week of life (Butte et al., 1973; Cote and Yasumura, 1975; Guillet and Michaelson, 1978; Haltmeyer et al., 1966; Tang and Phillips, 1977). It was later discovered that certain stressors and corticotrophin releasing factor (CRF) *can* elicit age-dependent but significant ACTH and/or CORT responses during the SHRP, and neonates have a functional negative feedback by CORT (Guillet and Michaelson, 1978; Schoenfeld et al., 1980; Walker et al., 1986; Walker et al., 1991). Furthermore, maternal separation longer than 24 hrs can enhance the sensitivity to ACTH during this period (Levine et al., 1991). Therefore, the SHRP in rats is context-dependent and the blunted CORT response may be due to an age-dependent suppression of response at the adrenal level.

Ontogeny of the stress response in non-human primates (non-altricial) differs from that of rodents. In rhesus macaques (*Macaca mulatta*), 2-day-old neonates can mount an adult-like

stress response to a brief maternal separation and rotation (Bowman and Wolf, 1965). In common marmosets (*Callithrix jacchus*), baseline cortisol and ACTH of 1-week-old neonates are significantly higher than adults (Pryce et al., 2002). In fact, baseline cortisol is approximately 10 times higher in neonates. This remarkably high level of cortisol is partly due to relatively large adrenals. Furthermore, cortisol and ACTH responses to isolation stress are higher in 2-month-olds compared to 12-month sub-adults. Extremely high levels of cortisol in addition to delayed shutdown of stress responses in young suggest a dampened cortisol negative feedback in this species.

Birds

The precocial-altricial spectrum mentioned above refers to a broad array of functional maturity at birth seen in birds and mammals (Starck and Ricklefs, 1998). Newly hatched nestlings and neonates differ in their mobility, sensory organ development, and feather development or thermoregulatory ability, leading to variation in extent of dependency to parents for feeding and thermoregulation. The ability to benefit from GCs depends on the state of maturation at hatching/birth and when they begin to leave the nest, forage, and become independent (Developmental Hypothesis) (Blas et al., 2006; Kitaysky et al., 2003; Schwabl, 1999; Sims and Holberton, 2000; Wada et al., 2007). Thus the development of the HPA axis should reflect this spectrum. In birds, it is hypothesized that precocial species with capacity to escape stressors may develop the HPA axis earlier than altricial species (nest-bound, parent-dependent), which would suffer only from detrimental effects of GCs. The altricial species should develop a functional HPA axis by the time it is beneficial to respond to a stressor, e.g. fledging.

Baseline CORT secretion before hatching is observed at least in domesticated precocial species, e.g. chickens (*Gallus domesticus*) and turkeys (*Meleagris gallopavo*) (Davis and Siopes, 1985; Jacobs, 1996; Scott et al., 1981; Wise and Frye, 1973) (reviewed in Jenkins and Porter,

2004) (Fig. 1.1b). Chicken embryos start to secrete CORT around 14 days of 21-day incubation (Scott et al., 1981; Wise and Frye, 1973) and the sensitivity to adrenocorticotropin hormone (ACTH) peaks around hatching (Carsia et al., 1987).

Some evidence suggests that chickens undergo a transient SHRP similarly to mammals. In embryonic stage, heating to 43°C for 2 hours significantly increases CORT in embryos in the last few days of incubation (Jacobs, 1996). In young, cold and/or handling stress can elicit stress response from 2- and 21-day post-hatch (dph) chicks but both stressors fail to stimulate a stress response in 1 dph chicks (Freeman, 1982; Freeman and Flack, 1980; Freeman and Manning, 1984) suggesting the stress hyporesponsive period in chickens lasts ~48 hours.

In non-precocious species, less data are available. There are no data on embryonic CORT secretion, thus existence of a true stress hyporesponsive period remains unclear. However, non-precocious species appear to develop the HPA axis later than precocial species (Fig. 1.1c). In semi-altricial and semi-precocial species, baseline CORT (Belthoff and Dufty, 1998; Heath, 1997; Love et al., 2003) (but also see Romero et al., 2006; Walker et al., 2005) and adrenocortical response to handling (Love et al., 2003; Walker et al., 2005) increase with age during nestling and fledgling period reaching an adult-like level before fledging. In altricial species, some increase the baseline CORT as they reach fledging (Kern et al., 2001; Schwabl, 1999) while others do not (Blas et al., 2005; Blas et al., 2006; Sims and Holberton, 2000). Similarly, white stork (*Ciconia ciconia*) (Blas et al., 2005; Blas et al., 2006), white-crowned sparrow (*Zonotrichia leucophrys nuttalli*) (Wada et al., 2007), and zebra finch (*Taeniopygia guttata*) (Wada et al., unpublished data) nestlings can respond to handling stress during nestling period while Northern mockingbird nestlings (*Mimus polyglottos*) (Sims and Holberton, 2000) and redpoll fledglings (*Carduelis flammea*) (Romero et al., 1998) cannot. Although early altricial nestlings cannot elicit a stress response to handling, they are sensitive to ACTH challenges (Sims and Holberton, 2000; Wada et al., 2007) indicating a dampened sensitivity at the level of pituitary or higher. There is variation in ontogeny of the stress response within

altricial species; however the HPA axis becomes functional later in life compared to precocial species.

Fish

In fish, there is a wide range in egg size, egg number, incubation period, developmental maturity, and body size at hatch (reviewed in Falk-Petersen, 2005). For instance, marine pelagic fish like Gadidae (e.g. cods and haddocks), Pleuronectidae (e.g. halibuts and flounders), and Sparidae (e.g. porgies) lay relatively small eggs, hatch with immature organs, and have long larval period, while Salmonidae (e.g. salmon and trout) lay large eggs, hatch at more developed state and larger size. The ontogeny of the HPA axis reflects such variation. Interrenals of fish from "small" eggs like Japanese sea bass (*Lateolabrax japonicus*) and yellowtail (*Seriola quinqueradiata*) do not appear until several days after hatching (20-25 days and 7 days post hatch, respectively) (Perez et al., 1999; Sakakura et al., 1998) (Fig. 1.1d) and the stress responses of tilapia (*Oreochromis mossambicus*) gradually increase from 5 days to 42 days after hatch (Pepels and Balm, 2004).

On the other hand, evidence shows some fish from "large" eggs can mount a stress response before hatching. Common carp (*Cyprinus carpio*) can increase cortisol within 5 minutes of handling stress just prior to hatching (Stouthart et al., 1998). Similarly, rainbow trout embryos (*Oncorhynchus mykiss*) secrete cortisol in response to ACTH *in vitro* (Barry et al., 1995b). However, the same species cannot mount a stress response to handling *in vivo* until 2 weeks post hatching (Barry et al., 1995a; Barry et al., 1995b) suggesting a possible stress hyporesponsive period at the level of the pituitary or higher. Egg size variation may also reflect on baseline cortisol content at hatch. "Large"-egg salmonids increase cortisol levels around hatching while "small"-egg non-salmonids often decrease cortisol until hatching (Ayson et al., 1995; de Jesus et al., 1991; de Jesus and Hirano, 1992; Feist and Schreck, 2001; Hwang and Wu, 1993; Hwang et al., 1992; Sampath-Kumar et al., 1995) (Fig. 1.1e). Similarly, "large"-egg

rainbow trout hatchlings had 3-6 times more cortisol than tilapia and more than 60 times those of yellowfin bream hatchlings (*Acanthropagrus latus*) (Hwang et al., 1992). However more studies are needed to determine the ontogeny of endocrine systems in marine fish (Falk-Petersen, 2005).

Reptiles and Amphibians

Far less data are available on the ontogeny of the stress response in reptiles and amphibians. In African clawed-frogs (*Xenopus laevis*), CORT is undetectable before hatching (Kloas et al., 1997). Baseline CORT begins to increase from stage 36 (soon after hatching) and peaks at stage 46 (right before premetamorphosis) (Glennemeier and Denver, 2002a; Kloas et al., 1997) (Fig. 1.1f). In Leopard frogs (*Rana pipiens*), the CORT level is low all though pre- and prometamorphosis which increases at metamorphosis climax and post-metamorphosis (Glennemeier and Denver, 2002a) (Fig. 1.1f). Pre- and prometamorphic tadpoles can mount a CORT response to confinement/shaking or intraspecific competition (Belden et al., 2003; Glennemeier and Denver, 2002a; Glennemeier and Denver, 2002b), although there are age differences in timing of response and negative feedback. Furthermore, even premetamorphic *X. laevis* and *R. pipiens* tadpoles are sensitive to ACTH challenges; sensitivity to ACTH is established later in prometamorphosis in Bullfrog (*Rana catesbeiana*) (Krug et al., 1983).

In reptiles, CORT increases from embryonic stage to hatching (Jennings et al., 2000; Medler and Lance, 1998) (Fig. 1.1g). There is an evidence that embryonic snake can respond to ACTH *in vitro* (Girling and Jones, 2006). By the time young reach a juvenile stage, turtles show an adrenocortical response to capture and restraint stress (Jessop and Hamann, 2005; Jessop et al., 2004).

BIRTH/HATCHING

Whether GCs are involved in birth/hatching appear to be determined by the developmental mode of each taxon. Fish and amphibians are non-amniotes and go through indirect development where larvae undergo metamorphosis into an adult form. On the other hand, mammals, birds, and reptiles are amniotes and go through direct development. Consequently, fish and amphibians are hatched at more immature stages and do not develop their adrenals/interenals until after hatching. Thus GC levels are low at hatching in most fish and amphibians.

Preparation for the world outside

In mammals, fetal GCs rise rapidly in the last days of gestation (see below). This elevation of GCs is critical for maturation of many organs, namely lung, small intestine, liver, adrenals, and kidney (reviewed in Liggins, 1994). Proper functions of these organs are vital for neonates' survival but were not necessary *in utero*. GCs stimulate development of these organs in anticipation of birth, a switch from continuous supply of oxygen and nutrition from placenta to breathing through lungs and intermittent feeding.

For example, it is well established that CORT promotes lung maturation at multiple levels. It enhances surfactant protein and phospholipids synthesis leading to secretion of surfactant (reviewed in Ballard, 1989; Liggins, 1994; Mendelson and Boggaram, 1991; Pepe and Albrecht, 1995). Surfactant is essential in extra-*utero* life, because it reduces surface tension within the alveoli preventing them from collapsing. CORT also facilitates structural development such as increasing compliance as well as glycogenolysis.

In birds, CORT has a similar effect on embryonic lung development. The embryonic lung of chickens shows a rapid and immense growth between 14 and 18 days of 21-day incubation (Hylka and Doneen, 1983). This coincides with the commencement of endogenous CORT secretion (Scott et al., 1981; Wise and Frye, 1973). *In vitro*, CORT, dexamethasone

(Dex, synthetic CORT), or epinephrine treatment all stimulate surfactant phospholipids synthesis in lung tissue (Hylka and Doneen, 1983; Sullivan and Orgeig, 2001). *In vivo*, hypophysectomy in the first days of incubation prevents a normal lung growth and decreases pulmonary surfactant phospholipids, which is partially rescued by CORT treatment or pituitary cell transplant later in development (Hylka and Doneen, 1983).

In respect to other critical organs, GCs stimulate Na^+-K^+ ATPase activities in the kidney tubules and in small intestine, synthesis of sucrase and alkaline phosphate in small intestine, and various enzyme activities necessary for glycogen synthesis and possibly gluconeogenesis in the liver (Liggins, 1994). In addition, GCs promote morphological maturation of small intestine.

GCs and parturition

Fetal GCs secretion signals and initiates a complex hormonal cascades leading to parturition in many mammalian species (reviewed in Challis et al., 2000; Challis et al., 2001; McLean and Smith, 1999; McLean and Smith, 2001; Renfree and Shaw, 1996; Thorburn and Liggins, 1994). Prior to birth, surges of ACTH, total and free fetal GCs are observed in sheep (Bassett and Thorburn, 1969; MacIsaac et al., 1985; Magyar et al., 1980; Norman et al., 1985), pigs (Heo et al., 2003), humans (Challis and Hooper, 1989; deM Fencl et al., 1980; Murphy, 1982; Murphy and Clifton, 2003; Yoon et al., 1998), and rodents (Martin et al., 1977; van Baelen et al., 1977). In addition, the followings may occur in concert: a decrease in fetal CBG (Martin et al., 1977) or maternal CRF binding protein (McLean et al., 1995), a positive feedback between placental CRF with fetal cortisol (Challis and Hooper, 1989), and/or decreased placental 11β - hydroxysteroid dehydrogenase activity (HSD) (Murphy and Clifton, 2003), all amplifying the elevation of GCs near term.

An unequivocal relationship between fetal cortisol and parturition is elegantly demonstrated in sheep. First, Dex infusion into fetus induces spontaneous delivery approximately 48 hours after the infusion (Liggins, 1969). A similar Dex infusion to pregnant

females, on the other hand, does not accelerate the parturition. Second, bilateral lesions of hypothalamic paraventricular nuclei, the site of CRF secretion, interfere with a normal preterm surge of fetal ACTH and cortisol and prolong the gestational period (Gluckman et al., 1991; McDonald and Nathanielsz, 1991). Similarly, infusion of CRF receptor antagonist into fetus delays parturition (Chan et al., 1998). Lastly, CRF infusion into fetus shortens the duration to parturition (Wintour et al., 1986). These lines of studies strongly suggest that the fetal HPA axis is crucial for the onset of hormonal cascades in normal parturition.

GCs and hatching

Similarly to mammalian parturition, CORT peaks around hatching in birds (Carsia et al., 1987; Davis and Siopes, 1985; Frigerio et al., 2001; Jacobs, 1996; Mashaly, 1991; Scott et al., 1981; Wentworth and Hussein, 1985) and reptiles (Jennings et al., 2000; Medler and Lance, 1998). Is CORT required for hatching in oviparous species? Some data suggest so. CORT administrations in turkey embryos 2 days before hatching significantly increase hatching success and trend for shorter incubation period (Wentworth and Hussein, 1985) (but also see Uller and Olsson, 2006). Furthermore, an administration of RU486 (CORT and progesterone receptor blocker) results in low hatching success of otherwise healthy embryos in tree lizards (*Urosaurus ornatus*) (Jennings et al., 2000) and chickens (Bordone et al., 1997; Nishigori et al., 2004). RU486 injection in late incubation also delays hatch date by 1 day in chickens (Bordone et al., 1997; Nishigori et al., 2004). Since RU486 specifically blocks CORT receptors in birds (Groyer et al., 1985), these data suggest endogenous CORT may control embryos' ability to hatch.

On the other hand, GC does not appear to be involved in hatching in fish with "small" eggs (except for rainbow trout). Cortisol either stays low until hatching (Perez et al., 1999; Szisch et al., 2005) or decline from fertilization to hatching (Barry et al., 1995a; de Jesus et al., 1991; Hwang and Wu, 1993; Hwang et al., 1992; Sampath-Kumar et al., 1995). In tilapia, this

decline is due to depletion of maternal cortisol before the young's HPI axis is functional (Hwang et al., 1992). Conversely "large"-egg species like salmon and carp increase ACTH and/or cortisol around hatchling (de Jesus and Hirano, 1992; Feist and Schreck, 2001; Stouthart et al., 1998). Thus it is possible that fish with k-selected traits (larger size, fewer offspring) have relatively mature HPI axis and elevate cortisol at hatching.

FLEDGING/DISPERSAL

Fledging

Fledging refers to an ontogenetic transition where young birds leave their nest. In some species, fledglings still cannot fly and are still fed by their parents. Regardless of fledglings' ability to fly, fledging poses young to sudden metabolic demands. Hormonal control or correlates of fledgling are still under debate but CORT is one of the promising candidates. Observational studies show that CORT increases prior to fledging in American kestrels (*Falco sparverius*) (Heath, 1997; Sockman and Schwabl, 2001), pied flycatcher (*Ficedula hypoleuca*) (Kern et al., 2001), canaries (*Serinus canaria*) (Schwabl, 1999), and Laysan Albatross (*Phoebastria immutabilis*) (Seabury Sprague and Breuner, 2005). Latter coincides with a decline in CBG, elevating free CORT even more. Furthermore, food supplementation to chicks delays both elevations of total and free CORT as well as fledging. Although it is difficult to compare capital and income breeders due to their diverse energetic demands in nestlings, CORT may be a common trigger for fledging.

Other species show heightened HPA activity during a fledgling period. In canaries, baseline CORT of 1 week fledglings is elevated compared to nestlings and adults (Schwabl, 1999). In American kestrels and zebra finches, fledglings have significantly higher stress-induced CORT than adults (Love et al., 2003) (Wada et al., unpublished data). In screech-owls (*Otus asio* and *O. Kennicottii*), baseline CORT peaks before and during an activity period associated with dispersal (Belthoff and Dufty, 1998). Thus, these relatively high CORT levels

may be related to increased locomotor, foraging activities, or changes in metabolism in preparations for fledging, dispersal, and independence.

Yet conflicting data exist in the roles of CORT in fledging. In these data, baseline CORT either does not change (Blas et al., 2005; Blas et al., 2006; Sims and Holberton, 2000) or decreases during the nestling period (Romero et al., 2006; Walker et al., 2005), or there is no difference between baseline CORT of fledged and non-fledged young (Love et al., 2003; Romero et al., 2006). Nesting location (ground or low brush vs. cavity or cliff) may contribute to the species variation seen in CORT near fledging (Heath, 1997; Romero et al., 2006). Fledglings of ground or brush nests may not take the first flight until days/weeks after fledging. On the other hand, fledglings of cavity or cliff nesters take the first flight as they leave the nest. Experimental manipulations on CORT levels are needed to determine the causal relationship between CORT and fledging.

Dispersal

Natal dispersal can be influenced by multiple factors and CORT is one of the proximate factors affecting propensity for dispersal. In birds, CORT enhances dispersal behavior in juveniles. Baseline CORT peaks before and during an activity period associated with dispersal in screech-owls (Belthoff and Dufty, 1998). In willow tits (*Parus montanus*), CORT implants in juveniles during flock establishment increase dispersal rates (Silverin, 1997). However, similar implants after flock establishment have no effect on dispersal, suggesting this effect of CORT on juvenile dispersal is limited to certain times of the year.

In addition to seasons, the timing of CORT elevation may determine its effect as well. Prenatal CORT exposure decreases dispersal in common lizards (*Lacerta vivipara*) (de Fraipont et al., 2000; Meylan et al., 2002) while postnatal CORT elevation has no effect on dispersal (Meylan et al., 2002). Body conditions of mothers and juveniles also affect the dispersal behavior: CORT decreases offspring dispersal only in ones from large mothers while good body

condition in juveniles increases dispersal (Meylan et al., 2002). This indicates that CORT modulates juveniles' dispersal behavior to one best adapted to the current environment, based on body condition and other factors.

METAMORPHOSIS AND SMOLTIFICATION

Metamorphosis

Thyroid hormones are known to be the main players of metamorphosis in amphibians and flatfishes. However, CORT acts in concert with thyroid hormones during the transformation of larvae. Triiodothyronine (T3) and/or thyroxine (T4) are elevated during metamorphosis in both taxa (e.g. de Jesus et al., 1991; Krain and Denver, 2004), and T3 or T4 alone can accelerate metamorphosis indicated by increased rate of tail resorption in tadpoles (Galton, 1990; Krug et al., 1983), dorsal fin ray resorption (de Jesus et al., 1990; de Jesus et al., 1998), and eye migration in flatfish larvae (de Jesus et al., 1990; Solbakken et al., 1999). Although CORT is also elevated in concert with metamorphosis (de Jesus et al., 1991; Glennemeier and Denver, 2002a; Krain and Denver, 2004; Krug et al., 1983; Sakakura et al., 1998; Szisch et al., 2005) (but also see Yamano et al., 1991), CORT alone fails to speed up the metamorphosis (Galton, 1990) (but also see de Jesus et al., 1990; Hayes et al., 1993). Yet, when tadpoles and fish larvae are treated with ACTH or CORT in addition to T3 or T4, combined treatments can elicit even faster metamorphosis than thyroid hormone alone (Brown and Kim, 1995; de Jesus et al., 1990; Galton, 1990; Krug et al., 1983), via further elevation of thyroid hormones (Galton, 1990) or increasing thyroid hormone receptors and conversions of T4 to T3 (Krain and Denver, 2004).

In amphibians, recent focus has shifted to CRF. Injections of CRF-like peptide accelerate metamorphosis (Boorse and Denver, 2002; Denver, 1993; Denver, 1997; Miranda et al., 2000) while CRF antagonist delays metamorphosis (Denver, 1997). The current view for the hormonal control of metamorphosis in amphibians is that CRF induces secretions of T3, T4, and CORT (reviewed in De Groef et al., 2006; Denver, 1998) and together orchestrate the

transformations in metamorphosis. It is important to note that other hormones like aldosterone, prolactin, and growth hormone are involved in this dramatic transition; the process is inhibited by prolactin and growth hormone.

Smoltification

Smoltification in anadromous fish prepares the young for the transition from freshwater to saltwater which requires a dramatic physiological, biochemical, and behavioral changes to adapt to the new salinity. Ontogeny of the seawater adaptation and its hormonal control are extensively studied and reviewed elsewhere (e.g. Mommsen et al., 1999; Varsamos et al., 2005) thus I will briefly review the role of cortisol in this process. During smoltification, multiple hormones are involved such as cortisol, prolactin, thyroid hormones, and growth hormone/insulin-like growth factor, among others.

Some of the main osmoregulatory physiology which cortisol regulates is the Na^+-K^+ ATPase activity and ion transport in gills and intestine. In salmonids, seawater tolerance, indicated by an increased Na^+-K^+ ATPase activity in the gills, is established while parrs are still in freshwater (Franklin et al., 1992; Ura et al., 1997). During the same time, an increase in cortisol is observed (Young, 1986). Not only are cortisol levels highly correlated with the gills' Na^+-K^+ ATPase activity (Franklin et al., 1992), but cortisol administrations significantly 1) increase gill Na^+-K^+ ATPase activity, gill Na^+-K^+-$2Cl^-$ cotransporter number, and gill chloride cells, 2) decrease plasma osmolarity and Na^+ concentrations, and 3) increase survival in seawater (Bisbal and Specker, 1991; Hwang and Wu, 1993; Madsen, 1990a; Madsen, 1990b; Mancera et al., 2002; Pelis and McCormick, 2001). Growth hormone and cortisol have additive effects on the ion transport; a combined hormone treatment results in significantly higher gill Na^+-K^+-$2Cl^-$ cotransporter abundance and Na^+-K^+ ATPase activity than when each hormone is administered alone (Madsen, 1990b; Pelis and McCormick, 2001).

CONCLUSION

Taken together, GCs have direct and permissive effects on various ontogenetic transitions in vertebrates (Table 1.1). GCs directly 1) promote maturation of critical organs before birth/hatch in mammals and birds, 2) initiate parturition events in mammals and possibly controls hatching abilities in birds and reptiles, 3) stimulate ion pump activity and abundance during smoltification in fish, and 4) CORT is a potential candidate for the timing of fledging in birds, although further studies are needed to determine the causal relationship. CORT also has a permissive action on thyroid hormones in amphibian and fish metamorphosis. However, cortisol does not appear to be involved in hatching in fish.

There is a wide range among taxa in the number of available studies concerning ontogeny of the HPA axis and the role of GCs in the ontogenetic transitions. Due to applications to medical and commercial practices, work has been mainly focused on mammals, cultured fish, and poultry. At the same time, relatively limited data are available in amphibians, reptiles, and wild caught birds, fish, and mammals. Future studies should encompass these taxa and groups, particularly in 1) the ontogeny of the HPA axis and 2) the role of CORT in organ maturation (Table 1.1). Toward this end, the present work investigates the ontogeny of the HPA axis in an altricial passerine.

Overall, GCs prepare organisms and trigger transitions into the subsequent life history stage. At the same time, there is variation in the pattern of HPA axis development within and across taxa. As mentioned earlier, the developmental strategy of each taxon/species is likely the determinant for this variation, such as direct vs. indirect development and r- vs. k-strategists. Future research should explore the common underlying mechanisms for GCs during development and evolution of these roles of GCs in vertebrate ontogeny.

WORKING HYPOTHESES

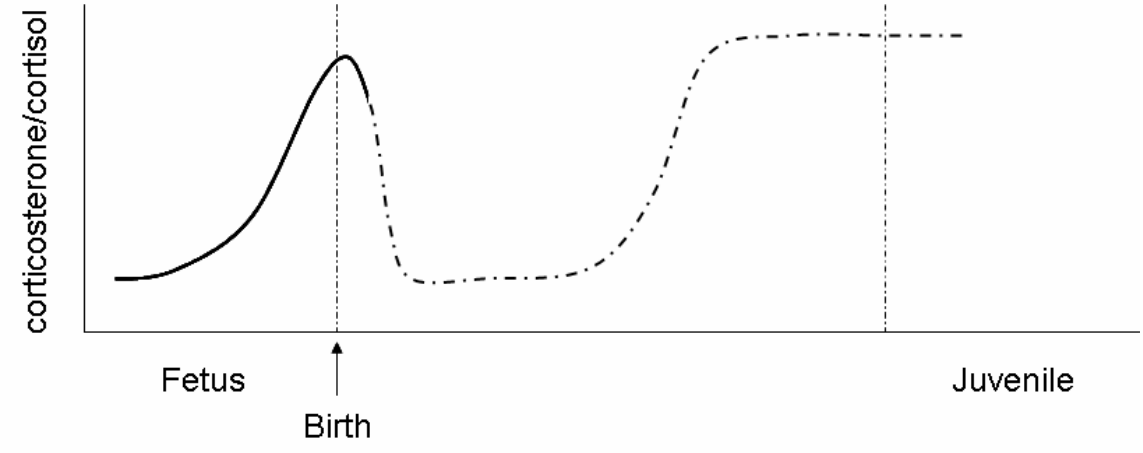
As mentioned above, developing animals may face a trade-off between the benefits and costs of GCs. To avoid deleterious effects of GCs, I hypothesized that animals going through substantial growth may have higher CBG levels, or enhance negative feedback/tonic inhibition on the HPA axis to keep GCs levels low. Here I investigated these hypotheses using altricial white-crowned sparrow nestlings. I examined 1) the ontogeny of the CORT response in total (bound and free) and free levels (Chapter 2), 2) changes in corticosteroid receptor levels in the brain with age (Chapter 3), and 3) effects of acute and extended elevation of CORT on behavior and growth (Chapter 4).

Table 1.1. Summary of direct and permissive effects of GCs on various ontogenetic transitions in vertebrates. ✓ and × denote the presence and the absence of the effects, respectively. '–' and '?' denote a lack of studies and a possible role of GCs during the ontogenetic event, respectively.

	Direct				Permissive
	organ maturation	parturition/ hatch	smoltification	fledging/ dispersal	metamorphosis
Mammals	✓	✓			
Birds	✓	?		?	
Fish	–	×	✓		✓
Amphibians	–	–		–	✓
Reptiles	–	?		✓	

Figure 1.1. Schematic representations of changes in baseline GCs during development.

a) mammals (dotted lines are for rodents)



b) precocial birds

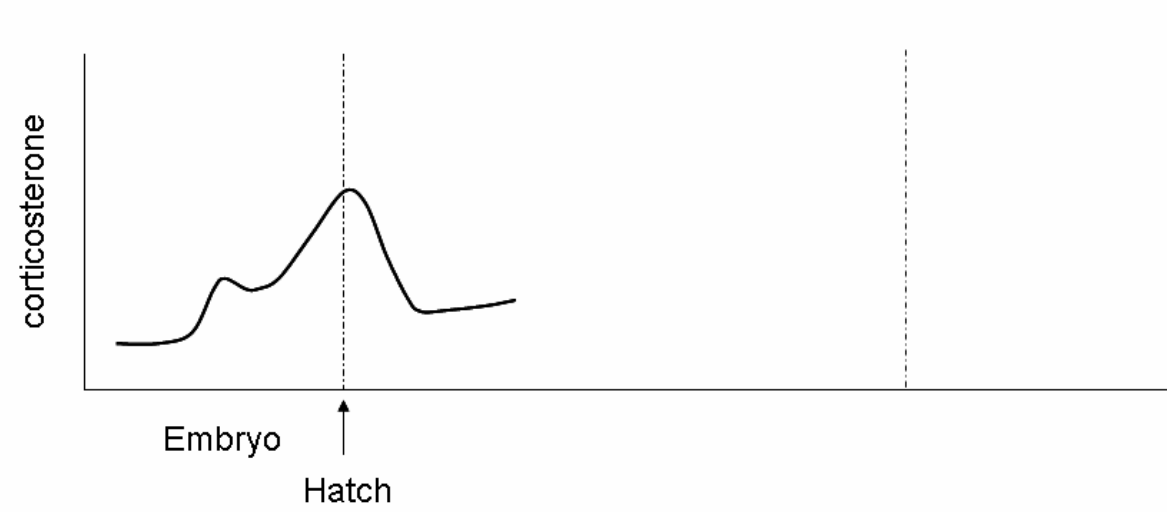
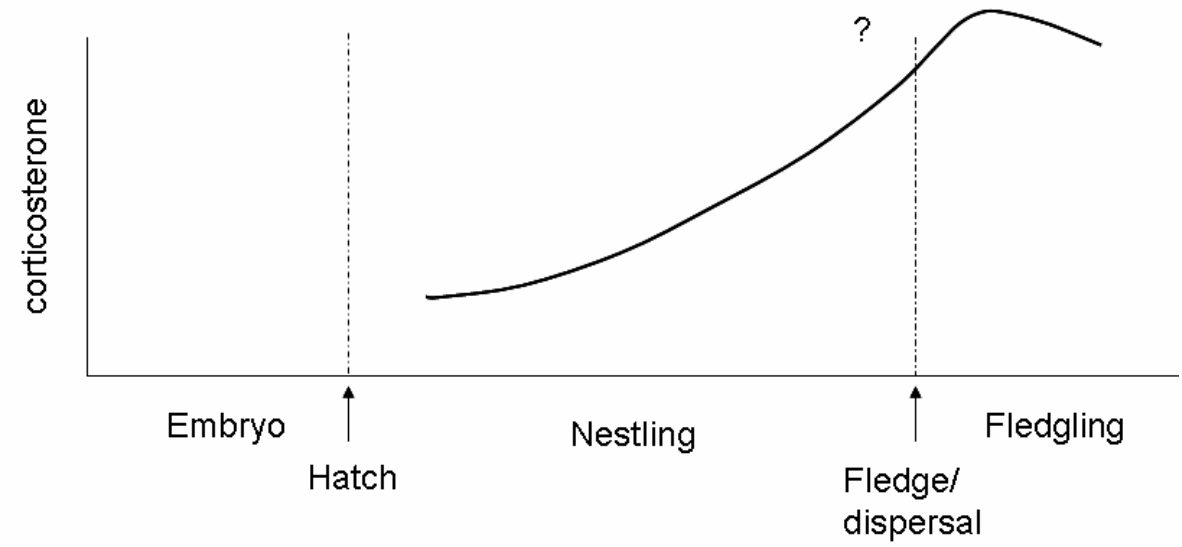


Figure 1.1.

c) non-precocial birds



d) non-salmonids fish

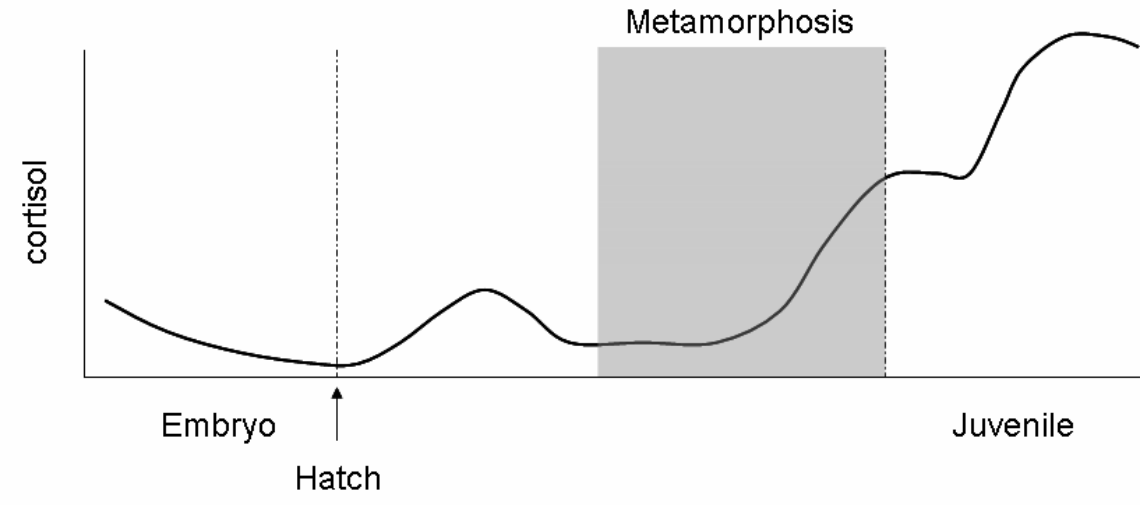
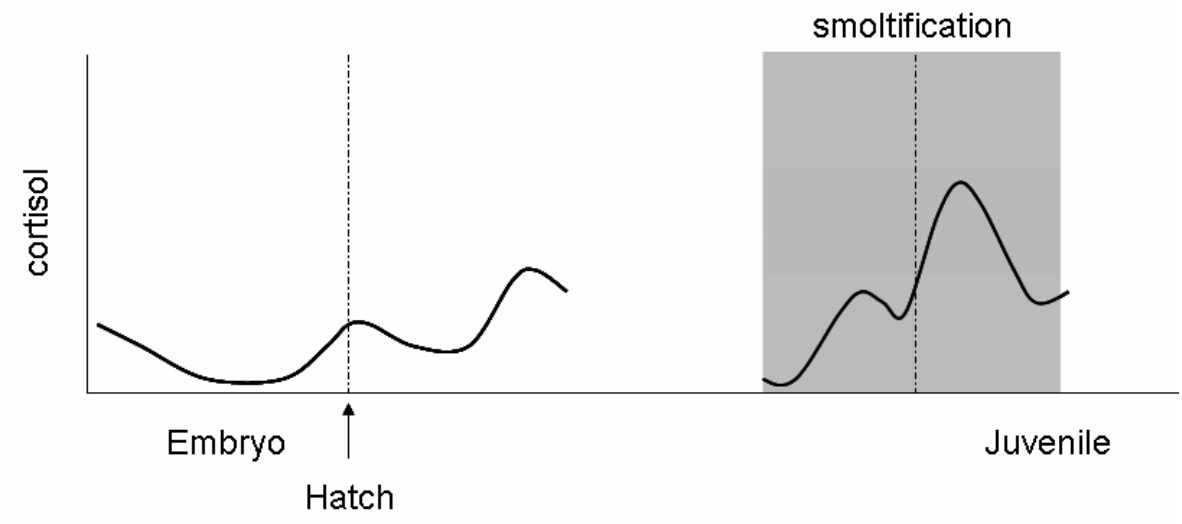


Figure 1.1.

e) salmonids



f) amphibians

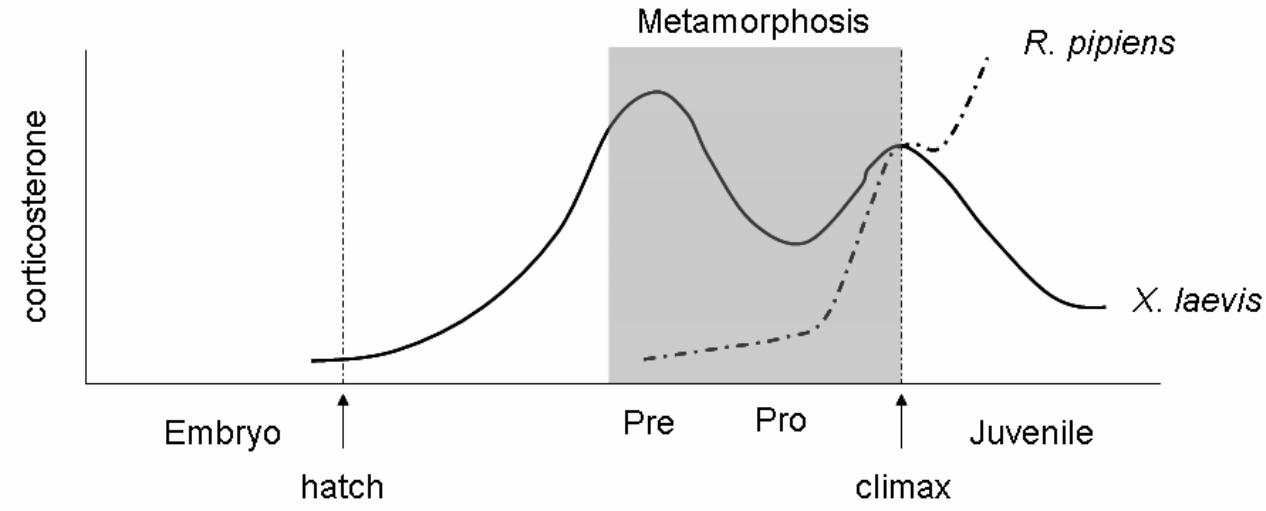
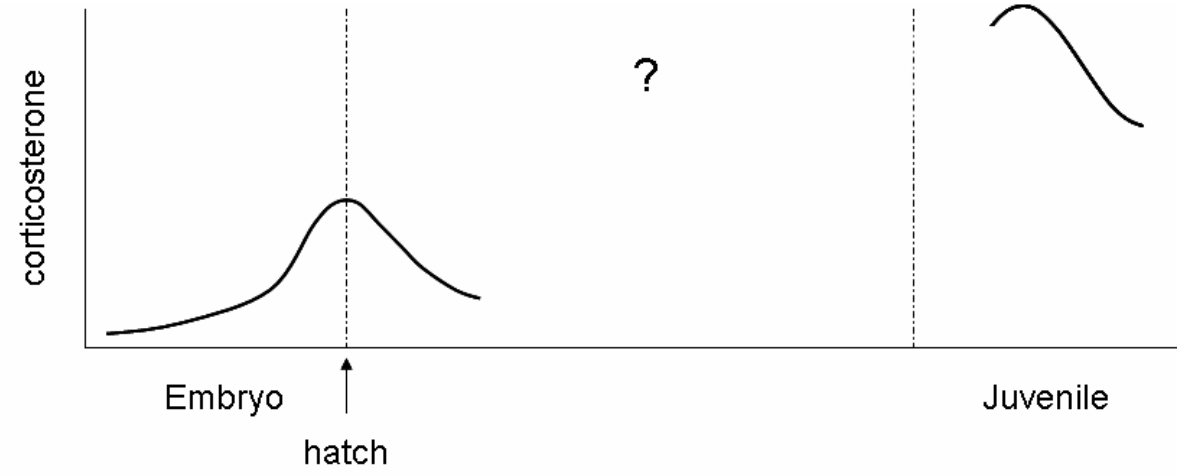


Figure 1.1.

g) reptiles



Chapter 2: Development of stress reactivity in white-crowned sparrow nestlings: total corticosterone response increases with age, while free corticosterone response remains low

INTRODUCTION

Free-living animals face a variety of challenges that can disrupt homeostasis. Activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to these disruptions is a well-conserved phenomenon. In adults, acute increases in glucocorticoids are thought to increase fitness (reviewed in Sapolsky et al., 2000), but extended secretion can have deleterious consequences (Petitte and Etches, 1991; Silverin, 1986; Tokarz, 1987; Wingfield and Silverin, 1986). In developing animals, this trade-off appears more severe. While glucocorticoid elevation has been shown to benefit young in obtaining food and transitioning to independence (Heath, 1997; Kitaysky et al., 2003; Kitaysky et al., 2001; Kitaysky and Wingfield, 2001), there are potent, long-term negative effects, such as decreasing growth (Morici et al., 1997), immune function (reviewed in McEwen et al., 1997), and neuronal cell number (Howard and Benjamins, 1975). Hence, the far-reaching consequences of elevated glucocorticoid secretion during development require tight HPA-axis regulation in young as compared to adults.

Previous studies indicate the presence of a stress hyporesponsive period in rat pups (Butte et al., 1973; Haltmeyer et al., 1966; Martin et al., 1977; Meaney et al., 1985a; Schoenfield et al., 1980) (reviewed in Sapolsky and Meaney, 1986) and in rainbow trout (Barry et al., 1995a). During this period, there is a reduction in the glucocorticoid response to stress. This reduction early in development is thought to minimize the long-term repercussions of elevated glucocorticoids. Although there is an extensive literature on the hyporesponsive period in rats, few studies address the phenomenon in birds.

In addition to suppression of the HPA axis, organisms have multiple mechanisms to regulate the amount of glucocorticoids reaching tissues. Corticosteroid binding globulin (CBG) may provide one such mechanism. The majority of plasma steroid is bound by binding globulins, with only 5-10% circulating unbound, or 'free' (Ekins, 1990). According to the free hormone hypothesis, only free hormones can enter tissues or be broken down; the free fraction is therefore thought to be the biologically active form of the steroid (Breuner and Orchinik, 2002; Ekins, 1990). If the free hormone hypothesis is correct, increasing CBG early in development may limit bioavailability of glucocorticoids during this critical period (Breuner et al., 2003; Lynn et al., 2003). However, the role of CBG in regulating bioavailability of glucocorticoids is currently under debate. In this light, we examine both total and free corticosterone (CORT) levels, allowing for interpretation of data based on either the total or free hormone hypotheses.

Toward this end, we explored the basic ontogeny of the adrenocortical response in nestling white-crowned sparrows, using standardized restraint stress (Wingfield, 1994). Furthermore, we performed adrenocorticotropic hormone (ACTH) challenges to assess developmental progression of the adrenals. These data will allow us to evaluate how total and free hormone levels are regulated in relation to crucial developmental stages in white-crowned sparrow nestlings. Periods of low stress reactivity would suggest protection from the deleterious effects of elevated corticosterone during critical developmental stages.

MATERIALS AND METHODS

Study Animals

Nuttall's white-crowned sparrow nestlings (*Zonotrichia leucophrys nuttalli*) were taken from nests at Bodega Marine Laboratory, University of California at Davis. Stress series samples (see below) were collected April through May of 2004, and ACTH challenges were performed April through June of 2003 and April through May of 2004.

White-crowned sparrow nestlings fledge around 10 days of age (Morton, 2002). During this 10-day nestling period, young go through dramatic developmental changes which include nearly an order of magnitude increase in body mass, feather growth, and acquisition of thermoregulatory ability, alertness, and coordinated movements (Morton, 2002). In this study, the nestling period is divided into three stages: days 1-3 (early nestling stage), days 4-6 (middle nestling stage), and days 7-9 (late nestling stage) (see discussion for description of the development during each stage).

Age of nestlings was estimated by 1) noting date of hatch (when nests are found in the egg stage) or 2) comparing developmental measures collected on the nestlings to the developmental schedule described in Morton (2002) and to personal observations of known age nestlings. Nests were randomly assigned to one of the three age categories for sampling. Each nest was sampled only once, and only one nestling per nest was sampled to avoid unequal representation of siblings in the three age groups. Sex was determined for late-stage nestling samples.

Stress Protocol

To measure reactivity of the HPA axis we used a standardized capture and handling protocol described in Wingfield (1994). Plasma corticosterone levels are

known to rise significantly within three minutes of capture (Wingfield et al., 1982). However, in this study corticosterone had still not begun to rise by four minutes after nest disturbance (Fig. 2.1). Therefore, blood samples collected within four minutes of nest disturbance are used as baseline samples. After the initial sampling, nestlings were placed in a cloth bag and additional samples were collected at 15, 30, and 60 minutes. To avoid potential complications caused by changing nestling body temperature under different field conditions, all were kept inside a jacket to keep them near human body temperature. To collect blood samples, the alar vein was punctured with a 26G needle and 20-30 μL of blood were collected into heparinized microhematocrit capillary tubes. The blood samples were kept cold on ice until the end of the day when they were centrifuged to separate plasma from the cellular fraction. The plasma samples were then kept at minus 20°C or below until the assays.

In addition to blood samples, developmental measurements (tarsus and wing length, body mass, feather development, and alertness) were taken. To minimize the time nestlings were away from the nest, those developmental measurements were collected between the 15 and 45 minute samples.

ACTH challenges

Similar to the stress response protocol, baseline samples were collected within four minutes of capture. After the initial sampling, nestlings and adults were weighed and injected with ACTH (Sigma: 100 I.U./kg body weight) in saline or saline alone into the jugular vein. The post-treatment samples were collected 30 minutes after the injection.

Measurement of corticosterone: EIA assay

Serum corticosterone levels were detected using Enzyme Immunoassay (EIA) kits (cat # 901-097, Assay Designs). In this assay, raw plasma is added directly to the wells without extraction; steroid displacement buffer (SDB) is added prior to the assay to degrade binding globulins. For every new species we optimize the assay using a protocol that is a bit more complicated than the usual RIA optimization method. To ensure that plasma is not interfering with hormone measurements, we strip plasma to remove any endogenous hormone and spike to a known amount of CORT. This way, as we dilute plasma (into a buffer containing the same amount of CORT) we will be able to measure directly how much interference is caused by the plasma, outside of variance introduced by unknown levels of hormone in the plasma. An additional complication is that Assay Designs provides a 'steroid displacement buffer' to degrade binding globulins. This SDB is a protease, and we have found that it can produce significant interference if used at too high a concentration. Therefore our optimization incorporates plasma dilutions at multiple concentrations of SDB.

Optimization Protocol: Plasma dilution and SDB concentration were optimized using stripped, spiked plasma (stripped with 1% norit A Charcoal and 0.1% dextran in water, then spiked to approximately 500pg/ml with CORT standard from the assay) diluted into a buffer of known corticosterone concentration (~500pg/ml). Samples were run against a standard curve at plasma dilutions of 1:10, 1:20, 1:40 and 1:60, each with 0, 1, and 2% SDB (% of raw plasma volume). Results are shown in Figure 2. Each point represents the value of CORT given by the assay at each plasma dilution for each concentration of SDB. The grey box represents the mean ± SEM measured from the dilution buffer alone (the CORT-spiked buffer used to dilute the plasma, so representing

the expected CORT level in the samples), giving a range of acceptable values for CORT from the assay.

For the corticosterone EIA of white-crowned sparrow plasma, a plasma dilution of 1:40 or greater with 1% SDB (per raw plasma volume) eliminated measurable effects of plasma on the assay (Fig. 2.2). Optimizing SDB concentration is critical, as higher concentrations appear to degrade antibody activity in the assay, artificially increasing estimated corticosterone amounts in wells.

To determine corticosterone levels, 10µl 1:100 dilution of SDB buffer were added to 10µl plasma. After 5 minutes, 380µl assay buffer were added to each sample, vortexed, and aliquoted to individual wells in the assay plate (100µl sample per well, each in triplicate). The standard curve was measured in triplicate as well, with six standards ranging from 2000pg/ml to 15.63pg/ml (100µl per well). A separate, external standard of 200pg/ml corticosterone was run in triplicate on every plate. For the first incubation with conjugated corticosterone and antibody, the plate was shaken for two hours in a 26ºC incubator. For the second incubation with substrate solution, the plate was incubated at the same temperature for one hour without shaking. After adding stop solution, the plate was read immediately on a Multiskan Ascent microplate reader at 405 nm corrected at 595 nm. Samples were completely randomized within and across plates. Inter- and intra-plate coefficient of variations were 21.5% and 7.98% respectively, and detectability was 6.5pg/well (2.6ng/ml). The detection limit was determined by taking two standard deviations away from the mean of the blank wells. In EIA assays, total binding wells (B0) receive only buffer, conjugate, and antibody. Thus we used these total binding wells as blanks to determine the detection limit. This limit was assigned to undetectable samples in the assay.

Measurement of corticosteroid binding globulin

Serum CBG levels were measured using a ligand-binding assay with tritiated corticosterone (described in Breuner et al., 2003). Previously, the CBG assay was characterized for white-crowned sparrow plasma (Lynn et al., 2003); optimal plasma dilution was 1:900 with an incubation period of 2 hours at 4°C. In these assays, total binding was determined using 50µL buffer, 50µL ^{3H}corticosterone, and 50µL stripped plasma. Non-specific binding was determined using 1µM unlabeled corticosterone instead of buffer. The affinity (K_d) of corticosterone for CBG at different ages was determined by equilibrium saturation binding assay. This was done using plasma pools for the three age classes and ^{3H}corticosterone between 0.30nM and 13.25nM. CBG capacity in individual samples was identified with a point sample assay using 18.9 nM ^{3H}corticosterone. Based on characterization assays, this ligand concentration should occupy 84.5% of the total binding sites. Therefore, we adjusted the CBG capacities to 100% for the free hormone analysis. Since CBG levels do not change within one hour of corticosterone elevation in white-crowned sparrows (Breuner et al., 2006), plasma from any time point within 60 minutes was used for the CBG assay. Intraassay variation for the point sample assay was 14.6%.

Free hormone levels were estimated using an equation by Barsano and Baumann (1989):

$$H_{free} = 0.5 \times \left[H_{total} - B_{\max} - 1/K_a \pm \sqrt{(B_{\max} - H_{total} + 1/K_a)^2 + 4(H_{total}/K_a)} \right]$$

where K_a is $1/K_d$ (nM), K_d is affinity of corticosterone for CBG, B_{max} is total CBG capacity, and H_{total} is total plasma hormone concentration. K_d was determined in equilibrium binding analysis using pooled plasma.

Statistics

The statistical analysis was done using SPSS 11.5 and GraphPad Prism 3.02, except for the 2-segment breakpoint analysis on free CORT (Fig. 2.8), which was completed in S+ 7. The effects of handling, age, and ACTH treatment on total and free corticosterone levels were determined by repeated measures ANOVA, followed by Bonferonni correction (stress response and ACTH studies were analyzed separately). CBG capacities were log transformed to correct for heteroscedasticity and the effect of age was determined using one-way ANOVA, followed by Tukey HSD. One-way ANOVA was used to compare the K_ds from 3 age groups. Data were considered to be significant when $P \leq 0.05$. Data are presented as mean ± SE.

When total corticosterone exceeds CBG capacity, it results in greatly elevated free corticosterone estimation. When the resultant free levels fell outside of two standard deviations of the mean, those values were excluded from the analysis (3 out of 94 total samples).

RESULTS

Total corticosterone levels

Overall, handling ($F_{3,18} = 17.23$, $P < 0.001$), age ($F_{2,19} = 20.38$, $P < 0.001$), and the interaction between handling and age ($F_{6,15} = 4.95$, $P < 0.001$) all contributed significantly to total corticosterone levels (Fig. 2.3). Pairwise comparison results show that the corticosterone levels of earlier two stages (D1-3 and 4-6) are significantly different from the later stage, D7-9 (D1-3 and D4-6, $P < 0.05$; see Table 2.1 for corrected and non-corrected P values). By 30 minutes, D7-9 corticosterone levels were significantly higher than those of the earlier two stages. Furthermore, while early-stage

nestlings (D1-3) show no significant increase in corticosterone over the 60 minute protocol (15, 30, and 60 minute samples against 0 minute sample: pairwise comparison $P > 0.05$; see Table 2.1 for pairwise comparison and bonferroni correction p values), nestlings from latter two stages responded to handling stress with significant increase in corticosterone (D4-6 and D7-9: $P < 0.05$; again, see Table 2.1). Baseline levels did not differ between age groups ($P > 0.05$). The low n in this study (combined with the bonferonni corrections) increases the potential for a Type II error. In addition, it is difficult to determine whether an absence of a statistically significant CORT response represents an absence of effect at the biological level (what increase in CORT is biologically relevant for the animal?). In this light, we choose to discuss the data in terms of 'limited stress reactivity' in the younger age groups, as opposed to 'no stress response' as indicated by the statistics.

Corticosteroid binding globulin

The affinity (K_d) of corticosterone for CBG did not change throughout the nestling period (Fig. 2.4, day 2-3, 4-6, and 7-9 nestlings = 3.13 ± 0.60 nM, 3.12 ± 0.33 nM, and 4.19 ± 0.67 nM respectively; $P = 0.304$). Additionally, while mean CBG capacity increased with age, this difference was not significant ($F_{2,21} = 2.06$, $P = 0.15$, Fig. 2.5).

Free corticosterone levels

There were significant overall effects of time (0-60 minutes: $F_{2,17} = 5.97$, $P = 0.001$) and interaction between time and age ($F_{6,13} = 2.53$, $P = 0.03$; Fig. 2.6), but no main effect of age ($F_{2,17} = 0.68$, $P = 0.52$). Pairwise comparisons determined that early and

middle-stage nestlings showed no significant response in free CORT levels (D1-3 $P > 0.05$; D4-6 $P > 0.05$; see Table 2.1), while late-stage nestlings showed a robust response ($P < 0.05$).

ACTH challenges

Overall, time, age, and treatment had significant effects on total corticosterone levels in nestlings challenged with ACTH ($F_{1,43} = 140.88$, $P < 0.001$; $F_{3,41} = 13.04$, $P < 0.001$; $F_{1,43} = 21.68$, $P < 0.001$ respectively, Fig. 2.7). In addition, there were significant interactions between time and age ($F_{3,41} = 7.77$, $P < 0.001$), and time and treatment ($F_{1,43} = 19.90$, $P < 0.001$).

All ages responded to ACTH injection with a significant increase in corticosterone ($F_{1,43} = 21.68$, $P < 0.001$). Nonetheless, the response in the early-stage nestlings was significantly lower than that of the late-stage nestlings ($P < 0.05$). Furthermore, the response in the late-stage nestlings was not significantly different from that of adults ($P > 0.05$). When ACTH and saline treatment groups were compared, ACTH elicited a significantly stronger response than saline in the later two stages ($P < 0.05$). The early-stage nestlings show a strong trend as well.

DISCUSSION

White-crowned sparrow nestlings show an extremely diminished corticosterone response to restraint during the first three days post-hatch. In addition, CBG capacity increases with age. This CBG increase with age further diminishes the glucocorticoid response to stress, especially in the middle age group. As a result, white-crowned

sparrow nestlings show low stress-reactivity (in free CORT) lasting through the first six days of the 10-day nestling period.

It is difficult to visually evaluate this extension of low reactivity from figures 3 and 6 (total and free CORT at 0, 15, 30, and 60 min). To more clearly exemplify the shift in patterns of CORT secretion between total and free, we have calculated an integrated measure of CORT.

Integrated CORT is a measure of the total amount of CORT secreted over the hour of restraint stress (expressed as ng/ml/hr); with only one value for each individual, it simplifies the comparisons between individuals, ages, and total or free (as used in Breuner et al., 1998). Regression analysis of **total** integrated CORT by age shows a linear increase over the 9 days of the nesting cycle (Fig. 2.8a: $R^2 = 0.66$, data were best fit by a linear model). However, regression analysis of free integrated CORT shows that CORT response to stress remains low over the first 6 days of development (Fig. 2.8b: R2 = 0.64, 2-segment linear regression (following Duggleby and Ward, 1991) identifies the best-fit breakpoint at 6.49 days). Hence, whether one favors the total or free hormone hypothesis will lead to vastly different conclusions on the development of stress reactivity in nestling white-crowned sparrows.

The hyporesponsive period has been described mainly in mammals and some in fish. In rainbow trout, larvae are resistant to handling and cold shock stress until two weeks post-hatch (Barry et al., 1995a). In rats, baseline cortisol levels decline after birth and remain low until the middle of the second week (Martin et al., 1977; Meaney et al., 1985b) (reviewed in Sapolsky and Meaney, 1986). At the same time, rat pups fail to respond to various stressors such as histamine (Butte et al., 1973), shock and heat (Haltmeyer et al., 1966), and ether (Schoenfield et al., 1980) during this time.

The patterns of CBG in postnatal development have been investigated in some mammals, but the literature shows mixed results. In neonatal pigs, cortisol remains constant from day one through ten; however, due to an increase in CBG, free cortisol levels decline with age (Heo et al., 2003) (note that it is not clear whether these samples represent baseline or stress-induced levels). Rat pups, on the other hand, have extremely low levels of CBG pre-weaning. 6-day old pup CBG is less than 3% of adult levels, but rises to 25% of adult levels by day 15 (Viau et al., 1996). Some studies suggest that cortisol and CBG follow very similar patterns resulting in similar changes in total and free cortisol (Henning, 1978). However, others indicate that an increase in CBG precedes the postnatal elevation of cortisol, which would keep the free levels low (Tinnikov, 1993).

In this study, we also observed a period of low stress reactivity (with no significant increase in CORT) in the first 3 to 6 days of development. This appears functionally equivalent to mammalian stress hyporesponsive period. This hyporesponsive period in young is thought to be adaptive due to the deleterious effects of glucocorticoids on growth and development (Sapolsky and Meaney, 1986). As noted above, chronically (weeks to months) elevated levels of glucocorticoids during development are known to suppress growth (Morici et al., 1997) and protein synthesis, damage neuronal cells (Howard and Benjamins, 1975), and alter development of cognition (Kitaysky et al., 2003) and the HPA axis (see Seckl, 2001). Thus, avoiding high levels of the hormone during a critical period in development may be adaptive (Sapolsky and Meaney, 1986). This may be especially important for species which undergo substantial developmental changes after birth/hatching.

In birds, a hyporesponsive period could be important for a variety of reasons. Many passerines gain 90% of adult body mass during the first 10-20 days of life

(Ricklefs, 1968). Similarly, altricial white-crowned sparrow nestlings go through major developmental changes within the first three days of their lives (Morton, 2002). This includes eye development and maximum increase in body size (Morton and Carey, 1971). During this period, nestlings show a nearly logarithmic increase in body mass. Therefore, avoiding exposure to corticosterone may be needed and adaptive since young are increasing tissue mass and developing essential organs.

The middle stage (D4-6) is critical for development as well. During this period, nestlings open their eyes, complete body mass growth, and increase alertness and coordination of movements (Morton, 2002; Morton and Carey, 1971). By the end of this stage, coordination of the movements is nearly established and the transition from ectothermy to endothermy has been made. Based on total corticosterone, the 'hyporesponsive' period is most extreme during only the first three days post-hatch. However, when free corticosterone is estimated, corticosterone response is indistinguishable between early and middle-stage nestlings. Thus, CBG may be one component of a mechanism to further protect the vulnerable tissues from corticosterone exposure during development.

One of the beneficial effects of corticosterone in adults is an increase in activity levels (Breuner et al., 1998). In the early-stage nestlings, induction of escape behavior is not adaptive (Sims and Holberton, 2000). Interestingly, the only age group that shows a robust increase in free corticosterone is the late nestling stage (D7-9). By this age, nestlings can fledge if they are threatened and can move away from the nest (Morton, 2002).

Corticosterone secretion in both sexes of late-stage nestlings appears to be identical until 30 minutes of handling (data not shown). The sample size is too small to

run statistics here; however, it is an intriguing question to explore when sex difference in adrenocortical response appear in altricial species.

The developmental status of newly hatched nestlings varies among species. Precocial nestlings, for instance, can walk, feed, and thermoregulate. On the other hand, altricial nestlings are nest-bound and completely dependent on parents for feeding and brooding. Considering the degree of development these nestlings go through after hatching, suppressed stress responses may be more important in altricial than precocial young. Several studies support this expectation. Semialtricial nestlings gradually increase adrenocortical response to handling with age, and show adult-like responses immediately before fledging (American kestrel *Falco sparverius*, Love et al., 2003; Magellanic penguin, *Spheniscus magellanicus*, Walker et al., 2005). In contrast, altricial Northern mockingbird nestlings (*Mimus polyglottos*, Sims and Holberton, 2000) show little or no stress response any time during the nestling period. In this study, we found that altricial nestlings *could* respond to restraint stress before fledging, however responses did not reach adult-like levels before fledging.

ACTH challenge

Early-stage white-crowned sparrow nestlings secreted little to no corticosterone in response to handling stress. ACTH challenge was used to understand the role of the adrenal during this hyporesponsive period. Early stage adrenals were unable to produce adult-like responses to ACTH, whereas late-stage adrenals could. These data indicate adrenals contribute to the low stress reactivity seen in these early-stage nestlings. However, early-stage adrenals did show a significant corticosterone response to ACTH, where little to no response occurred with restraint stress alone (similar to mammals; Cote

and Yasumura, 1975; Hiroshige and Sato, 1971). This indicates that the low reactivity is also due to dampened up-stream control of pituitary, hypothalamus, or neuronal input to hypothalamus.

We observed some discrepancy in baseline total corticosterone data in the different parts of our study. Stress series data suggest there was no increase in baseline corticosterone with age. However, ACTH data indicate that baseline levels increase significantly between the earlier two stages (D1-6) and the late stage (D7-9). Group means between the two studies are similar however variation is greater in the stress series study. When all the baseline data from two studies are pooled the baseline corticosterone levels increase significantly between D1-6 and D7-9 (one-way ANOVA, $F_{2,66} = 5.77$, $P = 0.005$).

Conclusions

This is one of the first studies to demonstrate that altricial nestlings can respond to a stressor before fledging. Nonetheless, white-crowned sparrow nestlings have a period of extremely low stress reactivity during the first three days of 10-day nestling period. This period is extended through day six when considering free corticosterone, due to increasing CBG. This may be adaptive to further protect developing tissue from excessive exposure to corticosterone.

Is this period of low stress reactivity due to specific regulation of corticosterone and binding globulins, or simply to the developmental progression of the HPA axis and liver? We are unable to assess whether the low levels of CORT after hatch are a suppression of a previously able adrenal, since we do not have CORT responses to stress pre-hatch. Chickens shows a decline in responsiveness just post-hatch (Freeman and Manning, 1984), but evidence from a precocial species is difficult to transfer to an

altricial one. In white-crowned sparrows, we hypothesize that the increasing stress reactivity is primarily due to development of the organs; however, as a result of their differential rates of development (CBG levels increase at a faster rate than total CORT), free CORT shows a more extreme hyporesponsive period later in development.

Table 2.1. Bonferonni corrected and non-corrected P values.

			Bonferonni	**Non-corrected**
Total Corticosterone	Age	D1-3 < D7-9	< 0.001	< 0.001
		D4-6 < D7-9	0.003	0.001
	Time (B0 to others)	D1-3	0.215, 1.00, and 1.00	0.036, 0.0190, and 0.816
		D4-6	0.035, 0.010, and 0.056	0.006, 0.002, and 0.009
		D7-9	0.006, <0.001, and <0.001	0.001, <0.001, and <0.001
	Baseline	D1-3 = D4-6 = D7-9	1.000, 1.000, and 1.000	0.662 and 0.808
Free Corticosterone	Time	D1-3	0.100, 0.329, and 1.00	0.017, 0.055, and 0.966
		D4-6	0.557, 0.141, and 0.114	0.093, 0.023, and 0.019
		D7-9	1.00, 0.004, and 0.147	0.646, 0.001, and 0.025
ACTH challenge	Age (overall)	D1-3 = D4-6	0.247	0.041
		D1-3, D4-6 < D7-9	<0.001 and 0.016	<0.001 and 0.003
		D7-9 < Adult	0.012	0.002
	Age (ACTH response)	D1-3 < D7-9	0.002	<0.001
		D7-9 = Adult	1.000	0.204
	ACTH vs. Saline	D1-3	0.063	0.063
		D4-6	0.005	0.005
		D7-9	0.001	0.001

Figure 2.1. Baseline corticosterone levels in relation to time after capture.

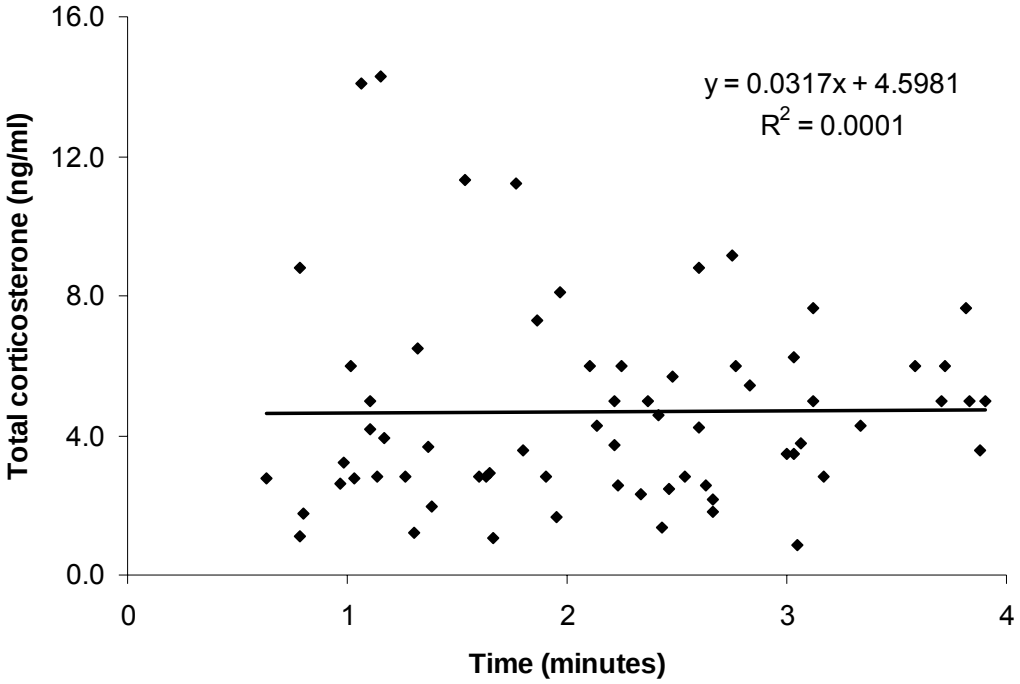


Figure 2.2. Optimization of EIA corticosterone assay for white-crowned sparrows testing plasma dilution and % steroid displacement buffer (SDB). Plasma was stripped and spiked to a known amount prior to the assay, and plasma was diluted into a buffer with the same value of CORT. As the dilution factor increases, the values become closer to the actual corticosterone levels added. The grey box represents the mean ± SEM measured from the dilution buffer alone (the CORT-spiked buffer used to dilute the stripped, spiked plasma, so representing the expected CORT level in the samples), giving a range of acceptable values for CORT from the assay. A plasma dilution of 1:40 or higher, with 1% SDB, eliminated any measurable effects of plasma in the assay.

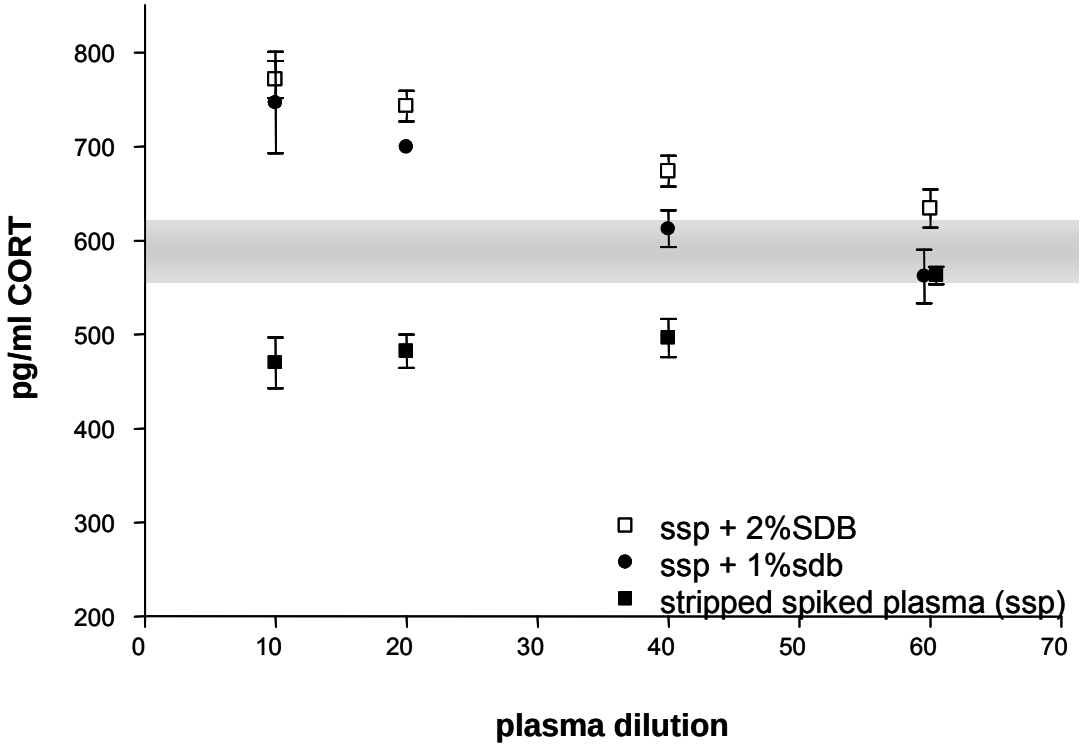


Figure 2.3. Changes in total corticosterone levels in response to handling stress. Data are means ± SE (n = 9, 8, and 7). Both handling and age had significant effects on total corticosterone levels.

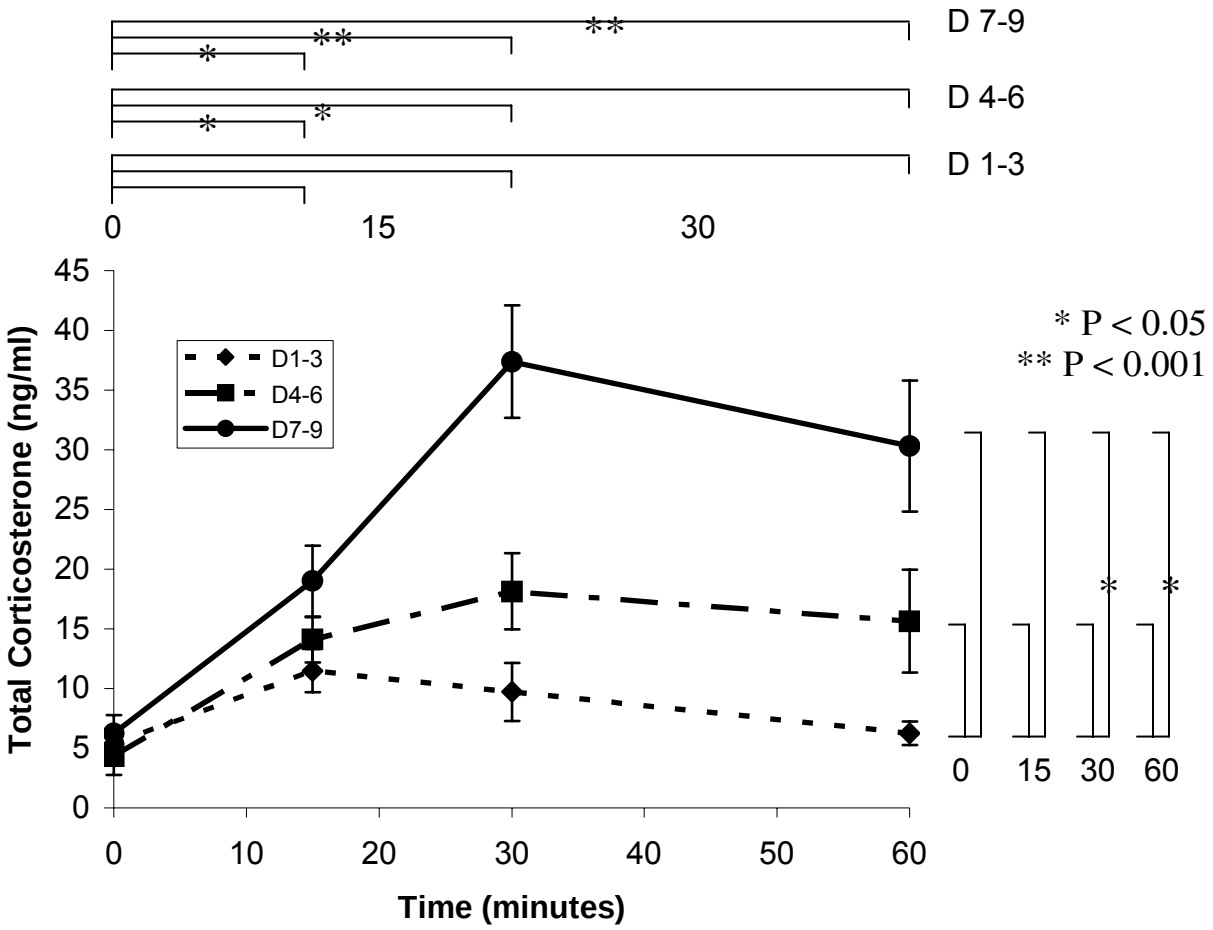


Figure 2.4. Equilibrium saturation binding of [^{3}H]CORT to nestling plasma at D2-3, D4-6, and D7-9. Data shown are specific binding (means ± SE at each concentration). Data are best fit by a one-site model, K_ds do not differ between age groups. Inset: Scatchard-Rosenthal replot of the data (for clarity, only D2-3 data is shown).

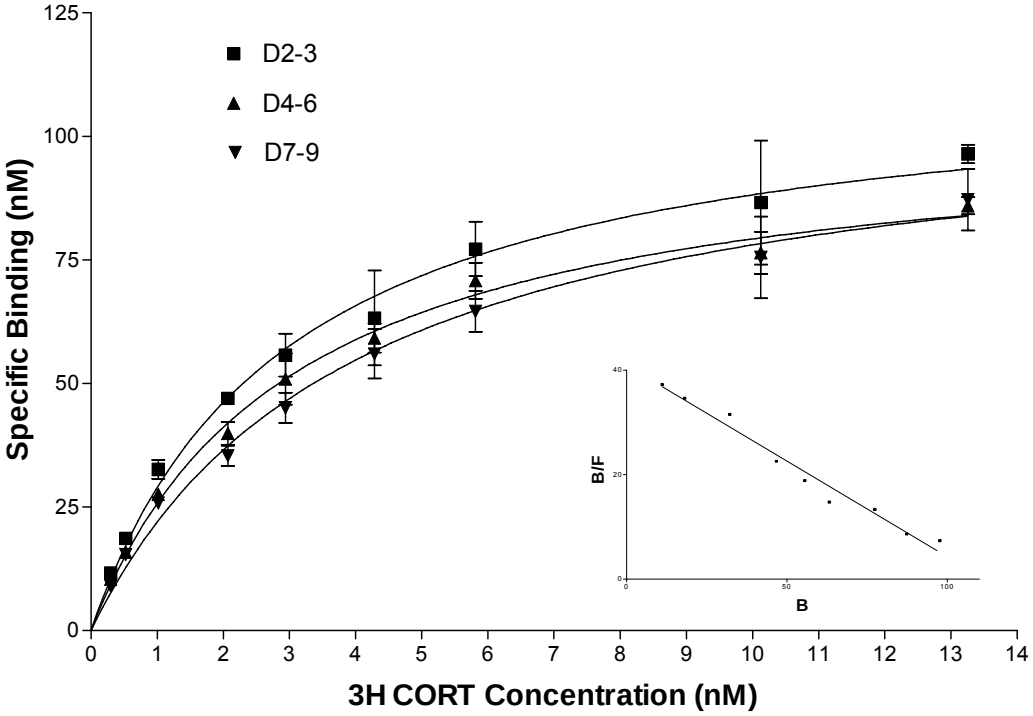


Figure 2.5. Corticosteroid binding globulin capacity during 10-day nestling period. Data are means ± SE (n = 9, 8, and 7). There was a trend towards increasing CBG with age.

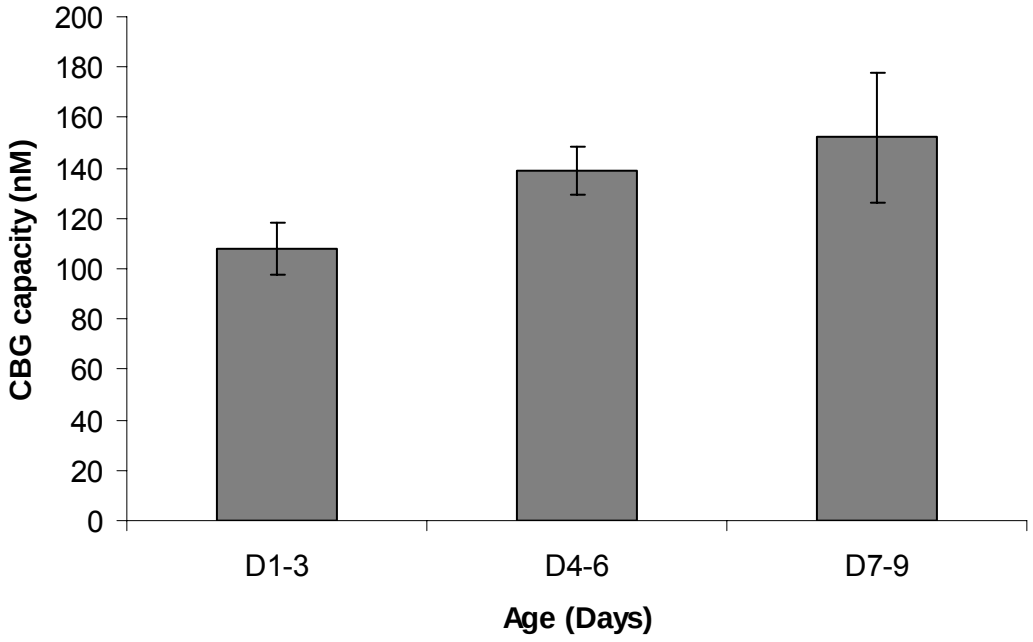


Figure 2.6. Changes in free corticosterone levels in response to handling stress during the 10-day nestling period. N = 9, 7, and 6.

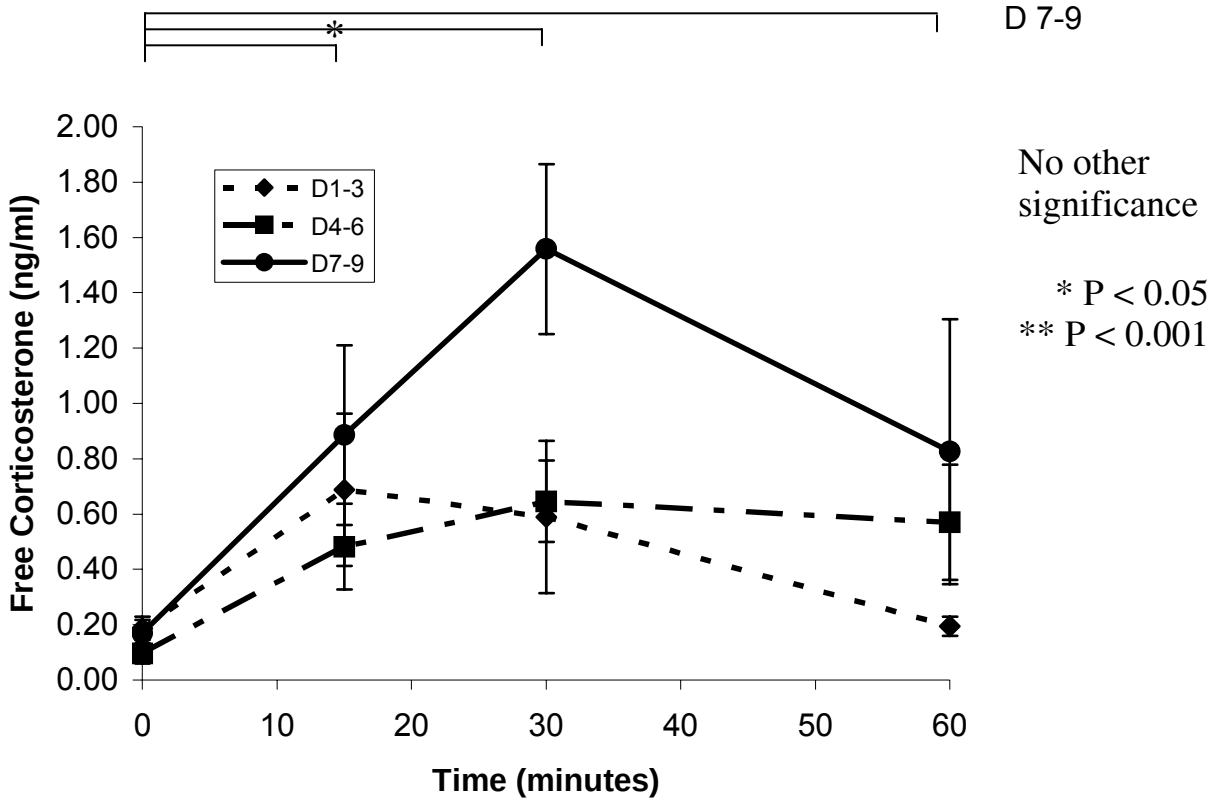


Figure 2.7. Corticosterone secretion in response to jugular injection of saline or ACTH. Data shown are means ± SE (sample size for 0', saline, and ACTH groups are: early nestling stage, 11, 6, and 6; middle nestling stage, 19, 9, and 11; late nestling stage, 15, 9, and 7; adult, 5 and 5 respectively). This graph combines 0' from both treatments, however, they were evaluated separately for statistics.

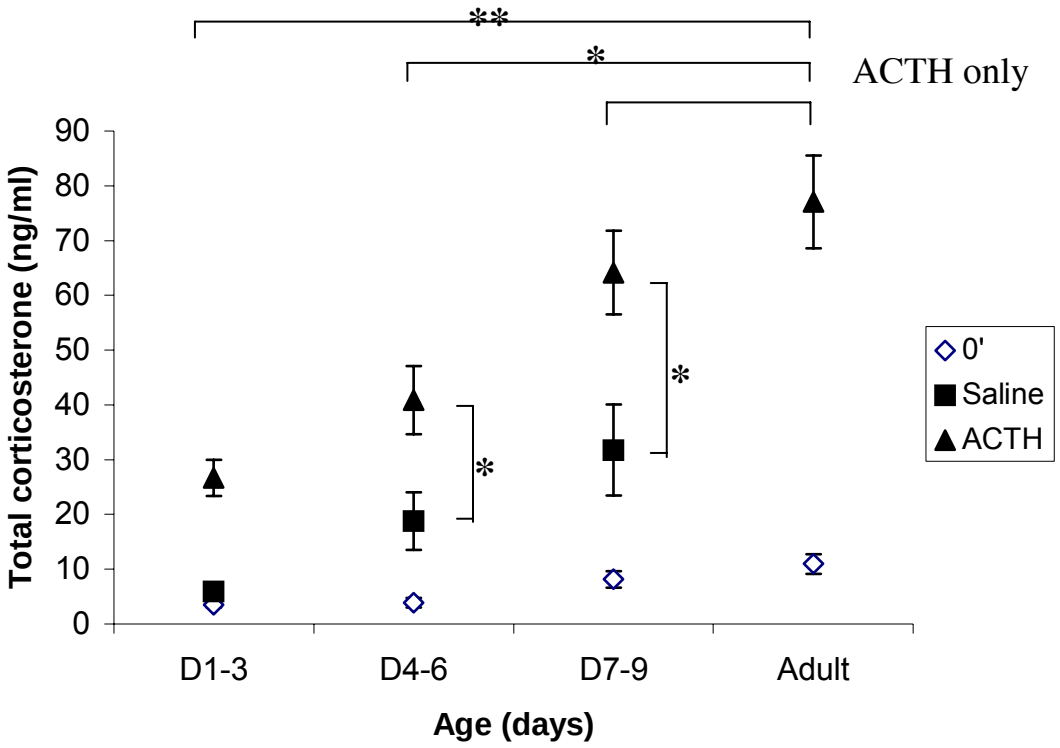
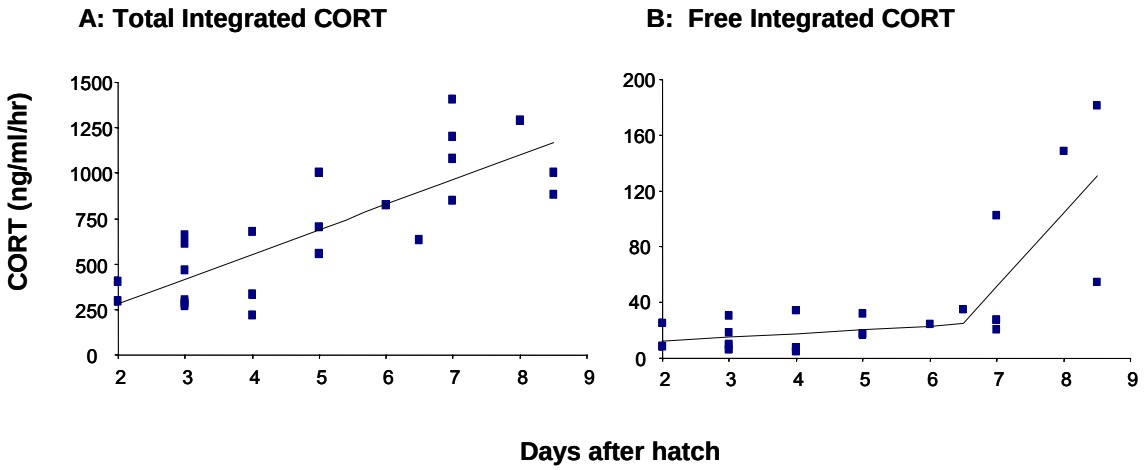


Figure 2.8. Integrated corticosterone: each point represents a calculation of the cumulative amount of corticosterone (either total (A) or free (B) corticosterone) secreted in each individual nestling over the hour of restraint stress. Data are plotted by age of nestling. These data help illuminate the extension of low stress-reactivity (into days 4-6) when free CORT is considered.



Chapter 3: Developmental changes in neural corticosteroid receptor capacity in altricial nestlings

INTRODUCTION

Several mammalian and fish species exhibit a period of hyporesponsiveness to certain stressors during a critical developmental phases (called a stress hyporesponsive period, SHRP) (reviewed in Sapolsky and Meaney, 1986; Vazquez, 1998; Walker et al., 2001). This is characterized by blunted corticotrophin and glucocorticoid secretion from the pituitary and adrenal/interrenals. Unfavorable effects of glucocorticoids on growth (Glennemeier and Denver, 2002c; Hayward and Wingfield, 2004; Janczak et al., 2006; Mashaly, 1991; Meylan and Clobert, 2005; Morici et al., 1997; Saino et al., 2005; Spencer et al., 2003; Wan et al., 2005), immune function (Morici et al., 1997; Rubolini et al., 2005), neuron formation (Howard and Benjamins, 1975), and survival (Janczak et al., 2006; Mashaly, 1991; Saino et al., 2005) (but also see Meylan and Clobert, 2005) led researchers to believe that the SHRP is an adaptive characteristic, serving to minimize detrimental effects of glucocorticoids during development.

In birds, little work has been done on a possible SHRP in altricial species. Although an embryonic hypothalamic-pituitary-adrenal (HPA) axis is yet to be examined, young do not respond to handling stress during early post-hatch period (Romero et al., 1998; Sims and Holberton, 2000; Wada et al., 2007). This low corticosterone (CORT) secretion early in development may be a result of immature HPA axis or enhanced control of the axis.

Negative feedback on the HPA axis largely controls the HPA reactivity to stress via corticosteroid receptors in hypothalamus and hippocampus (i.e., tonic inhibition, Herman et al., 1989; Sapolsky et al., 1984; Sapolsky, 1991). It is possible that the

dampened responses seen in early-stage altricial nestlings are due to enhanced corticosteroid receptor control of HPA axis. There are two receptor types for glucocorticoids; high affinity, mineralocorticoid receptors (MR, type I) and low affinity, glucocorticoid receptors (GR, type II). MR is occupied under basal conditions and suggested to mediate tonic inhibition during diurnal rhythm (Dallman et al., 1987; De Kloet and Reul, 1987; Reul and De Kloet, 1985), while GR is occupied during a stress response and facilitates negative feedback on elevated CORT (Reul and De Kloet, 1985). These two receptors differ in their distribution within the brain. MR is primarily found in the hippocampus and the dentate gyrus (Gerlach and McEwen, 1972; Stumpf and Sar, 1979). On the other hand, GR is widely spread throughout the brain with high density in cerebral cortex, hippocampus, thalamus and hypothalamus (Reul and De Kloet, 1986). These are also age-specific patterns in receptor densities; in rats, GR is most abundant in hippocampus in adults, while neonates have the highest number in cerebellum (Pavlik and Buresova, 1984).

In this study, we investigated neural GR and MR capacity through nestling development in white-crowned sparrows. We hypothesized that the dampened stress response observed in this species during early development may be due to higher receptor densities in hypothalamus and hippocampus, i.e. enhanced negative feedback and tonic inhibition. Using cytosol binding assays, we first explored the general ontogenetic patterns of GR and MR and then gained a more discrete pattern of development examining specific regions of the brain.

MATERIALS AND METHODS

Animals

Nuttall's white-crowned sparrow (*Zonotrichia leucophrys nuttalli*) nestlings were obtained from nests at Bodega Marine Reserve of University of California, Davis. Experiment 1 and 2 were conducted between April and June of 2004 and in May and June of 2006, respectively. In order to determine the nestlings' age, nests were checked every 2 to 3 days to note the date of hatching, or nestlings were compared to various physical characteristics of known aged nestlings. The nestling period (approximately 10 days) was divided into 3 age groups for these experiments: days 1-3, day 4-6, and day 7-9. White-crowned sparrow nestlings hatch with minimal down and eyes closed (Banks, 1959). During D1-3, the growth rate is the largest among the nestling period (Banks, 1959), and eyes start to open near the end of this period (personal observation). During D4-6, nestlings attain thermoregulatory ability and coordination of movement. During the last stage (D7-9), nestlings are more alert, can exhibit threat displays, and may fledge if disturbed. Energy is now allocated away from gaining mass and towards growing feathers and maintaining body temperature (Banks, 1959).

Endogenous CORT can interfere with receptor assays, hence we inhibited stress-induced CORT secretion with mitotane (ortho, para, dichlorodiphenyl dichloroethane), a pharmacological agent that reduces CORT production (Breuner et al., 2000). Each individual was weighed, and the appropriate volume of mitotane (300mg mitotane/mL peanut oil, 1.2g/kg bird) was injected into abdominal cavity or pectoralis muscle whichever was possible, 24-36 hrs prior to perfusion. The nestlings were then returned to their nests until the next day.

On the day of perfusion, birds were captured from their nests and the initial blood sample for baseline CORT was collected from the alar vein within 4 minutes of capture

(Wada et al., 2007). They were then transported into a laboratory in a nestbox covered with an opaque cloth. Immediately after collecting the second blood sample (pre-perfusion), birds were anesthetized with nembutal (Sodium Pentabarbitol, 50mg/mL, approximately 0.1g/kg bird). They were then perfused for approximately 5 minutes with 0.75% avian saline with heparin (1000usp unit/L).

For experiment 1, whole brain was removed and frozen at ≤ -40°C until the assay. For the assay, the brain was divided longitudinally, and each half was used in a separate assay (to gain two estimates of receptor number from each nestling). The specific binding from each assay was averaged for the data analysis. For experiment 2, five regions of the brain (optic tectum, cerebellum, hind brain, diencephalon, and hippocampus/HVC) were separated on ice immediately after perfusion, using a scalpel. They were put into separate eppendorf tubes and frozen at ≤ -40°C until the assay.

Cytosol preparation

Individual receptor levels for both experiments were determined using point-sample assay, following Breuner and Orchinik (2001). Affinities of CORT to GR and MR at different ages were determined using equilibrium saturation binding assay. All assay parameters (time of incubation, temperature, and protein concentration) were optimized for white-crowned sparrow nestling brain tissue.

In all assays, brain tissue was first homogenized in TEGMD buffer (10mM Tris, 1mM EDTA, 10% glycerol, 20mM molybdic acid, and 5mM dithiothreitol) with a glass homogenizer and vortexed with an equal volume of dextran-coated charcoal. The samples were then centrifuged for 1 hr at 4°C at 104,000 G. Supernatants of each sample were used in the following cytosolic receptor assays.

Cytosol Receptor Assay

Prepared cytosol was incubated with equal volume of ~15nM ^{3H}CORT and either TEGM buffer (GR & MR total binding), 100nM cold RU486 in TEGM (MR total binding), or 1□M cold CORT in TEGM (non-specific binding) for 3 hrs at room temperature. The solution volumes were 50□L in experiment 1 and the equilibrium saturation binding assay, and 25□L in experiment 2. After the incubation period, samples were filtered and rinsed through a Brandel harvester with 9mL TEM buffer to capture the ^{3H}CORT-receptor complex on a GF/B filter. Filters used in the assay were soaked in 3% PEI in TEM buffer for 1 hr at 4°C prior to harvesting.

In order to quantify the protein concentrations in each tissue sample, samples were incubated with 200□L of Bradford reagent and read against a standard curve at 595 nm with a Multiskan microplate plate reader.

MR specific binding was estimated from MR total binding minus non-specific binding. GR was estimated from GR&MR total binding minus non-specific binding and MR specific binding. Then the values were corrected for the protein concentrations in each sample to obtain fmol/mg protein. When non-specific binding was larger than total binding, or MR total binding was higher than total binding, they resulted in negative values. These were converted to 0 for the data analyses.

Sex determination

The DNA extraction and PCR protocol were modified after Freeman-Gallant et al. (2001). On a shaker, red blood cell samples were incubated with Tris-EDTA (TE) buffer, 20% SDS, and proteinase K at 65°C for 2 hours. DNA was extracted using phenol, phenol-chloroform mixture, and chloroform in 3 separate steps; DNA-reagent mixture was centrifuged for 10 min @ 13000rpm after each step. Ammonium acetate

and 100% ethanol was added to the supernatant of the last extraction step to precipitate the DNA. DNA was purified using 70% ethanol, then TE buffer was added to re-suspend the DNA. The DNA samples were amplified in a PCR machine with forward (gagaaactgtgcaaaacag) and reverse primers (tccagaatatcttctgctcc). Amplified DNA samples were run through an ethidium bromide stained agarose gel and read over a UV light. Adult DNA with known sex was run next to the nestling samples to confirm the sexing results.

Statistical analysis

Most analyses were completed with JMP 5.0.1 (Cary, NC); repeated measures ANOVA were performed in SPSS 12.0. Equilibrium saturation binding curves were drawn and K_ds were determined using GraphPad Prism 4 (San Diego, CA). The effect of age on K_d was determined by one-way ANOVA. For the point sample receptor data, homogeneity of variances was tested using Levene's test; when it resulted in p value of 0.05 or lower, data were transformed to fourth root. Due to a lower sample size and missing sex data on some individuals in experiment 1, sex difference was determined using t-test. Since there was no difference between the sexes, they were combined and analyzed for the effect of age using one-way ANOVA with Tukey HSD. For experiment 2, we used ANCOVA with age as a main factor and sex as a covariate. For the brain growth data, the effect of age was determined using one-way ANOVA followed by Tukey HSD. Data points deviating two standard deviations away from the mean were classified as outliers and excluded from the analysis (1 out of total 396 data points). Data are presented as mean $\pm$ SE. When $p \leq 0.05$, the null hypothesis was rejected.

RESULTS

Affinities of GR and MR

Equilibrium saturation binding shows that GR for D1-3, D4-6, and D7 is best fit by two-site, one-site, and one-site model, respectively (Fig. 3.1). MR for three age groups is best fit by one-site mode. One-way ANOVA shows neither GR nor MR K_d changes with age ($p > 0.05$).

Experiment 1: whole brain analysis

Mitotane treatment successfully suppressed CORT levels in the first two age groups (repeated measures ANOVA, pairwise comparisons, $p > 0.05$). The oldest age group retained a small but significant increase in CORT prior to perfusion ($p < 0.05$; Fig. 3.2). However, there was no correlation between pre-perfusion CORT levels and MR levels ($R^2 = 0.0026$), and no age difference in pre-perfusion levels of CORT (repeated measures ANOVA, pairwise comparisons, $p > 0.05$).

There was no difference between males and females either in GR or MR levels ($F_{1,14} = 0.0589$, $p = 0.812$; $F_{1,14} = 0.0691$, $p = 0.797$ respectively).

When both sexes were analyzed together, GR capacity did not change significantly with age ($F_{2,19} = 0.356$, $p = 0.705$) while MR capacity decreased with age ($F_{2,19} = 3.686$, $p = 0.044$) (Fig. 3.3). Pairwise comparison results show that D1-3 nestlings had significantly higher MR binding than D7-9 nestlings ($p \leq 0.05$).

Experiment 2: Five different regions of the brain

Overall, there was no effect of age on GR or MR in optic tectum, hind brain, diencephalon, or hippocampus/HVC, or GR in cerebellum (Table 3.1). However, MR

in cerebellum decreased significantly with age ($F_{2,31}$ = 5.650, p = 0.0087) (Fig. 3.4). Pairwise comparison results show that D1-3 nestlings had significantly higher MR levels than D4-6 or D7-9 nestlings ($p \leq 0.05$). There was a significant sex difference in hind brain MR ($F_{1,32}$ = 4.270, p = 0.048) and marginal difference in hippocampus/HVC GR ($F_{1,28}$ = 3.728, p = 0.067). Females had higher hind brain MR while lower GR in hippocampus/HVC.

DISCUSSION

White-crowned sparrows show a period of hyporesponsiveness to handling stress early in nestling development (Wada et al., 2007). We speculated that this blunt response is partly due to elevated negative feedback resulting from upregulation of corticosteroid receptors in hypothalamus and hippocampus, the major sites for negative feedback and tonic inhibition in adults (Herman et al., 1989; Sapolsky et al., 1984; Sapolsky et al., 1991). Supporting our prediction, corticosteroid receptor capacity decreased with age. However, MR capacity decreased with age, whereas GR capacity was not affected. In addition, this overall decline in MR levels is driven entirely by a decline in cerebellar MR. No age-related changes were observed in hippocampal or hypothalamic areas.

Age-related changes in corticosteroid receptors are often reflected in CORT secretion. An elevation of CORT in aging rats is shown to be a result of decreased corticosteroid receptors mainly in hippocampus, causing a dampened negative feedback (Meaney et al., 1988; Peiffer et al., 1991; Sapolsky et al., 1983). During SHRP, hippocampal GR receptors are relatively low (Meaney et al., 1985b; Olpe and McEwen, 1976; Rosenfeld et al., 1988), but low corticosteroid binding globulin (CBG) and CORT levels leads to an adult-like level of receptor occupancy under normal condition (Viau et

al., 1996). Furthermore, neonates' hippocampal GR is more sensitive to a same elevation in CORT than adults (Viau et al., 1996).

Relatively high corticosteroid receptor levels in the brain translate to higher sensitivity for actions of CORT, both negative feedback and detrimental actions of CORT. Neonatal handling in rats increases GR in hippocampus permanently (reviewed in Caldji et al., 2001; Meaney, 2001). These individuals have significantly lower adrenocortical responses to stress in adults due to an enhanced negative feedback. At the same time, relatively high receptor levels during development may increase vulnerability to excess CORT in certain areas of the brain (Benesova and Pavlik, 1989; Ferguson and Holson, 1999).

Our results in white-crowned sparrows differ from mammalian ontogenetic studies in that: 1) it was MR, not GR that changed with age, 2) MR levels *declined* over time and 3) this decline in MR capacity was most evident in the cerebellum. We do not know whether receptors in cerebellum are involved in negative feedback. Then why does MR in nestlings' cerebellum decline with age?

One possibility is that the high MR levels in early-stage nestling cerebellum are due to rapid development of the area, as CORT is involved in regulating cellular differentiation (De Kloet, 1991). In this species, cerebellum undergoes the most dramatic development during nestling period compared to optic tectum, hind brain, or diencephalons (Fig. 3.5). However, cerebellum may be more vulnerable to excess CORT due to the higher MR levels. In mammals, the cerebellum also undergoes substantial development during postnatal period (Bell et al., 1986; Rodier, 1988), containing highest levels of GR compared to other regions of the brain (Pavlik and Buresova, 1984). Dexamethasone (Dex) administration during this rapid cerebellum growth suppresses protein content in rat's cerebellum, in addition to reduction in body,

whole brain and regional weight (Benesova and Pavlik, 1989; Ferguson and Holson, 1999). While other regions of the brain recovered after Dex treatment, the cerebellum retained its deficiency in weight and protein content, again indicating its high sensitivity to glucocorticoids. It is worth noting that in white-crowned sparrows, MR levels go down by the time nestlings can respond to stress (middle to late staged nestlings) (Wada et al., 2007). This means that in optimal conditions an elevated CORT should not reach a nestlings' brain.

Alternatively, it is possible that endogenous elevation in CORT masked the true MR levels in the oldest age group. Mitotane treatment successfully suppressed CORT levels in the first two age groups, while the oldest age group retained a slight but significant increase in CORT prior to perfusion. However, such scenario is unlikely because there was no correlation between pre-perfusion CORT levels and MR levels, and no age difference in pre-perfusion levels of CORT. Additionally, MR levels were the same between the middle and oldest age groups, while endogenous CORT was only elevated in the oldest age group. And lastly, if endogenous CORT levels were masking MR in the older nestlings, MR levels would have declined in every brain area, not just the cerebellum.

There was also evidence of sex differences in receptor levels but only in MR levels in the hind brain and a marginal difference in GR levels in the hippocampus/HVC region. During rats' fetal and neonatal development, there are sex differences in temporal patterns of GR and MR in the brain (Owen and Matthews, 2003). In hypothalamus, only female fetuses show a decline in GR levels during gestation. Similarly, only female fetuses show an increase in GR and decrease in MR in hippocampus during gestation. However, no sex difference was observed in cerebral

cortex. Lack of sex differences in majority of the brain areas in white-crowned sparrow nestlings suggests that vulnerability to CORT is equal in both sexes.

Table 3.1. Summary of ANCOVA output for experiment 2. Significant effects are in bold. The significant age effect is shown in Fig. 3.3.

		Age		**Sex**	
		F	p value	F	p value
Optic tectum	GR	1.456	0.2503	0.532	0.472
	MR	1.879	0.1715	1.822	0.188
Cerebellum	GR	0.126	0.8822	0.127	0.725
	MR	5.6503	**0.0087**	0.0736	0.7882
Hind brain	GR	0.8603	0.4339	1.729	0.1992
	MR	0.8691	0.4303	4.2692	**0.0482**
Diencephalon	GR	0.5356	0.5912	0.2125	0.6484
	MR	0.325	0.7252	0.8283	0.3705
Hippocampus/HVC	GR	0.3594	0.7023	3.728	0.0671
	MR	0.2264	0.7993	1.0872	0.309

Figure 3.1. Equilibrium saturation binding of corticosteroid intracellular receptors at different ages. Data shown represent specific binding of 3H CORT with (filled) or without (open) 100nM RU486. Inset is the Scatchard-Rosenthal replot of the data. Only D1-3 data are shown for simplicity.

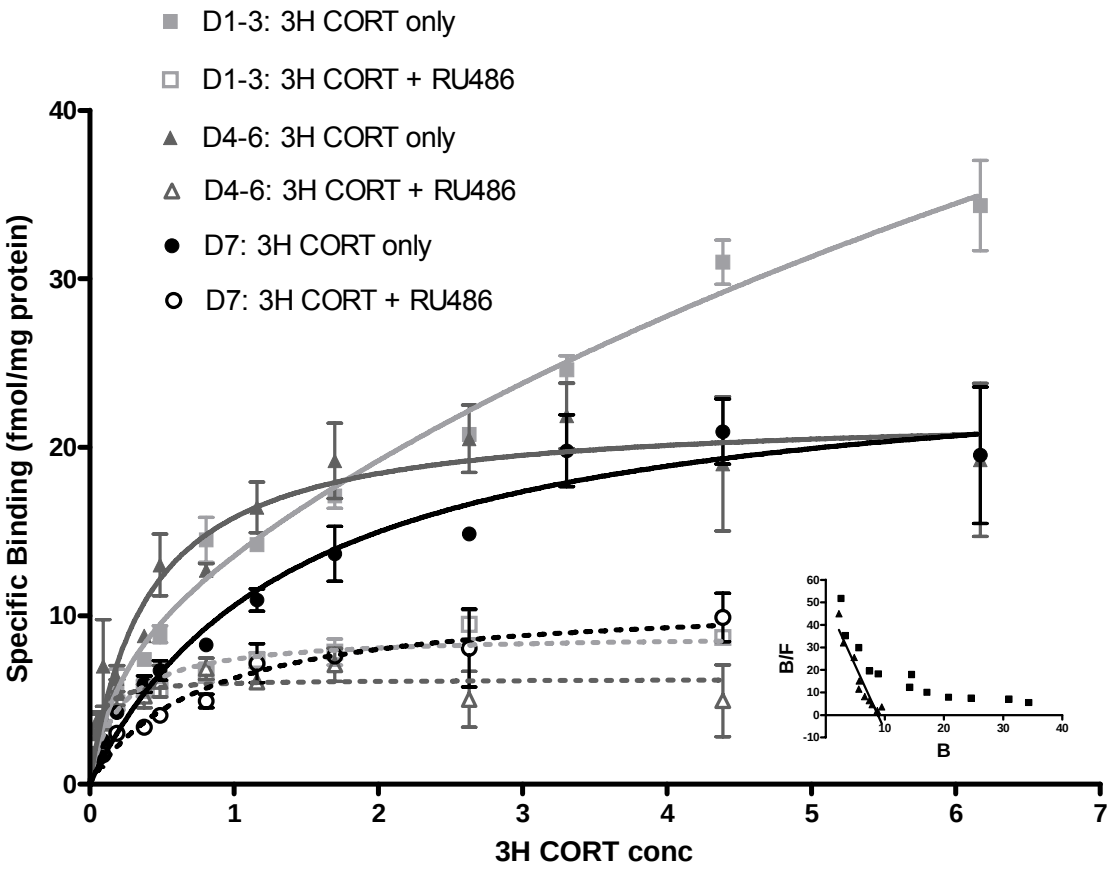


Figure 3.2. Baseline (0 min) and pre-perfusion levels of corticosterone in the whole-brain analysis. Stress-induced levels of corticosterone from the previous study were added for comparison (Wada et al., 2007). * $p \leq 0.05$.

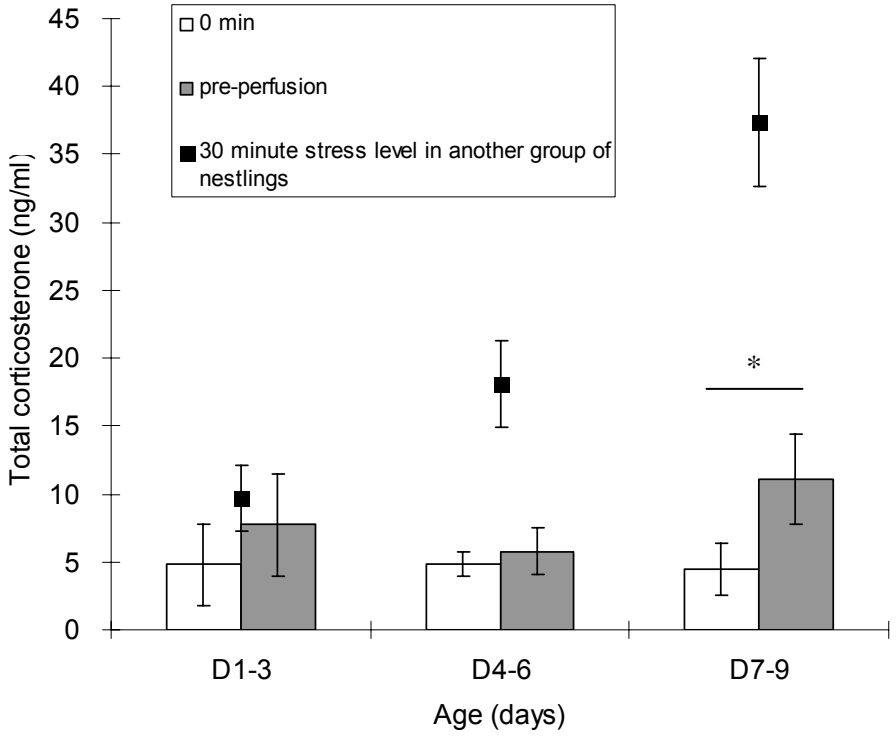


Figure 3.3. Changes in whole-brain GR and MR specific binding in relation to age. Different letters indicate a significance level of 0.05 between age groups.

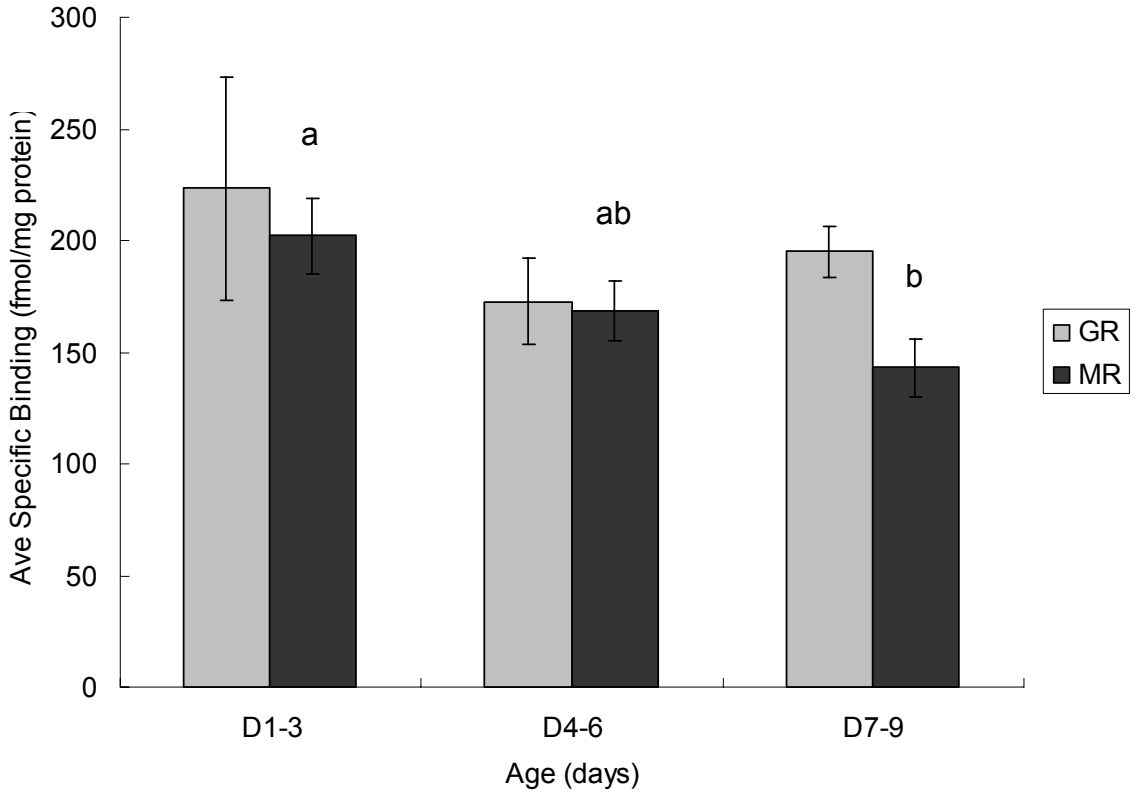


Figure 3.4. Changes in cerebellar GR and MR specific binding in relation to age. Different letters indicate a significance level of 0.05 between age groups.

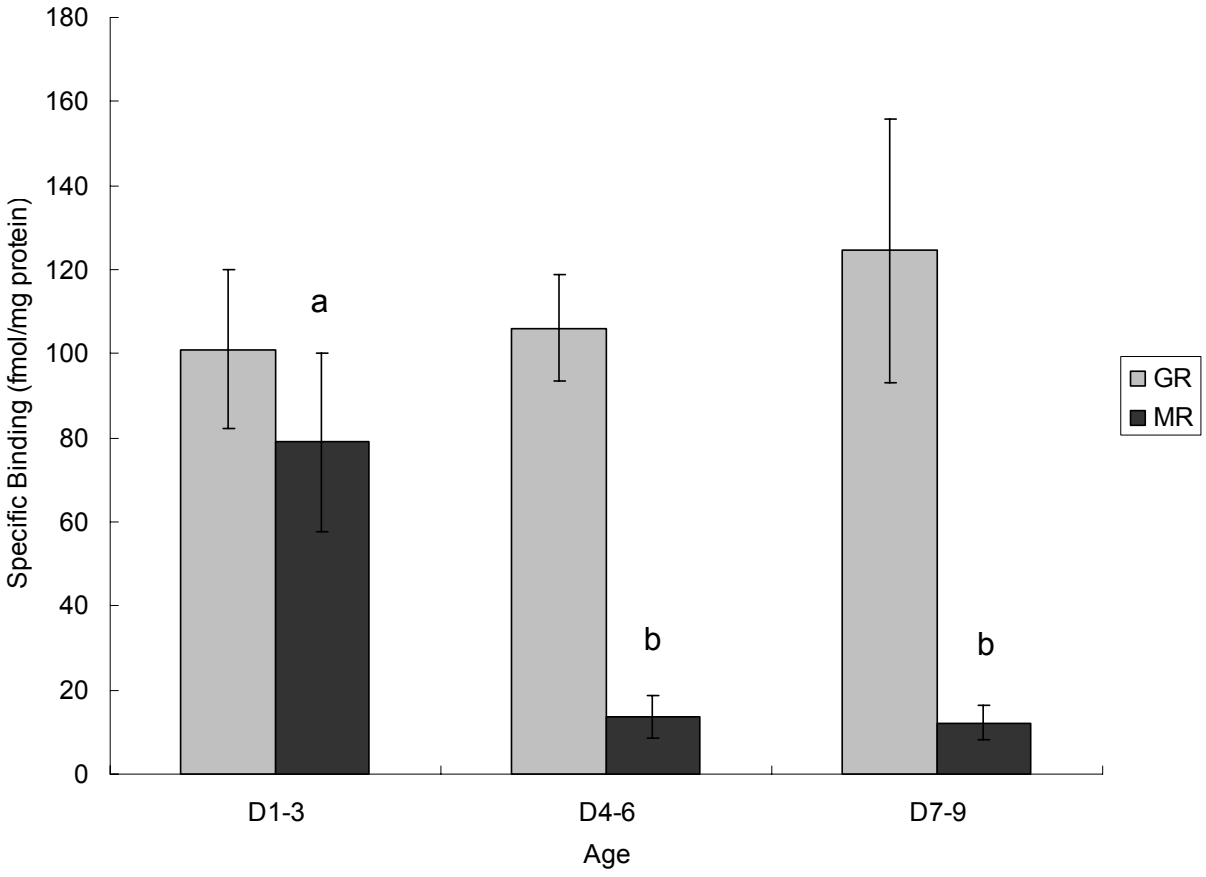


Figure 3.5a. Change in whole brain mass with age. Age had a significant effect on whole brain mass ($F_{2,19} = 40.75$, $p < 0.001$). Different letters indicate significant differences between groups ($p < 0.05$).

a)

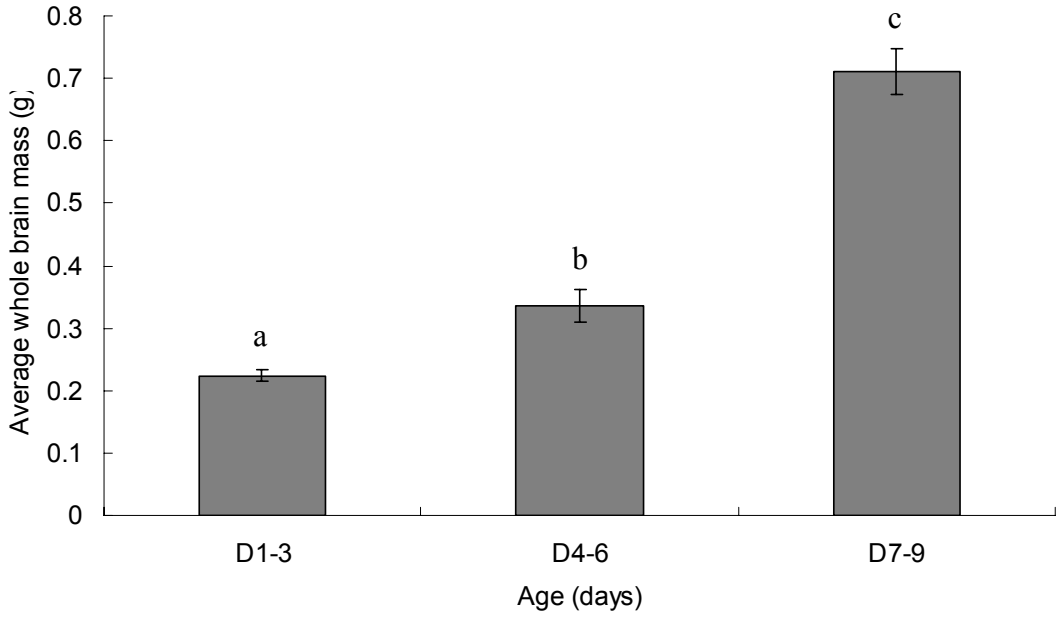
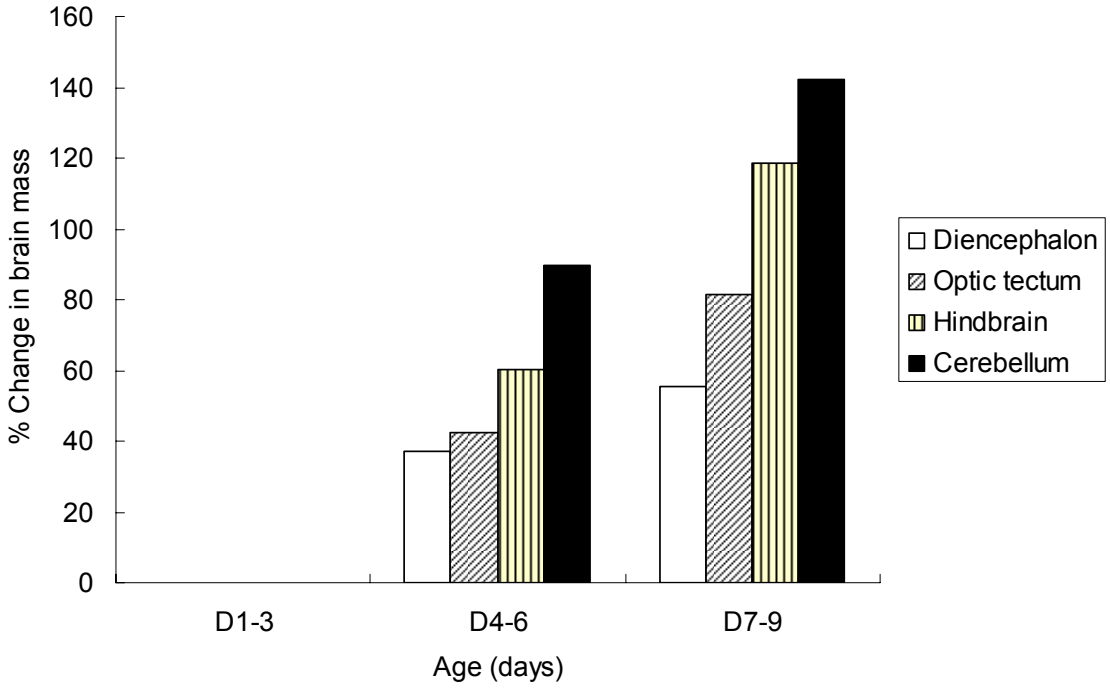


Figure 3.5b. Percent change in brain mass in different regions compared to D1-3.

b)



Chapter 4: Transient elevation of corticosterone alters begging behavior and growth of white-crowned sparrow nestlings

INTRODUCTION

Glucocorticoids present a cost/benefit tradeoff for vertebrate young. Glucocorticoids are essential during development; for example, they play critical roles in fetal organ maturation (reviewed in Liggins, 1994) and many life history stage transitions such as metamorphosis and fledging (Brown and Kim, 1995; de Jesus et al., 1990; Galton, 1990; Heath, 1997; Kern et al., 2001; Krug et al., 1983; Schwabl, 1999; Seabury Sprague and Breuner, 2005; Sockman and Schwabl, 2001). Yet at the same time, glucocorticoids can be detrimental for development. Prolonged exposure to glucocorticoids can cause increased mortality (Eriksen et al., 2006; Janczak et al., 2006; Mashaly, 1991; Saino et al., 2005) (but also see Meylan and Clobert, 2005), reduced growth and/or body condition (Eriksen et al., 2006; Hayward and Wingfield, 2004; Meylan and Clobert, 2005), and may result in a hypersensitive hypothalamic-pituitary-adrenal (HPA) axis as adults (Hayward and Wingfield, 2004). Recent studies also suggest corticosterone (CORT) hinders feather growth in adult European starlings (*Sturnus vulgaris*) (Romero et al., 2005) and barn swallow nestling (*Hirundo rustica*) (Saino et al., 2005) which can delay fledging. Thus, the duration, timing, and intensity of CORT exposure may be key factors determining the balance of cost/benefit tradeoffs during development.

Studies to date have utilized numerous methods for glucocorticoid administration during pre- and postnatal development. Researchers have used injections of CORT into eggs or mothers for transient, 'acute' CORT elevation (Dean and Matthews, 1999; Freire et al., 2006; Janczak et al., 2006; Rubolini et al., 2005; Saino et al., 2005; Uller and

Olsson, 2006). This prenatal exposure presumably mimics a maternal transfer of CORT to her offspring, especially when performed very early in development (Janczak et al., 2006; Rubolini et al., 2005; Saino et al., 2005). More prolonged, 'chronic', elevation of CORT is traditionally achieved by using subcutaneous implants; these often elevate hormone for weeks, and sometimes months (Catalani et al., 2000; Glennemeier and Denver, 2002c; Morici et al., 1997; Spencer et al., 2003). Some species may elevate CORT for such extended periods of time in nature, however, it is likely not biologically relevant for most. Thus it will be valuable to investigate the effect of short, moderate exposure to CORT, especially in species with shorter developmental periods (i.e. short-lived organisms).

During the early postnatal development in birds, a possible conflict exists between diverse effects of CORT; it can retard growth but can also facilitate begging. To investigate potential tradeoffs resulting from brief, moderate (physiologically relevant) elevations of CORT we evaluated CORT's effects on growth and begging through the nestling phase in Nuttall's white-crowned sparrows (*Zonotrichia leucophrys nuttalli*). In the first experiment, we tested the effects of an acute CORT elevation (25 min) on begging behavior, by feeding nestlings CORT- or oil- containing wax moth worms. In the second experiment, we artificially elevated CORT between 24 and 48 hours using a non-invasive dermal patch, and observed changes in growth.

MATERIALS AND METHODS

Animals

Nuttall's white-crowned sparrow nestlings (*Zonotrichia leucophrys nuttalli*) were captured from a free-living population on Bodega Marine Reserve, University of

California, Davis. Nestlings in this species develop from an egg to a fledgling, body mass of ~3g to over 20g, within ~10 days (Banks, 1959). For the purpose of the study, the ~10-day nestling period was divided into 3 age groups: days 1-3, 4-6, and 7-9. In D1-3 nestlings, eyes are closed or have just opened. During this period, nestlings gain mass in a near logarithmic fashion (Banks, 1959) and pin feather break occurs in 2.5 days. Eyes are fully open by D4-6. Nestlings of this age switch from ectothermy to endothermy, complete body mass gain, and increase alertness and coordination of movements (Morton, 2002; Morton and Carey, 1971). D7-9 nestlings switch from gaining mass to developing feathers. They are alert, show a fear reaction to an observer, and may be forcefully fledged if disturbed. Ages of the nestlings are estimated in two ways: 1) by monitoring the hatch date or 2) by comparing growth characteristics of nestlings with known ages. Experiment 1 (acute elevation of CORT) was conducted in spring of 2005 and experiment 2 (extended elevation of CORT) was conducted in spring of 2006.

CORT manipulation in nestlings

Each nest was randomly assigned to one of the 3 age groups for the experiment. On the day of the experiment, two nestlings (non-runt) from each nest were randomly selected for two treatments, control and experimental groups. Each nest and each individual was treated and observed only for that age group.

A. Experiment 1: Acute elevation and begging behavior

To deliver a transient increase of CORT non-invasively, we fed nestlings wax moth worms containing either CORT dissolved in peanut oil or peanut oil alone. This

method was modified after Breuner et al. (1998). The sample size for this experiment was 10, 13, 6 for the control and 12, 13, 9 for the CORT in D1-3, 4-6, and 7-9, respectively.

The concentration used in this study was 0.4mg corticosterone/ml peanut oil (Sigma). Peanut oil with or without CORT was injected into the worm using a 30-gauge needle mounted on a Hamilton syringe. The amount of solution injected into the wax worm was determined depending on the average mass of the two nestlings (Table 4.1a).

Nestlings were captured from their nests one at a time. Immediately after the capture, an initial blood sample and body mass were collected (Fig. 4.1a). All blood samples were obtained within 4 minutes of capture by puncturing the alar vein with a 26-gauge needle to measure the baseline levels of CORT (Wada et al., 2007). The nestling was then transported into the laboratory in a transportable nest box (a natural nest in a small cardboard box) covered by an opaque cloth and moved into the observation box upon arrival. The observation box consisted of a nest in a small box taped onto a larger box (Fig. 4.1b). The outside box had a ~3 cm slit where the experimenter could tap the small nest box inside with a finger without being seen by the nestling. A video camera was placed on a tripod just outside of the observation box aiming at the nest. The observation box plus the video camera were covered by black plastic cover with an eye hole for the entire duration of the behavioral observation. The room was kept dark except for inside the observation box. An electric body warmer was placed underneath the observation box to keep nestlings warm. Upon transferring the nestling into the observation box, they were fed wax worms depending on their body mass to bring all the nestlings to a similar fed state (17% of nestlings refused this first wax worm; we assumed those nestlings were fed and continued without the first feeding). The nest box within the observation box was then covered with an opaque cloth and nestlings were left

undisturbed for approximately 35 minutes. After the quiescence period, the nest box was uncovered to feed the nestling with a CORT/oil containing wax worm. Twenty-five minutes of behavioral observation (see below) immediately followed the wax worm ingestion. Another blood sample was collected after the behavioral observation to ensure the hormone manipulation was successful. Nestlings then had body size measures taken, and were returned to their nest.

Begging behaviors of the nestlings were videotaped for 25 minutes. During the observation, the nest box was tapped for 3 seconds every 5 minutes after an initial 5-minute acclimation period. Tapping mimics a signal of parents' return from their feeding trips and reliably elicited nestlings' begging behavior in a preliminary study (unpublished data). Videotapes were later analyzed for 4 parameters of begging behavior: latency to beg (time (sec) it took for nestlings to beg after the start of each tapping), duration of begging (sec), number of head lifts regardless of whether they resulted in actual begging, and number of peeping noises. The experimenter did not observe the behavior of the nestlings during the recording and the experimenter and the scorer did not know the treatment of the subjects during the experiment.

B. Experiment 2: Extended elevation and growth parameters

In the second experiment, CORT levels were artificially elevated for 24 to 48 hrs using a dermal patch containing CORT dissolved in peanut oil or peanut oil alone. This method was modified after Knapp and Moore (1997). The sample size in this experiment was 9, 10, and 10 for both treatment groups in D1-3, 4-6, and 7-9 respectively.

The concentration used in the dermal patches was 12.5mg corticosterone/mL peanut oil (Sigma). The amount of oil and the size of a patch were adjusted according

to the nestlings' mass (see Table 4.1b). The patch consisted of Johnson & Johnson clear Band-Aid, black vinyl electrical tape, and 3M Nexcare transparent dressing. Patches were assembled the night before or the morning of the application to avoid drying up. The peanut oil with or without CORT was loaded on the band-aid portion of the patch in the morning of the application using 20-gauge needle.

At the beginning of the experiment, an initial blood sample was drawn and growth parameters were measured as a baseline for the individual. All blood samples in this experiment were collected within 4 minutes of capture from the nest (Wada et al., 2007). After the growth measurement (see below), patches were applied between 2 ventral sternal/abdominal tracts. The skin was first cleaned using 70% ethanol. Patches were applied to the skin after dabbing peanut oil on the skin area to aid the transfer of oil from patch to skin. Nestlings were then returned to their nest. The subsequent blood and growth samples were collected approximately 1, 3, 6, 24, 30, and 48 hrs after the patch application. New patches were applied after the 24 hr sample was taken.

In this experiment, 5 growth parameters were measured: body mass (grams), tarsus (mm), first primary (mm, P1), and wing (mm) length, and developmental scores. The wing length measured here is slightly different from those measured in adults, which is traditionally measured as the length between a wrist joint and the tip of the longest primaries. Since bones are not yet defined in young birds, wing length was measured from the leading edge of the wing to the longest part of the primaries and secondaries. The developmental scores are the systematic scores of feather development on 5 parts of the body (wing, head, back, abdomen, and tail) on the scale of 0-5 (0 = no pin, 2 = pin, and 4 = sheath). As in experiment 1, nestling treatment was concealed from the experimenter for the duration of the study.

Blood sampling

The blood samples were kept on ice until they were spun in the centrifuge at the end of the day. Plasma and red blood cell samples were stored at -20°C or below until assay.

Corticosterone assays

Plasma CORT levels were determined using Enzyme Immunoassay (EIA) kits (cat # 901-097, Assay Designs). Plasma dilution and steroid displacement buffer values were optimized previously for this species (Wada et al., 2007). Samples were run in duplicate, while standard curves and standards were run in triplicate.

In 0.5mL eppendorf tubes, 7µL 1% SDB was added to the equal volume of raw plasma. After a 5-minute incubation, 266µL of assay buffer was added to the plasma (1:40 dilution). All plasma samples, standard curve, total binding, non-specific binding, and 500pg/mL standards were aliquotted into a 96-well plate; conjugated corticosterone and 2°antibody were added to each well, except for non-specific binding wells which received only antibody. The plate was incubated for 2 hrs on a shaker at 26°C. After the first incubation, the wells were rinsed 3 times with wash buffer. The plate was then incubated with substrate solution for 1 hr at 26°C (without shaking). After the second incubation, stop solution was added to each well and the plate was read at 405 nm, with correction at 595 nm (Multiskan Ascent microplate reader).

Samples from the first and the second experiments were run in 2 separate EIA assays. Samples from the first experiment were completely randomized within the assay while samples from the same nest were analyzed on the same plate for the second experiment. All the nests were, however, randomized within the assay. Detectability limits for the first and the second experiment were 0.64ng/mL and 0.87ng/mL

respectively (detectability = total binding % bound – 2 standard deviations, i.e., CORT values that were significantly different from blank wells). The detection limit of the plate was used when the levels of a sample fell under the limit. Inter-plate and intra-plate variations for the first and the second experiment were 3.6%, 6.6%, 5.4%, and 6.6% respectively.

Corticosteroid binding globulin assays

Plasma corticosteroid binding globulin (CBG) levels were determined using a ligand-binding assay with tritiated corticosterone (described in Breuner et al., 2003). Optimal assay parameters in WCS have been characterized previously (Lynn et al., 2003) and were validated for WCS nestlings (Wada et al., 2007). CBG levels of individual samples were measured in a point sample assay with 50µL 1:300 diluted plasma, 50µL ^{3H}CORT, and either 50µL 1µM cold CORT (non-specific binding) or 50mM tris assay buffer (total binding); tubes were then incubated for 2 hrs at 4°C. After the incubation period, samples were run through a Brandel harvester (to separate bound from free) followed by 3-3mL rinse with 25mM tris buffer. Filters were soaked with 25mM tris buffer with 3% PEI for 1 hr before harvesting. Intra-assay variation for the point sample assay was 22.1%.

Free hormone levels were estimated using an equation by Barsano and Baumann (1989):

$$H_{free} = 0.5 \times \left[H_{total} - B_{\max} - 1/K_a \pm \sqrt{(B_{\max} - H_{total} + 1/K_a)^2 + 4(H_{total}/K_a)} \right]$$

where K_a is $1/K_d$ (nM), K_d is affinity of corticosterone for CBG, B_{max} is total CBG capacity, and H_{total} is total plasma hormone concentration. K_d was previously determined in equilibrium binding analysis using pooled plasma (Wada et al., 2007).

Sex determination

The extraction and PCR procedure were modified after Freeman-Gallant et al. (2001). Red blood cells, Tris-EDTA (TE) buffer, 20% SDS, and proteinase K were incubated at 65°C for 2 hrs on a shaker. DNA was extracted in 3 steps: with phenol, phenol-chloroform mixture, then with chloroform. At each step of the extraction, a reagent-red blood cell mixture was spun down in a centrifuge for 10 min @13000rpm. At the end of the extraction, ammonium acetate and 100% ethanol was added to the supernatant. After purifying the DNA using 70% ethanol, TE buffer was added to re-suspend DNA. DNA samples were then run in PCR machine with forward (gagaaactgtgcaaaacag) and reverse primers (tccagaatatcttctgctcc). Post-PCR samples were run in an agarose gel stained with ethidium bromide and read with a UV light. Adult samples with known sex were run together to confirm the sexing results.

Data analysis

All data analyses were performed using SPSS 15.0. For experiment 1, the effects of treatment and age on CORT levels were determined using two-way ANOVA. The four parameters of begging behavior were reduced to three after principle axis factoring. Duration and number of head lift had factor loadings higher than 0.6; therefore they were combined by taking an average. The effects of treatment and age on 3 parameters of begging behavior were determined using MANOVA.

For the second experiment, the effect of treatment on CORT and CBG levels was analyzed using repeated measures ANOVA. The CORT levels and growth parameters were regressed using hierarchical multiple regression analysis. Five growth parameters were regressed separately to determine the effects of CORT on different types of growth. Prior to analyses, areas under the curve for both variables were calculated for each

individual. Since CORT and growth parameters did not always increase with time, this approach allowed us to incorporate both rates and direction of the change into one variable. In the multiple regression analysis, age was coded as the following: Age1 denotes for D1-3 nestlings (D1-3 = 1, D4-6 and 7-9 = 0), Age2 codes for D4-6 nestlings (D1-3 and 7-9 = 0, D4-6 = 1), and the oldest age group was a reference. Since sex and treatment did not have a significant effect on the CORT-growth regression in experiment 2, they were excluded from the further analyses.

Homogeneity of variance was tested using Levene's test. When results were $p \leq 0.05$, the data were log-transformed (begging behavior). Data were considered to be significant when $p \leq 0.05$. Data are presented as mean ± SE.

RESULTS

Acute elevation

A. CORT levels in nestlings

There was no significant effect of age ($F = 0.418$, $p = 0.66$) but a significant effect of treatment ($F = 26.54$, $p < 0.001$) on nestlings' CORT levels at the end of the behavioral observation (Fig. 4.2). CORT-treated nestlings had a significantly higher CORT than control nestlings. No significant interaction was observed between age and treatment ($F = 0.329$, $p = 0.721$).

B. Effects on begging behavior

There was no effect of treatment on any of the behaviors observed ($p > 0.05$). Significant effects of age were observed for all parameters: latency ($F = 15.88$, $p < 0.001$), duration-head lifts ($F = 8.169$, $p = 0.001$), and peeping ($F = 3.55$, $p = 0.035$). No

significant interactions were observed between age and treatment in duration-head lift or peeping ($p > 0.05$), however significant interaction was shown in latency to beg ($F = 4.27$, $p = 0.019$; Fig. 4.3). A pairwise comparison showed that in D4-6 nestlings, CORT-treated nestlings had longer latency to beg than controls.

Extended elevation

A. CORT and CBG levels in nestlings

Repeated measures ANOVA showed that there was a marginal effect of treatment on CORT levels ($F = 2.91$, $p = 0.094$, Fig. 4.4) and no effect of treatment on CBG levels ($F = 0.001$, $p = 0.976$, Fig. 4.5).

B. Effects on development

Overall, developmental measures were not explained by CORT levels alone ($p > 0.05$, Table 4.2). However some developmental measures showed a marginal correlation with CORT (P1 length and developmental scores, $p = 0.054$, 0.051 respectively). When age (Age1 and Age2) was added to the regression model, R^2 increased significantly for all 5 developmental measures ($p < 0.001$). When age*CORT interaction was added to the regression model, R^2 again increased significantly for mass, tarsus, and wing length ($p < 0.05$; Fig. 4.6-8). Standardized betas for all 3 moderations showed negative correlations.

DISCUSSION

Our study demonstrated that moderate, transient CORT increase can alter behavior and growth of white-crowned sparrow nestlings. In the first experiment, a

stress-response like increase in CORT over 25 min increased latency to beg in the middle-staged nestlings. In the second experiment, CORT levels of the nestlings were negatively correlated with mass, tarsus length, and wing length. The effect of CORT was apparent as early as 24 hrs after the treatment. These results suggest that even a moderate increase in CORT is detrimental for early postnatal development in white-crowned sparrows. Moreover, within the observed measures, CORT appears only costly for nestlings.

GLUCOCORTICOIDS AND BEHAVIOR

Both adult and developmental studies suggest that the effect of CORT on behaviors may be condition-dependent. More specifically, it may depend on duration, timing, and/or context of the exposure. In rodents, postnatal handling (brief separation) and maternal separation (3 hrs or more) have opposite effects on young's HPA reactivity in adulthood (see Anisman et al., 1998 for a review). Similarly, acute vs. chronic elevation of CORT may have opposite effects on begging behavior in avian young. Our study showed that transient increases in CORT suppress subsequent begging in middle-staged nestlings. An acute prenatal elevation of CORT also had a similar effect in yellow-legged gulls (*Larus michahellis*) (Rubolini et al., 2005), where begging rate was reduced in freshly hatched nestlings. On the other hand, chronically elevated CORT (for 1-3 days) increases begging behavior in black-legged kittiwakes (*Rissa tridactyla*) (Kitaysky and Wingfield, 2001). When conditions are unfavorable for a brief period of time, it may be beneficial for the young to conserve energy by reducing body movements. However, it may be more beneficial for young to increase begging when body goes into a negative energy balance. Distinct effects of CORT for acute and chronic elevation may be a mechanism for avian young to adjust energy balance during diverse types of stress.

Context dependency may also reflect the age of the nestling. In our study, acute CORT elevations only had an effect on middle-staged nestlings; this may reflect the physical and physiological stage of development. A similar phenomenon is seen in young domestic chickens (*Gallus domesticus*). CORT had an effect on anti-predatory and anxiety behaviors only when administered prenatally (d18 of incubation) and not postnatally (day1 post-hatch) (Freire et al., 2006). Across vertebrates, CORT is known to act in a highly context-dependent manner (Orchinik, 1998), and developmental stages may be one of those determinants for actions of CORT.

Many developmental studies acutely elevate CORT by giving a single injection into eggs or mothers (Dean and Matthews, 1999; Freire et al., 2006; Janczak et al., 2006; Rubolini et al., 2005; Saino et al., 2005; Uller and Olsson, 2006). It is important to note that this acute, prenatal exposure of CORT differs from our study in terms of timing of the treatment. In the former case, behaviors are observed days after the administration. This may reveal an organizational effect rather than a rapid action of CORT.

CORT levels in the experimental group reached between 9-12ng/ml in experiment 1. These are well within the physiological range of the species for their age, and equivalent to or less than those reached after a handling stress (Wada et al., 2007). However, effects of CORT are highly dose dependent (Breuner and Wingfield, 2000; Diamond et al., 1992), and the 'effective dose' may change with age. (Early staged nestlings have peak levels of ~11.5ng/ml after capture and handling stress, while late-staged nestlings reach ~37ng/ml). Hence, it is possible that the current dose was relatively low in the oldest group, and a higher dose of CORT would be necessary to stimulate changes in begging behavior.

Glucocorticoids and growth

It is generally accepted that chronically elevated CORT retards growth of young (Glennemeier and Denver, 2002c; Hayward and Wingfield, 2004; Morici et al., 1997; Spencer et al., 2003). However, CORT may alter growth rates more rapidly than previous studies have suggested. In studies demonstrating deleterious effects of CORT on growth, young are often exposed to CORT for extended period of time, ranging from 7 days to 3 months (Leonhardt et al., 2002; Morici et al., 1997). In others, embryos are exposed to CORT by a prenatal injection into eggs or mothers. Results from latter studies are mixed; some show significantly slower growth (Janczak et al., 2006; Saino et al., 2005) while others indicate no effect of CORT (Marion R. Preest, 2005; Rubolini et al., 2005; Uller and Olsson, 2006). The current study showed that negative relationships between CORT and mass, tarsus, and wing length were apparent after 24 hrs of patch application. It is important to point out that the greatest effects were seen in D1-3 and D4-6 nestlings. This is the time when nestlings of this species grow rapidly both in terms of body mass and structural size (i.e. skeleton) (Banks, 1959). During days 7-10, as they reach fledging, the development switches from mass gain to feather growth. When the length of the first primary was regressed against CORT levels, we only observed a marginal interaction between age and CORT. This suggests that CORT may have a stronger effect on mass and structural development than feather growth in this species.

However, feather growth is also important for young birds, especially for the transitions between nestling, fledgling, and independence. In adult European starlings, CORT is shown to inhibit feather growth (Romero et al., 2005). In young barn swallows, an acute prenatal exposure to CORT (single injection within 2 days of laying) slows the wing feather and rectrix growth (Saino et al., 2005). In our study, we

observed a negative relationship between wing length and CORT but not between P1 and CORT. Wing length in our study included carpometacarpus, patagium, and flight feathers. Hence, the significant effect of CORT in wing length may be a result of reduction in development rate for both bones and feathers.

CORT may serve as a mechanism to adjust to current body condition, as suggested above and by other researchers (Breuner and Hahn, 2003; McEwen and Wingfield, 2003). Food restriction is known to elevate CORT (Kitaysky et al., 2001). When this species responds to CORT by slowing growth, there may be a shift in energy allocation from growth to maintenance, until conditions improve. If so, the energy allocation may shift back to growth and there may be no permanent alteration in body size, cognition, or HPA reactivity. However if conditions do not improve, there may be irreversible changes, such as reduced body size/condition or song quality (e.g., Spencer et al., 2003). Such consequences of CORT elevation in this species are still not understood.

CORT levels observed in response to the patch application were moderate in experiment 2. The highest level observed was 19ng/ml of a middle-staged nestling. Virtually all individuals had CORT levels below the age-specific stress-induced levels for the whole duration of the study. We observed higher variation around the mean in experimental plasma CORT levels than expected. In addition, preliminary studies using adult white-crowned sparrows showed an extensive effect of CORT patches on plasma hormone levels (data not shown), while levels in nestlings changed little. We do not know the exact cause of this variation or the disparity between adults and young, however it may be due to a greater leakage, a differential skin diffusion rate or clearance rate, and physical interactions between siblings and parents in the field.

The current study demonstrated that brief and moderate increases of CORT can affect begging and growth in white-crowned sparrow nestlings. To our knowledge this is the first study to demonstrate 1) the rapid and negative effect of CORT on begging behavior and 2) the negative relationship between CORT and growth as early as 24 hrs after treatment. These results together indicate that both transient and extended CORT elevations are costly without any benefits in this species. Then again, effects of CORT appear to be highly context-dependent. Future studies are needed to determine the effects of more prolonged CORT elevations as well as effects on begging behaviors when a nestling's energy balance falls negative. These studies will help us understand whether CORT poses only cost-cost or both cost-benefit tradeoffs to sparrow nestlings during development.

Table 4.1. Volume of solution injected into mealworms (a) and size of patch used (b) in experiments 1 and 2, respectively.

a)

Mass (g)	Volume (μL)
6-8	10.5
8-10	13.5
10-12	16.5
12-14	19.5
14-16	22.5
16-18	25.5
18-20	28.5
20-22	31.5
22-24	34.5

b)

Mass (g)	Volume (μL)	Band-aid (mm)	Electrical tape (mm)	Dressing (mm)
0-10	5	2 x 4	6.5 x 8	11.5 x 25
10-15	10	4 x 4	7.5 x 8	13.5 x 25
15-20	15	6 x 4	7.5 x 9	13 x 30
>20	20	8 x 4	7.5 x 10	13 x 30

Table 4.2. Hierarchical multiple regression analysis outputs for CORT and five developmental measures. Age1 denotes for D1-3 nestlings (D1-3 = 1, D4-6 and 7-9 = 0), Age2 codes for D4-6 nestlings (D1-3 and 7-9 = 0, D4-6 = 1), and the oldest age group was a reference. The relationship was considered significant when $p \leq 0.05$.

	Model Summary				Coefficients		
Developmental measures	**Predictors**	**Adjusted R^2**	**R^2 change**	**Sig F change**	**Predictors**	**Std Beta**	**Sig**
Mass	CORT	-0.006	0.012	0.418	CORT	-0.013	0.841
	Age	0.881	0.875	*<0.001*	Age1	-0.916	*<0.001*
					Age2	-0.307	*0.015*
	Age*CORT	0.89	0.013	***0.044***	Age1*CORT	-0.194	***0.047***
					Age2*CORT	-0.239	***0.038***
Tarsus	CORT	0.001	0.018	0.311	CORT	0.001	0.982
	Age	0.922	0.908	*<0.001*	Age1	-0.924	*<0.001*
					Age2	-0.396	*<0.001*
	Age*CORT	0.931	0.011	***0.014***	Age1*CORT	-0.211	***0.007***
					Age2*CORT	-0.179	***0.05***
P1 length	CORT	0.048	0.065	0.054	CORT	0.048	0.295
	Age	0.939	0.878	*<0.001*	Age1	-0.994	*<0.001*
					Age2	-0.614	*<0.001*
	Age*CORT	0.943	0.006	0.068	Age1*CORT	-0.07	0.314
					Age2*CORT	-0.191	***0.022***
Wing length	CORT	0.031	0.048	0.1	CORT	0.032	0.468
	Age	0.941	0.897	*<0.001*	Age1	-0.983	*<0.001*
					Age2	-0.554	*<0.001*
	Age*CORT	0.947	0.007	***0.025***	Age1*CORT	-0.111	0.099
					Age2*CORT	-0.209	***0.01***
Developmental scores	CORT	0.05	0.066	0.051	CORT	0.068	0.126
	Age	0.946	0.883	*<0.001*	Age1	-0.98	*<0.001*
					Age2	-0.579	*<0.001*
	Age*CORT	0.949	0.004	0.114	Age1*CORT	-0.124	0.064
					Age2*CORT	-0.115	0.142

Figure 4.1a. Timeline for behavioral observations in experiment 1. Immediately after nestlings were captured from their nest, the initial blood sample was collected. Upon arrival at the lab, nestlings were fed wax moth worms (total worm weight scaled to chick body mass) and left undisturbed for ~35 minutes. After the quiescence period, nestlings were fed with 1 worm injected with peanut oil with or without CORT. 25-minute behavioral observation immediately followed. After collecting another blood sample and growth measures, nestlings were returned to their nest.

a)

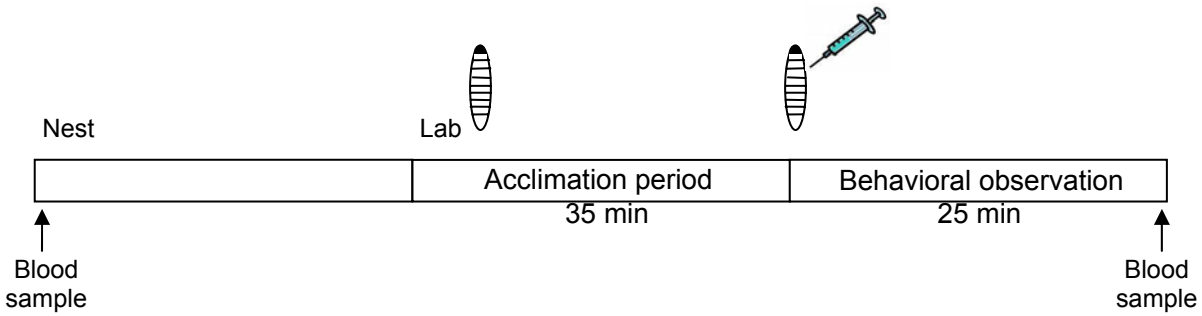


Figure 4.1b. The diagram for behavioral observations in experiment 1. Nestlings were placed in a natural nest within a small box taped onto a larger observation box. A small slit on the observation box allowed the experimenter to tap the nest box without seen by the subjects. A video camera was placed next to the observation box aiming at the nest box. An electric body warmer was place underneath the observation box to keep the nestlings warm.

b)

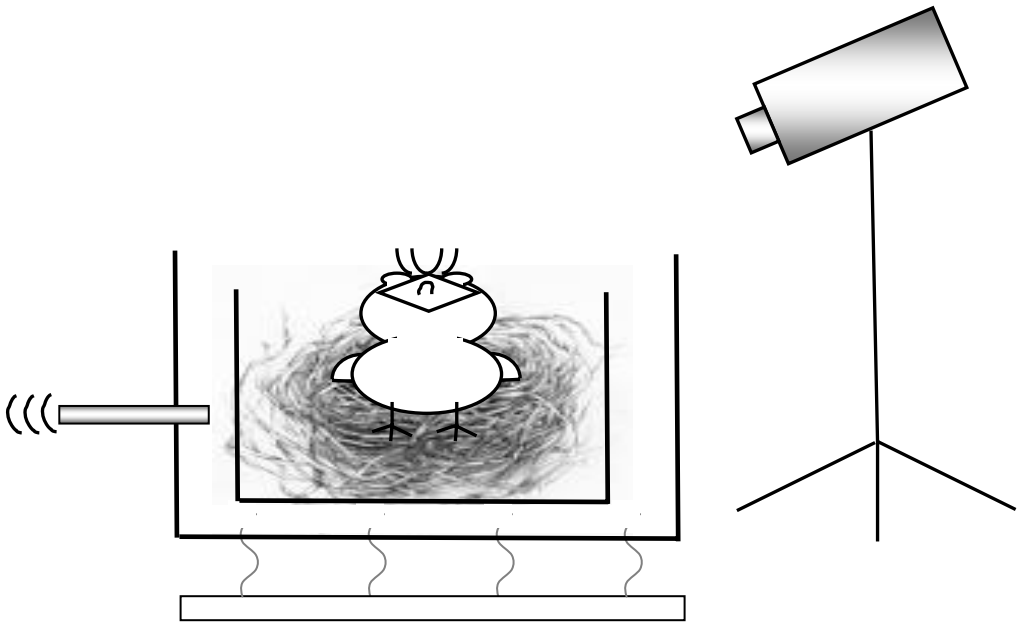


Figure 4.2. Total corticosterone levels at end of behavioral observation in control and CORT-treated nestlings in experiment 1. There was no effect of age but a significant effect of treatment on the hormone levels ($p < 0.001$). N = 9, 13, 8 for the controls and 10, 14, 13 for the CORT in D1-3, 4-6, and 7-9, respectively.

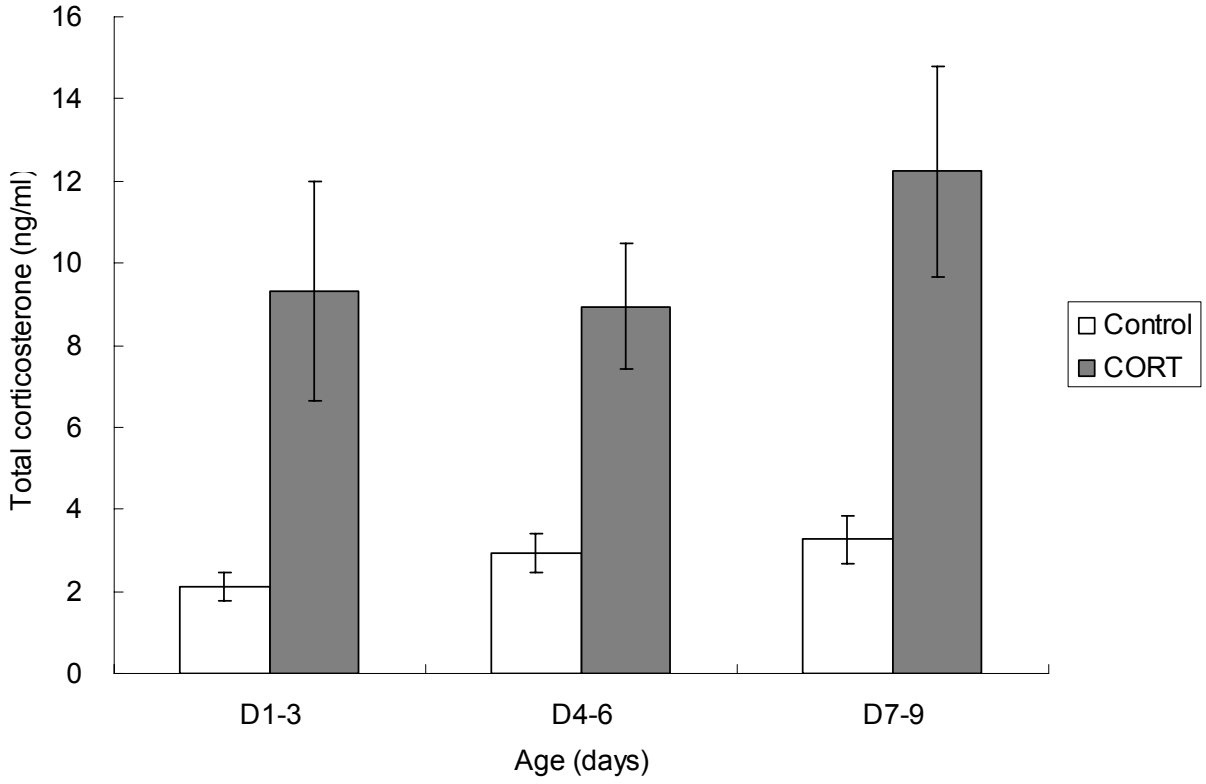


Figure 4.3. Latency to beg in 3 age groups. Latency was measured as the time it took for nestlings to beg after the start of tapping. N = 10, 13, 6 for the control and 12, 13, 9 for the CORT in D1-3, 4-6, and 7-9, respectively. *p < 0.05

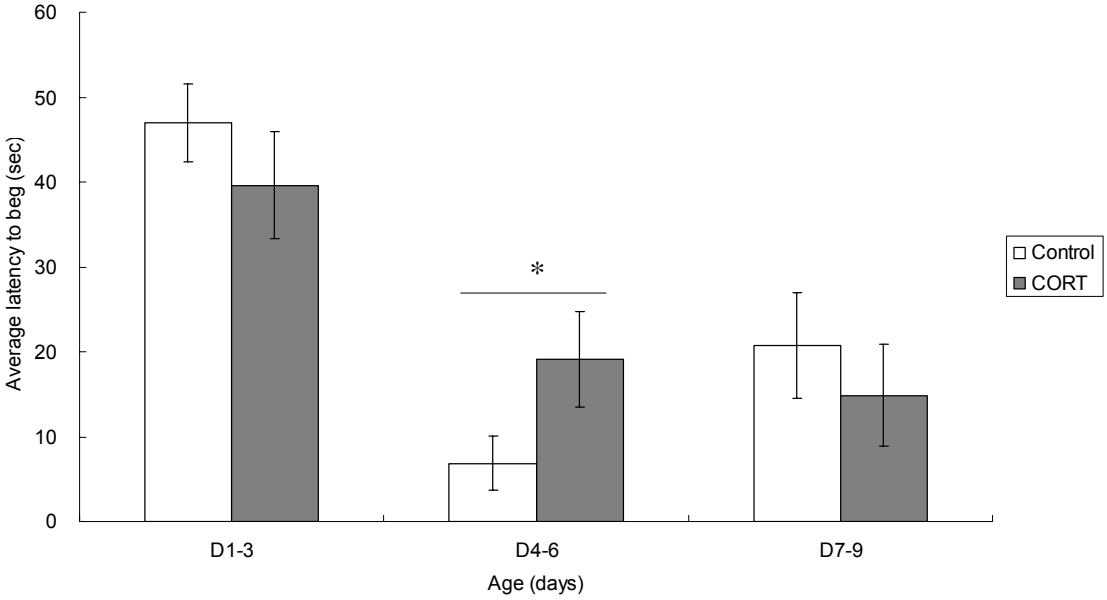


Figure 4.4. Changes in total corticosterone levels over 48 hrs of patch application in control and CORT-patched nestlings (experiment 2). Blood samples were collected prior to and 1, 3, 6, 24, 30, and 48 hrs after the patch application. After the 24-hr sample, a new patch was applied. There was a marginal effect of treatment ($p = 0.094$) on CORT levels.

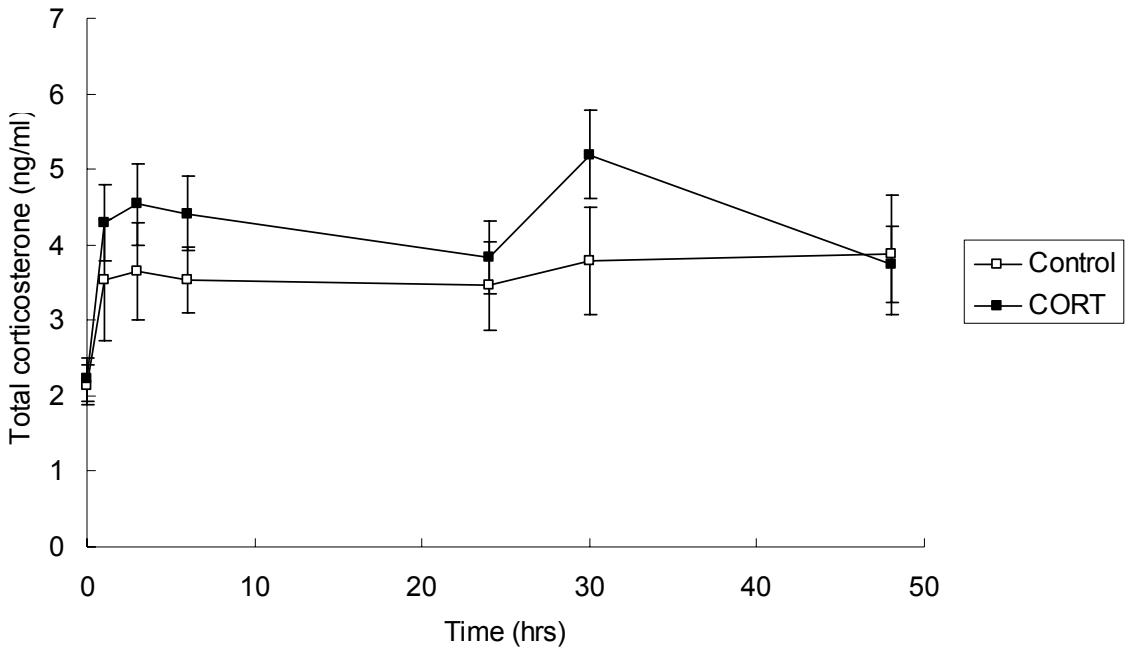


Figure 4.5. Changes in CBG levels over 48-hr period of patch application in control and CORT-patched nestlings (experiment 2). Treatment had no effect on plasma CBG levels ($p = 0.976$).

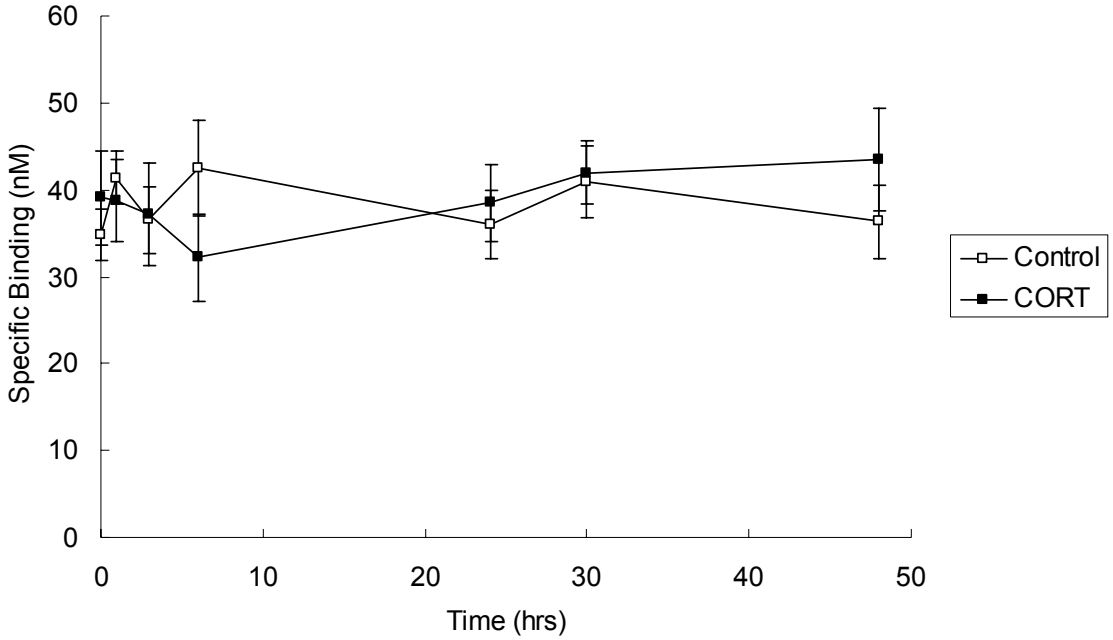


Figure 4.6. Integrated mass vs. integrated total corticosterone for the first 24 hrs. Integrated measures were used to incorporate both rate and direction of changes. Individuals from both treatments are plotted together. N = 18, 20, 20 for D1-3, 4-6, and 7-9, respectively. Trend lines are added for visualization.

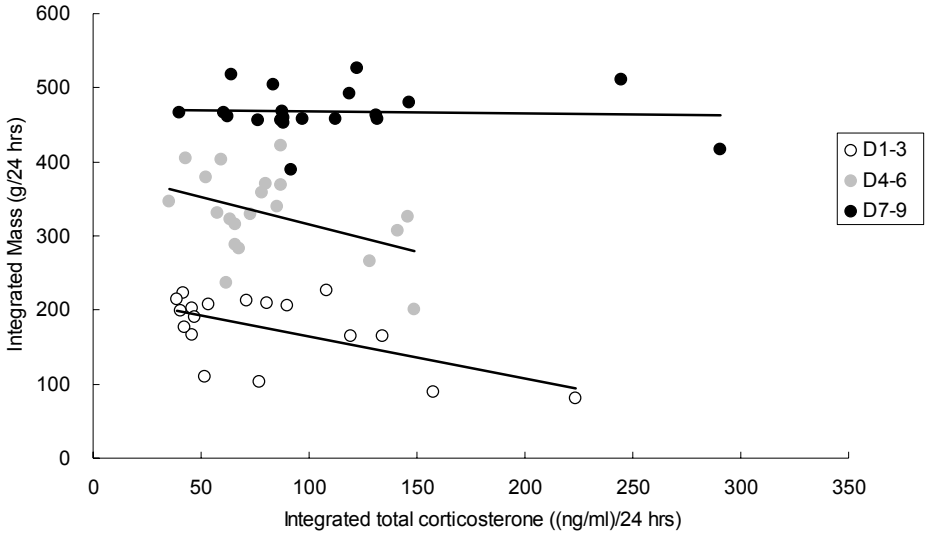


Figure 4.7. Integrated tarsus vs. integrated total corticosterone for the first 24 hrs. Integrated measures were used to incorporate both rate and direction of changes. Individuals from both treatments are plotted together. N = 18, 20, 20 for D1-3, 4-6, and 7-9, respectively. Trend lines are added for visualization.

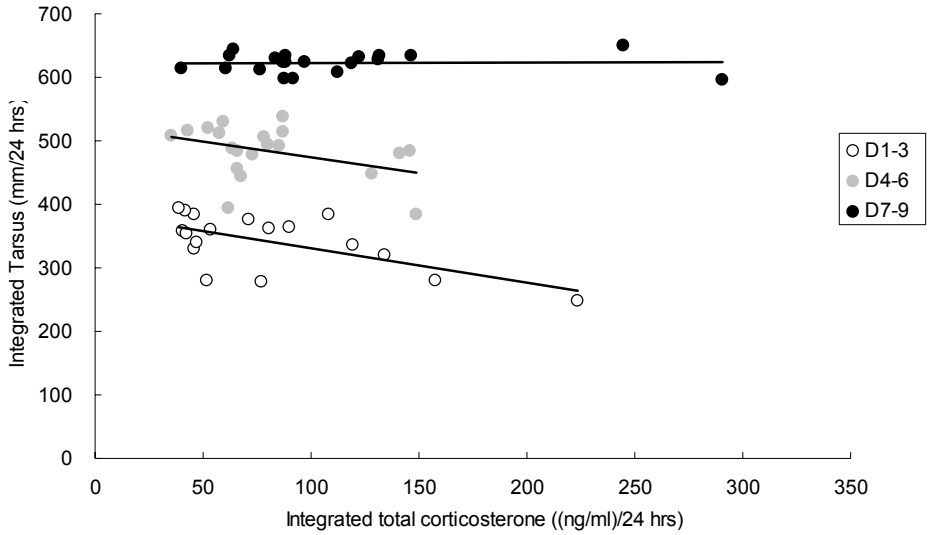
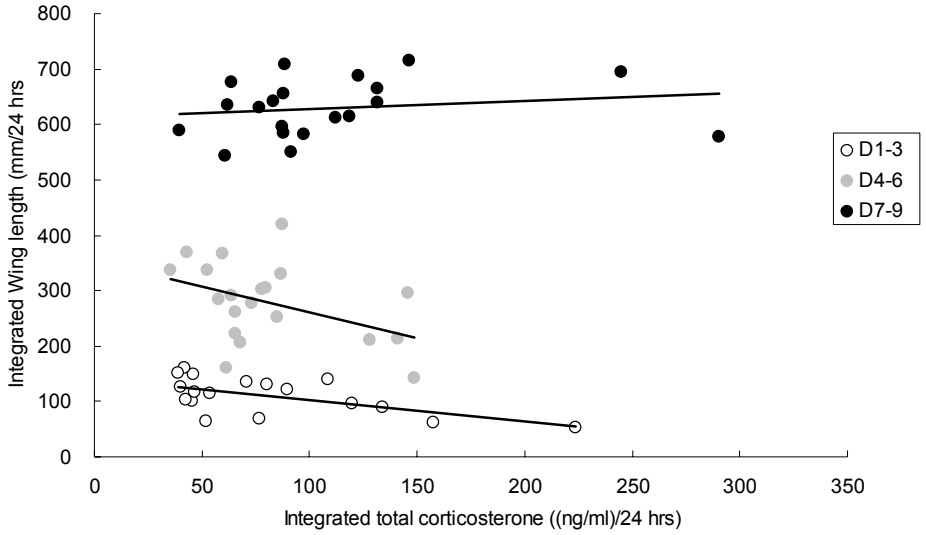


Figure 4.8. Integrated wing length vs. integrated total corticosterone for the first 24 hrs. Integrated measures were used to incorporate both rate and direction of changes. Individuals from both treatments are plotted together. N = 18, 20, 20 for D1-3, 4-6, and 7-9, respectively. Trend lines are added for visualization.



Chapter 5: Conclusion

Glucocorticoids may exert cost/benefit tradeoffs on animals during development. On one hand, glucocorticoids play critical roles in ontogenetic transitions and anticipatory physiological changes needed for those transitions (see chapter 1). In addition, they facilitate energy acquisition and possibly escape behaviors away from stressors via increases in locomotor activity (Crespi and Denver, 2004; Freire et al., 2006; Kitaysky et al., 2003; Kitaysky and Wingfield, 2001). On the other hand, exposures to excess glucocorticoids can result in high mortality rate, or shunted growth and permanently altered hypothalamic-pituitary-adrenal (HPA) sensitivity which can ultimately decrease fitness of individuals (Eriksen et al., 2006; Glennemeier and Denver, 2002c; Hayward and Wingfield, 2004; Janczak et al., 2006). The balance between the costs and benefits may be species-specific, depending on the mode of development, timing and duration of the hormone exposure.

In birds and mammals, there are variations in the mode of development within taxa. The altricial-precocial spectrum describes this range in functional maturity at birth/hatch (Starck and Ricklefs, 1998). These costs and benefits may be highly sub-stage specific; for example the costs of glucocorticoids may exceed benefits during a rapid growth phase before animals have ability to move. Reflecting this spectrum, the developmental hypothesis predicts that HPA axes of developing animals will not be responsive until they have ability to benefit from glucocorticoid secretion (Blas et al., 2006; Kitaysky et al., 2003; Schwabl, 1999; Sims and Holberton, 2000; Wada et al., 2007). There are several mechanisms which animals might use to protect sensitive tissues from high glucocorticoid exposure during critical developmental stages; in addition to the period of low stress reactivity (called stress hyporesponsive period) early

in development, animals may elevate corticosteroid binding globulin (CBG), enhance glucocorticoid clearance, or regulate the HPA axis more tightly through negative feedback. I have evaluated several mechanisms of corticosterone (CORT) regulation during development using an altricial passerine, the white-crowned sparrow (*Zonotrichia leucophrys nuttalli*).

White-crowned sparrows fledge from their nests at approximately 10 days post-hatching. For the series of experiments presented here, the 10-day nestling period was divided into three stages; early, middle, and late. Within the nestling period, nestlings gain mass, thermoregulatory ability, and coordination of movement whose energy allocation later switches from body mass gain to feather growth (Banks, 1959). Nestlings showed a period of low reactivity similar to the mammalian stress hyporesponsive period when early-staged nestlings did not respond to handling stress. Increasing CBG levels extended this period into the middle-stage. Although I observed an age-specific pattern in responses to exogenous adrenocorticotropic hormone (ACTH), all stages of nestlings could respond to the challenge. Next, I evaluated the ontogenetic changes in the negative feedback of the HPA axis by examining two types of corticosteroid receptor capacities in the brain; mineralocorticoid and glucocorticoid receptors (MR and GR). There was an overall decline in MR with age; however GR levels did not change with age. Furthermore, this decline is entirely due to a change in MR in the cerebellum, rather than in the hypothalamus or hippocampus, major sites of negative feedback (Herman et al., 1989; Sapolsky et al., 1984; Sapolsky et al., 1991). This suggests there is no ontogenetic change in HPA axis control at the level of brain receptors. Lastly, I artificially elevated nestlings' CORT levels by feeding CORT-injected worms and applying CORT-containing dermal patches. Both transient and

'intermediate' elevations of CORT delayed latency to beg and retarded growth, indicating CORT may be only costly during development in this species.

These results together have several implications. As in other altricial avian species (Blas et al., 2005; Blas et al., 2006; Romero et al., 1998; Sims and Holberton, 2000), white-crowned sparrow nestlings establish the HPA axis later than precocial species, which confirms the developmental hypothesis. However, timing in acquiring the HPA axis reactivity is similar to semi-altricial/precocial species (Love et al., 2003; Walker et al., 2005) rather than other altricial species such as redpolls (*Carduelis flammea*) (Romero et al., 1998) and Northern mockingbirds (*Mimus polyglottos*) (Sims and Holberton, 2000). Low stress reactivity in early-staged nestlings and patterns in CBG levels are considered to be some of the mechanisms to protect young from excess CORT. At the same time, this species does not appear to have an enhanced negative feedback on the HPA axis at the receptor level during development as there was no change in hippocampal or hypothalamic MR or GR capacities with age. Not surprisingly, effects of CORT are highly age-specific, possibly due to differences in types of development (e.g. gaining mass vs. growing feathers), HPA functionality, and receptor levels within nestling period.

Using the white-crowned sparrow as a model species, this research improves our understanding of the altricial developmental strategy. Altricial animals represent a unique system for investigating a relationship between CORT and development since substantial growth occurs in a potentially suboptimal environment (food availability, temperature, etc.) as opposed to precocial species. These series of experiments elucidated the ontogeny of the HPA axis in altricial nestlings regarding CORT, binding globulin, and receptor levels. Future studies should address whether stress responses are present in embryonic phase of altricial species. This will help us determine the

existence of a true stress hyporesponsive period in altricial avian species. Further studies are also needed to determine effects of CORT during fledgling period and when type I allostatic overload occurs during a nestling period due to low food availability (McEwen and Wingfield, 2003). For passerines, fledgling period is also an important developmental stage as birds memorize songs during this time (Marler, 1997) indicating brain development is still ongoing. This stage can also be vulnerable to excess CORT (nutritional stress hypothesis) (Nowicki et al., 2000; Nowicki et al., 1998; Nowicki et al., 2002).

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