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Seasonal Variation in Corticosterone, Stress Responses, and Physiology in the Northern Cardinal *Cardinalis cardinalis*

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The University of Southern Mississippi

SEASONAL VARIATION IN CORTICOSTERONE, STRESS RESPONSES, AND PHYSIOLOGY IN THE NORTHERN CARDINAL

CARDINALIS CARDINALIS

by

Benjamin Matthew Duckworth

A Thesis
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of The University of Southern Mississippi
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ABSTRACT

SEASONAL VARIATION IN CORTICOSTERONE, STRESS RESPONSES, AND PHYSIOLOGY IN THE NORTHERN CARDINAL

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Seasonal regulation of the adrenocortical response (e.g., 'stress response') appears to be ubiquitous in mid- to high-latitude vertebrates. Northern Cardinals (*Cardinalis cardinalis*) are a Temperate dwelling passerine of Tropical-descent with a wide species range (tropics to Northern Temperate zones). This species encounters a wide variety of environmental conditions and strong seasonality, however, corticosterone (CORT, the main adrenal stress hormone) has not been studied with regards to changes in seasonal levels. I initially analyzed samples from cardinals collected between 2007-2011 from the Lake Thoreau Environmental Research and Educational Center (Hattiesburg, Mississippi, USA). The data suggested seasonal differences of CORT, with higher plasma concentrations during the winter with decreases in pre-breeding, and even further decreases during breeding where the lowest average concentrations were observed. In 2012-2013 we used the same banded population of cardinals to monitor seasonal changes in stress responses. Using more stringent initial CORT (≤3min) samples, we observed an even more pronounced variation in seasonal CORT modulation which mirrored our larger 2007-2011 dataset. The data confirmed preliminary results that significant seasonal variation in CORT 'baseline' levels exist and now stress responses vary, particularly during breeding. In cardinals dampened breeding HPA (hypothalamic-pituitary-adrenal) activity resembles that of species breeding in harsh environments (blunted response to stress), instead of typical hormonal patterns exhibited by Temperate-breeders (robust response to stress). Lastly, we tested whether seasonal CORT levels impacted testosterone (T) concentrations throughout the annual cycle. Results indicated a significant effect of stress responses on initial T levels during pre-breeding. Testosterone levels were seasonally highest at this time of year while initial CORT levels were low. During pre-breeding, T is important for the development of secondary sexual characteristics and behaviors associated with breeding success (e.g. singing rates, courtship, and territoriality). Since CORT and T are known to negatively correlate with each other, it could be adaptive to decrease CORT levels at this time to prevent any hormonal interference that could negatively impact overall reproductive success. This research has helped to improve our overall understanding in how cardinals seasonally modulate two hormones that are very important for survival and reproduction.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropin hormone
Am. J. Physiol.	American Journal of Physiology
Am. Nat.	American Naturalist
Am. Sci.	American Scientist Online
Am. Zool.	American Zoologist
AR	Androgen receptor
Anim. Behav.	Animal Behavior
Ann. N.Y. Acad. Sci.	Annals of the New York Academy of Sciences
Ann. Rev. Ecolog. Syst.	Annual Review of Ecology, Evolution, and Systematics
ANOVA	Analysis of Variance
AVP	Arginine vasopressin
Behav. Ecol. Sociobiol.	Behavioral Ecology and Sociobiology
Biochem. J.	Biochemical Journal
Biol. Lett.	Biology Letters
Biol. Reprod.	Biology of Reproduction
BMR	Basal metabolic rate
Brain Res. Rev.	Brain Research Reviews
Br. Poult. Sci.	British Poultry Science
Can. J. Zool.	Canadian Journal of Zoology
CBG	Corticotropin-binding globulin
CCH	Capricious conditions hypothesis

Comp. Biochem. Phys. A	Comparative Biochemistry and Physiology A- Physiology; Molecular and Integrative
Comp. Biochem. Phys. B	Comparative Biochemistry and Physiology B- Biochemistry & Molecular Biology
Comp. Biochem. Phys. C	Comparative Biochemistry and Physiology C- Pharmacology Toxicology & Endocrinology
CORT	Corticosterone
CPM	Cycles per minute
CRH	Corticotropin releasing hormone
Dev. Neurobiol.	Developmental Neurobiology
E	Epinephrine
EIA	Enzyme immunoassay
Endocr. Rev.	Endocrine Reviews
Front. Behav. Neurosci.	Frontiers in Behavioral Neuroscience
Front. Neuroendocrinol.	Frontiers in Neuroendocrinology
FSH	Follicle stimulating hormone
Funct. Ecol.	Functional Ecology
GC	*Glucocorticoid*
Gen. Comp. Endocrinol.	General and Comparative Endocrinology
GLMM	General linear mixed model
GnIH	Gonadotropin inhibiting hormone
GnRH	Gonadotropin releasing hormone

GR	Glucocorticoid receptor
H^3	Tritiated
Horm. Behav.	Hormones and Behavior
HPA	Hypothalamic pituitary adrenal
HPG	Hypothalamic pituitary gonadal
HSD	Hydroxysteroid dehydrogenase
HSD (Tukeys)	Honest significant difference
ICB	Integrative and Comparable Biology
J. Androl.	Journal of Andrology
J. Avian Biol.	Journal of Avian Biology
J. Comp. Physiol.	Journal of Comparative Physiology
J. Endocrinol.	Journal of Endocrinology
J. Evol. Biol.	Journal of Evolutionary Biology
J. Exp. Zool.	Journal of Experimental Zoology
J. Exp. Biol.	Journal of Experimental Biology
J. Neurobiol.	Journal of Neurobiology
J. Neuroendocrin.	Journal of Neuroendocrinology
J. Neurosci.	Journal of Neuroscience
J. Ornithol.	Journal of Ornithology
J. Zool.	Journal of Zoology (London)
LH	Luteinizing hormone
Mol. Cell. Endocrinol.	Molecular and Cellular Endocrinology

MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
MT	Mesotocin
NE	Norepinephrine
Neurosci. Lett.	Neuroscience Letters
Neurosci. Biobehav. Rev.	Neuroscience & Biobehavioral Reviews
NT	Nucleus taeniae
Poult. Sci.	Poultry Science
Physiol. Behav.	Physiology & Behavior
P. Natl. Acad. Sci. USA.	Proceedings of the National Academy of Sciences of the United States of America
POMC	Pro-opiomelanocortin
PRL	Prolactin
Proc. R. Soc. Lond. Bio.	Proceedings of the Royal Society of London B Biological Science
PVN	Paraventricular nucleus
Rev. Biol.	Quarterly Review of Biology
SD	Standard deviation
SE	Standard error
T	Testosterone
Trends Ecol. Evol.	Trends in Ecology & Evolution
Wilson J. Ornithol.	Wilson Journal of Ornithology

CHAPTER I

GLUCOCORTICOIDS: PHYSIOLOGY, SEASONALITY, AND GEOGRAPHIC LOCALITY INFLUENCES

Introduction

What are Glucocorticoids?

Much is known about the glucocorticoids (GCs hereafter), often called the 'stress hormones' in a diverse spectrum of animal groups. They are a highly conserved family of steroid hormones that are secreted by the adrenal cortex upon activation of the hypothalamic-pituitary-adrenal (HPA) axis. One of the primary responsibilities of corticosterone (CORT, the main GC in birds) is to free stored energy reserves (Sapolsky et al., 2000) from fat tissues and convert it to glucose, a process known as gluconeogenesis. Glucocorticoids also play important roles in several processes such as locomotor activity, energy metabolism, and feeding behavior (Romero 2004). Low, fluctuating basal levels, which exhibit both daily (peak levels in morning prior to onset of activities, Tarlow et al., 2003) and seasonal variation (Romero 2002), aid in regulating general metabolism and activity levels (Sapolsky et al., 2000). In birds, basal CORT levels are often lowest during molt and peak during breeding (Romero and Remage-Healey 2000; Romero 2002).

How, and Why, is CORT Produced?

In addition to CORT providing energy for daily, general survival, it also regulates physiological changes during emergencies. The reason CORT is often referred to as a "stress hormone" is because blood concentrations can rise well above basal levels in response to a variety of stimuli or stressors, whether it is unfavorable weather, predation, or changes in social rank (McEwen and Wingfield 2003). For this paper'sake, stress can be defined as anything that disrupts ones' homeostatic norm, be it physical or psychological. Once a stressor, or even a non-stress related need for energy (e.g. increased general energy demands during reproduction), have been perceived, the synthesis and release of GCs is stimulated by the peptide hormone adrenocorticotropin (ACTH), which is produced by corticotropic cells of the anterior pituitary gland. The release of ACTH is stimulated by hypothalamic peptides like corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP, both of which are produced in the paraventricular nucleus or PVN). Continuing with the focus on stressful events like a predator attack, under acute stress the HPA-axis becomes highly activated which induces rapid increases (~5-10 min) in blood GC concentrations produced by tissues within the adrenal cortex (Sapolsky et al., 2000). The organism now has the energy in circulation to meet whatever challenge the environment has presented, while postponing processes unnecessary for immediate survival (e.g. growth, tissue repair, or ovulation). Dependent upon organismal needs, GCs in circulation communicate with the hypothalamus via negative/positive feedback loops and can increase/decrease how much more/less CORT is or is not produced, via binding to glucocorticoid receptors (GRs, discussed in more detail below). During a typical acute stress response, CORT's major duties include increasing glucose availability in circulation, inhibiting subsequent energy storage and/or gluconeogenesis, and decreasing glucose usage by non-essential tissues/physiological processes. Ultimately, this stress-induced release of GCs brings about a suite of physiological changes that enables an organism to react appropriately and increase its probabilities of surviving stressful encounters.

GCs Permissive and Suppressive Actions

Hormonal pleiotropy is defined as a single hormone having both positive and negative effects on different physiological systems or traits (Finch and Rose 1995; Hau 2007; Ketterson and Nolan 1999). A good example of this is how GCs exert permissive effects at target tissue sites at low basal levels, whereas, high concentrations of GCs in circulation can exert suppressive effects (e.g. inhibit reproductive physiology/behaviors, decrease immune functionality). The permissive and suppressive effects of GCs have been suggested to complement each other, the former preparing or priming defensive mechanisms for action and the latter, limiting these actions (Munck and Náray-Fejes-Tóth 1992). For example, GCs aid in limiting an inflammatory response and thus prevent problems associated with a defensive immune overshoot (e.g. auto-immune disorders).

How is CORT Production and Response Regulated?

As previously mentioned, many GC actions are not monotonic and act differently at target tissues at low versus high concentrations (Sapolsky et al., 2000). Part of how GCs are able to exert such widespread actions across numerous physiological systems is from binding with two different types of receptors. Glucocorticoids have two major receptors, type I (mineralocorticoid-receptor or MR) and type II (glucocorticoid-receptor or GR) receptors. Mineralocorticoid (type I) receptors bind at low, basal GC concentrations, regulate osmoregulation, and have a much greater affinity (x10) for binding GCs than type II receptors (Sapolsky et al., 2000). Type II receptors (GRs) only bind GCs after type I receptors become fully saturated, and play a larger role in responses to stress where they have been observed to impact food intake and energy metabolism (Landys et al., 2006). Coupling variation in GC levels both daily and annually with differentially active receptors allows this class of steroids to be very flexible in how they assist animals with energy metabolism and usage.

What Factors Contribute to Seasonal Fluctuation in CORT Secretion?

Steroid hormones, like CORT, are fat-soluble and therefore need to be bound to carrier proteins for transportation in circulation. The binding proteins for CORT are corticotropin-binding globulins (CBGs, Breuner and Orchinik 2001). Similar to how basal and stress-induced GC levels exhibit seasonal production/secretion changes, CBGs also have been observed to seasonally vary in concentrations and binding capacities (Breuner and Orchinik 2001; Breuner et al., 2003). Whenever GCs bind to a carrier protein it becomes deactivated in circulation, but whenever necessary, the hormone/protein complex is readily broken down and the steroid hormone is released/reactivated. This has been observed and is well-studied in Arctic species during breeding. Species breeding in harsh, unpredictable environments often have little time to raise young before severe winter storms occur. Due to how high CORT levels are often negatively correlated with breeding success, most birds breeding in such habitats suppress maximal CORT (stress-induced) production to avoid brood abandonment. This is supported by the ‘capricious conditions hypothesis’ (CCH), which states that reduced CORT secretion during stress coincident with parental phases of breeding, is necessary in harsh environments as a full response would otherwise trigger repeated nest abandonments (Cornelius et al., 2011). Dependent on the species, variable CBG binding capacities allow for an immediate response if the situation deteriorates (e.g. high CBG binding capacity) by breaking down the GC-bound CBG and reactivating the once-bound stress hormone while also keeping CORT from potentially interfering with reproduction when there are no stressful events to react to. By having an enhanced CBG-binding capacity, Arctic-breeders can still reproduce even though they elevated CORT by deactivating CORT via CBG-binding, but can readily reverse this process if life-threatening storms prompt a shift away from reproduction towards immediate survival. This is just one example of how and why CORT levels/responses vary in magnitude, not only between different species/populations, but within the same species/populations as well. Coupled with variation in GC receptor expression, this suggests that animals can have very complex patterns of CORT production and response.

Age has also been shown to significantly impact seasonal CORT production. Neonates across a wide range of species exhibit a period of stress-hyporesponsivity during which CORT produced during acute stress is reduced (Freeman and Flack 1980, 1981; Freeman and Manning 1984; Sapolsky and Meaney 1986), this is thought to protect the still-developing brain and immune system from deleterious effects associated with prolonged GC elevations (e.g., reduced glucose availability to these tissues and/or protein catabolism in these tissues, Soma and Wingfield 1998). In most species, stress responses become increasingly attenuated, in part because of acclimation. Not just age, but also the sex and mating system can also impact how CORT is secreted seasonally, especially during parental care within the breeding season. Furthermore, depending on mating system, whichever sex has the most parental responsibility will often dampen the magnitude of stress responses at that time of the year.

The environment an organism inhabits or breeds in is yet another important determinant in predicting how a population or species will vary seasonally in secreting/responding to GCs. Whether it is in the Arctic, tropics or Temperate zone (excluding extreme differences in elevation), each has distinct weather and climate patterns that temporally constrain breeding season length. High and low temperatures, variation in precipitation type and amount, and variation in food availability can each individually cause stress responses and changes in baseline CORT (linked to variation in daily energetic needs). In most cases environmental variation is compounded (e.g., it is cold and there is snow on the ground and food availability is reduced) making survival difficult and leading to changes in GC levels.

The last major factor contributing to seasonal variation in GC secretion is, the time of the year or season, more specifically the life history stage. Almost every bird on earth gradually progress through a series of life history stages (e.g. breeding, molt and migration) that are initiated by changes in environmental conditions (Jacobs and Wingfield 2000). As these seasonal stages progress, organisms coordinate the timing of each stage using predictable cues from the environment (e.g. seasonal changes in photoperiod, temperature). This enables them to match the relevant stage with its appropriate season. These seasonal stages were described by Jacobs (1996), where he states “organisms have a characteristic series of life history stages that makes up their life cycle”. These life history stages (LHSs) were further defined by Wingfield et al. (1997) as “progression of stages in the life cycles of individuals within populations”. A prominent role of hormones is to mediate transitions between life-history stages and regulate behavioral decisions as animals move between life history stages (Adkins-Regan 2005). Endocrine systems optimize life-history decisions by assimilating input regarding current conditions and adjusting physiology and behavior to optimize investment (Finch and Rose 1995). Hormones like CORT, contribute to endocrine regulation of seasonal changes within an organism (e.g. physiological, behavioral or morphological), and aid to anticipate predictable environmental fluctuations, ultimately inducing the necessary changes in each life-history stage. So dependent upon an organisms current condition, sex/mating system, age, habitat and LHS, variances in CORT production are in concordance with selective pressures of changing environments and are adaptive dynamic responses.

Collectively, stress hormones, like CORT, handle many permissive duties but if an individual perceives the need for additional energy, more CORT is synthesized and concentrations in circulation increase. Whether this additional energy is for securing food (e.g. foraging, chasing prey or fleeing a predator), finding a mate (e.g. singing to attract potential suitors, molting away old feathers for hot new breeding plumage), or even fighting (e.g. territorial dispute to defend/obtain mate/resources), whatever the need is CORT levels are constantly changing to provide an individual with energy to meet whatever daily and/or seasonal challenges to homeostasis the environment often presents.

The variation in stress hormones can be simply described using one word, ‘change’. Whether the variation in stress hormones stem from age, or sex differences, the changes an individual makes will be based on its condition, but more importantly what time of the year it is. For an example of changes in CORT levels, Table 1 (below) consists of over 35 years of collective research covering 53 studies and 56 different bird species. I have indicated with a mark (X) whenever a study found a significant change in CORT levels, whether it be age, sex or seasonal.

Table 1

Significant Differences in Corticosterone Levels by Species

Species	Age	Season	Sex	Source
Abert's Towhee *Pipilo aberti*		X	X	Wingfield et al., 1992
African Black Coucal *Centropus grillii*			X	Voigt and Goymann 2007
American Goldfinch *Carduelis tristis*			X	Li et al., 2012
American Kestrel *Falco sparverius*	X			Love et al., 2003
Black-throated Sparrow *Amphispiza bilineata*		X		Wingfield et al., 1992
Brown-headed Cowbird *Molothrus ater*		X		Dufty and Wingfield 1986
Bush Warbler *Cettia diphone*		X		Wada et al., 1999
Cactus Wren *Campylorhynchus brunneicapillus*		X		Wingfield et al., 1992
Canary (Atlantic/Island) *Serinus canaria*	X			Schwabl 1999
Chukar *Alectoris chukar*	X			Dickens and Romero 2010
Common Redpoll *Carduelis flammea*		X		Li et al., 2012
Common Tern *Sterna hirundo*	X			Heidinger et al., 2006
Curve-billed Thrasher *Toxostoma curvirostre*		X		Wingfield et al., 1992
Dark-eyed Junco *Junco hyemalis*		X		Deviche et al., 2000
Dusky Flycatcher *Empidonax oberholseri*		X	X	Pereyra and Wingfield 2003

Table 1 (continued).

Species	Age	Season	Sex	Source
Eastern Bluebird *Sialia sialis*	X			Lynn et al., 2013
Eurasian Kestrel *Falco tinnunculus*	X			Müller et al., 2010
European Blackbird *Turdus merula*		X	X	Partecke et al., 2006
European Starling *Sturnus vulgaris*		X		Dawson and Howe 1983
Gambel's White-crowned Sparrow *Zonotrichia leucophrys gambelii*		X		Romero and Wingfield 1998
Gouldian Finch *Erythrura gouldiae*	X	X		Maute et al., 2013
Gray Catbird *Dumetalla carolinensis*		X		Holberton et al., 1996
Greylag Goose *Anser anser*		X		Kotrschal et al., 1998
House Sparrow *Passer domesticus*		X	X	Breuner and Orchinik 2001; Romero et al., 2006
Inca Dove *Scardafella inca*			X	Wingfield et al., 1992
King Penguin *Aptenodytes patagonicus*	X			Corbel et al., 2010
Lapland Longspur *Calcarius lapponicus*		X	X	Astheimer et al., 1995; Romero et al., 1998b
Leach's Storm Petrel *Oceanodroma leucorhoa*	X			Kozlowski et al., 2010
Long-tailed Finch *Poepila acuticauda*	X	X		Maute et al., 2013
Magellanic Penguin *Speniscus magellanicus*			X	Hood et al., 1998
Northern Cardinal *Cardinalis cardinalis*	X	X	X	Duckworth and Jawor unpubl.
Northern Mockingbird *Mimus polyglottos*	X			Sims and Holberton 2000
Nuttall's White-crowned Sparrow *Zonotrichia leucophrys nuttalli*	X			Wada et al., 2007
Pectoral Sandpiper *Calidris melanotos*			X	O'Reilly and Wingfield 2001

Table 1 (continued).

Species	Age	Season	Sex	Source
Pied Flycatcher *Ficedula hypoleuca*		X	X	Silverin and Wingfield 1998
Pine Siskin *Carduelis pinus*			X	Li et al., 2012
Red Crossbill *Loxia curvirostra*		X		Cornelius et al., 2012
Red-footed Booby *Sula sula*		X	X	Lormée et al., 2003
Common Redpoll *Carduelis flammea*		X	X	Wingfield et al., 1994; Romero et al., 1998c
Red-winged Blackbird *Agelaius phoeniceus*		X		Johnsen 1998
Smith's Longspur *Calcarius pictus*			X	Meddle et al., 2003
Snow Bunting *Plectrophenax nivalis*		X		Romero et al., 1998a,d
Song Sparrow *Melospiza melodia*		X	X	Wingfield 1984, 85
Thin-billed Prion *Pachyptila belcheri*	X			Quillfeldt et al., 2007
Tufted puffin *Fratercula cirrhata*		X		Williams et al., 2008
Twite *Carduelis flavirostris*		X		Li et al., 2012
Wandering Albatross *Diomedea exulans*	X			Angelier et al., 2006
Western Sandpiper *Calidris mauri*		X	X	O'Reilly 1995; O'Reilly and Wingfield 2001
White-crowned Sparrow *Zonotrichia leucophrys*	X	X	X	Wingfield and Farner 1978; Romero and Wingfield 1998; Romero et al., 1997; Wada et al., 2007
White Stork *Ciconia ciconia*	X			Blas et al., 2006; Corbel and Groscolas 2008
Willow Ptarmigan *Lagopus lagopus*		X		Hannon and Wingfield 1990
Willow Tit *Parus montanus*		X		Silverin 1997
Wood Duck *Aix sponsa*		X		DuRant et al., 2013

Table 1 (continued).

Species	Age	Season	Sex	Source
Wood Thrush *Hylocichla mustelina*		X		Done et al., 2011
Yellow Warbler *Dendroica petechia*		X	X	Wilson and Holberton 2004, 07
Zebra Finch *Taeniopygia guttata*	X			Wada et al., 2008

Note. Experimental studies manipulating CORT levels (e.g. implants, pharmacological doses etc.) and results lacking significance were excluded from this table.

Summary

As the seasons change, wildlife inhabiting all types of environments experience physiological changes. The type of weather, at the same time of year, will never be uniform across Arctic, Temperate and Tropical zones. The individuals inhabiting these regions are also not uniform. Individual variation might be the single most difficult variable to account for in scientific studies. This becomes a challenge when studying stress physiology in free-ranging organisms. In part, this is because we do not know the individual histories of the wildlife we study. The past experiences of the wildlife we study affects their physiology today. How an organism perceives its surrounding environment has a direct impact on its condition which indirectly impacts how it regulates stress hormones, like CORT. The condition of an individual has a huge impact on how it decides to react to seasonal changes. Take a step back from condition, and age is another important building block in understanding stress physiology. Before reaching adulthood, one must first survive long enough to reach sexual maturity and in nature, dying from starvation, illness or depredation happens to young far more than not. One reason age impacts stress physiology brings the researcher back to condition, high GC levels for still-developing young can stunt and/or have deleterious alterations to how it develops. Taken together, understanding how and why changes occur in stress physiology is far from simple. But for simplicity, an organism must complete a few steps; first, it must survive long enough to reach sexual maturity. Second, directly tied to individual differences, is the current physiology state of an organism, or its condition. Lastly, if an organism is in good condition, depending on what time of the year it is, it makes physiological changes that best match the season and benefit the organism the most. Whether the decision is to find a mate and reproduce, or forage to increase energy stores to survive through the winter, it all comes down to the condition and time of year (season).

The work described here will focus on one species, the Northern Cardinal (*Cardinalis cardinalis*), and how this species regulates CORT across its life history. Our lab has been studying this species for a number of years and has documented high nest depredation and year-round territoriality in this species, both of which can be classified (by human observers) as stressors which could contribute to overly activating the HPA-axis and stress response physiology potentially leading to population declines. At the very least, the high activity patterns of cardinals demand high energy utilization which could also lead to an overly active HPA axis. However, when we catch cardinals and observe them in hand we see no signs suggesting they are being chronically stressed. In the next chapter of my research, I will be reporting on average CORT levels from our study population to determine how much, if any, seasonal variation exists in how much CORT is produced in cardinal

CHAPTER II

CORTICOSTERONE AND SEASONALITY

Introduction

In most avian species, corticosterone (CORT) concentrations fluctuate daily (the circadian rhythm is at peak in the morning prior to onset of activity, Tarlow et al., 2003) and seasonally; often with the lowest concentrations during molt and a peak in concentrations during breeding (Romero and Remage-Healey 2000; Romero 2002). Dependent upon many factors (e.g. sex, age, condition, time of year, life experiences, and habitat), the hypothalamic-pituitary-adrenal (HPA) axis, which controls CORT secretion, will vary in level of action, ultimately contributing to the seasonal variation observed in CORT secretion. One of CORT's primary responsibilities is to free stored energy reserves and increase glucose levels in circulation (Sapolsky et al., 2000). The coupling of energetically expensive behaviors, or changes in physiology, with higher levels of CORT is advantageous as the appropriate energy can be readily provided for all activities or physiological changes.

Patterns of Corticosterone Secretion

In birds, CORT concentrations are typically lowest during the winter (Romero et al., 1997) and molt (Astheimer et al., 1994), and highest during breeding. Elevated levels during reproduction may be related to the elevated energy demands associated with providing for offspring. In the fall it is somewhat counterintuitive to see decreases in CORT since the process of molting is an energetically expensive task involving the replacement of old feathers with new. One might assume that CORT levels would increase at this time. However, it has been suggested that lower CORT levels during molt are necessary for higher quality feather production (Romero et al., 2005) as elevated CORT can interfere with protein metabolism and usage (Landys et al., 2006). Since feathers are comprised of 95% protein (Murphy and King 1992), it is plausible to suggest that it is adaptive to produce overall lower concentrations of circulating CORT at this stage to protect against potentially deleterious impacts (here, poorer quality feather production) which may negatively impact an organisms survival. However, other studies have shown reduced CORT during feather replacement is a necessary prerequisite for a successful molt, at least in terms of feather growth rates, feather quality and potentially pigment deposition (Dawson et al., 2000; Dawson 2004; Ducrest et al., 2008). Taken together patterns of CORT changes over the annual cycle of living organisms can provide energy for expensive activities or, given the actions of CORT (reviewed in Chapter I), CORT levels can be altered in order to not interfere with some physiological activities or behaviors.

Another pattern of plasma CORT concentrations that becomes apparent when studying seasonal avian endocrine responses is how CORT production changes when faced with predictable and unpredictable environmental stressors (see Chapters I and III, i.e., any change in the environment that moves an individual out of their physiological steady state). A tradeoff between survival and reproduction is quite clear in many species and the degree of variance with which different species modulate responses to environmental changes using CORT is vast due to population and species variances in life-history traits such as developmental rates, age, fecundity and lifespan (Stearns 1992; Roff 2002). Such negative correlations have been seen between survival and reproduction and imply the existence of ubiquitous trade-offs (Charnov 1993). Proximate explanations for life history trade-offs are believed to result from limitations in the availability of critical resources (e.g. energy, nutrients, time), and require decisions on differential allocation of resources to costly traits (Ricklefs and Wikelski 2002; Zera and Harshman 2001). For example, individuals who invest their resources into reproductive function typically have fewer resources to invest in processes concerning self-maintenance, like the acquisition of additional fat (e.g. hyperphagia before migration) or elevated immunity (Sheldon and Verhulst 1996).

It has been suggested that these types of trade-offs are influenced by CORT modulation (which is influenced by individual variation) and that adrenocortical responses to environmental stressors occur through gene-environment interactions starting during development and continuing throughout different life cycles (Wingfield 2013). Levels of CORT, at both baseline and stress-induced levels are important for mitigating energy-expensive trade-offs and for protecting individuals during difficult and expensive environmental changes. This is a strong basis for the seasonal changes seen in both baseline and stress-induced levels of CORT.

The level of activation of stress responses often correlates with the overall health of an animal, and as a result CORT levels are often used to study organismal health at not just population levels, but for individuals as well (Romero 2004). Numerous studies have used CORT levels as an indicator of fitness (or lifetime reproductive success) in individuals and populations (Wikelski and Cooke 2006; Cabezas et al., 2007). Most often high concentrations of CORT (e.g., stress-induce levels) are negatively correlated with survival. For example, a long-term study on House Sparrows (*Passer domesticus*) by Koren et al. (2012), measured accumulated hormones in feathers grown during postnuptial molt and found that, when compared to controls, birds with significantly higher CORT perished over the following winter. Clearly CORT is an important hormone but its relationship with survival and reproduction is complex.

Factors Underlying Variation in Seasonal Corticosterone Production

The nature of the environment in which an organism inhabits has a significant impact on its physiology and behavior. Geographic factors (e.g. Temperate, Tropical, and Arctic localities), heavily influence seasonal variation in CORT secretion and regulation. It has been suggested that Arctic and Temperate species regulate CORT at different points along the HPA axis (Wingfield and Hunt 2002), and that the site of regulation often changes depending on the time of year (Breuner et al., 2003). A common finding is that birds vary strongly in their CORT levels based on geographic location and geography is known to strongly impact weather and resources.

Often species that breed at higher or more-northern altitudes/latitudes tend to have shorter breeding seasons with a limited number of reproductive attempts, due to temporal constraints from severe weather. The fewer clutches a bird has the more valuable each clutch becomes. Numerous studies support this pattern of populations breeding at high altitudes and/or northern latitudes often having increased value in broods and suppression of the magnitude of stress responses as prolonged stress can reduce reproductive output (Silverin et al., 1997; Silverin and Wingfield 1998; Wilson and Holberton 2004). This suppression or dampening of CORT production during reproductive periods is more often observed in Arctic breeding species rather than species breeding in Temperate and Tropical zones. This is mainly due to the short period of time Arctic-breeders have to reproduce before severe winter storms arrive.

Mating systems also impact how CORT is secreted seasonally, especially during parental care periods within the breeding season. Results from Bókony et al. (2009) suggest that whichever sex is responsible for the majority of parental care will often experience suppressed GC reactivity. For example, a well-illustrated study by O'Reilly and Wingfield (2001) compared several species of shorebird that exhibit three different mating systems: the polygamous Pectoral Sandpiper (*Calidris fulicaria*), the monogamous Semi-palmated Sandpiper (*C. pusilla*), and the polyandrous Red Phalarope (*C. fulicaria*). The authors predicted that the sex responsible for the majority of parental care would suppress GC reactivity during incubation and nestling phases. Supporting their predictions, GC production was suppressed in female Pectoral Sandpipers (female raises the brood alone), equal in male and female Semi-palmated Sandpipers (parental care is shared), and suppressed in male Red Phalaropes (male raises the brood alone).

The patterns of annual CORT secretion and the impacts of location are good generalities that cover a wide number of species. However evidence is mounting for a number of steroid hormones that there is not a 'one size fits all' explanation for circulating levels of hormones. Many species exhibit wide species ranges or a 'cross-over' in reproductive attributes/behaviors (e.g., have both Temperate and artic attributes) and more emphasis is needed on what this might mean for circulating levels of CORT and other hormones.

Preliminary Study and Study Species

The research described here profiles CORT levels that has been sectioned into broad ecological stages (breeding, pre-breeding & non-breeding) from compiled samples taken from a banded population of Northern Cardinals (*Cardinalis cardinalis*) that has been studied since 2007 at Lake Thoreau Environmental Research and Education Center. For this work, samples were taken and analyzed from passively captured individuals from 2007-2011. The data collected from these samples represents a starting point of investigations into CORT within this population and species. Cardinals are a multi-brooded species with a breeding season that can last over 6 months, with one pair having typically 2-4 broods (however up to 10 nests have been reported for pairs within a single breeding season, J.M. Jawor. unpubl. data), consisting of 2-4 eggs per clutch. Cardinals are a bi-parental species and both parents provide provisioning to their offspring, and contributing almost equally to offspring care, often fledging young 9-10 days after hatch. Additionally, nestling feeding rates of male cardinals appear to be unaffected by baseline or elevated levels of testosterone (DeVries and Jawor 2013). Cardinals are very territorial, usually keeping the same territory and mate year-round and both sexes participate in territory defense (Halkin and Linville 1999). Because cardinals tend to nest along forest edges, their nests sustain high depredation rates (~75% loss each year, M.S. DeVries, unpubl. data). Overall cardinals exhibit multiple behavioral characteristics similar to Arctic, Tropical, and Temperate breeders but whether they match one group specifically with respect to CORT secretion is not known.

From a human observers perspective it could seem like cardinals are under constant stressful conditions (e.g. continual territory defense, long breeding seasons with many young produced), but when captured we see no signs of chronic stress (e.g. muscle wasting, excessive amounts of parasites or abnormally high rates of infectious disease) indicative of suppressed immunity within this population of cardinals. This suggests either; a) they are not, in fact, experiencing stress at high levels, or b) they are somehow mitigating the impacts of potentially high CORT levels. By analyzing past samples from cardinals I first determined what, if any, variation exist seasonally in CORT levels as cardinals transition between annual life-history stages. I predicted that cardinals will have annual CORT profiles similar to that of other Temperate-zone breeders; on average, higher mean CORT in the breeding season and lower in nonbreeding, with females having lower CORT levels than males during breeding (to guard against elevated CORT deposition into eggs).

Methods

Cardinals were captured from 2007 to 2011 ($N = 265$; ♂ $n = 144$, ♀ $n = 121$) at the Lake Thoreau Environmental Research and Educational Center (Hattiesburg, MS, USA, 31.34°, -89.42°) where a banded population of cardinals has been under study since 2007. Mist nets and potter traps were deployed to catch cardinals between the hours 0600-1100 on capture days. Birds were captured in a variety of contexts; non-breeding at food resources, breeding at nests. Blood samples were obtained using a 25-gauge needle to puncture the alar wing vein, after which 100-200µl of blood was collected in heparinized micro-hematocrit tubes. Samples were kept on ice in the field and then later separated into plasma and red blood cell fractions by centrifugation and plasma was stored at -20°C until analyses.

To assess seasonal variation, CORT samples taken in the months of November – February were grouped as ‘non-breeding’, with March and April being ‘pre-breeding’ and ‘breeding’ during from May–August. During the breeding season, birds were captured during periods of incubation or while feeding nestlings, as opposed to passive captures with baited mist nets and walk-in traps, capture at food resources was the method used in all other seasons. Grouping months into seasons helps gauge how cardinals are modulating seasonal CORT, and allows assessment of levels between the sexes, while providing additional statistical power due to the three pairwise comparisons.

Study Issues

A few issues that could impact results will be discussed briefly. The samples analyzed here were not all taken within 3 min of capture (<3 min after capture more accurately reflects a non-stressed state; Romero and Reed 2005) and in many cases there was no pre-bleed handling time recorded. Initially, blood samples used in this study were not all collected for CORT analysis, but were part of other research studies. It is also important to note that some individuals experienced a chemical challenge (gonadotropin-releasing hormone [GnRH]) to force testosterone production from 2009-2011 for another aspect of this research. Corticosterone has been observed to negatively correlate to testosterone in some species and could have caused CORT concentrations to be lower than otherwise so. This work was done to better understand testosterone and behavior in this species and these samples have been assessed separately, however no work using GnRH challenges has found a correlation between CORT levels and T (DeVries et al. 2011, 2012; DeVries and Jawor 2013).

Corticosterone Analyses

Cardinal plasma CORT concentrations were determined using an ELISA immunoassay (EIA, Arbor Assays, Inc., #K014-H5) following modified techniques described for cardinals based on DeVries and Jawor (2013). In all plasma samples (10μl) we added 2000 cpm of tritiated CORT (H^3-CORT, PerkinElmer) for recovery calculations after extractions. Samples were extracted three times with diethyl ether, after which they were dried under nitrogen then re-suspended in 400µl of assay buffer. Samples on the EIA plate were assayed in duplicate quantities of 50µl, and we used an additional 100µl to determine recoveries (mean recoveries=94%). Following incubation with capture and identifying antibodies, plates were read using a microplate reader (Microplate Manager; Bio-Rad Laboratories, Inc.) and CORT concentration for each sample was calculated by the plate reader's built-in 4PLC software. Levels were corrected for initial sample volume and incomplete recovery. For each individual, all plasma samples were analyzed within the same assay and we randomly assigned all samples from the same individuals to plate wells. We randomly placed four Northern Bobwhite (*Colinus virginianus*) homogenized plasma samples throughout each plate to determine inter-assay and intra-assay variations. Plate correction factors were determined via comparison of intra-assay variation and applied using methods described by Jawor et al. (2006). Intra-assay coefficients of variation ranged from 7.9-11.2%, and inter-assay variation was 10.2%.

Statistical Analyses

Data were analyzed using JMP statistical software, version 10.0 (SAS Institute, Inc. 2012). Corrected CORT concentrations were log-transformed to meet normality requirements, Shapiro-Wilks goodness-of-fit test were used for verification. To test whether seasonal groups (non-breeding, pre-breeding & breeding) were significantly different from one another a One-way ANOVA was used. All stats are at the $p<0.05$ level, with data from results of statistical tests expressed as mean ± standard error (SE), and raw data (e.g., CORT levels represented in ng/ml) expressed as mean ± standard deviation (SD). One-way ANOVA was used to assess the relationship between CORT and mass.

Results

There was a significant effect of season on CORT levels at the $p<0.05$ level for 2 seasons ($F_{2,264}=7.16$, $P=0.0009$; $F_{2,262}=7.61$, $P=0.0006$). Post hoc comparisons using the Tukey HSD test indicated that the mean CORT for non-breeding (51.6±23) was significantly different than the breeding season (40.4±20.1). However, the pre-breeding levels (44.9±22.5) did not significantly differ from the non-breeding and breeding seasons levels (Figure 1).

When comparing the effect of season on CORT levels separately between the sexes, results suggest that males ($F_{2,141}=4.38$, $P=0.0143$) contribute to this effect (season) more so than females ($F_{2,118}=3.19$, $P=0.0444$). For both sexes, the significant difference was between the non-breeding and breeding seasons.

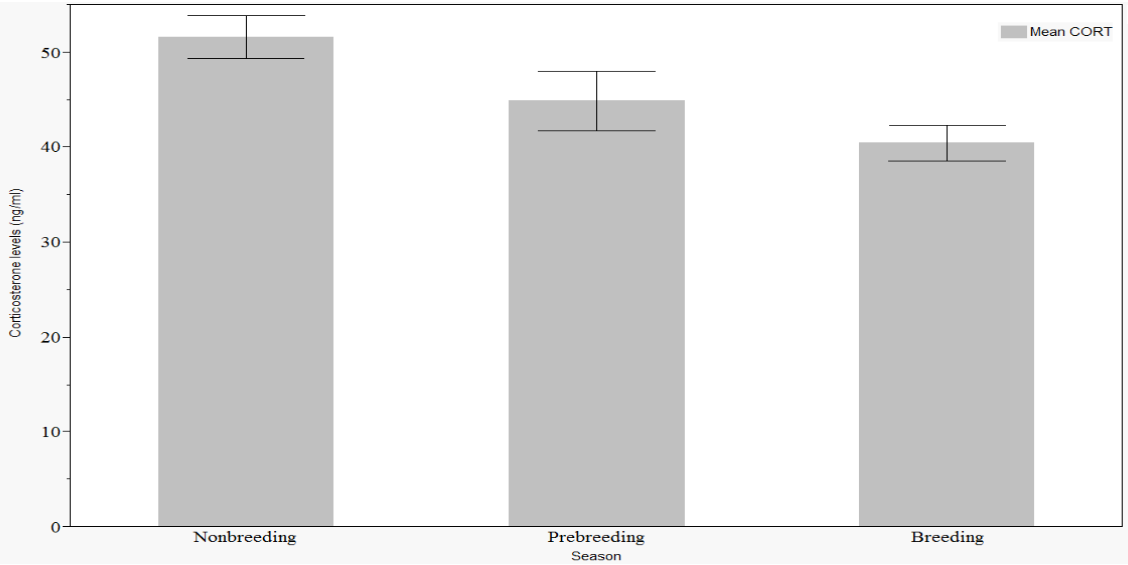


Figure 1. Corticosterone Levels Between the Seasons. Sexes grouped, during the non-breeding (n = 100), pre-breeding (n = 52), and breeding (n = 113) seasons.

Corticosterone Levels and Mass

There was a significant positive correlation between CORT and mass ($F_{1, 244}$ = 10.35, SE = 0.42, *P* = 0.0015, Figure 2), with heavier cardinals having higher average CORT levels, when compared to lighter individuals.

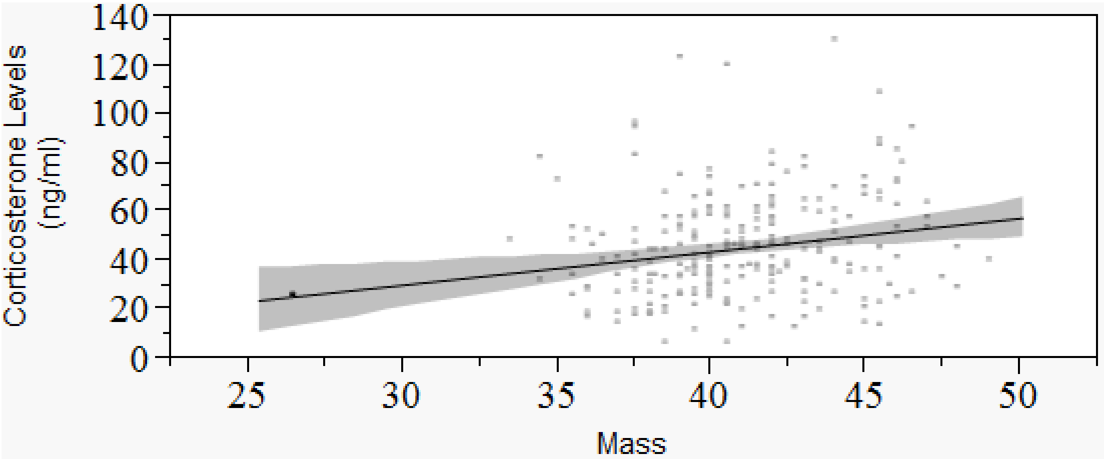


Figure 2. Positive Correlation Between Corticosterone Levels and Mass. Sexes combined. Average CORT concentrations positively correlate with mass (g). *N* = 246.

Discussion

Results from this data suggest that, on average, cardinals had higher levels of CORT during non-breeding seasons, as opposed to, pre- and breeding seasons. This was unexpected as a majority of resident species that are Temperate-breeders produce higher CORT levels while breeding during the spring and summer (Romero and Remage-Healey 2000; Romero 2002). Two possible factors explaining this type of CORT modulation are as follows; 1) elevated CORT during the colder winter months can be contributed to, at least in part, increases in basal metabolic rate (BMR) which has been suggested as a compensatory mechanism for the loss of body heat to the surrounding environment (Sgueo et al., 2012); and 2) suppressed CORT during the breeding season preserves energy consumption allowing for repeated nesting attempts. This species often builds nests along the forest edge often resulting in high nest depredation rates and frequent re-nesting. This type of energy constraint, or dampened HPA-axis responsivity during breeding could also be indicative of brood value (Bókony et al., 2009). This means that when the value of the current brood out-weighs that of potential future broods, an organism will attenuate HPA-axis responsivity to external stressors in order to prevent any behavioral interference with the current reproductive attempt (e.g. high CORT levels inducing brood abandonment), but further research will be necessary before this can be fully determined, in particular, basal and stress-induced CORT within the breeding season.

The results from this data suggest a significant positive correlation between mean CORT levels and mass (g), suggesting heavier (or healthier) cardinals possess the energetic reserves required to support higher CORT levels seasonally. Or maybe heavier cardinals are in better condition and able to more efficiently utilize reverted energy from higher CORT levels in circulation. Data from past studies have suggested strong links between body mass and BMR in birds, with heavier birds generally having higher BMR (McNab 2009), but this is not true for all species (McKechnie 2008). Maybe heavier or healthier cardinals have the additional energy to support elevated CORT whenever predictable/unpredictable environmental perturbations demand it from an individual. Future studies with cardinals inhabiting environments at higher latitudes (harsher winter weather) will increase our understanding of how much variation in cardinal non-breeding CORT levels can be contributed to changes in temperature and basal metabolic rates/capacities.

It is important to note that the HPA axis varies, both daily and seasonally, in levels of regulation so organisms can respond adaptively according to an individual's condition, age and time of year (e.g., seasons). After a stressor has been perceived, via sensory modalities, a hormonal response is transduced, and at target sites (e.g., brain, liver, adrenals) a response is generated (e.g., morphological, physiological, behavioral). Several pathways between the hypothalamus and the anterior pituitary function to release hormonal signals in reaction to a perceived threat (e.g., predation event). Once these chemical messengers reach target sites (e.g., brain, adrenals, liver), several paths exist to regulate levels of response. Metabolizing enzymes (e.g., 11β-HSD I/II) can increase binding of hormone-receptor complexes or deactivate the hormone and decrease hormone-receptor binding. These enzymes can change daily and seasonally, and act differently between closely-related individuals. Not just enzymes, but receptor types and densities experience a vast degree of change between individuals on a daily basis and according to time of year. Changes in hormone levels cannot be contributed to any one entity, it is far more complex. The HPA axis changes according to an individual's needs. The sensitivity in how stressors are perceived, variation in how that perceived threat is transduced and levels of response at target sites, all exhibit change both daily and seasonally. All of this change is coupled with variances among individuals; different heritable traits impact how they perceive and experience life (gene-environment) which ultimately contributes to how hormones are produced and in return, how the organism will respond.

In summary, from this initial annual profile we see that cardinals, like almost all other species assessed, show annual variation in circulating CORT; but contrary to what many Temperate-zone species exhibit, cardinals have the lowest annual CORT levels during breeding. This suggests that energetic demands vary throughout the year for cardinals, but perhaps not in a manner typical of Temperate-zone species. Given that cardinals display behavioral and physiological attributes characteristic of Tropical and Arctic breeders as well, this may not be particularly surprising. One problematic attribute here is that samples were not collected specifically to obtain levels closer to baseline CORT and no stress-induced levels were collected, both of which could show very different patterns than that was observed here.

CHAPTER III

SEASONAL VARIATION IN STRESS RESPONSES OF THE NORTHERN CARDINAL (*CARDINALIS CARDINALIS*); DOES RESPONSIVENESS VARY WITH TIME OF YEAR?

Introduction

What is Stress?

Defining stress and what stress encompasses has long been a topic of much debate (Sapolsky et al., 2000; Romero et al., 2009; Korte et al., 2005; McEwen and Wingfield 2003). This is largely due to individual variation, and the vast degree of variation both within and between avian populations and species, in perceiving potential stressors and in how robust stress responses are to perceived threats. For simplistic definition Chrousos and Gold (1992) stated, “stressors are stimuli that disrupt or threaten to disrupt homeostasis”. To better understand the complexities between species and what they might perceive as stressful it is, as Robert Sapolsky et al. (2000) said, “important to try and adopt the perception of the organism of interest, rather than ones’ own perception” and “it is always critical to appreciate an ethological setting within the framework of an organism, rather than the perception of the human studying that organism. A circumstance that might, to a human observer, appear to represent a stressful challenge to homeostasis might merely represent a normative life history stage for an animal with adequate metabolic reserves”. These statements together give researchers a strong clue that the stress response is not an easily defined phenomena, but it is one of critical importance.

Two major factors contributing to stress are the lack of control and the lack of predictability in events going on outside the animal (Sapolsky et al., 2000). It can be broken down even further into two types of stressors; predictable and unpredictable. Predictable (often acute) stressors (e.g. predation, unexpected drop in food availability, territorial disputes, daily temperature changes) are transient and can be anticipated (i.e. as the seasons move from summer to fall to winter colder temperatures are going to occur; predators exist). Predictable stressors can also take the form of daily physical, energetic, and social demands associated with the life history cycle (Landys et al., 2006).

Similar to predictable stressors, unpredictable stressors force an organism to make changes in order to survive, but vast differences are seen in how long it takes this type of stressor to be over. It is important to note that unpredictable stressors often do not present the individual with immediate life-threatening demands like many predictable stressors do. Instead, they present conditions that are deteriorating rapidly (e.g. decreased intake of food, and/or increased exposure to harsh elements), and this directly forces an organism to make adjustments to better its chances of survival. One major similarity between both types of stressors is that once a disturbance passes, a recovery phase is entered and the individual likely can return to its appropriate life history stage (Wingfield et al., 1998). Often with prolonged unpredictable stressors, the organism isn't given a chance to enter into the recovery phase, and this is where a line can be drawn between acute and chronic stress, with all the damages that can accrue over time as a result of chronic stress pathology (discussed in more detail later in this chapter).

What is the Stress Response?

During the stereotypical vertebrate stress response two phases of hormones, with their subsequent physiological/behavioral consequences, are produced. The first phase occurs once a stressor has been perceived; almost instantaneously the sympathetic nervous system becomes activated, resulting in an enhanced secretion of catecholamines (epinephrine [E] & norepinephrine [NE]) from the medulla of the adrenal glands, followed by corticotropin-releasing hormone (CRH) from the hypothalamus into hypothalamic-pituitary portal circulation. Corticotropin-releasing hormone will then enhance the secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary. Additionally, a decrease in gonadotropin-releasing hormone (GnRH) production from the hypothalamus occurs, followed by a reduction in pituitary gonadotropin release (follicle-stimulating hormone [FSH], luteinizing hormone[LH]), and lastly, increased pituitary prolactin (PRL) secretion and pancreatic secretion of glucagon also occur as stressors continue.

The next phase of hormone release aids an organism in fight or flight by providing a constant elevated feed of energy until normal homeostasis has returned and/or the stressor has been avoided or reduced. Glucocorticoids (e.g. corticosterone [CORT], the main GC in birds) are involved in this much slower second hormonal phase. The elevated hypothalamic CRH leads to elevated pituitary ACTH release, after which ACTH induces adrenal cortical tissues to release CORT. Adrenal GC secretion from the adrenal cortex is stimulated within a few minutes of a perceived stressor. All together this response to stress aids organisms by providing an immediate set of behavioral/physiological responses best suited for whatever challenge the environment has presented.

The two phases within the stress response differ strongly in means and times taken for each chemical messenger to reach its target tissue, with the first phase exerting most of its effects through rapid secondary messenger cascades at target tissues within seconds to a few minutes (Sapolsky et al., 2000). Conversely, few GC actions are evident until roughly an hour after an encounter with a stressor, due to steroid actions being mainly genomic (Sapolsky et al., 2000). Elevated GCs can lead to changes in blood glucose, body temperature, foraging and feeding activities, and even can alter how the body stores fat (Astheimer et al., 1992; Dawson et al., 1972; Gray et al., 1990; Harvey et al., 1986; Palokangas and Hissa 1971; Rogers et al., 1993; Siegel 1980; Wingfield et al., 1998) all of which aid in prolonging the response to stressor if need be and then the hopeful physical recovery from stress.

Acute versus Chronic Stress

Acute stressors are often predicable (e.g. predation, low food availability at certain times of the year, psycho-social stress [e.g. conspecific territorial disputes], daily temperature changes) and transient and most likely will not contribute significantly to long-term energy costs that have been described for stress responses (assuming organism survives, Wingfield 2013). Overall, GC-mediated mobilization of glucose is believed to provide the necessary energy to survive acute stress (Sapolsky et al., 2000). Redirecting energy distribution to target tissues in response to stress can have serious drawbacks, in particular, whenever homeostasis is being prevented from returning to its original, ‘unstressed’ state. At this time the pathological effects of chronic stress can wreak havoc on the body, often resulting in the death of an organism if unable to return to an 'unstressed' state (recovery of homeostatic norm).

Whenever a stressor prevents an organism from quickly re-establishing its homeostatic norm, CORT concentrations will continue to increase in circulation. During standardized capture and restraint stress protocols (designed to simulate a stressful event), maximal CORT concentrations occur within 30-60 min (Wingfield et al., 1982; Canoine et al., 2002). When these maximal concentrations continue to stay elevated instead of returning to 'basal' levels, it is termed "chronic" elevation. Exposure to prolonged CORT elevations can lead to a number of detrimental effects (Wingfield et al., 1994), including muscle loss (De La Cruz et al., 1981; Tomas et al., 1979), suppression of the immune system (Sapolsky et al., 2000), and increased susceptibility to disease (Ardia et al., 2003; Hanssen et al., 2004). With regards to chronic stress, the same hormones that help an organism survive acute stressors can, under less-than-ideal circumstances, wreak havoc inside the body. Differences in stress responses are known both between and within species/populations, these large-scale variations are, in part, due to the many factors underlying how and why phenotypes experience stressful events differently.

Factors Contributing to Seasonal Variation in Stress Responses

In birds, young breeders usually exhibit more robust stress responses when compared to older individuals (Angelier et al., 2007), and this can be partly contributed to older birds acclimating to the stressful conditions associated with reproduction. Additionally as individuals age their future reproductive events inevitably decrease. Therefore, the value older birds place on a brood should increase in each successive breeding season; acute stress responses are believed to be attenuated in older individuals in order to ensure that current reproduction is not inhibited (Breuner 2011). In this way, they avoid the long-term fitness (or lifetime reproductive success) costs of the GC stress response (Wingfield and Sapolsky 2003) by preventing behaviors associated with elevated CORT (brood abandonment). The impact age has on CORT regulation depends on a number of variables (e.g. organism's lifespan, breeding environment, mating system, length of breeding season), which can vary dramatically both within and between species and populations.

The sex of an organism is another factor contributing to variances observed in stress responses. Results from past studies suggest that whichever sex is responsible for a majority of parental care will exhibit a pronounced suppression of the stress response (see Chapter II; O'Reilly and Wingfield 2001). This is thought to protect against offspring desertion or to prevent against any immediate degradation in the condition of whichever sex (maternal/paternal) is providing the bulk of nestling care.

Adaptive reasoning for hormonal variation could stem from differential needs between the sexes, or within the same sex of different species, throughout different life-history stages. It is beneficial to an organism to be as flexible as possible when enduring constantly changing environments. Whether the flexibility stems from morphological, physiological or behavioral adaptations, all have the potential of enhancing an organisms ability to cope with extreme conditions and changing habitats. It seems plausible that selection would favor phenotypes possessing the survival capability for coping with, or buffering against, extreme conditions while maintaining some productive output. These adaptations have been called resilience, which is the ability of an individual to experience disturbance and maintain normal processes (Martin and Wiebe 2004). The GC stress response is thought to be partially heritable, and selection is therefore likely to act on this mechanism in order to optimize organismal fitness and provide resilience (Angelier et al., 2011; Evans et al., 2006).

In birds, studies often have mixed results which can be a testament to underlying physiological complexities. Some species have elevated CORT during breeding or summer months, others have higher levels during the wintering months or non-breeding season. Most variance within the breeding season stems from an organisms breeding habitat, which is often coupled with how long a breeding season lasts. Two possible explanations for this type of seasonal variation in CORT secretion are as follows; a) colder winter temperatures force an increase in energy expenditure to compensate for body heat lost to the external environment, and b) lower levels of CORT during breeding aids to avoid of the numerous deleterious effects elevated CORT levels have been shown to have when maternally-transferred to the developing embryo. Not only are negative maternal effects repressed, but also brood abandonment induced by elevated CORT levels are avoided as well.

Study

This study examines the annual CORT stress response profile (initial and stress-induced concentrations) in a banded population of Northern Cardinals (*Cardinalis cardinalis*) that has been under study since 2007. Cardinals are a resident species with a relatively long breeding season (6+ months, Halkin and Linville 1999). Socially monogamous pairs (often paired for life) are territorial year-round, and share parental duties (Halkin and Linville 1999). On average, cardinals lay 1-5 eggs (avg. 3-4) per clutch and typically produce 3-5 clutches in a season, but pairs have been documented having up to 10 broods during a single breeding season (J.M. Jawor unpubl. data). Due to their tendency to build nests along forest edges, these open-cup nesters often suffer high depredation rates (up to 75% reported within single breeding season, M.S. DeVries, unpubl. data). With almost six months of breeding, defending, building, raising and often losing offspring it's plausible to suggest that cardinals are subjected to seemingly high amounts of environmental stressors which could be reflected by plasma CORT concentrations, both at general circulating levels (non-stressed, hereafter initial CORT) and at levels following capture and restraint stress (stress-induced CORT). This is the first study to my knowledge that analyzes year-round initial CORT and stress responsiveness to capture and restraint in cardinals. I will be reporting not only seasonal changes and sex differences in initial CORT levels, but also sex differences and seasonality in stress responses as well.

Methods

Cardinals were captured using mist nets for initial (removed <3 min upon capture more accurately reflects unstressed condition, Romero and Reed 2005) and stress-induced CORT (after 30 min of restraint) from August-July 2012-2013 (N = 127, males n = 69; females n = 58) at the Lake Thoreau Environmental Research and Education Center (Hattiesburg, MS, U.S.A., 31.34°, -89.42°). Upon capture birds were removed from the net and a blood sample of 100-200μl was collected using a sterile 25-gauge needle after puncture in alar wing vein. Blood was then collected in heparinized micro-hematocrit capillary tubes. A second sample of 100-200μl (total amount taken represents < 1% of bird's mass in blood was collected) was taken following standard capture and restraint stress protocols (Wingfield et al., 1995). Birds were handled to record structural measurements (crest height, wing, tail & tarsus length [all in mm], mass [g]) after the first sample was obtained, then birds were restrained placing them securely into an opaque cloth bag for 30 min after collection of the initial sample, at which time the second sample was collected. A numbered metal band (U.S. Fish and Wildlife banding sub-permit #23479B) was then placed loosely around the bird's leg. Samples were then stored on ice until taken to the laboratory, where all samples were centrifuged (within 8 hours) for 5 min to allow for separation between plasma and red blood cells. Plasma was stored at -20°C until hormone levels were assayed.

Corticosterone Analysis

Cardinal plasma CORT levels were determined using EIA (Arbor Assays, Inc., #K014-H5) following modified techniques described for cardinals based on DeVries and Jawor (2013). In all plasma samples (10µl) we added 2000 cpm of tritiated CORT (H^3-CORT, PerkinElmer) for recovery calculations after extractions. Samples were extracted three times with diethyl ether, after which they were dried under nitrogen then re-suspended in 400µl of assay buffer. Samples were assayed in duplicate quantities of 50µl each on the EIA plate, and we used an additional 100µl to determine recoveries (mean recoveries=94%). Following incubation with capture and identifying antibodies, plates were read using a microplate reader (Microplate Manager; Bio-Rad Laboratories, Inc.) and CORT concentration for each sample was calculated by the plate reader’s built-in 4PLC software. Levels were corrected for initial sample volume and incomplete recovery. For each individual, all plasma samples were analyzed within the same assay and we randomly assigned all samples from the same individuals to plate wells. We randomly placed four Northern Bobwhite (*Colinus virginianus*) homogenized plasma samples throughout each plate to determine inter-assay and intra-assay variations. Plate correction factors were determined via comparison of intra-assay variation and applied using methods described by Jawor et al. (2006). Inter-assay and intra-assay coefficients of variation were 10.8 and 8.2%, respectively.

Statistical Analyses

To account for inter-assay variation in each assay we randomly placed multiple standards throughout each assay to determine and apply correction factors to all plasma samples on each plate. Months were grouped into 3 broad seasons (non-breeding [Sept.-Feb.], pre-breeding [Mar. & April] and breeding [May-Jun.]) for statistical analyses. Corrected CORT concentrations were log-transformed to meet normality requirements and allow for the use of parametric statistics, Shapiro-Wilks goodness-of-fit test were used for verification. One-way ANOVA was used to test whether seasonal groups (non-breeding, pre-breeding & breeding) were significantly different from one another. . To assess seasonal CORT responses (difference between stress-induced and initial levels), One-way analysis ANOVA tests were used, followed by Tukeys HSD post hoc tests for significant effects. To determine correlations between 'basal' or initial CORT levels and that of stress-induced concentrations with age (adult or juvenile), sex, mass (g), tarsus, crest, wing (all structural traits measured in mm), external temperature (°F), humidity (%), wind speed (mph) and weather condition (clear, scattered clouds, overcast) information obtained from National Oceanic and Atmospheric Administration website (NOAA.gov), general linear mixed models were used. All tests were done at the $p<0.05$ level, with data from results from statistical tests expressed as mean ± standard error (SE), and raw data expressed as mean ± standard deviation (SD).

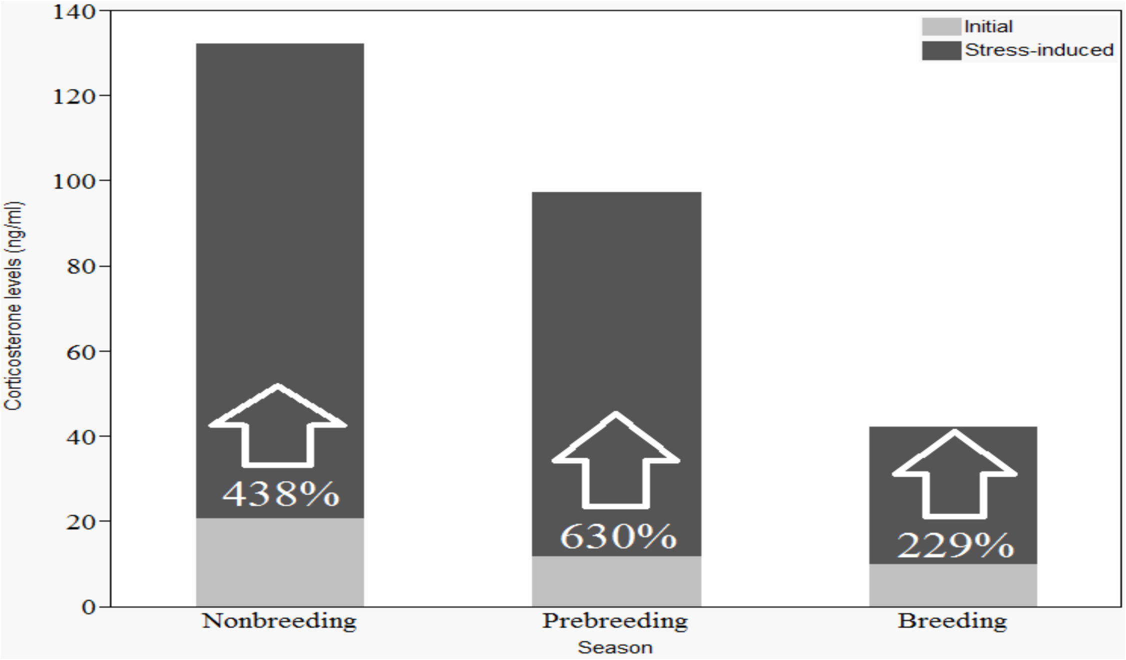
Results

Seasonal Variation in Initial and Stress-induced Corticosterone

One-way between subjects ANOVAs were conducted to compare the effect of initial and stress-induced CORT levels and sex. There was a significant effect of sex on CORT at the $p<0.05$ level for stress-induced levels ($F_{1,80}=4.66$, $P=0.0334$). Post hoc comparisons using the Tukey HSD test indicated that mean stress-induced CORT for males (98.99±64.1) was significantly different than that of female stress-induced levels (77.14±57.7).

Next we conducted a One-way between subjects ANOVA to compare the effect of seasons, for each sex, on CORT levels. For males, there was a significant effect of season on initial CORT ($F_{2,41}=8.46$, $P=0.0008$). Post hoc comparisons using the Tukey HSD test indicated that mean initial CORT in the non-breeding season (21.12±12.6) was significantly different than that of initial CORT in the pre-breeding (10.42±7.2) season. There was also a significant effect of season on stress-induced CORT ($F_{2,41}=4.83$, $P=0.0131$). Post hoc comparisons using the Tukey HSD test indicated that mean stress-induced CORT in the non-breeding (122.66±65) was significantly different than that of stress-induced CORT in the breeding (41.41±11.6) season.

For females, no significant effect found of season on initial levels of CORT ($F_{2,35}=2.36$, $P=0.109$). However, there was a significant effect of season on stress-induced CORT ($F_{2,35}=9.57$, $P=0.0005$). Post hoc comparisons using the Tukey HSD test indicated that mean stress-induced CORT in the non-breeding (99.82±59) was significantly different than that of stress-induced CORT in the breeding (26.4±8.52) season.



Seasonal Variation in Initial and Stress-Induced Corticosterone Between the Sexes

Results indicate that between the sexes, both initial (t_{38} = -2.019, *P*=0.05) and stress- induced (t_{38} = -0.873, *P*=0.387) CORT levels did not differ significantly during non-breeding and pre-breeding (Initial: t_{24} =1.449, *P*=0.16; Stress-induced: t_{24} =0.121, *P*=0.904) seasons. During the breeding season initial CORT levels between the sexes did not differ (t_{14} = -1.375, *P*=0.19), but stress-induced levels were significantly different ($t_{8.7}$ = -2.298, *P*=0.0478; see Tables 2 and 3).

Table 2

Mean and Range of Male Cardinals Corticosterone Levels (initial and stress-induced)

♂	Initial	Range	Stress-induced	Range
Non-breeding	22.74 ± 13.47	42.26	117.83 ± 69.89	177.12
Pre-breeding	10.42 ± 7.20	27.56	84.95 ± 49.85	171.48
Breeding	17.96 ± 17.16	18.42	43.29 ± 10.92	26.63

Table 3

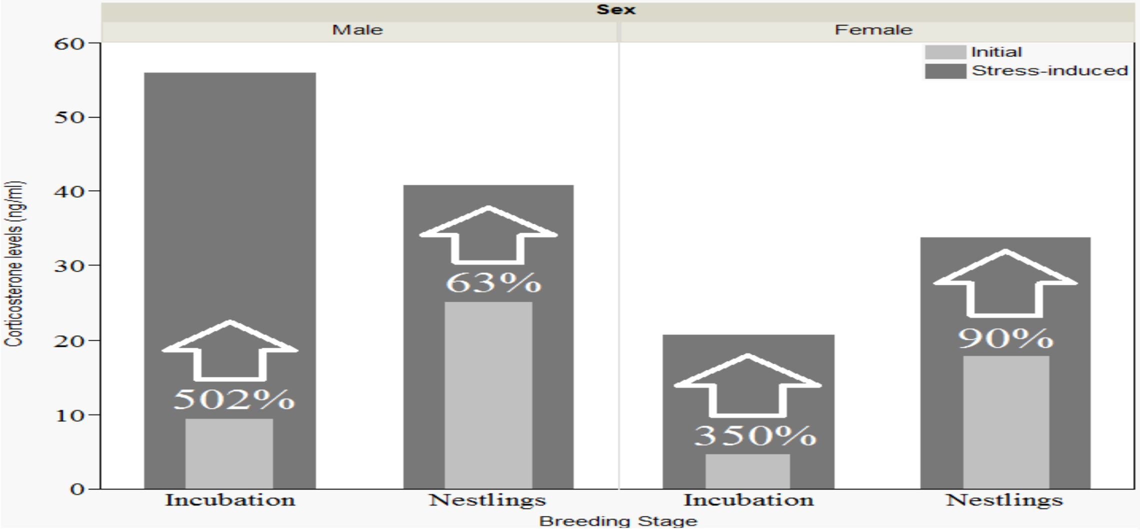
Mean and Range of Female Cardinals Corticosterone Levels (initial and stress-induced)

♀	Initial	Range	Stress-induced	Range
Non-breeding	15.33 ± 9.34	28.45	99.81 ± 60.08	214.75
Pre-breeding	15.29 ± 8.7	21.64	87.66 ± 52.78	144.11
Breeding	9.45 ± 8.15	22.38	29.19 ± 12.29	42.82

Sex Comparison Within the Breeding Season

In both sexes individuals assessed during incubation (only female incubate in cardinals but males do provide females with food during incubation, Jawor and Breitwisch 2004) stress responses were more pronounced than during nestling feeding and female responses were stronger during nestling care.

A One-way between subjects ANOVA was conducted to compare the effect of breeding stage (incubation & nestling) on initial and stress-induced CORT levels between the sexes. There were no significant effects of breeding stage on male initial and stress-induced levels but, an effect of breeding stage on female initial CORT was found ($F_{1,7}$=6.74, *P*=0.0356). Post hoc comparisons using Tukey HSD test indicated that mean initial CORT in incubation (5.05±2.52) was significantly different than initial CORT in nestling (18±3.57) phases. Although the effect of female stress-induced CORT on breeding stages is not significant, with a higher sample size that might change ($F_{1,7}$=5.52, *P*=0.0511; mean stress-induced CORT levels during incubation, 21.89±2.83 and nestling phase 34.16±4).



Age-related Differences in Initial and Stress-induced CORT Levels in Juveniles

Juveniles were individuals identified based on plumage in the non-breeding sample only, following their first molt juveniles are nearly indistinguishable from adults (Halkin and Linville 1999). A One-way between subjects ANOVA was conducted to compare the effect of age on CORT levels (initial and stress-induced), after controlling for sex. There was a significant effect of age on both sexes for initial CORT levels (male, $F_{1,47}$=8.08, P=0.0066; female, $F_{1,44}$=10.27, P=0.0025). Post hoc comparisons using the Tukey HSD test indicated that for both sexes, mean levels of initial CORT for juveniles (male, 37.76±5.31; female, 26.36±3.27) was significantly different than initial CORT levels in adults (male, 14.87±2.69; female, 13.55±2.05).

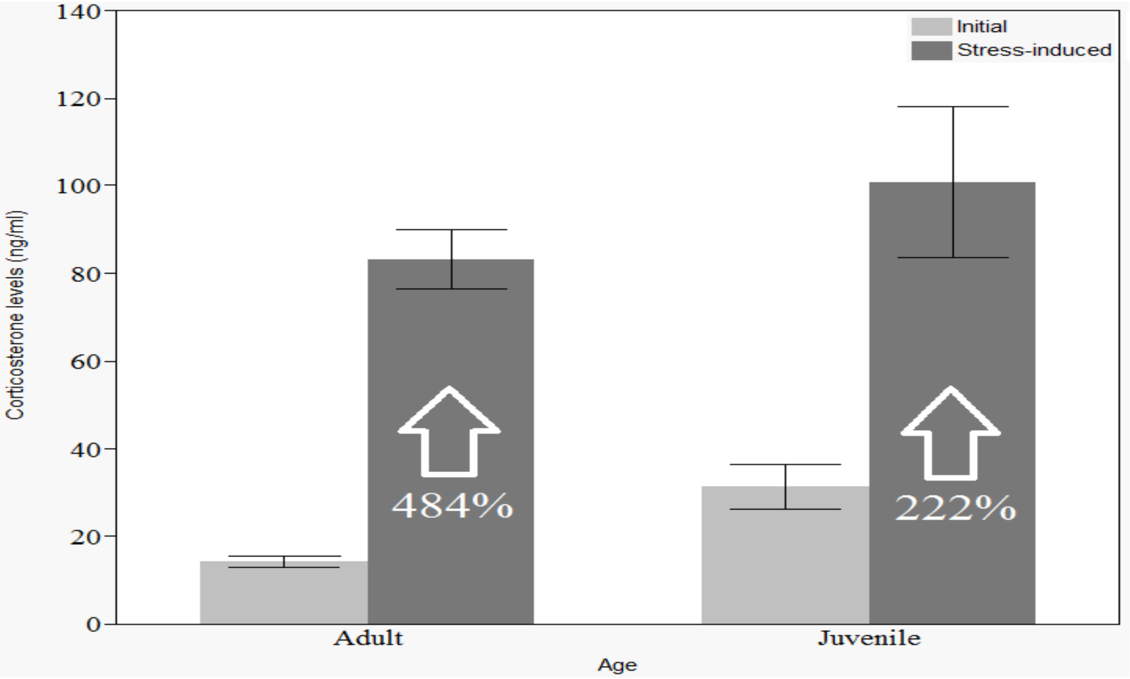


Figure 5. Age-related Differences in Initial and Stress-induced Corticosterone Levels. Adult (n=72) and juvenile (n=23) mean CORT levels.

Impact of Structural Morphology and Weather Variables on Levels of CORT

Sexes were analyzed separately due to significant variances, and juveniles were excluded from these statistical tests due to small sample size. For males, significant effects of temperature (F=4.47, *P*=0.0429) and humidity (F=7.41, *P*=0.0107) on initial CORT levels were found. No effects were found on stress-induced levels and overall magnitude in CORT response. For females, significant effects of tarsus (F=5.34, *P*= 0.0301; F= 4.63, *P*=0.0421) and temperature (F=11.89, *P*=0.0022; F=10.12, *P*=0.0042) were found on both initial and stress-induced CORT levels, respectively. For magnitude of CORT response, a significant effect was found for temperature (F=7.56, *P*=0.0114).

No significant effects were found for weather conditions (clear, scattered clouds, overcast) on initial ($F_{4,77}$=0.55, *P*=0.698), and stress-induced ($F_{4,77}$=1.22, *P*=0.307) levels, nor on the magnitude of stress responses ($F_{4,77}$=1.947, *P*=0.111). Also, no significant effects were found of wind speed on initial ($F_{10,77}$=0.76, *P*=0.665), and stress-induced ($F_{10,77}$=0.60, *P*=0.807) levels, or on the magnitude of stress responses ($F_{10,77}$=0.52, *P*=0.868). These relationships were not significant, even after controlling for age, sex and season.

Discussion

First, it is of note that the findings for initial (or pre-stress response) CORT levels using more stringent time controls for collecting initial samples, match the results from the year-round profile (described in Chapter II) with higher mean CORT levels during non-breeding, as opposed to, the pre- and breeding seasons. This supports earlier findings of Chapter II that general circulating levels of CORT vary in cardinals seasonally and they do so in a pattern not initially predicted. In this study, cardinals in this population displayed significant seasonal variation in both initial and stress-induced CORT levels. This could be due, in part, to changes in adrenal responsivity to adrenocorticotropic hormone (ACTH) and pituitary responsivity to corticotropin-releasing hormone (CRH, MacDougall-Shackleton et al., 2013) and arginine vasotocin, similar to that observed in other species (Romero et al., 1998c; Romero and Wingfield 2001), thus allowing individuals to modulate their CORT responses and appropriately modulate energy usage throughout an annual cycle where environment and activities vary throughout the year.

On average, we observed the highest annual levels of mean initial and stress-induced plasma CORT during the non-breeding season (winter). One possible explanation for this may be linked to an increase in basal metabolic rate (BMR), which has been observed in other species during winter, allowing for an enhanced cold tolerance, a process often involving substantial increases in the maximum resting thermogenic metabolic rate (Dawson and Marsh 1988; Liknes and Swanson 1996; Swanson and Liknes 2006). Results from a review on avian BMR indicate how many non-migrants adjust BMR seasonally, with winter BMR usually higher than summer BMR (McKechnie 2008), and our results show mechanistic support for this within our study population of cardinals (CORT can provide elevated glucose levels which is the fuel for increased BMR needs).

However, winter-onset increases in BMR are usually observed in species inhabiting higher latitudes than the ones where the current study site is located, where extremely cold winters are experienced, unlike sub-Tropical species where increases in BMR are often exhibited during the significantly warmer summer months (Smit and McKechnie 2010). It is suggested, that in sub-Tropical avian species, a summer increase in BMR is more likely to reflect the need for temperature regulation, and/or water conservation, than for cold tolerance (Smit and McKechnie 2010). The elevated 'baselines' and elaborate stress responses observed in this population and species during winter, while unexpected, may be a hallmark of cardinals as a Tropical descendent and may help explain their ability to expand into much more northern climates. Sgueo et al. (2012) found that more northern populations of cardinals do in fact have higher metabolic capacity during winter and that this species must operate at a higher percentage of their metabolic capacity to sustain higher relative effort (metabolic). It is possible that the loss of body heat to the external environment and the need to forage may be compensated for by elevations in CORT production.

Another significant finding in this study was recorded from pre-breeding to breeding seasons, where dramatic reductions were observed in the overall magnitude of stress responses. Nearly a 3-fold decrease was observed between CORT responses from the pre-breeding to breeding seasons. Similar initial CORT levels were recorded within this population during pre-breeding and breeding seasons (difference of <2 ng/ml), unlike the vast difference in stress-induced CORT levels following capture and restraint, suggesting a very different sensitivity in the HPA axis with time. One possible explanation for lower CORT during pre-breeding could be to decrease potential interferences often associated with elevations in CORT which have been shown to negatively impact reproductive and parental behaviors (typically through interference with testosterone, Greenberg and Wingfield 1987; Silverin 1986; Wingfield and Silverin 1986). Results from numerous studies have suggested that an inverse relationship exists between testosterone and CORT (Deviche et al., 2014). Glucocorticoids, in mammals have been shown to exhibit direct actions on testicular endocrine function by acting on testicular cells and inhibiting plasma testosterone production (Dong et al., 2004; Hu et al., 2008; Martin and Tremblay 2008). Given that testosterone promotes behaviors associated with breeding (e.g. increased singing rate, establishing and defending territories, courtship), potential selective pressures might be limiting the production of, or enhance the sensitivity to, circulating CORT at this stage to prevent interfering with these behaviors that are essential to successful reproduction (more detail on trade-offs between CORT and testosterone in Chapter IV).

In addition, within the breeding season, female cardinals had lower initial and stress-induced CORT levels, as opposed to males at this time. Explanations for why female cardinals have lower initial CORT and potentially suppress the magnitude of stress responses during the breeding season are centered on the potential deleterious impacts maternally elevated CORT levels can have once transferred to developing embryos. It has been reported that when gravid females are stressed they transfer their high CORT levels into the egg yolk where numerous deleterious effects (e.g. delayed egg hatching, loss of egg mass during incubation, reduced rate and loudness of late embryonic vocalizations, reduced chick begging display) have been observed (Rubolini et al., 2005). Another study found similar results when they experimentally injected CORT into the albumen which led to increased embryonic mortality rates, impaired development and post-hatch reductions in hatchling mass (Eriksen et al., 2003; Heiblum et al., 2001; Mashaly 1991). In addition to accruing embryonic damage, results from another study suggest that high concentrations of CORT can also negatively impact nestling survival through reduced growth rates and altered immunity (Saino et al., 2005).

The maternal-transfer of hormones like CORT is not limited to strictly having harmful impacts on developing embryos and nestlings, by doing so we ignore the possibility of short-term phenotypic changes allowing for possible increases in maternal fitness. Glucocorticoids can also be adaptive by preparing the developing embryo to best-suit the environment whenever it may hatch, and in doing so increasing the offspring's chances of survival. Results from a study by Love and Williams (2008), found that "low-quality" mothers (experimentally clipped wing feathers which altered foraging efforts) produced a higher proportion of female offspring, and male offspring produced grew at slower rates. One way this is adaptive is by producing a higher proportion of female offspring, the chances (assuming nestling survives to sexual maturity) females have to successfully reproduce are far better when compared to males (given male-male competition for mates). Secondly, the fewer and slower growing males required less food which better matched the poor-quality mothers' foraging ability. All-in-all, by poor-conditioned mothers producing broods that best match her capabilities, the maternal-transfer of elevated CORT to offspring can be adaptive by increasing the maternal survival probability 10-fold, and in doing so enhances fitness.

Another explanation for the pronounced reduction female cardinals' exhibit in CORT production during breeding may be indicative of investment differences between the sexes in parental care. Results from O'Reilly and Wingfield (2001) suggest that the more parental sex (sex with majority of parental duties) will experience overall lower glucocorticoid reactivity. Though only the female in this species is responsible for incubation, male cardinals constantly forage at this time to provide energy to incubating females and upon egg-hatch both parents provide food for their young. It could only be a small difference, if any, in the degree of parental effort both sexes exhibit but it is possible that females of this population and species are more invested in their offspring given how energetically expensive egg production can be. For males, CORT elevations observed after incubation most likely promotes an increased forage rate which can be adaptive due to potential increases in reproductive fitness via enhancing offspring survival probabilities from increases in nestling mass, and in return, expedite time until fledging.

Another way lower initial CORT and overall smaller magnitudes of stress responses exhibited during breeding may enhance reproductive fitness comes from a study by Edwards et al. (2013), which suggests that individuals with lower initial and stress-induced CORT levels returned to incubate more quickly after a disturbance. More time away from the nest, as a parent, could translate into increased exposure the eggs have to predation events which could be viewed as a reduction in overall fitness. It seems plausible that selection would favor lower circulating CORT concentrations and a reduction in magnitude of stress responses during breeding for this population and species given that up to 75% of nests are depredated. By selecting against the potentially harmful effects associated with elevations of this hormone at this time one may see an overall decrease in time away from nests and an overall increase in provisioning which may reduce the amount of time nestlings spend in the nest.

As cardinals progress through seasonal weather changes and variable reproductive bouts, they regulate their physiological response based upon numerous individual (e.g. number of successful fledglings already produced, age, physical condition) and environmental factors (e.g. length of breeding season, weather conditions, temperature changes, food availability, degree of predation). This allows them to progress from one LHS to the next in a way that balances self-maintenance (survival) with reproduction (number of successful offspring fledged within an annual breeding period) ultimately leading to enhance fitness and survival. Cardinals throughout their evolutionary history have successfully inhabited a wide range of geographic areas and variable environments (species range is from Central America to Canada and covers the entire eastern United States and the American southwest and they have been introduced in Hawaii, Halkin and Linville 1999). This is a testament to this species overall flexibility in physiology and behavior. The variation observed here in stress responses may likely be a part of this flexibility and adaptability by allowing cardinals to adapt to cooler environments, capitalize on a predator-heavy yet resource rich part of the environment, and block against any problems with maternally transmitted GC to offspring.

As organisms experience daily and seasonal events, and thus increase in age, they acclimate to environmental stressors. Through acclimation, organisms learn to cope with acute perturbations and in return, fewer stress hormones are necessary to respond adaptively. As nestlings, a period of HPA hypo-responsiveness occurs and this is believed to protect a vulnerable brain that’s still developing. As studies have shown elevated CORT levels can have deleterious effects on the developing brain. As young fledge, juveniles often mount more robust stress responses when compared to adults. As juveniles experience environmental stressors and learn what is, or isn’t a serious threat, they acclimate and overall robustness of stress responses decreases. Results from our study population suggests that juvenile cardinals are in line with previous findings and were found to exhibit higher baselines and stress-induced CORT in response to capture and restraint stress, when compared to adults.

CHAPTER IV

CORTICOSTERONE INTERACTIONS WITH OTHER ASPECTS OF PHYSIOLOGY IN NORTHERN CARDINALS

Introduction

Glucocorticoids (GCs), in particular corticosterone (CORT, main GC in birds), are central to a wide range of essential physiological processes. Glucocorticoid actions range from mediating daily energy levels at low basal concentrations; to mobilizing energy stores to exercising muscles at stress-induced levels (e.g. stress response; Sapolsky et al., 2000). The actions GCs induce in response to environmental challenges ultimately enable organisms to adaptively respond to acute challenges to homeostasis (e.g. severe weather, predation events). Stress hormones, like CORT, are able to exert broad-scale effects across major physiological systems by inducing different actions at target tissues at both low and high circulating levels. Most of GCs permissive effects are exerted at basal, or non-stressed levels, whereas its suppressive effects often occur after prolonged exposure to elevated (stress-induced) levels in circulation (chronic stress; severe storms lasting days to weeks). One well-studied area of GC physiology is in how, after prolonged elevated levels, they can disrupt reproduction.

One well-known suppressive GC action is the inhibition of gonadal hormone release (Sapolsky et al., 2000). Stress-induced GCs (e.g. elevated CORT levels in response to stressors) suppress the hypothalamic-pituitary gonadal (HPG) axis by inhibiting the activity of enzymes involved in the synthesis of androgens and/or stimulating the production of gonadotropin inhibitory hormones (GnIH, Chand and

Lovejoy 2011; Kirby et al., 2009; Wingfield and Sapolsky 2003). Results from Deviche et al. (2012) suggest that stress-induced CORT can significantly decrease circulating concentrations of the gonadal steroid testosterone (T) after 30 minutes of restraint (but not in all species, see Deviche et al., 2014). The relationship between T and CORT is complex; this is in part due to them both being steroid hormones and sharing structural properties which allow them to use similar carrier proteins while in circulation (Breuner and Orchinik 2009). Carrier proteins, like corticotropin binding globulin (CBG), can only actively bind a single hormone molecule at a time; this leads to competitive binding between CORT and T which often results in a negative correlation between the two steroids in circulation. Once bound to CBG, CORT or T become inactive until carrier proteins are broken down and then the hormone is released. These inactive-bound hormones in circulation can, whenever necessary, be broken down resulting in the newly unbound hormone becoming reactivated in circulation and free to bind to receptors and activate target tissue responses. Since both CORT and T are capable of binding to avian CBGs, changes in plasma levels of CORT (e.g. acute stress response) are likely to alter the amount of unbound or free plasma T in circulation and vice versa. For example, in response to acute stress (stress-induced), CORT levels reduce CBG availability by 50% and with fewer protein transports, more plasma CORT/T is free to enter into tissues, evoking responses at target tissue sites (Breuner and Orchinik 2009). This intrinsic relationship is further convoluted by physiological alterations associated with seasonal change and variation in energy demands throughout an individual's annual cycle.

Relationships like that between CORT and T are constantly changing as organisms gradually pass from one life history stage (LSH; e.g. breeding, molt, migration) to the next. Variations in seasonal demands contribute to appropriate subsequent physiological adjustments in the production of, and/or sensitivity to, specific hormones at that time or stage of an organism's life. Each LHS requires unique sets of behavioral and physiological adjustments, all of which are vital for an organism to succeed at that point in its life (survive and/or reproduce).

An example of the complexities of hormone interplay would be at the onset of spring when T levels in circulation are observed to dramatically increase in some avian species (Wingfield and Marler 1988; Wingfield et al., 1990). This upsurge of circulating T induces expression of reproductive accessory organs, and some secondary sexual characteristics, along with sperm production and maturation (Wingfield and Marler 1988; Wingfield et al., 1990). These underlying physiological mechanisms are responsible for initiating reproductive behaviors essential for breeding success. Testosterone plays an important role in reproduction by priming an individual for upcoming breeding opportunities, but in the weeks following copulation, T levels subside and another hormone increases in production. After copulation, pairs build a nest, lay eggs, incubate, and assuming no predation, raise young upon egg hatch. At this time the hormone prolactin (PRL) is thought to facilitate/stimulate the expression of parental care (Buntin 1996; Vleck 1998; Angelier and Chastel 2009). The expression of parental behaviors induced by PRL has been well documented in birds (Riddle et al., 1935; Nalbandov 1945; Buntin 1996). When plasma PRL levels rise in circulation, it is suggested to reinforce incubating behavior (El Halawani et al., 1986). Similar to the inverse relationship observed between CORT and T, PRL and CORT have also been proven to negatively correlate with one another in response to acute and chronic stressors (Chastel et al., 2005). Interplay between hormones that facilitate energetic availability, behavioral change, and physiological change are a hallmark of annual cycles. But, variation in environmental conditions can lead to significant changes away from typical annual cycles.

Significant changes in environment can lead to stress responses in individuals; here deterioration in environmental conditions can make it difficult for an adult individual to survive and elevations in the hormone CORT provide needed energy resources to survive negative changes. Once stress-induced CORT levels are reached, individuals can shift from exhibiting reproductive behaviors to behaviors associated with survival. When organisms shift priorities from reproduction to survival, the hormones associated with breeding behavior (T) and parental care (PRL) drastically decrease as circulating CORT levels surge. Short-term, these physiological and behavioral changes associated with stress-induced CORT levels prove to be very beneficial as they provide the organism with a facet of tools suited to enhance their survival. Stress-induced decreases in PRL levels in parental birds faced with environmental stressors (e.g. limited food availability) and can be considered adaptive by disturbing current parental effort to promote parental survival and prospects of future reproduction (Chastel et al., 2005). However, when the potential benefits associated with increased reproductive fitness out-weigh that of survival, an attenuation of the CORT/PRL response to stress may have evolved as an adaptive hormonal tactic permitting the maintenance of parental care, and as a result, maximizing current parental effort during stressful situations (Angelier and Chastel 2009). Studies have supported the hypothesis that birds attenuate PRL response to stress in order to maintain elevated PRL levels in stressful situations whenever current reproductive events have a large fitness value (incubation: Angelier et al., 2007; chick-rearing: Chastel et al., 2005). Due to how elevated levels of CORT can interfere with the hormones (e.g. T & PRL) and behaviors (e.g. proceptive/receptive, courtship, & parental care) vital for successful reproduction, selective pressures may screen this type of hormonal interference by enhancing the survival of those capable of decreasing the production of, or sensitivity to, CORT in circulation at times when it could reduce the overall fitness of an organism.

As noted earlier, most male birds exhibit maximal annual T production at the onset of breeding (Hau 2007). Cardinals in our study population produce T similarly, with minor increases in baseline levels but with strong abilities to elevate T during the pre-breeding season (March-April; Jawor 2007; DeVries et al., 2011). The timing of this surge in T production has been suggested to coincide with females becoming sexually receptive and exhibiting reproductive behaviors (e.g. courtship rituals) and with increased aggressive behavior (DeVries et al., 2011, 2012). These rising T levels are, in part, contributed to photostimulation (e.g. exposure to long days) which for males is a signal for the initiation of testes growth, growth of the song-control system (SCS; Smith et al., 1997), and initiation of reproductive behaviors.

Not only does T initiate physiological and behavioral changes vital for successful reproduction, it also plays an important role in signaling condition to potential mates. Studies have shown links for T enhancing male reproductive display (e.g. song production; Ball et al., 2002; Folstad and Karter 1992; Owens and Short 1995; Roberts et al., 2004), and this is important due to how display can positively correlate with high mating success for males (Andersson 1994). But what about the conflict between CORT and T, does this negative correlation result in a decrease to reproductive fitness? Sexually selected traits, for males, are often energetically taxing (Andersson 1994) and GCs play an important role in mediating energy balance and storage (McEwen and Wingfield 2003; Sapolsky 1992; Sapolsky et al., 2000; Wingfield and Sapolsky 2003). Substantial complementary evidence suggests that moderate increases in circulating GC levels are necessary for channeling the energy necessary for bolstering energetically expensive courtship displays (Buchanan 2000; Emerson 2001; Hau et al., 2010; McEwen and Wingfield 2003; Moore and Jessop 2003). Studies have presented evidence for moderate increases in circulating GC levels to aid in promoting the expression of sexually selected male traits (Breuner et al., 2008; Calisi et al., 2008; Emerson 2001; Leary et al., 2004, 2006, 2008; Moore and Jessop 2003) and the elaboration of courtship displays (Adkins-Regan 2005; Greenberg and Wingfield 1987; Sapolsky 1992; Sapolsky et al., 2000; Schoech et al., 2009) by mobilizing energy stores, but high GC concentrations are known to inhibit breeding behavior (Sapolsky et al., 2000).

The general concept is that moderate increases in circulating GC levels may be necessary to match the metabolic demands inevitably tied to subsequent elevations in intensity of such traits (e.g. concepts of the 'energy mobilization hypothesis'; Romero 2002). Some evidence shows support for high GC concentrations to also have a negative impact on courtship signal intensity which are pertinent for females in mate selection. For example, in male Barn Owls (*Tyto alba*), administration of CORT decreased melanin-based coloration with females preferring males with melanin-based color patterns indicative of males that were not exposed to high CORT levels during the period when melanin-based coloration develops (Roulin et al., 2008). Similarly, in Barn

Swallows (*Hirundo rustica*), males with the longest tails are preferred by females and males possessing these longer tails generally had lower CORT levels over shorter-tailed males (Saino et al., 2002). High circulating CORT can not only influence signals visually, but audibly as well. For example, results from work with Zebra Finches (*Taeniopygia guttata*) found a positive correlation between low CORT levels and song production, in which males produced songs that were longer in duration and produced more frequently when compared to males with high total and free basal CORT levels (Wada et al., 2008). Results from another Zebra Finch study suggested that when CORT was orally administered, it resulted in an increased pitch (Perez et al., 2012). Similarly, male Song Sparrows (*Melospiza melodia*) with the lowest CORT responses to restraint stress often produced more complex songs (Schmidt et al., 2012). Collectively, this suggests that CORT-mediated effects on vocalization may be an important influential determinant in female mate-choice for males with low CORT levels. In many Temperate zone vertebrates, levels of circulating T vary over the year and play crucial roles in regulating their breeding to the season that has beneficial offspring survival rates (Yoshimura 2010). All together CORT and T are essential for success as organisms adjust to seasonal and environmental changes, but clearly there are species differences in how this is managed and a strong negative impact of stress-induced glucocorticoids.

Study

Work presented in previous chapters focused on seasonal regulation of basal and stress-induced CORT, here I also investigate how concentrations of another hormone vary seasonally in response to standard capture and restraint stress and potentially how they interact with each other in Northern Cardinals (*Cardinalis cardinalis*). Here, using the same methods previously described (see methods in Chapter III) for standard capture and restraint stress protocol, we also analyzed testosterone (T) levels at the time of capture (< 3 min for baseline samples) and after 30 min of restraint (stress-induced) to observe the relationship between CORT, at both basal and stress-induced levels, with testosterone levels. Ultimately, we aim to increase our understanding of how cardinals seasonally regulate CORT secretion, and in return, how this type of modulation effects other aspects of physiology (T) throughout the annual cycle. Previous research that has compared T to CORT has done so following chemical challenges that force T production (DeVries et al., 2011, 2012). While no correlations were found between these two hormones the act of forcing T production may have negatively impacted endogenous patterns of hormone association.

Methods

Capture methods and hormonal analyses

All birds in this study were passively captured at the Lake Thoreau Environmental Research and Education Center (Hattiesburg, Mississippi) between 0600 and 1200 with mist nets. Blood samples were obtained, structural measurements were recorded and then birds were subjected to standard capture and restraint protocol (Wingfield et al., 1995) for stress-induced CORT levels (after 30 min restraint).

Cardinal plasma CORT levels were determined using EIA (Arbor Assays, Inc., #K014-H5) following modified techniques described for cardinals based on DeVries and Jawor (2013). In all plasma samples (10μl) we added 2000 cpm of tritiated CORT (H^3-CORT, PerkinElmer) for recovery calculations after extractions. Samples were extracted three times with diethyl ether, after which they were dried under nitrogen then re-suspended in 400µl of assay buffer. Samples were assayed in duplicate quantities of 50µl each on the EIA plate, and we used an additional 100µl to determine recoveries (mean recoveries=89.4%). Following incubation with capture and identifying antibodies, plates were read using a microplate reader (Microplate Manager; Bio-Rad Laboratories, Inc.) and CORT concentration for each sample was calculated by the plate reader's built-in 4PLC software. Levels were corrected for initial sample volume and incomplete recovery. For each individual, all plasma samples were analyzed within the same assay and we randomly assigned all samples from the same individuals to plate wells. We randomly placed four Northern Bobwhite (*Colinus virginianus*) homogenized plasma samples throughout each plate to determine inter-assay and intra-assay variations. Plate correction factors were determined via comparison of intra-assay variation and applied using methods described by Jawor et al. (2006). Inter-assay and intra-assay coefficients of variation were 10.8 and 8.2%, respectively.

Cardinal plasma T concentrations were determined using EIA (Enzo Life Sciences, Inc., #901-065) following modified techniques (Jawor et al., 2007) described for cardinals. In all plasma samples (10µl) we added 2000 cpm of tritiated T (H^3-T, PerkinElmer) for recovery calculations after extractions. Samples were extracted three times with diethyl ether, after which they were dried under nitrogen gas, and then re-suspended in 50µl of ethanol then diluted to 350µl with assay buffer. Samples were assayed in duplicate quantities of 100µl each on the EIA plate, and we used an additional 100µl to determine recoveries (mean recoveries=89.4%). Following incubation with capture and identifying antibodies, plates were read using a microplate reader (Microplate Manager; Bio-Rad Laboratories, Inc.) and T concentration for each sample was calculated by the plate reader's built-in 4PLC software. Levels were corrected for initial sample volume and incomplete recovery. For each individual, all plasma samples were analyzed within the same assay and we randomly assigned all samples from the same individuals to plate wells. We randomly placed three samples of known T levels (standards, not a plasma pool as for CORT analyses) throughout each plate to determine inter-assay and intra-assay variations to determine plate correction factors. Inter-assay coefficients of variation ranged from 8.2-10.8%, and inter-assay variation was 9.8%.

Statistical Analysis

Data were analyzed with JMP® (version 10, SAS Institute Inc., Cary, NC) software. To account for inter-assay variation in each assay we randomly placed multiple standards throughout each assay to determine and apply correction factors to all plasma samples on each plate. Months were grouped into 3 broad seasons (non-breeding [Sept.-Feb.], pre-breeding [Mar. & April] and breeding [May-Aug.]) for statistical analyses. Corrected CORT concentrations were log-transformed to meet normality requirements and allow for the use of parametric statistics, Shapiro-Wilks goodness-of-fit test were used for verification. One-way ANOVA was used to test whether seasonal groups (non-breeding, pre-breeding & breeding) were significantly different from one another, and when seasonal groups failed, months were tested separately. Results from statistical tests are expressed as mean ± standard error. To assess seasonal T responses (difference between stress-induced and initial levels), ANOVA Oneway-analysis tests were used, followed by Tukeys HSD post hoc tests for significant effects. We used general linear mixed models (GLMM) analyses to examine variability of hormonal production and relationships between basal and stress-induced CORT with T following standard capture and restraint stress protocol. Pairwise correlational tests were used for tests between T and CORT within individuals at initial and stress-induced stages.

Results

Testosterone Levels in Response to Capture and Restraint Stress

A significant seasonal difference was found in levels of T in response to 30 minutes of restraint stress ($t_{28} = -3.14$, $p < 0.002$; Figure 6.). No significant correlations between T and CORT within individuals were observed at both initial (F=3.07, P=0.331), and stress-induced levels (F=4.78, P=0.157). In almost all cases T decreased during the short term restraint of individuals. A One-way ANOVA was conducted to compare the effect of time of year (months) on initial and stress-induced testosterone (T) levels. There was no significant effect for month on stress-induced T levels, however a significant effect was found for initial T levels with month ($F_{5,18}$=6.69, P=0.0011). Post hoc comparisons using the Tukey HSD test indicated that the mean initial T levels in March (0.62±0.61) were significantly different from initial T levels in April (3.7±0.53).

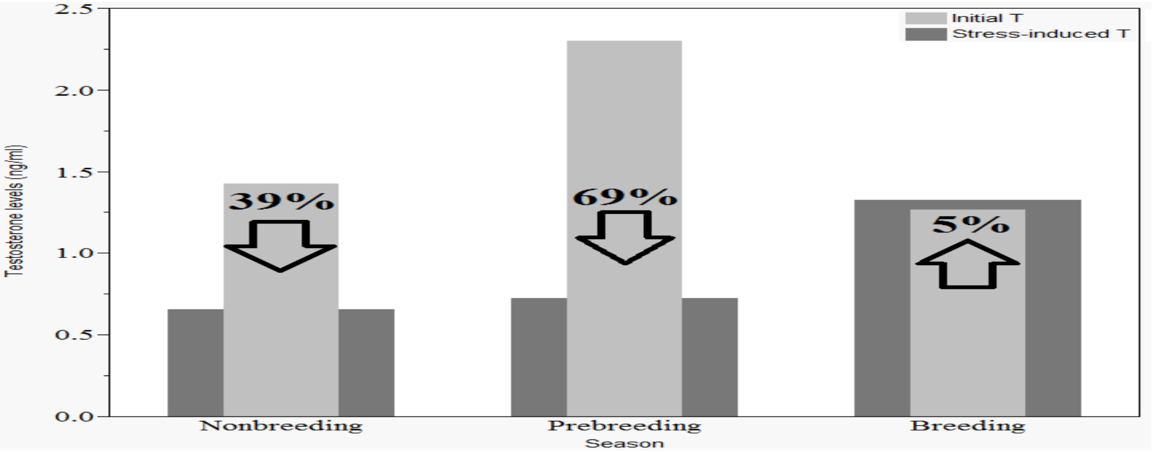


Figure 6. Testosterone Levels in Response to Capture and Restraint. Sexes combined, average T at initial (light gray) & stress-induced (dark gray) levels during non-breeding (n= 9), pre-breeding (n = 8) and breeding (n = 12) seasons. Percent (%) change indicative of increase or decrease in T levels from initial to stress-induced.

Testosterone Levels Between the Sexes During the Breeding Season

The only significant effect found on T levels within breeding was that of sex on levels during incubation ($F_{1,6}$=16.81, *P*=0.0027). Testosterone tended to increase in both sexes during incubation however it decreased during nestling care (not significant).

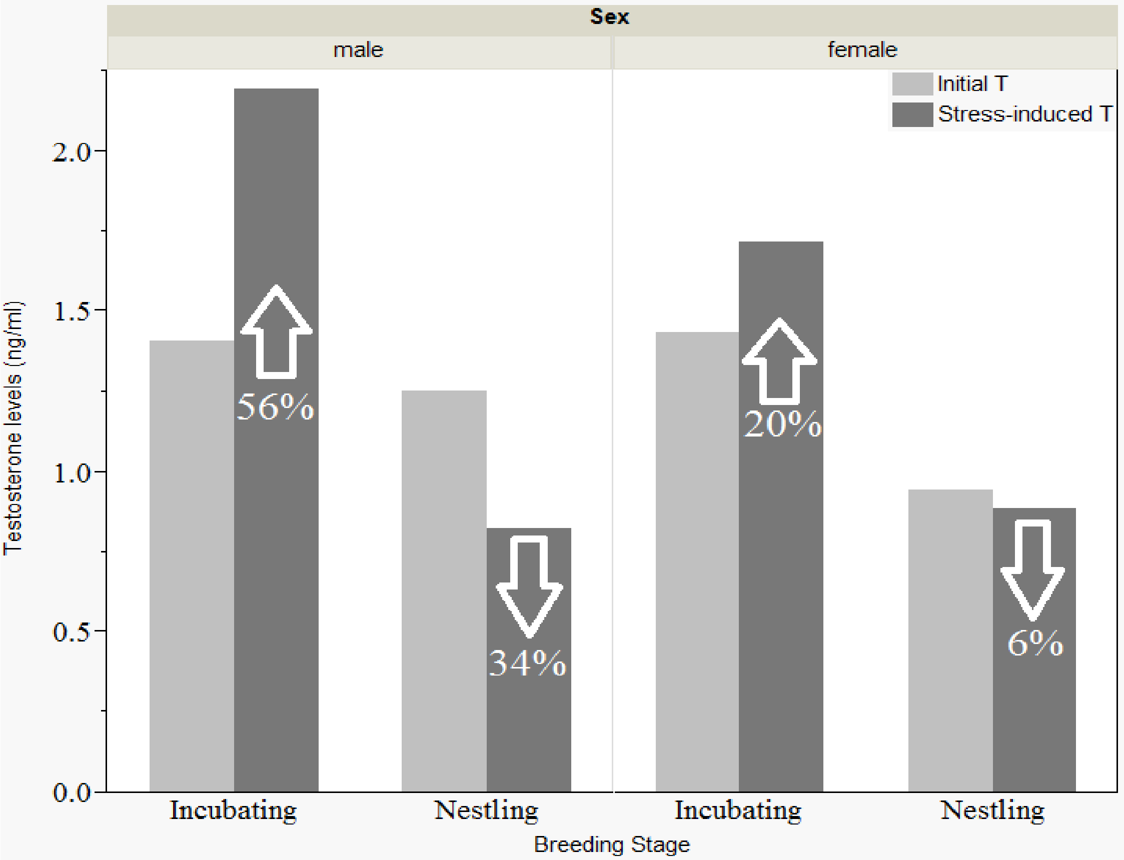


Figure 7. Testosterone Levels Between the Sexes at Different Parental Stages. Mean T levels for males caught while mate was incubating (*n* = 1), & nestling stage (*n* = 3), and females caught during incubation stage (*n* = 5), & nestling stage (*n* = 3).

Discussion

Results from previous work with cardinals (see Chapter III) found that CORT levels varied seasonally in response to capture and restraint stress. The aim of this study was to investigate whether CORT levels influence T levels, and whether any seasonal variation exists in how T is modulated. Seasonal variation was found on initial T levels during pre-breeding, particularly during the month of April. And this finding is in line with other studies (Deviche et al., 2014; Goymann et al., 2006), particularly for males in socially monogamous species (Wingfield et al., 1987, 1990; Beletsky et al., 1995). Pre-breeding was also when the largest difference in T levels were observed in response to capture and restraint stress (69% decrease). On the contrary, this was also when the greatest overall magnitude in CORT stress responses occurred (630% increase). In response to capture and restraint stress, CORT levels are known to decrease T levels (Deviche et al., 2012; Wingfield et al., 1982; Moore et al., 2002), but not in all species (Heiblum et al., 2000; Van Hout et al., 2010; Lynn et al., 2010). This could be partly contributed to changes in CBG, which are known to be seasonally regulated (Breuner and Orchinik 2001). It is known that CBG can bind ~90% of steroid hormones in circulation (Desantis et al., 2013). Any seasonal change in CBG could directly impact the amount of free CORT and/or T in circulation. This could result in more or less circulating CORT/T to pass through cell membranes of target tissues to induce a response.

Seasonal changes in CORT and T are not owed to a single factor, but more likely change at multiple levels. For example, changes in how environmental stressors are perceived and transduced into chemical responses in the brain. Just within the brain, several neuropeptides (e.g., CRH, AVT, MT) regulate POMC expression, which can be cleaved to produce ACTH. Seasonal changes in how these neuropeptides function and regulate parts of the HPA axis could indirectly impact the robustness in stress responses. Outside the brain, target sites of CORT (e.g., liver) and T (e.g., gonads) have multiple layers of response. Steroidogenic enzymes expressed at these target sites can convert CORT (e.g., 11β HSD I/II) and T (e.g., P450 aromatase, 5α reductase I/II) to other forms that can enhance or inhibit further binding and gene transcription. Lastly, once CORT/T binds to their respective receptors, co-activators and co-repressors can directly enhance or inhibit gene transcription. All of these levels of response can vary by day, season, sex, age, condition and by the individual, not only between and within different species, but populations as well.

It may be that cardinals can locally up- or down-regulate androgen receptors (ARs) and glucocorticoid receptors (GR) seasonally in certain regions of the brain to allow for strong behavioral responses without widespread negative physiological responses of circulating hormones with respects to breeding or parental care. For example, species that display year-round territorial defense like the Spotted Antbird (*Hylophylax naevioides*) which behaves aggressively during the non-breeding and breeding seasons, is able to achieve this without high circulating testosterone levels, by elevating mRNA expression of estrogen receptors in the preoptic area and AR in the nucleus taeniae (NT) during the non-breeding season (Canoine et al., 2007). Another example of this was found in female African Black Coucals (*Centropus grillii*), which have higher mRNA expression of ARs than males in the NT (Voigt and Goymann 2007). Additionally, it might prove beneficial, at times, to synthesize steroids locally instead of systemically to control behavior without widespread body response to a particular hormone (Soma 2006). The point is the same regulatory hormones are influencing behaviors in one or both sexes, but depending on the site of modulation along the neuroendocrine cascade, may have completely different results.

Glucocorticoids (in mammals) have been shown to exhibit direct actions on testicular endocrine function by acting on testicular cells and inhibiting plasma T production as a result (Dong et al., 2004; Hu et al., 2008; Martin and Tremblay 2008).

With lower circulating CORT, elevated T levels can enhance breeding success by inducing behaviors associated with courtship (e.g. increased singing rates), and by promoting aggressive behaviors related to territory and mate defense (Schwabl and Kriner 1991; Wingfield and Farner 1993). Corticosterone is also known to negatively correlate with another important hormone during breeding, prolactin (PRL). Prolactin has been indicated in behaviors associated with breeding such as nest provisioning, nest defense and brood guarding (Wang and Buntin 1999). Selective pressures might be limiting the production of, or enhance the sensitivity to, circulating CORT at this stage to prevent interfering with these behaviors that are essential to successful reproduction.

Conclusion

Acute stress imposing opposite effects on plasma CORT (increased) and T (decreased), particularly during breeding, suggests that birds, at this point, can prevent T levels from dropping below a 'minimum threshold' that is required to maintain certain behavior, morphology, and/or physiology that is T-dependent.

The hormonal and behavioral actions of cardinals are highly adaptive and allow great flexibility with respects to the environments they inhabit. With a range covering Tropical, sub-Tropical, and Temperate zones all the way to southern parts of Canada, they must be modulating hormones in a fashion that maximizes fitness. With high nest-depredation rates in a rather benign environment, one way cardinals may counter this is to treat each brood with intense parental care. It may be a hormonal way to compensate for losing a majority of nests by treating each as if it were its last. This relationship may fade as the breeding season reaches its end and many pairs have already fledged young.

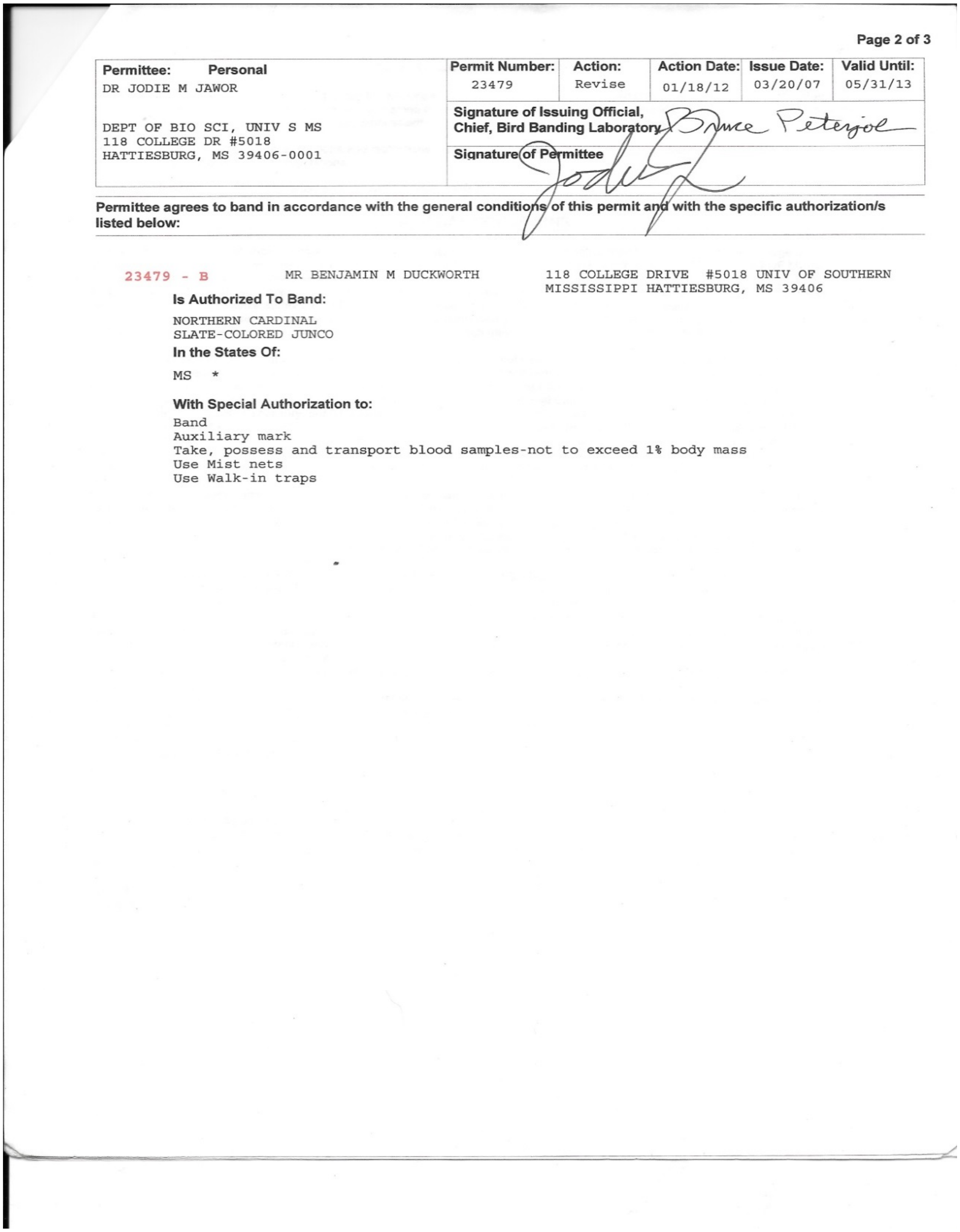
This may be part of why CORT increases in the winter in this species, which then may allow them to exploit cooler climates.

All together it seems like the Northern Cardinal has retained some of its Tropical ancestral hormonal correlates, but in addition to dampened HPA responsivity during breeding (very Arctic-like), may have diverted energy away from robust immune responses (necessary for Tropical life with higher infectious parasites) and invested more so in enhanced metabolic capabilities, thus allowing for an extended range into colder, more-northern latitudes (very Temperate-like).

For future research, more studies with cardinals, especially during breeding, investigating CORT/T with higher sample sizes, as well as, combining seasonal hormonal analyses with changes in receptor types and densities within the brain would allow for greater understanding of how HPA axis regulation is mitigated, and how T-dependent and CORT impacted behaviors are displayed. Additional work with near relatives of Northern Cardinals could allow for a determination of whether the unique hormone profiles observed in this species are part of what makes it unique or are symptomatic of the *Cardinalis* genus overall.

APPENDIX A

FEDERAL BIRD BANDING PERMIT, 2012-2013



Page 2 of 3

Permittee: **Personal** DR JODIE M JAWOR	**Permit Number:** 23479	**Action:** Revise	**Action Date:** 01/18/12	**Issue Date:** 03/20/07	**Valid Until:** 05/31/13
DEPT OF BIO SCI, UNIV S MS 118 COLLEGE DR #5018 HATTIESBURG, MS 39406-0001	**Signature of Issuing Official, Chief, Bird Banding Laboratory** Bruce Peterjohn				
	Signature of Permittee Jodie				

Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization/s listed below:

23479 - B MR BENJAMIN M DUCKWORTH 118 COLLEGE DRIVE #5018 UNIV OF SOUTHERN MISSISSIPPI HATTIESBURG, MS 39406

Is Authorized To Band:

NORTHERN CARDINAL
SLATE-COLORED JUNCO

In the States Of:

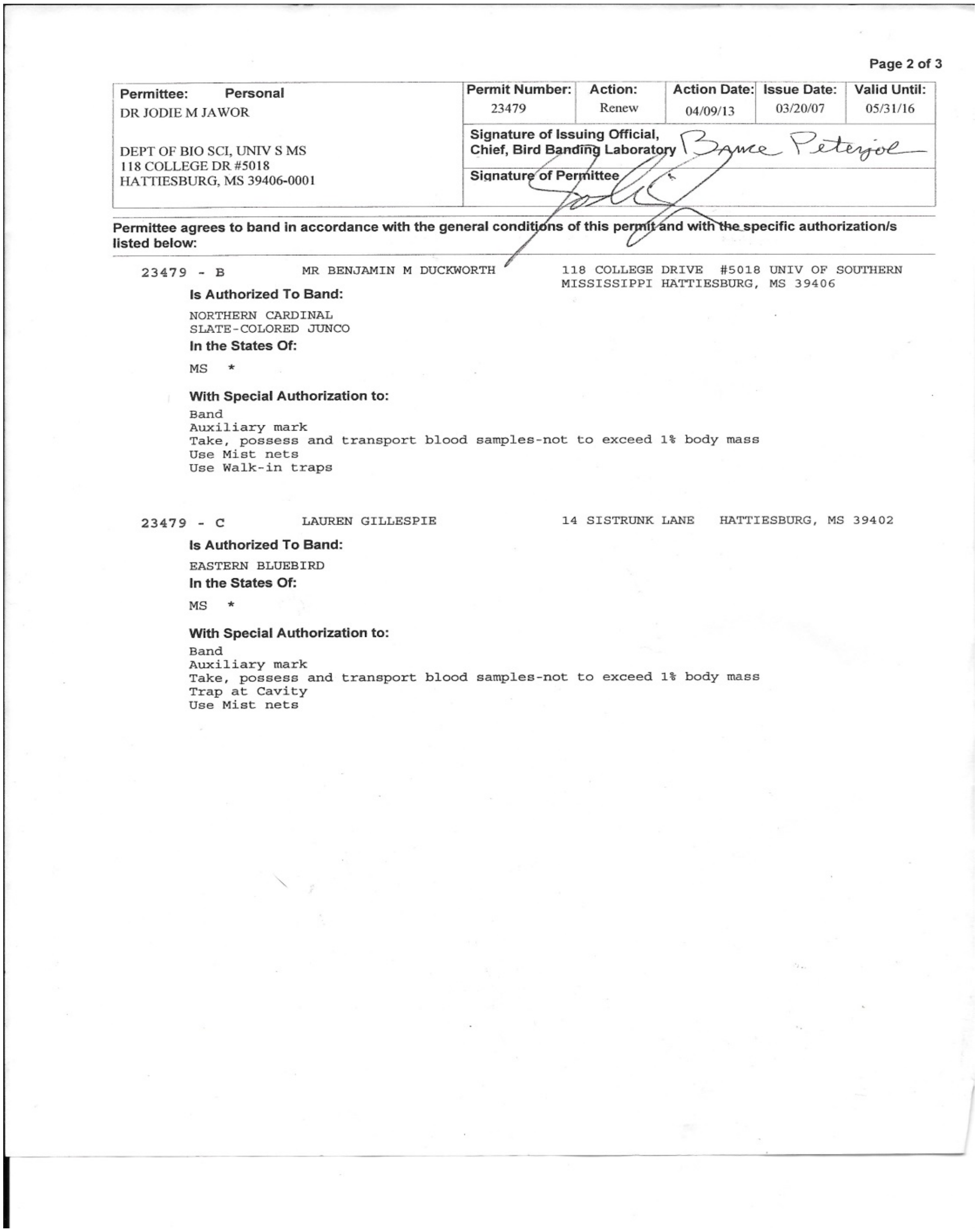
MS *

With Special Authorization to:

Band
Auxiliary mark
Take, possess and transport blood samples-not to exceed 1% body mass
Use Mist nets
Use Walk-in traps

APPENDIX B

FEDERAL BIRD BANDING PERMIT, 2013-2014



Page 2 of 3

Permittee: **Personal** DR JODIE M JAWOR	**Permit Number:** 23479	**Action:** Renew	**Action Date:** 04/09/13	**Issue Date:** 03/20/07	**Valid Until:** 05/31/16
DEPT OF BIO SCI, UNIV S MS 118 COLLEGE DR #5018 HATTIESBURG, MS 39406-0001	**Signature of Issuing Official, Chief, Bird Banding Laboratory** Bruce Peterjohn **Signature of Permittee**				

Permittee agrees to band in accordance with the general conditions of this permit and with the specific authorization/s listed below:

23479 - B MR BENJAMIN M DUCKWORTH 118 COLLEGE DRIVE #5018 UNIV OF SOUTHERN MISSISSIPPI HATTIESBURG, MS 39406

Is Authorized To Band:

NORTHERN CARDINAL
SLATE-COLORED JUNCO

In the States Of:

MS *

With Special Authorization to:

Band
Auxiliary mark
Take, possess and transport blood samples-not to exceed 1% body mass
Use Mist nets
Use Walk-in traps

23479 - C LAUREN GILLESPIE 14 SISTRUNK LANE HATTIESBURG, MS 39402

Is Authorized To Band:

EASTERN BLUEBIRD

In the States Of:

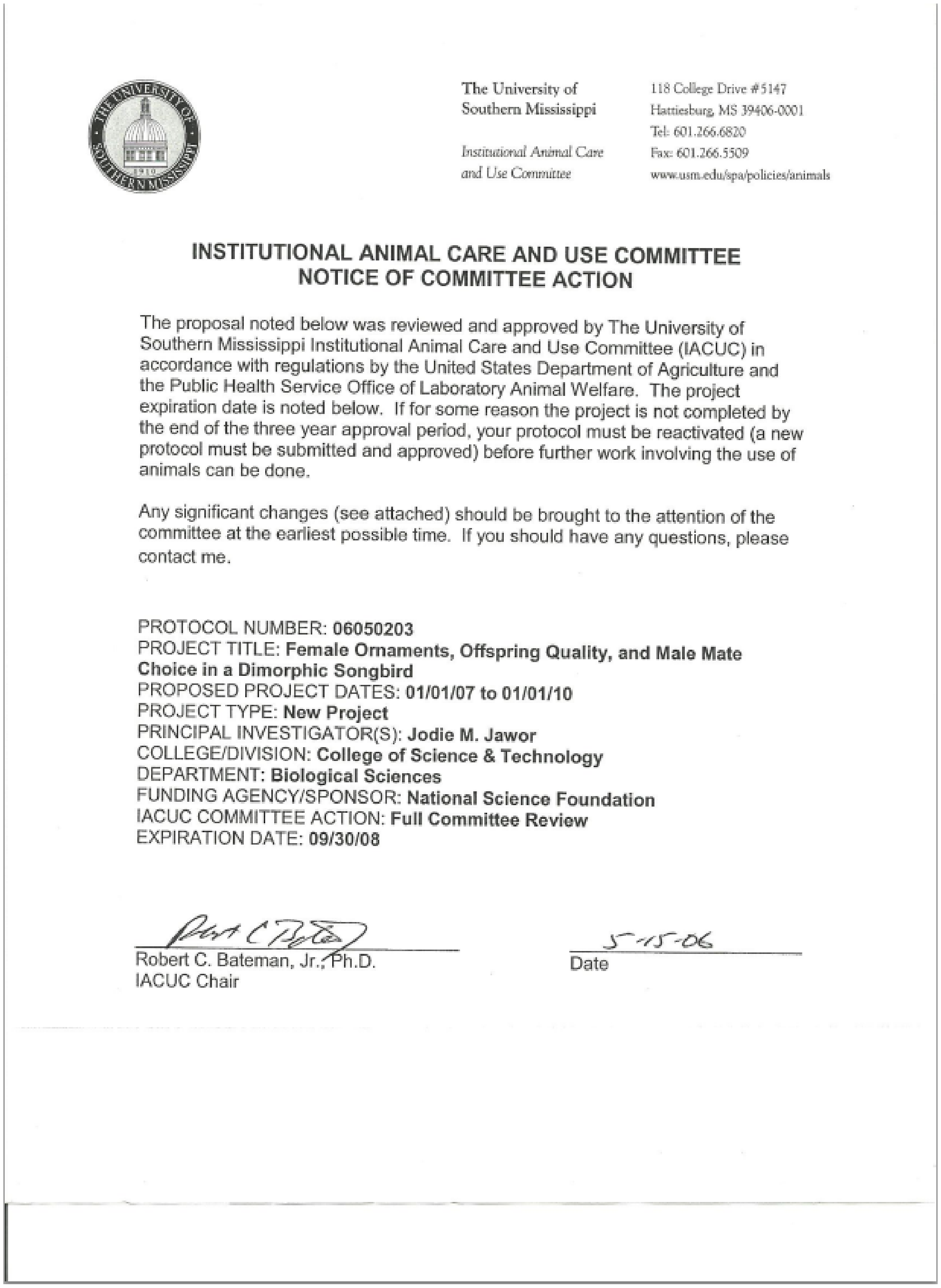
MS *

With Special Authorization to:

Band
Auxiliary mark
Take, possess and transport blood samples-not to exceed 1% body mass
Trap at Cavity
Use Mist nets

APPENDIX C

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION, 2007-2008



The University of
Southern Mississippi

*Institutional Animal Care
and Use Committee*

118 College Drive #5147
Hattiesburg, MS 39406-0001
Tel: 601.266.6820
Fax: 601.266.5509
www.usm.edu/spa/policies/animals

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

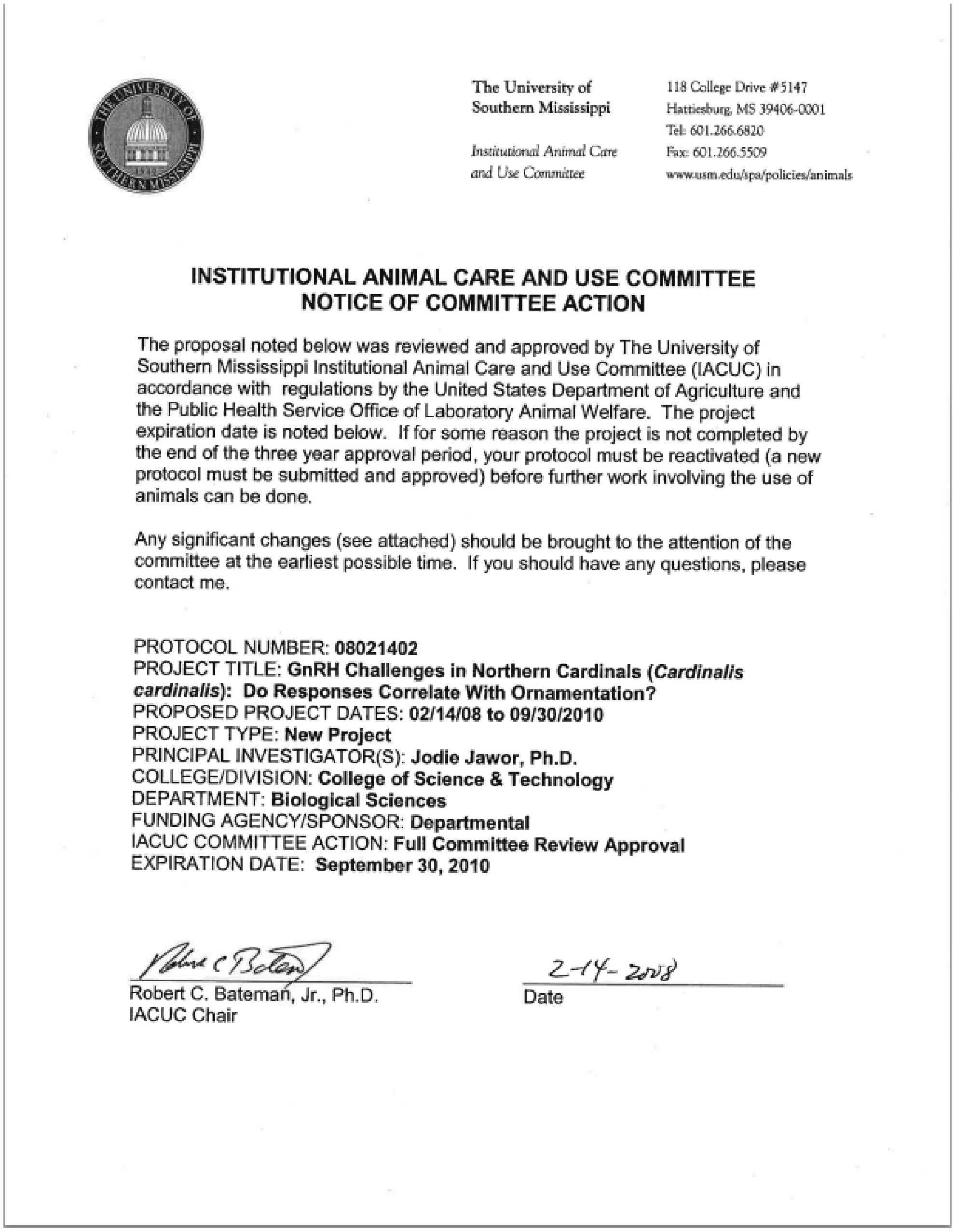
PROTOCOL NUMBER: **06050203**
PROJECT TITLE: **Female Ornaments, Offspring Quality, and Male Mate Choice in a Dimorphic Songbird**
PROPOSED PROJECT DATES: **01/01/07 to 01/01/10**
PROJECT TYPE: **New Project**
PRINCIPAL INVESTIGATOR(S): **Jodie M. Jawor**
COLLEGE/DIVISION: **College of Science & Technology**
DEPARTMENT: **Biological Sciences**
FUNDING AGENCY/SPONSOR: **National Science Foundation**
IACUC COMMITTEE ACTION: **Full Committee Review**
EXPIRATION DATE: **09/30/08**

Robert C. Bateman, Jr., Ph.D.
IACUC Chair

5-15-06
Date

APPENDIX D

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION, 2008-2010



The University of
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INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

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Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

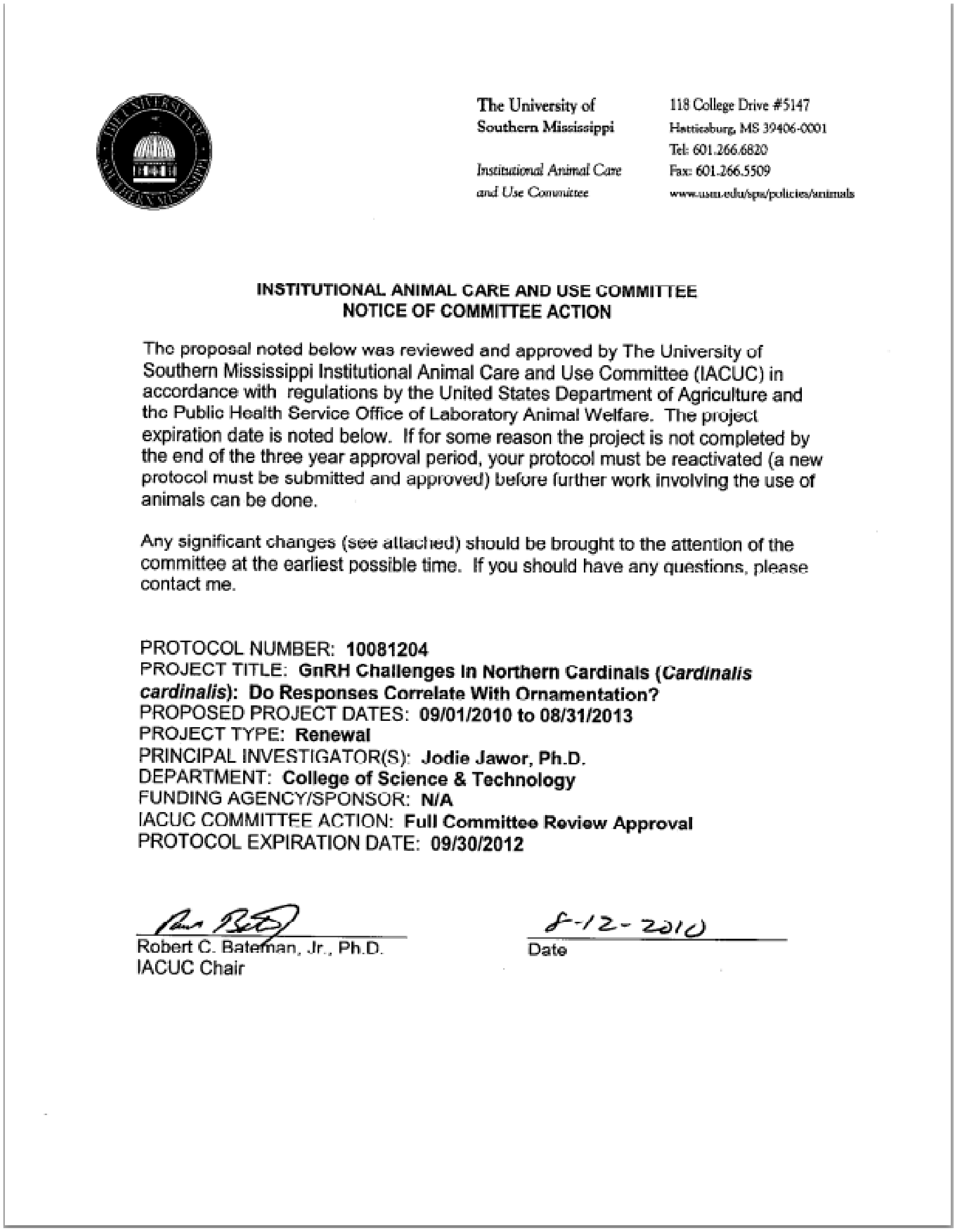
PROTOCOL NUMBER: **08021402**
PROJECT TITLE: **GnRH Challenges in Northern Cardinals (*Cardinalis cardinalis*): Do Responses Correlate With Ornamentation?**
PROPOSED PROJECT DATES: **02/14/08 to 09/30/2010**
PROJECT TYPE: **New Project**
PRINCIPAL INVESTIGATOR(S): **Jodie Jawor, Ph.D.**
COLLEGE/DIVISION: **College of Science & Technology**
DEPARTMENT: **Biological Sciences**
FUNDING AGENCY/SPONSOR: **Departmental**
IACUC COMMITTEE ACTION: **Full Committee Review Approval**
EXPIRATION DATE: **September 30, 2010**

Robert C. Bateman, Jr., Ph.D.
IACUC Chair

2-14-2008
Date

APPPENDIX E

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION, 2010-2013



The University of
Southern Mississippi

*Institutional Animal Care
and Use Committee*

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www.usm.edu/spa/policies/animals

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

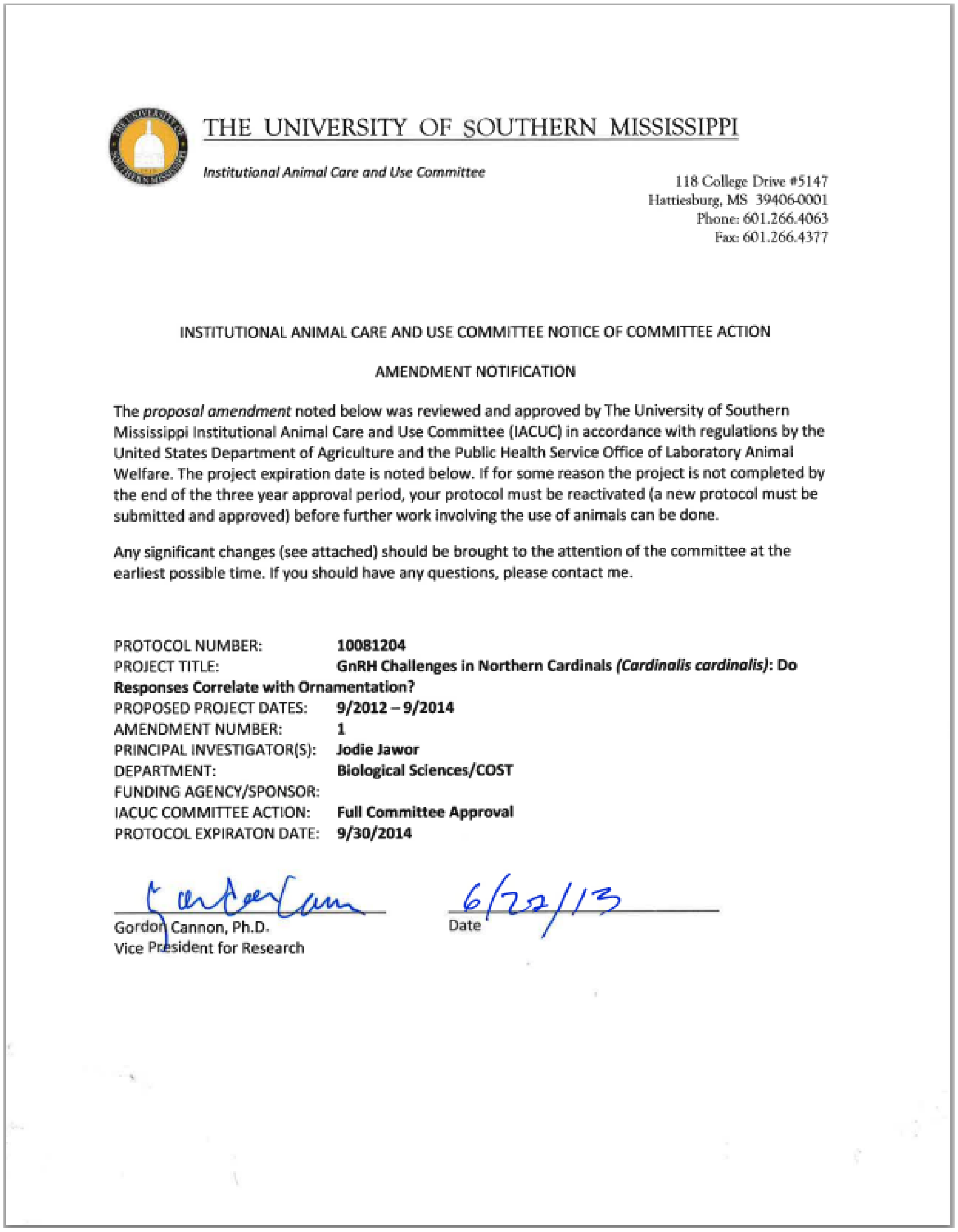
PROTOCOL NUMBER: **10081204**
PROJECT TITLE: **GnRH Challenges in Northern Cardinals (*Cardinalis cardinalis*): Do Responses Correlate With Ornamentation?**
PROPOSED PROJECT DATES: **09/01/2010 to 08/31/2013**
PROJECT TYPE: **Renewal**
PRINCIPAL INVESTIGATOR(S): **Jodie Jawor, Ph.D.**
DEPARTMENT: **College of Science & Technology**
FUNDING AGENCY/SPONSOR: **N/A**
IACUC COMMITTEE ACTION: **Full Committee Review Approval**
PROTOCOL EXPIRATION DATE: **09/30/2012**

Robert C. Bateman, Jr., Ph.D.
IACUC Chair

8-12-2010
Date

APPENDIX F

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION, 2013-2014



THE UNIVERSITY OF SOUTHERN MISSISSIPPI
Institutional Animal Care and Use Committee

118 College Drive #5147
Hattiesburg, MS 39406-0001
Phone: 601.266.4063
Fax: 601.266.4377

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

AMENDMENT NOTIFICATION

The *proposal amendment* noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

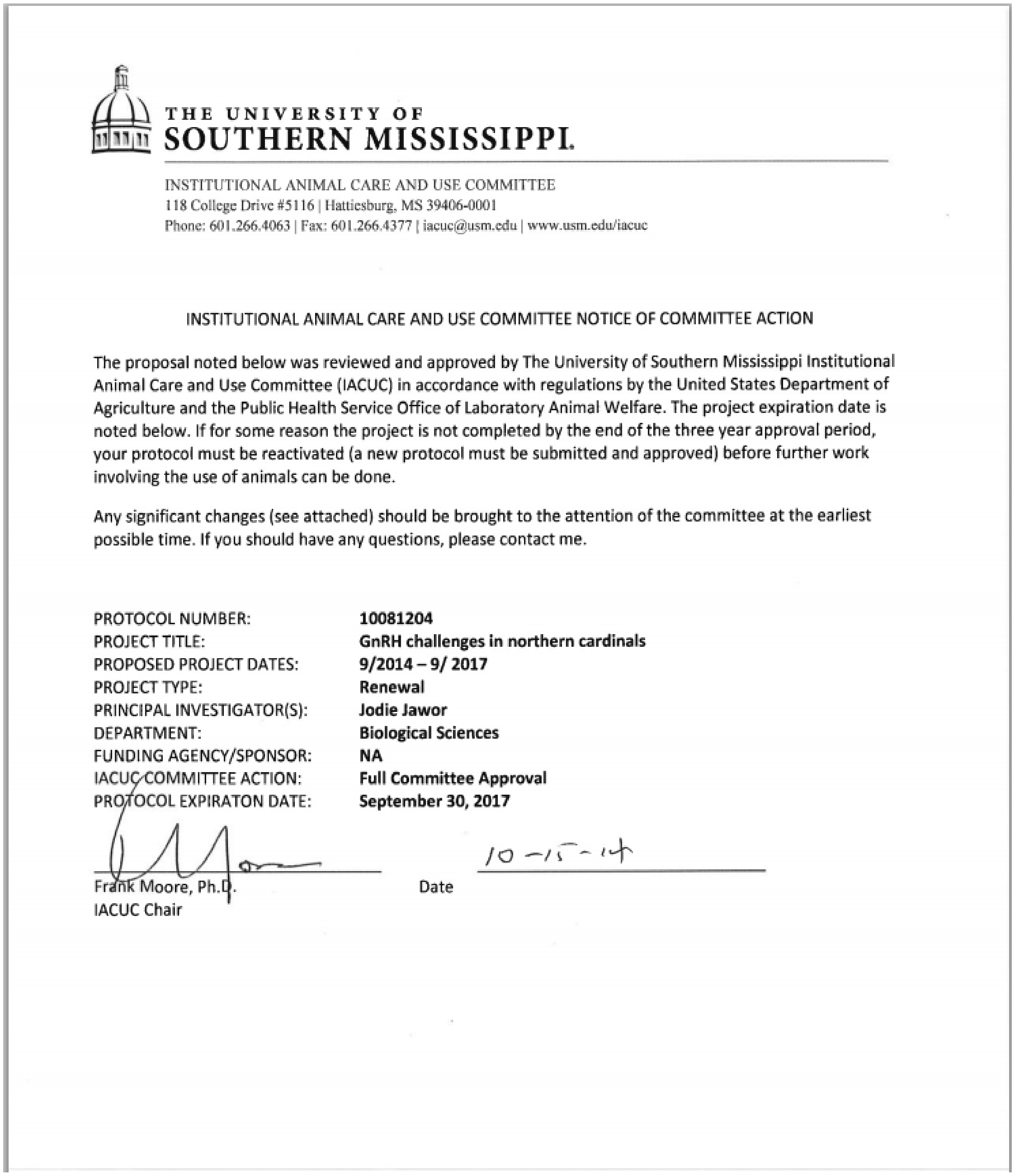
PROTOCOL NUMBER: **10081204**
PROJECT TITLE: **GnRH Challenges in Northern Cardinals *(Cardinalis cardinalis)*: Do Responses Correlate with Ornamentation?**
PROPOSED PROJECT DATES: **9/2012 – 9/2014**
AMENDMENT NUMBER: **1**
PRINCIPAL INVESTIGATOR(S): **Jodie Jawor**
DEPARTMENT: **Biological Sciences/COST**
FUNDING AGENCY/SPONSOR:
IACUC COMMITTEE ACTION: **Full Committee Approval**
PROTOCOL EXPIRATON DATE: **9/30/2014**

Gordon Cannon, Ph.D.
Vice President for Research

6/22/13
Date

APPENDIX G

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION, 2014-2015



THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.4063 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: **10081204**
PROJECT TITLE: **GnRH challenges in northern cardinals**
PROPOSED PROJECT DATES: **9/2014 – 9/ 2017**
PROJECT TYPE: **Renewal**
PRINCIPAL INVESTIGATOR(S): **Jodie Jawor**
DEPARTMENT: **Biological Sciences**
FUNDING AGENCY/SPONSOR: **NA**
IACUC COMMITTEE ACTION: **Full Committee Approval**
PROTOCOL EXPIRATON DATE: **September 30, 2017**

Frank Moore, Ph.D.
IACUC Chair

Date 10-15-14

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