

Ecological roles for corticosterone in birds: season, stages, habitat, and perturbations

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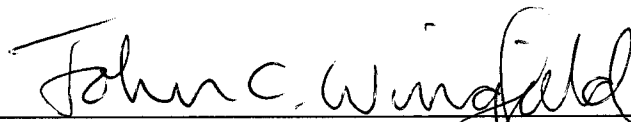
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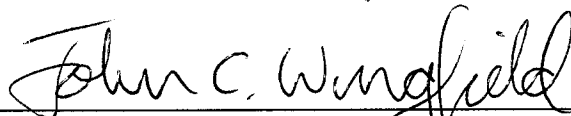
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Abstract

Ecological roles for corticosterone in birds: season, stages, habitat, and perturbations

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Glucocorticoid hormones have multiple purposes. At low levels, they function to maintain basic energy and salt balance. At high levels, they are key hormones in the emergency response to noxious stimuli. In the short term, glucocorticoids help the individual cope with a given challenge. Chronic exposure to high glucocorticoid levels can be detrimental to health and may increase mortality. In this dissertation, I explore how corticosterone (CORT) is modulated with life stage, season, habitat, and perturbations in song birds.

In rufous-collared sparrows (*Zonotrichia capensis costaricensis*), I studied the effects of season, breeding, and molt on baseline CORT levels and the CORT response to stress. Month explained most of the variation in the CORT data, with higher CORT levels occurring in the spring. Breeding individuals had a higher HPA axis response to stress than non-breeding individuals, most likely to favor self-maintenance over a current reproductive attempt. Unlike past research, we found that CORT levels did not change or were higher in molting birds.

I measured CORT levels, body condition, behavior, and hematocrit in song wrens (*Cyphorhinus phaeocephalus*) along a rainfall-induced habitat gradient. Birds living in drier habitat had lower body condition and were more likely to have an abnormally low hematocrit score. The relationship between rainfall and baseline CORT was not

significant, but birds with the highest baseline CORT levels lived at the dry edge of the range. Birds in better body condition and with lower baseline CORT levels were captured more quickly. Our results indicate that physiology and behavior can change with an environmental gradient.

To better understand the consequences of living in a disturbed environment, I studied the effects of repeated, acute pulses of CORT on the HPA axis and body condition in captive, wild Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*). CORT-treated birds had higher endogenous baseline CORT levels and failed to increase CORT levels with exposure to stress. Body mass, flight muscle, and food intake all declined with CORT treatment. CORT-treated birds expressed migratory restlessness but delayed the onset of molt. We conclude that frequent, acute CORT administration can create a chronic stress phenotype.

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DEDICATION

I dedicate this thesis to my family, friends, and teachers who have encouraged my growth and learning.

Chapter 1

Stress in a conservation context:

A review of how glucocorticoid levels change with conservation-relevant variables

Knowing the condition of individuals in a population is important for both evaluating the health of and developing management techniques for populations or species of conservation concern. Endocrinologists have developed non-invasive or minimally-invasive sampling techniques and methodologies to assess the condition of wild animals (Wingfield et al. 1997b, Berger et al. 1999, Millspaugh and Washburn 2004, Heath and Frederick 2005). There has been a call to arms within the endocrinology community to address pressing conservation questions with these tools (Cockrem 2005, Heath and Frederick 2005, Walker et al. 2005a, Wikelski and Cooke 2006). Researchers are increasingly measuring glucocorticoid hormones, which are often used as a measure of stress, in wild animals in a conservation context (Wasser et al. 1997, Wikelski et al. 2001, Walker et al. 2005b). This paper is written to 1) help conservation biologists understand the roles of glucocorticoids in basic physiology and 2) summarize conservation-relevant studies that have collected glucocorticoid data from wild populations.

Multiple actions of glucocorticoids

Glucocorticoid hormones, such as cortisol and corticosterone, have multiple functions that are regulated by the concentration of hormone (Wingfield et al. 1997a, Wingfield and Kitaysky 2002). A constitutive level of glucocorticoids (GCs) is needed to maintain life because GCs are vital for basic energy and salt management (Level A, Figure 1.1) (Norris 1997). Daily rhythms and changes in life stage cause baseline GCs level to rise and fall within a certain range (Level B, Figure 1.1) and these modulations are a part of the basic life cycle (Breuner et al. 1999, Dallman and Bhatnagar 2001, Wingfield and Romero 2001, Romero 2002). When GC levels rise above the “B” level into the “C” or

facultative range (Figure 1.1), they trigger an emergency response typically called “stress” (Wingfield et al. 1997a, Wingfield et al. 1998, Wingfield and Kitaysky 2002, Landys et al. in press). In the short term (minutes and hours), GCs levels in the “C” range promote survival behavior and physiology, helping the individual survive the given challenge (Table 1.1). However, in the long term (days and weeks), the actions of GCs at “C” levels are harmful to health and fitness of the individual (Table 1.1).

Three glucocorticoid receptors – the mineralocorticoid receptor (MR), glucocorticoid receptor (GR), and membrane-associated receptor (mGR) – manage the multiple roles of glucocorticoids (Sapolsky et al. 2000, Romero 2004, Landys et al. in press). Rapid actions of GCs are elicited by binding to the non-genomic mGR (Breuner et al. 1998, Breuner and Orchinik 2001, Landys et al. in press). These rapid effects include modulation of neuronal firing and behavior (review in Landys et al. in press). The MR has a higher affinity for GCs than the GR, and GCs preferentially bind to the MR when at low levels (Norris 1997). Binding of the MR regulates the basic body maintenance functions of GCs. Once GC levels are high enough to saturate the MR, they bind to the GR (Norris 1997, Landys et al. in press). Binding of the GR receptor stimulates the suite of behaviors and physiological changes characteristic of the emergency response.

Baseline GC levels

The primary function of GCs is to maintain adequate glucose and free fatty acid levels in the body. Baseline GC levels anticipate and track the body’s energy demand and are vital for maintaining the most basic biological functions (Dallman et al. 1993, Wingfield and Ramenofsky 1999, Sapolsky et al. 2000, Landys et al. in press). During an energetic challenge due to food deprivation or increased activity, GC levels will rise; this rise mobilizes energy stores to maintain basic functions (Dallman et al. 1993, Wingfield and Ramenofsky 1999, Wingfield and Romero 2001). If the body condition of the challenged animal declines below a given point and/or the animal experiences psychological stress, then higher GC levels will take on the additional role of facilitating

an emergency response (Wingfield et al. 1997b, Boonstra et al. 1998, Wingfield and Ramenofsky 1999, Romero and Wikelski 2001).

GC modulation

Daily

To prepare the individual for each day's predictable activities, GC levels in most animals rise and fall with 24 hr cycles (Figure 1.1, solid line). However, there are notable exceptions to this rule, such as the tuatara (*Sphenodon punctatus*), which do not modulate baseline GC levels with a daily cycle (Tyrrell and Cree 1998). Energy regulation (acquisition, deposition, and mobilization) is a major driver of the diel rhythms of basal GCs (Dallman et al. 1993). For example, in rats and birds, baseline GC levels peak before the active period and period of highest food intake (Dallman et al. 1993, Breuner et al. 1999, Romero and Remage-Healey 2000, Dallman and Bhatnagar 2001). GC levels fall over the period of activity, reaching their low at the beginning of the inactive period (Dallman et al. 1993, Breuner et al. 1999, Romero and Remage-Healey 2000, Dallman and Bhatnagar 2001). In contrast, free-living Galapagos marine iguana (*Amblyrhynchus cristatus*) have high basal GC levels during their period of activity and feeding (Woodley et al. 2003, Romero and Wikelski 2006). In addition, the daily GC cycle in this species is affected by the tides (Woodley et al. 2003, Romero and Wikelski 2006).

Life stages, season, and location

Just as GC levels vary with a daily cycle, baseline and stress-induced GC levels also change with seasons and life stage (Figure 1.1, solid line) (Wingfield and Romero 2001, Romero 2002, Reeder and Kramer 2005). For example, in most passerines, both baseline and stress-induced GC levels are reduced in molting individuals compared to non-molting individuals (Romero 2002, Romero et al. 2005). A review by Romero (2002) summarized the patterns of baseline and stress-induced GC levels in terrestrial vertebrates. He determined that the majority of terrestrial vertebrates had higher

baseline GC levels when breeding (Romero 2002). The influence of breeding on stress-induced GC levels is not clear-cut (Romero 2002). Recent work on the rufous-collared sparrows (*Zonotrichia capensis costaricensis*) showed that time of year, independent of life stage, can also affect baseline and stress-induced GC levels (Chapter 2).

Not all populations of the same species have similar GC profiles. Baseline and stress-induced GC levels also vary with latitude and habitat type (Dunlap and Wingfield 1995, Silverin et al. 1997, Silverin and Wingfield 1998, Breuner et al. 2003, Wilson and Holberton 2004). The details of GC modulation with life stage, season, and location are beyond the scope of this review. However, we emphasize that variation in GC levels with life stage, season, and location must be considered when measuring GC levels in wild populations.

Emergency life history stage

In response to noxious stimuli from the environment and social/psychological challenges, GC levels increase (Figure 1.1, dotted line) (Wingfield and Ramenofsky 1999, Wingfield and Romero 2001). The perspective of the animal determines whether a stimulus is “stressful” or not. For example, many animals are adapted to handle extreme conditions that seem unsuitable or “stressful” in the mind of a researcher. Animals living in the high-arctic do encounter environmental extremes, but they are a predictable part of the individual’s experience. GC levels rise in the “C” range (Figure 1.1) only when the conditions, be they food availability or weather events, become unpredictably severe or intense (Wingfield et al. 1983, Wingfield 1994, Wingfield and Kitaysky 2002).

Life stages such as migration, reproduction, or molt that are energy-demanding but predictable are often termed inappropriately termed “stressful” (Wingfield et al. 1998, Wingfield and Ramenofsky 1999). Such terminology represents a simple case of semantics and does not indicate a true rise in GCs to emergency levels. As mentioned

above, the GC response to a given stressor can be modulated. For example, life stage and body condition can affect the peak GC levels achieved in response to stressors, such as severe weather or capture (Romero et al. 2000, Woodley et al. 2003).

GCs in an ecological context

Most studies on GCs are conducted to understand the physiology of the individual on its own. Researchers are now using GCs levels as a way to measure animals' reaction to energetic demand and environmental variables, many of which are relevant to conservation issues.

The major threats to biodiversity are habitat destruction, alien species, pollution, overexploitation, disease, and global climate change (Wilcove et al. 1998, Easterling et al. 2000). Here we address how each of these variables, except for alien species, correlates with GC levels in wild animals.

Habitat variation

A number of studies have hypothesized that the “quality” of a habitat is reflected by the GC levels of the animals living within it. Marra and Holberton (1998) looked at corticosterone (CORT) levels in American redstarts (*Setophaga ruticilla*) that overwintered in either “good” quality habitat or in a “poorer”, more xeric, and potentially food-restricted habitat. The birds living in these two habitats had similar CORT levels on arrival to the wintering grounds, but, at the end of the winter, those living in the “poorer” habitat had higher baseline CORT levels than those birds overwintering in the “better” habitat. In addition, only the birds overwintering in the poorer-quality habitat failed to show a stress response to capture-and-restraint, indicating a down-regulated GC response to stress – a hallmark of chronic stress (Romero 2004).

In a similar study, Suorsa et al. (2003) looked at the effect of forest fragment size and forest age on treecreeper (*Certhia familiaris*) chicks. They found that chicks living in young, arthropod-poor habitat and small forest fragments had higher CORT levels than chicks living in mature forest and large forest fragments. Chicks with higher baseline CORT had a lower probability of survival than chicks with lower baseline CORT, indicating that CORT could have a predictive relationship with survival in this species. While this study focuses on variation in habitat, it is likely that differences in food availability mediate the observed “habitat” effects.

Carolina chickadees (*Poecile carolinensis*) living in disturbed recently-logged forests had elevated fecal CORT levels compared to chickadees living in undisturbed forests or residential sites (Lucas et al. 2006). There was no difference in fecal CORT among populations when the birds were brought into captivity. This result indicates that the variation in CORT among populations was not inherent to the population but rather to the conditions in which the population was living (Lucas et al. 2006).

The effect of habitat disturbance on GCs in the spotted owl (*Strix occidentalis*) is equivocal. In one study, male northern spotted owls showed increased fecal CORT levels if their territory was close to logging roads or recently harvested forests (Wasser et al. 1997). In this study, fecal CORT increased with increasing harvest intensity (Wasser et al. 1997). On the contrary, neither road, habitat characteristics, nor chainsaw sound correlated with fecal CORT levels in California spotted owls (Tempel and Gutierrez 2003, 2004).

Male spotted salamanders (*Ambystoma maculatum*) captured in disturbed habitat had lower baseline and stress-induced CORT levels compared to salamanders captured in undisturbed habitat (Homan et al. 2003). These results indicate that salamanders in disturbed habitat down-regulated the HPA axis in response to chronic stress. In the same study, Homan et al. (2003) documented that male salamanders had elevated

CORT levels after crossing a stretch of pavement with humans present but did not change CORT levels when migrating through forest habitat of different quality.

The fecal cortisol titers in grizzly (*Ursus arctos*) and black bears (*Ursus americanus*) did not increase with forest harvesting, open-pit coal mining, oil and gas exploitation, road use, poaching, or other contact with humans (von der Ohe et al. 2004, Wasser et al. 2004). Fecal cortisol levels in both species were, in fact, lower in an area of high disturbance and food abundance in Alberta, Canada compared to levels in an adjacent area of low disturbance and food abundance (Wasser et al. 2004). The abundance of humans at grizzly bear feeding sites in Alaska did not correlate with grizzly bear fecal cortisol levels (von der Ohe et al. 2004).

Weather

Severe weather events increase CORT levels in a variety of different species.

Unpredictable and unexpected stormy weather, including low temperature and high precipitation and wind, caused increased baseline CORT levels in male Puget Sound white-crowned sparrows (*Z. leucophrys pugetensis*), male eastern song sparrows (*Melospiza melodia*), Harris' sparrow (*Z. querula*), European blackbirds (*Turdus merula*), dark-eyed juncos (*Junco hyemalis*), cliff swallows (*Petrochelidon pyrrhonota*), and common diving petrels (*Pelecanoides urinatrix*) (Wingfield et al. 1983, Wingfield 1988, Rogers et al. 1993a, Smith et al. 1994, Wingfield 1994, Wingfield et al. 1998, Raouf et al. 2006). Breeding lapland longspurs (*Calcarius lapponicus*) captured during a severe spring snow storm did not have higher baseline CORT levels (potentially due to the presence of food in the traps in which they were caught), but, compared to birds in calm weather, had higher stress-induced CORT levels (Astheimer et al. 1995).

Unusually high temperatures can also increase baseline CORT levels as was observed in Abert's towhee (*Pipilo aberti*) and the black-throated sparrow (*Amphispiza bilineata*), both residents of the Sonoran desert (Wingfield et al. 1992).

The extent to which poor weather conditions elevate GC levels may depend on the life stage of the individual. During molt in three arctic bird species (the common redpoll, snow bunting, and Lapland longspur), increased baseline and stress-induced CORT levels were highly correlated with weather patterns (Romero et al. 2000). During breeding, only the Lapland longspur showed a relationship between weather and CORT (Romero et al. 2000).

Food limitation

A shortage of good-quality food can change elevate baseline GC levels and alter the stress response in both adults and chicks. Baseline and stress-induced CORT levels increased significantly in Galapagos marine iguana (*Amblyrhynchus cristatus*) exposed to a severe food shortage (Romero and Wikelski 2001). In response to low food availability, breeding black-legged kittiwakes (*Rissa tridactyla*) had higher baseline CORT levels and a damped CORT response to stress (Kitaysky et al. 1999b, Lanctot et al. 2003). Both black-legged kittiwake and red-legged kittiwake chicks (*Rissa brevirostris*) increase baseline CORT levels in response to food restriction or low quality food, but, in contrast to adults, they also show elevated stress-induced CORT levels (Kitaysky et al. 1999a, Kitaysky et al. 2001a). Magellanic penguin (*Spheniscus magellanicus*) chicks that were food deprived at the mid-point towards fledging had increased baseline CORT levels, but chicks that were food deprived when about to fledge did not (Walker et al. 2005c). In addition, the food-deprived penguins about to fledge also had a suppressed stress response to capture, indicating a down-regulation of the stress axis (Walker et al. 2005c).

Predators

GC levels increase in response to a single exposure to a predator and are modulated with chronic exposure to predators. Breeding pied flycatchers (*Ficedula hypoleuca*) increased CORT levels in response to a robotic, stuffed weasel (*Mustela vulgaris*, a predator on adult flycatchers) but not to a robotic, stuffed woodpecker (*Dendrocopus*

major, a predator on nestlings) (Silverin 1998). CORT levels in free-living great tits (*Parus major*) were not different 30 to 60 min after exposure to a stuffed owl mount (Cockrem and Silverin 2002). However, captive, wild great tits did have increased CORT levels in response to exposure to the same mount, indicating that the ability of the bird to move away from the predator affected its GC response (Cockrem and Silverin 2002). Male east African stonechat (*Saxicola torquata axillaris*) that share their territory with a predatory fiscal shrike (*Lanius collaris*) had higher baseline CORT than birds living in territories without this predator, indicating an effect of chronic exposure to predators (Scheuerlein et al. 2001).

Snowshoe hare (*Lepus americanus*) had higher baseline levels of CORT and increased GC responsiveness to stress during the decline and low point of the their population cycle (Boonstra et al. 1998). Boonstra et al. (1998) posit that when predation rates are high, hares are in a psychologically-induced state of stress due to 1) a constant state of high vigilance induced by failed attacks on themselves, 2) the experience of hearing the screams of nearby attacks on other hares, and/or 3) more frequently encountering predator signs. Boonstra et al. (1998) found that food supplementation alone and in conjunction with a reduction in predator pressure reduced and/or eliminated the state of chronic stress.

Nesting western song sparrows responded to predator pressure and food supplementation in a similar way (Clinchy et al. 2004). Sparrows with low predation pressure and high food abundance had low baseline and stress-induced CORT levels; sparrows with high predator pressure and low food abundance had high baseline and stress-induced CORT levels (Clinchy et al. 2004).

Disease

Chronically elevated GCs can compromise the immune system (Sapolsky et al. 2000, Saino et al. 2003). However, the role of baseline GC levels in affecting susceptibility and response to infection is not well understood.

Fecal GC levels were similar in female wild Rocky Mountain big-horn sheep (*Ovis canadensis canadensis*) regardless of whether or not they were infected with lungworm (*Muellerius capillaris* or *Protostrongylus* ssp.) (Goldstein et al. 2005). Adult and juvenile cliff swallows had increased baseline CORT levels in response to haematophagous ecto-parasites (Raouf et al. 2006). On the contrary, juvenile male black iguana (*Ctenosaura similis*) infected with blood-borne or ecto-parasites had lower CORT levels than non-infected individuals (Hanley and Stamps 2002). Infected iguana increased CORT levels with captivity while non-infected individuals did not, indicating that infected individuals may be more susceptible to stressors (Hanley and Stamps 2002). Similarly, fence lizards (*Sceloporus occidentalis*) infected with malaria (*Plasmodium mexianum*) had higher CORT levels in response to stress than non-infected individuals (Dunlap and Schall 1995).

Pollution

Pollution can increase GC levels for a number of reasons, including fouling of the skin/feathers, direct toxic effects on tissues, and reduction of food supply. For example, male Magellanic penguins fouled by a petroleum spill had significantly higher baseline CORT levels than non-oiled birds (Fowler et al. 1995). In addition, baseline CORT levels correlated positively with percent coverage of the body by crude oil (Fowler et al. 1995). Similarly, exposure of Galapagos marine iguana to trace oil pollution significantly increased their baseline CORT levels (Wikelski et al. 2001, Wikelski et al. 2002).

Exposure to heavy metals from coal combustion waste (Cd, Ar, Se, Cr, Cu, Ba) caused a rapid elevation in CORT levels in the Southern toad (*Bufo terrestris*) (Hopkins et al. 1997). In contrast, exposure to a similar suite of heavy metals (mainly Cu, Zn, Ni, Pb, As) did not affect CORT levels in pied flycatcher males or nestlings or great tit females and nestlings (Eeva et al. 2003, Eeva et al. 2005). Mercury loads did not correlate with CORT levels in the white ibis (*Eudocimus albus*), even though the metal did disrupt some aspects of reproduction (Heath and Frederick 2005). However, the CORT levels in this study were not indicative of baseline and differences in baseline CORT could have been eliminated by the initiation of the stress response in the study subjects.

The literature on the effects of pollutants in fish is extensive, and cannot be adequately reviewed here. In brief, aquatic pollutants can have severe consequences on the physiology of exposed fish, including disruption of the HPA axis and a decrease in plasma CORT levels (Brodeur et al. 1997, Laflamme et al. 2000, Eeva et al. 2003).

Human disturbance

Human disturbance can change GC levels through either physical or psychological stressors. Both wolves (*Canus lupus*) and elk (*Cervus elaphus*) increased fecal GC levels in a dose-dependant manner with exposure to snowmobile activity (Creel et al. 2002). Elk living in Custer State Park, South Dakota had significantly higher fecal GC levels in the summer months when tourist activity was greatest and the temperature highest than in the fall during hunting season, though it is uncertain how much of this variation was due to the annual cycle (Millspaugh et al. 2001). The authors believe that the high tourist activity and high temperatures may have created a chronic stress situation and resultant high GC levels. In contrast, they believe that fall hunting was too brief and infrequent a stressor to cause population-level changes in GC levels. Similarly, mourning doves (*Zenaida macroura*) did not show population-level changes in GC levels with hunting (Roy and Woolf 2001).

In contrast, hound hunting caused extremely high GC levels in some red deer (Bateson and Bradshaw 1997, Mason 1998). Repeated pursuit of cougars (*Felis concolor*) by domestic dogs reduced stress-induced GC levels, indicating a down-regulation of the stress axis and potentially impairing the cougars' ability to respond appropriately to stressors (Harlow et al. 1992).

The effect of ecotourism on CORT in birds is mixed. At hatch, Magellanic penguin chicks exposed to tourists had higher stress-induced CORT levels than unexposed chicks (Walker et al. 2005b). However, before fledging, exposure to tourists had no effects on GC levels (Walker et al. 2005b). In contrast, nestling hoatzin (*Opisthocomus hoazin*) exposed to tourists had similar CORT levels to nestlings not exposed to tourists (Müllner et al. 2004). Before fledging, exposure to tourists caused higher stress-induced GC levels (Müllner et al. 2004).

Adult Magellanic penguins with chronic exposure to tourists habituate and do not increase CORT with each visit (Walker et al. 2006). Opposite to chicks, tourist-exposed adults had lower stress-induced CORT levels (Walker et al. 2006). Similar results were found with Galápagos marine iguana (Romero and Wikelski 2002). Tourist-exposed and naïve iguana had similar baseline CORT levels, but tourist-exposed iguana had lower stress-induced CORT levels (Romero and Wikelski 2002).

Directions for future research

This review has indicated that the change in GCs is not uniform within or across species nor within or across environmental perturbations. However, the strength and predictability of patterns in GCs with different variables should increase with the collection of more data. We encourage researchers to conduct studies similar to the ones discussed here.

GC measures are relevant to conservation-related questions other than those discussed in this review. We are not aware of any studies that explore the impact of alien species on GC levels in native populations and know of just one study on how GC levels affect the population dynamics of an introduced species (Moore et al. 2005b). We encourage more research along this avenue. GC measures can also be useful for captive-rearing and reintroduction projects as an indicator of the physiological and psychological impact of manipulations and release to the wild (Adams et al. 2005, Hartup et al. 2005).

Our knowledge of the relationship between GC levels and survival in wild animals is limited. Three recent studies indicate the GCs may provide information about future survival in certain circumstances: survival was best with intermediate baseline CORT levels in cliff swallows, negatively correlated with baseline CORT levels in treecreeper chicks, and negatively correlated with stress-induced CORT levels in Galapagos marine iguana (Romero and Wikelski 2001, Suorsa et al. 2003, Brown et al. 2005). More studies are necessary to determine the generality of these results. However, if GCs are reliably indicative of future survival, GC measurements could be a powerful tool for those interested in managing wild and released populations.

Conclusions

In this paper, we have discussed the roles of GCs in basic functioning and reviewed the literature on GC levels in wild animals exposed to perturbations. Exposure to poor habitat, severe weather events, food limitation, predation, disease, and human disturbance can alter baseline GC levels and the GC response to stressors. The exposure of wild populations to these factors will, most likely, increase with global climate change and large-scale land-use change and habitat alteration. As such, we advocate the use of GCs as a way to understand how changing environments affect wild populations.

Although the reviewed studies address the population level, the individual level is often the most important for conservation applications (Cockrem 2005). Variation in

endocrine profiles among individuals is the rule not the exception in most studies (Sapolsky 1983, Wingfield et al. 1994, Wingfield and Romero 2001). Much of this variation in the stress response is due to the inherent coping style of the captured individuals (Koolhaas et al. 1999). However, the individuals with high baseline GC levels and low stress-induced GC levels are the sentinels for a population that is potentially at risk to the effects of environmental change.

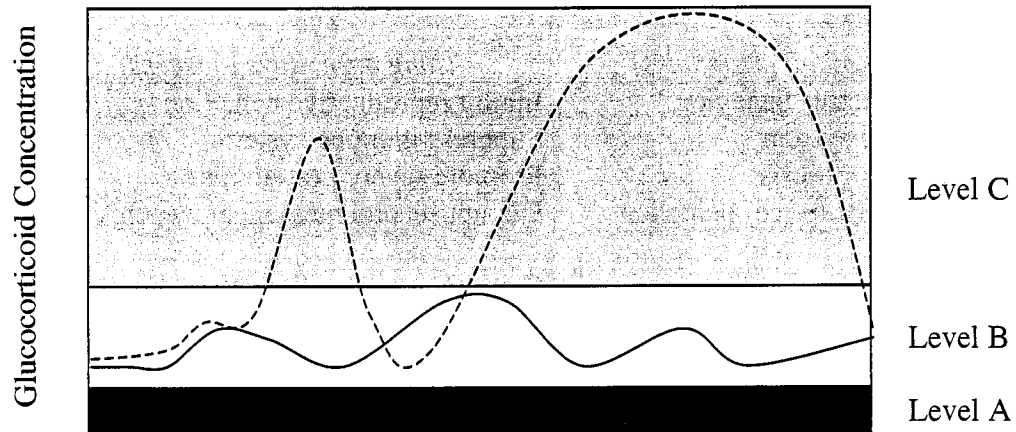


Figure 1.1. Glucocorticoid (GC) hormones have different actions at different levels (after Wingfield et al. 1997a). A small amount of glucocorticoids is necessary for basic glucose and salt regulation, indicated by the black bar or Level A. Glucocorticoids rise and fall with daily, tidal, seasonal, life history cycles (solid line) within the B range (clear bar). With noxious physical or psychological stimuli, glucocorticoid levels will increase into the C range (gray bar, dotted line). Each incursion into the C range indicates an emergency response.

Table 1.1: Effects of glucocorticoids during a rapid or chronic “stress” response (from Wingfield 1994, Wingfield et al. 1998). The rapid effects of glucocorticoids promote survival but the chronic effects can be a detriment to survival or lethal.

<i>Rapid</i> (short term, minutes to hours)	<i>Chronic</i> (long term, days to weeks)
Suppress reproductive behavior	Inhibit reproductive behavior
Regulate immune system	Suppress immune system
Increase gluconeogenesis	Promote severe protein loss
Promote escape (irruptive) behavior during day	Disrupt second messenger systems
Promote night restfulness by lowering standard metabolic rate	Cause neuronal cell death
Promote recovery on return to normal life history stage	Suppress growth and metamorphosis

Chapter 2

Modulation of the hypothalamo-pituitary-adrenal cortex axis in Costa Rican rufous-collared sparrow (*Zonotrichia capensis costaricensis*)

Introduction

Glucocorticoid hormones have different functions at different levels: 1) At low levels, they are responsible for basic glucose and salt regulation and 2) At high levels, they modulate the emergency response to stressful stimuli (Dallman et al. 1993, Wingfield et al. 1998, Sapolsky et al. 2000, Landys et al. in press). The low or baseline levels of glucocorticoids vary with such factors as the animals' energetic demand and environmental conditions (Dallman et al. 1993, Romero et al. 2000, Romero 2002). Studying these baseline levels in a population can give us insight into the allostatic load of the animal at different life stages and seasons (McEwen and Wingfield 2003, Landys et al. in press).

Stimulation of the hypothalamo-pituitary-adrenal cortex (HPA) axis by noxious stimuli results in elevated glucocorticoid levels. Short-term elevations of glucocorticoid levels into the range that triggers the emergency response promote survival behavior and physiology (Wingfield et al. 1998, Sapolsky et al. 2000). For example, elevated glucocorticoid levels are permissive to foraging behavior, increase glucose conversion in body typically from protein stores, regulate immune system, suppress reproductive behavior, and promote escape/irruptive behavior (Wingfield et al. 1998, Wingfield and Romero 2001). Although these physiological and behavioral alterations are advantageous for immediate survival, they can have negative consequences on the successful completion of different life stages. In this paper, we explore baseline glucococorticoid levels and the sensitivity of the HPA axis to stress in a population of

the rufous-collared sparrow (*Zonotrichia capensis costaricensis*). We separate three of the major factors thought to affect the functioning of the HPA axis in birds (time of year, molt, and breeding stage) in order to better understand the constraints on these birds under different environmental and internal states.

A trade-off for all animals lies between investment in self and investment in offspring (Stearns 1976, 1992). Recent research indicates that glucocorticoid hormones may play a role in this trade-off (Wingfield et al. 1995b, Kitaysky et al. 2001b, Wingfield and Romero 2001, Holberton and Wingfield 2003, Love et al. 2004). High glucocorticoid levels decrease reproductive behavior, including territoriality and care for young, and promote activities necessary for self survival, such as foraging and irruptive movement (Silverin 1986, Wingfield 1994, Wingfield and Ramenofsky 1997, Wingfield et al. 1998, Wingfield and Romero 2001, Breuner and Hahn 2003). To avoid high glucocorticoid levels, some birds that breed in extreme environments or that have limited breeding opportunities decrease their HPA axis response to stressful events (“stress response”) (Wingfield et al. 1992, Wingfield et al. 1995b, Wingfield et al. 1998, O'Reilly and Wingfield 2001, Holberton and Wingfield 2003).

The suppression of the stress response during breeding can have drawbacks for the individual because glucocorticoids regulate the physiological and behavioral changes that promote short-term survival (Wingfield et al. 1998, Sapolsky et al. 2000). For example, after a severe spring snowstorm in northern Alaska, most lapland longspurs (*Calcarius lapponicus*) abandoned their nests and joined food-searching flocks (Astheimer et al. 1995). However, one female was found dead on her nest, having never stopped incubating her eggs (JCW, personal observation). In contrast to this scenario, if breeding opportunities are ample and the environment non-extreme, breeding birds will maintain or even up regulate the ability to release glucocorticoids in response to stressful conditions (Wingfield et al. 1992, Wingfield and Ramenofsky 1999, O'Reilly and Wingfield 2001, Wingfield and Kitaysky 2002). Thus, in the face of additional

stressors, they will trade a current reproductive attempt for increased survival and future reproduction.

In all seasonal birds studied to date, the HPA axis is down regulated in molting individuals (Romero 2002). This down regulation during molt is thought to prevent the catabolic effects of glucocorticoids on protein deposited in the feathers (Romero et al. 2005). The pigeon (*Columba livia*) is the one known exception that does not down regulate the HPA axis during molt (Romero and Wingfield 2001). However molt in this species can occur throughout the year and takes many months more than it does in other, more seasonal species (Johnston 1992, Romero and Wingfield 2001).

All but one study on HPA axis modulation in birds has been conducted on species living in temperate and arctic zones (Romero 2002, Wada et al. in press). In species living at high latitudes, life stage is tied to environmental conditions as seasons change. Thus, determining which aspects of HPA axis modulation are due to the physiology of each life stage and which are due to environment is difficult. Although laboratory studies on captive birds in environmental chambers could tease apart life stage and environment effects on the HPA axis, experimenters cannot alter the facts that 1) captivity alters the HPA axis and 2) seasonal birds have evolved to live in different climates in their different life stages (Romero and Wingfield 1999, Wingfield 2005).

The rufous-collared sparrow, *Z. capensis costaricensis*, exhibits flexibility in the timing of breeding, with synchronicity being higher within a pair than in the population as a whole (Miller 1962, Stiles et al. 1989, Moore et al. 2005a). Although breeding has been documented in this species year-round, populations in both Costa Rica and Colombia have two defined breeding periods per year (Miller 1962, Wolf 1969, Stiles et al. 1989). Males have recrudesced testes for 4 months per breeding bout in a 6 mo reproductive cycle (Miller 1959a, 1962). Thus, they have reproductive capacity for 8 months of the

year. Females maintain the ovary in a ready state year-round, with ova approximately 7-10 days from their culminating ovulation phase at all times (Miller 1962).

In Colombia, the two breeding peaks center around mid-January and mid-July (Miller 1962). They occur during both the shortest and longest days of the year, span the periods with the annual temperature minimum and maximum, and correlate with the latter end of the region's two wet periods (Miller 1962). Similarly, Costa Rican *Z. capensis costaricensis* have breeding peaks between February-April and June-August (Wolf 1969, Stiles et al. 1989). These peaks occur during increasing and decreasing photoperiod, hot and cold temperature extremes, and the dry season and period of reduced rainfall in the wet season (Wolf 1969, Coen 1983). (Each year, Costa Rica has a regular *veranillo* or variable period of reduced rain in the middle of the wet season that occurs in July or August (Coen 1983).) Because of the species' two breeding peaks in Costa Rica and the variability in timing of active reproduction and/or molt within populations, we were able to test for effects of season alone on the HPA axis. We believe that this is the first study in birds to isolate the effects of environment/season from life stage when exploring HPA axis modulation.

Although some overlap of molting and the end of breeding is typical in temperate and arctic birds, molt is usually considered its own life stage (Morton and Morton 1990). Considerable overlap of molt and breeding has been documented in this and other *Z. capensis* populations and is not unusual in tropical species (Table 1, Miller 1961, Wolf 1969, Foster 1975). Similar to its 6 mo reproductive cycle, *Z. capensis costaricensis* also undergoes two complete prebasic molts a year in Colombia with an approximate 6 mo cycle (Miller 1961). Each molt takes about 2 mo (ranging from 1 mo and 20 days to 2 mo and 20 days) and typically begins 3 to 4 weeks after the beginning of a dry season (Miller 1961, Wolf 1969). In Costa Rican *Z. capensis costaricensis*, an additional partial prealternate molt (some body feathers) may occur between January and March (Wolf 1969). Molt may be more precisely timed than breeding in some populations of *Z.*

capensis costaricensis, as it is in many higher latitude species (Miller 1961, 1962, Dawson et al. 2001).

Because both molt and reproduction are relatively asynchronous within our study population of *Z. capensis costaricensis*, we were able to sample birds in all combinations of breeding and molting (breeding and molting, breeding and not molting, not breeding and molting, not breeding and not molting). During breeding in both males and females, we expected that baseline corticosterone (CORT) levels would be elevated due to the high energetic demand of this life stage. We hypothesized that birds living in relatively benign environments with ample opportunity to breed would maintain or increase their HPA responsiveness to stress during reproduction in order to favor self-maintenance over the care of young (Wingfield et al. 1992, O'Reilly and Wingfield 2001, Breuner et al. 2003, Holberton and Wingfield 2003). Thus, we expected breeding males and females would have a similar or more responsive HPA axis than non-breeding birds caught in the same month. We did not expect the sexes to have differential modulation of the stress response with breeding because both sexes should have a similar predisposition to favor investment in self over investment in offspring.

Because molting passerines, including this species, have consistently low baseline CORT levels and reduced HPA axis responsiveness (Astheimer et al. 1994, Romero et al. 1998d, c, b, Romero and Wingfield 2001, Romero 2002, Wada et al. in press), we expected that molting *Z. capensis costaricensis* of both sexes would have low baseline CORT levels and a reduced HPA axis response to stress compared to non-molting birds. If birds were both breeding and molting, we expected that 1) baseline CORT levels would be elevated in reaction to the demands of simultaneously maintaining two life stages and 2) the HPA axis response to stress would respond similarly to that of breeding birds because higher levels of CORT would promote self survival over reproductive behaviors (Wingfield et al. 1998, Sapolsky et al. 2000). We did not expect

that season would affect the HPA axis in this population because climatic conditions are not highly variable or extreme at our montane study sites, even between seasons.

Methods

We conducted this study on the rufous-collared sparrow (*Z. capensis costaricensis*), a small Central and South American emberizine sparrow that thrives in open areas, especially those that are altered by humans. We collected samples during four field seasons at two locations. In 2002, we collected all samples at the Cuerici Biological Station from July 8-19 (N 09° 33' 13.9", W 83° 40' 04.1", elevation=2585m). In 2004, 2005, and 2006, we collected samples from birds at both Cuerici Biological Station and Finca dos Lados (N 10° 10' 8.4', W 84° 17' 2.0", elevation=1780m) from July 11-25, April 19-May 3, and March 12-23 respectively. The majority of the *Z. capensis* habitat at the Cuerici Biological Station is organic blackberry (*Rubus sp.*) fields. The station also contains cow pastures and a working farm with small crop fields and domestic animals. Most of the birds we captured at this site were near the houses, crop fields, and pens for the resident animals, where *Z. capensis* congregates to feed. The habitat at Finca dos Lados is mostly former cow pasture that is being reforested. It contains low shrubs with some wild, native blackberry bushes. A few farm animals are kept at the farm, and a small area is used to grow vegetables and trees for the reforestation work. We captured all of the *Z. capensis* at this site near the farm's cabin.

Seasonality is defined by rainfall in Costa Rica, with the wet season occurring from May to November and the dry season from December to April (Coen 1983). However, rainfall and higher humidity levels are not uncommon in the dry season at the high elevation sites where we conducted our work (Coen 1983). *Z. capensis* has been recorded breeding in all months of the year in Costa Rica, with breeding peaks in the dry season from February to April and in the wet season from June to August (Stiles et al. 1989). Our sampling occurred during these two, more defined breeding seasons and, thus, both the wet and dry seasons. We captured both breeding and non-breeding,

molting and non-molting birds at each site during each of our field seasons. Table 2.1 details the breeding and molting state of birds that we captured in more than one season/year.

We used baited potter traps or mist nests to capture the birds. The first blood sample from each bird was taken within 3 min after capture in a mist net or approach in a potter trap. We used this first sample to measure baseline CORT levels, as it takes at least 3 min in birds for CORT to rise after disturbance (Wingfield et al. 1982). We took additional samples at 5, 10, 30 and 60 min after capture to measure the responsiveness of each individual to a standardized disturbance of capture, handling, and restraint, termed a “stress series” (Wingfield et al. 1992). We held the birds in a cloth bag in the shade between sampling points. If we did not observe when a bird was captured in a mist net, we did not use data from the animal for baseline CORT levels, but did conduct a standardized “stress series” on the individual to determine its maximum CORT levels.

We used two measurements to assess the ability of the HPA axis to respond to stress: the maximum level of CORT achieved in response to a standardized stressor and the rate of increase in CORT during standardized stressor. These measures provide related though different insights into the functioning of the HPA axis. All blood samples were stored on ice until the end of each day when we separated the plasma and red blood cells. We froze the samples and transported them to the University of Washington on dry ice.

Birds in reproductive condition were sexed via external anatomy (cloacal protuberance for males and brood patch for females). In 2002, we conducted a unilateral laparotomy for measurement of the gonads on all birds, except for females with an active brood patch. The correlation between the size of the cloacal protuberance and testis size was strong in this population of *Z. capensis costaricensis* (linear regression, $R^2=0.61$, $p=0.0001$), a population in Ecuador (I. T. Moore, unpublished data), and a population in

Colombia (Miller 1959a). After verifying the relationship between cloacal protuberance and testis size, we scored male reproductive state only with the size of the cloacal protuberance. We noted birds with a swollen cloacal protuberance of at least 5 mm height as reproductive (Miller 1959a). We defined the reproductive cycle of females by the following brood patch states, feathered chest: not reproductive, defeathering chest: preparing to breed, chest bare of feathers and skin flat: preparing to lay eggs, chest bare of feathers and swollen with edema: incubating eggs, chest bare of feathers and skin wrinkled: caring for and feeding young, chest refeathering: ending reproductive phase.

We genetically sexed non-reproductive birds on which we did not conduct a laparotomy. We froze packed red blood cells after collection and extracted the DNA from them using Qiagen DNeasy tissue kits. We used primers P2 and P8 with polymerase chain reaction (PCR) to amplify a portion of the CHD-W gene and its homolog the CHD-Z gene, which are found on the avian sex chromosomes (Griffiths et al. 1998). These primers result in a large size difference between the target fragments. We used the “touchdown” thermal cycler profile as described in Fridolfsson and Ellegren (1999) for the PCR reaction. We separated the PCR product and visualized it on an agarose gel.

We classified birds as molting only if they had growing primary feathers.

CORT assays

We measured CORT levels via direct radioimmunoassay following the methodology of Wingfield *et. al* (1992). Briefly, we added a small aliquot of tritiated CORT (2,000 counts/min) to each sample for determination of extraction efficiency and incubated the samples overnight. We extracted each sample with 4 ml of redistilled dichloromethane for at least 2 hrs before aspirating and drying the dichloromethane fraction and resuspending the solutes in phosphate-buffered saline with gelatin. We assayed all samples in duplicate and corrected hormone values for the initial plasma volume and

percent recovery of the initial aliquot of tritiated CORT. We ran blank and standard samples with each assay to verify its performance.

We ran 5 separate assays to analyze the samples for this project, all conducted by DSB. The limit of detection for the assays ranged from 1.1-2.72ng/ml depending on the amount of plasma used for the assay (12-20 μ l). Interassay variation for the 5 assays was 18% and interassay variation in the hands of DSB was 8.54%.

Statistical analyses

We analyzed data sets from males and females separately and, if necessary, log transformed them to achieve normality. We sampled some individuals in multiple years and included them multiple times in the analyses. Because each sampling point was separated by at least 8 mo, including multiple samples from the same individual should not be problematic. We used chi-squared tests to determine if frequency of molt or reproductive state varied with month. To explore the relationship between month, reproductive state, and molt on baseline and maximum CORT levels we used least mean squares models. We tested for the effects of these same factors on the responsiveness of the HPA axis to capture-and-restraint with repeated-measures MANOVA. We used Tukey HSD or student's t-tests for post-hoc analyses with significant least mean squares model outputs and contrast analysis for post-hoc analysis with significant repeated-measures MANOVA outputs. We conducted all analyses with JMP 6.0 (SAS Institute 2005).

Results

HPA axis in females

More females were breeding in March and April than in July (Figure 2.1a and b, $\chi^2=31.02$, $N=57$, $p=0.0006$). A similar number of females were molting in each months ($\chi^2=2.61$, $p=0.27$, $N=57$, ave. 29% molting, Figure 2.1b). Baseline and maximum CORT levels were higher in the spring than in the summer and these levels were not

affected by reproductive state or molt (Table 2.2, Figure 2.2). HPA axis responsiveness to capture-and-restraint sampling was higher in the spring (Table 2.2, Figure 2.3a). Birds caring for chicks tended to have a higher HPA axis response to stress (Table 2.2, Figure 2.3b). Molt did not affect the sensitivity of the HPA axis to stress (Table 2.2). We did not use an interaction term between breeding state and molt for the female statistical models because, with the inclusion of an interaction term, the models had too few degrees of freedom. When the breeding state of the females was reduced to presence/absence, there was still no affect of breeding state on CORT levels or the stress response.

HPA axis in males

Significantly more males were in breeding condition in March and April than in July (Figure 2.1c, $\chi^2=11.69$, $N=127$, $p=0.0029$), but month did not affect the number of animals in molt ($\chi^2=4.29$, $p=0.12$, $N=134$, ave. 21% molting, Figure 2.1c). Although baseline CORT levels were significantly affected by month, molt, and an interaction between molt and reproductive stage, post-hoc analysis reveals that baseline CORT level were higher only in molting birds compared to non-molting birds (Table 2.2, Figures 2.2 and 2.4). Maximum CORT levels were higher in March than in April or July (Table 2.2, Figure 2.2). The HPA axis response to a stress was higher in the spring than in July (Table 2.2, Figures 2.5a). Breeding males had a higher HPA response to acute stress than non-breeding males, with males that were simultaneously breeding and molting having the highest HPA axis response to acute stress (Table 2.2, Figure 2.5b).

Natural History

Due to the unusual and flexible seasonality of *Z. capensis costaricensis*, we believe that further documentation of its natural history is necessary to gain insight into this and other future studies. Here we present background information and observations that we believe are of biological import.

At the Cuerici Biological Station, we have captured an actively breeding female that was over four years old. In contrast, we have also documented a male with a fully developed cloacal protuberance that was still in his juvenile plumage (DSB, personal observation). Because birds start to molt out of their juvenile plumage at 54 days old and finish at about 4 mo (Miller 1961), we can assume that this breeding juvenile either failed to molt into adult plumage or had reached sexual maturity within the first couple months of life. We have caught 2 juveniles molting into adult plumage in the spring and neither were reproductively mature.

Due to higher densities and a greater proportion of non-breeding birds in the summer months, we believe that the birds may move to the Cuerici farm in flocks to feed (DSB, personal observation). Flocking has been reported in Costa Rica before (Wolf 1969).

Repeat captures between years were common. The majority of the birds captured in multiple years (potentially residents) were in breeding condition in July, when only about half of the captured individuals were breeding (Table 2.1, Figure 2.1). This observation indicates that the non-breeding individuals caught in July could be vagrants. We have documented some movement of birds within the valley in which we work at the Cuerici Biological Station. Banded individuals were located at farms as far as 4 km away from our study site, and a more systematic survey of movement and dispersal is underway (C. Solano, personal observation).

Discussion

We designed our study to test for the effects of season, breeding condition, and molt on baseline and maximum levels of CORT and the responsiveness of the HPA axis to stress in male and female *Z. capensis costaricensis*. We conducted the study during the two annual breeding peaks of this species in Costa Rica. The responsiveness of the HPA axis to a standardized stressor was higher in March and April in both males and females. Maximum CORT was higher in the spring (March and April) in females and in

March in males. Females also had higher baseline CORT levels in March. Thus, *Z. capensis* in Costa Rica modulated the HPA axis seasonally.

Breeding males, especially those that are molting, had a higher HPA axis response to capture-and-restraint than non-breeding males. Females had a similar trend for higher HPA axis responsiveness when tending to chicks. Maintenance of or an increase in HPA axis responsiveness to a standardized stressor during breeding is common in birds that have a long breeding seasons and do not breed in extreme environments (Wingfield et al. 1992, O'Reilly and Wingfield 2001, Breuner et al. 2003). However, this is the first time that the increase in HPA axis responsiveness has been attributed to breeding alone and not also season. Molting males had higher baseline CORT levels than non-molting males, but molt did not affect the HPA axis in females. In all other cases in which CORT has been measured in seasonally molting song birds, the molting birds have a down-regulated HPA axis (Romero 2002).

Season

We were surprised to find that season explained most of the variation in the HPA axis in these populations of *Z. capensis costaricensis*, and that this variation was not related to breeding per se. In both males and females, maximum CORT levels and HPA axis responsiveness to stress were higher in the spring (dry season) than in the summer (wet season). In females, baseline CORT levels were also higher in the spring. CORT levels and HPA axis responsiveness may be higher in the spring due to the temperature extremes that occur during these months. In Costa Rica, the annual temperature minima occurs in February and the annual temperature maxima in March or April (Coen 1983). Frost is an occasional occurrence at Cuerici during the dry season (December-April), and was present on two of the mornings when we were at the site in March 2006. Higher CORT levels during the spring could help the birds mobilize enough glucose and consume enough food to meet energetic demand (Sapolsky 1983b, Dallman et al. 1993, Wingfield et al. 1998). A stronger HPA axis response could also facilitate

irruptive movements to better locales, most likely down slope, if environmental conditions deteriorate (Wingfield and Ramenofsky 1997, Breuner and Hahn 2003, Wingfield 2003).

Z. capensis costaricensis in Costa Rica has two breeding and molting seasons a year. However, the two breeding seasons during which we sampled the populations had opposite photoperiod dynamics: in the spring, photoperiod was increasing and in the summer, it was decreasing. Similarly, in the Ecuadorian Andes, the breeding seasons of two populations of *Z. capensis costaricensis* do not seem to be regulated by photoperiod and instead are correlated with the phase-shifted pattern of local rainfall (Moore et al. 2005a, Moore et al. 2006). Although photoperiod does not seem to be a major factor in controlling life cycle timing in this species, *Z. capensis costaricensis* can detect and respond to days longer and shorter than they experience in their tropical home (Miller 1959b, 1965, Epple et al. 1972). Thus, *Z. capensis costaricensis* could be responding to photoperiod with a change in the HPA axis. *Z. capensis* is thought to be the basal species in the *Zonotrichia* genus (Zink and Blackwell 1996). Whether the species evolved at a tropical or temperate latitude is unknown. If the HPA axis in these Costa Rican populations of *Z. capensis* was indeed responding to photoperiod cues and if the species originated in the temperate zone, the modulation may be a biological legacy from the species or genus when it lived at higher latitudes and bred, molted, and potentially migrated once a year. This hypothesis should be considered further and tested. However, modulation of the HPA axis should remain only if it is adaptive in some way. For example, these birds could be responding to photoperiod because change in day length may alter the timing and length of the foraging period.

Further studies could explore whether the seasonal pattern of HPA axis modulation that we have detected in the tropics is also seen in *Z. capensis* living at higher latitudes to the south. We know already that molt affects the HPA axis of *Z. capensis costaricensis* differently in Costa Rica (up regulation in males, no change in females) than in Ecuador

(down regulation) (Wada et al. in press). This difference in response to molt could be due to the degree of seasonality at the study locales or other environmental attributes that vary with latitude and climate. Latitude is known to affect the HPA axis in *Z. leucophrys* (Breuner et al. 2003). For example, *Z. leucophrys gambelii*, an arctic breeder, shows marked variation in stress response in different seasons and life history stages as does *Z. leucophrys oriantha*, which breeds in the western mountains of the US (Romero et al. 1997, JCW unpublished data). In contrast, *Z. leucophrys nutalli*, a non-migratory race living in California, and *Z. leucophrys pugetensis*, a short distant migrant that breeds on the coast of the US Pacific Northwest, show no seasonal variation in stress response except a down regulation of the axis during molt (JCW unpublished data). Modulation of the HPA axis with latitude also occurs in other non-*Zonotrichia* passerines, such as breeding willow warblers (*Phylloscopus trochilus*), yellow warblers (*Dendroica petechia*), pied flycatchers (*Ficedula hypoleuca*), and bush warbler (*Cettia diphone*) (Wingfield et al. 1995a, Silverin et al. 1997, Silverin and Wingfield 1998, Breuner et al. 2003, Wilson and Holberton 2004).

Breeding

Breeding birds may maintain or up-regulate the stress response so that they are more likely to abandon the nest when conditions deteriorate (Wingfield et al. 1995b, Silverin et al. 1997, Wingfield and Ramenofsky 1997, Romero 2002, Wingfield and Kitaysky 2002, Richardson 2003, Love et al. 2004). Their increased HPA axis sensitivity to stress would favor self-survival and the potential for future reproductive events over the survival current offspring. In this population of *Z. capensis costaricensis*, breeding increased the responsiveness of the HPA axis to stress in males, even when they are molting, and there was a trend for a similar pattern in females. That both sexes increase the HPA axis responsiveness to stress with breeding was expected because both members of a pair feed nestlings and fledglings in this species (Miller and Miller 1968, O'Reilly and Wingfield 2001). In a sedentary species with a relatively high survival

rates and ample opportunity for subsequent breeding attempts, this favoring of self over young is adaptive.

However, because the primary role of CORT in the body is to regulate glucose levels, the HPA axis in breeding birds may release more CORT to promote feeding behavior and in response to increased energetic demands (Dallman et al. 1993, Wingfield et al. 1998, Sapolsky et al. 2000, Romero 2002). The data from males simultaneously breeding and molting, which have higher HPA axis responsiveness than other groups, support the hypothesis that energy demands play a role in reactivity of the HPA axis.

Due to the asynchrony of reproduction in this population, we were unable to determine the breeding substage of the males (mate laying eggs, mate incubating eggs, feeding nestlings, feeding fledglings) that we sampled in this study. Although most of the HPA axis modulation in breeding males occurs within breeding substages (Wingfield and Farner 1978a, Astheimer et al. 1995, Romero et al. 1997, Romero 2002, Holberton and Wingfield 2003, Wilson and Holberton 2004), the HPA axis in a seasonal Ecuadorian population of *Z. capensis costaricensis* sampled in both early and mid-breeding season is similar (Wada et al. in press). Similarly, temperate yellow warblers (*Dendroica petechia*) have no change in the HPA axis response to stress between preparental and parental breeding stages (Wilson and Holberton 2004). Thus, our conclusions may not be compromised by our failure to assess breeding substage.

Only one other study that we are aware of describes the HPA axis in breeding females during the many substages of breeding (Love et al. 2004). Love *et al.* (2004) found that European starling (*Sturnus vulgaris*) females do not modulate baseline CORT with breeding stage. However, maximum CORT levels are higher in this species during lay than during incubation and chick rearing (Love et al. 2004). We found no effect of reproductive stage on baseline or maximum CORT alone, but did find that reproductive stage affected HPA axis responsiveness. Opposite of the starling findings, females

caring for young tended to have a greater stress response than females in other stages (Love et al. 2004). Not all species modulate the HPA axis response to stress with breeding substage: arctic breeding tree sparrow (*Spizella arborea*), white-crowned sparrow, and yellow warbler have similar HPA axis response to stress when preparental (nest building, laying, incubation) and parental (feeding nestlings and fledglings) (Holberton and Wingfield 2003, Wilson and Holberton 2004).

Molt

Body and wing molt occur in both breeding seasons and in birds of all stages of reproduction. In females, molt had no effect on the HPA axis. This result is similar to temperate pigeons (*Columba livia*) in which molt does not affect baseline or maximum CORT levels or the response to capture stress (Romero and Wingfield 2001). However, in pigeons one molt lasts most of the year, and in *Z. capensis* molt is completed after 2 mo (Miller 1961, Romero and Wingfield 2001).

Compared to non-molting males, molting males had higher baseline CORT levels. This result is opposite to those found in all other seasonally molting birds, including *Z. capensis costaricensis* in the Ecuadorian Andes (Romero 2002, Wada et al. in press). Our finding may be spurious and due to the low sample size of molting, non-breeding males (N=4). However, if the result is valid, we must consider the costs and benefits to elevated baseline CORT levels during molt. In birds, high CORT levels slow the progress of molt and can degrade developing proteins (Romero et al. 2005). The cost of a long molt for the Costa Rican *Z. capensis costaricensis* may be little to none because 1) the asynchrony of molt in this population indicates that the conditions for molt are not limited to specific points in time and 2) the pressure for these birds to complete molt quickly is small due to the population's sedentary nature.

Elevated baseline CORT levels might in fact be beneficial for the process of molt and the individual's short-term survival by increasing immediate energy stores, promoting

foraging behavior, and enabling irruptive movements to more benign locales in the mountain valleys (Wingfield and Ramenofsky 1997, Wingfield et al. 1998, Wingfield and Ramenofsky 1999, Romero et al. 2000, Breuner and Hahn 2003). High baseline levels of CORT in molting males may be a response to the energetic demands of feather growth and of maintaining flight and body temperature with incomplete feathers (Murphy and King 1992, Romero et al. 2000, Romero et al. 2005). Further work on the responsiveness of the HPA axis during molt in sedentary, flexible species would be needed to test these hypotheses. However, the lack of HPA axis down-regulation during a forced molt in starlings supports our hypothesis that low CORT levels might not be crucial to molt nor beneficial to the bird in all circumstances, though it should be noted that this forced molt is not part of the predictable life cycle (Romero et al. 2005).

In this study, we did not measure CORT binding globulin (CBG) levels. Changes in CBG levels throughout the seasons and between molt/non-molt and breeding/non-breeding groups could alter the patterns that we see in free CORT levels. In an Ecuadorian population of *Z. capensis*, CBG levels change seasonally, and seasonal changes observed in total CORT levels do not exist in free CORT levels (Wada et al. in press). Studies on other species have found that CBG levels are higher early in the breeding season, most likely due to increases in plasma testosterone, than they are later in the breeding season or during molt (Silverin 1986, Romero et al. 1998c, Romero and Wingfield 1998, Deviche et al. 2001). However, new evidence on the role of CBG-bound CORT makes us question the biological meaning free CORT levels (Adams 2005, Hammes et al. 2005).

Conclusions

This is the first study to show that season alone affects the HPA axis in a wild song bird. While the adaptive role of this modulation is unclear, further research may elucidate our results. However, our findings indicate that researchers should consider the role of both life stage and season when interpreting data on the HPA axis. As

expected given the natural history of this population, breeding birds of both sexes up regulated the HPA axis. This up regulation may indicate that in emergency conditions, adults favor self-survival and future reproductive attempts over current reproductive events. We found that molting males had higher baseline CORT levels than non-molting males and that males that were both molting and breeding had the strongest HPA axis response to an acute stressor. This up regulation of the HPA axis during molt is counter to findings in all other birds that molt seasonally (Romero 2002). CORT's primary role for molting *Z. capensis costaricensis* in this population may be one of energy mobilization and acquisition. To truly understand how the HPA axis interacts with season, breeding, and molt in birds, future studies should focus on systems in which each of these factors can be isolated and combined factorially.

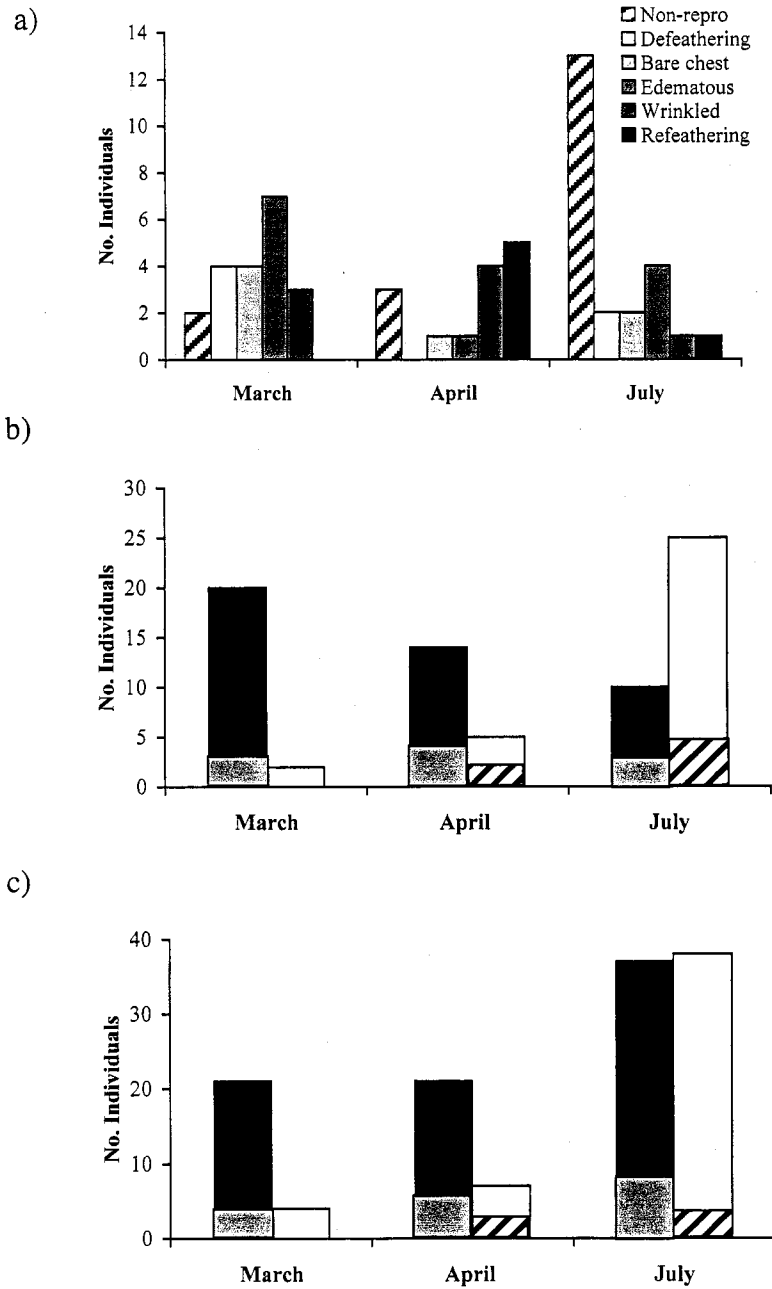


Figure 2.1. Variation in a) reproductive stage of females, b) reproductive and molt stage of females, and c) reproductive and molt stage of males with month. The proportion of females in each reproductive state changed significantly with month. The proportion of molting females did not vary with month. The proportion of males breeding but not molting varied significantly with month.

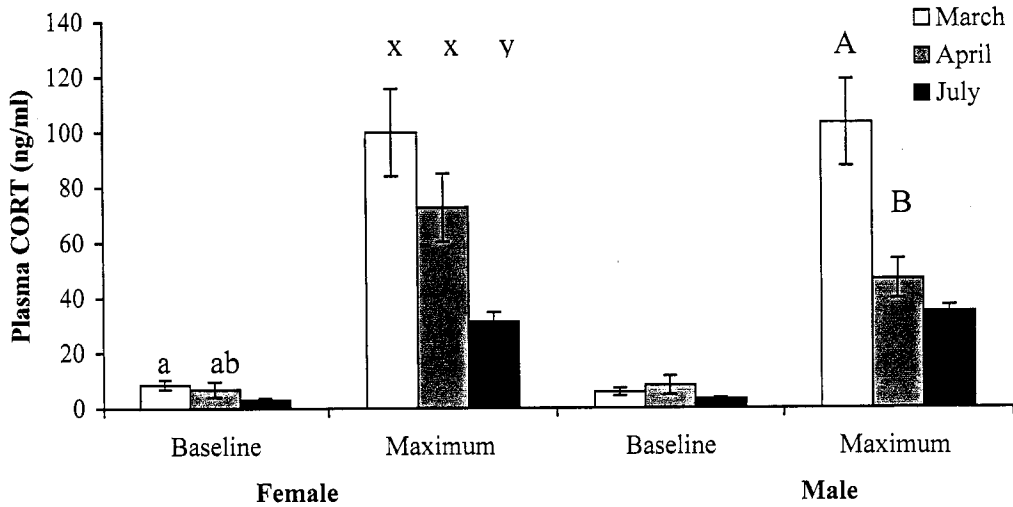
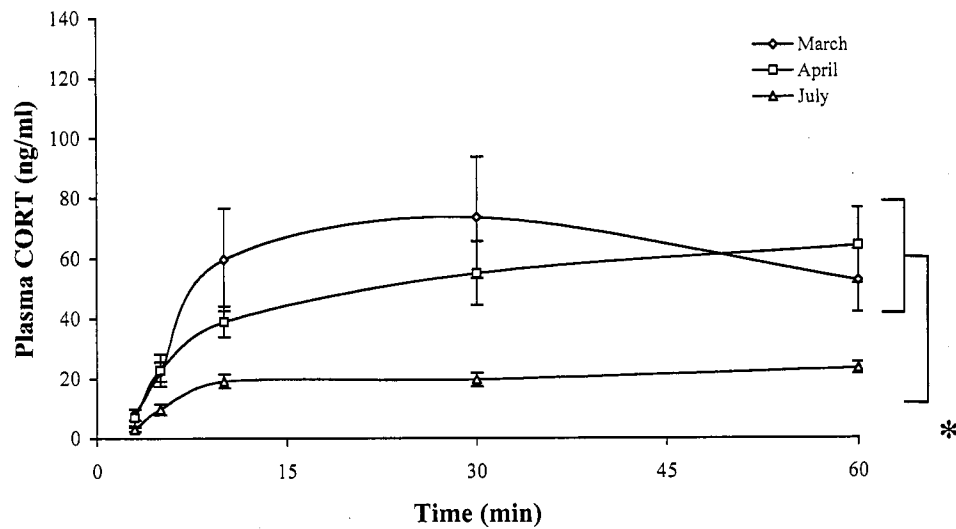


Figure 2.2. Mean baseline and maximum plasma CORT levels \pm SE in male and female birds in each month. The results of post-hoc tests on the effect of month in each data set are presented with letters. Groups with different letters are significantly different from each other ($p < 0.05$). Sample size for baseline CORT in March, April, and July in females was 15, 14, 21 and in males was 9, 5, 37. Sample size for maximum CORT in March, April, and July in females was 18, 14, 23 and in males was 12, 6, 40.

a)



b)

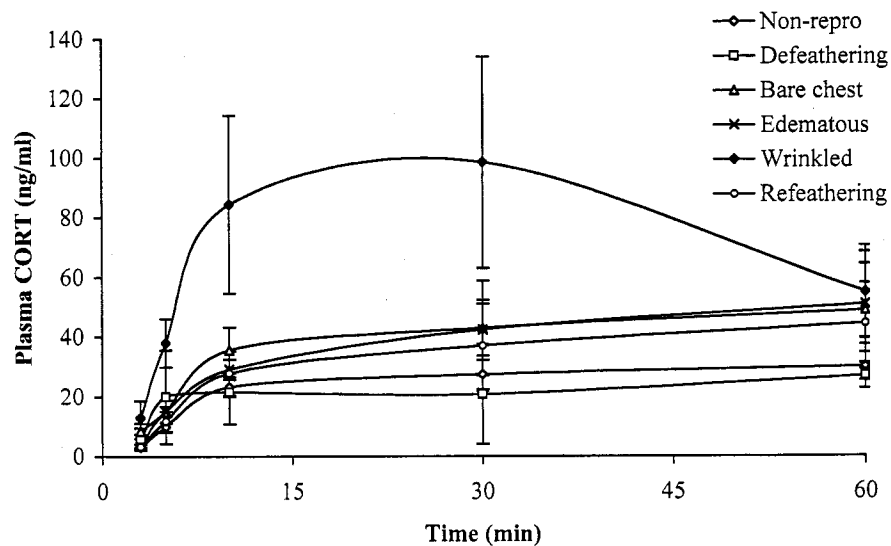


Figure 2.3. Mean plasma CORT levels \pm SE in response to capture-and-restraint sampling in females by a) month and b) breeding stage (as indicated by brood patch state). The HPA axis response to stress was higher in females in the spring (March and April) than in the summer (July), as indicated by an asterisk. There was a trend for HPA axis response to vary with breeding stage, with birds feeding chicks (“wrinkled” brood patch) having the most pronounced response. Sample sizes were as follows a) March=12, April=13, July=21 and b) non-repro=13, defeathering=2, bare chest=6, edematous=11, wrinkled=7, refeathering=7.

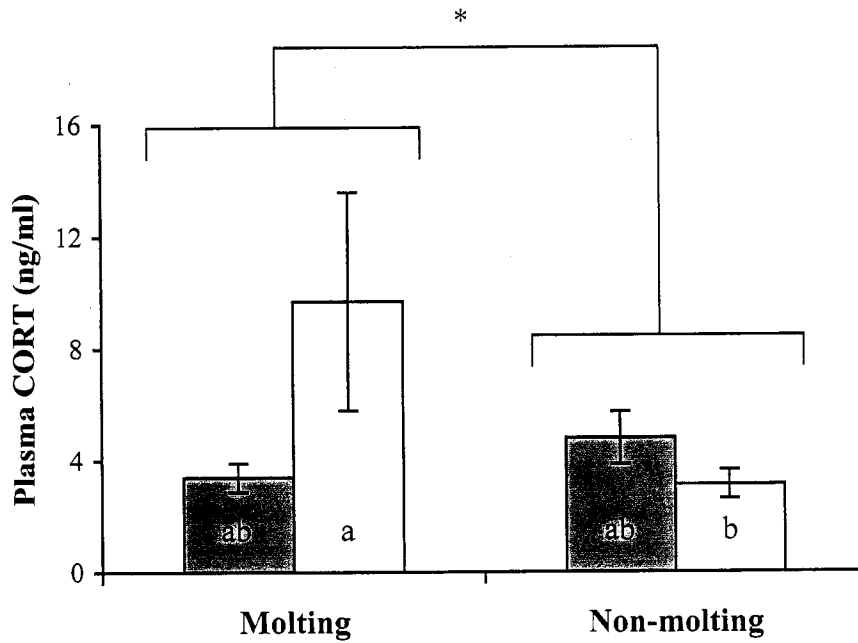


Figure 2.4. Mean baseline plasma CORT level \pm SE in breeding (gray bars) and non-breeding (white bars) males that were molting or non-molting. ANOVA analysis revealed that molting birds had significantly higher baseline CORT levels than non-molting birds, as indicated by the asterisk. Post-hoc analysis on the significant interaction between molting and breeding indicated that non-breeding, molting birds had higher baseline CORT levels than non-breeding, non-molting males. Significant differences are indicated by groups that do not share letters. Sample sizes were as follows: molting, breeding=7; molting, non-breeding=4; non-molting, breeding=16; non-molting, non-breeding=23.

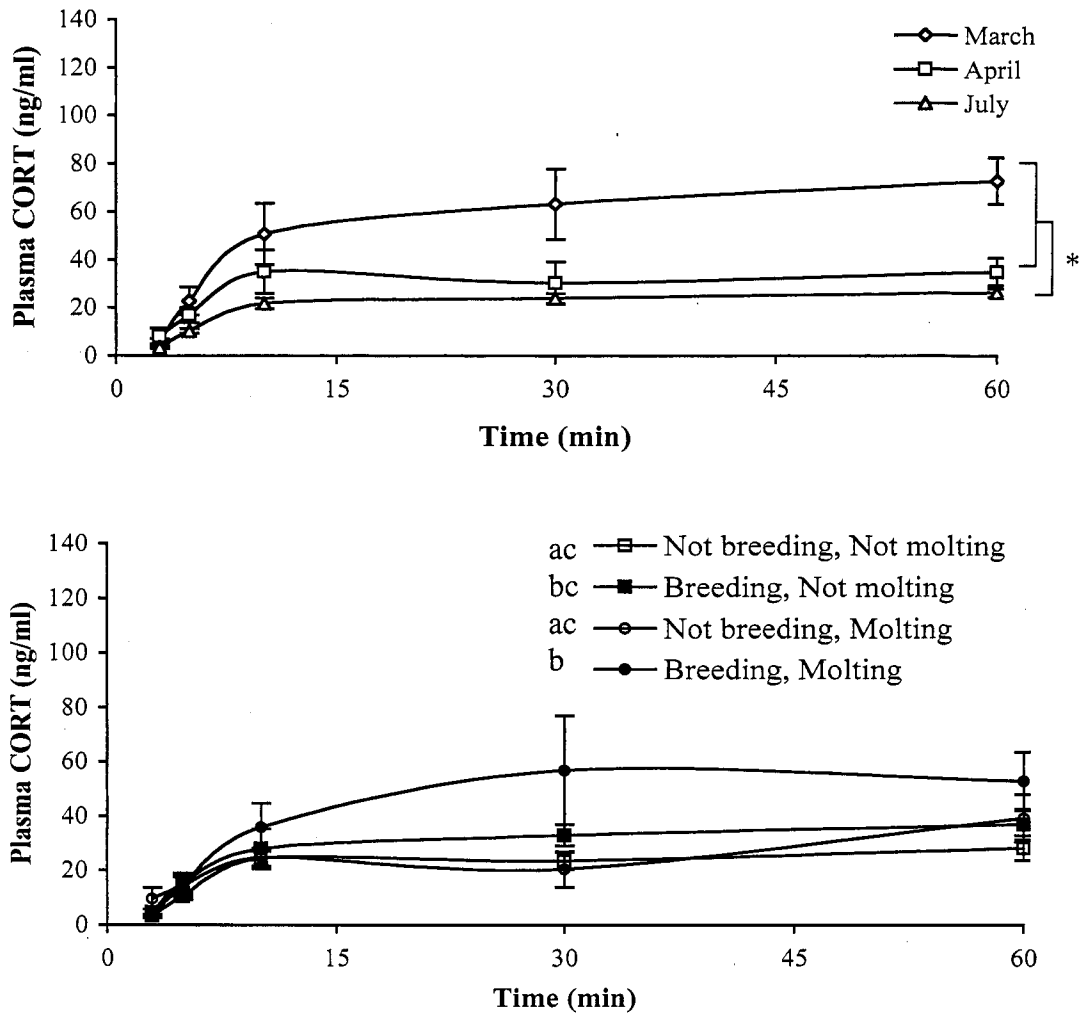


Figure 2.5. Mean plasma CORT levels \pm SE in response to capture-and-restraint sampling in males by a) month and b) breeding and molt status. The HPA axis response to stress was higher in males in the spring (March and April) than in the summer (July), as indicated by an asterisk. Breeding males had a higher HPA axis response to stress than non-breeders, and males that were both breeding and molting had the highest response. Significant differences via a post-hoc tests are indicated by groups that do not share letters. Sample sizes were as follows a) March=9, April=5, July=37 and b) molting, breeding=7; molting, non-breeding=4; non-molting, breeding=16; non-molting, non-breeding=23.

Table 2.1. Reproductive and molt state of males and females caught in multiple seasons. If males had a cloacal protuberance longer than 5 mm, the individual was scored as breeding. Female breeding stage was scored by the state of the brood patch. The 6 brood patch conditions are: 0) feathered chest: not reproductive, 1) defeathering chest: preparing to breed, 2) chest bare of feathers and skin flat: preparing to lay eggs, 3) chest bare of feathers and swollen with edema: incubating eggs, 4) chest bare of feathers and skin wrinkled: caring for and feeding young, 5) chest refeathering: ending reproductive phase. If a bird was molting primary (1°), secondary (2°), or "tertiary" (3°) wing feathers, the progression of molt was categorized by those feather tracts. If there was no wing molt, birds were scored as molting body feathers (including the tail) or just the tail. We captured one male (605) during its post-juvenile molt and one female (218) when it was a fledgling.

Band No.	March 2006		April 2005		July 2002/2004	
	<i>Breeding</i>	<i>Molting</i>	<i>Breeding</i>	<i>Molting</i>	<i>Breeding</i>	<i>Molting</i>
MALES						
105	Yes	Tail	No	Body	Yes	2°
325	Yes	No	Yes	1°	Yes	No
605	Yes	1°	No	Postjuv.		
267			No	1°	Yes	No
355			Yes	None	Yes	1°
506			Yes	1°	Yes	No
763			No	1°	No	Body
791			Yes	None	Yes	No
822			Yes	?	Yes	1°
116					Yes	?
					Yes	Body
187					Yes	Body
					No	Tail
FEMALES	<i>BP</i>	<i>Molting</i>	<i>BP</i>	<i>Molting</i>	<i>BP</i>	<i>Molting</i>
218	3	Body	3	None	Fledgling	
217	2	Body	4	None		
368	2	None	4	None	4	3°
387	4	None			None	1°
395	3	None			None	Body
401	3	None			1	1°
522	3	1°	None	1°	1	Body
614	4	None	3	?		
645	3	1°	None	2°		
137			2	None	5	1°
189			5	1°	5	Body
359			4	Body	None	Body
789			3	None	None	1°

Table 2.2. Results of ANOVA tests from least mean squares models or repeated-measures MANOVA tests for HPA axis attributes of males and females. Significant results are given in bold. Trends are in italics. The results of post-hoc analysis are given in Figures 2.2-2.5.

<u>Females</u>				<u>Males</u>			
ANOVA	F	df	p	F	df	p	
<i>Baseline CORT</i>							
Model	2.90	8,41	0.01	Model	1.32	5,44	0.01
Month	4.38	2	0.02	Month	3.38	2	0.04
Repro. State	1.42	5	0.24	Repro. State	0.66	1	0.42
Molt	0.08	1	0.78	Molt	5.76	1	0.02
				Repro. State*Molt	6.36	1	0.02
<i>Maximum CORT</i>							
Model	7.04	8,46	<0.0001	Model	14.19	5,51	<0.0001
Month	17.13	2	<0.0001	Month	29.34	2	<0.0001
Repro. State	1.46	5	0.22	Repro. State	0.55	1	0.46
Molt	0.30	1	0.59	Molt	1.54	1	0.22
				Repro. State*Molt	0.03	1	0.87
<i>Repeated-measures</i>							
MANOVA	F	df	p	F	df	p	
<i>Stress response</i>							
Month	10.53	2,37	0.0002	Month	13.59	2,44	<0.0001
Repro. State	2.36	5,37	0.06	Repro. State	1.17	1,44	0.28
Molt	0.13	1,37	0.73	Molt	1.12	1,44	0.30
Time	15.89	4,148	<0.0001	Repro. State*Molt	0.35	1,44	0.55
Time*Month	0.80	8,148	0.60	Time	13.48	3,130	<0.0001
Time*Repro. State	1.05	20,148	0.40	Time*Month	0.46	6,130	0.84
Time*Molt	0.77	4,148	0.55	Time*Repro. State	2.73	3,130	0.047
				Time*Molt	2.37	3,130	0.08
				Time*Repro. State*Molt	3.22	3,130	0.03

Chapter 3

Living on the edge: does proximity to a geographical range boundary influence corticosterone levels in tropical song wrens (*Cyphorhinus phaeocephalus*)?

Introduction

Understanding why species can live in some locales but not others has long interested biologists (Allee 1926, Root 1988, Root et al. 2003). Identifying the factors that delineate species range borders is challenging, often because the factors vary from interactions with other species to a diverse number of abiotic variables (Gaston 2003). Moisture and humidity can be important variables in determining avian distributions (Smith 1977, Karr 1980, Brawn et al. 1998). For example, in the Panama Canal region, bird species richness is higher in areas with higher annual rainfall (Robinson et al. 2004).

Tropical avian understory insectivores are particularly impacted by habitat changes that affect forest moisture and humidity, such as forest fragmentation and degradation (Thiollay 1992, Stouffer and Bierregaard Jr. 1995, Canaday 1997, Stratford and Stouffer 1999, Sekercioglu et al. 2002). This guild of birds could be responding to either 1) a decrease in their invertebrate prey base due to the prey's insensitivity to habitat conditions or 2) a physiological intolerance to the habitat conditions. There is evidence supporting both of these hypotheses. For example, terrestrial insectivores follow their prey to a greater degree than other guilds, with their abundance correlating well ($R^2=0.57$) with prey abundance (Karr and Brawn 1990). However, species in many guilds avoid xeric areas, supporting the physiological constraints hypothesis (Karr and Freemark 1983).

In this study, we investigated the effects of a rainfall gradient on the physiology of the song wren (*Cyphorhinus phaeocephalus*), a terrestrial, understory insectivore of lowland tropical forests (Ridgely and Gwynne Jr. 1989). By looking for signs of physiological stress in the song wrens at the dry end of a rainfall gradient, we explore the role of physiological constraint hypothesis in helping define their range border. We conducted our work across the isthmus of Panama alongside the Panama Canal. A strong rainfall gradient occurs across the isthmus of Panama, with Pacific coast average annual rainfall being 1700-1800mm and Caribbean coast average annual rainfall being 3100-3200mm (Condit et al. 2001, Pyke et al. 2001, Robinson et al. 2004).

Approximately 90% of the annual rain falls between May and November (Windsor 1990). In the Canal corridor of Panama, the song wren is widespread on the wet, Caribbean side of the continental divide, but is not found to the south on the dry, Pacific side (Ridgely and Gwynne Jr. 1989) Thus, a prominent geographic range edge passes through the isthmus and is coincident with the occurrence of the rainfall gradient. A potential limit to the wrens' distribution, therefore, is the change in moisture and humidity along the rainfall gradient. Past research on this species in the Panama Canal area indicates that dry microhabitats affect both the local distribution and behavior of the song wren (Morton 1978, Karr and Freemark 1983).

We measured hematocrit, body condition, and the hormone corticosterone (CORT) – a systemic marker for physiological demands related to energy. CORT and other glucocorticoids are released in response to a diversity of stressors and other events that increase energetic demand, such as inclement weather, food or water shortages, parasitism, and psychological challenges (Cain and Lien 1985, Wingfield et al. 1998, Sapolsky et al. 2000). CORT levels can also be elevated if an animal is living in sub-optimal habitat (Wasser et al. 1997, Marra and Holberton 1998, Suorsa et al. 2003).

In the short term, glucocorticoids alter physiology and behavior in a way that helps the animal survive acute challenges (Astheimer et al. 1995, Wingfield et al. 1998,

Wingfield and Ramenofsky 1999, Sapolsky et al. 2000). However, chronically high levels of glucocorticoids are non-adaptive and can be lethal, for example causing muscle wasting, neuronal cell death and inhibited reproductive capabilities (Wingfield et al. 1998, Sapolsky et al. 2000, Korte et al. 2005). A couple studies in wild animals have found that high baseline CORT levels are negatively correlated with survival (Romero and Wikelski 2001, Brown et al. 2005).

We are aware of one study that sampled CORT in a species throughout its range (Dunlap and Wingfield 1995). The western fence lizard (*Sceloporus occidentalis*) lives throughout most of the western USA, but its southern distribution seems to be limited by temperate and humidity. Dunlap and Wingfield (1995) found that stress-induced CORT levels were highest in the populations at the range edge and at hotter, drier times of year. However, the effect of season on maximum CORT levels was due in part to reproductive stage at sampling time and the effect of range was due in part to inherent differences among the populations, as tested by exposure to common garden conditions (Dunlap and Wingfield 1995).

We tested whether baseline and maximum (stress-induced) CORT levels in the song wren varied with the rainfall gradient across the isthmus of Panama. Because baseline CORT levels are elevated and maximum CORT levels lowered in birds with chronically higher energy demand, we predicted that song wrens living closer to the dry range edge would have elevated baseline CORT levels and lowered maximum CORT levels compared with song wrens living away from the range edge (in wetter habitats). We also tested if the body condition and hematocrit levels of the wrens would vary with the rainfall gradient, predicting that birds living closer to the dry range edge would have lower body condition and lower hematocrit due to either living in suboptimal habitat or responding to elevated levels of CORT. We did not expect our technique for capturing birds (conspecific playback) and the behavior it elicited to affect CORT levels. The absence of physiological differences in birds along the environmental gradient could

indicate that either the rainfall gradient did not affect the physiological traits we chose to measure or individuals in this species were adapted to their local environment.

Methods

The song wren is a non-migratory, understory insectivore of lowland tropical forests from Honduras to western Ecuador (Ridgely and Gwynne Jr. 1989). The species forages on the forest floor, eating arthropods in the leaf litter (Skutch 1940, Stiles et al. 1989, Robinson et al. 2000). In Panama, the song wren's reproductive season coincides with the locale's rainy season, starting in June and ending in December (Robinson et al. 2000). Pairs are socially monogamous and maintain year-round, all-purpose territories. Young birds stay with their parents for about 8 months, and family groups roost together in dormitory nests and forage together throughout the day (Robinson et al. 2000).

We sampled 59 song wrens in lowland moist forest north of the continental divide along the Panama Canal (Republic of Panama, 9°N 79°W) in Camino de Cruces and Soberania National Parks, the Fort Sherman area, and forest close to Achote Road. All samples were taken during the rainy season in August and September 2002. The song wren's breeding season occurred at this time, and we did sample actively breeding birds (Robinson et al. 2000). Birds were located using playback with a Sony tape player (WM-FS473) and Radio Shack speakers (Mini-amplifier speaker, catalogue number 277-1008C). Once we located a bird or family group, we set up a mist-net close to the responding bird(s) and used playback to draw the bird(s) into the net. Our playback recording included many of the different song types of the song wren and was recorded from many individuals. We recorded the total time that each bird was exposed to playback before capture.

Upon capture, each individual was subjected to standardized capture, handling, restraint, and sampling, termed a "stress series" (Wingfield et al. 1992). Within 3

minutes of capture, we took a small sample (40 μ l) of blood from the alar vein of the wing and used this sample to measure baseline CORT levels (Wingfield et al. 1982). Additional 40 μ l samples were taken from each bird at 5, 10, 30 and 60 minutes after capture. These additional samples were used to measure maximum CORT levels. Between each sampling time, the individual was placed in a cloth bag. After blood sampling, we collected data on mass and tarsus, wing, and tail length. Mass, tarsus length and wing length were measured to the nearest 0.1 mm or g and tail length to the nearest 1 mm. Blood samples were stored on ice until the end of the day, when they were centrifuged and the plasma collected and stored at a maximum of -20°C . Samples were transported to the University of Washington on dry ice, and hormone levels measured using the radioimmunoassay technique described in Wingfield *et al.* 1992. Inter-assay variation was 13.28% and intra-assay variation in the hands of DSB is 8.5%.

We defined body condition as the residuals from a regression of log bird mass against principal component 1 of an analysis with tarsus, wing, and tail length. We used data from 144 song wrens for the analysis. The first principal component (PC1) of the principal components analysis model explained 53% of the variation in the data set. The loading of PC1 was: tarsus = 0.49, wing = 0.66, tail = 0.57. We regressed PC1 for each bird against its mass ($r^2 = 0.27$, $p = 0.0001$), and used the residuals as the body condition index. Although this technique of using residuals of an ordinary least squares regression to calculate body condition has been criticized in the literature, recent analyses find it more robust than other techniques, especially since inter-individual size variation in this species is low (Green 2001, Schulte-Hostedde et al. 2005).

To estimate annual rainfall at the sites where we caught birds, we estimated the distance of each capture site to the continental divide. Using data collected by the Panama Canal Authority at multiple sites along the isthmus, we calculated the approximate annual rainfall at each site of capture using a linear regression of distance from the continental divide and average annual rainfall (Panama Canal Authority, Meteorology and

Hydrology Branch, Republic of Panama). Our estimations were verified by comparison with data from Robinson *et al.* (2004).

We used PATH analysis to explore the relationships among rainfall at the locales where we sampled birds, body condition, the amount of time the birds were exposed to playback prior to capture, and baseline or maximum CORT levels. For both baseline and maximum CORT, we first built a model with all possible interactions among our four variables and then took away relationships between variables until we found the simplest and most significant model to describe the data. Error variance terms were included for each dependent variable, though these terms are not shown in the presented model figures. A model was accepted if it was significantly different than the independence model, most regressions between data sets were significant, the Tucker-Lewis Index (TLI) was above 0.95, and the root mean square error of approximation (RMSEA) was below 0.06 (Hu and Bentler 1999, ITS University of Texas at Austin 2002). Normality in the data sets was tested with a Shapiro-Wilk W test and, if necessary, log transformed to achieve normality. T-tests assuming unequal variance were used to test if males and female have different baseline or maximum CORT levels.

We used a linear regression to explore the relationship between hematocrit score (% packed red-blood cell volume) and either body condition score or position along the rainfall gradient. The hematocrit score of birds is quite variable among species (ranging from 30-70 in healthy individuals of different species), thus making it difficult to define anemia in species that are not well studied. In order to define a low hematocrit state, potentially indicating anemia, we took the mean ($x=46$) and standard deviation ($SD=4$) of the hematocrit scores for all of our samples. We then subtracted the standard deviation from the mean ($x=42$), and categorized if each bird was above or below this value. Those below the value could potentially be anemic. We used a non-parametric Wilcoxon test to determine if birds with an abnormally low hematocrit experience different rainfall regimes than those birds with more normal hematocrit scores.

Analyses were conducted using JMP 6.0 or AMOS (ITS University of Texas at Austin 2002, SAS Institute 2005).

Results

Baseline or maximum CORT levels did not differ between males and females (Figure 3.1, baseline: $t_{19}=1.54$, $p=0.14$, power 0.36; maximum: $t_{18}=-2.01$, $p=0.06$, power=0.59), so the data from males and females was pooled for the PATH analyses. We recognize that the power for these tests was low, and our assumption of equality between sexes could be suspect.

For baseline CORT, the best-fit model includes the following relationships, with the standardized regression coefficient listed in parentheses: rainfall and body condition (0.41), body condition and exposure to conspecific playback (-0.33), exposure to conspecific playback and baseline CORT (0.34), and rainfall and baseline CORT (-0.18). All correlations were significant except for that between rainfall and baseline CORT. The relationship between rainfall and body condition was the strongest, though not much more so than the other significant relationships. The best-fit model explained a small amount of the variation in each data set: 17% for body condition and baseline CORT and 11% for exposure to conspecific playback. The model's fit was $\chi^2=1.1$, $df=2$, $p=0.57$, $TLI=1.27$, $RMSEA=0.00$. This model and the rejected models are presented in figure 3.2 and the regressions of the relationships in the best-fit model are presented in figure 3.3. Notably, neither of the models in which baseline CORT acted as an independent variable was chosen as the best model.

For maximum CORT, the chosen best-fit model included the following relationships, with the standardized regression coefficient listed in parentheses: rainfall and body condition (0.41), body condition to exposure and conspecific playback (-0.34), and exposure to conspecific playback and maximum CORT (-0.17). The regression of

exposure to conspecific playback with maximum CORT level was not statistically significant. The significant relationships in the model explained a small amount of the variation in each data set: 17% for body condition and 12% for exposure to conspecific playback. The fit of this best model was: $\chi^2=2.5$, $df=3$, $p=0.47$, $TLI=1.2$, $RMSEA=0.00$. It is presented along with the rejected models in figure 3.4, and the regressions of the relationships in this model are presented in figure 3.2.

Hematocrit increased slightly as rainfall increased across the gradient (Figure 3.5, $R^2=0.10$, $F_{55}=5.81$, $p=0.02$) and with body condition ($R^2=0.10$, $F_{44}=4.56$, $p=0.04$). Birds with abnormally low hematocrit score ($N=7$) were significantly more likely to live at the dry end of the rainfall gradient than birds with a normal hematocrit score (Figure 3.5, Wilcoxon test: $Z=-2.16$, $p=0.03$, $mean\pm SD$ rainfall for normal hematocrit= 2433 ± 390 mm and for below normal hematocrit= 2088 ± 106).

Discussion

We predicted that song wrens living at the drier end of a rainfall gradient would have higher baseline CORT levels and lower maximum CORT levels, body condition, and hematocrit. We did not expect that exposure to conspecific playback would affect hormones levels. In support of our predictions, we found that birds living in drier habitat had lower body condition and were more likely to have an abnormally low hematocrit score. The low hematocrit score could indicate a higher rate of anemia at the dry edge of the species' range. Although we are uncertain of the cause of this anemia, it could be due to low resources. We found significant relationships between physiological measures (body condition and CORT levels), behavior, and rainfall, although the strength of these relationships was limited. Given that we conducted this study during the wet season, we expect there to be less variation in abiotic conditions and food resources than during the dry season. Thus, the fact that we observed the predicted patterns between environment and physiology is interesting, and indicates a link between an environmental gradient and physiological traits.

Our results may have been confounded by applying linear models to non-linear relationships. In other studies of range edges, researchers have found that conditions are suitable for some distance away from the edge and then decline rapidly near the edge (Fagan et al. 2003). However, given the linear nature of the decline in rainfall from the Caribbean Sea to our final sampling point near the isthmus' continental divide, we believe that our assumption of linear response was valid (Robinson et al. 2004).

Rainfall and body condition and hematocrit

In the models for baseline and maximum CORT, rainfall was correlated with body condition. Direct effects of humidity on body function could have caused this relationship, but the mechanisms for such an effect are not apparent. The effect of rainfall on body condition could also be mediated by prey biomass (Strong and Sherry 2000).

Four separate studies on leaf-litter arthropod abundance in the Panama Canal region have shown that abundance is low in the dry season and higher in the wet season (Willis 1976, Gradwohl and Greenberg 1982, Levings and Windsor 1982, Robinson 2000). Currently, we have limited information about how leaf litter arthropod abundance varies across the rainfall gradient within a given season. A sweep net study conducted in Costa Rica suggests that arthropod abundance in the dry season is higher in wetter areas (Janzen and Schoener 1968). Soil and leaf-litter humidity can have a large effect on the survivorship of the arthropod community living within it: litter arthropods are at risk of desiccation and a small reduction in average relative humidity can cause death (review in Levings and Windsor 1982). Thus, the following two hypotheses are reasonable: 1) arthropod abundance decreases with the decline in rainfall across the isthmus and 2) birds near the drier range edge are food limited.

We found a significant, though very weak, positive relationship between hematocrit and annual rainfall. We would expect the opposite result were the birds at the dry side of the gradient suffering from dehydration. When we sampled the birds in the middle of the rainy season, it was unlikely that birds at any locale were moisture limited. A difference in hematocrit due to the effects of water restriction in the dry season was also unlikely because prior experience with water-restriction and arid environments have no effect on hematocrit in quail and fence lizard, respectively (Dunlap 1995, Goldstein 1995).

As predicted, the lowest hematocrit scores occur at the dry end of the rainfall gradient, closer to the southern range edge. We recognize that the greater number of low hematocrit scores may be due to the greater number of data points at the dry end of the gradient. However, the birds at the dry edge of the range could also have a higher incidence of resource- or condition-induced anemia. Supporting this hypothesis, we found that birds living in drier habitat were significantly more likely to have an abnormally low hematocrit score.

Body condition and time to capture

The better a song wren's body condition, the quicker we captured it. This relationship makes intuitive sense in that a bird with greater internal stores of energy will have more energy available to spend on territory defense. The more actively a bird interacted with playback, the more likely it was to fly into the net/s we set up to catch the focal bird/s. Furthermore, a bird in good condition is more likely to be willing to engage in a physical fight with a challenging intruder. Thus, avid territory defenders were more likely to fly within close proximity of the playback and into the net/s set above the speaker.

Although the relationship between body condition and aggression seems intuitive, there is very little data in the literature to validate our hypothesis. White-crowned sparrows (*Zonotrichia leucophrys*) compromised by an immune challenge that can cause a

decline in body condition have reduced response to simulated territorial intrusion (Owen-Ashley et al. 2006). Dominant individual/s in communally-living species are often in the best condition, especially at the time at which they fight to achieve their new social status (Sapolsky 1987, Renison et al. 2002). Although neither of these examples provides direct evidence for a positive relationship between body condition and aggression in a non-communal wild species, the rationale behind this relationship is apparent.

Factors affecting corticosterone

The effect of exposure to conspecific playback on CORT levels in birds is mixed (Wingfield 1985, Sorenson et al. 1997, Wingfield et al. 1997b, Romero et al. 1998a, Silverin 1998, Astheimer et al. 2000, Canoine 2001, Canoine and Gwinner 2005). We found that the longer song wrens were exposed to conspecific playback, the higher their baseline CORT levels.

Three hypotheses could explain the positive relationship between baseline CORT and exposure to conspecific playback. First, the exposure to conspecific playback could increase CORT levels. For many birds that hold territories just to breed, territory loss may result in reproductive failure for one breeding season. Some species increase androgens as they engage in aggressive behaviors towards a challenging intruder, though this relationship between territory defense and androgens has little support in this and other resident tropical birds (Wingfield et al. 1990, Wingfield et al. 1991, Levin and Wingfield 1992, Wikelski et al. 1999, Hau et al. 2004, Moore et al. 2004, Fedy and Stutchbury 2006). The song wren holds its territories throughout the year and the territory provides all of the resources the bird needs to survive (food, water, dormitory and breeding nests, knowledge of safe foraging locations). The loss of such a territory in this species could result in both reproductive failure and reduced survivorship due to an inability of the bird to find appropriate places to eat and sleep. Because the negative impact of territory loss to song wren survival might be greater than in other species,

song wrens may see social challenge as a “stressful” event to which they increase baseline CORT levels.

Second, the primary action of glucocorticoids is energy regulation (Dallman et al. 1993, Norris 1997). If song wrens engaging in territory defense were more active than normal, they might have had higher CORT levels in order to mobilize usable energy.

Third, the exposure to playback could have no effect on hormone levels. Birds with higher baseline CORT levels may have been less aggressive, less active, and, thus, less likely to be captured by our net. Prior research has shown that birds implanted with CORT are less aggressive (Wingfield and Silverin 1986), It was possible that the relationship between CORT and exposure to playback was indirect. Namely, it could have been a reflection of the fact that birds with low body condition were not readily captured.

Elevated baseline CORT levels are typical in birds that experience chronic stress (Wingfield et al. 1997b). Given the assumption that birds living at the dry edge of the species’ distribution would be living in more marginal habitat and the probability that more birds there would experience a chronically stressful environment, we predicted that rainfall would have a direct, negative effect on baseline CORT levels. This relationship was not significant in our PATH model. However, where rainfall was lower, we found more birds with higher baseline CORT (Figure 3.3d). This pattern supports our initial hypothesis that birds at the dry edge of their range were more likely to show signs of stress. The data also indicated that some individuals are not affected by marginal habitat. That the relationship was not statistically significance does not indicate that this pattern between rainfall and baseline CORT was not biologically relevant.

Because our data were purely correlative, we cannot be certain about why baseline CORT levels were variable. The increased variation at the dry end of the isthmus could be because we collected more samples from that area and, thus, the probability of observing outliers was higher. Also, the forest in which we sampled birds could have been more disturbed on the drier side of the distribution, leading to increases in baseline CORT due to habitat variables other than rainfall. A number of times, we caught more than one member of a family group. We found that baseline CORT levels in one family member can be markedly different than that of his/her mate or young. This finding leads us to believe that the elevations in baseline CORT seen at the dry end of the rainfall gradient were not due to poor overall territory quality alone. Finally, variation in the abundance of breeding versus non-breeding birds across the isthmus might have affected the CORT levels (Wingfield and Sapolsky 2003). However, because we did not detect a difference in baseline or maximum CORT levels between females with and without brood patches (unpublished data), we doubt the validity of this argument.

No factor in this study was significantly correlated with maximum CORT levels, as induced by capture-and-restraint. Thus, position on the rainfall gradient, body condition, and exposure to conspecific playback were not correlated with the sensitivity of the hypothalamo-pituitary-adrenal cortex axis to stress. In some scenarios, prior stress causes either a heightened response to subsequent stressors or a down-regulation of the HPA axis as a whole (Wingfield and Romero 2001, Romero 2004, Rich and Romero 2005). Neither of these phenomena occurred in a significant manner with the variables that we measured.

Conclusions

Our data provide modest support for the hypothesis that physiological measures, such as CORT, body condition, and hematocrit, can indicate the sub-optimal conditions that occur at a geographical range edge. We found that body condition and hematocrit were lower in song wrens at the dry edge of their range. CORT levels were not directly

affected by the rainfall gradient, but we did find more individuals with higher baseline CORT levels closer to the dry range edge. If moisture and humidity do affect song wren condition and range, the distribution of the species might change with the current drying of the isthmus of Panama (Condit 1998).

We encourage other researchers studying endocrine, ecological, or behavioral patterns in nature to take a more integrative approach to their question at hand. In this study, we found that an environmental variable correlated with body condition, which correlated with behavior, which correlated with hormone levels. Had we not considered these different types of variables, we would have had little insight into factors affecting the distribution of the song wren.

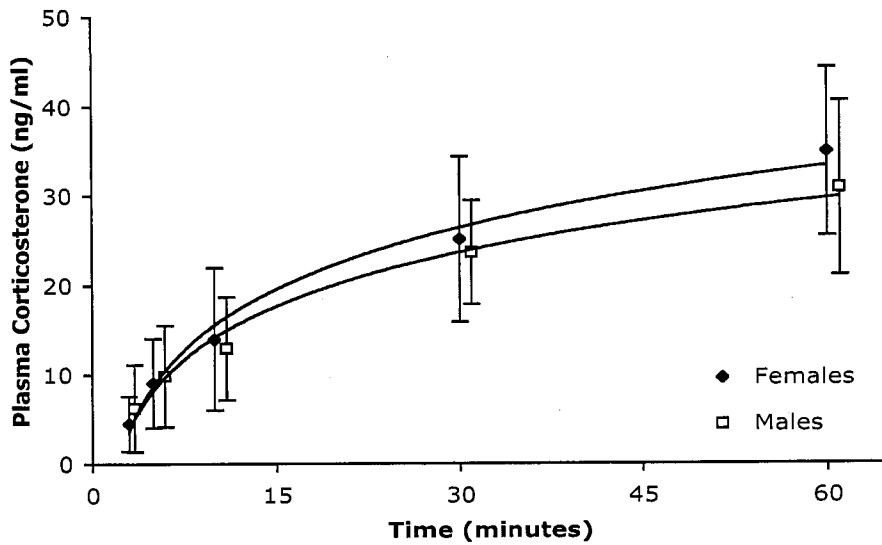
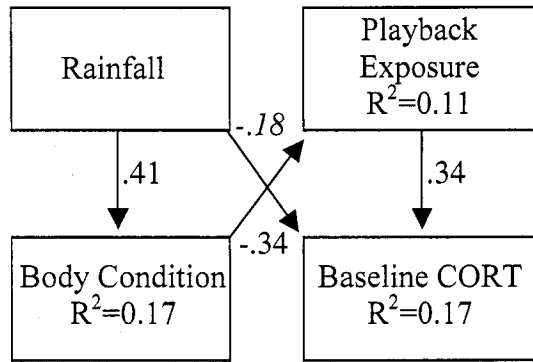
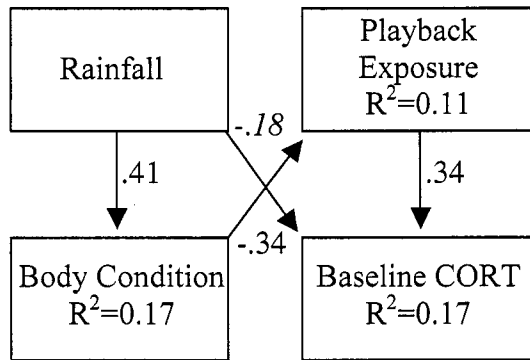


Figure 3.1. Plasma CORT levels in male and female song wrens in response to capture and restraint. The data are presented as means \pm standard deviation, with females denoted by solid diamonds and males by empty squares. Though samples were taken at the same time for the two sexes, the data markers are off-set for clarity. Males and females have similar baseline or maximum CORT levels.

a) $\chi^2=1.1$, $df=1$, $p=0.29$, $TLI=0.92$, $RMSEA=0.05$



b) $\chi^2=1.1$, $df=2$, $p=0.57$, $TLI=1.27$, $RMSEA=0.00$



c) $\chi^2=2.9$, $df=3$, $p=0.40$, $TLI=1.0$, $RMSEA=0.00$

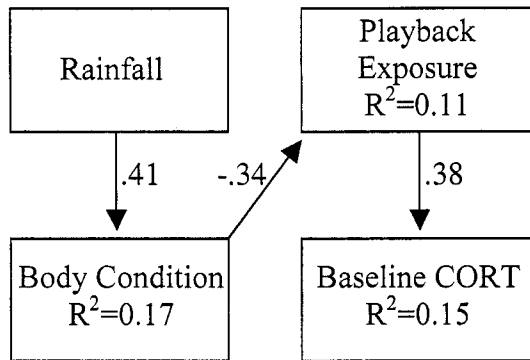
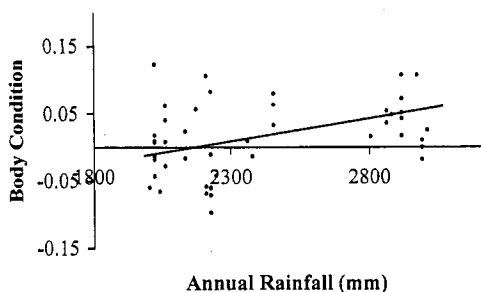
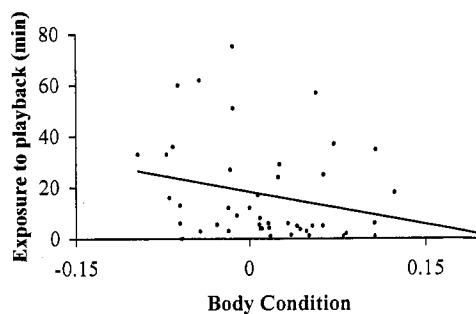


Figure 3.2. PATH models showing relationships between average annual rainfall at location of capture, time of exposure to conspecific playback, body condition, and baseline CORT levels of male and female song wrens. The data above each model indicate its fit. The text adjacent to each arrow is its standardized regression coefficient. Significant regressions ($p \leq 0.05$) are shown in plain text and non-significant regressions ($p > 0.05$) are in italics. The R^2 values in each box indicate the amount of variation in the dataset explained by both direct and indirect relationships within the model. Model *b* was chosen as the best model for the data.

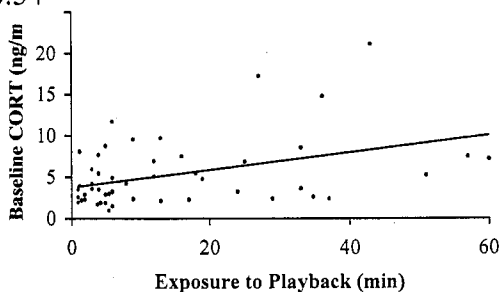
a) 0.41



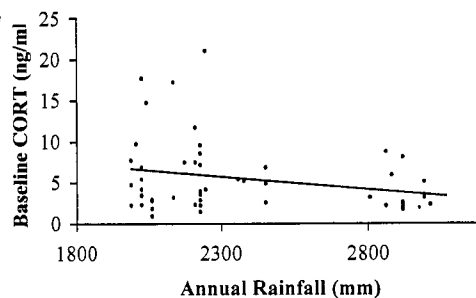
b) -0.33



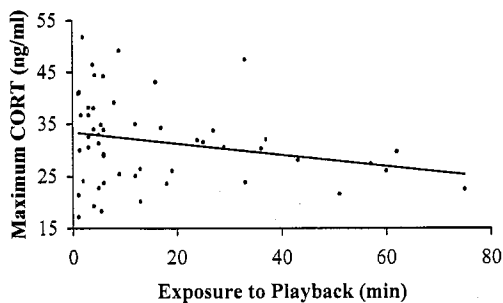
c) 0.34



d)



e)



f)

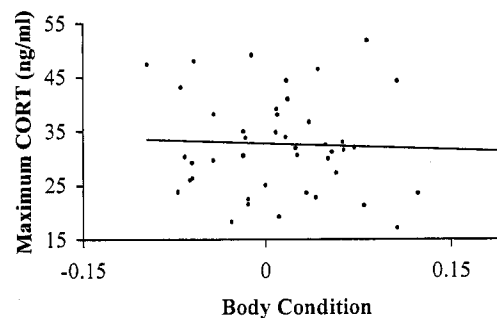
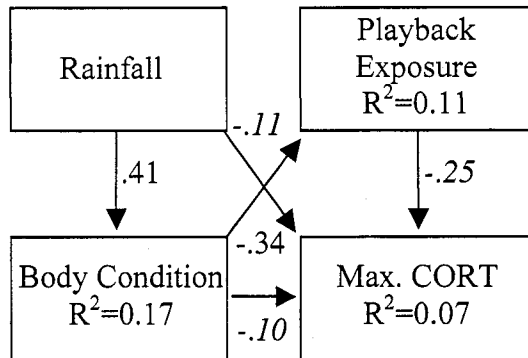
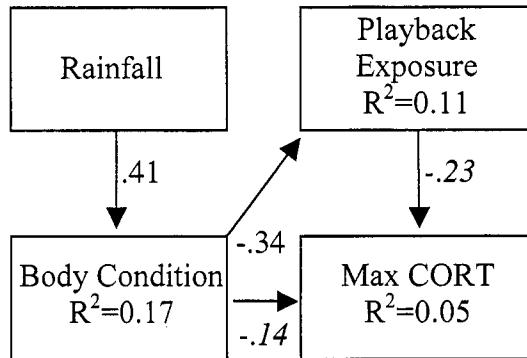


Figure 3.3. Linear regression relationships between rainfall, body condition, time of exposure to playback, and baseline or maximum CORT. Graphs *a* and *b* are included in the PATH analysis for both baseline and maximum CORT. Graph *c* is specific to the baseline CORT PATH analysis and figures *d* and *e* to the maximum CORT PATH analysis. Graph *f* was not included in the PATH analysis but is discussed in the text. The standardized regression coefficient for significant relationships from the chosen PATH models is given above each figure. Analyses were performed with log-transformed data, but untransformed data are depicted here.

a) $\chi^2=1.1$, $df=1$, $p=0.29$, $TLI=0.85$, $RMSEA=0.05$



b) $\chi^2=1.7$, $df=2$, $p=0.44$, $TLI=1.19$, $RMSEA=0.00$



c) $\chi^2=2.5$, $df=3$, $p=0.47$, $TLI=1.18$, $RMSEA=0.00$

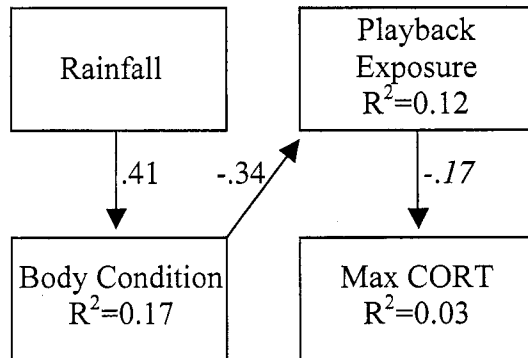


Figure 3.4. PATH models showing relationships between average annual rainfall at location of capture, time of exposure to conspecific playback, and body condition and maximum CORT levels of male and female song wrens. The data above each model indicate its fit. The text adjacent to each arrow is its standardized regression coefficient. Significant regressions ($p \leq 0.05$) are shown in plain text and non-significant regressions ($p > 0.05$) are in italics. The R^2 values in each box indicate the amount of variation in the dataset explained by both direct and indirect relationships within the model. Model c was chosen as the best model for the data.

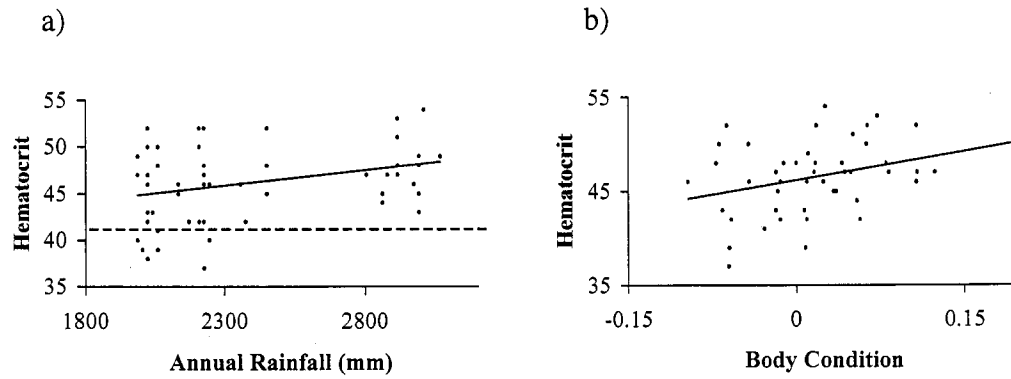


Figure 3.5. Linear regressions with hematocrit and annual rainfall ($R^2=0.1$, $p=0.02$) or body condition ($R^2=0.1$, $p=0.04$). The dotted line in *a* indicates the threshold between low and normal hematocrit.

Chapter 4

Effects of repeated, acute corticosterone administration on the hypothalamo-pituitary-adrenal axis in the white-crowned sparrow

(Zonotrichia leucophrys gambelii)

Introduction

Disturbance is a natural part of life for any wild animal. In response to most unexpected, challenging events, vertebrates release glucocorticoid hormones (Harvey et al. 1984, Wingfield 1994, Sapolsky et al. 2000, Wingfield and Romero 2001). For example, exposure to predators, unfavorable weather conditions, humans, pollution, food restriction, and habitat variation all stimulate the hypothalamo-pituitary-adrenal (HPA) axis (Smith et al. 1994, Fowler et al. 1995, Marra and Holberton 1998, Wingfield et al. 1998, Scheuerlein et al. 2001, Lynn et al. 2003, Suorsa et al. 2003, Walker et al. 2006). In the short term, this elevation of glucocorticoids promotes activities and behaviors that enhance survival, but in the long-term elevated glucocorticoids can be harmful and potentially lethal (Harvey et al. 1984, Wingfield et al. 1998, Sapolsky et al. 2000, Greenberg et al. 2002). As researchers explore the effects of anthropogenic disturbance on natural populations, they are increasingly turning to the glucocorticoid hormones as a way to measure the response of animals to the energetic demand of their environment (Wasser et al. 1997, Creel et al. 2002, Müllner et al. 2004, Walker et al. 2005b). In this paper, we study the effects of frequent, acute administration of a glucocorticoid hormone on the HPA axis of captive, wild birds.

Biomedical studies compose the vast majority of what we know about glucocorticoids and stress in general (Romero 2004). The contribution of these works to our knowledge base is enormous and extremely valuable. However, as ecologically-oriented research begins to incorporate the techniques and concepts of stress physiology, laboratory

studies more relevant to ecological questions are needed to interpret and elucidate findings from the field.

To manipulate glucocorticoids levels in vertebrates, the vast majority of studies use chronic subcutaneous implants of hormone (Gray et al. 1990, Astheimer et al. 1992, but see Breuner et al. 1998, Hiebert et al. 2000, Romero et al. 2005). These implants elevate glucocorticoids levels to constantly high levels for a series of days. They override the hypothalamo-pituitary-adrenal (HPA) axis' control of endogenous glucocorticoid levels and, thus, do not allow for any of the modulation that naturally occurs on a daily cycle (Dallman et al. 1993, Breuner et al. 1999). Such a hormone dosing technique is appropriate for modeling a prolonged, severe stressful event, such as a multi-day snowstorm. However, the majority of stressful events in the wild are probably not as severe nor as prolonged, thus rendering the implant-induced chronic stress model unsuitable to many research questions.

A more ecologically-relevant model for a "stressful" environment is one in which stressful events are brief but more frequent. For example, a wild animal living in non-optimal habitat could encounter predators, climatic extremes, food shortages, and/or anthropogenic disturbance more frequently than they are accustomed to. Some of these encounters might cause a small rise in glucocorticoid levels. In a laboratory setting, one way to model the type of environment in which short stressors are frequent is to dose animals with small pulses of glucocorticoids.

Another way of modeling chronic stress in captive animals is to use frequent periods of disturbance or noxious stimuli that elicit anxiety, the emergency response, and/or elevate glucocorticoid levels (Sapolsky et al. 2000, Dallman and Bhatnagar 2001, Retana-Marquez et al. 2003b, Rich and Romero 2005). These models are valuable in that they incorporate the vast majority of the psychological and physiological components of the stress response. However, because so many variables change within

the body of the study subjects in response to the treatment(s), the precise role for each component of the stress response is difficult to pinpoint. In addition, standardizing the glucocorticoid levels that the animals are exposed to over time is difficult; animals can change their response to stressors with the time of day or chronic exposure and animals have different HPA axis responses to different stressors (Dobráková et al. 1993, Canoine et al. 2002, Retana-Marquez et al. 2003b, Landys et al. 2004b, Rich and Romero 2005). By isolating the stress hormone corticosterone (CORT) in our work, we can more accurately define the role of this hormone in the development of the chronic stress phenotype. Because this hormone and the closely related hormone cortisol are the most studied components of the stress axis in wild animals, understanding the effects of the glucocorticoid hormones is relevant to interpret the findings and guide the design of field studies.

We characterized how various aspects of the HPA axis of a captive sparrow in breeding condition responded to repeated, short pulses of CORT. We sampled the birds a number of times during the experiments to measure endogenous baseline levels of CORT. Elevated baseline CORT is a classic indicator of chronic stress (Fleshner et al. 1995, Wingfield et al. 1998, Sapolsky et al. 2000, Romero 2004). We predicted that our CORT-treated birds would indeed have elevated baseline CORT levels, indicating that our treatments created a chronic stress phenotype.

We also collected samples for CORT after stimulating the HPA axis by either capture-and-restraint or injection of adrenocorticotropin (ACTH), the hormone that induces the synthesis and release of glucocorticoids from the adrenal gland (Wingfield et al. 1992, Norris 1997). Knowledge of the functional capabilities of the HPA axis is an important component of understanding a chronic stress phenotype. For example, in some scenarios of chronic disturbance, vertebrates will down-regulate the stress response, potentially to avoid the costs of high glucocorticoids levels (Harlow et al. 1992, Rich and Romero 2005, Walker et al. 2006). In other scenarios, just the opposite occurs:

exposure to chronically stressful stimuli causes increased glucocorticoid levels (facilitation) or changes the duration of the hormone elevation (disrupted negative feedback) (Boonstra et al. 1998, Dallman and Bhatnagar 2001, Romero 2004). We predicted down-regulation of the HPA axis response to stress in our CORT-treated birds. This down-regulation would result in a lower CORT response to acute stress and ACTH challenge (Rich and Romero 2005).

CORT is just one part of the HPA axis, so we measured a number of other variables to better characterize the reaction of the sparrows to CORT treatments. CORT treatment could affect hepatic CORT metabolism (Jamieson et al. 1999). In this study, we provide some of the first data in birds exploring whether hepatic CORT metabolism is modulated with CORT-treatment. We predicted that CORT-treated birds would show increased hepatic CORT clearance.

Mammals and birds experiencing chronic stress often respond with adrenal hypertrophy (see review in Harvey et al. 1984, Rogers et al. 1993b, a, Boonstra et al. 1998, Retana-Marquez et al. 2003b). We measured the size of the adrenal gland in CORT and control-treated birds, and expected that we would observe adrenal hypertrophy in the CORT-treated individuals.

Corticosterone binding globulin (CBG) is the primary transport molecule for CORT in the blood and CORT bound to CBG is not metabolized. The action of CBG-bound CORT is currently under debate. The free-hormone hypothesis postulates that free, not protein-bound, hormone concentrations affect, but are not the only driver of, intracellular hormone concentrations (Mendel 1989). Recently, Hammes et al. (2005) found that hormone-binding globulin complexes can bind to a membrane receptor which brings the complex into the cell and that binding protein-steroid complexes have a functional role in the body. Thus, it is probable that CBG-CORT complexes have a biological activity. Past studies have shown that high glucocorticoid levels due to

exposure to stress lowers CBG levels (Fleshner et al. 1995, Boonstra et al. 1998, Deak et al. 1999, Lynn et al. 2003). We measured CBG levels in study subjects and predicted that repeated, acute pulses of CORT would also cause CBG level in breeding sparrows to drop.

Methods

Our study subject was the Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelli*), a small migratory song bird that breeds in the boreal zone and winters in the southwest United States (Cortopassi and Mewaldt 1965, Chilton et al. 1995a). We captured these birds during their autumnal migration through Sunnyside Game Reserve in Sunnyside, Washington (46.1°N, 119.5°W). Sparrows were housed in outdoor aviaries at the University of Washington (Seattle, WA) until use in our experiments.

Pre to early breeding season experiment

In January 2005, we brought 36 adult male and 3 adult female *Z. leucophrys* from outdoor aviaries into two environmental chambers with a light schedule of 10L:14D. Each bird was housed in its own cage. The birds were acclimated to cages for two weeks and were handled twice a day (approximately 1 min/handling) to acclimate them to capture and handling (see Figure 4.1 for chronology of experiment). After this two-week period, we took blood samples using a "capture-and-restraint" protocol. Briefly, blood was taken from all birds within 3 minutes of entering the environmental chamber and used to measure baseline CORT levels (Wingfield et al. 1982). Additional samples, taken at 10, 30, and 60 minutes, were used to measure CORT (Wingfield et al 1992) and a sample taken at 10 min was used to measure CBG.

The following day, we began our treatments. Our dosage technique and validation studies are presented in Boyd et al. (in preparation). Briefly, we used DMSO as the vehicle for CORT dosage since DMSO readily dissolves this steroid and draws the steroid into the body if the DMSO-CORT solution is placed on the skin (Williams and

Barry 2004). DMSO is a safe chemical that is frequently used in veterinary and human medicine, and is often used to give medicines to horses. At each treatment time, every animal was handled and dosed topically with either 20 μ g CORT in 20 μ l DMSO or a control of 20 μ l DMSO. This dose raised plasma CORT levels to approximately 12.6ng/ml, a level that was above baseline but well below maximal CORT levels (Boyd et al. in preparation). All doses were placed on the back of the neck at the featherless tract above the jugular vein. Fifteen birds that were housed together in one chamber were dosed once a day (1X), with 8 birds receiving CORT doses and 7 birds acting as DMSO controls. The remaining 24 birds in the second chamber were dosed three times a day (3X), with 12 birds receiving CORT doses and 12 birds acting as DMSO controls. To prevent our study subjects from predicting the time of treatments and to more adequately simulate the random nature of environmental stressors, the dosing times were haphazardly selected throughout the day. There was no regular interval between doses, and the shortest time period between doses was 1.5 hours.

We treated the birds before the stimulation of the breeding life stage to study if repeated, acute CORT administration could alter the development of this life stage. After four days of dosing birds in a short-day, winter life stage, we changed the light regime to 19L:5D to stimulate the birds to transition into the breeding life-history stage. During this fifth day, the birds were not dosed. We repeated this five-day cycle (4 days treatment, 1 day no treatment) three times. On the 15th or 16th days, we took blood samples using the same capture-and-restraint sampling technique discussed above. On the 16th day, 6 CORT-treated and 6 control birds from the 3X group were sacrificed. The adrenal gland in these birds was measured *in situ*.

The remaining birds were then treated with three more 5-day cycles of dosing. A capture-and-restraint blood sample was taken from all birds on the 31st or 32nd days. On the day following the blood sampling, each bird received a jugular injection of 2.8IU porcine ACTH (Sigma) in 20 μ l lactated Ringer's solution. This injection quantity was

approximately 100IU/kg and has been validated and used in other studies on this species (Romero and Wingfield 1998, 1999). After injection, the birds were placed in a cloth bag and blood samples collected at 30 and 80 min post injection. We did not include a saline-injection control in this study because, with the capture-and-restraint blood samples taken two days prior, each bird could act as its own control. In addition, we were primarily interested in differences in response to ACTH challenge among the treatment groups not the absolute response to ACTH challenge.

We dosed the birds for one more day before we sacrificed them. After sacrifice, the adrenal was measured *in situ*. We chose to measure the adrenal *in situ* because of the difficulty of removing the adrenal intact in song birds. Adrenal volume was calculated using the formula for an ellipsoid cylinder ($4/3\pi a^2b$, where a is half the adrenal width and b half the length). We recognize that this assumption of shape may have introduced variation in our measures and that the adrenal does not necessarily have an ellipsoidal shape. However, this shape assumption was appropriate given the outline of the exposed gland *in situ*. In addition, we assumed that any variation in size of the adrenal gland among treatment groups would be reflected with our calculations. The liver was collected from each bird at the end of the experiment for the *in vitro* work described below.

In vitro liver CORT metabolism

The techniques for determining hepatic CORT metabolism *in vitro* were modeled after work by Doell et al. (1981), Lindberg et al. (1972), and Edwards et al. (2005). Each liver was stored on ice in avian Ringer's solution until we had collected all livers. We took 50mg and 100mg aliquots from each liver and minced the tissue with scissor action of two scalpels. We centrifuged the homogenate at 1000 rpm for 5 min, aspirated the supernate, suspended the tissue in 0.5ml avian Ringer's and immediately plated it in a 16-well plate on ice. Once we prepared all samples, we placed the plates in a gently-rotating, O₂ infused, incubator at 30°C. We allowed 2 hrs for the tissue to acclimate to

the culture setting, and then added 2.65×10^6 cpm tritiated CORT in 10ul ethanol to each sample (2% EtOH final solution). We incubated the plates the CORT for 15 min, and then immediately placed them on ice to stop the metabolism of CORT. We collected the tissue slurry (duration of 30 min to complete all samples) and froze the samples at -80°C for later analysis.

The liver metabolizes CORT into two major breakdown products, deoxycorticosterone and 11-dehydrocorticosterone (Norris 1997). Using the tritium marker, we could measure how much of the tritiated CORT the liver homogenate metabolized into these two compounds. To do so, we extracted each sample with redistilled dichloromethane for 2 hrs and then with a 9:1 redistilled dichloromethane:methanol mixture for another 2 hrs. We dried the extractants and resuspended the solutes with 1ml of a 9:1 ethyl acetate:methanol mixture. After this point the samples were stored at room temperature. We fractionated the samples using thin layer chromatography. We scored 2.5cm lanes onto plastic-backed silicone coated plates and spotted spotted the samples at the origin. Deoxycorticosterone, 11-dehydrocorticosterone, and CORT standards were included on each plate. We ran the plates in two 100ml baths: first in 4:1 hexanes:ethyl acetate and second in 9:1 chloroform:methanol with 0.5ml distilled H_2O . We imaged the standards on each plate using short-wave UV light. We cut out the bands for each column and extracted them with 4ml 1:1 ethyl acetate:methanol for 30 min. We reconstituted each fraction with 0.5ml 1:1 ethyl acetate:methanol and added 4.5ml scintillation fluid to each sample. We then counted the vials with on a Beckman scintillation counter. For each birds, we expressed the quantity of tritium in each of the metabolite bands (deoxycorticosterone and 11-dehydrocorticosterone) as a fraction of the tritium in the CORT band.

Late breeding season to autumnal molt experiment

In February 2004, we brought 21 white-crowned sparrows, a mix of adults (8) and juveniles (13) and males (13) and females (8), from an outdoor aviary into

environmental chambers on a short day photoperiod (8L:16D). We housed each bird in its own cage. Twelve days later, we induced the breeding life stage by switched the photoperiod to a long day (20L:4D). For two weeks prior to the experiment, we handled the birds 3 times a day to acclimate them to capture and brief holding (<1 min/handling) (see Figure 4.2). We then randomly assigned the birds into three treatment groups. One set of birds (n=7), termed “control”, was handled 3 times a day and given a topical dose of 20 μ l DMSO on the neck. The second set of birds (n=8), termed “CORT”, was handled three times a day and given a topical dose of 20 μ g CORT dissolved in 20 μ l DMSO. These two groups were housed together in two environmental chambers. The third set of birds (n=6), termed “undisturbed”, was housed in its own chamber and was not subject to either daily handling or to topical DMSO. On the day before treatments began, we collected blood using the capture-and-restraint protocol described above. Briefly, we took blood samples at <3, 10, 30, and 60 min after we first entered the birds’ environmental chamber.

We dosed the birds three times a day (3X) for a period of four days. On the fifth day, no doses were given. We repeated this schedule three times, so that there were a total of 12 days of treatment and 3 days without treatment. Dosing times were haphazardly selected throughout the day so that there was no set time of dosing or regular interval between doses. The shortest time period between doses was 1.5 hours. On April 30, the second day after the last treatment, we collected blood using the previously described capture-and-restraint protocol. A final capture-and-restraint blood sample was collected from each bird between 2 and 12 days after the individual completed its molt.

Assays

We measured CORT levels in the plasma using a direct radioimmunoassay following the techniques described in Wingfield *et al* (1992). For each sample, we incubated a small aliquot of plasma (20 μ l for most samples) overnight with distilled water (200 μ l total volume) and 20 μ l tritiated CORT (10,000cpm/100 μ l). We extracted this mixture

with 4.5ml redistilled dichloromethane for 2 hrs, aspirated the dichloromethane, and dried the solution in a heated incubator with N₂ gas. We reconstituted each sample with 550 μ l phosphate buffered saline with gelatin (PBSG). We separated a 200 μ l aliquot of this solution to measure the amount of CORT lost in the extraction process. The average recovery values for the 3 assays in 2004 was 88.62% and for the 3 assays in 2005 was 90.86%. We used two 100 μ l aliquots of hormone-PBSG solution to perform the radioimmunoassay. We incubated each aliquot overnight with 100 μ l of tritiated CORT and 100 μ l CORT antibody (Endocrine Sciences). To separate the bound and free hormone, we incubated each sample for a maximum of 12 min with 0.5ml of a dextran-coated charcoal solution (2.5g charcoal/1L PBSG) and centrifuged it for 10 min at 2,000 rpm. We decanted the solution containing free hormone and added 4.5ml of scintillation fluid (UltimaGold, Perkin Elmer) to it before counting it on a Beckmann scintillation counter.

Each assay included blanks, samples of a standard CORT solutions, a 9 sample standard curve, and controls to measure assay performance (total binding, non-specific binding, and total radioactivity added to each tube). DSB conducted all assays. Intra-assay variation, as measured the coefficient of variation of 11 standard samples is 8.5%. Inter-assay variation, as measured by coefficient of variation of standard solution included in each assay, is 13.39%.

To measure CBG, we used a point sample analysis similar to the one described in Lynn *et al.* (2003), except that we used 3.86nM [³H] CORT for the assay. Briefly, to strip samples of native hormone, we 1) incubated the samples for 20 min at room temperature with an equal volume of dextran-coated charcoal solution (0.1% dextran, 1% Norit A charcoal in 50nM Tris) and then 2) centrifuged them for 10 min at 4500 rpm, 4°C. To perform the assay, we incubated 50 μ l of diluted, striped plasma (1:900 final dilution), 50 μ l 3.86nM [³H] CORT, and either 50 μ l 50mM Tris buffer or 50 μ l unlabeled CORT (2000pg/100 μ l) on ice for 2 hr. We separated bound and free hormone

using rapid vacuum filtration over a glass fiber filter (Whatman GF/B) that had been soaking for 1 hr prior to filtering in 25 mM Tris with 0.3% PEI. We extracted the radioactive CORT bound to the filters with 100 μ l ethanol and added 5 ml scintillation fluid (UlitmGold, Perkin Elmer) to each sample before counting them on a Beckmann liquid scintillation counter. We ran all samples in triplicate and, to avoid the complications of inter-assay variation, ran all samples from one individual on the same plate. We calculated the amount of free CORT per sample using the following equation Barsano and Baumann (1989):

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

For our calculations, we used $K_d=4.35$, the average K_d found in WCS as per Breuner and Orchinik (2002). Samples from the early breeding season underwent two freeze-thaw cycles before we assayed them for CBG and samples from the end late breeding season experiment underwent three freeze-thaw cycles before we assayed them for CBG.

Statistics

We performed statistical analyses using JMP 6.0 (SAS Institute 2005). When datasets were non-normal, we log transformed them to attain a normal distribution. If the log transformation did not achieve normality, we used non-parametric statistics. We used ANOVA or Kruskal-Wallis tests to determine if the treatment groups differed in baseline CORT, maximum CORT, CBG, or metabolite:CORT ratio at specific sampling point. We used Tukey-Kramer post-hoc tests to determine which groups significantly differed.

We used two-way repeated measures MANOVA tests for the early breeding season experiment and one-way repeated measures MANOVA tests for the late breeding season experiment to explore how the HPA axis changed over the course of the

experiment with treatment and/or treatment dosage. We tested for differences in the CORT response to capture-and-restraint among treatment group at each sampling point with a repeated-measures MANOVA. We tested for differences in plasma CORT levels among treatment groups after 30 min of just restraint or ACTH injection plus restraint with paired t-tests.

We used a t-test to explore the impact of treatment on adrenal size of the 3X groups sacrificed at the midpoint of the early breeding season experiment. We tested for the impact of both treatment and dose per day on adrenal size at the end of the same experiment with a two-way ANOVA. We used a t-test to test for differences between CORT metabolism between the sample preparation with 50 or 100mg liver tissue. We conducted Wilcoxon or t-tests to determine if baseline CORT, maximum CORT, or CBG levels varied between years for the replicated treatment groups.

Results

Early Breeding Season Experiment

Endogenous CORT levels

Baseline CORT levels increased with CORT treatment, the number of CORT doses per day, and time (Table 4.1). CORT-treated birds had a greater increase of CORT with time, and this increase was more rapid in 3X than 1X birds (Table 4.1). Baseline CORT levels were similar in all groups before the experiment began (Table 4.2, Figure 4.3). At the mid-experiment sample, baseline CORT was elevated in the 3X CORT-treated birds compared to the controls (Table 4.2, Figure 4.3). At this time, baseline CORT levels in the 1X CORT-treated birds were similar to both control groups and the 3X CORT-treated birds. At the end of the experiment, both 3X and 1X CORT-treated birds had higher baseline CORT levels than the control groups (Table 4.2, Figure 4.3).

Maximum CORT levels increased with time in all treatment groups, but were not affected by treatment, dose per day, or an interaction between dose per day and treatment

(Tables 4.1 and 4.2, Figure 4.3). However, the power for these tests was low (Table 4.2).

At the beginning of the study, all treatment groups showed a significant increase in CORT with capture-and-restraint protocol (Table 4.3, Figure 4.4). The stress response did not vary among groups in response to treatment or dose per day, but did vary with an interaction of treatment and dose per day (Table 4.1). This difference is most likely due to type II error, namely spuriously high maximum CORT levels in the 3X DMSO group.

After hormone treatment was initiated, CORT-treated birds differed from controls in their response to capture-and-restraint (Tables 4.1 and 4.3, Figure 4.4). The number of CORT doses per day also affected this response. Mid-experiment, treatment, time, an interaction between time and treatment, and an interaction between time, treatment, and dose per day affected the stress response (Table 4.1, Figure 4.4). Namely, CORT-treated birds did not change CORT levels over the 60 min sampling time, but control birds did (Table 4.3, Figure 4.4). 3X CORT-treated birds show a non-significant decline in CORT levels during the 60 min sampling time, while 1X CORT-treated birds show a non-significant increase in CORT levels. CORT-treated birds had elevated baseline CORT levels compared to the control groups (Table 4.2, Figure 4.3).

Similarly, at the end of the experiment, treatment, time, an interaction between time, and an interaction between treatment and time, treatment, and dose per day significantly affected the stress response (Table 4.1, Figure 4.4). Again, CORT-treated birds did not show the significant change in CORT levels over time that the control groups did (Table 4.3, Figure 4.4). 3X CORT-treated birds had a non-significant decline in CORT levels over the 60 min (Table 4.3, Figure 4.4). CORT levels in 1X CORT-treated birds were level (Table 4.3, Figure 4.4). CORT-treated birds had elevated baseline CORT levels compared to the control groups (Table 4.2, Figure 4.3).

ACTH challenge

Plasma CORT levels were higher 30 min after ACTH injection than 80 min after injection, and the response to ACTH challenge was similar among the different treatment and dosage groups (Table 4.1, Figure 4.5). In CORT-treated birds, plasma CORT levels were higher 30 min after the ACTH injection plus capture-and-restraint than 30 min after just capture-and-restraint (Figure 4.5, paired t-test, 3X CORT: $t=-3.55$, $p=0.02$; 1X CORT: $t=-2.54$, $p=0.04$; 3X control: $t=-1.24$, $p=0.28$; 1X control: $t=-2.03$, $p=0.09$).

CBG

CBG levels were not affected by treatment or dose per day, but they did increase slightly throughout the experiment (Table 4.1, Figure 4.3). Because we found no difference in CBG levels, we assume that changes in free CORT levels parallel changes in total CORT levels and do not present free CORT data here.

Adrenal size

At the midpoint of the experiment, adrenal size in 3X birds was similar between treatment groups (t-test: $t=-1.19$, $DF=9$, $p=0.27$). Treatment and dose per day did not affect adrenal volume at the end of the experiment (Two-way ANOVA, $F=0.68$, $DF=3,23$, $p=0.57$).

In vitro liver CORT metabolism

The amount of tissue used in the preparation did not affect the ratio of metabolites to CORT (deoxycort ratio: $t=-1.21$, $DF=48$, $p=0.23$; 11 dehydrocort ratio: $t=-1.78$, $DF=47$, $p=0.08$). For both tissue amounts, treatment and dose per day had no effect on the ratio of CORT metabolites to CORT (Table 4.1). Because the data for the 100mg tissue preparation have less variance, we chose to present those data (Figure 4.3).

*Late breeding season experiment**Endogenous CORT levels*

Endogenous baseline CORT levels fluctuated over time, but were significantly higher in CORT-treated birds after the treatment period (Table 4.2 and 4.4, Figure 4.6). Before the experiment began and after molt, baseline CORT levels among the three treatment groups were similar (Table 4.2).

Time but not treatment significantly affected maximum CORT levels, and the treatment groups varied in how they changed over time (Table 4.4, Figure 4.6). Maximum CORT levels were similar among the treatment groups before the experiment began and after the birds completed molt (Table 4.2, Figure 4.6). Directly after the treatment period, maximum CORT levels in CORT-treated birds were higher than in DMSO-treated and undisturbed birds (Table 4.2, Figure 4.6).

The three treatment groups had similar CORT response to the capture-and-restraint sampling protocol before the experiment began and after the termination of molt (Tables 4.3 and 4.4, Figure 4.7). After the treatment period, treatment, time, and an interaction of time and treatment significantly affected the response to capture-and-restraint (Table 4.4, Figure 4.7): CORT-treated birds did not show increased CORT levels with the 60 min capture-and-restraint, but the two control groups did (Table 4.3, Figure 4.7).

CBG and free corticosterone

CBG levels did not vary with time or treatment alone, but these factors did interact significantly such that CBG levels increased after the treatment period only in the CORT-treated birds (Table 4.4, Figure 4.6).

Baseline and maximum levels of free CORT fell over time in all treatment groups and were not significantly affected by treatment (Table 4.4, Figure 4.6). However, baseline and maximum free CORT levels were elevated in CORT-treated birds after the treatment period (Table 4.4, Figure 4.6).

Differences between studies

Baseline but not maximum CORT levels were higher before treatment in the early breeding season experiment than in the late breeding season (baseline: Wilcoxon, $Z=-2.31$, $p=0.02$; maximum: t-test, $t=1.57$, $DF=46$, $p=0.12$). However, baseline and maximum CORT levels did not differ between years in 3X CORT-treated birds after approximately 2 weeks of treatment (baseline: Wilcoxon, $Z=0.35$, $p=0.73$; maximum: t-test, $t=-1.38$, $DF=11$, $p=0.20$). Maximum CORT levels were higher in DMSO-treated birds after approximately 2 weeks of treatment in the early breeding season experiment compared to the late breeding season experiment (baseline: Wilcoxon, $Z=0.59$, $p=0.56$; maximum: $t=3.08$, $DF=11$, $p=0.01$).

Before the treatment period, CBG levels were higher in the early than in the late breeding season (t-test, $t=4.55$, $DF=33$, $p<0.0001$). CBG levels in 3X DMSO-treated birds but not the 3X CORT-treated birds were higher after approximately two weeks of treatment in the early breeding season experiment compared to the late breeding season experiment (DMSO-treated: $t=5.01$, $DF=14$, $p<0.0002$; CORT-treated: $t=1.19$, $DF=10$, $p=0.26$).

Discussion

We found that frequent pulses of CORT were sufficient to cause two classic hallmarks of chronic stress: an increase in endogenous baseline CORT levels and a down regulation of the stress response. Unlike many chronically-stressed animals, the adrenal gland was not hypertrophied. Birds responded to CORT treatment in a dose-dependant manner. We found no difference among treatment groups in 1) responsiveness of the

HPA axis to ACTH or 2) hepatic CORT metabolism. CORT treatment produced mixed effects on CBG levels: CBG levels increased in the late but not the early breeding season experiment and CBG levels were higher in early than the late breeding season.

CORT levels

Endogenous baseline CORT levels were significantly elevated by CORT-treatment in both experiments. From these results, we conclude that frequent, small pulses of CORT are sufficient to create a “chronic stress” phenotype during breeding. We emphasize that each CORT dose caused an elevation of CORT that was low and well within the physiological range (Boyd et al. in preparation). The effects of CORT pulses on the HPA axis were reversible, as seen by the similarity of baseline CORT levels among treatment groups after molt.

In birds, chronically stressful conditions cause an increase in baseline CORT levels (Fowler et al. 1995, Scheuerlein et al. 2001, Clinchy et al. 2004). Interestingly, captive starlings chronically exposed to a variety of noxious stimuli showed the opposite pattern: baseline CORT levels decreased due to exposure to stressors (Rich and Romero 2005). The variable results between our study and Rich and Romero’s (2005) point to differences between repeated administration of noxious stimuli and repeated administration of CORT itself. These differences could be due to the HPA changes brought about by exogenous CORT. The differences could also be linked to the experience of the birds in the two studies: the noxious stimuli experienced by the starlings caused increases in CORT, but they were familiar. The sparrows in our study could not control the CORT pulses and could not develop a familiarity with the stressful events our CORT doses mimicked.

The effect of CORT-treatment on endogenous baseline CORT levels was dose dependent: birds treated 3X had higher baseline CORT levels than birds treated 1X. Baseline CORT levels in 3X CORT-treated birds were significantly elevated compared

to controls at both the mid-point and end of the experiment. Baseline CORT levels in 1X CORT-treated birds were elevated compared to controls only at the end of the experiment. The dose-dependant response of these birds to CORT pulses has interesting application for both clinical and natural studies. Our dosing technique could be used to for studies exploring the effects of various levels of endogenous CORT. Our results also give insight into the mechanisms underlying elevated baseline CORT levels in populations subject to frequent stressors (Wasser et al. 1997, Boonstra et al. 1998, Marra and Holberton 1998, Creel et al. 2002).

Baseline CORT levels in the three control groups (1X DMSO, 3X DMSO, and undisturbed) changed slightly over time, but did not increase markedly post-treatment. The similar baseline CORT levels in DMSO and undisturbed control groups indicated that neither the DMSO vehicle nor frequent handling chronically elevated endogenous CORT levels. The lack of difference between the 1X and 3X DMSO-control groups supports our claim that frequent handling did not affect endogenous levels of baseline CORT.

Pre-treatment baseline CORT levels were higher in the early breeding season than the late breeding season, which is consistent with data collected in the field (Romero et al. 1997). While the majority of variation in baseline CORT levels with life stage is absent in captivity, some variation does still occur (Romero et al. 1997, Breuner et al. 1999, Romero and Wingfield 1999, Landys et al. 2004b). After approximately two weeks of treatment, baseline CORT levels in the 3X CORT-treated groups and the 3X DMSO-treated groups were similar between experiments. The equalization of baseline CORT levels between experiments is due to CORT administration in the CORT-treated groups and the progression through different life stages (wintering, early breeding, late breeding, molting) during the sampling period in both the CORT and DMSO-treated birds.

Treatment and the dosage of treatments per day did not affect maximum CORT levels in the early breeding season, but treatment increased maximum CORT levels in the late breeding season. The reason for the up-regulation of the entire HPA axis in one breeding sub-stage and not the other is unclear. The white-crowned sparrow may be more susceptible to the effects of elevated CORT in the late breeding season when baseline CORT and sex steroid levels are naturally low. Differences in susceptibility to CORT could also be driven by variation in CBG levels (see below) or receptor fields between the early and late breeding season.

Maximum CORT levels increased with time in both experiments. In the early breeding season experiment, maximum CORT levels changed in all treatment groups. This change was most likely due to transition between life stages and/or substages, as discussed above (Romero et al. 1997, Romero and Wingfield 1999). In the late breeding season experiment, the increase was most likely due to the elevated maximum CORT levels in CORT-treated birds after the treatment period.

In both the early and late breeding season, CORT-treated birds did not show the typical increase in endogenous CORT levels with capture-and-restraint stress. This result indicates that repeated, acute CORT administration resulted in the loss of the birds' ability to respond to novel stressors. Two likely reasons for the lack of response in CORT-treated are that 1) the adrenals could not produce more CORT than was produced basally and 2) the HPA axis was down regulated. Changes in the receptor fields of the HPA axis may have also caused the lack of response to capture and restraint. Finally, other psychological and physiological factors, such as altered perception of capture and restraint as a stressful event or modified HPA axis feedback mechanisms, could be involved in hormone release under capture-and-restraint sampling.

Down regulation of the HPA axis is a classic indicator of the chronic-stress phenotype (Romero 2004) and has been observed in wild animals living in disturbed environments (Walker et al. 2006). If, under chronic stress, CORT levels are at a maximum under resting conditions, what chemical signal can animals use to trigger the behavioral and physiological changes that promote survival during a challenge? The answer to this question may in fact be that chronically stressed animals are unable to cope with additional challenges because they lack the CORT signal (Wingfield 2003).

ACTH challenge

In the early breeding season experiment, CORT levels after the ACTH challenge were not affected by treatment. Thus, maximum adrenal output was similar among the treatment groups.

CORT levels in the two control groups (DMSO-treated) were similar with and without ACTH injection, as has been found before in this subspecies (Astheimer et al. 1994). This result indicates that 1) capture-and-restraint alone was enough to maximally stimulate the HPA axis and 2) ACTH injection did not stimulate a greater negative feedback response than capture-and-restraint alone. In contrast, in both 1X and 3X CORT-treated birds, CORT levels were higher after 30 min of restraint in ACTH-treated birds than in non-injected birds. We assume that the higher CORT levels after the ACTH injection were due to the action of ACTH on adrenal CORT release. From this observation, we can conclude that the adrenal in CORT-treated birds was indeed still sensitive to the ACTH hormone signal. The lower CORT levels in non-injected, CORT-treated birds could indicate a weaker HPA axis response to capture-and-restraint, stronger negative feedback to CORT levels, or increased CORT clearance. The maximum CORT levels in response to a stressor may be controlled at the level of the brain (via ACTH or CRF release) in CORT-treated birds. These birds may be appropriately tailoring the emergency response to their compromised condition.

There is no clear pattern of how chronically-stressed birds and mammals will respond to an ACTH challenge. Chronically stressed baboons do not change their response to ACTH, snowshoe hares increase their response, and starlings and cougars decrease their response (Sapolsky 1983, Harlow et al. 1992, Boonstra et al. 1998, Rich and Romero 2005). Thus, the functioning of the HPA axis in chronically-stressed vertebrates may be dependant on the animal's context and the nature and severity of the chronic stressor.

We did not include a saline-injection control in this study because we had collected samples from the birds using a capture-and-restraint protocol two days prior. Using these samples, we were able to treat each individual as its own control. The primary question for this portion of the study was not the effect of ACTH on CORT levels but the difference in response to ACTH among the treatment groups. With this question, the need for a saline-injected control group was reduced.

Adrenal size

Adrenal size usually increases under chronic stress conditions (Rogers et al. 1993a, Miller and Tyrrell 1995, Boonstra et al. 1998, Retana-Marquez et al. 2003a, Retana-Marquez et al. 2003b). However, treatment had no affect on adrenal size in the early breeding season experiment. Although baseline CORT levels in the CORT-treated birds were higher than in the control groups, maximum CORT production was the same. These data indicate that no change in adrenal size was necessary to sustain the high CORT production observed in the CORT-treated animals. However, treatment with exogenous CORT and potential alteration of the negative feedback system may have prevented hypertrophy.

We do not know if CORT treatment affected adrenal size in the late breeding season experiment. Because both baseline and maximum CORT levels were elevated in CORT-treated birds, we expect that the adrenals hypertrophied in these birds to support increased hormone production.

Liver metabolism

We know very little about the dynamics and potential modulation of hepatic CORT metabolism in birds. Because we observed a dip in CORT levels 10 min after capture-and-restraint in CORT-treated birds, we hypothesized that CORT-treated birds increased hepatic CORT clearance. Our data did not support this hypothesis: treatment had no effect on hepatic CORT metabolism.

We consider it unlikely that our *in vitro* or thin layer chromatography methods were flawed because we modeled them after published studies (Lindberg et al. 1972, Doell et al. 1981, Edwards et al. 2005). However, we recognize that our techniques may not have been precise enough to measure fine-scale changes in metabolism. Conditions other than those that regulate the HPA axis may be responsible for modulation of hepatic CORT metabolism (Drake et al. 2005). It is also possible that birds do not regulate hepatic CORT metabolism. Further research is necessary to support such a claim. However, recent work on sex steroids in lizards indicates that hepatic metabolism of steroids does not vary with sex or season/reproductive condition (Edwards et al. 2005).

CBG and free corticosterone

CORT-treated birds increased CBG levels in the late but not the early breeding season. In the early breeding season experiment, CBG levels in all but the 3X DMSO group were highest at the mid-point of the experiment. CBG levels are typically elevated early in the breeding season, most likely due to the rise in sex hormones at this time (Silverin 1986, Romero and Wingfield 1998, Deviche et al. 2001, T. Sperry and S. O'Brien unpublished data on *Z. leucophrys gambelli*). Why CBG levels were not elevated at the mid-point of the study in the 3X DMSO group is unclear.

In the late breeding season experiment, CBG levels again changed over time and the dynamics of this change was affected by CORT treatment. CBG levels in both control groups were low post-experiment. The opposite pattern was seen in CORT-treated birds: CORT-treated birds increased CBG levels. However, even with higher CBG levels, CORT-treated birds still had higher baseline and maximum free CORT levels than the two control groups.

Administering chronic stressors typically causes CBG levels to fall and free CORT levels to increase (Fleshner et al. 1995, Boonstra et al. 1998, Deak et al. 1999, Breuner and Orchinik 2002, Lynn et al. 2003). We are not aware of studies that discuss the effects of CORT treatment on CBG levels in birds. Mammals do not show a consistent CBG response to CORT treatment (see review in Breuner and Orchinik 2002), and the same inconsistency may occur in birds. CORT might not be the major regulator of CBG levels in birds. Instead, CBG levels may fluctuate to regulate levels of sex hormones, especially since birds have no known dedicated sex hormone binding globulins (Breuner and Orchinik 2002).

CBG levels were higher early in the breeding season than late in the breeding season, a result consistent with past work in birds (Silverin 1986, Romero et al. 1998c, Romero and Wingfield 1998, Deviche et al. 2001). However, we are uncertain why the pre-breeding birds in the early breeding season experiment would have higher CBG levels than breeding birds. Considering that females tend to have lower CBG levels than males, this difference may due to the fact that we included more females in the experiment late in the breeding season than in the experiment early in the breeding season (Silverin 1986, Deviche et al. 2001). However, no obvious differences in CBG levels occurred between sexes in either experiment (sample sizes were too small to analyze statistically).

The difference in CBG levels between the two studies may be due in part to the treatment of the plasma samples; the late breeding season samples went through three rather than two freeze-thaw cycles before we measured CBG levels. Freeze-thaw cycles are known to denature proteins, effecting their concentration in the sample.

CBG levels in the CORT-treated birds were similar between the two studies. This similarity could indicate that CBG titers rise to a certain level in response to exogenous CORT pulses or endogenous baseline CORT levels.

Conclusions

We found that frequent, acute CORT administration can create a chronic stress phenotype. Specifically, birds treated with CORT both once a day and three times a day had increased endogenous baseline CORT levels and failed to increase CORT in response to a stressor. Both of these alterations of the HPA axis are classic indicators of chronic stress (Romero 2004). However, unlike most chronically-stressed vertebrates, the birds in our early breeding season study did not increase adrenal size.

The results of this study have implications for the effect of disturbance on wild animals. We posit that in a degraded habitat or one with common disturbances, animals would frequently mount stress responses. Because we administered only CORT in this study, we cannot suggest that our techniques mimicked the entire nature of the stress response. However, the data indicated that frequent, acute pulses of CORT alone were sufficient to cause HPA axis changes similar to those in chronically-stressed animals. Thus, frequent, acute environmental stressors could create a chronically-stressed individual. If this hypothesis is true, the implications of this study have special relevance for conservation biology. Namely, frequent, small disturbances could indeed alter the HPA axis of an individual in a way that could compromise its fitness (Wingfield 1994, Sapolsky et al. 2000, Wingfield and Romero 2001).

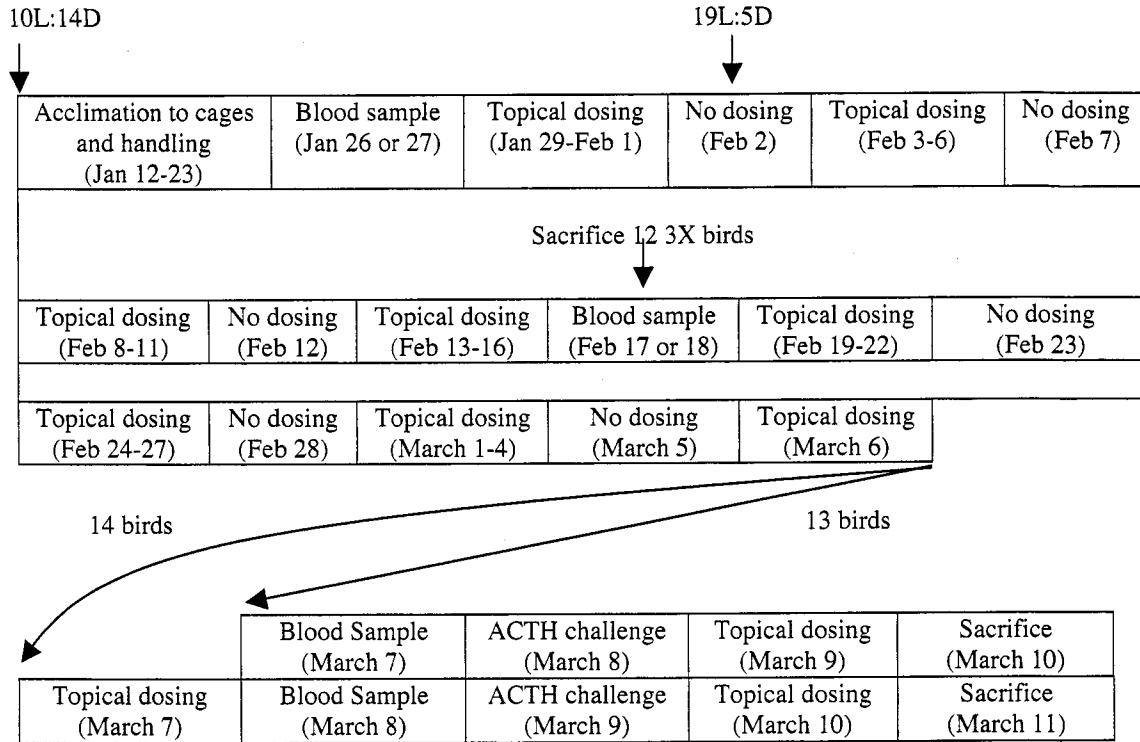


Figure 4.1. Schedule of treatments for birds in the early breeding season experiment.

Acclimation to handling (March 30-April 13)	Blood sampling (April 14)	Topical dosing (April 15-18)	No dosing (April 19)	Topical dosing (April 20-23)
No dosing (April 24)	Topical dosing (April 25-28)	No dosing (April 29)	Blood sampling (April 30)	Blood sampling between 2-12 days after completion of molt (July 13-August 26)

↑
Photorefractory

Figure 4.2. Schedule of treatments for birds in the late breeding season experiment.

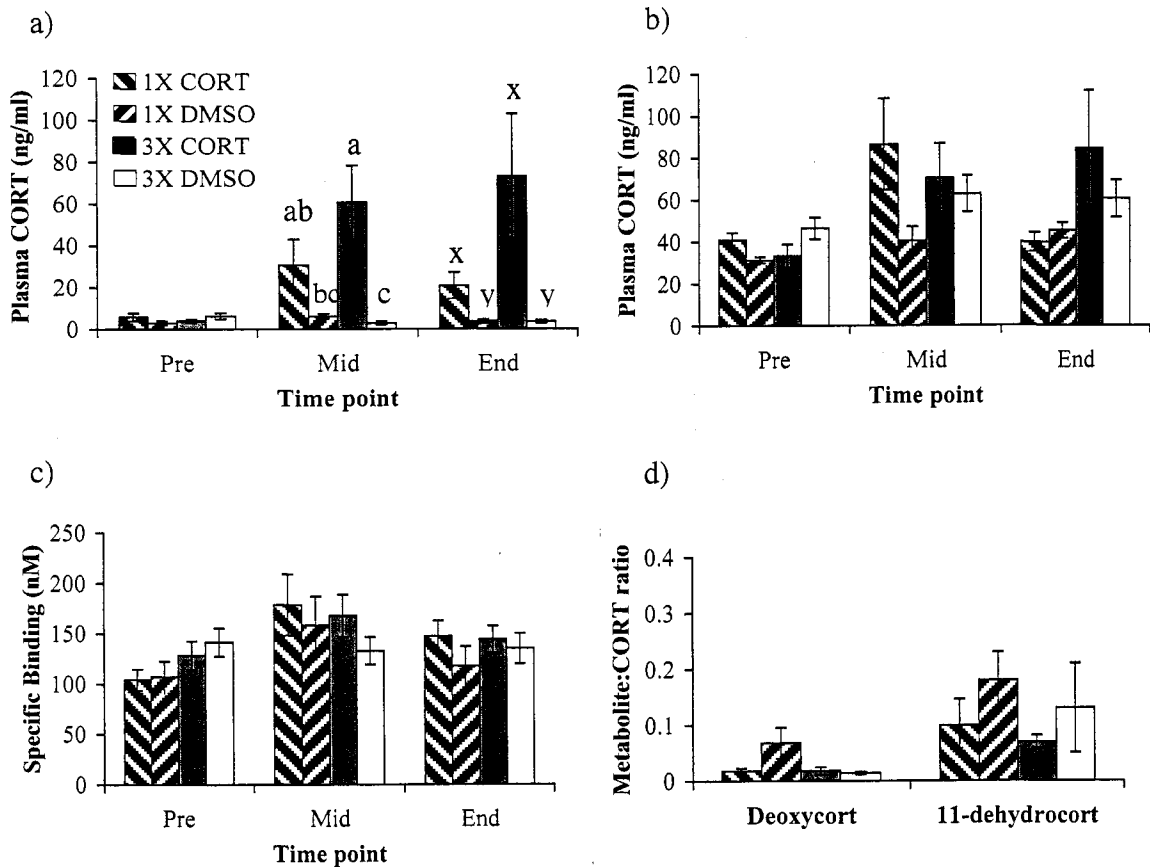


Figure 4.3. Baseline (a) and maximum (b) plasma CORT levels and CBG levels (c, as indicated by CBG specific binding) before, at the mid-point, and at the end of the early breeding season experiment. Levels were compared among treatment groups at each point in the experiment. Baseline CORT levels were significantly higher in CORT-treated birds at the mid-point and end of the experiment, and these differences are denoted by letters. Maximum CORT levels and CBG levels were similar at all time points. Treatment did not affect hepatic CORT breakdown (d).

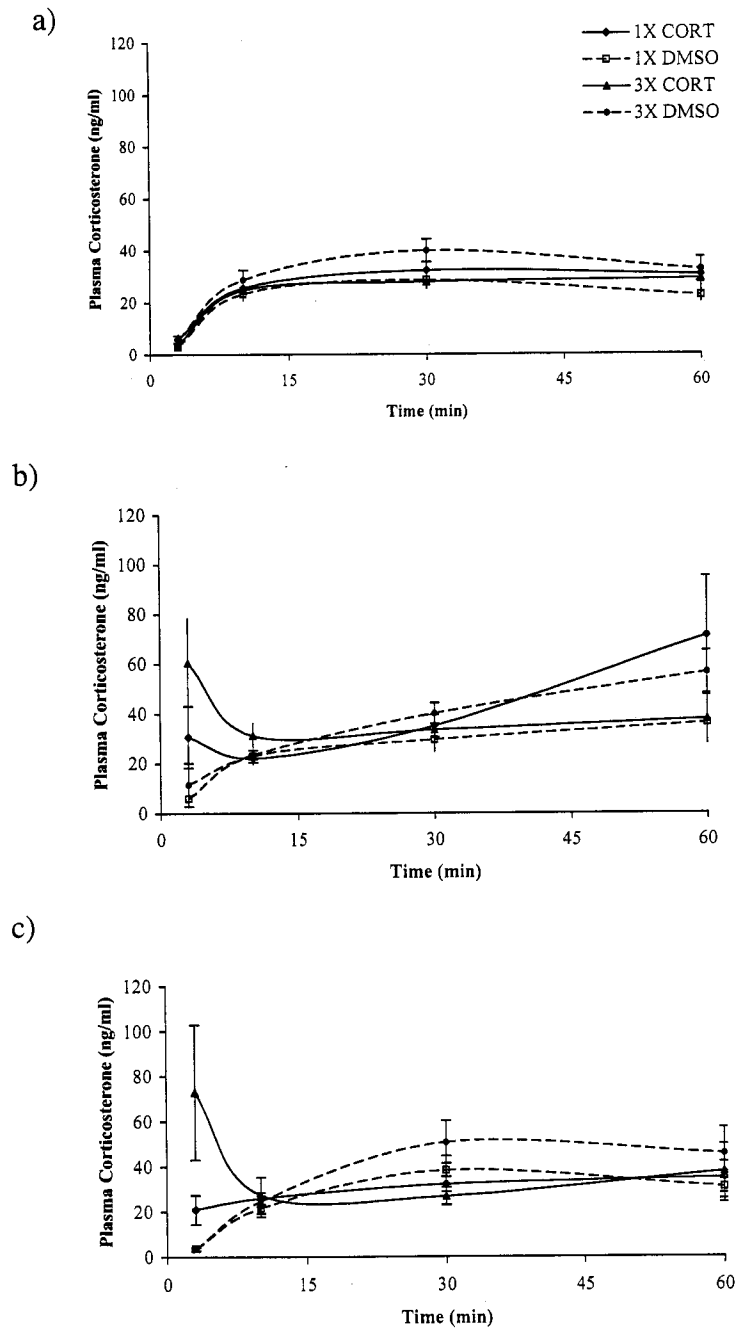


Figure 4.4. Plasma CORT levels in response to capture-and-restraint sampling before the initiation (*a*), at the mid-point (*b*), and at the end of the early breeding season experiment (*c*). The CORT response to stress was similar among treatment groups before the initiation of the experiment. The two CORT-treated groups differ from the control groups at both the mid-point and end of the experiment, failing to increase CORT over time.

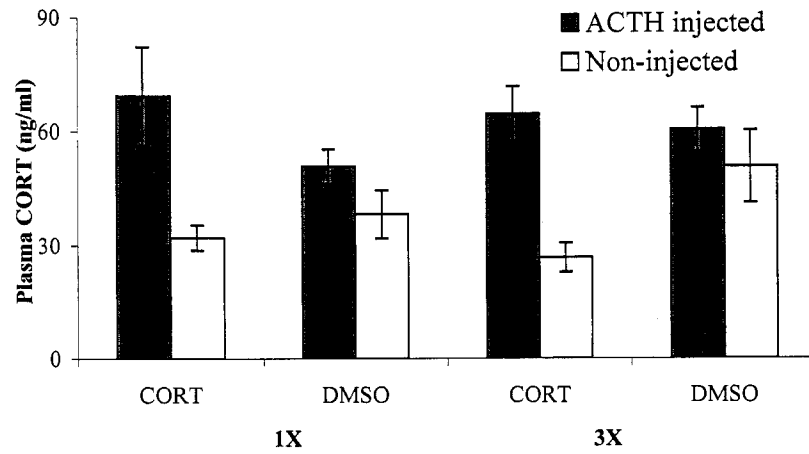


Figure 4.5. Plasma CORT levels 30 min after ACTH injection and capture-and-restraint (“ACTH injected”) or just capture-and-restraint (“non-injected”) in DMSO or CORT-treated birds dosed either once per day (1X) or three times pre day (3X). Plasma CORT levels were significantly higher after ACTH injection in the CORT-treated birds.

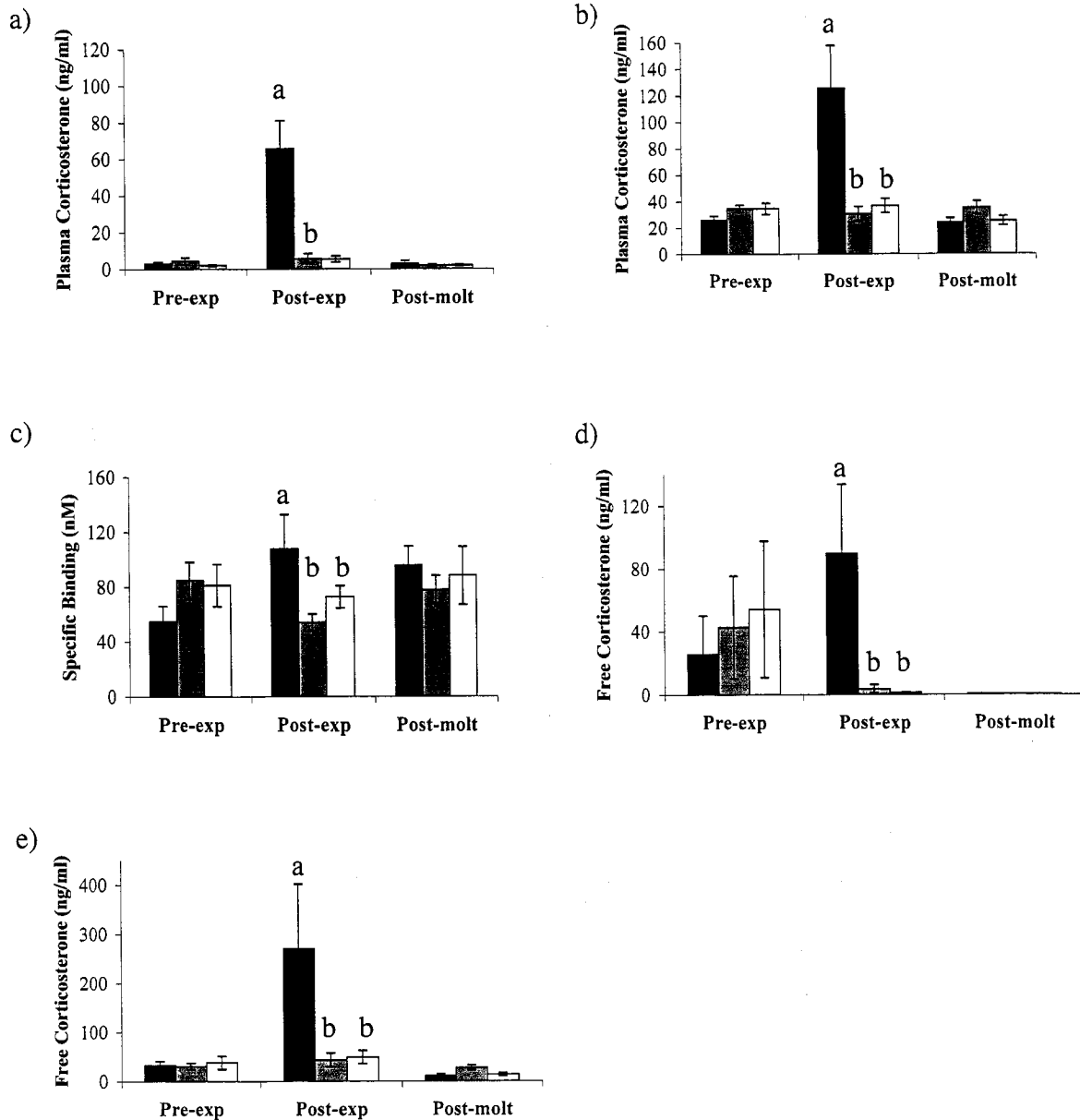


Figure 4.6. Baseline (*a*) and maximum (*b*) plasma total CORT levels, CBG levels (*c*, as indicated by CBG specific binding), and baseline (*d*) and maximum (*e*) free CORT levels before treatment, after treatment, and after molt in the late breeding season experiment. CORT and CBG levels were compared among treatment groups at each point in the experiment. Baseline and maximum total and free CORT levels and CBG specific binding levels were significantly higher in CORT-treated birds after treatment, as denoted by letters in the figures.

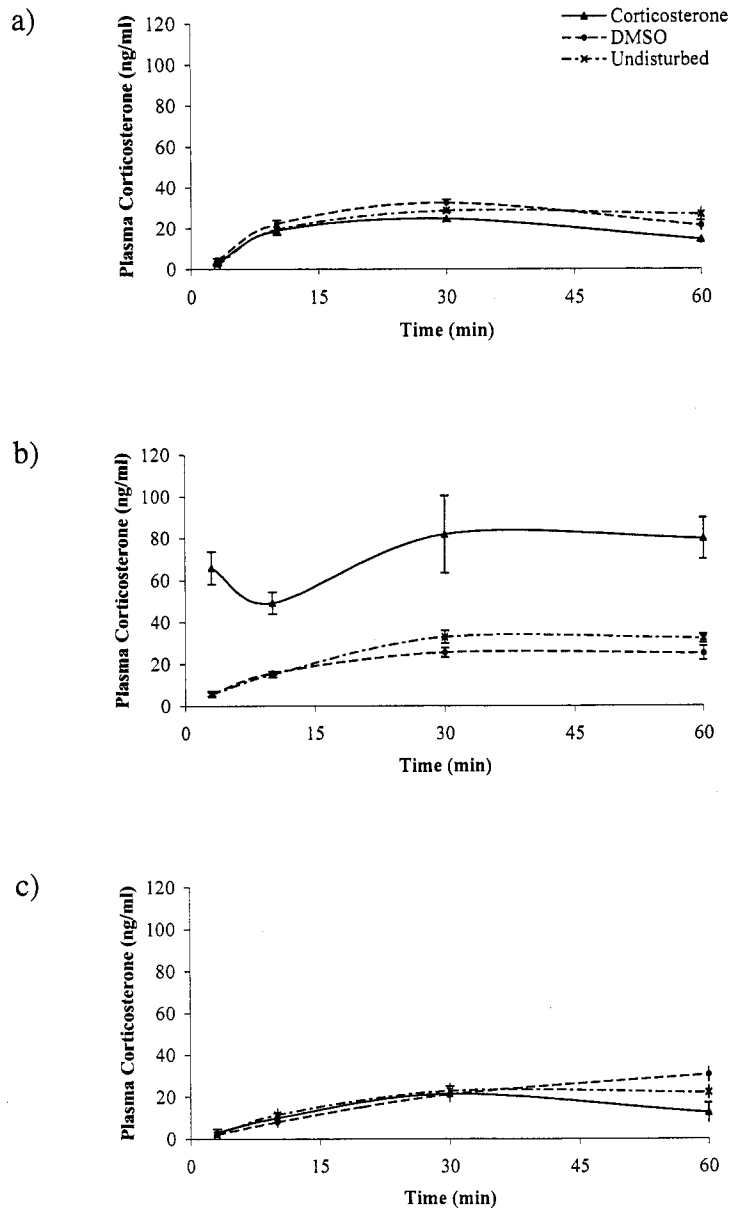


Figure 4.7. Plasma CORT levels in response to capture-and-restraint sampling before treatments (a), after treatments (b), and after molt (c) in the late breeding season experiment. CORT response to stress was similar among treatment groups before treatment and after molt. The CORT-treated group differs from the control groups after treatments, failing to increase CORT over time.

Table 4.1. Results of statistical tests from the early breeding season experiment. Each row represents a separate test. Baseline and maximum CORT levels, CBG levels, CORT data from the ACTH challenge, and CORT data from the stress series were analyzed with two-way repeated measure MANOVA. Time in the stress series analyses represents the 60 min of the capture and restraint protocol. Time in the other analyses represents the course of the experiment. Hepatic CORT metabolism data were analyzed with three-way ANOVA. Significant results are in bold text and trends are italicized.

	Treatment			Dose per day			Treatment* Dose per day			Time		
	F	DF	p	F	DF	p	F	DF	p	F	DF	p
Baseline CORT	28.47	1,23	<0.0001	4.87	1,23	0.04	0.52	1,23	0.48	25.45	2,22	0.0001
Max CORT	0.27	1,23	0.61	<i>3.67</i>	<i>1,23</i>	<i>0.07</i>	1.92	1,23	0.18	8.09	2,46	0.001
CBG	0.93	1,28	0.34	0.06	1,28	0.81	0.02	1,28	0.89	5.70	2,56	0.005
ACTH challenge	0.16	2,21	0.69	0.05	1,21	0.82	0.001	1,21	0.97	4.61	1,21	0.04
<i>Stress series</i>												
Pre-exp	0.09	1,33	0.77	0.94	1,33	0.34	4.89	1,33	0.03	98.74	3,31	<0.0001
Mid-exp	12.97	1,35	0.001	0.45	1,35	0.51	0.03	1,35	0.86	30.02	2,83	<0.0001
Post-exp	5.98	1,22	0.02	1.15	1,22	0.30	0.001	1,22	0.97	13.18	3,66	<0.0001
<i>CORT metabolism</i>												
Deoxycort:CORT												
50mg tissue	1.22	1,21	0.28	0.05	1,21	0.83	0.63	1,21	0.44			
100mg tissue	1.37	1,25	0.25	3.92	1,25	0.06	2.52	1,25	0.12			
11-dehydrocort:CORT												
50 mg tissue	0.34	1,21	0.57	0.004	1,21	0.95	0.04	1,21	0.84			
100mg tissue	0.64	1,25	0.43	2.23	1,25	0.15	0.75	1,25	0.39			

Table 4.1 continued

	Time*Treatment			Time*Dose per Day			Time*Treatment* Dose per Day		
	F	DF	p	F	DF	P	F	DF	p
Baseline CORT	32.12	2,22	0.0001	0.44	2,22	0.65	10.03	2,22	0.0008
Max CORT	2.05	2,46	0.14	1.14	2,46	0.33	2.46	2,46	0.10
CBG	0.91	2,56	0.41	2.43	2,56	0.10	0.32	2,56	0.73
ACTH challenge	0.09	1,21	0.77	0.78	1,21	0.39	0.47	1,21	0.50
<i>Stress series</i>									
Pre-exp	1.26	3,31	0.31	0.45	3,31	0.72	1.29	3,31	0.30
Mid-exp	20.38	2,83	<0.0001	0.20	2,83	0.86	6.83	2,83	0.0009
Post-exp	25.81	3,66	<0.0001	1.68	3,66	0.18	2.86	3,66	0.04

Table 4.2. Results of ANOVA or Kruskal-Wallis tests on baseline and maximum CORT levels among treatment groups at each point of both experiments. The significant results of post-hoc Tukey-Kramer tests are given for tests with significant results. Significant results are in bold text and trends are italicized.

Early Breeding	Pre-experiment			Mid-experiment			Post-experiment		
	F	DF	<i>p</i>	F	DF	<i>p</i>	F	DF	<i>p</i>
Baseline CORT	1.59	3,33	0.21	27.05	3,34	<0.0001	14.82	3,23	<0.0001
				P<0.05 for only 3X CORT vs 1X and 3X DMSO 1X CORT vs 3X DMSO			P<0.05 for only 1X and 3X CORT vs 1X and 3X DMSO		
Maximum CORT	2.65	3,35	0.06	1.72	3,35	0.18	1.87	3,23	0.16
				Power=0.41			Power=0.42		
Late Breeding	Pre-experiment			Post-experiment			Post-molt		
	χ^2	DF	<i>p</i>	F	DF	<i>p</i>	χ^2	DF	<i>p</i>
Baseline CORT	1.71	2	0.43	20.90	2,18	<0.0001	0.007	2	1.0
				P<0.05 for only CORT vs DMSO CORT vs undisturbed					
Maximum CORT	F	DF	<i>p</i>	F	DF	<i>p</i>	F	DF	<i>p</i>
	2.24	2,18	0.14	7.25	2,18	0.005	2.05	2,17	0.16
				P<0.05 for only CORT vs DMSO CORT vs undisturbed					

Table 4.3. Results of repeated-measures MANOVA tests on the CORT response to capture-and-restraint for each treatment group at each point of both experiments. Groups that significantly increased CORT with capture-and-restraint are in bold text.

Early Breeding	<i>Pre-experiment</i>			<i>Mid-experiment</i>			<i>Post-experiment</i>		
	F	DF	<i>p</i>	F	DF	<i>p</i>	F	DF	<i>P</i>
1X CORT	24.47	3,21	<0.0001	2.66	3,5	0.16	5.02	3,4	0.08
1X DMSO	47.52	2,13	<0.0001	28.50	3,4	0.004	28.48	3,4	0.004
3X CORT	89.58	2,23	<0.0001	1.61	3,9	0.26	2.28	3,3	0.26
3X DMSO	43.78	3,27	<0.0001	38.19	3,9	<0.0001	115.0	3,3	0.001

Late Breeding	<i>Pre-experiment</i>			<i>Post-experiment</i>			<i>Post-molt</i>		
	F	DF	<i>p</i>	F	DF	<i>p</i>	F	DF	<i>p</i>
3X CORT	29.83	2,13	<0.0001	0.15	3,21	0.93	14.92	3,15	<0.0001
3X DMSO	14.14	3,18	<0.0001	10.68	1,9	0.006	57.41	3,15	<0.0001
Undisturbed	41.34	3,15	<0.0001	21.58	3,15	<0.0001	57.11	3,15	<0.0001

Table 4.4. Results of repeated-measure MANOVA tests on HPA axis data from the late breeding season experiment. Each row represents a separate test. Time in the stress series analyses represents the 60 min of the capture-and-restraint protocol. Time in the other analyses represents the course of the experiment. Significant results are in bold text.

	Treatment			Time			Treatment * Time		
	F	DF	p	F	DF	p	F	DF	p
Baseline CORT	12.02	2,18	0.0005	13.78	1,21	0.0009	11.18	2,21	0.0003
Baseline free CORT	0.16	2,15	0.85	29.58	2,30	<0.0001	3.65	4,30	0.02
Maximum CORT	0.54	2,17	0.59	8.96	2,34	0.0008	9.67	4,34	<0.0001
Max. free CORT	0.17	2,15	0.85	25.53	2,30	<0.0001	8.70	4,30	<0.0001
CBG	1.50	2,16	0.25	1.80	2,32	0.18	6.19	4,32	0.0008
<i>Stress series</i>									
Pre-exp	0.63	2,18	0.54	70.67	3,54	<0.0001	0.86	6,54	0.53
Post-exp	7.66	2,18	0.0039	10.94	3,54	<0.0001	4.50	6,54	0.0009
Post-molt	1.07	2,16	0.37	97.00	3,48	<0.0001	1.96	6,48	0.09

Chapter 5

Repeated, acute pulses of corticosterone create a chronic stress phenotype in Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*)

Introduction

In vertebrates, the adaptive response to an unexpected stressor is to activate coping mechanisms such as the hypothalamo-pituitary-adrenal (HPA) axis (Wingfield 1994, Wingfield et al. 1998, Wingfield and Ramenofsky 1999, Sapolsky et al. 2000). Levels of the glucocorticoid hormones, such as cortisol and corticosterone (CORT), increase as an animal attempts to cope with a variety of different disturbances and unpredictable events (Wingfield et al. 1998, Sapolsky et al. 2000). The stress response is adaptive in that it helps animals survive a given challenge and return to homeostatic set points relevant to the time of year or life stage after the challenge passes (Astheimer et al. 1995, Wingfield and Ramenofsky 1999, Sapolsky et al. 2000). However, the stress response is designed for coping with acute challenges. Due to catabolic effects on the body and inhibition of many processes and behaviors, chronic activation of the stress response, as assessed by high glucocorticoid levels, is maladaptive (Wingfield et al. 1998, Sapolsky et al. 2000, Korte et al. 2005). Recently, researchers have measured CORT in wild animals as an indicator of physiological stress induced by the environment and of the animal's body condition (Wasser et al. 1997, Wingfield et al. 1997b, Boonstra et al. 1998, Marra and Holberton 1998, Scheuerlein et al. 2001, Creel et al. 2002, Romero 2004, Reeder and Kramer 2005, Walker et al. 2005a).

Glucocorticoids are not only involved in coping with perturbations, they also regulate energy mobilization for annual and daily cycles; they stay above a constitutive levels needed for proper glucose balance and below a level that triggers an emergency "stress" response (Wingfield et al. 1997b, Breuner et al. 1999, Romero and Wingfield 1999, Wingfield and Ramenofsky 1999, Romero 2002). For most animals, perturbations that

increase glucocorticoids to stress levels, those levels that trigger an emergency response, are infrequent. Constant elevation of glucocorticoids to “stress” levels are rare in nature and compromise the animal, sometimes to the point of facilitating its death. Long, severe storms and food depletion events are examples of phenomena that can cause this constant, high elevation of glucocorticoids, and this high hormone scenario has been studied using hormone implants.

Though much is known about stress physiology when glucocorticoids are at high levels constantly, little is known about how wild animals respond to repeated, acute stressors that we posit would cause repeated, acute pulses of glucocorticoids. Nor do we fully understand when frequent, acute stress becomes chronic stress. In this study, we present a methodology for simulating an environment with frequent, acute stressors and discuss the results of two studies in which we applied frequent pulses of CORT to a captive group of wild, migratory white-crowned sparrows (*Zonotrichia leucophrys gambelii*). In doing so, we explore the role of frequent, acute pulses of CORT as a mechanism mediating frequent, acute stressors. In addition to documenting the effect of frequent, acute stressors, we also explore 1) whether the effects of the hormone manipulation varied with time of year (as expressed by progression of the breeding season), 2) whether the response of the study subjects differed given the number of stressors they experienced per day, and 3) if prior experience of frequent stressors affected the development and progress of subsequent life-history stages.

A number of studies have created models for studying chronic stress by repeatedly exposing animals to noxious stimuli that elicit an increase in CORT levels. These studies are numerous in the captive rodent literature (Sapolsky et al. 2000, Dallman and Bhatnagar 2001), but are rarer in “non-model”, wild-caught animals. Rich and Romero (2005) recently published a study in which they exposed wild-caught, captive starlings (*Sturnus vulgaris*) to repeated, acute stressors (4-5 30 min stressors/day), such as loud noise, restraint, crowding, and disturbance to their cage. They found that the starlings

down-regulated their HPA axis at the level of ACTH release from the pituitary, resulting in lower basal and stress-induced CORT levels. These animals lost a significant amount of mass due to exposure to the stressors. In a similar study, Lankford et al. (2005) found that the stress-induced maximum but not baseline CORT levels in juvenile green sturgeon (*Acipenser medirostris*) increased after 7, 14, and 21 days of a chronic stress regime that included events such as chasing, water depth reduction, and confinement. The authors do not report data on body condition of these fish, but they found that the maintenance metabolic rate of the fish increased. They posit that the additional resources needed by the animals to cope with chronic stress could leave fewer resources for reproduction, the immune system, growth, and replenishment of energy stores.

Stressors change the levels of many hormones. Unlike the two studies discussed above that challenge their subjects with stressful events, we designed our study to better understand the direct role of glucocorticoids in the stress response and the actions of glucocorticoids alone on body condition. Specifically, we examined what repeated, acute pulses of glucocorticoids did to tissues. In addition, because we dosed our experimental animals with CORT itself, our study was not confounded at the population or individual level by how the subjects perceived stress or by acclimation of our animals to the chosen stressors.

We predicted that repeated, acute CORT administration would cause declines in body condition similar to those caused by CORT implants and chronic stress. We predicted that compared to controls, CORT-treated birds would 1) decrease body mass, 2) increase fat stores, and 3) decrease flight muscles (Gray et al. 1990, Dallman et al. 1993, Sapolsky et al. 2000). We expected no change in feeding rate between CORT- and control-treated birds (Astheimer et al. 1992) and hypertrophy of the gallbladder, as occurs in fish subjected to chronic social subordination (Earley et al. 2004). Because CORT-implanted birds increase night time restfulness, we predicted that CORT-treated

birds would express less migratory behavior than control birds and potentially might not express any migratory behavior (Buttemer et al. 1991). We expected no effect of CORT administration on molt due to the temporal separation of CORT treatment and molt. Finally, we expected that the effects of CORT treatment would be similar in the early and late breeding season and that the birds would respond to CORT in a dose-dependant manner.

Methods

Pre to early-breeding season

We captured the Gambel's white-crowned sparrows used in both of our experiments during autumnal migration through Sunnyside Game Reserve in Sunnyside, Washington (46.1°N, 119.5°W). The birds were housed in outdoor aviaries at the University of Washington until use in our experiments.

In January 2005, we brought 36 adult male and 3 adult female white-crowned sparrows into two environmental chambers with a light schedule of 10L:14D. The birds were acclimated to individual cages for two weeks and were handled twice a day (<1 min/handling) to acclimate them to capture and brief holding (see Figure 1, Wingfield et al. 1982, Dobrakovova et al. 1993). After this two-week period, we measured food intake for a 24-hour period. Three days later, we measured the body condition of all animals by assessing body weight, abdominal and furcular fat score (0-5 scale as per Helms and Drury (1960) and Wingfield and Farner (1978b)), and condition of the flight muscle. The condition of the flight muscle was assessed by noting the curvature of the muscle around the sternal keel with a score of 0=extremely concave, 1=concave, 2=neither concave or convex, 3=convex, as based on Gosler (1991).

The day after body condition assessment, we began our treatments. Our dosage technique and validation studies are presented in Boyd et al. (in preparation). Briefly, we used DMSO as the vehicle for CORT dosage since DMSO readily dissolves this

steroid and effectively draws the steroid into the body if the DMSO-CORT solution is placed on the skin (Williams and Barry 2004). DMSO is a safe chemical that is frequently used in veterinary and human medicine, and is often used to give medicines to horses. For treatments, each animal was dosed topically with either 20 μ g CORT in 20 μ l DMSO or a control of 20 μ l DMSO. The experimental dose raised plasma CORT levels to approximately 12.6ng/ml, a level that was above baseline but well below maximal CORT levels (Boyd et al. in preparation). All doses were placed on the back of the neck at the featherless tract above the jugular vein.

Fifteen birds housed in the same chamber were dosed once a day (1X), with 8 birds receiving CORT doses and 7 birds acting as DMSO controls. The remaining 24 birds housed in a second chamber were dosed three times a day (3X), with 12 birds receiving CORT doses and 12 birds acting as DMSO controls. To prevent our study subjects from predicting the time of treatments and to more adequately simulate the random nature of environmental stressors, we haphazardly selected the dosing times. There was also no regular interval between doses. The shortest time period between doses was 1.5 hours and the longest time period between doses was 9 hours.

We treated the birds before the initiation of the breeding life stage (as induced by photoperiod) to study if repeated, acute CORT administration could alter the development of this life stage. After four days of dosing birds in a short-day, winter life stage, we changed the light regime to 19L:5D to stimulate the birds to transition into the breeding life-history stage. During this fifth day, the birds were not dosed, and we measured body condition and the amount of food eaten during this 24-hour period. We repeated this five-day cycle (4 days treatment, 1 day no treatment and body condition/food intake assessment) six times.

We recorded the migratory activity of 4 3X-DMSO and 4 3X-CORT-treated birds during the onset (13th or 14th days after photostimulation) and full expression of

migratory behavior (23rd or 24th days after photostimulation) (King and Farner 1963, Landys et al. 2004b). We videotaped each bird for 40 min after lights off and scored its activity every 20 sec. Activities were tallied as migratory (beak-up, beak-up flight) or non-migratory (resting, jumping, flying, feeding) (Ramenofsky et al. 2003, Agatsuma and Ramenofsky In press).

All birds were sacrificed at the end of the study. We collected the gastrointestinal tract including the liver and gall bladder to determine if CORT treatment affected gallbladder function. While the gall bladder was intact on the gastrointestinal tract, we extracted bile from it and measured the quantity using a Hamilton syringe (μ l). We then dissected the liver from the body, patted it dry, and weighed it (g).

End of breeding season to autumnal molt

In February 2004, we brought 21 white-crowned sparrows, a mix of adults (8) and juveniles (13) and males (13) and females (8), from an outdoor aviary into environmental chambers on a short day photoperiod (8L:16D). Twelve days later, we switched the birds to a long-day cycle (20L:4D) to induce the breeding life stage. For two weeks prior to the experiment, the birds were handled 3 times a day to acclimate them to capture and brief holding (<1 min/handling) (see Figure 2). We then randomly assigned the birds into three treatment groups. One set of birds (n=7), termed “control”, was handled 3 times a day and given a topical dose of 20 μ l DMSO on the neck. The second set of birds (n=8), termed “CORT”, was handled 3 times a day and given a topical dose of 20 μ g CORT dissolved in 20 μ l DMSO. These two groups were housed together in two environmental chambers. The third set of birds (n=6), termed “undisturbed”, was housed in its own chamber and was not subject to either daily handling or to topical DMSO.

On the day before treatments began, we measured the body condition of all animals by assessing body weight, abdominal and furcular fat score, and flight muscle score. The

treated birds were dosed three times a day (3X) for a period of four days. On the fifth day, we did not administer treatments and measured the body condition and 24 hr food intake of all birds. This schedule was repeated three times, so that there were a total of 12 days of treatment and 3 days without treatment. Dosing times were haphazardly spaced throughout the day so that there was no consistent time of dosing or regular interval between doses. The shortest time period between doses was 1.5 hours and the longest time period between doses was 9 hours.

Body condition measurements and molt score were taken every 5 days until each animal completed molt. To score molt, we estimated the percent length of each new primary and secondary feather on the left wing as it grew. If a feather had recently dropped and there was no visible replacement, we scored it as 0%. We noted old feathers as not molting. After all birds had finished molting, we plucked the first (P1) and last (P9) primary feathers. We measured the length of these feathers and counted their number of fault bars (areas of low tissue deposition). We measured molt intensity as the length of primary and secondary feather material missing from a wing. To obtain molt intensity at each time point, we calculated the fraction of each growing feather that was missing and multiplied it by the average length of that feather when full grown. Average feather length was determined by measuring and averaging the length of each primary and secondary in 10 newly-molted captive, individuals housed in the UW aviaries.

Data analyses

We performed all analyses using JMP 5.1. When datasets were non-normal, we log transformed them to attain a normal distribution. If the log transformation did not achieve normality, we used non-parametric statistics. We used one or two-way repeated measures MANOVA tests to explore the impacts of our treatments on the various aspects of body condition and molt intensity. To confirm significant differences between groups, we used post-hoc ANOVA or Kruskal-Wallis tests with additional post-hoc Tukey-Kramer or t-tests. We tested the affect of treatment on gallbladder

function with a two-way ANOVA. To test for differences in the timing of molt initiation and termination with treatment, we used Holm-adjusted χ^2 tests. We tested the duration of molt, feather characteristics, and maximum number of molting feathers with ANOVA or Kruskal-Wallis tests.

Results

Pre to early-breeding season

Mass

At the midpoint of the experiment, mass had increased over time for all birds (Table 5.1, Figure 5.3). By the end of the experiment, the two main effects on mass were time and an interaction of treatment and dose per day, with 3X CORT birds failing to increase mass as the other treatment groups did. When we conducted separate analyses for the dose per day groups, both 1X and 3X birds had significant changes (both increases and decreases) in mass over time (Table 5.1, Figure 5.3). 3X CORT-treated birds did not gain mass like the other treatment groups and showed a slight decline in mass over time (Table 5.1, Figure 5.3).

Fat stores

Fat stores were not affected by treatment or dose per day, but changed significantly with time at both the mid-point and end of the experiment (Table 5.1, Figure 5.3). Fat stores in the 1X birds increased over time and in the 3X birds fluctuated over time (Figure 5.3).

Flight muscle

At the mid point of the experiment, treatment alone did not affect flight muscle score, but 3X birds did lose flight muscle with time (Table 5.1, Figure 5.3). At the end of the experiment, 1X and 3X CORT-treated birds had less flight muscle than the controls and both 3X groups had less flight muscle than the 1X groups (Table 5.1, Figure 5.3).

Food intake

Food intake at the midpoint and end of the experiment was not affected by treatment or dose per day alone (Table 5.1, Figure 5.3). However, time and an interaction of treatment and dose per day did affect food intake. The 1X groups and 3X DMSO-treated group increased food intake over time but the 3X CORT-treated group showed no change in food intake. When we ran separate analyses for birds treated with different doses per day, we found that all birds dosed 1X increased food intake with time (Table 5.1, Figure 5.3). Food intake in the 3X CORT-treated birds was lower than that in the 3X DMSO-treated birds (Table 5.1, Figure 5.3). At the midpoint of the experiment in 3X birds, food intake in the DMSO-treated birds increased with time but food intake in the CORT-treated birds stayed constant with time (Table 5.1, Figure 5.3).

Gall bladder bile retention

The ratio of bile volume/liver mass was not significantly affected by treatment, dose per day, or an interaction of the two factors, but 3X birds tended to have more bile volume/liver mass than 1X birds (Figure 5.4, Two-way ANOVA, treatment: $F_{1,18}=0.71$, $p=0.41$; dose per day: $F_{1,18}=4.05$, $p=0.06$; treatment*dose per day: $F_{1,18}=1.96$, $p=0.18$).

Migration

CORT and DMSO-treated birds displayed migratory behavior during both observation times. The small sample size prevents us from conducting statistical analyses on the data. However, the percent of time spent migrating appears similar in both treatment groups at each sampling point (Table 5.2).

End of breeding season to autumnal molt

Mass

All birds lost some mass over the course of the experiment (Table 5.3, Figure 5.5). During hormone treatment, CORT-treated birds lost more mass than the other groups (Table 5.3, Figure 5.5). When each time point during the treatment period was analyzed

separately, CORT-treated birds weighed significantly less after 4 and 8 days of treatment but not 12 days of treatment (Table 5.3). Birds in all treatment groups showed moderate to no mass loss for 20 days post-treatment, after which mass in all treatment groups increased (Table 5.3, Figure 5.5). Although we followed the mass of birds for longer than 55 days post-treatment, after this point some animals had finished molting and were no longer comparable to those in molt.

Fat

There was no significant affect of treatment alone on total fat score (Table 5.3). Fat scores in all treatment groups changed significantly over time (Table 5.3, Figure 5.5). The two control groups steadily lost fat stores (Table 5.3, Figure 5.5). The CORT-treated birds showed an initial drop in fat scores followed by a complete recovery to pre-treatment levels (Table 5.3, Figure 5.5). For 55 days post-treatment, fat stores in all birds changed significantly with time (Table 5.3, Figure 5.5). The two control groups showed a fall and then rise of fat stores while fat stores in the CORT-treated birds steadily declined (Figure 5.5).

Flight muscle

During the treatment period, CORT-treated birds lost flight muscle and the two control groups increased flight muscle (Table 5.3, Figure 5.5). 5 days after the last treatment, flight muscles scores were similar between the control and CORT-treated birds (Table 5.3). In the 55-day period post-treatment, the flight muscle score in all groups changed over time, with most birds having less muscle at the end of the trial (Table 5.3, Figure 5.5).

Food intake

Treatment alone did not affect food intake during the experiment (Table 5.3). However, CORT-treated birds showed a rise in food intake over time and the two control groups had fairly constant food intake over time (Table 5.3, Figure 5.5).

Molt

The initiation of molt in CORT-treated birds was delayed by 10 days (Tables 5.4, 5.5). Treatment did not affect duration and termination of molt or the maximum number of feathers that birds had growing at once (Tables 5.3, 5.4, 5.5). Molt intensity was not affected by treatment and decreased over the course of molt in all treatment groups (Table 5.3, Figure 5.5). There was no difference among treatment groups in the length or number of fault bars in the P1 and P9 feathers (Table 5.3).

Notes on multiple statistical test

In this study, we conducted 139 statistical tests. We did not do Bonferroni corrections. We assume a 5% chance of finding a significant result by chance alone. Thus, we assume that 7 of our significant results are invalid, though it is impossible to determine which results these are. That we found 54 significant results in the study as a whole indicated to us that most of the observed, significant effects of hormone treatment were real.

Discussion

The effects of chronically-elevated CORT on body condition have been well-studied in birds and other vertebrate taxa and are the background against which we examined the effects of repeated, acute CORT administration (Dallman et al. 1993, Sapolsky et al. 2000, Wingfield and Romero 2001). CORT implants have a mixed effect on body mass, causing decline in some songbird studies but not others (Wingfield 1988, Gray et al. 1990). However, birds and rodents exposed to chronic stressors (instead of just CORT) reliably lose weight (Marra and Holberton 1998, Dallman and Bhatnagar 2001, Müllner et al. 2004, Rich and Romero 2005). Mass loss in chronically-stressed fish is partially attributed to a significant increase in the gallbladder's bile retention, which hinders energy assimilation in the gut (Earley et al. 2004). CORT implants increase fat stores in both wild and captive birds (Wingfield and Silverin 1986, Wingfield 1988, Gray et al.

1990). Glucocorticoids have a catabolic action on skeletal muscle (Chester Jones et al. 1972, Norris 1997), and songbirds implanted with CORT show wasting in the flight muscles (Gray et al. 1990, Honey 1990, Astheimer et al. 2000, Bauchinger and Biebach 2001, Wingfield and Romero 2001).

The effect of CORT on feeding rate in birds is equivocal (see review in Astheimer et al. 1992). The current theory is that CORT is permissive to food intake in birds but that high CORT levels do not always increase food intake (Gray et al. 1990, Astheimer et al. 1992, Landys et al. 2004a, Landys et al. in press). It is unclear whether this lack of CORT effect on food intake is due to inaction of the hormone or the presence of *ad libitum* food in the captive setting overriding the motivation for CORT-treated animals to feed (Astheimer et al. 1992). On the contrary, evidence from fish suggests that the glucocorticoid-induced increases of glucose, amino acids, and free fatty acids in the blood may in fact suppress the appetite (Gregory and Wood 1999).

Body condition and repeated, acute CORT pulses

Repeated, acute elevations of CORT caused a decline in body condition in both experiments. CORT-treated birds had lower body mass and reduced muscle stores but did not have increased fat stores. The loss of muscle and body mass parallel each other in both experiments. Because CORT-treated birds did not increase fat stores, the loss of muscle mass was probably the cause for the loss of body mass, as has been suggested in chronically-stressed stonechats (*Saxicola torquata*) (Scheuerlein et al. 2001). As expected given their decline in body mass, CORT-treated birds ate less than control birds. Birds dosed with CORT 3 times a day had decreased body condition but birds dosed once a day did not, indicating that CORT's effects on body condition were dose dependent.

In the early breeding season study, the mass of 3X CORT-treated birds diverged from the mass of control birds with the onset of migratory restlessness. CORT treatment is

usually associated with increased nighttime restfulness in birds (Buttemer et al. 1991). The expression of migratory restlessness makes this energy-saving restfulness impossible and could be responsible for initiating the observed decline in mass.

Why we did not see an affect of CORT treatment on fat is unclear. The most common result in CORT implant studies is to see no change in body mass and an increase in fat stores (Wingfield and Silverin 1986, Gray et al. 1990). In our study, CORT treatment caused a decline in mass. Because our birds lost weight in response to CORT, they likely did not have the available internal stores to convert into fat.

The release of free fatty acids in response to glucocorticosteroid treatment can be dose-dependent (Mukherjee and Mukherjee 1973). Our low-level CORT pulses may have been insufficient to cause much free fatty acid release. However, if these pulses did cause free fatty acid release, the released energy could have accommodated increases in activity or metabolism, potentially resulting in a balance between the lipid breakdown and deposition actions of CORT (Breuner et al. 1998, Landys et al. in press). We attempted to quantify the behavioral effects of the CORT-treatment in this experiment, but our observations were confounded by the disturbance of handling the animals while dosing (Christine Askew, unpublished data). Why the CORT-treated birds did not respond to their endogenously high baseline CORT levels with increased fat stores is curious (Chapter 4). The pulsatile nature of endogenous CORT release versus the tonic release in CORT-implanted birds may affect the development of fat stores (Freedman et al. 1986, Wingfield and Silverin 1986, Gray et al. 1990).

We found no significant effect of CORT treatment on bile storage. However, our ability to detect differences in bile storage among groups may have been compromised by the fact that all treatment groups had *ad libitum* access to food before sacrifice. The trend for greater bile retention in 3X birds than in the 1X birds is curious. The more frequent

handling of the 3X group most likely caused more frequent rises in CRF (Boyd et al., unpublished data), which may have increased bile retention (Earley et al. 2004).

The recovery of body condition after CORT dosing was rapid. Five days after the cessation of treatment, muscle stores were once again equivalent among the treatment groups. However, CORT treatment delayed molt by at least 10 days, indicating that exposure to repeated, acute doses of CORT can have effects beyond the dosing period. This delay may be due to preferential deposition of protein in re-growing skeletal muscles or the persistent effects of CORT-mediated changes in gene expression (Romero et al. 2005). Given CORT's inhibitory effect on feather growth (Romero et al. 2005), the high endogenous baseline CORT levels in CORT-treated birds could have delayed molt onset (Chapter 4).

A delay in molt could have serious implications in the wild. Gambel's white-crowned sparrow breeds in the high-arctic king (Cortopassi and Mewaldt 1965, Chilton et al. 1995b). Individuals with delayed migration are more likely to encounter fall storms that could cause reductions in internal energy stores and, thus, an impaired ability to migrate south. However, a delay in molt initiation birds does not necessarily equate with a delay in molt completion. Due to the effects of decreasing day length, birds that initiate molt later molt faster (Morton and Morton 1990, Dawson et al. 2000, Dawson 2004). Faster-molting birds produce lower quality primaries that are lighter, shorter, more asymmetrical, less rigid, have thinner rachis, and are less resistant to wear (Dawson et al. 2000, Dawson 2004). Our failure to find differences in molt duration or intensity or in feather quality (fault bars and feather length) was most likely because we did not change day length during our experiment. We assume that, with more natural light cues, the CORT-treated birds with delayed molt would have suffered negative consequences on their flight performance and thermoregulatory costs due to quickly-grown, poor-quality feathers (Dawson 2004).

Effects of Life Stage

The negative effect of CORT on body and flight muscle mass were immediate in the late breeding season but not the early breeding season. We suspect that modulation of the HPA axis was partially responsible for the differential timing of body condition decline. The HPA axis changes within the breeding stage and with other life stages in most vertebrates, including the white-crowned sparrow (Romero and Wingfield 1999, Romero 2002). In addition, the response to HPA axis manipulation in this species also varies with life stage (Astheimer et al. 1994, Landys et al. 2004a).

Corticosterone binding globulin (CBG) levels also change seasonally in songbirds (Breuner and Orchinik 2001). We found that, before treatment, CBG levels were higher at the beginning of the breeding season than at the end of the breeding season (Chapter 4). The more rapid and severe drop in body condition at the end of the breeding season could have been due to the birds' lower CBG levels. High CBG levels could have protected birds in the early breeding season from the effects of CORT. This protective effect was likely lost as baseline CORT levels in CORT-treated birds rose without a concurrent increase of CBG levels (Chapter 4).

Variation in receptor fields with season could also help explain why the effects of CORT treatment differ in birds in different life-history stages (Breuner and Orchinik 2001). For example, the loss of GR receptors suppresses appetite, a behavioral phenomenon observed in this study (Landys et al. 2004a).

Causation of Body Condition Decline

Our study revealed a conundrum in terms of the relationship between chronic stress and body condition: do frequent stress responses change the HPA axis to cause elevated baseline CORT levels, which then cause decreased body condition? or do frequent stress responses and resultant high pulses of CORT cause decreased body condition, which then causes elevated baseline CORT levels? This conundrum highlights the

interplay between CORT's protective role during unpredictable stressors and its role in maintaining basic energy balance in the body.

Birds treated 1X showed little change in body condition even though they had elevated baseline CORT levels (Chapter 4). Because endogenous baseline CORT levels are not correlated with body condition degradation in these birds, we believe that the frequent peaks of CORT are responsible for degrading body condition. Thus, the number of times a day an animal mounts a stress response may affect its body condition and, possibly, survival. An example from the field provides further evidence for this hypothesis: hoatzin fledglings displayed a dose-response effect to the stress of tourist activity, with survival being higher in a year when tourist activity was low compared to other years of higher tourist exposure (Müllner et al. 2004).

Study Design

In this study, we focused on the actions of CORT alone. By administering just CORT, we did not account for the effects of other hormones in the HPA axis, namely CRF and ACTH. CRF potentiates changes in behavior, digestion, and metabolism (Rothwell 1990, Lowry and Moore 1991, Richardson et al. 2000, Earley et al. 2004). For example, in the white-crowned sparrow, intracerebroventricular CRF decreases food intake in wintering birds and inhibits territorial behavior in free-living, breeding males (Romero et al. 1998a, Richardson et al. 2000).

By dosing the animals with the same amount of CORT over time, our experimental design did not accommodate for the fact that an animal's perception of a stressor can change over time. Prior experience, learning, and the ability to predict or control stressful events are all known to affect how animals react to a given novel or familiar stimulus (Sapolsky 1987, Dallman and Bhatnagar 2001, Greenberg et al. 2002). Though our experimental model did not account for the dynamic nature of the stress response and its interaction with the animal's physical and psychological condition, it was a

fairly conservative design for our question of how frequent exposure to elevated levels of CORT can affect an animal.

The exposure and response to capture of all handled birds likely affected their psychological state and HPA axis. However, because the handled DMSO-control birds in the late breeding season experiment had similar body condition to the birds that were not regularly disturbed and handled, we assume that the stimulation due to handling did not result in chronic stress.

Conclusion and Implications

Our study demonstrated both the effectiveness of a novel way to administer small doses of CORT and the implications of repeated, acute pulses of CORT. We found that frequent pulses of CORT lowered body condition. We demonstrated that prior experience of frequent pulses of CORT can impact future life stages. Our limited study on the migratory behavior of 3X birds suggested no difference in vernal migratory behavior between the CORT and control-treated birds, indicating that the urge to migrate is expressed even in animals with poor body condition.

Our work demonstrated a threshold response to CORT administration: body condition declined little with 1X CORT but declined significantly with 3X CORT. In addition, life stage affected the response to CORT administration. Birds dosed with CORT in the late breeding season showed an immediate decline in body condition while birds dosed with CORT in the early breeding stage were more resistant to declines in body condition. Variation in sensitivity to CORT has been documented in this and other species before (Breuner et al. 1998, Astheimer et al. 2000, Landys et al. 2004a).

The use of physiological tools to study ecology and behavior provides an understanding of the mechanisms behind the patterns that we see in nature (Romero 2004, Wikelski and Cooke 2006). As more studies use endocrine techniques to explore the impacts of

humans on wild animals, the need for valid and relevant laboratory models with which field data can be interpreted increases.

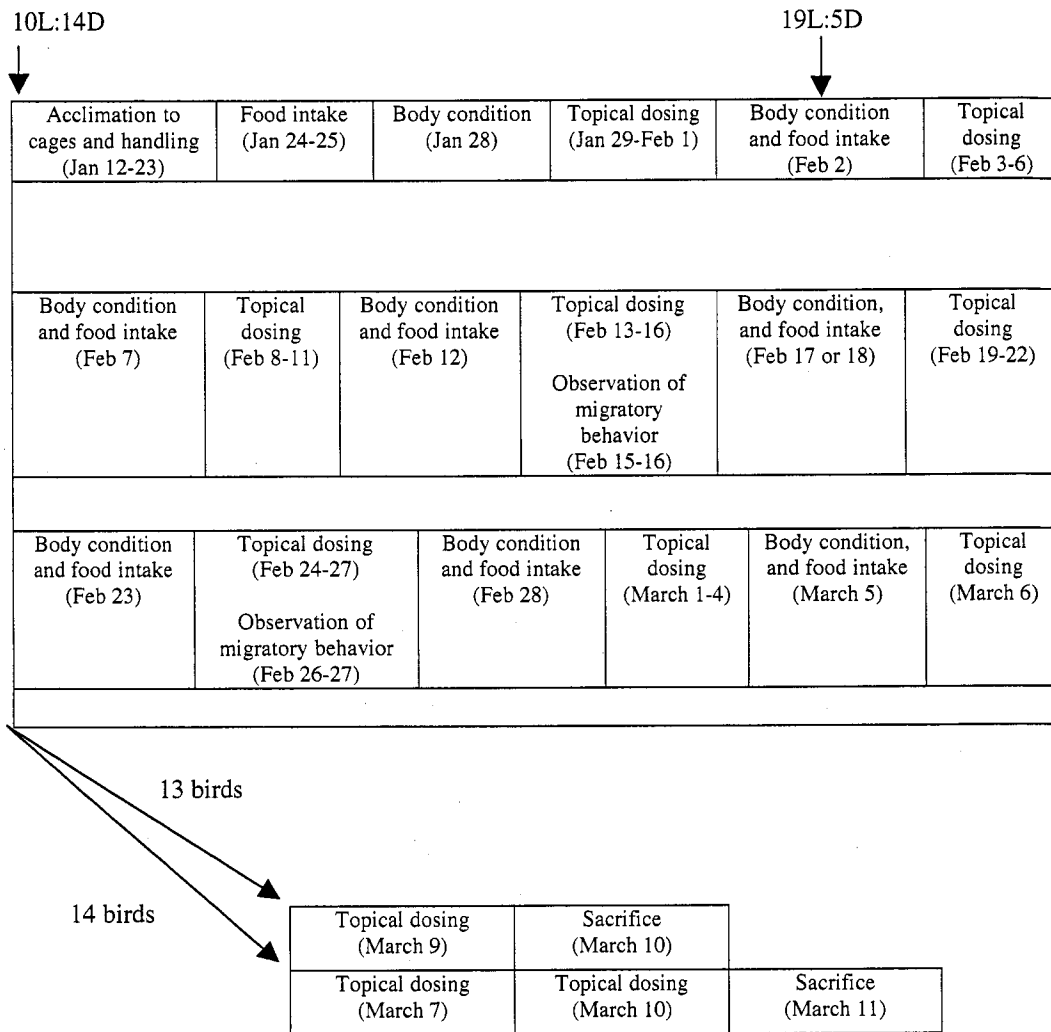


Figure 5.1. Schedule for the early breeding season experiment.

Acclimation to handling (March 30-April 13)	Body condition (April 14)	Topical dosing (April 15-18)	Body condition and food intake (April 19)	Topical dosing (April 20-23)
Body condition and food intake (April 24)	Topical dosing (April 25-28)	Body condition and food intake (April 29)	Body condition and molt once every 5 days (May 1-August 26)	

↑
Photorefractory

Figure 5.2. Schedule for the late breeding season experiment.

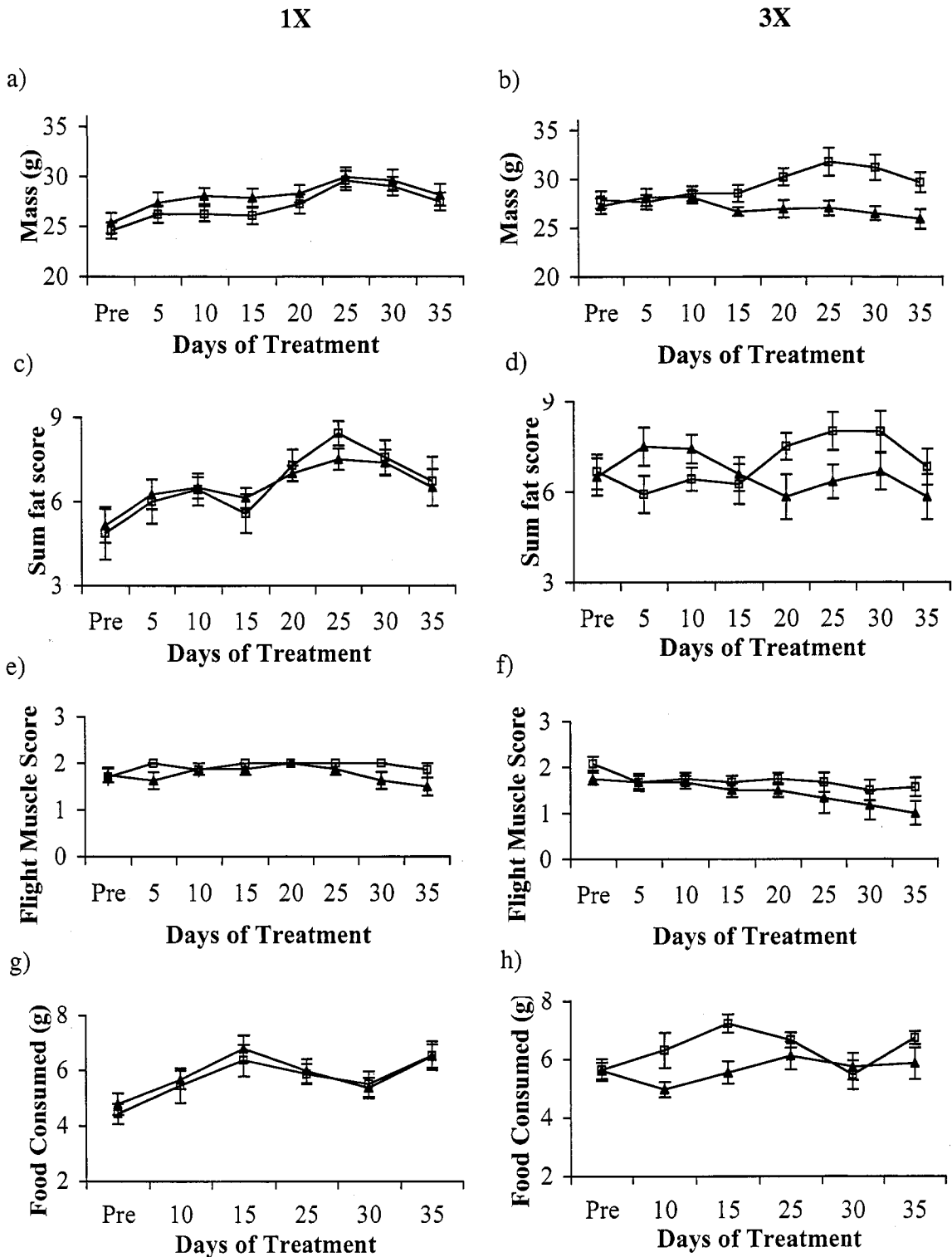


Figure 5.3. Mass (a, b), fat stores (c, d), flight muscle score (e, f), and food intake (g, h) measures in birds treated once (left) or three (right) times a day with CORT dissolved in DMSO (triangles) or DMSO only (squares) in the early breeding season. The data are presented as a mean \pm standard error. Statistical analyses are discussed in the text.

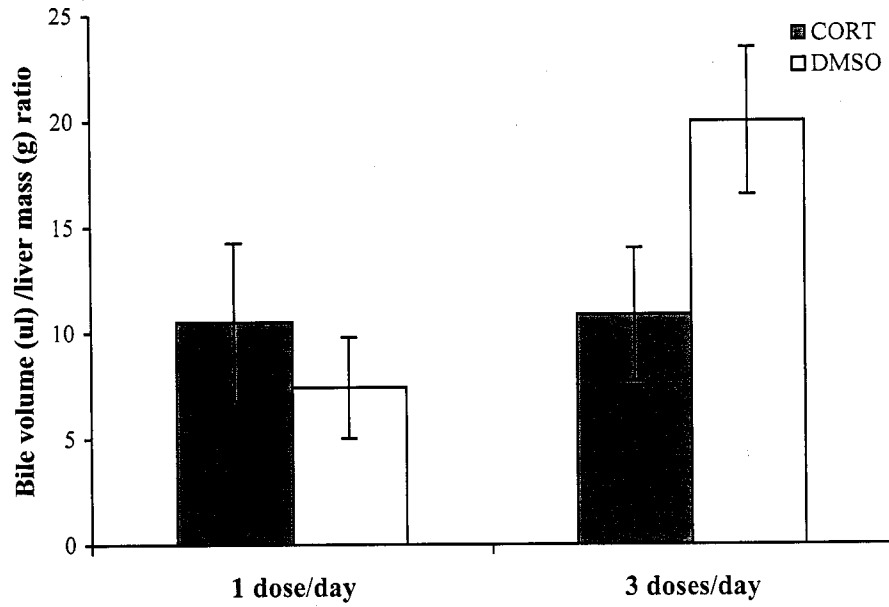


Figure 5.4. Bile volume/liver mass ratio \pm standard error in birds treated either once or three times a day with CORT dissolved in DMSO (CORT) or only DMSO. 3X birds tended to retain more bile per unit liver tissue.

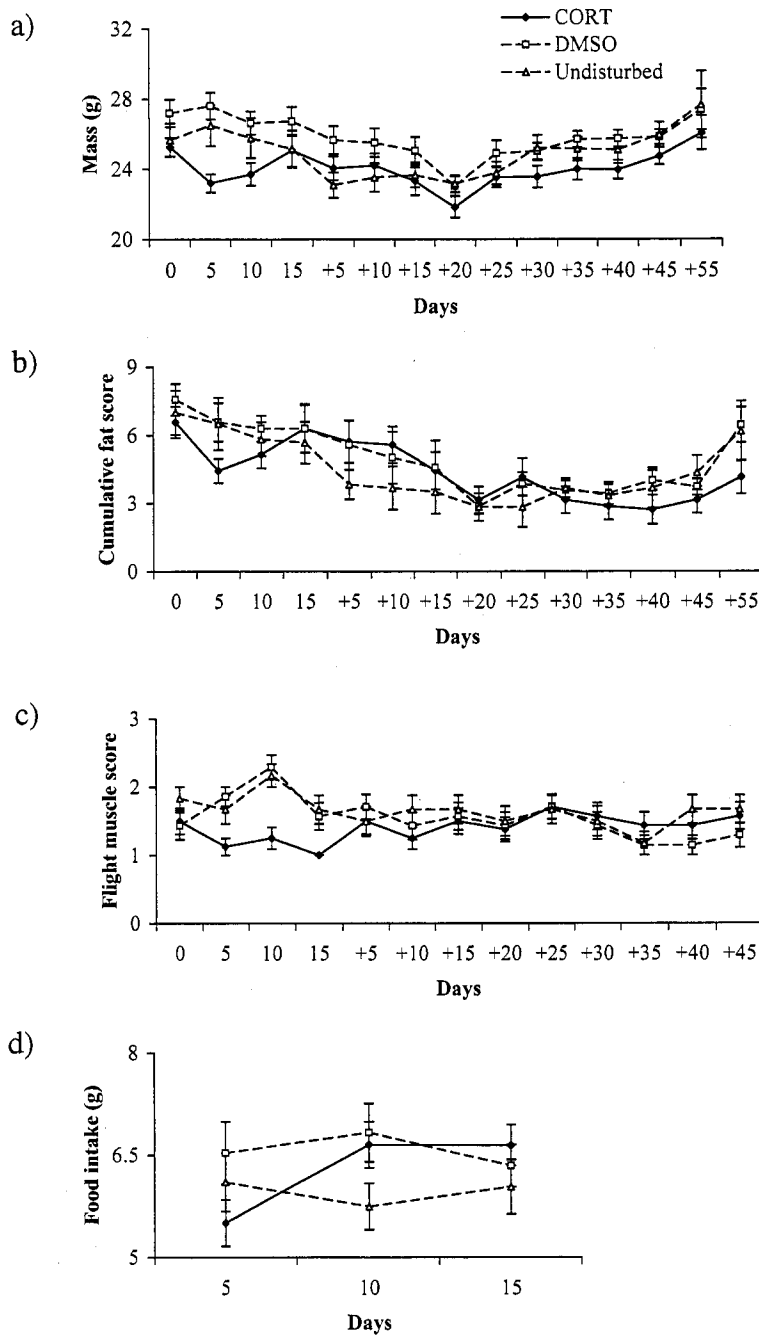


Figure 5.5. Mass (a), fat (b), flight muscle score (c) and food intake (d) in birds that were not handled (Undisturbed) or were treated three times a day with CORT dissolved in DMSO (CORT) or DMSO only (DMSO) in the late breeding season. Treatment started on day 1 and continued until day 15. All days after treatment are noted with a "+". The data are presented as a mean \pm standard error. Statistical analyses are discussed in the text

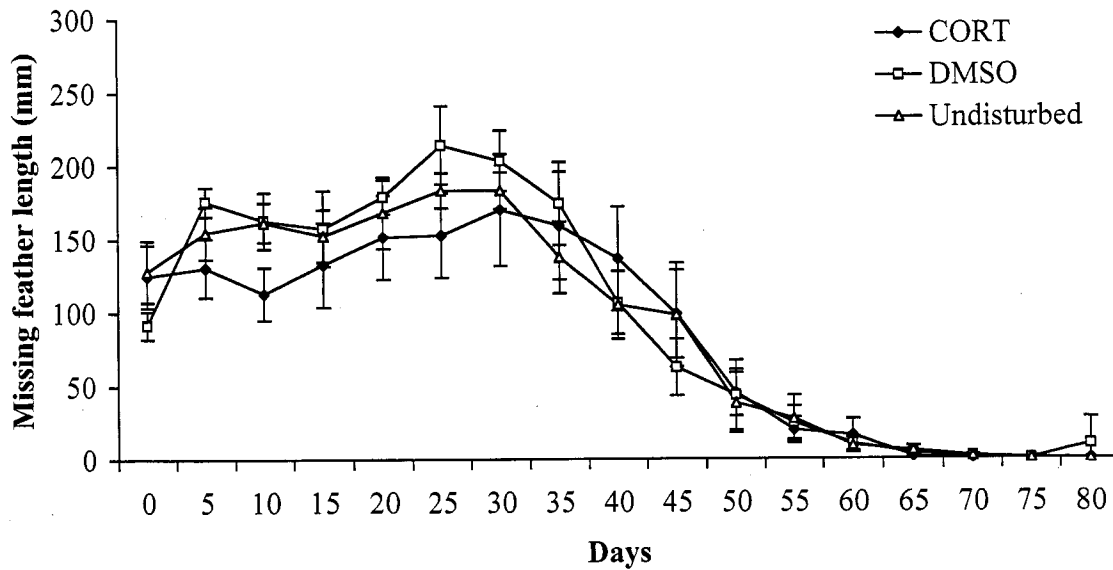


Figure 5.6. Molt intensity over time in birds that were not handled (Undisturbed) or treated with CORT dissolved in DMSO (CORT) or DMSO alone (DMSO). Day 0 is the first day that molt was observed in each bird. Molt intensity is the amount of feather tissue missing in the primary and secondary feathers on one wing. The data are presented as a mean \pm standard error bars. Statistical analyses are discussed in the text.

Table 5.1. Results of one or two-way repeated measures MANOVA from the early breeding season experiment. Each row represents a different statistical test. Significant results are in bold and trends are italicized.

		Treatment			Dose per day			Treatment*Dose per day			Time		
		F	DF	p	F	DF	p	F	DF	p	F	DF	p
<i>Mass</i>													
	Mid	0.02	1,35	0.90	3.92	1,35	0.056	3.63	1,35	0.06	4.86	4,32	0.0036
	End	1.65	1,23	0.21	0.62	1,23	0.44	6.90	1,23	0.01	15.28	7,17	0.0001
	1X	0.92	1,13	0.35							11.96	7,7	0.002
	3X	7.87	1,10	0.02							5.83	7,4	0.05
<i>Flight muscle</i>													
	Mid	2.18	1,35	0.15	3.84	1,35	0.058	0.20	1,35	0.66	0.22	4,32	0.92
	End	4.24	1,23	0.05	8.98	1,23	0.006	0.26	1,23	0.61	1.84	7,17	0.14
<i>Fat</i>													
	Mid	0.24	1,35	0.63	1.89	1,35	0.18	0.003	1,35	0.95	5.14	4,32	0.0026
	End	1.11	1,23	0.30	1.24	1,23	0.28	0.75	1,23	0.40	5.39	7,17	0.0022
<i>Food intake</i>													
	Mid	1.31	1,35	0.26	0.97	1,35	0.33	4.99	1,35	0.03	13.05	2,70	<0.0001
	End	2.41	1,23	0.13	1.3	1,23	0.27	4.5	1,23	0.04	5.68	5,115	0.0001
	1X – mid	0.63	1,13	0.44							9.37	2,26	0.0009
	1X – end	0.16	1,13	0.69							7.19	5,65	<0.0001
	3X – mid	6.51	1,22	0.02							3.7	2,44	0.03
	3X – end	7.04	1,10	0.02							1.03	5,50	0.41

Table 5.1 continued

		Time*Treatment			Time*Dose per Day			Time*Treatment* Dose per Day		
		F	DF	p	F	DF	p	F	DF	p
<i>Mass</i>										
	Mid	0.84	4.32	0.51	1.22	4.32	0.32	0.72	4.32	0.58
	End	0.77	7.17	0.65	0.82	7.17	0.59	0.73	7.17	0.65
	1X	1.16	7.7	0.43						
	3X	0.64	7.4	0.72						
<i>Flight muscle</i>										
	Mid	0.24	4.32	0.91	2.69	4.32	0.05	0.60	4.32	0.67
	End	0.93	7.17	0.51	0.71	7.17	0.67	0.27	7.17	0.96
<i>Fat</i>										
	Mid	2.06	4.32	0.11	1.7	4.32	0.17	0.90	4.32	0.47
	End	1.2	7.17	0.35	1.74	7.17	0.16	0.87	7.17	0.55
<i>Food intake</i>										
	Mid	1.3	2.70	0.28	2.7	2.70	0.07	1.31	2.70	0.27
	End	0.97	1.23	0.55	1.5	1.23	0.20	1.7	1.23	0.14
	1X - mid	0.03	2.26	0.97						
	1X - end	0.15	5.65	0.98						
	3X - mid	3.63	2.44	0.03						
	3X - end	2.09	5.50	0.08						

Table 5.2. Percent migratory behavior during the onset and full expression of migratory restlessness in the early breeding season. Four birds in each treatment group were observed every 20 sec for the 40 min immediately after nightfall.

<i>Treatment</i>	<i>% Migratory Activity</i>	
	<i>Onset of Migration</i>	<i>Full Migration</i>
CORT	26.10±19.63	65.61±28.60
DMSO	35.27±20.39	59.98±15.22

Table 5.4. Results from Holm-adjusted χ^2 analyses testing for differences in the timing of molt initiation and termination among treatment (undisturbed, CORT-treated, and DMSO-treated). The two significant results are in bold and indicate that molt initiation was delayed in the CORT-treated group.

	χ^2	<i>DF</i>	<i>p</i>
Initiation			
May 10	3.44	2,18	0.18
May 15	22.94	2,18	<0.005
May 20	9.36	2,18	0.037
May 25	7.35	2,17	0.076
May 30	4.63	2,17	0.20
Termination			
July 5	5.30	2,17	0.47
July 10	2.29	2,17	0.58
July 15	2.78	2,17	0.58
July 20	5.09	2,17	0.47
July 25	1.27	2,17	0.58
July 30	1.09	2,17	0.58
Aug 4	2.79	2,17	0.58

Table 5.5. Number of birds molting or non-molting in each treatment group at initiation and termination of molt. Dates with significant difference between groups are in bold.

	<u>CORT-treated</u>		<u>DMSO</u>		<u>Undisturbed</u>	
	<i>Molting</i>	<i>Non-molting</i>	<i>Molting</i>	<i>Non-molting</i>	<i>Molting</i>	<i>Non-molting</i>
Initiation						
May 10	0	8	2	5	1	5
May 15	0	8	6	1	6	0
May 20	4	4	7	0	6	0
May 25	4	3	7	0	6	0
May 30	5	2	7	0	6	0
Termination						
July 5	7	0	4	3	4	2
July 10	6	1	4	3	3	3
July 15	6	1	4	3	3	3
July 20	6	1	2	5	3	3
July 25	5	2	2	5	3	3
July 30	3	4	2	5	1	5
Aug 4	2	5	1	6	0	6

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VITA

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