The Evolution of the Stress Axis in Ground Squirrels

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Ecology and Evolutionary Biology
University of Toronto

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Abstract

The hypothalamic-pituitary-adrenal (HPA) axis, or stress axis, is a key physiological system that mediates the relationship of the organism with its environment. Because activation of the HPA axis mobilizes energy stores for immediate use, but sustained activation can have deleterious effects on survival, the HPA axis has been implicated in the tradeoff between reproduction and survival. In this thesis, I investigate whether there is an association between one life history trait, reproductive lifespan, and the functioning of the HPA axis as predicted by the “adaptive stress hypothesis”.

The adaptive stress hypothesis predicts that species adopting life history strategies characterized by short lifespans and early reproduction should maximize the energy available for reproduction through high levels of circulating glucocorticoids caused by the dysregulation of the HPA axis in the breeding season, whereas those characterized by long lifespans and extended reproduction should maintain a functioning HPA axis with low levels of glucocorticoids throughout life. To test this hypothesis, I studied five species of ground squirrels that vary dramatically in male reproductive lifespan: arctic, Richardson’s, Columbian, thirteen-lined, and Franklin’s ground squirrels (*Urocitellus parryii*, *U. richardsonii*, *U. columbianus*, *Ictidomys tridecemlineatus*, and *Poliocitellus franklinii*).

I used a stress profile to characterize the HPA axis of male ground squirrels immediately before and immediately after the breeding season. The stress profile included measures of plasma glucocorticoid concentrations, determinants of plasma glucocorticoid concentrations (corticosteroid binding globulin levels, adrenal sensitivity/capacity, negative feedback, and intrinsic restraint), and markers of the biological effects of glucocorticoids (energy mobilization,
health, and immune function). Contrary to the adaptive stress hypothesis, I found no relationship between reproductive lifespan and postbreeding glucocorticoid levels. Species also varied significantly and unexpectedly in how determinants of glucocorticoid levels changed over the breeding season, and in how glucocorticoids levels translated into biological effects. I also observed unexpected patterns of individual variation within species. Thus, life history alone did not predict HPA axis functioning. My results suggest that the HPA axis is so flexible in its functioning, that we will need to adopt a much more detailed model of the HPA axis in order to fully understand the relationship between the HPA axis and life history variation.
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Chapter 1

1 Introduction

Life history traits are evolved traits that contribute directly to an individual’s fitness; they include age at first reproduction, number of offspring produced, and reproductive lifespan. The combination of life history traits in any species or group of individuals within a species is the life history strategy. Although the diversity of life history strategies in nature is impressive, we do not observe all possible combinations of traits. For example, most species are either long-lived with low reproductive rates or short-lived with high reproductive rates, leading to the conclusion that there are constraints on the evolution of life history strategies (Ricklefs and Wikelski 2002).

One possible reason we do not see some combinations of life history traits would be the presence of a tradeoff between two traits. For example, there is considerable evidence that a tradeoff between reproduction and survival is ubiquitous in nature (Reznick 1985; Reznick 1992). However, the physiological basis for such tradeoffs remains elusive (Ricklefs and Wikelski 2002; Stearns 2000).

My research focuses on the impact and functioning of the hypothalamic-pituitary-adrenal (HPA) axis and its role in life histories. The HPA axis is one of the key physiological systems that mediates the relationship of the organism with its environment, both in terms of short-term responses and long-term evolutionary responses to particular ecological and environmental pressures. Glucocorticoids orchestrate a whole-organism response by influencing the expression of thousands of genes that are involved in controlling metabolism, growth, repair and reproduction, manage resource allocation (Phuc Le et al. 2005). Given its central role, the HPA axis has been implicated in the tradeoff between reproduction and survival (Wingfield and Sapolsky 2003). Activation of the HPA axis plays a critical role in mobilizing energy stores for immediate use, but sustained activation can have deleterious effects on survival (Sapolsky et al. 2000).

In this thesis, I investigate whether there is an association between one life history trait—reproductive lifespan—and the functioning of the HPA axis. In this Introduction, I provide an overview of the physiology of the HPA axis, followed by a brief review of the effect of glucocorticoids (GCs) on life history traits in mammals. Next, I review the characteristics of
ground squirrels that make them an excellent group in which to test the role of GCs in supporting or constraining life history strategies. Finally, I lay out the specific objectives of each chapter.

1.1 The physiology of the HPA axis

The HPA axis is typically portrayed as shown in Figure 1.1. Under this simplified model of the HPA axis, circadian rhythms or the perception of a stressor stimulate specific neurons in the hypothalamus to release corticotropin-releasing hormone and arginine vasopressin, which stimulate the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) into the blood. ACTH then stimulates the adrenal cortex to produce GCs. Once in the blood, most of the hormone is normally bound by corticosteroid binding globulin (CBG). Because CBG is a large molecule, bound hormone cannot pass out of the circulatory system under normal circumstances (Mendel 1992); this renders bound hormone biologically inactive. The unbound (free) hormone is able to leave the circulatory system and exert biological effects on every tissue in the body, primarily through binding to cytosolic GC receptors.

Under non-stress conditions, GCs fluctuate at relatively low concentrations in a circadian rhythm primarily governed by the hippocampus (Bamberger et al. 1996). As GC levels increase, the hormone activates mineralocorticoid receptors in the hippocampus, which suppresses further release of GCs (de Kloet et al. 1998; Joëls et al. 2008). At these basal levels, GCs are central to daily rhythms. For example, changes in basal GCs are involved in stimulating appetite and food-searching behaviour (Sapolsky et al. 2000). However, when an organism perceives a stressor—any threat to homeostasis coming from the internal or external environment—the sympathetic nervous system causes the release of catecholamines to generate immediate physiological effects (over a period of seconds to minutes) while the HPA axis begins a neuroendocrine cascade that ultimately causes the release of relatively high GC concentrations over a period of several minutes to hours (Sapolsky et al. 2000). Under acute stress conditions, negative feedback is accomplished by activation of GC receptors in the pituitary, hypothalamus and hippocampus which eventually terminate further GC production (de Kloet et al. 1998; Keller-Wood and Dallman 1984). Thus, acute stress concentrations of GCs help the animal respond to threats but are also self-terminating, allowing the animal to return to its basal conditions.

Stress-induced GC levels have a variety of effects, including promoting escape behaviours (Wingfield et al. 1998) and suppressing growth (Sapolsky et al. 2000). However, the hallmark
role of stress-induced GC levels is their metabolic effects. These include reducing uptake of glucose in peripheral tissues, thereby increasing blood glucose levels and delivery to exercising muscles, promoting lipolysis and protein catabolism to fuel hepatic gluconeogenesis (i.e. conversion of glycerol and amino acids into glucose), and promoting glycogen deposition in the liver (Sapolsky et al. 2000). These effects are aimed at increasing the immediate availability of stored energy, but this energy comes at some expense. Reproductive and food-seeking behaviour is quickly suppressed in most species (Sapolsky et al. 2000) and prolonged GC exposure begins to have detrimental effects such as impairing immune function, and catabolism of tissues that will need to be rebuilt when the stressor finally ends (Sapolsky 2002; Sapolsky et al. 2000).

1.2 Reproductive lifespan and the adaptive stress hypothesis

For males of many species, reproduction is an inherently stressful time. This is especially the case for those species with a single, intensely competitive, annual breeding season (Boonstra 2005). In such species, the HPA axis poses a challenge and an opportunity. In the face of a predictable stressor, activation of the HPA axis can mobilize energy to support reproductive efforts. However, sustained exposure to GCs over the breeding season can potentially inhibit reproduction and have detrimental effects on long-term survival.

The ability of the HPA axis to mobilize stored energy, at a cost to future survival, makes GCs a prime candidate for mediating the tradeoff between reproduction and survival (Wingfield and Sapolsky 2003). If this is the case, then we expect to find a predictable pattern in GC levels (i.e. plasma GC concentrations) and life history strategy. An example of just such a pattern comes from a series of studies of dasyurid marsupials in Australia. In at least 10 dasyurid species the entire male population dies at the end of their first breeding season (Bradley 2003; Lee and Cockburn 1985). In at least four of these species (Antechinus stuartii, A. swainsonii, A. flavipes, and Phascogale calura) there is a common physiological progression among males. As the breeding season approaches, free GC levels increase due to the cumulative effects of increased total GC production, decreased CBG levels, and (at least in P. calura and A. swainsonii) failure of negative feedback (Bradley 1987; Bradley 1990; Bradley et al. 1980; Lee and Cockburn 1985; McDonald et al. 1981). The elevated free GC levels lead to gastric ulcers, suppression of immune and inflammatory responses, increased parasitism, and shifts in hematological
parameters (Barker et al. 1978; Bradley et al. 1980; Cheal et al. 1976) that ultimately result in the male die-off. In contrast, iteroparous marsupials like the fat-tailed dunnart, *Sminthopsis crassicaudata*, show no relationship between testosterone and CBG levels, and although cortisol concentrations fluctuate over the breeding season, they are lower than the maximum CBG binding capacity, resulting in low free GC levels (McDonald et al. 1981). This research suggested a link between the evolution of male reproductive lifespan and HPA axis function.

The distinct endocrine profile of the semelparous marsupials led Lee and Cockburn (1985) to propose that the gluconeogenic effect of cortisol allow these species to derive energy from the breakdown of muscle, thereby freeing them to spend time seeking mates instead of foraging during a time of food scarcity. They also predicted that this strategy could apply to other small mammals and could explain the spring population declines observed in a number of vole species (Braithwaite and Lee 1979; Lee and Cockburn 1985). This “adaptive stress hypothesis” focused on the potential for GC excess caused by the dysregulation of the HPA axis to support life history strategies characterized by short lifespans and early reproduction. However, subsequent studies have failed to find a similar role for GCs in the semelparous didelphid marsupial, the Virginia opossum (*Didelphis virginiana*; Woods and Hellgren 2003), or in other mammal species with short male reproductive lifespans like the meadow vole (*Microtus pennsylvanicus*; Boonstra and Boag 1992). Boonstra and Boag (1992) pointed out that, although male meadow voles are short-lived, they typically have more than one opportunity to mate because of an extended breeding season, postpartum insemination in females, and a short gestation period. They therefore proposed that the adaptive stress response would be limited to those species in which there is a single annual breeding season as well as a low survival rate between years. In contrast, they proposed that species with longer reproductive lifespans should have a “homeostatic” stress response characterized by intact negative feedback, maintenance of CBG levels, and a more modest increase in free GCs. Under this hypothesis, we predict a negative relationship between reproductive lifespan and GCs in the breeding season.

My primary objective was to test the adaptive stress hypothesis using a comparative approach with five species of North American ground squirrels that vary in the likelihood of males surviving for more than one breeding season.
1.3 Ground squirrels as study organisms

Life history traits are determined by numerous internal and external factors, including phylogenetic constraints, physiological tradeoffs among life history traits, and ecological and environmental impacts on survival and reproduction (Stearns 2000). Thus, a tradeoff mediated by stress physiology is only one of several factors shaping a trait like male reproductive lifespan. To isolate the role of the HPA axis as much as possible, it is important to select a study system in which the other factors shaping life history traits are controlled for as much as possible. Ground squirrels are excellent study species in this regard.

I studied five species of ground squirrels that have mean male reproductive lifespans ranging from 1.1 to 2.7 years: arctic, Richardson’s, Columbian, thirteen-lined, and Franklin’s ground squirrels (Urocitellus parryii, U. richardsonii, U. columbianus, Ictidomys tridecemlineatus, and Poliocitellus franklinii). Two recent independent phylogenies of these species based on mitochondrial DNA (Harrison et al. 2003; Herron et al. 2004) show that all five species are relatively closely related. The analysis by Harrison et al. (2003) suggests that these species diverged from 10.7 to 1.3 million years ago. Figure 1.2 shows a simplified phylogeny to highlight the relative relatedness of the species to each other. By selecting closely related species, we hoped to minimize the role of phylogenetic constraints in shaping male reproductive lifespan.

The selected species are among the best-studied ground squirrels, so we know a great deal about their basic biology. They share very similar environmental and ecological features, with all five species living in environments characterized by long, cold winters during which the animals hibernate. The males emerge prior to females and the single, short annual breeding season begins a matter of days after the first females begin to emerge. Males in all species mate with multiple females. Thus reproduction is a similar challenge for all five species: males must emerge from hibernation ready to begin breeding with as many females as they can. These commonalities make these ground squirrels ideally suited to this type of comparative study.

1.4 Approach to measuring the HPA axis

One of the main approaches to studying how the HPA axis functions, particularly in the avian literature, is the capture stress protocol. This involves taking an initial blood sample within a
few minutes of capture, followed by one or more blood samples over the next 30 min to 1 h to measure the acute stress response (e.g. Wingfield 1995). Because it takes several minutes for plasma GCs to rise following an acute stressor, the initial blood sample provides baseline GC levels as they were prior to capture. The subsequent blood samples measure the acute stress response. In many mammals, however, trapping methods or the wariness of the animals preclude researchers from being close enough to the trap to obtain an initial blood sample within 3 min of capture (Boonstra 2005; Sheriff et al. 2011). Because logistics and the wariness of most of my species prevented me from obtaining blood samples within 3 min, I adopted a hormone challenge approach to characterizing the HPA axis.

The hormone challenge protocol is an attempt to probe several aspects of both the HPA axis and the downstream effects of stress in order to infer the recent experience of the animal in the field. Under my standard trapping protocol, the first blood sample was taken after ≥1 h of captivity and therefore provides GC levels under sustained acute stress. Immediately after taking the initial blood sample, I injected the animals with dexamethasone (DEX), an artificial GC that suppresses endogenous GC production by activating GC receptors in the pituitary (Cole et al. 2000; de Kloet et al. 1998). This probes one aspect of the negative feedback system; if GC levels have not fallen 2 h after the DEX injection, then we conclude that negative feedback is impaired (Boonstra 2005; Sapolsky and Altmann 1991). After taking a blood sample 2 h after the DEX injection, I injected adrenocorticotropic hormone (ACTH) and took three blood samples over the next two hours. By calculating the area under the GC response curve (the AUC), we can assess adrenal sensitivity/capacity. Adrenal mass is another rough indicator of adrenal capacity based on the assumption that adrenal hypertrophy should reflect greater capacity to produce adrenal steroids. I also used the ratio of the maximum free GC level in response to ACTH to initial free GC to measure “intrinsic restraint” which I define as the degree to which negative feedback at the level of both brain and pituitary, and sensitivity of hypothalamus and pituitary restrain GC production relative to the maximum adrenal production as measured by ACTH-induced GC levels. Because ACTH directly stimulates the adrenals, the maximum free GC levels following the ACTH injection are not tempered by negative feedback or reduced hypothalamic-pituitary sensitivity and thus represent the maximum ability of the adrenals to respond to ACTH stimulation. In contrast, the free GC level in the initial blood sample is the result of ongoing stimulation of the HPA axis as a result of capture stress but it is modulated by both
hypothalamic-pituitary sensitivity and negative feedback by GCs. Hence the ratio of these two levels (i.e. maximum/initial) represents a measure of the intrinsic restraint of the stress axis when challenged by a stressor. The higher the ratio, the stronger the intrinsic restraint.

In addition to the foregoing measures of the HPA axis itself, I wanted to assess the downstream effects of GCs (i.e. effects on the physiology of GC-sensitive tissues). By observing how downstream markers changed from the prebreeding to postbreeding session, I could draw inferences about the extent to which the HPA axis was activated during the breeding season. I assessed three factors in my downstream measures: energy mobilization, an index of condition, and an index of immune function. To assess the ability of the body to mobilize energy in response to sustained acute stress, I measured blood glucose and free fatty acid levels of animals acutely stressed by capture. Because GCs stimulate hepatic gluconeogenesis and decrease peripheral uptake of glucose (Dallman et al. 1993), chronic exposure to elevated GCs increases glycogen stores that can be rapidly converted to glucose when a stressor (like capture in live-traps) is encountered (Boonstra et al. 1998). Thus, an increase in stress-induced glucose levels from one trapping session to another indicates that the animal has experienced chronically elevated GC levels between sessions. In contrast, I expected that animals exposed to long-term elevations of GCs would have reduced free fatty acid levels due to the catabolism of fat stores. I measured hematocrit levels as a general indicator of health status (Boonstra et al. 2001; Clinchy et al. 2004; Kim et al. 2005) and white blood cell counts as a general indicator of immune function (e.g. Boonstra and McColl 2000); in both cases, a decrease was assumed to be an indication of chronic GC exposure.

1.5 Thesis objective and hypotheses

The overall objective of my thesis was to ask whether the functioning of the HPA axis during the reproductive season changes in a predictable way based on male reproductive lifespan or other traits in a group of closely related ground squirrel species. I was interested not only in how GC levels changed over the breeding season, but also in the mechanism of any increase or decrease in GCs and in the downstream effects of the GCs. Because I had to trap multiple species each field season, I could not monitor any one species over the entire course of the breeding season. Instead, I trapped males of each species in a prebreeding session (before females emerged) and a postbreeding session (immediately at the end of the breeding season). I then compared the
functioning of the HPA axis in the prebreeding and postbreeding animals to determine whether the changes matched those predicted by the adaptive stress hypothesis. Throughout my thesis, my emphasis is on the change in levels from prebreeding to postbreeding sessions, not the absolute levels in any one session.

Ideally, a description of the stress response would include baseline measures of both GCs and the downstream variables of interest. I could not obtain baseline measures for most species, but for variables like GC, glucose and free fatty acid levels, one would naturally expect sudden increases in response to the stress of capture (that is, after all, a major point of the stress response). For these variables I am missing that baseline data and, therefore, when I see a change in stress-induced values for these variables, I cannot infer that baseline values change in the same way. For other variables, like CBG, hematocrit, and white blood cell counts, it is not clear whether they will change rapidly in response to capture stress. For those variables that change over the course of capture, I am only able to report how stress-induced levels change from pre- to postbreeding and the changes in baseline values remain unknown. For those variables that do not change rapidly in response to capture stress, the changes I observe over the breeding period are the only changes happening; there are no unknown “baseline” changes. It was therefore important to determine which downstream variables under the sustained acute stress of capture differed from baseline conditions. In chapter 2 (published in General and Comparative Endocrinology; Delehanty and Boonstra 2009) I examine the effects of capture on the measures that I used to characterize the physiological state of the ground squirrels. In this study, I caught a number of Richardson’s ground squirrels from which I took baseline blood samples (collected in < 3 min after capture). I compared the GC levels and downstream measures of GC action in the baseline samples with the same measures obtained using my standard trapping procedure. This let me determine which measures were sensitive to capture stress, and which ones reveal only seasonal changes.

I also address the question of whether changes in acute stress GC levels mirror changes in baseline levels (chapter 3). At low GC concentrations negative feedback is primarily regulated by mineralocorticoid receptors in the hippocampus, whereas at higher GC levels such as those associated with acute stress, negative feedback is primarily regulated by glucocorticoid receptors in the hippocampus, hypothalamus and pituitary (de Kloet et al. 1998). In this way, basal GC and acute stress GC levels can be regulated independently. If baseline levels change
independently of acute stress levels (e.g. for reasons of change in reproductive state, season, social state; see Romero 2002), then missing a change in baseline levels could mask some important biological changes in the HPA axis. In Chapter 3 (submitted to General and Comparative Endocrinology) I compare how baseline GC levels are related to the stressed GC levels that I obtained using my standard trapping methodology in order to help me interpret the results of my main study (in Chapters 4 and 5). I obtained baseline and acute stress GC levels in Richardson’s ground squirrels in both the prebreeding and postbreeding trapping sessions and asked what we can learn from obtaining baseline blood samples that I did not uncover using stressed blood samples.

With the first two studies providing a context for the physiological measures I was using, in chapter 4 I test the adaptive stress hypothesis in arctic ground squirrels, a species in which most males live through one breeding season and some live through two breeding seasons (Delehanty and Boonstra 2011; published in Physiological and Biochemical Zoology). Previous work on the stress physiology of this species led Boonstra et al. (2001) to suggest that arctic ground squirrels showed some features of an adaptive stress response. I made a direct test of this hypothesis by measuring the physiological progression of male arctic ground squirrels over the course of the breeding season.

In Chapter 5 I test the adaptive stress hypothesis by comparing the physiology of all five species. I measured the changes in the HPA axis and in downstream physiology over the breeding season. I looked for a correlation between male reproductive lifespan and GC levels, and I also made a qualitative comparison of the direction of change in measures of the HPA axis and in downstream physiology.

In the concluding chapter, I summarize my overall conclusions and discuss how my results fit into the larger literature on the role of stress physiology in the evolution of life history strategies. In particular, I discuss some recent reviews in the avian literature and argue that physiological ecologists need to incorporate a more complex model of the HPA axis in order to resolve some of the questions raised by my results and those of other researchers.
Figure 1.1: A basic model of the vertebrate HPA axis. A stressor is perceived by the hypothalamus, which releases corticotropin releasing hormone (CRH) and arginine vasopressin (AVP; present in mammals, arginine vasotocin in other vertebrates). These hormones stimulate the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) which, in turn, stimulates the adrenal gland (in reptiles, birds and mammals, interrenal gland in fish and amphibians) to produce glucocorticoids (cortisol, corticosterone, or both, depending on species).

Glucocorticoids (GCs) enter the circulation where, in most species, they are mostly bound by corticosteroid binding globulin (CBG). Bound hormone cannot pass out of the circulatory system under normal circumstances (Mendel 1992), rendering bound hormone biologically inactive for as long as it is bound. The unbound (free) hormone is able to leave the circulatory system and exert biological effects on tissues, affecting reproduction, immunity and metabolism. Circulating GCs also act as negative feedback signals at the pituitary, hypothalamus and hippocampus, inhibiting further GC production.
Figure 1.2: A simplified phylogeny of the ground squirrel species used in this study, including estimated times of divergence (branch lengths are not to scale; mya = millions of years ago). Species not included in this study are not shown. Based on Harrison et al. (2003).
References


Chapter 2

2 Impact of live trapping on stress profiles of Richardson's ground squirrel (*Spermophilus richardsonii*)

2.1 Introduction

The hypothalamic-pituitary-adrenal axis (HPA axis or stress axis) is a critical neuroendocrine system that allows vertebrates to respond adaptively to diverse physical, social, and environmental stressors. Because the HPA axis is closely tied to the physiological controls of reproduction, aging, and immunity, the HPA axis is of particular interest to researchers studying the physiological ecology of wild populations and the evolution of physiological traits (Boonstra, 2005; Mateo, 2007; Reeder and Kramer, 2005).

Because the term “stress” is often used vaguely, it is important to clearly define both the conception of stress being used and the measures used to quantify and characterize stress. In our research, we define “stress” simply as a state of physiological challenge to homeostasis that has activated the HPA axis, resulting in the release of glucocorticoids (GCs). Because of the diverse range of targets and effects of GCs, stress is most informatively quantified and described using an array of physiological measures that, taken together, provide a comprehensive understanding of an animal’s physiological response to stressors that it is or has been experiencing. We therefore define a “stress profile” as a suite of physiological measurements that describe both the current state of HPA axis functioning and the impact of HPA activation on GC targets.

Specifically, our direct measures of HPA activation are total cortisol levels and corticosteroid binding globulin (CBG) concentration (our measure being maximum corticosteroid binding capacity – MCBC) from which we calculate the biologically active free cortisol level. Our measurements of glucocorticoid targets include energy mobilization (blood glucose and free fatty acid levels), immune function (white blood cell counts and neutrophil to lymphocyte ratio) and general health (hematocrit).

A common problem for studies of stress in wild animals is that the stress of capture and handling often prevents researchers from obtaining a “true base” stress profile. There are now numerous
wildlife studies showing that plasma GC levels increase significantly after 2-5 minutes from capture (Boonstra et al., 1998, 2001a; Boonstra and Singleton, 1993; Cash et al., 1997; Mueller et al., 2006; Place and Kenagy, 2000; Romero et al., 2008; Romero and Reed, 2005; Romero and Romero, 2002; Wada et al., 2007). However, it is often impractical or impossible to obtain blood samples from wildlife this quickly under field conditions, especially where cage-type traps are used for capture (e.g. Lynn and Porter, 2008). Instead, researchers are often limited to obtaining “nominal base” blood samples collected after an animal has been trapped for periods often measured in hours. Ultimately, though, we are interested in how the animal was functioning under natural conditions so it is important to know how much capture and handling have shifted the stress profile of the animal and whether stress profile has shifted in a way that would compromise the interpretation of the results (e.g. if the response to capture maximized the capacity of the animals to respond, thereby masking differences between animals in their natural state).

In a larger study looking at the stress response of breeding male ground squirrels (*Spermophilus* spp.), we were interested in comparing how the stress profiles of males changed between the pre-breeding period (prior to females emerging from hibernation) and the end of the intense, competitive breeding season. In that larger study, we used a hormone challenge protocol in which hormone injections are used to override the stress of capture and probe the responsiveness of the animal’s HPA axis (Boonstra, 2005). However, even when using a hormone challenge protocol, it is often valuable to look at the measurements made after capture but before the administration of hormones (the nominal base measurement). In order to properly interpret the nominal base results, it is helpful to have some data on true base values because some, but not all, parameters are likely to change significantly in response to capture and handling. The goals of this study were to examine what components of the stress profile of Richardson’s ground squirrels (*Spermophilus richardsonii*) were affected by our capture protocol, to quantify those effects, and to understand how those effects would impact our interpretation of our base and hormone challenge results. We were also interested in comparing how Richardson’s ground squirrels differed in their response to capture stress from the closely related Arctic ground squirrel (*Spermophilus parryii*) studied by Boonstra et al. (2001a).

There is some evidence that nominal base GC levels are still capable of revealing seasonal or inter-annual changes (Boonstra et al., 1998; Place and Kenagy, 2000) or experimental treatment
effects (Boonstra and Singleton, 1993). That is, although capture raises GC levels above true base levels, the seasonal, annual or experimental changes in GC levels in these studies were sufficiently large that they were not masked by the fact that the nominal base samples were elevated compared to true base values. However, there are no studies that have looked at how capture and handling affect the full suite of parameters that we include in our stress profile in a single species.

2.2 Methods and materials

2.2.1 Study Area

The study was conducted at the University of Alberta’s Kinsella Ranch (53°N 111°33’W), 150 km southeast of Edmonton, Alberta. We trapped in rolling elk and cattle pastures of mixed grasses and forbs, low shrubs and occasional stands of aspen poplar. All procedures were carried out under University of Toronto animal use protocol 200006524 and as approved by the University of Alberta Faculty Animal Policy and Welfare Committee.

2.2.2 Trapping and Field Sampling

Two groups of Richardson’s ground squirrels were live-trapped in home made burrow traps (Wobeser and Leighton, 1979) to measure blood parameters, the first to provide true base estimates and the second to provide nominal base estimates. In all cases, we arrived at the trapping site prior to the majority of animals emerging for the day and placed traps in burrows that animals were seen to have entered, as well as in any nearby burrows that may have been connected to the first burrow.

Nominal base animals were trapped between March 25, 2007 and March 27, 2007. Traps were set and checked frequently (<30 min). When an animal was found in a burrow trap, it was weighed and transferred to a Tomahawk live trap (Tomahawk Live Trap Company, Tomahawk, WI, USA), covered with a pillowcase, and placed in a quiet central holding area. Once a sufficient number of animals were captured, the caged squirrels were brought back to a mobile laboratory and placed in a cool, quiet location, with each trap still covered by a pillowcase. After 1 hr, the first animal was retrieved, released from the trap into a pillowcase, and anesthetized using isoflurane (IsoFlo, Abbott Laboratories, Saint-Laurent, Quebec, Canada). A 0.6 ml blood sample was taken from either the suborbital sinus or by cardiac puncture (see Results). Because
these animals were to be used in another study, they were placed back in the Tomahawk trap and returned to the holding area. All animals captured in the same day were sampled within 30 min of each other. The maximum time in a trap before the nominal base sample was taken was 4 hours.

True base animals were trapped on March 28, 2007. Traps were set in the same manner as for nominal base animals, except fewer traps were set and the traps were monitored continuously. As soon as an animal was in the trap, we immediately approached the trap, released the animal from the trap into a pillowcase, and anesthetized the animal with isoflurane using a nose cone method. A 0.6 ml sample of blood was taken by cardiac puncture. The time from when the animal was seen to be trapped to the time the blood sample was obtained ranged from 105 sec to 153 sec.

In all cases, blood samples were placed in 1.3 ml lavender tip microtube vials (Sarstedt, Germany) once collected. Glucose levels (mg/dl) were immediately measured using a FreeStyle glucose meter (Abbott Laboratories, Alameda, CA, USA) using residual blood in the syringe.

2.2.3 Necropsy

After the completion of the blood sampling (for the true base animals) or after the hormone challenge protocol (for the nominal base animals), the animals were euthanized by halothane overdose and necropsied. Adrenal weights were measured to the nearest milligram, and testes were weighed to the nearest 0.01 g.

2.2.4 Laboratory Analyses

After all blood samples were collected, two microhematocrit tubes were filled (approximately 75 µl each) from the lavender tip vials and spun at 13460×g for 5 min in an IEC MB microhematocrit centrifuge (International Equipment Company, Needham Heights, MA, USA). The packed cell volume was recorded. Of the blood remaining in the lavender tip vial, a sample of approximately 0.15 ml was placed in a 0.6 ml microcentrifuge tube and spun at 8800×g for 8 min. The plasma from the microhematocrit tubes and the microcentrifuge vial was placed in a clean vial and stored at -20°C until returning to the laboratory where it was stored at -80°C. The final 0.3 ml portion of each blood sample was retained in the lavender tip vial and sent away for a complete blood count (Vita-tech Veterinary Laboratory Services, Markham, ON, Canada).
Upon return to the laboratory, plasma samples were analyzed for cortisol, maximum corticosteroid binding capacity, androgens, and free fatty acids.

We measured total cortisol by radioimmunoassay following the methods of Etches (1976) as modified by Boonstra et al. (2001b), and as further modified here. Briefly, 40 µl of ultrapure water and 20 µl of NH₄OH were added (to saponify triglycerides) to duplicate 10 µl samples of plasma in 12x75 mm polypropylene test tubes. We added 2 ml dichloromethane (Fisher) and vortexed for 4 min. Samples were then centrifuged at 1000 rpm for 5 min, and the aqueous layer was aspirated. One 600 µl aliquot of the dichloromethane extraction was removed to a fresh test tube (unless there was insufficient plasma to run the sample in duplicate, in which case two 600 µl aliquots were removed to separate test tubes and treated as duplicates from this point onward) and dried under filtered air. Next, 300 µl of phosphate buffer (pH 7) was added to each tube and allowed to equilibrate at room temperature for 1 h, at which point 100 µl of diluted [1,2,6,7-³H] cortisol (Amersham Biosciences, USA) and 100 µl of diluted anti-cortisol antibody A-155 obtained from Western Chemical (Fort Collins, CO) were added to each tube. Cross reactivities of this antibody are provided in Boonstra et al. (2001b). Samples were left overnight at 4°C. The following morning, 200 µl of dextran-coated charcoal was used to separate bound and free hormone. After a 30 min incubation at 4°C, the samples were centrifuged at 2000×g for 12 min and 500 µl of supernatant was added to 2 ml scintillation fluid (ACS, Amersham Biosciences, USA) and left in the dark at room temperature for at least 7 hours before being read in a scintillation counter (Packard Tri-Carb 2900TR, Boston, MA, USA). The intra- and inter-assay coefficients of variation were 4% and 8%, respectively. This method has a reported mean recovery of 105% (SE=1.2%, range 100%-107%) and a detection limit of 10 pg/10 µl (Boonstra and McColl, 2000).

To estimate the amount of protein-bound cortisol, we calculated the maximum corticosteroid binding capacity (MCBC) in duplicate 10 µl samples of plasma using the techniques described in Boonstra et al. (2001b). This technique requires knowing the dissociation constant of CBG, which we calculated by performing a saturation analysis following Hammond and Lähteenmäki (1983). Using the saturation curve (data not shown), we calculated the dissociation constant using nonlinear regression (SAS PROC NLIN) to fit the equation \( y = B_{max} (x/(x + K_d)) \), where \( y \) equals the amount of hormone bound, \( x \) equals the amount of tritiated hormone added, \( B_{max} \)
equals the maximum binding capacity, and $K_d$ equals the dissociation constant. The $K_d$ for Richardson’s ground was calculated to be 20.9nM (the $K_d$ for Arctic ground squirrels reported by Boonstra et al. 2001b was 22.2nM). We assumed that the albumin concentrations and ratio of albumin-bound to free cortisol were comparable to those calculated for Arctic ground squirrels in Boonstra et al. (2001b). Briefly, we added a known specific activity of $[1,2,6,7\text{-}^{3}H]$ cortisol, non-tritiated cortisol, and 400 µl of phosphate buffer (pH 7) to duplicate 10 µl plasma samples in 12x75 mm polypropylene test tubes. Samples were vortexed and equilibrated overnight at 4°C. The next morning, 200 µl of dextran-coated charcoal was added to the tubes, which were centrifuged at 2000×g for 12 min after a 30 min incubation time at 4°C. A 500 µl aliquot of the supernatant was removed to 2 ml of scintillation fluid and left in the dark at room temperature for at least 7 hours before being read in a scintillation counter. This assay was run concurrently with the cortisol assay for the same samples.

The antibody used in our testosterone assay (P43/11, from Croze and Etches, 1980) has a 62% cross reactivity with dihydrotestosterone, so we refer to “androgen levels” rather than testosterone levels in this paper. We used the radioimmunoassay procedure described in Boonstra et al. (2001b). Our intra- and inter-assay coefficients of variation were 5% and 5.5% respectively. Recovery rates for this assay is reported as 96.5% (SE=0.7%, range 92%-102%) with a detection limit of 10 pg/25 µl (Boonstra et al. 2001a).

Free fatty acids were assayed using a commercially available kit (NEFA-C, Wako Chemicals USA Inc., Richmond VA, USA), modified to be used in a 96 well microtitre plate (Johnson and Peters, 1993). Reagents were made up according to kit instructions, but Reagent A was then diluted with 13.3 ml of 0.5 M phosphate buffer and Reagent B was diluted with 33.3 ml buffer. Because an initial trial using 5 µl of plasma were found to have higher concentrations than the supplied standard solution, we used 2.5 µl of plasma per well. After adding plasma or standard solutions to wells, 95 µl of Reagent A was added to each well, shaken for 1 min and then incubated at room temperature for 30 min. Near the end of the incubation period, the plate was pre-read (absorbance at 550 nm) in a spectrophotometer in order to compensate for absorbance by hemolysed plasma samples. At the end of the 30 min incubation, 195 µl of Reagent B was added to each well. The plate was shaken for 1 min and then incubated at room temperature for 30 min before being reading the absorbance at 550 nm in the spectrophotometer. Intra- and
inter-assay coefficients of variation were 6.6% and 10.0%, respectively. The detection limit was considered to be the lowest concentration on the standard curve, 0.06mM.

2.2.5 Statistics

All data are expressed as means ± SE unless otherwise stated. All data were analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA). Data were examined for normality using PROC UNIVARIATE NORMAL. The raw white blood cell count data was non-normal but were normalized with a natural log transformation. The neutrophil and lymphocyte counts were not normal and transformations did not make them normal, we therefore used the Wilcoxon-Mann-Whitney non-parametric two sample test using the EXACT option that generates a Monte Carlo-based exact p-value. All other variables were compared with PROC TTEST. In the case of free fatty acids, the variance of nominal base samples was significantly greater than the variance of the true base samples so the Satterthwaite adjustment for unequal variances was used. Variances of all other variables did not differ between true and nominal base samples, so we used the standard pooled variance t-tests for those data.

2.3 Results

There were no differences between true and nominal base animals in body mass, adrenal mass or mass of testes (Table 2.1). We collected blood from the first 6 nominal base animals from the suborbital sinus, but for the next 9 nominal base animals we collected blood by cardiac puncture. Therefore, before comparing true and nominal base animals, we compared the blood parameters of nominal base animals from suborbital versus heart bleeds. The only parameter that showed a significant difference was MCBC (suborbital: N=6, 112.0±8.5 ng/ml; heart: N=9, 82.2±4.9 ng/ml; t=-3.27, p=0.006). Accordingly, we excluded the suborbital bleeds from comparisons with the true base animals for the MCBC and free cortisol comparisons. For consistency and to make the total cortisol values directly comparable to the free cortisol values, we also excluded the suborbital bleeds from the comparison of total cortisol levels in true and nominal base animals.

The results are summarized in Table 2.1. Total cortisol levels were 39.6% higher in nominal base animals than in true base animals. However, because nominal base animals had lower MCBC than true base animals (nominal base levels were 78.5% those of true base) free cortisol
levels in nominal base animals were 212% those of true base animals. Androgen levels showed signs of rapid suppression: nominal base animals had androgen levels that were just 57.4% those of true base animals.

In terms of energy mobilization, our standard handling protocol of keeping animals in traps for at least an hour prior to taking the nominal base blood sample resulted in significant increases in glucose and free fatty acid levels. Glucose levels in nominal base animals were 22.2% higher than in true base animals and free fatty acid levels were 221.0% higher.

In contrast to the hormone and energy parameters, there were no dramatic differences in the other blood parameters. Packed cell volume in true base animals was slightly higher than in nominal base animals, but the difference was not statistically significant (p=0.087). Cell counts also showed no difference between true and nominal base animals, but the N:L ratio in nominal base animals was 58.7% that of true base values.

2.4 Discussion
2.4.1 The Importance of Measuring Free Cortisol
As expected, total cortisol levels were affected significantly by our standard capture and handling protocol. However, the magnitude of the effect varies considerably depending on whether or not one assesses the effect of corticosteroid binding globulin (CBG). Under the “free hormone hypothesis”, only those GCs not bound to carrier proteins like albumin or CBG are biologically active (Rosner, 1990; Siiteri et al., 1982). It has been clear for some time that this understanding is not strictly true because CBG acts in some cases as a carrier protein, delivering GCs to specific tissues, and there is evidence that the CBG-GC complex can interact with specific cell membrane receptors (see reviews in Hammond 1995 and Breuner and Orchinik 2002). However, in terms of the core GC functions that are typically of interest to physiological ecologists (e.g. its role in maintaining allostatic [McEwen and Wingfield 2003] and playing a central role in the “flight or fight” response), the evidence suggests that circulating free GC levels are the critical measure.

In our study, total cortisol levels increased by 39.6% in response to capture stress, but free cortisol increased by 212% due to a rapid drop in MCBC. The interaction between cortisol and binding capacity allow Richardson’s ground squirrels to mount an effective cortisol response that
is five times greater than that suggested by the total cortisol levels. In contrast to these results, in
a comparison of Arctic ground squirrel males that were either shot (true base) or had been
trapped for 20 min to 90 min prior to having blood taken (nominal base), Boonstra et al. (2001a)
found a three- to fourfold increase in free cortisol as a result of capture stress but the increase
was strictly a result of increased total cortisol production as there was no change in MCBC
levels. Boonstra and McColl (2000) found a 4% drop in Arctic ground squirrel binding capacity
after 4 hours, whereas we observed a 21% drop in Richardson’s binding capacity in the same
time frame. Rapid (<1 hr) changes in GC binding capacity were observed in 5 of 9 species of
birds studied by Breuner et al. (2006) and in snowshoe hares (Lepus americanus) (Boonstra and
Tinnikov, 1998). However, CBG levels in other species only respond to stress after a period of
6-24 hours (rats: Fleshner et al., 1995; Marti et al. 1997) or appear to be resistant to chronic
stress (starlings, Sturnus vulgaris: Cyr et al. 2007). Thus, while it is known that species differ in
how quickly their CBG levels respond to stress, it is somewhat surprising that two species as
closely related as Richardson’s and Arctic ground squirrels differ so much in this respect.

Richardson’s and Arctic ground squirrels are closely related, belonging to the same clade, having
diverged an estimated 1.3 million years ago (Harrison et al., 2003). They are both obligate
hibernators living in highly seasonal environments with long, cold winters. The males of both
species emerge prior to the females at times when there is no new food available and the animals
subsist on food cached in their burrows the previous year (Buck and Barnes, 1999; Gillis et al.,
2005; Michener, 1992). Males of both species are short-lived; the annual disappearance rate of
males for both species is about 80% (Gillis, 2003; Michener, 1998). Only about 20% of males in
a Richardson’s population were 2 years old and fewer than 5% were 3 or 4 years old (Michener,
1998). Thus, the majority of males in both species will only live long enough to participate in
one breeding season. The difference in how these two species respond to capture stress has
implications for interspecific comparisons of stress profiles. For example, Richardson’s and
Arctic ground squirrels differ in how their maximum corticosteroid binding capacity is affected
by capture stress: the GC binding capacity of Richardson’s ground squirrels drops whereas that
of Arctic ground squirrels stays constant. In the wild, then, an Arctic ground squirrel’s free
cortisol response to a natural stressor will be a direct function of its total cortisol production
whereas the free cortisol response of a Richardson’s ground squirrel will depend not only on
total cortisol production, but also on how quickly binding capacity falls. If binding capacity falls
more slowly than total cortisol production rises, then the higher baseline binding capacity in Richardson’s ground squirrels will buffer them from the effects of acute stressors in comparison to Arctic ground squirrels initially. Furthermore, our standard capture protocol results in squirrels being stressed for at least an hour, but if natural stressors are typically more fleeting rather than that, it is possible that binding capacity in Richardson’s does not fall and that their stress response is more muted than that of Arctic ground squirrels. These results and those of Boonstra et al. (1998), Boonstra and Tinnikov (1998) and Breuner et al. (2006) highlight the importance of measuring GC binding capacity and not just relying on total GC levels.

2.4.2 Rapid Suppression of Androgens

The HPA axis is intricately tied to the hypothalamic-pituitary-gonadal (HPG) axis (Wingfield and Sapolsky, 2003) and because we are interested in how the HPA axis is adapted to support alternative reproductive strategies, it is informative to include androgen levels as part of the stress profile. Surprisingly few studies have measured GC and androgen levels of wild animals simultaneously, and in those, the results are variable. Boonstra et al. (2001a) found that shot Arctic ground squirrel males (true base) had significantly lower androgen levels than males subject to capture stress. Place and Kenagy (2000) found a similar pattern of increased testosterone in male yellow-pine chipmunks (*Tamias amoenus*) subject to capture stress, but only in the spring; in the fall, they found no difference between true base and stressed animals (although testosterone levels were very low at this time of year, being <0.5ng/ml). In contrast, Boonstra and Singleton (1993) found that androgen levels were lower in snowshoe hares subject to capture stress compared with true base animals, as did Lance et al. (2004) in their study of American alligators (*Alligator mississippiensis*), and Moore et al. (1991) in their study of the tree lizard, *Urosaurus ornatus*. Moore and Mason (2001) and Cease et al. (2007) found no effect of exogenous GCs and no effect of capture stress, respectively, on testosterone in red-sided garter snakes (*Thamnophis sirtalis parietalis*), but Moore et al. (2000) found an increase in corticosterone and decrease in testosterone in red-sided garter snakes in response to a 4 hour capture stress protocol.

Given the similarities between Richardson’s and Arctic ground squirrels, we had expected that Richardson’s would respond to the stress of capture by increasing androgen levels as Boonstra et al. (2001a) found in Arctic ground squirrels. Instead, we found a dramatic decrease from
9.4 ng/ml to 5.4 ng/ml. This raises interesting questions about the adaptive significance of how androgen levels responds to stress. Boonstra and Boag (1992) and Wingfield and Sapolsky (2003) have predicted that species with compressed breeding seasons and those that may only be able to breed once in their lifetime should show reproductive resistance to stress. One mechanism for preventing the decline of sex hormones in response to stress is compensatory stimulation of the gonadal axis in times of stress (Wingfield and Sapolsky, 2003), and it appears that some form of compensatory androgen production is used by Arctic ground squirrels (Boonstra et al., 2001b). Clearly, however, Richardson’s ground squirrels are either not resistant to stress or they have a different mechanism for resisting stress; further study is required to understand this unexpected observation.

2.4.3 Mobilization of Energy

We measured blood glucose levels and plasma free fatty acid levels as measures of energy mobilization. Stress-induced levels of glucocorticoids stimulate gluconeogenesis in the liver, inhibit insulin activity, decrease peripheral tissue uptake of glucose, and ultimately promote the breakdown of protein and lipids to produce substrates for gluconeogenesis in the liver (Sapolsky et al., 2000). We therefore expected to see an increase in glucose levels in nominal base animals compared with true base, as indeed we did (Table 2.1). Our findings are consistent with those of other studies that have looked at the effect of capture stress on glucose levels (American alligator, Lance et al. 2004; snowshoe hares, Boonstra and Singleton 1993; and meadow voles (Microtus pennsylvanicus), Fletcher and Boonstra 2006).

Both ACTH and GCs have lipolytic actions (Boonstra and Tinnikov, 1998; Sapolsky et al., 2000) so we would expect to see an increase in FFA levels in response to capture stress. The only study that has looked at the effect of capture stress on FFA levels is Handasyde et al. (2003). In that study, 5 female and 2 male platypuses (Ornithorhynchus anatinus) in the non-breeding season showed increased FFA levels in the stressed sample compared with true base values. We found that nominal base animals had FFA levels that were an average of 21% higher than those of the true base samples.

Both of our measures of energy mobilizations indicate that by the time we take our nominal base samples under our standard protocol, the animals have greatly increased their glucose and FFA levels. Although fewer studies have looked at energy mobilization than cortisol response to
capture stress, all studies show a consistent pattern of energy mobilization as one would expect given the central role of rapid energy mobilization in the stress response.

2.4.4 Blood Parameters

Hematocrit (the proportion of blood volume comprised of red blood cells) is sometimes used as a measure of anemia and a general indicator of health (Boonstra et al., 2001b; Clinchy et al., 2004; Kim et al., 2005), the idea being that a low packed cell volume is indicative of anemia, possibly caused by blood loss due to gastric ulcers. However, hematocrit can also be affected by short-term fluctuations in plasma volume unrelated to anemia (e.g. Dawson and Bortolotti 1997) as well as the release of red blood cells from the spleen (Guntheroth and Mullins, 1963; Opdyke, 1970). Therefore, hematocrit readings need to be interpreted with some caution. In the present study, for example, any change in hematocrit would more likely be the result of a change in plasma volume rather than being evidence of blood loss.

Our nominal base animals had slightly lower mean hematocrits compared with true base animals, but the difference was not statistically significant. Previous studies that have examined the effect of acute capture stress on hematocrit have found increases of approximately 10% (snowshoe hares, Boonstra et al. 1998; meadow voles, Fletcher and Boonstra 2006) or no effect (Atlantic sharpnose shark (*Rhizoprionodon terraenovae*), Hoffmayer and Parsons 2001).

The second hematologic indicator of stress that we looked for was a stress leukogram: an increase in neutrophils with a concomitant decrease in lymphocytes (Cattet et al., 2003) often reported as an increased neutrophil:lymphocyte (N:L) ratio (e.g. Kim et al. 2005) or, in birds, an increase in the heterophil:lymphocyte (H:L) ratio (e.g. Kilgas et al. 2006). The rapid increase in circulating neutrophils is thought to result from decreased adherence of neutrophils to vascular walls (Cronstein et al., 1992), whereas the decrease in lymphocytes is due to the redistribution of lymphocytes out of circulation and into tissue (Dhabhar and McEwen, 1997). Thus, the N:L ratio can potentially be altered quite quickly. We found a statistically significant increase in neutrophils, but no statistically significant change in lymphocytes (Table 2.1). The significant increase in the N:L ratio therefore appears to be due to the increase in neutrophils. However, the p-values for the neutrophils and N:L ratio are both approximately 0.04. Because we did not apply any Bonferroni corrections, and because we did not see a decrease in lymphocytes, we
consider this increase in N:L ratio to be a preliminary finding that should be interpreted cautiously.

Davis (2005) found a significant decrease in house finch (*Carpodacus mexicanus*) leukocyte numbers between base blood samples and samples taken at 30 or 60 minutes post-capture. He also found an increase in H:L ratio, but it appeared to be an artifact of repeat blood sampling rather than the stress of capture. We found no such effect on white blood cell counts (Table 2.1).

### 2.4.5 Conclusion

The two most striking findings in this study are how different the Richardson’s ground squirrels are from the closely related Arctic ground squirrels in Boonstra et al. (2001a), and the fact that 8 out of 11 measures in our stress profiles changed significantly in response to capture stress within a period of 4 hours. Of those parameters that changed in response to capture stress, the majority changed in predictable ways (e.g. total cortisol, glucose, free fatty acids all increased as one would expect), but the sudden decline in MCBC and testosterone were unexpected based on what we knew about the response of Arctic ground squirrels to capture stress. Although it may not be practical to alter standard trapping protocols in order to obtain true base measurements from wild study organisms, our results suggest that in cases where researchers are interested in more than just total cortisol levels, having at least a small sample of true base readings may be valuable when it comes to interpreting nominal base results.
Table 2.1: Comparison of physiological parameters measured in true base (blood taken in <3 min from time of capture) and nominal base animals (blood taken up to several hours after capture), with significant differences highlighted in bold. All comparisons are two-tailed t-tests with pooled variances except * = Satterthwaite adjustment for unequal variances and † = Wilcoxon-Mann-Whitney non-parametric two sample test calculated in SAS PROC NPAR1WAY with the EXACT option to produce exact p-values.

Abbreviations: MCBC = maximum corticosteroid binding capacity; FFA = free fatty acids; WBC = white blood cells.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>True Base (mean±SE)</th>
<th>Nominal Base (mean±SE)</th>
<th>t</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>372 ± 14.6</td>
<td>370 ± 9.9</td>
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<td>18</td>
<td>0.918</td>
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<td>Adrenal Weight (mg)</td>
<td>73.2 ± 8.2</td>
<td>77.3 ± 5.3</td>
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<td>Testes Weight (g)</td>
<td>2.16 ± 0.19</td>
<td>2.051 ± 0.08</td>
<td>0.62</td>
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<td>0.546</td>
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<tr>
<td>Total Cortisol (ng/ml)</td>
<td>126.24 ± 19.3</td>
<td>176.22 ± 7.5</td>
<td>-2.89</td>
<td>12</td>
<td>0.014</td>
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<tr>
<td>MCBC (ng/ml)</td>
<td>104.7 ± 6.5</td>
<td>82.2 ± 4.9</td>
<td>2.76</td>
<td>12</td>
<td>0.017</td>
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<tr>
<td>Free Cortisol (ng/ml)</td>
<td>32.7 ± 8.6</td>
<td>69.2 ± 3.2</td>
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</tr>
<tr>
<td>Androgen (ng/ml)</td>
<td>9.42 ± 1.23</td>
<td>5.41 ± 0.67</td>
<td>2.96</td>
<td>18</td>
<td>0.008</td>
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<td>Glucose (mg/dl)</td>
<td>103.2 ± 4.1</td>
<td>126.13 ± 5.4</td>
<td>-2.34</td>
<td>18</td>
<td>0.031</td>
</tr>
<tr>
<td>FFA (mM)</td>
<td>0.2458 ± 0.021</td>
<td>0.5432 ± 0.067</td>
<td>-4.27*</td>
<td>16.3*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
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<td>46.7 ± 0.91</td>
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<tr>
<td>Neutrophils (×10⁹/l)</td>
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<td>2.46 ± 0.41</td>
<td>S = 63.5 †</td>
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<tr>
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<td>1.29 ± 0.17</td>
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<td>WBC (×10⁹/l)</td>
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<td>4.10 ± 0.57</td>
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<td>N:L Ratio</td>
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<td>2.067</td>
<td>2.25</td>
<td>18</td>
<td>0.040</td>
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</tbody>
</table>
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Chapter 3

3 The benefits of baseline glucocorticoid measurements: maximal cortisol production under baseline conditions revealed in male Richardson’s ground squirrels (*Urocitellus richardsonii*)

3.1 Introduction

The vertebrate hypothalamic-pituitary-adrenal (HPA) axis is a dynamic system that plays a number of critical roles in baseline physiology and in responding to stressors (Sapolsky et al. 2000). One challenge for those wanting to describe the status of the HPA axis in an animal is choosing measures that capture biologically relevant features (Sheriff et al. 2011). There are two main approaches taken by researchers who are interested in measuring plasma hormone levels. The first is the “capture stress protocol” in which glucocorticoid (GC) levels are measured first in a baseline blood sample and then in an acute stress blood sample. This method is especially prevalent in avian studies in which it is relatively easy to obtain a baseline blood sample within 3 min—quickly enough that the stress of capture and handling has not yet caused a change in plasma GC levels (Romero and Reed 2005). An acute stress blood sample is typically taken 15-30 min after capture (Breuner et al. 2008) once GC levels have peaked, although sometimes multiple blood samples are taken after the baseline sample in order to track the rate of increase in GC levels (Wingfield et al. 1992), thereby providing some information on the sensitivity of the adrenal gland. By measuring both baseline and acute stress GC levels, one can assess both how the animal is responding to its normal environment and the strength of the animal’s acute stress response. In the latter case, a greater acute stress increase over baseline levels is presumed to indicate a more intense biological response to natural stressors.

Unfortunately, in many wildlife species it is impractical or impossible to get a baseline blood sample within minutes of capture. For example, most mammals are trapped in live traps that are left set for many minutes or hours, and many mammals are unlikely to enter traps if human observers are nearby. In these situations the first blood sample is an acute stress sample; frequently the stress of capture has been ongoing for several hours. We call this the “sustained stress protocol.” Sometimes it is possible to obtain true baseline samples by shooting animals
(e.g. Boonstra and Singleton 1993) but there are instances where this is not possible (e.g. very small or rare animals, or field sites in areas where killing animals is prohibited). Studies using a sustained stress protocol may use a hormone challenge protocol to probe how the HPA axis is functioning (Boonstra 2005). However, hormone challenge protocols cannot replace true baseline blood samples.

Not measuring baseline levels is a potential problem for studies asking questions about how HPA axis function changes from one time to another, such as breeding and nonbreeding periods. At low GC concentrations negative feedback is primarily regulated by mineralocorticoid receptors in the hippocampus, whereas higher GC levels such as those associated with acute stress engage negative feedback by glucocorticoid receptors in the hippocampus, hypothalamus and pituitary (de Kloet et al. 1998). In this way, basal GC and acute stress GC levels can be regulated independently. If baseline levels change independently of acute stress levels (e.g. for reasons of change in reproductive state, season, social state; see Romero and Romero 2002), then missing a change in baseline levels could mask some important biological changes in the HPA axis. Few studies that rely on acute stress GC levels have tried to determine whether changes in acute stress GC levels mirror baseline changes. Boonstra and Singleton (Boonstra and Singleton 1993) found that seasonal changes in baseline GC levels of snowshoe hares (*Lepus americanus*) were reflected in sustained acute stress GC levels. Likewise, Place and Kenagy (Place and Kenagy 2000) and Kenagy and Place (Kenagy and Place 2000) found that sustained acute stress GC levels of both male and female yellow-pine chipmunks, *Tamias amoenus*, reflected baseline GC changes.

We studied five species of North American ground squirrels in a study that included comparing the functioning of the HPA axis in males before and immediately after the breeding season. The logistics of trapping multiple species in a year, as well as the natural wariness of most of our ground squirrel species prevented us from collecting baseline samples routinely. However, the population of Richardson’s ground squirrels (*Urocitellus richardsonii*) that we studied were more easily trapped than the other species, and with extra effort it was possible to get blood samples in less than 3 min (e.g. Delehanty and Boonstra 2009). Taking advantage of the trapability of this species, we had two main objectives in the present study. First, we wanted to test whether the sustained acute stress blood sample changed in tandem with baseline GC levels as they do on snowshoe hares and yellow-pine chipmunks. Second, we wanted to determine
whether acute stress blood samples obtained following our standard protocol (i.e. after a variable amount of time from about 1.5 h up to 5 h) provide the same information as the acute stress samples taken in the capture stress protocol (i.e. 30 min after capture).

3.2 Materials and Methods

Male RGS were captured in burrow traps at the University of Alberta Kinsella Ranch (53° 0’ 16” N, 111° 31’ 38” W). Prebreeding males were trapped in 2009 between 29 March and 2 April, prior to females emerging (with the exception of one female that emerged on 1 April). The postbreeding animals were trapped on 19 and 20 April, by which time most or all females had emerged and breeding was largely over. We started trapping when animals became active—typically around 09h00. We set one or more traps at burrow entrances in the immediate vicinity of where we saw an animal enter a burrow and we then monitored the traps closely, but inconspicuously. As soon as an animal entered a trap, we started a timer, rushed to the trap and emptied the animal into a pillowcase. We anesthetized the animal by placing a vial containing cotton soaked with isoflurane (IsoFlo, Abbott Laboratories, Saint Laurent, QC, Canada) over the nose. Using battery powered clippers, we shaved the hair from the inside of the animal’s hind leg and took a 0.3 mL blood sample from the medial saphenous vein using a heparinized 29 gauge insulin syringe. We noted whether the sample was taken within 3 min of capture. Samples taken within 3 minutes were designated as BASE bleeds and were assumed to reflect the animal’s state prior to capture. After taking the blood sample, the animals was weighed and placed in a Tomahawk live trap (Tomahawk #102, Tomahawk Live Trap Company, Tomahawk, WI, USA), which was then covered by the pillowcase. The animal was then carried in the covered trap for the next 30 min as we continued trapping. After 30 min, we removed the animal from the trap, anesthetized it, and took a second blood sample, designated the ACUTE sample. The animal was once again returned to the trap, which we covered with the pillowcase and placed in a cool, quiet location until trapping was completed. After we caught several animals, and no later than 13h00, we returned the animals to the field laboratory where they were allowed to acclimate in a quiet location for 1 h, after which we anesthetized one animal at a time and took a third blood sample, designated the SUSTAINED sample (for “sustained acute stress”).

We measured cortisol concentrations (Richardson’s ground squirrels have negligible corticosterone levels; Delehanty and Boonstra, unpublished data) in all blood samples using a
commercial cortisol kit (Diasorin GammaCoat Cortisol Kit, Stillwater, MN, USA) according to kit instructions. To calculate free GC levels (i.e. GC not bound to corticosteroid binding globulin or albumin) we used the formula provided in Barsano and Baumann (Barsano and Baumann 1989). This formula requires the equilibrium dissociation constants for CBG/cortisol, which we calculated using the methods described in Delehanty and Boonstra (2011). The equilibrium dissociation constant (±SE) for Richardson’s ground squirrels was 5.4±0.56 nM. The Barsano and Baumann (Barsano and Baumann 1989) equation also requires the maximum corticosteroid binding capacity of CBG in each individual sample. We used the procedures laid out in Delehanty and Boonstra (Delehanty and Boonstra 2009) to calculate maximum corticosteroid binding.

Because we were not able to collect all samples from each animal, we used SAS PROC MIXED, which permits missing values in repeated measures designs. The free hormone data was non-normal, but natural logarithm transformation improved overall normality, so the analysis of the free hormone data was performed on ln-transformed data. We also used the Satterthwaite method of calculating degrees of freedom for all comparisons because postbreeding sample sizes were smaller and had higher variance relative to prebreeding samples. There is some debate over the appropriateness of correcting for multiple post hoc comparisons (e.g. Nakagawa 2004)). In the present case, however, we are making so many pairwise comparisons from a single dataset that we felt it was important to present the corrected P-values. We specified the Tukey-Kramer method for multiple comparisons and report those P-values. Where a significant unadjusted P-value is made insignificant, we report both values.

A subset of prebreeding (n=9) and postbreeding (n=8) animals were euthanized and we measured the mass of free abdominal fat (i.e. non-mesenteric fat deposits).

### 3.3 Results and Discussion

In the prebreeding session we caught 27 individual males and obtained 20 BASE, 27 ACUTE and 22 SUSTAINED blood samples. In the postbreeding session we caught 9 males and obtained 6 BASE, 9 ACUTE and 9 SUSTAINED blood samples. The lower sample size in the postbreeding session was due, in part, to the fact that we were catching females as well as males and we had limited time available for trapping. It may also result from the fact that some males die during the course of the breeding season (Michener and Locklear 1990).
Most prebreeding animals have extremely elevated GC levels in the baseline bleed

BASE bleed GC levels in prebreeding males were elevated almost to stress-induced levels. There was little to no difference between BASE and ACUTE GC levels in the prebreeding session for either total or free GC (Fig. 3.1). Without adjusting for multiple post hoc comparisons, the p-values for the prebreeding BASE to ACUTE pairwise comparisons are significant or nearly significant (t_{23.2} = -2.33, p = 0.028 for total GC and t_{23.3} = -1.98, p = 0.060 for free GC) but the Tukey-Kramer adjusted p-values of p = 0.22 for total GC and p = 0.38 for free GC suggest that there is no difference between BASE and ACUTE GC levels. As measured by the change in mean GC levels, the increase from BASE to ACUTE was only 17% (total GC) or 33% (free GC), which is small compared to the increase in the postbreeding period (below, section 3.2) and compared to similar data from 2007 (where total GC increased 40% and free GC increased 112%; Delehanty and Boonstra 2009).

Mean values can be deceptive, though, and we can get a better picture of what is happening by looking at how individual values are distributed (Fig. 3.2) and at individual measures of reactivity (i.e. the change in GC levels of individual animals from their BASE to ACUTE blood samples; Fig. 3.3). In Figure 3.2 we see that in the prebreeding session, individuals are almost all clustered at high total and free GC levels in both the BASE and ACUTE bleeds, suggesting that the elevated GC levels (>120 ng/mL total, > 50 ng/mL free) are an almost uniform feature of prebreeding animals. However, the range of GC concentrations within these clusters is still considerable (a twofold range in total GC and fourfold in free GC). As a result, when we look at the plots of reactivity in Figure 3.3, we see that the individual reactivity varies significantly. Most animals have low or even negative reactivity, but several prebreeding animals increasing GC levels by ≥50 ng/mL. Using just the individual reactivity data, we see that the mean reactivity is greater than zero (total GC mean ± SE 24.2±11.3 ng/mL: t_{17} = 2.13, p = 0.048; free GC mean ± SE 25.7±10.0 ng/mL: t_{17} = 2.57, p = 0.02). From this we conclude that reactivity is not eliminated in all prebreeding males but most have, at best, a limited ability to mount a GC reaction to the stress of capture.

This is truly remarkable: most males have as much or almost as much GC circulating when they emerge from their burrows in the morning as when they are in the clutches of a potential predator. We can think of two non-exclusive explanations for this. First, it is possible that the
BASE GC levels are reflecting the presence of a persistent stressor that affects all animals. To generate a sustained GC response it seems most likely that the stressor would be an environmental factor, such as the absence of food, rather than social which would presumably result in more variability in GC levels. Because male RGS consume food that they stored in the fall prior to emerging above ground in the spring (Michener 1992), they emerge with considerable fat stores. Prebreeding free abdominal fat stores in 2009 were (mean ± SE) 2.5% ±0.3% of body mass (n=9) which is significantly lower than the 4.0% ±0.3% of body mass (n=14) found in 2007 males (2007 data from chapter 5; \( t_{21} = 3.40, p = 0.0027 \)). This could indicate that the 2009 animals were facing greater energetic challenges and having to mobilize their fat stores more quickly than the 2007 animals. But intuitively, it seems unlikely that hunger should generate such a strong stress response: hunger may require dipping into the body’s reserves, but the best strategy would presumably be to mobilize only as much energy as absolutely necessary, thereby maximizing the probability of surviving until food becomes available. Thus, although environmental conditions may help to explain the high BASE GC levels in 2009, we do not think they are sufficient.

The second explanation for hugely elevated BASE GC levels is the “basal stress hypothesis” that we proposed in Delehanty and Boonstra (2011) in respect of arctic ground squirrels (Urocitellus parryii). Under the basal stress hypothesis, BASE GC levels are elevated not in response to stressors, but due to a change in homeostatic set point (consistent with the concepts of allostasis in McEwen and Wingfield 2003 and reactive scope in Romero et al. 2009). In Delehanty and Boonstra (2011) we hypothesized that high basal GC levels could be achieved by suppressing CBG levels and downregulating mineralocorticoid receptors in the hippocampus (which are involved in regulating basal GC levels—de Kloet et al. 1998; vanHaarst et al. 1997). This would elevate basal free GC levels even in the absence of a stressor. We suggested that this could be a means of mobilizing stored energy reserves in the prebreeding period, a time when no new food is available (as is the case in both arctic and Richardson’s ground squirrels) and in anticipation of the coming intense intrasexual competition. In the present study, CBG levels (measured as maximum corticosteroid binding capacity) are reduced in the prebreeding period relative to postbreeding as predicted by the basal stress hypothesis (Table 3.1). A key untested prediction for this hypothesis, however, is the downregulation of hippocampal mineralocorticoid receptors.
3.3.2 Most postbreeding animals have reduced baseline GC levels but reactivity varies

Postbreeding BASE GC levels were significantly lower than prebreeding levels. BASE total GC levels fell by 52% between sessions and free GC fell by 94% (Fig. 3.1; \( t_{28.2} = 4.77 \), adjusted \( p = 0.0007 \) and \( t_{23.5} = 6.44 \), adjusted \( p < 0.0001 \) for total and free GC, respectively). However, as Figure 3.2 shows, some postbreeding animals still had considerably elevated GC levels more characteristic of prebreeding animals. This suggests that there is inter-individual variability in when (or if) animals switch to lower their baseline GC levels. The drop in BASE GC levels in the postbreeding session opened up the potential for animals to regain stress reactivity. Indeed, using mean values in our repeated measures ANOVA analysis, mean GC levels increased from BASE to ACUTE in the postbreeding session (total GC: \( t_{22.7} = -4.78 \), adjusted \( p = 0.001 \); free GC: \( t_{22.7} = -7.49 \), adjusted \( p < 0.0001 \)). This picture of increased total GC response to acute stress is repeated when we look at plots of reactivity (Fig. 3.3); there we see that total GC reactivity is very strong compared to the prebreeding period, with all postbreeding animals increasing total GC levels by >80 ng/mL. This means that postbreeding animals—unlike the prebreeding individuals—are able to respond to capture stress with significant GC production beyond their baseline levels.

Free GC reactivity is a very different story. Overall, CBG levels (measured by maximum corticosteroid binding capacity) increased from the pre- to postbreeding session (Table 3.1), which, in combination with lower total GC levels, meant BASE free GC levels were <16 ng/mL for 5 of the 6 postbreeding males (Fig. 3.2). The increase in binding capacity and decrease in GC levels meant that several animals had so much extra binding capacity that even during the ACUTE bleed, free GC levels of 5 of 9 animals remained <16 ng/mL (Fig. 3.2). For these animals, reactivity remains low, but for the opposite reason as in the prebreeding season: instead of being maximally stimulated, these individuals start with very low BASE GC levels and generate almost no free GC response because of high CBG levels. However, other individuals mount strong free GC responses, with concentrations reaching levels similar to those of prebreeding animals (Fig. 3.2). Plotting the individual reactivity reveals the same range of response (Fig. 3.3). We interpret this as indicating that individuals can have very different experiences in the breeding season that shape their HPA axis accordingly. Among the possible factors that could be behind the individual differences are age, behavioural traits (“personality”),
and mating success. Longer term studies that include behavioural observations would contribute significantly to our understanding of the basis for the individual variation that we observed.

### 3.3.3 Measuring short-term acute stress tells a similar, but not identical, tale as measuring sustained acute stress

The ACUTE bleeds were taken after 30 min of captivity, whereas the SUSTAINED bleed was taken ≥1.5 h (up to 5 h) after capture. We wanted to know whether these two blood samples are equivalent despite the difference in length of time to sampling. We compared total and free GC levels in these two bleeds within a trapping session (i.e. change from ACUTE to SUSTAINED) and we compared how each sample changed from pre- to postbreeding trapping sessions.

There was a slight drop in total GC levels from the ACUTE bleed to the SUSTAINED bleed in the prebreeding session ($t_{33.2} = 3.11$, adjusted $p = 0.041$) but not the postbreeding session ($t_{29.4} = -0.41$, adjusted $p = 1.0$). There was no change in free GC levels from the ACUTE to SUSTAINED bleed in either session (prebreeding: $t_{34.1} = 1.78$, adjusted $p = 0.49$; postbreeding: $t_{30.2} = -0.75$, adjusted $p = 0.97$). The drop in total GC from the ACUTE to SUSTAINED bleed in the prebreeding session could indicate that adrenal GC production has fallen somewhat over the course of capture. This could be the result of habituation to the stress of capture or adrenal “exhaustion”. Neither one of these hypotheses offers an intuitive explanation for the lack of a change in the postbreeding session. Another explanation for the decrease in total GC levels from the ACUTE to SUSTAINED bleeds is that ACUTE GC levels represent a transient level that then decreases by the SUSTAINED bleed as negative feedback by GC receptors in the pituitary and the brain reach equilibrium with the ongoing stimulation of the HPA axis by continued captivity. If negative feedback strength increases in the postbreeding session, the equilibrium would be reached sooner, explaining the lack of change between ACUTE and SUSTAINED bleeds in the postbreeding session. Although the reasons for the difference between prebreeding and postbreeding sessions are unknown, we can at least conclude that the ACUTE and SUSTAINED bleeds are broadly similar, but not identical, measures.

The second question we asked is whether the ACUTE and SUSTAINED blood samples tell us the same thing about how animals respond to capture stress in the pre- and postbreeding sessions. Total GC levels in the ACUTE bleed possibly declined from pre- to postbreeding (Fig. 3.1; $t_{33.4} = 2.59$, unadjusted $p = 0.014$, adjusted $p = 0.13$), but SUSTAINED bleed total GC levels
definitely did not change from pre- to postbreeding (Fig. 3.1; \( t_{29.9} = 0.49 \), adjusted \( p = 1.0 \)). The same ambiguity is encountered when comparing free GC levels. Free GC levels in the ACUTE bleed dropped by 80% between pre- and postbreeding sessions (Fig. 3.1; \( t_{35.1} = 4.25 \), adjusted \( p = 0.0019 \)), and SUSTAINED free GC levels fell 63%, but the difference in SUSTAINED free GC levels was only significant before the Tukey-Kramer adjustment (Fig. 3.1; \( t_{31.1} = 2.73 \), \( p = 0.010 \), adjusted \( p = 0.097 \)). Part of the statistical ambiguity is due to the highly variable ACUTE and SUSTAINED GC levels (Fig. 3.2), and it is this individual variability that seems to us to be the critical feature of our data. Some individuals had very low free GC levels in both the ACUTE and SUSTAINED samples: four of the 9 postbreeding animals had SUSTAINED free GC levels in the range of 3-14 ng/mL due mostly to very high CBG levels and 5 of 9 animals had ACUTE bleed levels <16 ng/mL (discussed in section 3.3.2). Most of the remaining individuals had high free GC levels comparable to prebreeding levels, which led to high variance in the postbreeding samples.

If we were only to compare the changes in mean GC levels of ACUTE and SUSTAINED blood samples, we might conclude that the ACUTE and SUSTAINED bleeds provide different information. However, the mean values in this case seem less important than the individual variation. Some individuals become low-responders in the post-breeding season (i.e. they have very low free GC levels even in response to acute stress), whereas others respond to acute stress by increasing GC levels to levels similar to those experienced in the prebreeding season. In this respect, both the ACUTE and SUSTAINED blood samples reveal this feature.

### 3.3.4 Prebreeding animals in 2009 differed from prebreeding animals in 2007

In Delehanty and Boonstra (2009) we compared “true base” blood samples (what we call BASE in this paper) to “nominal base” blood samples (which are equivalent to the SUSTAINED blood samples in this paper). The animals used in Delehanty and Boonstra (2009) were trapped in 2007 at a location about 1.5 km from where the animals for the present study were caught. In our 2009 paper, we found that total and free GC levels were lower in “true base” than in “nominal base” animals. In other words, the 2007 prebreeding animals maintained stress reactivity during the prebreeding period and were able to respond to the additional stress of capture by elevating GC levels by 40% for total GC and 112% for free GC (Delehanty and Boonstra 2009). Comparing prebreeding BASE to SUSTAINED GC levels in our present data,
there is no difference in total or free GC (total: $t_{26.2} = 1.21$, adjusted $p = 0.83$; free: $t_{29.5} = 0.06$, adjusted $p = 1.00$).

The direction of change of acute stress GC levels from prebreeding to postbreeding sessions were also different in 2007 and 2009. In chapter 5 I report the 2007 pre- and postbreeding GC levels. In that study, we used our standard sampling methodology, which means that those data are equivalent to the SUSTAINED blood samples in this study. Total and free GC levels increased (by 28% and 51%, respectively) from the pre- to postbreeding period in 2007, suggesting that postbreeding animals were mounting a stronger response to capture stress at the end of the breeding season than before breeding. In contrast, the 2009 animals in the present study showed no change in total GC and a possible drop in free GC (see section 3.3.3), suggesting a constant or decreasing response to capture stress from pre- to postbreeding.

We speculate that the difference in GC dynamics between years were caused either by variations in weather from year to year (although both years seemed comparable to us) or by habitat differences between the two sites (the 2009 site had visibly less varied vegetation than the 2007 site, but not to such an extent that we had expected them to be important). Alternatively, the difference in how the means change could be a byproduct of the high inter-individual variability that we observed in both this study (see sections 3.3.2 and 3.3.3) and in 2007 (chapter 5). Multi-year studies of individual animals in a single field site would be of great value in trying to understand the source of such dramatic variation in GC patterns.

### 3.4 Conclusion

Unlike snowshoe hares and yellow-pine chipmunks, acute stress GC levels (ACUTE or SUSTAINED) of male Richardson’s ground squirrels did not change in tandem with baseline GC levels from pre- to postbreeding trapping sessions. As a result, relying on our standard trapping protocol means that we could be missing some potentially very important information about the dynamics of the stress response in Richardson’s ground squirrels. In particular, the very high baseline GC levels in the 2009 animals and the fact that 2007 animals did not have such elevated levels are important and intriguing observations, but would be completely missed under our standard protocol. Moreover, the fact that baseline levels vary from year to year (or from one location to another) suggest that it would be valuable to always make the effort to collect at least some baseline samples even if they cannot be routinely collected.
We also conclude that ACUTE and SUSTAINED blood samples do not tell us identical stories about the average physiological response to capture stress, but they do provide a consistent story about the importance of individual variation in the postbreeding period. More data are required to fully understand the significance of the difference in mean ACUTE and SUSTAINED GC levels: first, to resolve the statistical uncertainty resulting from our use of Tukey-Kramer adjustments for post hoc comparisons; and second, to determine whether any differences between ACUTE and SUSTAINED GC levels are large enough to have any biological significance.
Table 3.1. Main effects and interactions for total GC, maximum corticosteroid binding capacity (MCBC), and free GC levels using SAS PROC MIXED with Satterthwaite-adjusted denominator degrees of freedom. “Bleed” refers to the sequential bleeds, BASE, ACUTE and SUSTAINED (see Methods) and “Session” refers to prebreeding and postbreeding trapping sessions. Total and MCBC data are untransformed, but free GC levels were natural log-transformed for analysis.

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Figure 3.1: Changes in total and free glucocorticoid levels from pre- to postbreeding sessions for the BASE, ACUTE and SUSTAINED blood samples. Error bars represent 95% confidence intervals. Note that because free glucocorticoid levels were analyzed using natural logarithm–transformed data, the means presented are geometric means and the confidence intervals are asymmetrical.
Figure 3.2: Plots of total and free glucocorticoid levels from the BASE, ACUTE and SUSTAINED blood samples, showing individual values, means (large horizontal line) and 95% confidence intervals (vertical and small horizontal lines). Because free hormone levels were natural logarithm–transformed, the means presented are geometric means and the confidence intervals are asymmetrical.
Figure 3.3: Stress reactivity in pre- and postbreeding trapping sessions. The y-axis represents the change in cortisol levels from the BASE to ACUTE blood samples.
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Copyright Acknowledgements

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Chapter 4

4 Coping with intense reproductive aggression in male arctic ground squirrels: the stress axis and its signature tell divergent stories

4.1 Introduction

The hypothalamic-pituitary-adrenal axis (HPA axis) has been of longstanding interest to physiological ecologists because of its central role in permitting vertebrates to respond to a wide range of stressors from temperature extremes to social interactions and predation risk (Wingfield and Romero 2001). At the onset of an acute stressor that threatens an individual’s homeostasis, the sympathetic nervous system causes the release of catecholamines to generate immediate physiological effects (over a period of seconds to minutes) while the HPA axis begins a neuroendocrine response that ultimately causes the releases of glucocorticoids (GCs, cortisol (CORT) and/or corticosterone) over a period of several minutes to hours (Sapolsky et al. 2000). Glucocorticoids are generally described as promoting the immediate survival of the individual (Boonstra et al. 1998; Wingfield et al. 1998; Sapolsky et al. 2000). They do this by redirecting the body’s resources away from physiological processes not essential to immediate survival, including reproduction and growth (Sapolsky et al. 2000; Charmandari et al. 2005), thereby freeing up energy to be devoted to overcoming or escaping the stressor.

The negative effects of chronic stress (by which we mean chronic activation of the HPA axis) on reproduction have been well described (Sapolsky et al. 2000; Wingfield and Sapolsky 2003). In northern and Arctic species, the interactions between the HPA axis and the hypothalamic-pituitary-gonadal axis (reproductive axis) become critical because chronic stress during a narrow reproductive window could have a disproportionately large impact on the animal’s fitness. Nowhere is this truer than in those northern and Arctic species in which males aggressively compete for mating opportunities. When the breeding season also coincides with limited food availability, it is likely that reproductive success will be highly influenced by the ability to maximize the energy they can devote to obtaining mates.

The mobilization of stored energy is a major feature of the HPA axis, making it potentially useful as a means for supporting reproductive effort; but the chronic activation of the HPA axis
(e.g. for a breeding season lasting days or weeks) is typically seen as detrimental to an animal’s survival (McDonald et al. 1981; Boonstra et al. 1998; Sapolsky et al. 2000; Cyr et al. 2007; Dickens et al. 2009a). However, it has been hypothesized that chronic activation of the HPA axis during the breeding season can be an adaptive strategy in certain circumstances. By maintaining a robust, or even an enhanced, stress response during the breeding season, an individual could maximize fitness by increasing the energy available for reproduction even if it reduces survival. This strategy has been called an “adaptive stress response” (Boonstra and Boag 1992) and is dependent on the ability to avoid or overcome the negative effects of stress on reproduction. The alternative strategy was the “homeostasis stress response” in which the normal feedback mechanisms of the HPA axis remain intact over the breeding season; this was proposed to be characteristic of iteroparous species. The adaptive stress response is best exemplified by a group of semelparous marsupials in Australia. In at least 10 species of dasyurid marsupials the entire male population dies off at the end of their first breeding season (Lee and Cockburn 1985; Bradley 2003). The endocrine profiles of males have been studied in great detail in four of these species (Antechinus stuartii, A. swainsonii, A. flavipes, and Phascogale calura). Each one shares a common physiological progression. As the breeding season approaches, free GC levels increase in males. Free GC refers to GC not bound by corticosteroid binding globulin (CBG) and it is widely believed that only free hormone can leave the circulatory system and exert a biological effect on tissues – see Rosner (1990), Ekins (1992), Mendel (1992), and Malisch and Breuner (2010). The increase in free GC is the result of several factors: first, blood GC levels increase because adrenal GC production increases; second, testosterone drives down CBG production which lowers the proportion of bound GC, and, third, the negative feedback that normally shuts down GC production after the onset of the stress response, fails (at least in P. calura and A. swainsonii) (Bradley et al. 1980; McDonald et al. 1981; Lee and Cockburn 1985; Bradley 1987 1990). The elevated GC levels lead to gastric ulcers, suppression of immune and inflammatory responses, increased parasitism, and shifts in hematological parameters (Cheal et al. 1976; Barker et al. 1978; Bradley et al. 1980) that ultimately result in the male die-off. The adaptive stress hypothesis predicts that this type of response should evolve in species that have low survival between breeding seasons (Boonstra and Boag 1992).
The arctic ground squirrel (*Urocitellus parryii* [Helgen et al. 2009], formerly *Spermophilus parryii* [Helgen et al. 2009]), is one such species that shows a life history broadly similar to that of the semelparous marsupials (Boonstra 2005). Although they are not semelparous, males have low annual survival rates with approximately 62% of males living through only one breeding season, and <1% surviving beyond a second breeding season (estimated from figures in Gillis 2003). The once-a-year breeding opportunity combined with a relatively low between year survival rate for males led Boonstra et al. (2001b) to hypothesize that breeding male arctic ground squirrels would exhibit an adaptive stress response that promotes reproductive success at the expense of survival, whereas non-breeding males and juvenile males would exhibit a homeostatic stress response.

To test their hypothesis, Boonstra et al. (2001b) compared the stress response of breeding, non-breeding, and juvenile male arctic ground squirrels. Although they found some evidence that reproductive males had a chronically activated HPA axis, their predictions were only partly borne out (Table 4.1). One of the difficulties with that study was the fact that they compared the HPA axis at three very different life stages: post-breeding adults (near or at the end of the breeding period), non-breeding adults (trapped in mid- to late-summer, well after completion of the breeding season), and juveniles (trapped in late summer). Because the adaptive stress hypothesis relates specifically to changes in the HPA axis over the breeding season, a better test of the hypothesis is to examine whether the functioning of the HPA axis becomes dysregulated over the course of the breeding season as the hypothesis predicts. We therefore set out to make this direct test of the adaptive stress hypothesis by comparing the stress response of male arctic ground squirrels immediately before the breeding season with that at the end of the breeding season (a span of three weeks).

The biology of arctic ground squirrels makes them particularly well suited to testing this hypothesis. Male arctic ground squirrels emerge from hibernation before the females, at a time when snow still covers most of the ground (Buck and Barnes 1999). However, they have already been euthermic for the previous 2-3 weeks, during which time their testes grow and spermatogenesis begins (Barnes and Ritter 1993). During this time they remain underground and consume cached food stores, regaining all their body mass lost over winter (Buck and Barnes 1999, Gillis et al. 2005). As a result, when they emerge above ground, they have significant fat reserves (Buck and Barnes 1999). Being well fed and having been sequestered in their burrows away from any aggressive interactions, we predicted that male arctic ground squirrels should be
relatively unstressed when first emerging. However, approximately one week after the first males emerge from hibernation, the females begin to emerge and rapidly enter estrus (Buck and Barnes 1999). Males defend territories encompassing the burrows of several females (Lacey and Wieczorek 2001), but aggressive encounters are frequent as males attempt to obtain matings with females not within their territory (Lacey et al. 1997). These encounters can result in severe wounding (e.g. gaping wounds, severed testes, lost eyes) and even death (Carl 1971; Holmes 1977; Gillis 2003; this study). Given the intensity of the mating competition among males over 2-3 weeks, we predicted that males would show signs of chronic stress and a breakdown of negative feedback in the HPA axis by the end of the breeding season.

We made the following predictions about the change in male physiology from pre-breeding (the unstressed state) to post-breeding (the chronically stressed state) sampling periods. First, we predicted that relative to pre-breeding males, those at the end of the breeding season would show signs of chronic activation of the HPA axis over the breeding season by having:

(a) higher free CORT levels (in accordance with the free hormone hypothesis, free hormone levels determine the amount of hormone immediately available for uptake by tissues, so we made no specific predictions about how total CORT levels should change);

(b) lower CBG levels as a result of high testosterone levels (shown to suppress CBG in some semelparous marsupials: McDonald et al. (1981) and Bradley (1987)) or chronic stress (found to reduce CBG levels in other species: Dallman et al. (1987) Armario et al. (1994) Fleshner et al. (1995) and Boonstra et al. (1998)); and

(c) attenuated negative feedback in response to adrenal production of CORT as seen in semelparous marsupials (McDonald et al. 1986; Bradley 1990)

Next, because one of the primary roles of GCs is to mobilize energy in times of need, we considered how the chronic activation of the HPA axis over the breeding season would alter the metabolic profiles of arctic ground squirrels. Because CORT reduces the peripheral uptake of glucose, promotes lipolysis and protein catabolism, and promotes hepatic gluconeogenesis so that the stores of glucose and glycogen in the liver are increased under chronic stress (Boonstra et al. 1998; Sapolsky et al. 2000), we predicted that males at the end of the breeding season would show a different metabolic profile characterized by:
(a) higher stress-induced blood glucose levels; and

(b) depleted fat stores and lower stress-induced free fatty acid levels.

Finally, we used two hematological measures to assess general health and immune function. We predicted that post-breeding males would show:

(a) lower hematocrit levels than pre-breeding animals because, although hematocrit can change in response to a number of factors, a number of wildlife studies have found that higher hematocrit levels indicate better body condition (Franzmann and LeResche 1978; Lochmiller et al. 1986; Hellgren et al. 1993; Boonstra et al. 1998; Boonstra et al. 2001b; but see Dickens et al. 2009b); and

(b) decreased white blood cell counts due to the immunosuppressive effect of chronic stress (Sapolsky et al. 2000).

4.2 Materials and methods

4.2.1 Study area

In 2007 we trapped ground squirrels 50 km west of Pelly Crossing, Yukon at the Pelly River Ranch (62°50’N 137°18’W) in pasture and oat fields. Snow still covered about 70% of the land on April 10 and had disappeared entirely by April 27. All procedures were carried out under University of Toronto Animal Use Protocol 200006524, issued in accordance with the Canadian Council on Animal Care guidelines.

4.2.2 Trapping and field sampling

We trapped males during a pre-breeding session from April 10-12, prior to the emergence of any females. Based on the fact that we observed no squirrels on a visit to the site on April 5 and the landowner observed the first squirrel on April 7, we are confident that our trapping session caught animals that had emerged only days before capture at most. We returned for a post-breeding trapping session 2 weeks later (April 27-30, 2007). Animals were trapped by placing homemade burrow traps (Wobeser and Leighton 1979) or cage traps (Tomahawk #102, Tomahawk Live Trap Company, Tomahawk, WI, USA) in or next to burrows that animals were
seen to have entered. Traps were monitored at least every 30 minutes and captured males were then kept in cage traps covered with a pillowcase in a quiet location until trapping was completed. We started trapping soon after animals emerged in the morning (typically between 0800-0900 hours) and we trapped for at most 4 hours or until we had as many animals as we could process at one time (6-9 animals).

At the completion of trapping, animals were brought to a central processing area where they were placed in a cool, quiet location with pillowcases still covering the individual traps and left for at least 1 hour. Then, one animal at a time was removed from its trap, weighed, and anesthetized with isoflurane (IsoFlo, Abbott Laboratories, Saint Laurent, QC, Canada). We took a 0.6 mL “stressed baseline” blood sample. We could not obtain true baseline samples from unstressed animals because we found these arctic ground squirrels to be very reluctant to enter traps if we were nearby; hence there was no practical way to obtain blood samples within the 3-5 minutes typically needed to obtain true baseline values (see Delehanty and Boonstra 2009). Approximately 0.3 mL of the first blood sample was collected in a lavender-tip tube and sent for a complete blood count (including white cell counts) at a commercial lab (Vita-tech Veterinary Laboratory Services, Markham, ON, Canada). We also used this first blood sample to measure blood glucose levels (mg/dL) with a FreeStyle glucose meter (Abbott Laboratories, Alameda, CA, USA) and hematocrit levels, in duplicate, using microhematocrit tubes and a microhematocrit centrifuge. The remaining blood collected in this sampling and all blood from subsequent samplings were centrifuged and plasma was collected and frozen at -20°C until returning to the laboratory where it was stored at -80°C.

After taking the first blood sample, we started a hormone challenge protocol consisting of an injection of dexamethasone (“DEX”) followed two hours later by an injection of adrenocorticotropic hormone (“ACTH”). Dexamethasone is an artificial glucocorticoid that normally suppresses endogenous cortisol production by activating the glucocorticoid receptors in the pituitary (DeKloet et al. 1998), inhibiting ACTH production and down-regulating GC production. Chronic stress can impair this negative feedback system, and animals whose GC levels do not fall as much or as rapidly in response to DEX treatment are described as “dexamethasone resistant” (Bradley 1990; Sapolsky and Altmann 1991). We injected 0.1, 0.2, or 0.4 mg/kg DEX (Sabex, Montreal, Canada; dilutions with physiological saline) into the heart, then returned the animal to the covered trap and processed the next animal. Two hours after the
dexamethasone injection, we anesthetized the animal again and took a 0.2-0.3 mL blood sample (“DEX bleed”). Because previous studies used 0.4 mg/kg and found little evidence of dexamethasone resistance, we tried the lower doses to test whether the 0.4 mg/kg was too high to detect resistance. We found no correlation between dose and either total or free CORT in the DEX bleed (data not shown), indicating that a maximal response was achieved with the lowest dose. We therefore ignored dose as a factor in our analysis of dexamethasone resistance.

After taking the DEX blood sample, we injected 4 IU/kg of ACTH (ACTH; Synacthen, Novartis Pharmaceuticals Canada, Dorval, Quebec) intramuscularly into the thigh. Post-ACTH blood samples of 0.2 mL each were taken at 30, 60 and 120 minutes (“P30”, “P60” and “P120” bleeds).

After the P120 bleed, the animal was euthanized by anesthetic overdose and decapitation. On necropsy, we extracted and weighed paired adrenal gland mass to the nearest 1 mg (as another rough measure of adrenal capacity). As a gross measure of lipid energy stores we weighed free abdominal fat to the nearest 0.1 g. Free abdominal fat was defined as fat that was readily extracted from the abdominal cavity and did not include mesenteric fat. To illustrate the intensity of the breeding season, we examined the skin internally and externally, for evidence of wounding. We scored the degree of wounding according to the number of punctures (bite marks that penetrated the skin, usually only visible from the inside of the hide) and externally visible wounds (tears in the skin that were open to the muscle below or had scabbed over). Our scoring categories were: “None” for no wounds, “Minimal” for <10 punctures and <2 cm² of externally visible wounding, “Moderate” for ≥10 punctures and/or >2 cm² but <7 cm² of externally visible wounding, and “Severe” for ≥7 cm² of externally visible wounding and/or significant damage to muscle, testes or eyes.

4.2.3 Laboratory procedures

We measured total CORT and estimated maximum corticosteroid binding capacity (MCBC, a measure of CBG level) using the radioimmunoassay methods described in Delehanty and Boonstra (2009). In this procedure, we used dextran-coated charcoal to separate bound from unbound hormone. The MCBC results are sensitive to the length of time to which the hormone mixture is exposed to the charcoal because hormone/CBG complexes can dissociate during the incubation, thereby causing a loss of bound hormone to the charcoal. We used a 30 min incubation with charcoal whereas Boonstra et al. (2001b) used a 10 min incubation. We
calculated that samples lost 11% of bound hormone over the additional 20 min of incubation, and we therefore adjusted our MCBC values accordingly to make our results directly comparable to previously published data. In addition to binding by CBG, CORT also binds to albumin but with very low affinity. Based on a number of lines of evidence, some endocrinologists have concluded that albumin-bound hormone should be treated as free (Tait and Burstein 1964; Ekins 1992). The equation in Barsano and Baumann (1989) allows one to calculate non-CBG-bound hormone levels (i.e. free plus albumin-bound) given only the total CORT concentration, the maximum corticosteroid binding capacity, and the CBG/CORT equilibrium dissociation constant ($K_d$). Boonstra et al. (2001b) calculated the $K_d$ for arctic ground squirrel CBG as 22.2 nM using a dialysis technique. However, because this study was part of a larger comparative study of 5 species of ground squirrels, we recalculated the $K_d$ of arctic ground squirrels using the same technique that we would be using for the other species. Using methods adapted from Hammond and Läähteenmäki (1983), we calculated the $K_d$ as (mean±SE) 4.0±0.42 nM (see electronic supplement for details).

Because testosterone has been shown to affect CBG levels (Bradley 1987; McDonald et al. 1981), we measured androgen levels to see if they could explain any changes in MCBC that we observed. We measured androgen levels in the stressed baseline plasma sample by radioimmunoassay according to the procedures in Delehanty and Boonstra (2009). We refer to “androgen” levels because our antibody (P43/11; Croze and Etches 1980) had a 62% cross-reactivity with dihydrotestosterone (DHT) and 16% with androstenedione.

Both GCs and ACTH can be lipolytic (Kiwaki and Levine 2003; Vehiopoulos and Herzig 2007), and because the hallmark of the adaptive stress response is maximizing the availability of energy even at the expense of longer term survival, we expect these animals to very rapidly deplete fat stores and also to catabolize muscle as an energy source. Therefore, we predicted that pre-breeding animals should be able to mobilize free fatty acids (FFAs) in response to capture stress, whereas post-breeding animals should have virtually no fat reserves left and should, therefore, show a reduced FFA response to capture stress. We measured FFAs using a NEFA-C kit (Wako Chemicals USA Inc., Richmond VA, USA) modified to be used with a 96-well plate (Johnson and Peters 1993; see Delehanty and Boonstra 2009).
4.2.4 Statistics

All data were analyzed using SAS 8.2 (SAS Institute Inc., Cary, NC, USA). Where appropriate, data were first examined for normality using PROC UNIVARIATE NORMAL. We compared most variables using t-tests (PROC TTEST). Where pre- and post-breeding data had non-homogeneous variances, we use the Satterthwaite test. Where non-normal data could not be normalized with a transformation, we used the Wilcoxon-Mann-Whitney non-parametric two sample test using the EXACT option to generate a Monte Carlo-based exact p-value. Other statistical tests are described in the Results. All data are presented as means ± standard error.

4.3 Results

A total of 18 males were captured in the pre-breeding session and 17 were captured in the post-breeding session. Due to limited plasma volumes not all hormone assays could be performed on all individuals or all blood samples from an individual, so sample sizes for each comparison vary.

4.3.1 The toll of the breeding season

To put our main results in context, we first present our data on changes in mass and wounding to illustrate the intensity of the breeding season (Fig. 4.1). From the pre- to the post-breeding sessions, male arctic ground squirrels lost 31% of their body mass ($t=9.15$, df=34, $p<0.0001$) and 88% of free abdominal fat reserves ($t=11.35$; Satterthwaite adjusted df=14.2; $p<0.0001$). Over the same period, males suffered an increasing number of wounds, with the percentage of severe wounds increasing from 0 to 23% (Fisher’s exact test, $n=30$, $p<0.001$). None of the males we captured in the post-breeding session were free of wounds.

4.3.2 Hormonal changes

From the pre- to post-breeding trapping session, total CORT levels in the stressed baseline sample increased by 22% ($t=-2.20$, Satterthwaite df=25.6, $p=0.037$), but there was no net change in free CORT levels ($t=-0.13$, Satterthwaite df=23.1, $p=0.90$; Fig. 4.2) contrary to our predictions. Free CORT did not increase because there was a simultaneous 41% increase in MCBC ($t=-3.83$, df=33, $p=0.0006$) which was also contrary to our predictions. Individual
animals’ MCBC levels did not change over the course of the hormone challenge protocol (repeated measures ANOVA using SAS PROC MIXED, F_{4,33}=1.03, p=0.40)

The mean free CORT level 2 hours after the injection of dexamethasone was 0.31 ± 0.03 ng/mL in the pre-breeding session and 0.22 ± 0.02 ng/mL in the post-breeding session. The difference was statistically significant (Wilcoxon-Mann-Whitney two-sample test S=192.0, p=0.003). However, we think it is unlikely that the difference of 0.1 ng/mL has any biological significance given that they are only 2% and 1% of stressed baseline levels respectively. If the difference is real, it is in the opposite direction as predicted. However, because arctic ground squirrels in both sessions remained highly responsive to dexamethasone compared to semelparous marsupials, in which dexamethasone in post-breeding males elicits only a 10%-27% drop in CORT levels (McDonald et al. 1986; Bradley 1990), we conclude that there is no evidence of any meaningful change in dexamethasone resistance from the pre- to the post-breeding state. This, too, is contrary to our prediction.

### 4.3.3 Downstream measures of stress

To measure the effects of CORT levels on target tissues we measured blood glucose, free fatty acids, white blood cell counts, and hematocrit (Fig. 4.3). Post-breeding blood glucose levels were 10% higher than in the pre-breeding session (t=-2.18, df=33, p=0.037) and free fatty acid levels were 46% lower (Wilcoxon-Mann-Whitney two-sample test S=177.5, p=0.0002). White blood cell counts were 30% lower (t = 3.04, Satterthwaite adjusted df = 21.7, p = 0.0061), and hematocrit levels (% packed cell volume) were 15% lower (t = 5.68, df = 33, p<0.0001). All of these results were consistent with our predictions.

### 4.3.4 Explanatory data

Because our CORT and MCBC results were completely contrary to our predictions, we examined some of the other hormonal data we collected in order to better understand the causes of the CORT changes we observed. We looked at adrenal mass and the total CORT AUC in response to ACTH stimulation. Measuring total CORT levels over time in response to ACTH injection (i.e. the area under the curve—AUC—measured from the DEX bleed through the P120 bleed, using the DEX bleed CORT concentration as a baseline) provides an integrated measure of the animals’ sensitivity and capacity to produce CORT. This information helps us to
understand why we may see an increase or decrease in CORT levels. For example, an increase in AUC suggests that the adrenal glands are either more sensitive to ACTH or have a greater capacity to produce CORT. However, we found no change in adrenal mass; paired adrenal gland weights were 187±9.9 mg (n=12) in the pre-breeding session and 197±13.4 mg (n=17) in the post-breeding session (t=-0.57, df=27, p=0.57). The AUC was also constant between sessions. In the pre-breeding session the AUC was 282.7 ± 21.2 ng·h/mL (n=13) and in the post-breeding session it was 314.1 ± 11.9 ng·h/mL (n=15) (t=-1.33, df=26, p=0.19; Fig. 4.4).

We also measured androgens to see if they play a role in determining MCBC levels. There was no change in stressed baseline androgen levels (pre- and post-breeding levels were 11.6 ± 0.6 ng/mL and 10.2 ± 0.7 ng/mL respectively; t=1.66, df=33, p=0.11), but to examine the relationship between androgen levels and MCBC more closely, we ran an ANCOVA (SAS PROC GLM). Controlling for session (pre- or post-breeding), there was a positive relationship between MCBC levels and androgens (ANCOVA, F_{1,32}=4.32, p=0.046), but androgens only explained an additional 8% of the variation in MCBC levels after taking into account trapping session.

### 4.4 Discussion

We predicted that over the course of the 2-3 week breeding season male arctic ground squirrels would show an adaptive stress response similar to that observed in semelparous male marsupials. The dramatic increase in severe wounding and loss of body mass and abdominal fat (Fig. 4.1) indicated that this period was intensely costly for males. However, none of our three hormonal response predictions were borne out: we predicted higher post-breeding free CORT levels but found no change; we predicted lower MCBC in post-breeding animals but found MCBC increased from pre- to post-breeding sessions; and we predicted greater dexamethasone resistance in post-breeding animals but found very slight resistance in pre-breeding animals instead. Far from showing an adaptive stress response in which free CORT levels soar, male arctic ground squirrels appear to have a homeostatic response in which total CORT production (during acute stress) increases to compensate for an increase in MCBC, keeping the free CORT response constant throughout the breeding season. In contrast, all four of our predictions for downstream effects (higher glucose levels, lower free fatty acids, lower hematocrits, and lower white blood cell counts) were borne out (Table 4.2). Thus, if we look at the hormonal data, the
animals appear to have a homeostatic stress response, but if we look at the downstream physiology, they appear to have an adaptive stress response and be chronically stressed. We therefore reject the adaptive stress hypothesis; the HPA axis of male arctic ground squirrels does not show the characteristic features of surging free GC levels and falling MCBC associated with the adaptive stress response characteristic of semelparous marsupials.

We are left with the task of trying to make sense of these results. To do this, we first examine the CORT data in more detail to try to understand how the HPA axis changed over the breeding season. Then we propose several new hypotheses that can help to reconcile the CORT and downstream results.

4.4.1 Cortisol response

To interpret our results, it helps to think about the sequence of events leading up to the collection of our first blood sample. In the field, the animal starts from a basal unstressed CORT level. Upon capture, the HPA axis is stimulated and CORT production over resting levels begins. As free CORT levels increase, negative feedback at the level of the brain and pituitary act in opposition to the ongoing stimulation of the HPA axis by the continued captivity. We assume that by the time we take our stressed baseline blood sample, about 2-4 h later, an equilibrium exists between stimulation and negative feedback. The fact that we see no change in stressed baseline free CORT suggests that, from the pre- to post-breeding period, the HPA axis is not changing its functioning in response to acute capture stress. So why do post-breeding animals have higher total CORT levels? It is not because the adrenal glands are producing more CORT in response to stimulation: adrenal mass did not change and total CORT AUC in response to ACTH did not differ between trapping sessions, both of which indicate that adrenal sensitivity/capacity remained stable. Nor is there evidence of impaired negative feedback: pre- and post-breeding animals all responded strongly to dexamethasone, indicating that negative feedback continued to be robust. We therefore conclude that the most likely explanation is that free CORT levels determine the degree of negative feedback and, because MCBC is higher in post-breeding animals, more total CORT is required to reach free CORT concentrations that provide the same degree of negative feedback as in the pre-breeding session.

This consistency in the free CORT response is remarkable considering the dramatic changes that occur over the breeding season. The pre-breeding animals had extensive fat reserves and
physical aggression in those first few days above ground was relatively limited as evidenced by the low wounding scores (Fig. 4.1). However, three weeks later in the post-breeding session, mean body mass had decreased 31%, abdominal fat mass had decreased 88%, and the vast majority of animals had moderate to severe wounding. The fact that animals in such different conditions maintain an identical free CORT response suggests that they are adopting a homeostatic stress response. But if these animals are maintaining a homeostatic stress response, why does MCBC increase over the breeding season?

Two factors are usually cited as affecting CBG concentrations and, therefore, MCBC: testosterone and chronic stress. In semelparous marsupials, increasing testosterone over the breeding season causes CBG levels to decline (McDonald et al. 1981; Bradley 1987). In contrast, arctic ground squirrel CBG levels (measured indirectly as MCBC) increased over the breeding season (Fig. 4.2) despite unchanged androgen levels. Moreover, when we included androgen levels as a covariate in our comparison of pre- and post-breeding MCBC levels, we found a small positive effect of androgens on MCBC levels. We therefore conclude, as did Boonstra et al. (2001b), that CBG levels in arctic ground squirrels are not suppressed by testosterone.

Chronic stress has also been implicated in reducing CBG (Dallman et al. 1987; Armario et al. 1994; Fleshner et al. 1995; Boonstra et al. 1998). Boonstra et al. (2001b) measured an MCBC of 84.9 ng/mL in breeding male arctic ground squirrels (trapped at the equivalent time as our post-breeding males which had almost identical levels: 102.1 ng/mL) and an MCBC of 264.0 ng/mL in non-breeding males trapped in mid- to late-summer. They suggested that the lower MCBC in breeding animals reflected the stress of breeding. Our results do not support this hypothesis. We found that pre-breeding males, trapped prior to the onset of most male aggression at a time when they had ample fat reserves and little wounding, actually had lower MCBC values than the post-breeding males at the end of two weeks of intensive fighting and mating. CBG levels in male arctic ground squirrels appear to be determined by some factor other than androgen levels and chronic stress, and they change in a manner that is inconsistent with an adaptive stress response.

Thus, the HPA axis of male arctic ground squirrels is incompatible with both the adaptive stress and homeostasis models proposed by Boonstra and Boag (1992), and alternative explanations need to be examined. In the appendix, we propose five non-exclusive alternative hypotheses that
help to explain our observations, but here we focus on the two hypotheses that can explain our most significant observation, the increase in MCBC.

4.4.2 Alternative hypotheses

We call the first explanation the “basal stress hypothesis”. According to this hypothesis, male arctic ground squirrels maintain a constant acute stress response over the active season, but alter their basal physiology during the breeding season such that they have unusually high free CORT concentrations both during the pre- and post-breeding periods compared with non-breeding periods. We suggest that they do this by maintaining very low CBG levels in the breeding season so that a greater proportion of basal CORT (i.e. CORT levels in the absence of stressors activating the HPA axis) is free. By maintaining elevated free CORT levels in the basal state, the animals could be constantly evoking responses from CORT-sensitive target tissues, thereby causing the symptoms of glucocorticoid excess that we observed in our post-breeding downstream measures of stress (glucose, free fatty acids, white blood cell count and hematocrit). A phenomenon similar to this has been observed in rats. Fleshner et al. (1995) found that a single acute stress session caused basal CORT levels to be elevated for up to 96 hours and CBG levels were decreased for up to 48 hours, thereby resulting in several days of increased free CORT exposure even in the absence of stressors. A similar effect over 24 hours has been observed in Japanese quail (Malisch et al. 2010).

Our data and that of Boonstra et al. (2001a and 2001b) provide some support for the basal stress hypothesis. First, MCBC is at its lowest during the breeding season and then increases fourfold by mid-summer (this study and Boonstra et al. 2001b). Second, male arctic ground squirrels have unusually high levels of free CORT in the breeding season. Boonstra et al. (2001a) collected basal blood samples from male arctic ground squirrels shot in late April and early May (a time equivalent to the post-breeding sample in this study) and found that an astonishing 75% of total CORT was free. In contrast, free CORT in numerous other species is typically <10% of total CORT when animals are unstressed (e.g. approximately 2% in male zebra finches, *Taeniopygia guttata* [Breuner et al. 2006], between 2-6% in male house sparrows, *Passer domesticus* [Breuner and Orchinik 2001], 8-9% in stallions, *Equus caballus*, [Alexander and Irvine 1998], and around 6% in humans [Lewis et al. 2005]). The high level of free CORT in male arctic ground squirrels during the breeding season is therefore noteworthy. Unfortunately,
we have not been able to test a key prediction of the basal stress hypothesis: that male arctic
ground squirrels in mid-summer should have basal total CORT concentrations similar to those of
breeding season animals, but a greatly reduced proportion of free CORT. Another key prediction
is that negative feedback by hippocampal mineralocorticoid receptors (which regulate basal GC
levels; de Kloet (1998)) must be down-regulated during the period that MCBC values are low,
otherwise basal CORT levels would decline due to the negative feedback of the high free CORT
levels.

The second hypothesis that attempts to explain both the increase in MCBC and the downstream
indicators of chronic stress is the “reservoir hormone hypothesis”. Under this hypothesis,
although CBG-bound CORT is initially not available to target tissues, it acts as a reservoir of
CORT that is gradually released after the termination of the stressor (Rosner 1990, Hammond
1995, Breuner and Orchinik 2002). The higher total CORT and MCBC in post-breeding animals
relative to pre-breeding animals means that the post-breeding animals experience a more
sustained exposure to CORT as the bound fraction is released over time. Because hormone that
is bound to CBG at the time of stress (e.g. during aggressive interactions) will gradually be freed
after the end of the stressor, this hypothesis predicts that the downstream measures of stress will
be proportional to total CORT rather than to free CORT. This conclusion is contrary to many
studies (including Delehanty and Boonstra 2009) that assume that the free hormone fraction
measured at a single point in time provides the most meaningful measure of the downstream
impacts of stress (see the appendix for a discussion of how this hypothesis impacts the free
hormone hypothesis). This would explain the downstream signs of chronic stress observed in the
post-breeding animals in our study. Under this hypothesis, the increase in MCBC over the
course of the breeding season can be seen as a strategy to increase overall CORT exposure of
target tissues at an energetically demanding time. This hypothesis has been proposed as the
explanation for a seasonal pattern in corticosterone levels in house sparrows (*Passer
domesticus*). Breuner and Or nichik (2001) found that baseline and stressed total corticosterone
levels were higher during nesting compared to molt and winter, but free corticosterone levels
remained constant throughout the year because MCBC increased during molt as well. They
proposed that energetic needs during nesting were greater and by increasing the CBG-bound
hormone pool during the nesting season the birds would have a reservoir of corticosterone to
regulate energy availability without further activation of the HPA axis (Breuner and Orchinik
Some evidence that CBG plays a role in sustaining energy mobilization comes from the fact that humans who lack CBG experience chronic fatigue (Torpy et al. 2001) and mutant mice strains that lack CBG show reduced activity (Petersen et al. 2006). What has not been demonstrated, though, is whether this energetic effect is a function of plasma CBG or tissue-based CBG.

This is an attractive hypothesis in our study because it is a much simpler explanation for the increased MCBC and the downstream indicators of stress than is the basal stress hypothesis. However, there is one main hurdle to this hypothesis as it applies to male arctic ground squirrels: their MCBC values double between the breeding season and mid- to late-summer (Boonstra et al. 2001b), and yet summer is a time when males are focused on gaining weight and there are abundant food resources. If higher MCBC values are a way to increase the net exposure of target tissues to CORT, it is difficult to understand why the animals would be increasing their exposure at a time of plentiful food and little evidence of aggression (Boonstra, pers. comm.). A longitudinal study that tracks the functioning of the HPA axis and downstream measures of stress would be essential for testing this hypothesis.

4.4.3 Conclusion

Male arctic ground squirrels have a life history that is broadly similar to that of the semelparous marsupials, but they do not exhibit the marsupial adaptive stress response characterized by an unrestrained HPA axis that leads to elevated free GC levels and classic symptoms of GC excess. Instead, symptoms of chronic stress develop in male arctic ground squirrels over the course of the breeding season despite constant free CORT levels. The mechanism by which symptoms of chronic stress develop despite a constant stress response remain unclear.

Our results also illustrate the importance of measuring more than just CORT levels when trying to understand the role of the HPA axis in an animal’s biology. Had we only measured total CORT, we would have reported an increase in CORT production over the course of the breeding season and interpreted it as increased stress responsiveness. By including the MCBC assay, we were able to show that the biologically active free CORT levels actually remained constant. Had we limited our measures of stress to CORT and MCBC, we would have concluded that stress responsiveness was constant and the animals were unaffected by the breeding season. It is only by including some downstream measures of stress that the complex dynamics of the HPA axis
begin to be revealed. Finally, our results have also highlighted the potential importance of longitudinal studies that cover several different periods in an animal’s life. Our study examined the HPA axis over the intense 3-week period of emergence and mating. On its own, the slight increase in MCBC over the breeding period is curious, but when we compared these levels to the much higher MCBC levels that Boonstra et al. (2001) found in mid-summer we were able recognize that the pre- and post-breeding levels were both extremely low.

Although our understanding of how the HPA axis supports the reproductive life history strategies of the male arctic ground squirrels is still incomplete, we now have several testable hypotheses to explore the complex relationship between the HPA axis and animal ecology.
**Table 4.1:** Physiological status of breeding male arctic ground squirrels as predicted by the adaptive stress hypothesis (Boonstra and Boag 1992) and observed by Boonstra et al. (2001b). The predicted response refers to the predicted status of breeding males in comparison with non-breeding, pre-hibernating adult males and juvenile males.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Predicted Response</th>
<th>Observed Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Cortisol</td>
<td>Higher</td>
<td>No difference or higher*</td>
</tr>
<tr>
<td>Response to dexamethasone</td>
<td>Dexamethasone Resistance</td>
<td>Slight resistance</td>
</tr>
<tr>
<td>Corticosteroid binding globulin</td>
<td>Lower</td>
<td>Lower</td>
</tr>
<tr>
<td>Glucose</td>
<td>Higher</td>
<td>Lower</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>Higher</td>
<td>Lower than non-breeding males, but the same as juveniles</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>Lower</td>
<td>Lower than non-breeding males, but the same as juveniles</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>Lower</td>
<td>Lower</td>
</tr>
<tr>
<td>Response to antigen challenge</td>
<td>Poorer</td>
<td>Poorer</td>
</tr>
</tbody>
</table>

* Boonstra et al. (2001a) used a hormone challenge protocol that involved taking 5 blood samples over the course of two hormone treatments (the same protocol we used - see Methods for details). The first blood sample was taken after the stress of capture and handling, but before the administration of the hormones. The three classes of squirrels showed no difference in this baseline free cortisol level. However, in a repeated measures ANOVA analysis, there was a significant effect of class (with breeding males having higher free cortisol levels than non-breeding adult males and juveniles).
Table 4.2: Predictions of the adaptive stress hypothesis and observed changes in this study. Arrows indicate increases or decreases from the pre- to post-breeding sessions in the responsiveness of the HPA axis, energy mobilization and condition, and the immune response of male arctic ground squirrels. Abbreviations: CORT, cortisol; DEX, dexamethasone; MCBC, maximum corticosteroid binding capacity (our measure of corticosteroid binding globulin levels).

<table>
<thead>
<tr>
<th>Predicted Change</th>
<th>Observed Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stressed Baseline</td>
<td>⬆ None</td>
</tr>
<tr>
<td>free CORT</td>
<td></td>
</tr>
<tr>
<td>DEX resistance</td>
<td>⬆ (⬇)*</td>
</tr>
<tr>
<td>MCBC</td>
<td>⬇ ⬆</td>
</tr>
<tr>
<td>Glucose</td>
<td>⬆ ⬆</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>⬇ ⬇</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>⬇ ⬇</td>
</tr>
<tr>
<td>White Blood Cells</td>
<td>⬇ ⬇</td>
</tr>
</tbody>
</table>

* Although there was a statistically significant change in DEX free CORT levels, the difference was so small that there is likely of no biological significance.
Figure 4.1: Changes in (a) body mass, (b) free abdominal fat and (c) wounding in male arctic ground squirrels from the pre-breeding (n = 19, 14, and 13 respectively) to post-breeding (n = 17 for all measures) trapping session. All changes were statistically significant (see Results). Error bars are standard errors. See Methods for wound scoring.
Figure 4.2: Plasma cortisol and maximum corticosteroid binding capacity (MCBC) in male arctic ground squirrels in the pre-breeding (n=18) and post-breeding (n=17) sessions measured at initial bleed (mean ± SE). Total CORT and MCBC are significantly different in pre- and postbreeding sessions; free CORT is not.
Figure 4.3: Changes in metabolic and blood parameters in male arctic ground squirrels over the breeding season (means ± 1 SE). All data come from the initial bleed of the hormonal challenge and all changes are significantly different (P < 0.05). Sample sizes are n=18 pre-breeding males and n=17 post-breeding males except for free fatty acids (FFA) (n=16 post-breeding) and white blood cells (n=15 pre-breeding).
Figure 4.4: Total CORT response in male arctic ground squirrels to capture and the hormone challenge protocol in pre-breeding (solid line, n=13) and post-breeding (dashed line, n=15) sessions (mean ± SE). For the area under the CORT curve in response to the ACTH challenge, individual CORT concentrations were standardized by subtracting the animal’s total CORT level at the DEX bleed (taken immediately prior to injecting ACTH) from each reading. The area under the ACTH response curve did not differ between sessions.
References


Appendix 1

In this appendix, we include details of our method for calculating the equilibrium dissociation constant ($K_d$) of CBG, we expand our discussion of the various alternative hypotheses that we believe could play a role in explaining our results, and we discuss how the reservoir hormone hypothesis relates to the free hormone hypothesis.

Methods

Our method for calculating the $K_d$ was adapted from the saturation binding of Hammond and Lähteenmäki (1983). Plasma was stripped of CORT with activated charcoal (70 mg per 1 mL plasma) left for 4 hours at 37°C. After centrifuging to obtain clean plasma, 50 µL of diluted plasma (diluted 1/25 with 0.01 M phosphate buffered saline containing 0.1% gelatin, pH 7.4) was then incubated with 50 µL of a series of eight concentrations of radioactive $[1,2,6,7-^3H]$cortisol (Amersham Biosciences, USA). Concentrations ranged from 0.5 to 30 nM either with (non-specific binding tubes, in duplicate) or without (total binding tubes, in duplicate) 200 pmol of non-tritiated CORT. A single set of 8 tubes (total count tubes) received the tritiated CORT and 750 µL buffer. All tubes were allowed to equilibrate for >3 h at 37°C on a shaker. After this incubation period, we placed the non-specific binding tubes and total binding tubes in a saline ice bath (0°C) and we added 700 µL ice-cold dextran-coated charcoal (1.25 g charcoal with 0.125 g T70 dextran dissolved in buffer) to the tubes within 1 min. These tubes were then vortexed briefly. All tubes were then placed in a centrifuge at 0°C. Ten minutes after the charcoal was added to the last tube, the centrifuge was started and the tubes were spun at 2000 × g for 12 min. We pipetted 500 µL of supernatant from each tube into 2.5 mL of scintillation fluid (Biosafe II, Research Products International, USA). These were then counted in a scintillation counter (Packard Tri-Carb 2900TR, Boston, USA). Specific binding at each hot cortisol concentration was calculated by subtracting the counts per minute (cpm) of the non-specific binding tubes from those of the total binding tubes. Specific binding was plotted against free hormone concentration in the total binding tubes. Free hormone (the concentration of hormone neither specifically nor non-specifically bound at equilibrium) was calculated by multiplying the concentration of hot cortisol added (i.e. from 0.5 to 30 nM) by [1 - (cpm of the total binding tube)/(cpm of the total count tube)]. We calculated the equilibrium dissociation constant using non-linear regression
(SAS PROC NLIN) to fit the equation \( y = B_{\text{max}} \frac{x}{(x + K_d)} \) where \( y \) is the activity of bound hormone in cpm, \( x \) equals the concentration of free hot cortisol in nM, \( B_{\text{max}} \) equals the maximum specific binding capacity of the CBG in counts per minute, and \( K_d \) is the equilibrium dissociation constant in nM.

**Proposed Alternative Hypotheses**

We can conceive of five explanations as to how male arctic ground squirrels can show downstream indications of chronic stress despite seeming to maintain a constant stress response:

1. **Wrong Metric Hypothesis**: our downstream measures of stress are, in fact, not indicative of chronic CORT exposure and instead reflect other physiological changes unrelated to stress.

2. **Frequency of Activation Hypothesis**: despite a consistent free hormone response to acute stressors, the cumulative effect of repeated acute stressors over the breeding season could cause downstream CORT target tissues to be chronically exposed to elevated CORT.

3. **Sensitivity Hypothesis**: target tissues are upregulating their CORT sensitivity as the breeding season progresses by increasing glucocorticoid receptor density or increasing the degree to which receptor-CORT complexes affect gene expression.

4. **Basal Stress Hypothesis**: CBG is being downregulated during the breeding season such that in the unstressed basal state (i.e. the male is not being challenged by another male or by live-trapping) a large proportion of the total CORT circulates as free CORT, thereby continually eliciting a stress-like response from CORT sensitive target tissues.

5. **Reservoir Hormone Hypothesis**: the increase in CBG-bound CORT in the post-breeding session provides a reservoir of CORT that is released over time after the HPA axis is no longer stimulated, effectively increasing the duration of the stress response and the net exposure of CORT-sensitive tissues to CORT.
We address the basal stress and reservoir hormone hypotheses in the main paper, so here we will address only the first three hypotheses.

The first hypothesis, that our downstream measures are not actually indicative of stress, supposes that our metabolic and blood measurements have changed for reasons other than chronic CORT exposure. It is certainly true that each of our measures is influenced by factors other than CORT. However, these measures have been successfully used in other studies of chronic stress (e.g. Boonstra and Singleton 1993; Boonstra et al. 1998; Ackerman et al. 2000; Clinchy et al. 2004), and their use as indicators of chronic stress has a sound underpinning in the basic physiology of the HPA axis. Moreover, we cannot conceive of another physiological explanation for these outcomes; at a time when male arctic ground squirrels are losing so much mass and engaged in such severe aggressive competition for mates, it is difficult to imagine that another physiological response would be more prominent than the stress response. We believe that our results are true indications of chronic stress, and the remaining four hypotheses represent more probable, non-exclusive explanations for our observations.

The second hypothesis focuses on the frequency of activation of the acute stress response. Under this hypothesis, stressful interactions are frequent enough during the breeding season that CORT-sensitive tissues are chronically exposed to transient elevations in CORT, thereby producing the downstream indications of chronic stress. We have very little evidence either for or against this hypothesis. Typically, repeated acute stressors lead to changes in the acute response as well: either amplifying the free CORT response (e.g. in rats, Retana-Márquez et al. 2003) or reducing it (e.g. in starlings, *Sturnus vulgaris*, Rich and Romero 2005). We saw no change in the free CORT response of male arctic ground squirrels, but this is not strong evidence against the frequency hypothesis. This hypothesis could be tested by observing individual male behaviour through the breeding season and looking for correlations between the frequency of aggressive interactions and measures of chronic stress. Another test would be to compare the downstream measures of stress in animals that are exposed to “intruder” males in staged encounters with those that are not as Buck and Barnes (2003) did (see also Scott (1987) who performed a similar experiment in *Antechinus*).

Under our third hypothesis, the sensitivity hypothesis, the HPA axis responds to stressors in a consistent manner throughout the year, but certain CORT-responsive tissues increase their
sensitivity over the course of the breeding season. The increased sensitivity of tissues, along with the increased frequency of acute stressors, could have the same effect as chronic exposure to high CORT concentrations. There are several levels at which tissue sensitivity could be enhanced. First, tissues could up- or downregulate the expression of 11β-hydroxysteroid dehydrogenases (which interconvert CORT and biologically inactive forms, thereby either amplifying or reducing the CORT signal being received by the tissue). Second, glucocorticoid receptor density could be altered. Third, the efficiency of CORT signal transduction could be changed. Receptor density and 11β-hydroxysteroid dehydrogenase activity can be measured (Jamieson et al. 1999; Cole et al. 2000; Mai et al. 2005) in key tissues like adipose, muscle and liver, and would provide a good test of this hypothesis. One potential benefit of this strategy is that the animals could vary the sensitivity of specific tissues, thereby tailoring the stress response to particular seasonal or environmental circumstances. For example, during the breeding season, an animal could increase the sensitivity of tissues that promote the mobilization of energy but keep reproductive tissues comparatively insensitive to CORT so as not to suppress reproductive activity.

Is the Reservoir Hormone Hypothesis Consistent with the Free Hormone Hypothesis?

Under the reservoir hormone hypothesis, we suggested that total GC levels are a better predictor of downstream effects of stress than free GC levels. Whether this contradicts the free hormone hypothesis depends on exactly what one means by “free hormone hypothesis”, and the literature is not always consistent on this point.

An excellent starting point for any discussion of the free hormone hypothesis is Mendel (1992). There, he points out that the “free hormone hypothesis” had (at the time he was writing) often been worded along the lines of “intracellular hormone concentrations (and therefore biologic activity) are dependent on the concentration of free rather than protein-bound hormone in the plasma” (Table 2 in Mendel 1992). Mendel (1992) points out that this conflates two separate issues: what hormone fraction is able to leave the circulatory system to enter tissues, and what measure of circulating hormone best predicts downstream biological responses. He then defines the “free hormone transport hypothesis” as the simpler proposition that only free hormone can
pass out of the circulatory system. Using several lines of evidence, Mendel (1992) argues persuasively that the free hormone transport hypothesis is generally valid whereas the proposition that plasma free hormone levels determine biological effects is true only in certain instances (based on such things as tissue-specific uptake rates and capillary flow rates).

Following this terminology, the reservoir hormone hypothesis is contrary to the free hormone hypothesis (because downstream effects reflect total rather than free hormone concentrations), but not the free hormone transport hypothesis (because it is only as bound hormone gradually dissociates from CBG that it enters the tissues and exerts it effect). However, Mendel’s nomenclature is not widely used in the comparative literature; in fact, most of the recent literature uses the term “free hormone hypothesis” in its original sense to refer to what Mendel called the “free hormone transport hypothesis”. For example, Breuner and Orchinik (2002) define the free hormone hypothesis as follows (at p. 100):

“According to the free hormone hypothesis, steroid bound to plasma binding globulins is unavailable to tissues; the ‘free’ (unbound) hormone is the biologically active fraction, able to enter cells, activate intracellular or membrane receptors, and also be available for metabolism in the liver.”

Thus, when Breuner and Orchinik (2002) discuss the potential role of CBG as a reservoir of hormone, they describe it as “[c]onsistent with the free hormone hypothesis” (p. 100). We have adhered to this definition of the free hormone hypothesis in this paper, so we do not see our results as contrary to the free hormone hypothesis (in the sense of Mendel’s free hormone transport hypothesis).

Of course, to focus only on what hormone leaves the capillaries is to miss the larger issue that most physiological ecologists want to address: how do we get a meaningful measure of the state of an animal’s HPA axis? Should we be focusing on free or total hormone levels? What is the significance of changes in CBG levels? Implicit in many studies that measure free hormone levels, is the assumption that these are the biologically relevant values, and that the slow dissociation of bound hormone has a negligible effect on GC target tissues. If viewed through the lens of the reservoir hormone hypothesis, our results suggest that this is not true. However, additional evidence is needed before we can distinguish between the several alternative hypotheses that we have proposed.
Literature Cited


Copyright Acknowledgement

A version of this paper has been previously published (with the appendix as an electronic supplement): Delehanty, B., and R. Boonstra. 2011. Coping with Intense Reproductive Aggression in Male Arctic Ground Squirrels: The stress axis and its Signature Tell Divergent Stories. Physiological and Biochemical Zoology, 84:417-428. © 2011 The University Of Chicago, reproduced with permission.
Chapter 5

5 The evolution of the HPA axis: Reproductive lifespan fails to predict breeding season function in ground squirrels

5.1 Introduction

Life history traits are evolved traits that bear directly on an organism’s fitness; they include traits such as age at first reproduction, number of offspring produced, and reproductive lifespan. The combination of life history traits in any species or group of individuals within a species is the life history strategy. The diversity of life history strategies in nature is impressive, but not all possible combinations of traits are observed. Most species are either long-lived with low reproductive rates or short-lived with high reproductive rates, leading to the conclusion that there are constraints on the evolution of life history strategies (Ricklefs and Wikelski 2002). One explanation for this is the existence of tradeoffs between these two traits. There is considerable evidence that a tradeoff between reproduction and survival is ubiquitous in nature (Reznick 1985; Reznick 1992) but the physiological basis for such a tradeoff remains elusive (Reznick et al. 2002; Stearns 2000).

The vertebrate HPA axis (the hypothalamic-pituitary-adrenal axis in birds and mammals and the hypothalamic-pituitary-interrenal axis in amphibians, reptiles, and fish) is highly conserved in its basic functioning across all vertebrates. The activation of the HPA axis plays a critical role in mobilizing energy stores for immediate use but sustained activation can have deleterious effects on survival and reproduction (Sapolsky et al. 2000). Because of this it has been hypothesized (Boonstra and Boag 1992; Lee and Cockburn 1985; Ricklefs and Wikelski 2002; Wingfield et al. 1998) that the HPA axis could mediate the tradeoff between current reproduction and survival (i.e. future reproduction).

The basic model of the HPA axis is illustrated in Fig. 5.1(a). Under non-stress conditions, glucocorticoids (GCs) fluctuate at relatively low concentrations in a circadian rhythm, primarily governed by negative feedback at the level of the hippocampus (Bamberger et al. 1996). As GC
levels increase, the hormone occupies the high affinity mineralocorticoid receptors in the hippocampus, which suppress further release of GCs (de Kloet et al. 1998; Joëls et al. 2008). However, when an organism perceives a stressor—any threat to homeostasis coming from the internal or external environment—the sympathetic nervous system causes the release of catecholamines to generate immediate physiological effects (over a period of seconds to minutes) while the HPA axis begins a neuroendocrine cascade that ultimately causes the release of GCs at concentrations above basal levels over a period of several minutes to hours (Sapolsky et al. 2000). Under acute stress conditions, negative feedback is accomplished by activation of lower affinity GC receptors in the pituitary, hypothalamus and hippocampus, eventually terminating further GC production (de Kloet et al. 1998; Keller-Wood and Dallman 1984).

At acute stress levels, GCs are generally described as promoting the immediate survival of the individual (Sapolsky et al. 2000; Wingfield et al. 1998). They do this reducing uptake of glucose in peripheral tissues thereby increasing blood glucose levels and delivery to exercising muscles, promoting lipolysis and protein catabolism to fuel hepatic gluconeogenesis (i.e. conversion of free fatty acids and amino acids into glucose), and promoting glycogen deposition in the liver (Sapolsky et al. 2000). However, prolonged GC exposure begins to have detrimental effects on survival, such as impairing immune function, and catabolism of tissues that will need to be rebuilt when the stressor finally ends (Sapolsky 2002; Sapolsky et al. 2000). In other words, the effects of GCs that promote short-term survival can start to have negative effects on the animal’s fitness by threatening reproduction and survival over the longer term (e.g. Boonstra et al. 1998; Clinchy et al. 2004).

However, research on a group of Australian marsupials shows that chronic activation of the HPA axis can be adaptive for some life history strategies. Semelparity is at one extreme of the current/future reproductive spectrum, in which an individual’s lifetime reproductive success is determined during a single intense mating period. In at least 10 species of semelparous dasyurid marsupials the HPA axis functions very differently from that in iteroparous species. In the semelparous species, males experience a rapid increase in total and free GC levels over the short, intense breeding season. The increase in free GC is the result of several factors: adrenal production increases, testosterone drives down corticosteroid binding globulin (CBG) production which lowers the proportion of bound GC, and, in at least two of these species, *Phascogale calura* and *Antechinus swainsonii*, the negative feedback system fails (Bradley 1987; Bradley
Elevated free GC levels, lead to gastric ulcers, suppression of immune and inflammatory responses, increased parasitism, and shifts in hematological parameters (Barker et al. 1978; Bradley et al. 1980; Cheal et al. 1976) that ultimately result in male die-off. Braithwaite and Lee (1979) hypothesized that the sustained free GC exposure is adaptive because it allows the males to maximize the amount of energy devoted to breeding by obtaining it from internal resources and thus minimize the amount of time to get energy from external, environmental sources. Breeding in these dasyurids occurs at a time when food resources are scarce in their highly seasonal environment. In contrast to these semelparous species, iteroparous dasyurids like the fat-tailed dunnart, Sminthopsis crassicaudata, show no relationship between testosterone and CBG levels, and although cortisol concentrations fluctuate over the breeding season, total GC levels are always lower than the maximum CBG binding capacity; thus the free, biologically active GCs remain low (McDonald et al. 1981).

The distinct endocrine profile of the semelparous marsupials led Lee and Cockburn (1985) to propose that the gluconeogenic effect of cortisol allow these species to derive energy from the breakdown of muscle, thereby freeing them to spend time seeking mates instead of foraging during a time of scarce food. They also predicted that this strategy could apply to other small mammals and could explain the spring population declines observed in a number of vole species (Braithwaite and Lee 1979; Lee and Cockburn 1985). This “adaptive stress hypothesis” focused on the potential for GC excess, caused by the disregulation of the HPA axis, to support life history strategies characterized by short lifespans and early reproduction. However, subsequent studies have failed to find a similar role for GCs in the semelparous didelphid marsupial, the Virginia opossum (Didelphis virginiana; Woods and Hellgren 2003), or in other mammal species with short male reproductive lifespans like the meadow vole (Microtus pennsylvanicus; Boonstra and Boag 1992). Boonstra and Boag (1992) pointed out that, although male meadow voles are short-lived, they are iteroparous, typically having multiple mating opportunities as females have multiple litters during an extended breeding season. They therefore proposed that the adaptive stress response would be limited to those species in which females raise a single litter each year (as in the dasyurid marsupials) and in which all animals have a low survival rate between years. In contrast, they proposed that species with longer reproductive lifespans should have a
“homeostatic” stress response characterized by intact negative feedback, maintenance of CBG levels, and a more modest increase in free GCs.

We set out to test the adaptive stress hypothesis using a comparative approach. We studied five species of North American ground squirrels that vary in their mean reproductive lifespan (ranging from 1.1 to 2.7 years; see Table 5.1). We studied arctic (AGS) Richardson’s (RGS), Columbian (CGS), thirteen-lined (TLGS), and Franklin’s (FGS) ground squirrels (Urocitellus parryii, U. richardsonii, U. columbianus, Ictidomys tridecemlineatus, and Poliocitellus franklinii, respectively, following the reclassification by Helgen et al. 2009). The five species are closely related based on two independent phylogenies using mitochondrial DNA (Harrison et al. 2003; Herron et al. 2004; see a simplified phylogeny in Fig. 1.2), and they have similar natural histories. All five species live in environments characterized by long, cold winters during which the animals hibernate. Males emerge prior to females and the short annual breeding season begins a matter of days after the females begin to emerge. Males of all species mate with multiple females. Thus reproduction is a similar challenge for all five species: males must emerge from hibernation ready to begin breeding with as many females as they can. In light of this similarity the adaptive stress hypothesis supposes that, over the course of the breeding season, the functioning of the HPA axis in these species will be shaped primarily by male reproductive lifespan.

The adaptive stress hypothesis generates two predictions about the how the HPA axis should vary among species. The first, a quantitative prediction, is that there will be a negative correlation between reproductive lifespan and GCs in the breeding season because species with long reproductive lifespans cannot afford to risk the detrimental effects of high GC levels. The second, a qualitative prediction, is that males of species with shorter reproductive lifespan should show a progression of the HPA axis over the breeding season similar to that of the semelparous dasyurids (i.e. long term exposure to GCs), whereas species with longer reproductive lifespans should show a homeostatic stress response (in which exposure to GCs is tightly controlled by CBG and strong negative feedback). We made the following specific predictions about the change in male physiology from pre-breeding (prior to the challenge of securing mates) to post-breeding (immediately at the end of the breeding season) sampling periods. First, because the hallmark of the adaptive stress response is a strong increase in free hormone levels leading to
chronic GC exposure over the breeding season, we predicted that trends in GC and CBG levels from pre- to postbreeding samples would include:

(a) shorter-lived species would show greater increases in free GC levels than longer-lived species;

(b) shorter-lived species, but not longer-lived species, would show decreases in CBG levels as a result of high testosterone levels (shown to suppress CBG in some semelparous marsupials: Bradley 1987; McDonald et al. 1981) or chronic stress (found to reduce CBG levels in other species: Armario et al. 1994; Boonstra et al. 1998; Dallman et al. 1987; Fleshner et al. 1995); and

(c) shorter-lived species would show attenuated negative feedback in response to adrenal production of GC as seen in semelparous marsupials (Bradley 1990; McDonald et al. 1986), whereas longer-lived species would show intact negative feedback.

Next, because one of the primary roles of GCs is to mobilize energy in times of need, we considered how the HPA axis would influence the metabolic profiles of ground squirrels over the breeding season. Because GC reduces the peripheral uptake of glucose, promotes lipolysis and protein catabolism, and promotes hepatic gluconeogenesis so that the stores of glucose and glycogen in the liver are increased under chronic stress (Boonstra et al. 1998; Sapolsky et al. 2000), we predicted that shorter- and longer-lived males would show different changes from pre- to postbreeding session in their metabolic profiles:

(a) shorter-lived species would show greater increases in stress-induced blood glucose levels than longer-lived species; and

(b) shorter-lived species, but not longer-lived species, would show a decline in stress-induced free fatty acid levels.

Finally, we used two hematological measures to assess general health and immune function. First, we used hematocrit levels as a measure of condition. Although hematocrit can change in response to a number of factors, a number of wildlife studies have found that higher hematocrit levels indicate better body condition (Boonstra et al. 1998; Boonstra et al. 2001b; Franzmann and Leresche 1978; Hellgren et al. 1993; Lochmiller et al. 1986; but see Dickens et al. 2009)).
Second, we used a decline in white blood cell counts as a coarse measure of stress-induced immunosuppression (Sapolsky et al. 2000). We predicted the following changes over the breeding season:

(a) a decline in hematocrit levels in shorter-lived, but not longer-lived species; and

(b) decreased white blood cell counts in shorter-lived but not longer-lived species.

5.2 Materials and Methods

5.2.1 Study Species

Life history traits are determined by numerous intrinsic and extrinsic factors, including phylogenetic constraints, tradeoffs among life history traits and ecological and environmental impacts on survival and reproduction (Stearns 2000). Thus, a tradeoff mediated by stress physiology is only one of several factors shaping a trait like male reproductive lifespan. To help identify the relationship between reproductive lifespan and HPA axis functioning, we studied a group of closely related species that experience similar environments and reproductive challenges to isolate the role of the HPA axis as much as possible. The analysis by Harrison et al. (2003) suggests that these species fall into two clades (RGS, AGS and CGS in one clade; FGS and TLGS in the other) that diverged 10.7 million years ago. The TLGS and FGS diverged 9.1 million years ago, the CGS diverged from the RGS and AGS 4.4 million years ago, and the RGS and AGS diverged 1.3 million years ago (Harrison et al. 2003). The AGS, RGS and FGS all have shorter-lived males, whereas CGS and TLGS have longer-lived males. Even among closely related species, though, there are numerous differences in ecological factors that cannot be controlled but which might influence GC levels. We therefore selected species that, as much as possible, have well described natural histories to help us interpret our results. Table 5.1 sets out some of the key aspects of the biology of our five species.

5.2.2 Components of the Stress Profile

A stress profile is a collection of physiological measures that describe the functioning of the HPA axis and the effects of GCs on other physiological systems (“downstream effects”). By obtaining stress profiles in prebreeding and postbreeding animals in our 5 ground squirrel species
we are able to observe how the functioning of the HPA axis has changed over a 2-3 week period when reproduction is the central focus of male ground squirrels. For a basic description of the functioning of the HPA axis, there are two main pieces of information that we would ideally like to have. First, we would like to measure baseline GC levels, in which GC levels are not being affected by acute stressors such as capture, a predation attempt, or aggressive social encounters. This tells us what level of GCs the animal is being exposed to under conditions as close to basal as possible (basal levels imply the absence of any stressors whatsoever, but in wild populations we can rarely be sure that the animal’s HPA axis is not being activated by persistent stressors like low food abundance; hence, a “baseline” measure is an approximation of “basal” levels). Second, we would like to measure the GC response to a standardized acute stressor to see how strongly the animal’s HPA axis reacts when it encounters a stressor. These two pieces of information are most commonly obtained through the “capture stress protocol.” This involves taking an initial blood sample within a few minutes of capture, followed by one or more blood samples over the next 30 min to 1 h to measure the GC response to capture (e.g. Wingfield et al. 1995). Because it takes several minutes for plasma GCs to rise following an acute stressor, the initial blood sample provides baseline GC levels as they were prior to capture. The subsequent blood samples measure the strength of the acute stress response. This procedure is most common in avian studies, because many birds are caught in mist nets that allow for the continual monitoring of the net and rapid removal of captured birds. In many mammals, however, trapping methods or the wariness of the animals typically preclude researchers from being close enough to the trap to obtain an initial blood sample within 3 min of capture (Boonstra 2005; Sheriff et al. 2011). Because logistics and the wariness of most of our species prevented us from obtaining blood samples within 3 min, we could not use the capture stress protocol and instead adopted a hormone challenge approach to characterizing the HPA axis.

The hormone challenge protocol is an attempt to probe the strength of the stress response and negative feedback, and to measure some downstream impacts of GC exposure. Our first blood sample was taken after ≥1 h of captivity and therefore gives us GC levels under sustained acute stress. It is acute in the sense that capture is a sudden event that is intensely stressful, but because it is longer in duration than any conceivable natural predation attempt we call it “sustained”. With this sample, we measured total GC, CBG levels, and, based on those values, we estimated free GC (details in section 5.2.4 Laboratory Procedures). Next we injected the
animals with dexamethasone (DEX), an artificial GC that suppresses endogenous GC production by activating GC receptors in the pituitary (Cole et al. 2000; de Kloet et al. 1998). This lets us probe one aspect of the negative feedback system; if GC levels have not fallen 2 h after the DEX injection, then we conclude that negative feedback is impaired (Boonstra 2005; Sapolsky and Altmann 1991). After taking the DEX bleed 2 h post-injection, we injected adrenocorticotropic hormone (ACTH) to directly stimulate adrenal production of GCs and took three blood samples over 2 h. By calculating the area under the GC response curve (the AUC), we can assess adrenal sensitivity/capacity. This is similar to measuring the increase from baseline to acute stress GC levels in the capture stress protocol, which is a measure of the strength of the stress response. Adrenal mass is another rough indicator of adrenal capacity based on the assumption that adrenal hypertrophy should reflect greater capacity to produce adrenal steroids. We also use the ratio of the maximum free GC level in response to ACTH to initial free GC to measure “intrinsic restraint” of the HPA axis. Because ACTH directly stimulates the adrenals, the maximum free GC levels following the ACTH injection are not tempered by lack of drive in the hypothalamus or pituitary or by negative feedback. The ACTH response represents the maximum ability of the adrenals to respond to ACTH stimulation. In contrast, the free GC level in our initial blood sample is the result of ongoing stimulation of the HPA axis as a result of capture stress but it is modulated by any insensitivity to stressors at the level of the brain or pituitary and to negative feedback of the GCs. Hence the ratio of these two levels (i.e. maximum/initial) represents a measure of the degree to which the HPA axis produces a less-than-maximal GC response when challenged by a stressor (i.e. “intrinsic restraint”). The higher the ratio, the stronger the intrinsic restraint.

In addition to the foregoing measures of the functioning of the HPA axis, we were interested in determining how GCs were actually impacting other aspects of the animals’ physiology (for convenience, we refer to these as “downstream effects” of GCs). We assessed three factors: energy mobilization, an index of condition, and an index of immune function. To assess the ability of the body to mobilize energy in response to sustained acute stress, we measured blood glucose and free fatty acid levels at the initial blood sample. Because GCs stimulate hepatic gluconeogenesis and decrease peripheral uptake of glucose (Dallman et al. 1993), an increase from one trapping session to another in stress-induced glucose levels (from capture in live traps) indicates that the animal has experienced chronically elevated GC levels prior to the capture
experience and thus has greater glycogen stores that can rapidly be converted to glucose when a stressor is encountered (Boonstra et al. 1998). In contrast, we expect that animals exposed to long-term elevations of GCs will have reduced free fatty acid levels due to the catabolism of fat stores. We also measured hematocrit as a general indicator of health status (Boonstra et al. 2001b; Clinchy et al. 2004; Kim et al. 2005) and white blood cell counts as a general indicator of immune function (e.g. Cattet et al. 2003).

5.2.3 Trapping and Field Sampling

In all cases, we trapped males during a prebreeding session (within a few days of first emerging from hibernation in spring) and then returned for a postbreeding trapping session 2-3 weeks later. In 2007 we trapped Richardson’s ground squirrels (RGS) at Kinsella, Alberta (53°N 111°33’W) from March 25-27 for the prebreeding sample and from April 15-16 for the postbreeding sample, arctic ground squirrels (AGS) 50 km west of Pelly Crossing, Yukon (62°50’N 137°18’W) from April 10-12 for the prebreeding sample and from April 27-30 for the postbreeding sample, and Columbian ground squirrels (CGS) at Kananaskis, Alberta (51°2’N 115°2’W) from April 22-24 for the prebreeding sample and from May 7-9 for the postbreeding sample. In 2008 we trapped thirteen-lined ground squirrels (TLGS) from April 15-19 for the prebreeding sample and from May 5-11 for the postbreeding sample and Franklin’s ground squirrels (FGS) from May 6-11 for the prebreeding sample and May 28-30 for the postbreeding sample, both near Portage La Prairie, Manitoba (TLGS at 50°7’N 98°23’W, FGS at 50°12’N 98°14’W). Animals were trapped by placing homemade burrow traps (Wobeser and Leighton 1979) or cage traps (Tomahawk #102, Tomahawk Live Trap Company, Tomahawk, WI, USA) in or next to burrows that animals were seen to have entered. Traps were monitored at least every 30 minutes and captured males were then kept in cage traps covered with a pillowcase in a shaded, quiet location until trapping was completed. We started trapping soon after animals emerged in the morning (typically between 07h00-09h00) and we trapped for at most 4 hours or until we had as many animals as we could process at one time (6-9 animals).

At the completion of trapping, animals were brought to a central processing area where they were placed in a cool, quiet location with pillowcases still covering the individual traps and left for at least 1 hour to habituate to the field laboratory. Then, one animal at a time was removed from its trap, weighed, and anesthetized with isoflurane (IsoFlo, Abbott Laboratories, Saint
Laurent, QC, Canada). We took an initial 0.6 mL “capture stress” blood sample and injected 0.1 or 0.4 mg/kg dexamethasone (Sabex, Montreal, Canada), then returned the animal to the covered trap and processed the next animal. As we found no difference in dexamethasone response to the different doses in 2007 (unpublished data), we administered 0.1 mg/kg to all animals in 2008. Two hours after the dexamethasone injection, we anesthetized the animal again and took a 0.2-0.3 mL blood sample (“DEX” bleed) and then injected 4 IU/kg of adrenocorticotropic hormone (ACTH; Synacthen Depot, Novartis Pharmaceuticals Canada Inc, Dorval, Quebec). Post-ACTH blood samples of 0.2 mL each were taken at 30, 60 and 120 minutes (“P30”, “P60” and “P120” bleeds). Each time a blood sample was taken, we measured blood glucose levels (mg/dL) with a FreeStyle glucose meter (Abbott Laboratories, Alameda, CA, USA). Blood samples were centrifuged and plasma was collected and frozen at -20°C at the field site until returning to the university laboratory where it was stored at -80°C. After the P120 bleed, the animal was euthanized by anesthetic overdose and decapitation.

On necropsy, we cut out and weighed free abdominal fat to the nearest 0.1 g and mass of the adrenal glands to the nearest mg on an electronic balance. Free abdominal fat was defined as fat that was easily removed from the abdominal cavity and did not include fat in the mesentery. To quantify the intensity of inter-male aggression during the breeding season, we examined the skin internally and externally for evidence of wounding. The degree of wounding was scored according to the number of punctures (bite marks that penetrated the skin, usually only visible from the inside of the hide) and externally visible wounds (tears in the skin that exposed the muscle below or had scabbed over). Our scoring categories were: “None” for no wounds, “Minimal” for <10 punctures and <2 cm² of externally visible wounding, “Moderate” for ≥10 punctures and/or >2 cm² but <7 cm² of externally visible wounding, and “Severe” for ≥7 cm² of externally visible wounding and/or significant damage to muscle, testes or eyes. Because FGS were not very abundant, we did not kill the pre-breeding animals. As a result, we do not have pre-breeding fat or adrenal masses for this species, and their wound scores were based solely on an external examination.

5.2.4 Laboratory Procedures

We measured total cortisol levels in RGS and CGS by radioimmunoassay using the methods described in Delehanty and Boonstra (2009), but switched to a commercial cortisol kit (Diasorin
GammaCoat Cortisol Kit, Stillwater, MN, USA) to measure cortisol levels in TLGS and FGS according to kit instructions. In a validation run (unpublished data), the cortisol levels measured by the kit and our previous protocol were highly correlated ($r^2 = 0.97$ for 18 samples ranging from 22 ng/mL to 452 ng/mL), although the Diasorin kit tended to give values about 7 ng/mL higher than our previous procedure. This difference could possibly be attributed to the fact that our initial procedure involved a dichloromethane extraction whereas the Diasorin kit did not.

Tests on an initial subset of individuals revealed that cortisol is the dominant GC in RGS and CGS, with corticosterone being undetectable, or nearly so, in most individuals. Boonstra et al. (2001a) also found a similar pattern in AGS. However, we found that TLGS had corticosterone levels that were typically 10-20% those of their cortisol levels, and that FGS had corticosterone levels around 30-40% of their cortisol levels. Thus, in both of these species we measured corticosterone levels using the procedures in Boonstra and Boag (1992). Because plasma samples were limited for some individuals, we only measured corticosterone levels in 5 pre-breeding and 5 post-breeding individuals from each species, for all 5 hormone challenge plasma samples (initial, DEX, P30, P60, and P120). We calculated corticosterone as a percentage of total GC at each bleed in these animals. We used these means to estimate amount of corticosterone in the remaining animals.

Although some researchers rely on total GC levels as measures of downstream biological impacts, most vertebrates have CBG circulating in their blood, which binds GCs with high affinity. Any GC molecules bound to CBG cannot leave the circulatory system meaning that, while bound, they cannot enter GC-sensitive tissues nor can they be metabolized by the liver (Mendel 1992) and are, with some exceptions, biologically inert while bound. Because CBG levels can vary seasonally or with respect to reproduction (e.g. Boonstra et al. 2001a; Bradley et al. 1980; Love et al. 2004; Williams et al. 2008) or decline in response to acute or chronic stress (e.g. Armario et al. 1994; Boonstra et al. 1998; Breuner et al. 2006; Dallman et al. 1987; Spencer et al. 1996) some researchers measure free hormone concentrations on the basis that they are more biologically meaningful measures of immediate GC exposure than are total GC levels. We adopted this approach, and to calculate free hormone concentrations we measured maximum corticosteroid binding activity (MCBC) using the procedures in Boonstra et al. (2001a). The MCBC assay provides an estimate of CBG levels, but is expressed in the maximum amount of hormone that can be bound by the CBG, rather than in the actual amount of protein. Briefly, 20
ng cortisol and 0.1 ng of radioactive cortisol ([1,2,6,7-\textsuperscript{3}H] Cortisol, Amersham Biosciences, USA) was added to 10 μL plasma in 500 μL phosphate buffered saline (PBS) and allowed to reach equilibrium at 4°C overnight. To separate bound from unbound hormone, we added 200 μL of dextran-coated charcoal (6.25 g/L charcoal in PBS with 0.625 g T70 dextran) at 4°C and after 30 min we centrifuged the samples at 2000 \( \times g \) for 12 min. The radioactivity in 500 μL of the supernatant of each sample (containing CBG-bound hormone) was compared to the radioactivity in an equal volume from “total” tubes (containing 0.1 ng radioactive cortisol in 710 mL PBS). Using the proportion bound allowed us to calculate the amount of hormone bound by CBG in each sample tube. We then used the equation in Barsano and Baumann (1989) to calculate free hormone levels. This formula also requires the equilibrium dissociation constants for CBG/cortisol for each of the species, which we calculated using the methods described in Delehanty and Boonstra (2011). We calculated the equilibrium dissociation constants (±SE) for AGS, RGS, CGS, TLGS and FGS as 4.04 ± 0.42 nM, 5.4 ± 0.56 nM, 4.73 ± 0.29 nM, 17.8 ± 2.3 nM, and 26.9 ± 10.0 nM, respectively (unpublished data). For thirteen-lined and Franklin’s ground squirrels, we assumed that the dissociation constants of CBG for cortisol and corticosterone were equal and that the two GCs could be treated as a single GC pool.

5.2.5 Statistics

All data were analyzed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). For our regression of postbreeding GC levels against reproductive lifespan, we did not test for or control for phylogenetic nonindependence. Our small sample size precluded using phylogenetic contrasts: tests for phylogenetic independence perform poorly on small datasets (e.g. Abouheif 1999), and if our species were not independent, our sample size would be reduced by one in order to do the contrast. We feel our decision is further justified by the fact that we selected very closely related species, and because previous comparative studies of GC levels have found that much more diverse species groupings are phylogenetically independent (Bókony et al. 2009; Hau et al. 2010).

For the pre- and postbreeding comparisons, data were first examined for normality. Where significantly non-normal data could not be normalized with a transformation, we used the Wilcoxon-Mann-Whitney non-parametric two sample test using the EXACT option to generate a Monte Carlo-based exact \( P \)-value. Because we released pre-breeding Franklin’s ground squirrels
and some of our post-breeding samples came from recaptured individuals, we analyzed differences between pre- and postbreeding sessions using a mixed model in SAS PROC MIXED, with subjects as a repeated measure. All other species comparisons used PROC TTEST; where pre- and post-breeding data had non-homogeneous variances based on the Folded-F test, we use Satterthwaite-adjusted degrees of freedom for the t-tests. Unless otherwise specified, we present means with 95% confidence intervals.

5.2.6 Approvals and permits

All protocols were approved by the University of Toronto Animal Care Committee using Canadian Council on Animals Care guidelines (protocol numbers 20006524 and 20007022). These protocols were also approved by the University of Alberta Faculty Animal Policy and Welfare Committee for the Richardson’s ground squirrels captured at the University of Alberta Kinsella Ranch. Appropriate wildlife research permits were obtained from the governments of Alberta, Yukon and Manitoba.

5.3 Results and discussion

In this section, we first discuss the association between reproductive lifespan and GC level, and then we provide the qualitative descriptions of each of the five species individually, placing our results in the context of their life history and ecology. We finish with a general discussion of the significance of our results taken as a whole.

5.3.1 Correlation between GC levels and reproductive lifespan

We found no significant association between mean male reproductive lifespan and postbreeding free or total GC levels (SAS PROC GLM, total GC: $F_{1,3} = 1.0$, $p = 0.39$; free GC: $F_{1,3} = 0.75$, $p = 0.45$). We tried controlling for prebreeding GC levels, litter size, and body mass, but additional variables did not make the regression significant. With only 5 species and a limited range of reproductive lifespans, it is possible that we just did not have a large enough sample size to detect a relationship. However, in light of our results from the qualitative portion of our study, below, it seems unlikely that adding more species would reveal a relationship.

5.3.2 Arctic ground squirrel

Male AGS are found throughout the mainland arctic tundra of North American and in alpine
areas and forest meadows of northwest North America. They hibernate for 7-8 months of the year and mate in a brief, intense, breeding season early in the spring (Karels et al. 2000). Males emerge from hibernation before the females, at a time when snow still covers most of the ground (Buck and Barnes 1999). In the 2-3 weeks prior to emergence, the squirrels have been eutheremic, but they remain underground and consume cached food stores (Barnes and Ritter 1993; Buck and Barnes 1999; Gillis et al. 2005). As a result, when they do emerge, they have significant fat reserves (Buck and Barnes 1999). Adult males have relatively low annual survival rates. In a study of Arctic ground squirrels in the southwest Yukon, about 60% of yearling males died before their second breeding season, and <1% of males survived to a third breeding season (Gillis 2003), resulting in a mean expected reproductive lifespan of 1.4 years (Table 5.1).

Body mass declined over the breeding period by 31% and free abdominal fat declined by 88% (Table 5.2). Wounding rates and severity increased, with severe wounds increasing from 0% in the prebreeding session to 23% in the postbreeding session (Fig. 5.2). Our results indicate that the breeding season is a period of intense aggression and is energetically expensive for male AGS, leading us to predict in Delehanty and Boonstra (2011) that AGS males should show an adaptive stress response. However, in Delehanty and Boonstra (2011) we concluded that, despite having relatively short-lived males, they did not conform to the predictions of the adaptive stress hypothesis. Although they showed signs of chronic GC exposure, their GC and CBG levels did not change as predicted. We include some of our previously published arctic ground squirrel data in this paper for ease of comparison with the other four species.

We found that total GC increased from pre- to postbreeding, but due to an increase in CBG levels, free GC levels remained constant (Fig. 5.3a, b, and c). We found no change in GC response to ACTH stimulation, no change in adrenal gland mass, and we concluded there was no biologically significant change in DEX response (Table 5.2; Delehanty and Boonstra 2011). There was no difference in intrinsic restraint as measured by the ratio of maximum GC in response to ACTH to free GC at the initial bleed at either time (Table 5.2). This constancy in the stress response from the pre- to postbreeding periods is contrary to the adaptive stress hypothesis. However, all of the downstream measures indicated a chronic exposure to GCs as would be expected under the adaptive stress hypothesis. Glucose increased, free fatty acids decreased, hematocrit decreased and white blood cells decreased (Table 5.2). We concluded that the breeding season is clearly very costly for male AGS, and they show clear signs of chronic
GC exposure despite the fact that they do not appear to be altering their acute stress response
over the course of the breeding season.

5.3.3 Richardson’s ground squirrel

The natural history of the RGS is very similar to that of the AGS, but the stress profiles are not at
all alike. Like the AGS, male RGS at our site emerged in early spring when the ground was still
mostly covered in snow and no new plant growth had begun. Prior to emergence, males become
euthermic but stay underground, consuming cached food and gaining weight (Michener 1992),
suggesting that lack of food (which can be a stressor: Clinchy et al. 2004) is unlikely to be a
stressor in the prebreeding period. Upon emergence in late March, males defend territories
encompassing several females and when the females emerge, males compete aggressively for
mating opportunities (Michener 1983; Michener 1992). From the pre- to the postbreeding
sessions, male RGS lost 13% of their body mass and 88% of free abdominal fat reserves (Table
5.2). Over the same period, males suffered an increasing number of wounds, with the percentage
of animals having moderate to severe wounds increasing from 7% to 67% (Fig. 5.2; Fisher’s
exact test, n=30, p=0.006). The mean expected reproductive lifespan of males is 1.3 years
(Table 5.1). These data support our view of the breeding season as an intensely stressful period
for male RGS and is comparable to that of AGS, which led us to predict that males would show
an adaptive stress response.

As a consequence of the breeding season stressors, postbreeding RGS had higher total and free
GC levels in response to capture stress as compared to prebreeding animals: total GC levels
were 28% higher and free GC levels were 51% higher (Fig. 5.3(d) and (f)). There was no change
in CBG levels, however (Fig. 5.3(e)). The increase in mean total GC AUC in response to the
ACTH stimulation from the pre- to the postbreeding session was just below statistical
significance at p=0.052 (see Table 5.2). This is suggestive of an increase in adrenal
sensitivity/capacity, which could explain the increase in GC levels, but more data is needed to
reach a firm conclusion. There was no change in adrenal mass (Table 5.2), indicating no adrenal
hypertrophy, which is sometimes associated with increased GC secretion (Boonstra et al. 1998;
Harvey et al. 1984). There was no change in the strength of negative feedback exerted at the
level of the pituitary in response to the dexamethasone, nor was there any difference in intrinsic
restraint (Table 5.2).
The overall picture of the HPA axis of male RGS is one of increasing GC responsiveness to stressors but no change in CBG levels or intrinsic restraint. This suggests that as the breeding season progresses males benefit from an increase in their GC response to stressors. Because RGS males are very similar in natural history to AGS males, we expected to see the same downstream effects of chronic stress as we observed in AGS. In fact, the only downstream effect that matched that of the AGS was a decreased ability to mobilize free fatty acids (Table 5.2). All other downstream indicators of stress are different from AGS and contrary to our predictions: we saw no change in glucose or hematocrit levels, and white blood cell counts actually increased (Table 5.2). Thus, despite the outward appearance of being chronically stressed over the course of the breeding season (loss of mass, significant wounding) and despite producing more GCs in response to stressors (increasing GC levels, although with no change in intrinsic restraint or CBG levels), male RGS show few downstream indications of that stress. One explanation for this pattern is that the prebreeding animals have already been exposed to chronically elevated GC levels, and so the prebreeding measures are already indicative of GC exposure. We assumed that the prebreeding period was not stressful due to the fact that there was little aggression at that time and the animals were consuming or had just finished consuming abundant cached food. However, it is possible that the pre-breeding RGS males are adopting the same “basal stress” strategy that we hypothesized for AGS in Delehanty and Boonstra (2011). Under that hypothesis, males have elevated basal GC levels as a result of downregulating mineralocorticoid receptors in the hippocampus. By doing this, they maintain elevated GCs in the breeding period even in the absence of stressors. We hypothesized that this strategy would allow males to mobilize a ready supply of easily accessible energy in the form of liver glucose and glycogen. Under this scenario, male RGS have elevated GC levels even prior to emergence, as they are consuming their cached food. Thus, RGS are functioning in a state of preparedness for the coming mating challenge. When they emerge, their glucose levels are already elevated, and their white blood cell counts and hematocrit levels are depressed. Free fatty acids would still be higher in the prebreeding session simply because of the ample fat stores at that time. This hypothesis finds some support in the fact that when we caught animals and took baseline samples in <3 min in Delehanty and Boonstra (2009), 26% of their GCs were free. This is a higher proportion of free hormone than we see in many species; free hormone levels are typically <15% of total hormone (2% in male zebra finches, *Taeniopygia guttata* (Breuner et al. 2006); between 2% and 6% in male house sparrows, *Passer domesticus* (Breuner and Orchinik 2001); 8%–9% in
stallions, *Equus caballus* (Alexander and Irvine 1998); and around 6% in humans (Lewis et al. 2005)). Moreover, in 2009 we found that prebreeding animals had 44% free hormone at baseline and their baseline levels were so high that capture failed to increase GC levels above baseline (chapter 3). Interestingly, we found that total and free GC levels did not change, or possibly even declined from pre- to postbreeding periods in our 2009 animals (chapter 3).

Another key feature of our data is the variation in responses among individuals. In both RGS and AGS males, variability of GC concentrations increased considerably from pre- to postbreeding sessions. In male RGS, prebreeding total and free GC levels are more tightly clustered around the mean than they are in postbreeding animals (Folded F, total GC: $F_{14,14}=5.76$, $p=0.002$; free GC: $F_{14,14}=7.61$, $p=0.0005$). Looking at Fig. 5.3(d) and (f), about half of the animals remain within the prebreeding range of hormone levels, whereas the other half have much higher GC levels than what is found in the prebreeding session. Although we do not have pre- and postbreeding measures for individual animals, if our sampling is representative then some individuals pass through the breeding season without changing their GC response to acute stress, while other individuals become hyper-responsive. We found a similar pattern in male AGS (Fig. 5.3(a) and (c); Delehanty and Boonstra 2011). The need to investigate individual variation in endocrine traits has recently been highlighted (Williams 2008), and our data suggest that studying the sources of individual variation in male RGS and AGS would be fruitful. We looked for correlations that could explain the variation in GC levels, but we found no relationship between postbreeding GC levels and body mass, free abdominal fat, or wounding. Age may well be a key determinant in how individuals respond to stress, but unfortunately we were not able to age our ground squirrels despite trying to use eye lens weights and sectioned femurs and jaws. Studies on populations with animals of known age will be critical for understanding the causes of individual variation in GC levels.

### 5.3.4 Columbian ground squirrel

Male CGS are the longest lived of our five species, with a mean expected reproductive lifespan of 2.7 years, twice that of AGS and RGS (Table 5.2). They are also the only species to delay sexual maturity; males first reproduce at 3 years of age (Neuhaus and Pelletier 2001). In many respects, though, male CGS face similar challenges to those of AGS and RGS. Males emerge from hibernation in mountain valleys in late April when snow still covers much of the ground
and there is no fresh plant growth to eat (Neuhaus et al. 1999 and pers. obs.), suggesting that energy is likely to be a limited resource for breeding male CGS just as it is for AGS and RGS. Indeed, mean body mass of male CGS declined 10% and mean free abdominal fat mass declined 90% (Table 5.2). In contrast to AGS and RGS, however, caching by reproductive male CGS is not nearly as universal; some researchers have found no evidence of food caches (Young 1990), whereas others have found caches in some, but not all, adult male burrow (Shaw 1926). We do not know whether our population had cached food sources, but we can at least conclude that AGS, RGS and CGS all share the characteristic of having a negative energy balance over the course of the breeding season. Also like RGS and AGS, male CGS engage in defence polygyny and breeding involves some physical aggression as evidenced by the increase in wounding from pre- to postbreeding sessions (Fig. 5.2; Fisher’s exact test, n=23, p<0.0001). However, no CGS experienced severe wounding, and only 2 of 13 postbreeding animals had wounding classed as moderate—the rest had only minor wounds. This is in stark contrast to the dramatic wounding found in the AGS and RGS. Thus, the CGS breeding season can best be characterized as energetically challenging but involving less physical damage than the AGS and RGS.

On the basis of their long reproductive lifespan we had predicted that of all our species, male CGS would be the least likely to have a stress response conforming to the adaptive stress hypothesis. However, male CGS showed the most extreme increase in GC response of all species we studied. From pre- to postbreeding, total GC response to capture stress increased 120%, and despite a 140% increase in CBG, free GC levels still doubled (Fig. 5.3(g), (h), and (i)). The increase in GC production was at least partly due to an increase in adrenal sensitivity/capacity: total GC AUC in response to the ACTH stimulation increased 76% and adrenal mass increased 37% (Table 5.2). There was no change in the strength of negative feedback exerted at the level of the pituitary in response to the dexamethasone (Table 5.2). Our estimate of intrinsic restraint supports the DEX results; there was no difference in the ratio of maximum free GC to initial free GC levels (Table 5.2). The high mean and standard error in postbreeding total feedback (17.91±10.2) was due primarily to one outlier animal that had a ratio of 136. With that animal removed, the postbreeding mean (8.0±3.0) was much closer to the prebreeding (7.4±1.8).

The increase in adrenal sensitivity/capacity, the increase in total and free GC levels, and the lack of any change in negative feedback or intrinsic restraint all portray a species that is actually
trying to increase its GC response to acute stress as the breeding season progresses, a hallmark of the adaptive stress hypothesis. Despite this, downstream measures showed inconsistent signs of chronic GC exposure. Glucose levels increased 29% (Table 5.2), which suggests that sustained GC exposure resulted in substantial gluconeogenesis by the liver and hence larger glycogen stores. The only other species to show an increase in glucose levels was the AGS, and they only increased by 10% (Delehanty and Boonstra 2011). However, CGS free fatty acids did not change from pre- to postbreeding, suggesting that despite their loss of free abdominal fat, CGS do not deplete their ability to mobilize fat as quickly as do AGS and RGS, which both saw significant drops in free fatty acid levels (this study and Delehanty and Boonstra 2011). We found no change in hematocrit, but did observe a decline in white cell counts (Table 5.2). The CGS results are more similar to the adaptive stress response than we had predicted, with a dramatic increase in circulating GCs and some evidence of chronic GC exposure over the breeding season. However, the most striking feature of the downstream results is how different they are from RGS despite both species showing increases in both total and free GC levels. This suggests that these species differ in how specific tissues perceive or respond to the increase in circulating GCs.

As with the AGS and RGS, the variance around mean total GC, MCBC, and free GC levels all increased significantly from pre- to postbreeding (Folded F, total GC: $F_{12,15}=6.73$, $p=0.0009$; MCBC: $F_{12,15}=16.6$, $p<0.0001$; free GC: $F_{12,15}=4.62$, $p=0.0066$; Fig. 5.3(g), (h) and (i)). However, whereas there was considerable overlap in the pre- and postbreeding ranges in AGS and RGS, there is very little overlap in the CGS data. This means that, in contrast to some RGS that maintain a consistent GC response from the pre- to the postbreeding period (i.e. no change), all male CGS change their GC response. The increase in variance means that some individuals alter their response more than others. As with RGS, though, no relationship between postbreeding GC levels and body mass, free abdominal fat mass or wounding were revealed when we performed ANCOVAs (not shown). If age plays a role in determining the GC response, then it does so only in determining the degree of increase in GC levels.

5.3.5 Thirteen-lined Ground Squirrel

The TLGS is the smallest of our five species, and its range is the largest of all of our species—it extends throughout much of the American Great Plains as far south as the Texas gulf coast
(Streubel and Fitzgerald 1978). In some of its range, therefore, TLGS do not experience long, cold winters. However, as in much of the Canadian prairies and aspen parkland, our TLGS population lived side by side with RGS and FGS populations, and therefore they experience the same local climate. Whereas male RGS had emerged in our study area by late March 2008, the TLGS did not emerge at our site until mid-April. Local residents reported that this was fairly typical. Despite a high first year mortality rate, males that survive that first breeding season stand a good chance of breeding for another 2 or 3 years (Table 5.1; Erlien and Tester 1984). We estimated their mean reproductive lifespan at 1.9 years—the second highest of our 5 species. We therefore expected the TLGS would show a homeostatic stress response.

Unlike the RGS, CGS and AGS populations we studied, the male TLGS emerged at a time when snow cover had receded and fresh vegetation and insects were just beginning to emerge (pers. obs.). We could not find any published data indicating that TLGS cache food for consumption in the spring, but the comparatively low fat stores in our prebreeding sample suggests that they do not rely on fat stores after emergence to the same extent as do AGS, RGS and CGS. The mean body mass of TLGS males upon emergence was 204.3±5.7 g, and their mean free abdominal fat mass was 3.4±0.28 g (1.6% of their body mass, compared to 3.9% in RGS, 3.4% in CGS and 7% in AGS). Although free abdominal fat reserves declined by 48% (postbreeding: 2.1±0.26 g; \( t=3.23, \text{df}=27, \text{p}=0.003 \)), there was no difference in mean body mass of postbreeding animals compared to prebreeding animals (postbreeding: 211.1±4.4 g; \( t=-0.92, \text{df}=27, \text{p}=0.36 \)). This suggests that even if male TLGS continue to catabolize their free abdominal fat stores, it is not due to a negative energy balance. Rather, they are able to find enough food so as to allocate energy to increasing peripheral fat (i.e. non-abdominal), increasing muscle mass, or to growth over the course of the breeding season. Whether TLGS males have no cached food or just consume it gradually over the breeding season, it is clear that TLGS have a very different energy balance in the breeding season when compared to AGS, RGS, and CGS.

Male TLGS also differ from RGS, CGS and AGS in that they experience almost no wounding over the breeding season. We found very little wounding of male TLGS and there was no significant change in wounding rates from pre- to postbreeding trapping sessions (Fig. 5.2; Fisher’s exact test, n=29, p=0.08). This is consistent with Schwagmeyer and Woontner’s (1986) finding that direct conflict was sporadic and only rarely left squirrels with any injuries. This is typical of scramble-competition polygyny, in which competition among males is primarily based
on mate-finding ability and sometimes sperm competition, rather than on aggression. Male TLGS, however, range over comparatively large areas to locate and mate with females (Schwagmeyer and Woontner 1986). Schwagmeyer (1988) found males had home ranges of almost 5 ha in the breeding season, in contrast to about 1 ha in RGS (Davis and Murie 1985) and 0.3 ha in CGS (Murie and Harris 1978). Thus, although the mating season is far less physically challenging than it is for RGS, AGS, or even CGS, it may be energetically challenging as they search for receptive females.

In AGS, RGS and CGS, the toll of the breeding season was clear from the weight loss and wounding, which led us to see the breeding season as intensely stressful for these species. In contrast, the overall picture of the breeding season for male TLGS is one of neutral energy balance and low aggression, which raises the question of whether the breeding season is stressful for male TLGS; that is, does the breeding season pose a physiological challenge that requires a response by the HPA axis, or does the HPA axis remain unchanged through the breeding season? Despite the consistency in physical condition of males, their hormonal milieu changed markedly from pre- to postbreeding sessions, suggesting that modulation of the HPA axis supports reproduction. Total GC in response to capture stress decreased by 59% and CBG decreased by 47% (Fig. 5.3(j) and (l)). Free GC also declined significantly—by 39%—but the most notable feature of the TLGS GC data is the fact that unlike any of the other species, male TLGS have more GC binding capacity than total GC levels (Fig 5.3(k)). This means that a much smaller proportion of their total GC is free: comparing pre-breeding sessions, TLGS have 17% free, whereas every other species that we studied had about 50% free (this study and Delehanty and Boonstra 2011). Maintaining CBG binding capacity in excess of total GC production suggests that male TLGS are buffering against the immediate effects of acute stress; that is, they appear to be minimizing their exposure to free GC during acute stress. Accordingly, this looks like a “resistance to stress” strategy (Wingfield and Sapolsky 2003) in which a species adopts a strategy of minimizing GC exposure during the reproductive period in order to avoid the negative effects of GCs on reproduction. Moreover, two lines of evidence suggest that TLGS may actually be intensifying their resistance to stress over the course of the breeding season. The first is that even though MCBC declines, it remains high enough that postbreeding free GC levels are significantly lower than prebreeding levels (that being said, the fact that the difference in free GC from pre- to postbreeding was only 1.7 ng/mL causes us to question if that difference
is biologically meaningful). The second, more compelling, line of evidence for increasing resistance to stress is the fact that intrinsic restraint strengthened from pre- to postbreeding. Even though we saw no sign of dexamethasone resistance the ratio of maximum free GC to initial free GC increased up to 6-fold (Table 5.2; excluding an outlier ratio of 322 brings the mean ± SE down from 54.4 ± 23.8 to 32 ± 8.9, which makes it a 3-fold increase). A third way to decrease GC exposure is to decrease the sensitivity/capacity of the adrenal glands, but the total GC AUC in response to ACTH stimulation did not change (Table 5.2). Curiously, adrenal mass increased (Table 5.2). We have no explanation for the increase in adrenal mass given that GC production is declining and total GC AUC remains fixed. It is possible that the increase in adrenal mass is related to changes in other adrenal functions.

The decrease in total and free GC levels in response to capture stress, along with the increased intrinsic restraint led us to conclude that, as the breeding season progresses, male TLGS decrease their sensitivity to stressors. When we look at the downstream measures of stress, the balance of the evidence suggests no chronic exposure to GCs. We found no change in glucose levels (Table 5.2). Free fatty acid levels fell, but the difference was just beyond the threshold for significance at p=0.06 (Table 5.2). The adaptive stress hypothesis predicts a drop in free fatty acids at the same time as GC levels increase on the basis that fat stores are mobilized rapidly, followed by the catabolism of muscle. The decline of free fatty acids in TLGS, however, occurred in the context of decreasing GCs. This means that the drop in free fatty acids could simply be the result of a decrease in stress-mediated lipolysis. Accordingly, the possible drop in free fatty acids does not indicate chronic exposure to GCs. Hematocrit levels dropped, which is suggestive of chronic stress over the breeding season, but white blood cell count increased which suggests the contrary, that stressors lessened from pre- to postbreeding (Table 5.2). As for the variance in GC measures, the variance in total GC, MCBC and free GC are all constant (Folded F, total GC: F_{14,13}=1.39, p=0.56; MCBC: F_{14,13}=2.52, p=0.10; free GC: F_{14,13}=1.20, p=0.74; Fig. 5.3(j), (k) and (l)), suggesting that all individuals adopt the same hormonal strategy, and individuals do not gain any advantage from either increasing or decreasing their GC response relative to other individuals.

We conclude that the male TLGS stress profile resembles a homeostatic strategy, and possibly even a resistance strategy in the sense of Wingfield and Sapolsky (2003). The overall picture of
the TLGS stress profile is one that avoids downstream consequences of chronic GC exposure by reducing GC production.

5.3.6 Franklin’s ground squirrel

Franklin’s ground squirrels typically live around marshes and forested areas of the Canadian prairies and aspen parkland. This is the least studied of our 5 species, and it was also the least abundant. Our estimate of expected mean reproductive lifespan of 1.1 years is based on a large multi-year study by Erlien and Tester (1984), which found 90% of males died after a single breeding season, and none survived for more than two breeding seasons, but previous work in our study site found that a few males could live up to 4 years (James Hare, pers. comm.). However, the evidence indicates that male FGS have short reproductive lifespans and we predicted that their stress profile would fit the adaptive stress hypothesis.

Like TLGS, male FGS emerge at a time when new food is becoming available. In our study area, FGS typically emerge in the first week of May (James Hare, pers. comm.). In 2008 the males began appearing around May 6, at which time vegetation and insects had already begun to emerge. Like the TLGS, male FGS did not lose any body mass over the breeding season (Table 5.2). Unfortunately we do not have data on free abdominal fat reserves, but the lack of any change in body mass and the lack of change in wounding rates (Fisher’s exact test, n=16, p=0.31) combined with the low severity of observed wounding (Fig. 5.2) all suggest that the breeding season experience for FGS is similar to that of TLGS. As with the TLGS, male FGS seem to have adequate food resources during the breeding season and do not seem to rely on extensive fat reserves during this period.

Despite the similarities between FGS and TLGS, though, we once again find that their stress axes function quite differently from each other and from the other species. As with TLGS, total and free GC levels decreased from pre- to postbreeding (by 29% and 40% respectively; Fig. 5.3(j) and (l)) but in FGS the CBG levels remained constant (Fig. 5.3(k)). The drop in GC levels appears to be due both to decreasing adrenal sensitivity/capacity and increased intrinsic restraint. Adrenal sensitivity/capacity as measured by total GC AUC in response to ACTH fell 15% and intrinsic restraint increased even though we saw no sign of dexamethasone resistance (Table 5.2).
Based on the physical and hormonal data, the breeding season for male FGS appears to be one of neutral energy balance, moderate physical aggression, and decreasing GC exposure due both to lower GC production and more intrinsic restraint. As with the TLGS, this is consistent with a “resistance to stress” approach, whereby the male FGS is decreasing its responsiveness to stressors over the course of the breeding season. In the case of the FGS, though, the prediction that downstream measures should show no sign of GC excess under this type of strategy is fully borne out. We found no change in glucose or free fatty acid levels, nor did we see any change in hematocrit (Table 5.2). White blood cell counts increased, as we would expect from the falling GC levels (Table 5.2). Thus, of our 5 species, the species with the shortest reproductive lifespan actually has a stress profile most consistent with a homeostatic or resistance strategy.

5.4 Synthesis

Table 5.3 summarizes the direction of change in the means and variances of the physical, HPA axis and downstream measures from this study and from Delehanty and Boonstra (2011). At the outset of this study we predicted that post-breeding GC levels would be correlated with male reproductive lifespan, and we predicted that the shorter the male reproductive lifespan, the more that species’ stress profile would resemble an adaptive stress response instead of a homeostatic response. As much as possible, our choice of species controlled for ecological and phylogenetic differences, yet we found no correlation between reproductive lifespan and GC levels, and we did not find that our qualitative descriptions of stress profiles reflected reproductive lifespan either. We therefore conclude that, contrary to our hypothesis, ground squirrel life histories do not predict stress physiology. However, our data do provide two additional insights into the evolution of the HPA axis in ground squirrels.

The first is that we found that species differed in the mechanisms used to change capture-stress GC levels over the breeding season. Total GC levels increased in AGS, RGS and CGS and declined in TLGS and FGS, but there was little uniformity in how those changes came about and how they translated into free hormone levels. Some species altered intrinsic restraint, some altered CBG levels, and some altered adrenal sensitivity/capacity. If the only important measure is whether free GC levels increase or decrease, then why is there such diversity in the mechanisms behind those changes? Similar diversity has been observed in 4 species of songbirds. Romero et al. (Romero et al. 1998a; Romero et al. 1998b; Romero et al. 1998c) and
Romero and Wingfield (1998) found that all 4 of their study species showed seasonal variation in GC regulation, but they used different mechanisms: one species regulated release at the adrenal, one at the pituitary, and two at the hypothalamus. We interpret the fact that closely related species that face very similar challenges use different methods for increasing or decreasing their acute stress response as evidence that the means by which GC levels change is significant and may represent distinct strategies. For example, both CBG and ACTH could have important biological effects in their own right. Some authors have hypothesized that CBG-bound hormone can act as a reservoir of GC that is gradually freed and exerts biological effects as it is freed from CBG (Breuner and Orchinik 2002; Hammond 1995; Rosner 1990). Likewise, ACTH does not merely stimulate the production of GCs, it also acts directly on some tissues (e.g. adipose tissue, Boston 1999). If such effects are of a sufficient magnitude, this would imply (using just one hypothetical example) that an increase in acute stress GC levels from pre- to postbreeding accomplished by an increase in adrenal sensitivity (which would leave ACTH levels unchanged) versus a decrease in GC receptors in the brain and pituitary (which would slow down negative feedback and increase ACTH levels) are physiologically distinct strategies despite the fact that GCs increase by the same amount in both scenarios. To understand the relationship between the HPA axis and life history, we would need to look not only at whether GCs increase or decrease, but also at the means by which that change is accomplished. This implies that natural selection may be tailoring the HPA axis of species to very specific needs by acting on several components of the HPA axis and not just on GC levels. Although it is premature to speculate about the adaptive significance of the diverse mechanisms of GC increase and decrease that we found, the simple fact that there is such diversity suggests this is an area worthy of further study.

The second insight provided by our data is that biological effects of GCs are not necessarily proportional to GC levels. The fact that our downstream measures of GC exposure varied among even those species that had similar trends in GC levels leads us to conclude that these ground squirrel species may vary in how GC signals are translated into biological effects. We are aware of only one other comparative study that has directly looked at downstream regulation of GC exposure. Breuner et al. (2003) found that in three subspecies of white-crowned sparrow (*Zonotrichia leucophrys*) GC receptor density in brain and liver varied among subspecies. They found that the *oriantha* subspecies had about half the circulating free GC levels that *pugetensis* subspecies did, but after taking into account differences in glucocorticoid receptor densities, they
estimate that receptor occupancy in *oriantha* was about 90% and 80% that of *pugetensis* in the brain and liver respectively (estimated from their Fig. 5). Although incorporating receptor density did not alter the pattern amongst species that emerged based on free GCs, it did greatly reduce the magnitude of the apparent difference between two species, making simple GC levels a misleading index of the actual physiological state of the animals.

Our results and those of Breuner et al. (2003) challenge the validity of the assumption—common in the comparative literature—that GC levels are “functionally significant”. Tissue-specific regulation of GC effects may occur through a combination of several well-known mechanisms. These include the up- and downregulation of glucocorticoid and mineralocorticoid receptors, interconversion of active GCs with their inactive forms by 11β-hydroxysteroid dehydrogenase (11β-HSD) types 1 and 2, the presence of extracellular and intracellular CBG, active uptake of hormone by cells, and regulation of nuclear transcription/repression effects of receptor/hormone complexes in the nucleus (see Fig. 5.1(b) and references cited therein). Under this more complex model of the HPA axis, GC levels are not necessarily indicative of the magnitude of biological effects on GC-sensitive tissues. These mechanisms are well described in the biomedical literature that shows tissue-specific regulation of GC levels occurs in humans and lab animals. For example, 11β-HSD1 converts inactive GC (cortisone or 11-dehydrocorticosterone) to the active GC (cortisol or corticosterone) in a variety of tissues including the liver, adipose, and brain, and the presence and activity of this enzyme in a given tissue varies substantially between mice, rats and humans (Tomlinson et al. 2004). The conversion of active GC into the inactive form by 11-β-HSD2 occurs in tissues having a role in sodium transport, especially the kidney, thereby letting aldosterone, rather than GCs, bind to mineralocorticoid receptors (Ferrari 2010). Another example of localized GC effects is the enzymatic cleaving of CBG at sites of inflammation (Lin et al. 2010), thereby increasing local GC concentration independently of circulating GCs.

We are not the first to propose that these downstream factors may be critical to understanding how hormonal systems support (or constrain) the evolution of life history strategies. Several researchers have acknowledged that their downstream determinants of GC effects could affect the interpretation of their results (Landys et al. 2006; Malisch and Breuner 2010; Romero 2002),
but to date, there has been no concerted attempt to pursue this possibility. Our data suggest that this, too, is an area worthy of future research.
Table 5.1  Summary of the major biological characteristics of the 5 species in this study. We assumed that yearlings were reproductive if we did not catch any non-scrotal males in either the pre- or postbreeding sessions. The expected number of breeding seasons is the average number of breeding seasons a male can expect to survive through based on the given mortality rates. For thirteen-lined ground squirrels, we used the average first year survival rate of two studies, 60%. Where no other source is provided, the data are from this study.

<table>
<thead>
<tr>
<th></th>
<th>Arctic</th>
<th>Richardson’s</th>
<th>Columbian</th>
<th>Thirteen-lined</th>
<th>Franklin’s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average male mass (g) at emergence</td>
<td>891</td>
<td>373</td>
<td>529</td>
<td>204</td>
<td>387</td>
</tr>
<tr>
<td>Location and Habitat (our study)</td>
<td>Yukon - boreal forest clearings</td>
<td>Alberta - aspen parkland</td>
<td>Alberta - subalpine meadows</td>
<td>Manitoba - prairie</td>
<td>Manitoba - marsh and hardwood forest</td>
</tr>
<tr>
<td>Age of first reproduction</td>
<td>Yearling</td>
<td>Yearling</td>
<td>3 year-old(^a)</td>
<td>Yearling(^b)</td>
<td>Yearling</td>
</tr>
<tr>
<td>Annual mortality rate of reproductive males</td>
<td>1 yr - 62(^%)</td>
<td>1 yr - 80(^%)</td>
<td>3 yr - 25(^%)</td>
<td>1 yr – 50(^%)-70(^%)</td>
<td>1 yr - 90(^%)</td>
</tr>
<tr>
<td>≥2 yr - 97(^%)(^c)</td>
<td>2 yr - 80(^%)(^d)</td>
<td>4 yr - 29(^%)</td>
<td>2 yr – 35(^%)</td>
<td>3 yr - 30(^%)</td>
<td>2 yr - 100(^%)(^e)</td>
</tr>
<tr>
<td>6 yr - 43(^%)(^a)</td>
<td>4 yr - 80(^%)(^b,e)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expected breeding number of seasons</td>
<td>1.4</td>
<td>1.3</td>
<td>2.7</td>
<td>1.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Mating system(^f)</td>
<td>Defence</td>
<td>Defence</td>
<td>Defence</td>
<td>Scramble competition</td>
<td>Scramble competition(^g)</td>
</tr>
<tr>
<td>Emergence of Males</td>
<td>early</td>
<td>early</td>
<td>early</td>
<td>late</td>
<td>late</td>
</tr>
<tr>
<td>Average litter size(^b)</td>
<td>5.0-7.5</td>
<td>6.2-9.8</td>
<td>2.7-5.4</td>
<td>8.1-8.9</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Sources:  (a) Neuhaus and Pelletier (2001); (b) Schwagmeyer (1984); (c) estimated from Gillis (2003); (d) Michener (1989); (e) estimated from Erlien and Tester (1984); (f) Dobson (1984); (g) assumed based on personal observations of male home ranges and lack of aggression; (h) reviewed in Murie et al. (1980).
Table 5.2  Comparisons of body condition and physiological parameters measured in prebreeding and postbreeding animals in each of the five species in our study (most arctic ground squirrel data is from Delehanty and Boonstra 2011). Statistically significant differences are highlighted in bold. All comparisons are two-tailed t-tests with pooled variances except * = Satterthwaite adjustment for unequal variances; † = Wilcoxon-Mann-Whitney non-parametric two sample test, and ‡ = t-test on natural logarithm-transformed data, but untransformed means and SE are presented. For Franklin’s ground squirrels, ANOVAs using SAS PROC MIXED were used instead of t-tests due to repeated measures (see Methods). Abbreviations: AUC = total GC area under the curve in response to adrenocorticotropic hormone; DEX = Post-dexamethasone blood sample; FFA = free fatty acids; GC = Glucocorticoid; WBC = white blood cell count.

<table>
<thead>
<tr>
<th>Species</th>
<th>Parameter</th>
<th>Prebreeding mean±SE (n)</th>
<th>Postbreeding mean±SE (n)</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body mass (g)</td>
<td>891.2±20.8 (19)</td>
<td>614.1±22.0 (17)</td>
<td>9.15</td>
<td>&lt;0.0001</td>
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<tr>
<td>Arctic</td>
<td>Abdominal fat (g)</td>
<td>70.0±5.4 (14)</td>
<td>7.2±1.1 (17)</td>
<td>14.1*</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ground Squirrel</td>
<td>AUC (ng·h·mL⁻¹)</td>
<td>282.7±21.2 (13)</td>
<td>314.1±11.9 (15)</td>
<td>-1.33</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>Adrenal Weight (mg)</td>
<td>187.2±9.9 (9)</td>
<td>197.4±17 (17)</td>
<td>-0.57</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>DEX free GC (ng/mL)</td>
<td>0.31±0.03 (17)</td>
<td>0.22±0.02 (16)</td>
<td>S=192.0</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Total Feedback Ratio</td>
<td>2.60±0.31 (13)</td>
<td>2.57±0.39 (15)</td>
<td>0.06</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>Glucose (mg/dL)</td>
<td>115.1±3.3 (18)</td>
<td>126.5±4.0 (17)</td>
<td>-2.18</td>
<td>0.04</td>
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<tr>
<td></td>
<td>FFA (mM)</td>
<td>0.44±0.04 (18)</td>
<td>0.24±0.06 (16)</td>
<td>S=177.5</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>Hematocrit (%)</td>
<td>48.1±1.0 (18)</td>
<td>41.0±0.74 (17)</td>
<td>5.68</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>WBC (×10⁹/L)</td>
<td>9.4±0.83 (15)</td>
<td>6.57±0.44 (17)</td>
<td>3.04*</td>
<td>0.006</td>
</tr>
</tbody>
</table>

<p>|                  | Body mass (g)      | 370.0±9.9 (19)         | 322.0±9.7 (15)          | 3.46   | 0.0017 |
| Richardson’s     | Abdominal fat (g)  | 14.9±1.2 (14)          | 1.9±0.45 (15)           | 10.54* | &lt;0.0001|
| Ground Squirrel  | AUC (ng·h·mL⁻¹)    | 388±12.2 (15)          | 431±18.1 (13)           | -2.04  | 0.052  |
|                  | Adrenal Weight (mg)| 77.3±2.1 (15)         | 80.2±2.1 (15)           | -0.38  | 0.71   |
|                  | DEX free GC (ng/mL)| 1.02±0.15 (15)        | 7.62±4.06 (13)          | -1.63* | 0.13   |
|                  | Total Feedback Ratio | 2.19±0.18 (15)        | 3.00±1.15 (13)          | -0.70* | 0.50   |
|                  | Glucose (mg/dL)    | 126.1±5.4 (15)        | 115.3±4.8 (15)          | 1.51   | 0.14   |
|                  | FFA (mM)           | 0.54±0.07 (15)        | 0.36±0.03 (15)          | 2.54*  | 0.02   |
|                  | Hematocrit (%)     | 46.7±0.91 (15)        | 46.2±0.61 (15)          | 0.46   | 0.65   |
|                  | WBC (×10⁹/L)       | 4.1±0.57 (12)         | 6.7±0.77 (15)           | -2.54  | 0.02   |</p>
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Prebreeding mean±SE (n)</th>
<th>Postbreeding mean±SE (n)</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body mass (g)</strong></td>
<td>529.4±11.7 (16)</td>
<td>478.1±9.9 (13)</td>
<td>3.25</td>
<td>0.0013</td>
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<td>Abdominal fat (g)</td>
<td>18.1±1.7 (10)</td>
<td>2.0±0.4 (12)</td>
<td>9.23*</td>
<td>&lt;0.0001</td>
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<td>AUC (ng·h·mL⁻¹)</td>
<td>313.1±20.9 (10)</td>
<td>550.7±30.4 (13)</td>
<td>-6.05</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Adrenal Weight (mg)</td>
<td>71.5±3.8 (10)</td>
<td>98.0±5.7 (13)</td>
<td>-3.58</td>
<td>0.002</td>
</tr>
<tr>
<td>DEX free GC (ng/mL)</td>
<td>0.68±0.08 (16)</td>
<td>3.1±2.0 (13)</td>
<td>-1.21*</td>
<td>0.25</td>
</tr>
<tr>
<td>Total Feedback Ratio</td>
<td>7.40±1.8 (10)</td>
<td>17.91±10.2 (13)</td>
<td>S=132.0†</td>
<td>0.48</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>122.1±4.2 (16)</td>
<td>157.2±6.7 (13)</td>
<td>-4.62</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FFA (mM)</td>
<td>0.28±0.02 (16)</td>
<td>0.28±0.04 (13)</td>
<td>-0.05*</td>
<td>0.96</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>45.7±1.1 (16)</td>
<td>45.5±1.1 (13)</td>
<td>0.11</td>
<td>0.91</td>
</tr>
<tr>
<td>WBC (×10⁹/L)</td>
<td>7.6±0.56 (16)</td>
<td>5.6±0.65 (13)</td>
<td>2.30</td>
<td>0.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Columbian Ground Squirrel</th>
<th>Thirteen-lined Ground Squirrel</th>
<th>Franklin’s Ground Squirrel</th>
<th>F₁,₁₄&lt;0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g)</td>
<td>204.3±5.7 (15)</td>
<td>211.1±4.5 (14)</td>
<td>204.3±5.7 (8)</td>
<td>2.6±0.25 (14)</td>
</tr>
<tr>
<td>Abdominal fat (g)</td>
<td>3.4±0.3 (15)</td>
<td>2.1±0.3 (14)</td>
<td>n/a</td>
<td>11.1±1.7 (8)</td>
</tr>
<tr>
<td>AUC (ng·h·mL⁻¹)</td>
<td>81.4±6.4 (15)</td>
<td>90.6±6.6 (15)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Adrenal Weight (mg)</td>
<td>28.5±1.5 (15)</td>
<td>37.3±1.7 (14)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>DEX free GC (ng/mL)</td>
<td>0.50±0.09 (15)</td>
<td>0.28±0.08 (14)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Total Feedback Ratio</td>
<td>9.50±1.7 (15)</td>
<td>54.4±23.8 (13)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>146.4±5.8 (15)</td>
<td>155.0±4.7 (14)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>FFA (mM)</td>
<td>0.85±0.16 (9)</td>
<td>0.46±0.10 (8)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>48.5±0.80 (11)</td>
<td>45.0±1.21 (14)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>WBC (×10⁹/L)</td>
<td>2.6±0.25 (14)</td>
<td>3.9±0.30 (14)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F₁,₁₄=6.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F₁,₁₄=6.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F₁,₁₄=1.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F₁,₁₄=0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F₁,₁₄=3.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F₁,₁₃=14.40</td>
</tr>
</tbody>
</table>
Table 5.3  Summary of changes in means of the main variables used to characterize the HPA axis and downstream effects of glucocorticoid (GC) exposure in this study and in Delehanty and Boonstra (2011). Up arrows indicate an increase from pre- to postbreeding sessions, down arrows a decrease, and an equal sign indicates no change. Arrows with question marks indicate a non-significant difference that was close to the 0.05 threshold of significance. For the measures of total GC, maximum corticosteroid binding capacity (MCBC) and free GC, an asterisk indicates an increase in variance around the mean from pre- to postbreeding; no asterisk indicates equality of variances.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Significance</th>
<th>Arctic Ground Squirrel</th>
<th>Richardson’s Ground Squirrel</th>
<th>Columbian Ground Squirrel</th>
<th>Thirteen-lined Ground Squirrel</th>
<th>Franklin’s Ground Squirrel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total GC (Capture-stress)</td>
<td>Based on the initial bleed, taken after ≥1 h of captivity. This measures the amount of GC produced in response to the standardized stressor of capture. GC levels represent an equilibrium between stimulation and negative feedback.</td>
<td>↑*</td>
<td>↑*</td>
<td>↑*</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>MCBC</td>
<td>MCBC is a measure of CBG and is used to calculate free GC. The more CBG present, the greater the proportion of GC will be bound and not available for immediate uptake, but bound hormone will be made available for uptake when it is released as GC levels fall.</td>
<td>↑</td>
<td>=</td>
<td>↑*</td>
<td>↓</td>
<td>=</td>
</tr>
<tr>
<td>Test</td>
<td>Description</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free GC (Capture-stress)</td>
<td>Also based on the initial bleed, free GC is a measure of how much GC is immediately available for uptake by tissues. Free GC is estimated based on total GC, the MCBC, and the species-specific equilibrium dissociation constant of CBG (see Methods).</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC response to ACTH</td>
<td>This is a measure of adrenal sensitivity/capacity. Because ACTH acts directly on the adrenals, the GC response to ACTH shows how much GC can be produced in the absence of negative feedback.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenal mass</td>
<td>This is a measure of adrenal capacity based on the assumption that hypertrophied adrenal glands have an increased capacity to produce GCs.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-DEX free GC</td>
<td>Dexamethasone is a synthetic GC that activates GC receptors and suppresses endogenous GC production. Reduced suppression (higher post-DEX free GC levels) indicates impaired negative feedback at the level of the pituitary.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intrinsic Restraint</td>
<td>This is the ratio of the maximum free GC level obtained from the ACTH stimulation (i.e., in the absence of negative feedback) to the initial free GC (in which stimulation is balanced by negative feedback and reduced hypothalamic-pituitary drive). The higher the ratio, the greater the negative feedback.</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Interpretation</td>
<td>The HPA axis appears to follow the homeostatic model of maintaining a consistent free GC response through the breeding season.</td>
<td>The HPA axis resembles an adaptive stress response because of the increase in GC response to capture, possibly through increased adrenal sensitivity/capacity, but there is no reduction in intrinsic restraint.</td>
<td>The increase in total and free GC brought about by the increase on adrenal sensitivity/capacity resembles an adaptive stress response despite the long reproductive lifespan of this species. But the increase in MCBC is contrary to the adaptive response.</td>
<td>The decrease in GCs and increased intrinsic restraint strength suggests a weakening of the stress response over the breeding season, consistent with a &quot;resistance to stress&quot; strategy (Wingfield and Sapolsky 2003).</td>
<td>The decrease in GCs and adrenal sensitivity/capacity and the increase in intrinsic restraint all suggest a weakening stress response over the breeding season. This is consistent with a &quot;resistance to stress&quot; strategy (Wingfield and Sapolsky 2003).</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>An increase in GC response to a standard stressor suggests an increase in liver glucose stores, which is indicative of stress-induced gluconeogenesis in the recent past.</td>
<td>↑</td>
<td>=</td>
<td>↑</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>Free fatty acids are mobilized by ACTH-induced and GC-induced lipolysis. A drop in free fatty acid levels suggests a loss of fat reserves.</td>
<td>↓</td>
<td>↓</td>
<td>=</td>
<td>=</td>
<td>(↓?)</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>Hematocrit is a measure of packed red cell volume expressed as a percentage of total blood volume. Decreases in hematocrit are used as an indicator of overall poor health.</td>
<td>□</td>
<td>□</td>
<td>=</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>White blood cell counts are used as a general index of immune function. A decrease in counts is taken as an indication of compromised immune function.</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Interpretation</td>
<td>Downstream measures all indicate chronic stress, despite the apparent homeostatic stress response of the HPA axis.</td>
<td>Downstream measures are mixed, but are consistent with the basal stress hypothesis (Delehanty and Boonstra 2011) in which prebreeding basal GC levels are elevated even without external stressors.</td>
<td>Downstream measures are mixed, with glucose and white blood cells suggesting chronic exposure to GCs, but hematocrit and free fatty acids not responding as predicted by the increase in GCs.</td>
<td>Downstream measures are mixed, but the lack of change in glucose and the increase in white blood cells are consistent with a decline in the strength of the stress response over the breeding season.</td>
<td>Downstream measures are consistent with the &quot;resistance to stress&quot; interpretation of the GC results, in which the GC response declines over the breeding season.</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.1  A basic model of the HPA axis of a vertebrate (a) and a more comprehensive model of the tissue-specific regulation of glucocorticoid (GC) effects (b). In panel (a), a stressor is
perceived by the hypothalamus, which releases corticotropin releasing hormone (CRH) and arginine vasopressin (AVP). These hormones stimulate the anterior pituitary to secrete adrenocorticotropin hormone (ACTH), which in turn stimulates the adrenal gland to produce glucocorticoids (cortisol, corticosterone, or both, depending on species). Glucocorticoids (GCs) enter the circulation where, in most species, they are mostly bound by corticosteroid binding globulin (CBG). The unbound (free) hormone is able to leave the circulatory system and exert biological effects on tissues, affecting reproduction, immunity and metabolism. Circulating GCs also act as negative feedback signals at the pituitary, hypothalamus and hippocampus, inhibiting further GC production. Whereas the model in panel (a) assumes that reproduction, metabolism, and immunity are affected in proportion to plasma GC concentrations, panel (b) illustrates the many ways in which tissues can up- or down-regulate the GC signal that actually bring about a response. In panel (b), the solid circles represent active GCs (cortisol or corticosterone) and the open circles represent their inactive variants (cortisone or 11-dehydrocorticosterone).

Corticosteroid binding globulin (CBG) is represented by the semicircles. CBG can be found in the plasma, the extra-cellular matrix, and in the cytosol (Malisch and Breuner 2010). In circulation, CBG prevents GC from diffusing out (Mandel 1992) whereas circulating free hormone can enter cells, bind to glucocorticoid (or mineralocorticoid) receptors (represented by hearts) and proceed to up- or downregulate gene expression in the nucleus. All other things being equal, the density of receptors can alter the biological effects of GC levels (compare tissue 1 with tissue 2). CBG in the extra-cellular matrix can prevent GC from entering cells (ii), or it can bind to cell surface receptors (diamonds) that proceed to have non-genomic effects (iii). Circulating cortisone or 11-dehydrocorticosterone can enter cells and be converted by 11β-hydroxysteroid type 1 (11β-HSD1) into active GC (iv). Conversely, glucocorticoid receptors can be protected from GCs by conversion of active GCs to their inactive forms by 11β-HSD2 (v). Cytosolic CBG (sometimes called transcortin) can also reduce glucocorticoid receptor activation (v). An additional layer of complexity is created by the fact that genomic actions can be inhibited or enhanced by various corepressors and coactivators (Horwitz et al. 1996) that have not been illustrated here. Abbreviations: ACTH = Adrenocorticotropic hormone; AVP = arginine vasopressin (found in mammals, arginine vasotocin in non-mammalian vertebrates); CRH corticotropin releasing hormone.
Figure 5.2 Prevalence and severity of wounding in pre- and postbreeding animals. All bars represent to 100% of the data and the proportion of the bar made up by the various categories are indicated by the size of the subset of the bar. Wounding was based on the number and size of wounds. Categories were: “None” for no wounds, “Minimal” for <10 punctures and <2 cm² of externally visible wounding, “Moderate” for ≥10 punctures and/or >2 cm² but <7 cm² of externally visible wounding, and “Severe” for ≥7 cm² of externally visible wounding and/or significant damage to muscle, testes or eyes.
**Figure 5.3** Plots of pre- and postbreeding total glucocorticoid (GC) levels, maximum corticosteroid binding capacity (MCBC) and free GC levels in all 5 species of ground squirrels. Arctic ground squirrel plots are adapted from Delehanty and Boonstra (2011). Vertical bars represent 95% confidence intervals. Test statistics and p-values for each panel are as follows:

(a) $t_{25.6} = -2.2$, $p = 0.04$; (b) $t_{33} = -3.83$, $p = 0.001$; (c) $t_{22.7} = -0.41$, $p = 0.68$; (d) $t_{18.7} = -2.61$, $p = 0.02$; (e) $t_{28} = -0.73$, $p = 0.47$; (f) $t_{17.6} = -2.5$, $p = 0.02$; (g) $t_{14.9} = -5.6$, $p < 0.001$; (h) $t_{13.2} = -5.5$, $p = 0.01$; (i) $t_{16.2} = -2.92$, $p = 0.01$; (j) $t_{27} = 5.0$, $p < 0.0001$; (k) $t_{27} = 2.5$, $p = 0.02$; (l) $t_{27} = 5.26$, $p < 0.0001$; (m) $F_{1,14} = 9.09$, $p = 0.01$; (n) $F_{1,14} = 1.02$, $p = 0.33$; (o) $F_{1,14} = 20.5$, $p = 0.01$. 
References


Chapter 6

6 General Discussion and Conclusions

There is a growing interest in the study of stress in wildlife. Conservation biologists are increasingly looking to measures of stress to understand the impact of human development on wildlife species (Busch and Hayward 2009; Fokidis et al. 2009; Homyack 2010; Tracy et al. 2006), and the field of physiological ecology has begun to focus more on understanding how the HPA axis affects fitness (Bókony et al. 2009; Bonier et al. 2009; Breuner et al. 2008; Hau et al. 2010). To pursue these goals, it is essential that we have a reliable model of how the HPA axis functions in wildlife species. The biomedical literature gives us a highly detailed understanding of the mechanisms of GC production, action, and metabolism, but because the biomedical literature is based almost exclusively on laboratory animals, it offers little insight into how these mechanisms can evolve to meet real-world challenges. In contrast to the biomedical literature, physiological ecologists have generally ignored the mechanistic intricacies of the HPA axis by adopting a simplified model of the HPA axis (Figure 5.1(a)) in which GC levels are seen as the sole endpoint of the HPA axis and the downstream biological impacts are assumed to be directly proportional to the GC concentrations. Adopting this approach in my thesis, I found no relationship between the HPA axis and male reproductive lifespan, but instead found that the HPA axis in ground squirrels varies substantially among species, suggesting the HPA axis is much more finely tuned to the specific challenges of individual species and individual animals. My data suggest that the simplified model of the HPA axis is inadequate for tackling questions about the role of the HPA axis in supporting the life history strategies of vertebrates, and it suggests four main avenues for future research.

6.1 Downstream markers of stress are critical

The vast majority of stress studies measure total GC levels and rely on that as a measure of how “stressed” the animal is. That is, the biological effects of GCs are assumed to be directly proportional to GC concentrations. In studies that focus on short-term changes in GC levels (≤1 day), this assumption may be generally valid because the downstream determinants of GC effects are proteins whose abundance are likely to change more slowly than GC levels (e.g. Jamieson et al. 1999 show that 11β-hydroxysteroid dehydrogenase levels change over a period of ≥1 day).
However, in studies like mine that compare the HPA axis over a period of weeks, it is reasonable to expect that the downstream determinants of GC action will change, making GC levels unreliable indicators of downstream effects. For example, between the prebreeding and postbreeding sessions in my study, we know that plasma CBG levels changed in 3 of 5 species. The biological significance of plasma CBG levels is not currently understood, with some speculating that CBG-bound hormone acts mostly as a reservoir of hormone that is released gradually after termination of the stress response (Breuner and Orchinik 2002; Hammond 1995; Rosner 1990), and others speculating that it simply serves to facilitate the delivery of a hydrophobic hormone in blood (e.g. Breuner and Orchinik 2002). Therefore, even with this easily measured and widely discussed determinant of GC action it is not clear how CBG affects the downstream effects of GCs. The other, more difficult to measure, determinants of GC action such as extracellular and intracellular CBG, 11β-hydroxysteroid dehydrogenases, GC receptor density, and transcription cofactors (Fig. 5.1) could also change over the course of weeks and do so in a way that varies among tissues and species. One way to account for this possibility is to directly measure downstream effects.

I used glucose, free fatty acid level, hematocrit level, and white blood cell counts to assay the downstream effects of GCs. This allowed me to make qualitative predictions about how species exhibiting an adaptive stress response, characterized by chronic GC exposure, should differ from species exhibiting a homeostatic stress response, characterized by more constrained GC exposure. It is important to note that throughout my research, it was the change in levels from prebreeding to postbreeding sessions that were of interest, not the absolute levels in any one session. By observing how these downstream markers changed over the breeding season, I could draw inferences about the extent to which the HPA axis was activated over the breeding season.

One important caveat needs to be addressed before discussing my findings on downstream markers. Throughout this thesis, I have focused on the HPA axis. However, the stress response also includes an immediate response by the sympathetic-adrenomedullary (SAM) system, which causes the release of catecholamines to generate immediate physiological effects (over a period of seconds to minutes). The SAM response is closely integrated with the HPA axis (Charmandari et al. 2005), and catecholamines can have impacts on many of the same physiological systems as GCs. For example, catecholamines affect immune function (Padgett and Glaser 2003), glucose release (Bonga 1997, Sapolsky et al. 2000), and lipolysis (e.g.
Hamann et al. 2003). Due to the difficulties in measuring catecholamine levels under field conditions (Reeder and Kramer 2005), most comparative studies of the stress response—including mine—have focused on the HPA axis and largely ignored the SAM response. However, by measuring downstream effects of GCs in my thesis, it becomes important to consider whether changes in the SAM system are at least partly responsible for some of the observed changes that I have attributed to GCs. If future comparative studies give greater consideration to downstream effects of GCs, greater attention must also be given to distinguishing downstream effects mediated by catecholamines and those resulting from GC action.

In Chapters 4 and 5 I showed that changes in these downstream measures varied dramatically among the 5 species, and there were few similarities even when the direction of change in GC levels were the same. This suggests that the species differ in downstream sensitivity to the effects of GCs. My measures of downstream effects are not without problems, though. Glucose and free fatty acid levels climbed rapidly in response to capture stress (Table 2.1), as expected given the role of the HPA axis in mobilizing energy. The fact that there are short-term changes in these variables means that my observations of changes in glucose and free fatty acid levels in Chapters 4 and 5 represent only the change in the ability to mobilize these substrates under acute stress. They do not necessarily reflect how these substrates look under baseline conditions. As I explain in section 6.4, below, studying changes in basal physiology may be critical to understanding how the HPA axis supports life history strategies. Hematocrit and white blood cell count did not change in response to short-term capture stress in RGS (Table 2.1), which means that obtaining baseline blood samples isn’t critical for these measures. However, these are both very coarse measures of health and immune function. Unfortunately, most techniques for more accurate measures of immune function require either multiple captures or immediate laboratory processing of samples, neither of which was feasible for my project.

The fact that I found no relationship between the direction of change in GC levels and the downstream effects (summarized in Table 5.3) suggests that species differ in how GCs affect downstream physiology. If this is correct, then the biological significance of GC levels cannot simply be inferred from GC concentrations, at least over a time span of days and weeks. Ball and Balthazart (2008) reviewed the avian literature on the effects of sex steroid hormones and they concluded that attempts to explain reproductive and aggressive behaviour as functions of
plasma hormone levels have generally failed. They, too, emphasize the likely importance of
downstream determinants of hormone action. Hau (2007) proposes two conceptual models of
the linkages between testosterone and life history traits. The first, the “evolutionary constraint
hypothesis”, views testosterone levels, responsiveness of target tissues, and life history traits as
tightly linked, resulting in a dichotomy between high testosterone/high reproductive effort and
low testosterone/high self-maintenance. This is analogous to the assumption that GCs are
functionally significant under the simple model of the HPA axis. Hau’s (2007) second model is
the “evolutionary potential hypothesis,” which suggests that testosterone levels and the
mechanisms that can influence target tissue response (e.g. receptor density, conversion to
inactive forms, interaction with the HPA axis) can evolve independently from each other
resulting in a much more finely adaptable system. Hau (2007) concluded that the evidence
supports the evolutionary potential model of testosterone action. My results suggest that
physiological ecologists need to take a similar approach to the stress axis and its relationship to
life history traits. Future research will need to describe the variation in downstream determinants
of GC effects, and then develop a suite of downstream physiological measures that better
describe the biological impacts of GCs.

6.2 Patterns may be less general than expected

Any given life history trait is shaped by numerous internal and external factors, which means that
simple regressions of a life history variable against any one physiological measure is likely to be
subject to considerable noise. This appears to be the case in the avian literature; birds are, by far,
the best studied taxonomic group in this regard. Two recent reviews have tested hypotheses that
the stress response of reproducing birds should reflect the importance of current reproduction to
fitness. Specifically, birds whose lifetime reproductive success is primarily dependent on the
successful rearing of the current brood (i.e. “high brood value”) should have an attenuated stress
response so as to maintain reproductive effort despite stressors. This is essentially the inverse of
the adaptive stress hypothesis, which predicts increasing GC levels as the importance of current
reproduction increases. In the first review Bókony et al. (2009) found that, contrary to the
hypothesis, baseline GC levels were actually higher in high brood value species and acute stress
GC levels were unrelated to brood value when the authors examined the data in bivariate models.
However, when they controlled for baseline GC levels and breeding latitude, they found an
inverse relationship between brood value and acute stress GC concentrations, although it was
very weak. Bókony et al. (2009) acknowledge that the relationship between acute stress levels and brood value was weak and they raise the possibility that this noise may be due to downstream determinants of GC effects (i.e. by tissue-specific regulation of GC effects by variable GC receptor levels, conversion of active GC to inactive forms, and other factors as described in Figure 5.1).

In the second review, Hau et al. (2010) also looked at baseline and acute stress GC levels in birds, but they limited their review to adult male passerines. They used their own data and passerine data culled from the literature (including Bókony et al. 2009). The life-history variables they considered were length of breeding season (a measure of renesting potential) and annual adult survival rates, but they also included body mass in their analyses. They then looked for relationships between these variables and baseline GC, maximum GC in response to capture, and testosterone. Using model selection methods, Hau et al. (2010) found that baseline GC levels were mostly correlated with length of breeding season (although when using one subset of published data, body mass was also a significant factor), with longer breeding seasons being associated with lower baseline GC levels ($r^2$ values—the proportion of variation explained by the model—ranged from 0.11 to 0.29 depending on the dataset used). In contrast, maximum GC levels were best explained by body mass and adult survival rate ($r^2$ values ranged from 0.20 to 0.39 depending on the dataset analyzed), with smaller species reaching higher maximum GC levels than larger ones and species with higher survival rates having higher maximum GC levels than species with lower survival rates. The higher coefficients of determination ($r^2$ values) in Hau et al. (2010) compared to Bókony et al. (2009) suggest that life history can successfully predict GC levels as long as one focuses on a small phylogenetic group (passerines only), and control for other factors that shape GC levels like sex and body mass. In other words it seems that GC levels may be correlated with life history traits only over certain phylogenetic ranges and after controlling for other ecological or physiological factors that also influence the HPA axis.

In my study, I was able to control for some of the major confounding factors. I selected species that are phylogenetically quite closely related (Harrison et al. 2003 and Herron et al. 2004; Figure 1.2), and all species were studied in locations that face very similar environmental conditions. All five species also face similar reproductive challenges: males need to emerge from hibernation, ready to mate, and then secure matings with as many females as possible. This
shared biology involves a concentrated period of time (2-3 weeks) during which males are preoccupied with reproduction, leading me to assume that their stress physiology during this time should be likewise be geared toward maximizing reproduction. I was also able to statistically control for body mass, prebreeding GC levels and litter size when searching for a correlation between GCs and reproductive lifespan. Despite this, I did not find significant associations between postbreeding GC levels and reproductive lifespan, and no qualitative similarities among species that shared similar reproductive lifespan and similar ecologies (Chapter 5). It is possible that my sample size was too small and the range in reproductive lifespan too small to uncover a relationship between reproductive lifespan and the HPA axis. However, the fact that the stress physiology of each species was so different from one another makes it seem unlikely that a meaningful pattern will emerge simply by studying more species. Instead, it seems more likely that ground squirrels vary considerably in their downstream regulation of GC effects, and patterns will only emerge when we understand these effects more fully.

6.3 Variability may teach us more than mean values

Williams (2008) makes a cogent case for paying attention to variability in hormone levels rather than focusing simply on mean values. He points out that endocrine hormone levels can vary 5- to 15-fold among individuals, and that this is more variability than most other physiological traits. Moreover, the variability of the stress response is not just a matter of degree. Guimont and Wynne-Edwards (2006) studied individual variation in the response of the Djungarian dwarf hamster (Phodopus campbelli) to a fixed stressor. They took a baseline blood sample, applied a fixed restraint stress, and then took three additional blood samples over the next 120 min. If the resulting data were viewed only as means and standard errors, it appears that there was a reliable plasma GC increase of 50 ng/mL over baseline levels by 10 min after restraint stress. This increase was followed by a return to pre-stress levels. However, when individual values were examined, hormone responses varied from mirroring the mean trend, to no change over the entire protocol, to a continuous decline in GC levels over the protocol.

Individual variation was also a prominent feature in my results. In 3 of the 5 species I studied, both total and free GC levels were more variable in the postbreeding period than in the prebreeding period (Table 5.3). In both AGS and RGS in 2007, the postbreeding variation overlapped considerably with prebreeding values (Figure 5.3), and in RGS the pattern emerged
also emerged in 2009 (Figure 3.2; folded F test, total GC: $F_{8,21} = 3.0$, $p = 0.04$; free GC: $F_{8,21} = 4.7$, $p = 0.004$) even though the changes in total and free GC levels were different between years (see section 3.3.4). The increased variability in GC levels suggests that some postbreeding animals appear to have a consistent stress response over the breeding season, whereas other animals increase their GC responsiveness. The CGS were also more varied in their stress response in the postbreeding session compared to the prebreeding session, but there was less overlap between prebreeding and postbreeding GC levels (Figure 5.3). Both TLGS and FGS maintained consistent inter-individual variation throughout the breeding season.

There are two main lines of research to pursue based on these results. The first is an attempt to understand the basis for the observed individual variation. This would require a multi-year study that tracks individual animals closely. Three of the most critical things to pursue would be behaviour (do social interactions or personality traits determine GC levels?), mating attempts and paternity (are GC levels correlated with the number of matings attempted or the number of offspring sired?) and age (are older animals adopting different GC strategies because future reproduction contributes less to their lifetime reproductive fitness?). The second line of research suggested by my data is why inter-individual variation in some species—like the TLGS and FGS—is consistent over the breeding season. What is it about these species that constrains the range of individual stress responses? Again, multi-year studies looking at behaviour, reproductive success and age effects would help to shed light on this question. Ideally, these studies would be replicated over the range of each species so that the effects of habitat and climate can be better elucidated.

### 6.4 Baseline glucocorticoid levels may reveal patterns that are not present in acute stress levels

Seasonal variation in baseline GC levels is widespread in vertebrates. Romero (2002) reviewed seasonal changes in baseline and acute stress GC levels in the literature and found that baseline (but not acute stress) GC levels were most commonly elevated during breeding relative to pre- or postbreeding periods in reptiles, amphibians and birds. The pattern was less clear in mammals, largely because of the relatively scarcity of mammalian studies with seasonal data. Romero (2002) considered three adaptive explanations for seasonal variation in GC levels. The first was that GC levels peak during times of increased energy demands (the energetic hypothesis). The
second was that GC levels fluctuate based on the desirability of the behavioural effects of increased GCs, such as increased mobility and decreased parental behaviour (the behavioural hypothesis). The third explanation, developed by Romero, was that the increase in baseline GCs performed a preparative function (the preparative hypothesis). The preparative effects of GCs were documented by Sapolsky et al. (2000), and are defined as effects that modulate later responses to stressors. Thus, Romero (2002) proposed that seasonal increases in GCs could be priming the organism for adverse conditions they are about to encounter and suggests that this strategy would be available to species that can predict certain stressors such as competitive breeding seasons and the likelihood of adverse weather.

Moreover, Romero (2002) notes that the preparative and energetic hypotheses can be complementary when predictable stressors are accompanied by increased energetic demands. This reasoning seems to apply directly to the AGS and RGS males in my research. One of the unexpected and most intriguing findings of my research was evidence that baseline GC levels were elevated in the prebreeding period relative to the postbreeding period in both AGS and RGS (the only species for which I have relevant data). I measured baseline GC levels in male RGS in both the prebreeding and postbreeding trapping sessions in 2009. I found that in the prebreeding session baseline GC levels were elevated to acute stress levels (Figs. 3.1 and 3.2), but baseline levels declined dramatically by the postbreeding session. In AGS, I had to rely on less direct information, but based on the trends in CBG levels and on previously published baseline GC and CBG data (Boonstra et al. 2001), I argue in Chapter 4 that AGS seem to be maintaining low CBG levels and high free GC levels in the prebreeding period, and then increasing CBG levels by mid-summer. On the basis of these observations, I proposed the “basal stress hypothesis” in which animals increase their basal GC levels to a point that would normally be associated with a stress response (perhaps not as dramatically as the 2009 RGS, but still more than basal levels in the non-breeding season). The benefit of this strategy is that before the stressors of the breeding season are encountered, fat and protein are being catabolized and converted into glucose and glycogen in the liver. Thus, the basal stress hypothesis is consistent with Romero’s (2002) energetic and preparative explanations for seasonal variation in GCs.

Under the basal stress hypothesis, the key hormonal changes occur in the basal levels and do not necessarily require stress-induced GC levels to follow suit. It is quite possible for the acute stress response to remain constant or to fluctuate differently from baseline levels (e.g. chapter 3);
Romero (2002) also documents exactly such a phenomenon. The lack of a relationship between GC levels and reproductive lifespan in my research may be explained by the fact that we were measuring acute stress GC levels. Thus, despite the difficulties involved in obtaining baseline GC levels in many mammals (Boonstra 2005; Kenagy and Place 2000), my research suggests that measuring changes in baseline GC levels may be essential to understanding the evolution of the HPA axis in ground squirrels.

6.5 Conclusion

Although it is abundantly clear that GCs can affect key life history traits (e.g. Blas et al. 2007; Romero and Wikelski 2001, Romero and Wikelski 2010; Sheriff et al. 2009), the relationship between GCs and life history strategies continues to be murky. In male ground squirrels, even during a period when reproduction is their central focus, knowing the reproductive lifespan of various species does not allow one to predict how the HPA axis will function. Instead, it seems that the HPA axis is so flexible in its functioning, that we will need to adopt a much more detailed model of the HPA axis—one that incorporates downstream determinants of GC effects and their potentially tissue-specific regulation—before we can hope to understand the connections between the HPA axis and life history variation. Because the behaviour and ecology of several ground squirrel species have been intensively studied for decades, ground squirrels continue to be excellent organisms in which to pursue these questions.
References


