Chronically elevated corticosterone levels, via cocoa butter injections of corticosterone, do not affect stress response, immune function, and body condition in free-living painted turtles (*Chrysemys picta*)

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Acronyms and symbols used in the text:

[CORT], corticosterone concentration
BCI, body condition index
CBG, corticosteroid-binding globulin
CORT, corticosterone
F, female
g, gram
GC, glucocorticoid
h, hour
H:L, heterophil-to-lymphocyte ratio
M, male
mg, milligram
min, minute
mL, milliliter
mm, millimeter
n, sample size
ng, nanogram
pg, picogram
PL, plastron length
RBC, red blood cell
SD, standard deviation
SE, standard error
t, time
T_b, body temperature
WBC, white blood cell

ABSTRACT

Chronic stress can result in elevated circulating levels of glucocorticoid hormones in vertebrates, which can affect their stress response, their immune function, and eventually their fitness. I tested the effect of chronic corticosterone (CORT) elevation on the acute stress responsiveness, immune function, and body condition of free-living painted turtles (*Chrysemys picta*) in Gatineau Park, using slow-release exogenous CORT administration. While Silastic implants did not predictably elevate circulating CORT concentrations in painted turtles, injections of CORT-laden cocoa butter kept circulating levels elevated for up to 3 weeks, to concentrations likely physiologically and ecologically relevant for the species. I measured the acute CORT stress response, parasitaemia, heterophil-to-lymphocyte ratios, and total leukocyte counts after 1 week and 3 weeks, and determined body condition after 1 week, 3 weeks, and 1 year. Compared to sham and control turtles, I observed no effect of treatment on these hormonal, immune, and body condition metrics of stress, possibly because CORT mediates resource allocation only in the presence of additional immune or energy challenges, because of the masking effect of extrinsic factors, or because free, not total, CORT appears to be biologically active.

RÉSUMÉ

Le stress chronique peut entraîner des concentrations élevées d'hormones glucocorticoïdes chez les vertébrés, ce qui peut affecter leur réponse au stress, leur fonction immunitaire et, éventuellement, leur aptitude. J'ai testé l'effet d'une augmentation chronique de corticostérone (CORT) sur la réponse au stress aigu, la fonction immunitaire et la condition corporelle chez des tortues peintes (*Chrysemys picta*) en milieu naturel, dans le parc de la Gatineau, à l'aide de l'administration de CORT à libération prolongée. Les implants Silastic n'ont pas entrainé d'augmentation des concentrations de CORT en circulation chez les tortues peintes, mais des injections de beurre de cacao chargé de CORT ont maintenu les niveaux élevés jusqu'à 3 semaines, à des concentrations probablement physiologiquement et écologiquement pertinentes pour l'espèce. J'ai mesuré la réponse au stress, la parasitémie, les ratios hétérophile-lymphocyte et le nombre de leucocytes après 1 et 3 semaines, et déterminé la condition corporelle après 1 semaine, 3 semaines et 1 an. En comparaison aux groupes contrôle et placébo, je n'ai observé aucun effet du traitement sur ces mesures du stress qu'elles soient hormonales, immunitaires ou de condition corporelle, possiblement parce que la CORT intervient dans l'allocation des ressources seulement en présence de demandes immunitaires ou énergétiques accrues, à cause de l'effet masquant de facteurs extrinsèques, ou parce que la CORT libre, et non totale, semble être celle biologiquement active.

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GENERAL INTRODUCTION

Vertebrate stress response

Hormones are released by vertebrates facing a stressful stimulus (a stressor) to cope with and survive adverse conditions. Activation of the hypothalamic-pituitary-adrenal (HPA) axis (in tetrapods, or hypothalamic-pituitary-interrenal – HPI – axis in fish) is a significant aspect of the vertebrate stress response. This process culminates in glucocorticoid (GC) release several minutes following exposure to a stressor (Sapolsky et al., 2000). The main GCs are cortisol (in fish and most mammals) and corticosterone (CORT, in amphibians, reptiles, birds, rodents, and lagomorphs) (Norris, 2007). GCs act on muscles, liver, and adipose tissue and affect energy metabolism to increase circulating glucose concentrations (Norris, 2007; Sapolsky et al., 2000). An array of behavioural and physiological effects are provoked by GCs, including suppression of immunological and inflammatory responses, increases in circulating glucose concentrations and feeding behaviour, mediation of the cardiovascular stress response, and suppression of reproductive behaviour (reviewed in Sapolsky et al., 2000). It is believed these effects support short-term survival of the organism. Within hours of exposure to an initial stimulus, the effects of an acute stressor usually diminish. However, sustained elevated levels of GCs have caused negative long-term physiological effects (Sapolsky et al., 2000), especially on immunity, growth, body condition, and reproduction (Dickens and Romero, 2013; Guillette et al., 1995; Norris, 2007). Ultimately, chronic stressors can impact negatively the overall fitness of organisms and, eventually, their population dynamics (Bonier et al., 2009; Crespi et al., 2013).

Interpretations of GC levels are complicated by the fact that they have at least two receptors, which when bound to GCs have different physiological and behavioural consequences (Romero, 2004). Permissive effects are usually activated at baseline levels by the high affinity type I corticosteroid receptor (or mineralocorticoid receptor, MR), while stimulatory and inhibitory effects, such as anti-inflammatory and immunosuppressive actions, are generally activated at stress-induced levels by the low affinity type II corticosteroid receptor (or glucocorticoid receptor, GR) (Sapolsky et al., 2000). Finally, the CORT response to stressors might be regulated by plasma corticosteroid-binding globulins (CBG) (reviewed by Breuner and Orchinik, 2002; Romero, 2002). A restricted, though growing, number of stress studies now measure CBG levels (e.g. Busch et al., 2008; Cyr and Romero, 2007). There is ongoing discussion regarding the exact role of CBG: binding of CORT to CBG would seem to regulate the availability of free hormone to target tissues, serve as a tissue buffer against potentially negative effects of elevated CORT levels, alter CORT clearance rates, and modulate CORT actions by altering local CORT concentrations (Breuner and Orchinik, 2002). It has been suggested by Malisch and Breuner (2010) that free CORT is the biologically active fraction in the plasma (immediately usable to tissues), while the bound fraction exists as a source of potential free CORT, a relevant reservoir for periods of greater metabolic need. Breuner and colleagues (2013) have recently reviewed studies that present robust evidence for this free hormone hypothesis. Furthermore, they suggested that downstream metrics such as body mass changes or immune function should be used to evaluate physiological changes in CORT and CBG, in addition to the typically measured CORT levels. Desantis and colleagues (2013) concluded that high levels of CBG bind ~90% of the GCs, which can act as a circulating reservoir of GCs, in most vertebrate species.

Several variables have been shown to impact CORT levels. As ectotherms, reptiles experience important body temperature (T_b) variations. Studies on tuataras (*Sphenodon punctatus*) (Tyrrell and Cree, 1998), marine iguanas (*Amblyrhynchus cristatus*) (Woodley et al., 2003), common geckos (*Hoplodactylus maculatus*) (Cree et al., 2003; Girling and Cree, 1995), and Galápagos tortoises (*Geochelone nigra*) (Schramm et al., 1999) have indicated a positive relationship between baseline CORT and T_b or environmental temperature. Dunlap and Wingfield (1995), however, found no such correlation in western fence lizards (*Sceloporus occidentalis*). It was suggested by Cree and colleagues (2003) that the strong positive correlation between baseline CORT levels (0.5-9.5 ng/mL) and T_b (range: 9-30°C) observed in common geckos indicates a normal metabolic activity increase and that, in reptiles, T_b should always be analysed for a possible predictive influence on plasma CORT. A more recent study, however, found higher baseline and stress-induced CORT concentrations in Children's pythons (*Antaresia children*) in cold vs. warm conditions (Dupoué et al., 2013). The authors discussed the possibility that the organism remain alert at suboptimal temperatures with the help of high CORT concentrations, although it remains unclear how increased CORT concentrations relate to cold temperatures.

Chronic stress, baseline CORT levels, and acute stress responsiveness

The term "chronic stress", although subject to a certain degree of debate, can generally be defined as multiple, frequent exposure and/or long-term constant exposure to stressors (Cyr and Romero, 2009). Examples of chronic stressors are prolonged inclement weather and food deprivation, social interactions, disturbances to habitat, and human activities. In the past, another widely supported definition was long-term allostatic overload, where GC levels stay above baseline (i.e., within the range usually related to acute stress,

with type I and II GC receptors bound; Bonier et al., 2009). Johnstone and colleagues (Johnstone et al., 2012b) defined chronic stress as a state of elevated stress that is prolonged or severe enough that it is fitness reducing (i.e. prolonged distress) and stated that an animal can recover from chronic stress. Boonstra (2013) argued that a stressor should be defined as acute or chronic depending on the duration of its consequences on the physiology of the animal, and not of the stressor itself. He presented an interesting analysis of the role of chronic stress in nature, and suggested that chronic stress is not the unavoidable result of a persistent, severe stressor: wild animals will evolve to be chronically stressed if it is adaptive and if it is not, they will only respond acutely.

In free-living animals, exposure to frequent and repeated and/or chronic stressors may result in elevated circulating baseline CORT levels (see Romero, 2004; Sapolsky et al., 2000). For example, elk (*Cervus elaphus*) and wolf (*Canis lupus*) populations near snowmobile roads in national parks had higher fecal GC levels (being an approximation of baseline levels) (Creel et al., 2002), as did male northern spotted owls (*Strix occidentalis*) nesting near major logging roads and timber harvest activity (Wasser et al., 1997). A decrease in baseline CORT levels following chronic stress, however, has also been observed in some studies. Rich and Romero (2005) found that both basal and stress-induced CORT responses were significantly reduced in chronically stressed captive European starlings (*Sturnus vulgaris*). Similarly, female starlings subjected to a chronic psychological stress protocol (loud radio, predator calls, novel objects, and predator decoys) showed lower baseline CORT levels than control birds (Cyr and Romero, 2007). The authors initially concluded that such reduction in CORT levels was contrary to the general assumption that higher concentrations of circulating GCs indicate stress, suggesting that more research is

needed before CORT levels can be used to assess chronic stress accurately in field studies. They later suggested, however, that the observed reduced GC levels are explained better by physiological desensitization (Cyr and Romero, 2009) (see below for details).

To explore how repeated, but short-lived disturbances (like repeated tourist visits or repeated passage of recreational vehicles) may affect animal health, Busch and colleagues (2008) found that repeated administration of CORT to captive white-crowned sparrows (*Zonotrichia leucophrys gambelii*) resulted in higher endogenous baseline CORT levels and a down-regulation of the endogenous adrenocortical response to a standardized stress. Therefore, the authors suggested that 1-3 perturbations per day can result in higher baseline CORT levels similar to those observed during chronic stress and can affect responsiveness to additional stressors. In a number of field studies, such stress response down-regulation to acute stressors has been linked to exposure to chronic stressors (Romero, 2004). One study found that, compared to individuals in an undisturbed pond, spotted salamanders (*Ambystoma maculatum*) from a breeding pond experiencing an environmental disturbance (a recent housing development having partly surrounded it) had a lower stress responsiveness to capture and handling (Homan et al., 2003). Moreover, breeding Magellanic penguins (*Spheniscus magellanicus*) exposed to moderate levels of human visitation had increased stress-induced CORT levels likely caused by human presence, while penguins exposed to very high levels of tourism did not respond hormonally to human presence, but baseline CORT levels were similar for the two groups (Fowler, 1999). Since the moderately visited birds demonstrated severe behavioural responses (alarm state and aggression) to human presence, while penguins in the tourist area did not, these results suggest that in some conditions animals can habituate to human visitation (Fowler, 1999). Similarly, Galápagos

marine iguanas (*Amblyrhynchus cristatus*) from a site heavily exposed to tourism had reduced stress-induced CORT levels compared to animals from a site undisturbed by humans, which suggest that iguanas in tourist areas are not chronically stressed by current levels of tourism, and therefore avoid the negative effects potentially caused by chronically elevated CORT levels (Romero, 2002). The authors suggested that the tourist-exposed iguanas' long-term survival may be compromised, however, since their lowered CORT response to a stressful stimulus may limit the beneficial effects of acute CORT release. In another study, it was found that free-ranging copperhead snakes (*Agkistrodon contortrix*) captured on public roads showed a lower stress response than snakes captured in the forest, although their baseline CORT levels were the same (Owen et al., 2014).

Cyr and Romero (2009) defined habituation as "a decrease in response intensity as a novel stressor becomes familiar with repetition". They emphasized that in field studies of stress, hormonal habituation is often put forward as the main interpretation when animals show a reduced intensity of their hormonal response to repeated or chronic stressors (Cyr and Romero, 2009). Falsely concluding that individuals or populations habituated to a stressor when they did not, however, may lead researchers and wildlife managers to ignore stressors, which would negatively impact the population. They proposed three alternative explanations for an attenuated stress response to repeated stimuli: seasonal and life-history variations in physiological responses, desensitization of the physiological response without habituation to the stressor, and exhaustion. They provided potential mechanisms for physiological desensitization: down-regulation of receptors, dysregulation of negative feedback, down-regulation of hormone production, changes in concentration of synthetic enzymes, and

changes in rates of hormone clearance. They also pointed out that desensitization may be costly as it may compromise the animal's ability to deal successfully with other stressors.

The influence of ecological factors on GC stress response variation among species is largely unknown, but models by Jessop and colleagues (2013) indicated that combinations of variables including body mass (as a surrogate for metabolic rate), net primary productivity, and latitude influenced GC stress responsiveness in reptiles and birds. They concluded that evolution to species-specific traits as well as large-scale environmental variation influence GC stress response variation among species.

Patterns of GC secretion in wild animals are not well known, though more and more studies are being conducted to define these patterns. In a recent review, however, Dickens and Romero (2013) revealed that a consistent, predictable, endocrine response is not necessarily exhibited by chronically stressed wild animals. They concluded that the direction of the changes is less important than the fact that the response changes at all. Johnstone and colleagues (2012b) made several suggestions to help address the problem of confounding factors in the interpretation of indices of physiological stress. For example, measuring multiple stress indicators, such as hormone and leukocyte levels, reduces the risk of confusion with trapping stress. Measuring other condition indices, such as body condition and parasite loads, can supplement the stress data, with the typical expectation that a higher level of physiological stress should be associated with poorer body condition and higher parasite loads.

Leukocyte profiles as an indicator of stress

A leukocyte profile is composed of the proportions of each white blood cell (WBC) type, and is usually obtained by light microscope examination of 100 leukocytes in a stained

blood smear (Davis et al., 2008). Evidence suggests that leukocyte profiles are related to GC levels in all vertebrate taxa, including reptiles, in response to either natural stressors or exogenous administration of stress hormones (reviewed by Davis et al., 2008). GCs are said to cause changes in lymphocyte redistribution from the blood to other body compartments, and provoke an influx of neutrophils (heterophils in birds and reptiles; the primary phagocytic leukocyte) from the bone marrow to the blood. As a result, the number and proportion of neutrophils increase, and the number of lymphocytes, which contribute to an array of immunological functions, decreases. The N:L or H:L ratio, that is the relative proportion of neutrophils/heterophils to lymphocytes, can therefore constitute a composite measure of the stress response: high GC levels would be reflected by a high N:L or H:L ratio. Measuring N:L or H:L ratios offers certain advantages over direct GC measurement. Unlike the hormonal response to stress, the initial leukocyte response time varies from hours to days, being slower in ectothermic animals owing to their temperature-dependant metabolism, which allows more time for sampling than is the case for baseline GC levels. Such leukocyte indicators are therefore more resistant to possible effects of trapping or handling stress. This technique is also relatively inexpensive, requiring only microscope slides, stain, and a light microscope. Finally, the blood sample necessary to make a smear is very small, so this technique can be used on small or newly hatched animals. Selman and colleagues (2013) used this technique to establish that yellow-blotched sawbacks (*Graptemys flavimaculata*), an imperiled species of freshwater turtle, had notably lower H:L levels at an undisturbed site compared to those found at a site disturbed by recreational boating. For their part, Johnstone and colleagues (2012a) found that, compared to those inhabiting

unfragmented forest, agile antechinus (*Antechinus agilis*) living in forest fragments showed greater chronic stress, poorer condition, and higher N:L ratios.

Immune function during chronic stress

GCs are generally immunosuppressive under chronic conditions. Sapolsky and colleagues (2000) concluded that most GC actions on immune and inflammatory reactions are suppressive, even under conditions of exposure to basal GC levels. GCs lower circulating levels of the majority of WBC types, and inhibit synthesis, release or efficacy of cytokines and other mediators aiding immune and inflammatory reactions.

Immune function can be measured in a number of ways. For example, the rate of cutaneous wound healing is considered to be an integrated measure of stress-sensitive innate immune function (French et al., 2006). It is also a biologically relevant measure of immune function in many free-living animals, where predation attempts and intraspecific combat result in frequent wounding. French and colleagues (2006) found that captive male tree lizards (*Urosaurus ornatus*) submitted to daily restraint stress healed more slowly and had higher CORT levels than controls. They concluded that stress compromises healing and that the effects of stress are at least partly mediated by CORT. Later, French and colleagues (2007) tested alternative hypotheses examining the effects of CORT (through implants) on wound healing during reproduction, using female tree lizards. They hypothesized that CORT may act directly, in which case all CORT-treated animals should have suppressed wound healing regardless of diet or energy state, or indirectly, in which case CORT would suppress wound healing to conserve resources only under energetically limiting conditions. In pre-reproductive state, only animals that were both CORT-treated and on a restricted diet had suppressed healing. In vitellogenic (reproductive) animals, CORT treatment suppressed

healing rate regardless of food treatment. These findings suggest that CORT is a mediator of physiological trade-offs between the reproductive and immune systems, with its action apparently depending on energy availability.

The physiological effect of chronically elevated CORT levels on immunosuppression has not been extensively researched in wild animals. A study on wild Galápagos marine iguanas (*Amblyrhynchus cristatus*) by Berger and colleagues (2005) did, however, directly test the immunosuppressive role of CORT. The authors either restrained the animals or injected CORT in reproductive males, and found that such experimental elevation of CORT markedly reduced immune activity (measured by a phytohemagglutinin swelling-response (PHA) test), demonstrating that immunosuppression can be caused by CORT.

Parasites are naturally found in a variety of free-living animals, including turtles (Readel et al., 2008). Leeches are one of the most commonly observed parasites of freshwater turtles (discussed in Readel et al., 2008). Hematophagus leeches are vectors for hemoparasites such as the coccidian parasites *Haemogregarina* spp. and the hemoflagellates *Trypanosoma* spp. (Mann, 1962; Siddall and Desser, 1991, 2001) and can transmit these parasites between turtles, both intra- and interspecifically (Siddall and Desser, 2001). Parasites are known to strongly affect the fitness of their hosts by influencing important physiological and behavioural processes. For example, hemoparasites affect lizard fitness by decreasing hemoglobin levels, resting oxygen consumption, running speed, and cell regeneration (Oppliger et al., 1996; Oppliger and Clobert, 1997). Because the immune system is costly to maintain, immunosuppression can occur if energy is diverted from immune defence to cope with a stressful situation. Therefore, stressed individuals should be more susceptible to infection by parasites (Lafferty and Holt, 2003). For example, the density

of stress factors (crowding, limited refuges and food resources, simulated predation) had a significant effect on the intensity of both natural and experimentally-induced parasitaemia in common lizards (*Lacerta vivipara*) (Oppliger et al., 1998). In common side-blotched lizards (*Uta stansburiana*) experiencing variable levels of environmental stress, elevated physiological stress was associated with suppressed immunity, measured as bactericidal ability and cutaneous wound healing (Lucas and French, 2012).

CORT implantation and its relevance

Manipulating circulating levels of GC hormones is one way to ascertain the consequences of elevated GCs on organisms' immune function, stress response, or reproduction, amongst others. The increase in circulating GCs, a key element of the stress response, can be examined through CORT implantation, although it should be noted that it cannot mimic stress perfectly (Denardo and Licht, 1993). By eliminating the perceptual aspect of the stress response, the direct manipulation of CORT simplifies stress-related questions (Denardo and Sinervo, 1994a, b), allowing for the study of the effect of stress on various fitness indicators, immunity, or reproductive success separately from any confounding effect of the actual stressor (e.g., habituation) or other related factors (e.g., parasite density or habitat variations).

Objectives

Freshwater turtles are fairly abundant in eastern North America, and the biomass of their communities can reach or surpass that of fishes (Congdon et al., 1986; Iverson, 1982). As anthropogenic disturbance of natural habitats is likely to continue to rise, documenting the impact of chronic stressors on native species such as reptiles (proportionally the most threatened vertebrate group in Canada) is relevant to conservation. When the link between

baseline physiological traits and fitness is known, conservation managers can use physiological traits to predict and anticipate future problems (Wikelski and Cooke 2006).

In this context, I aimed to determine how chronically elevated GC levels (from exogenous CORT implants) affect the acute stress response, immune function (measured as parasitaemia, H:L ratios, and total WBC counts), and body condition in a free-living reptile, the painted turtle (*Chrysemys picta*).

I used the painted turtle as my study species for several reasons. First, the painted turtle is found in relatively large populations at our latitude, and therefore individuals are present in sufficient numbers at a given location. Second, the painted turtle is an aerial-basking species, which implies that individuals are closer to the surface and would be more susceptible to potential anthropogenic chronic stressors. Third, painted turtles have a body size large enough to collect the needed blood sample without significantly impacting the total blood volume of the animal.

In Chapter 1, I assessed the effectiveness of two CORT administration methods (Silastic® implants and cocoa butter injections) in chronically elevating circulating CORT levels in the painted turtle. Several techniques have been used to chronically administer CORT to animals, commonly through Silastic implants and cocoa butter injections. In several reptile and bird species, Silastic implants containing CORT have been used, although formal validation of the effectiveness of these implants in the long-term appears to be lacking. Cocoa butter has been widely used as a vehicle for corticosteroid injections in fish, mostly in cold water, but evidence suggests that this method could be successfully used in other ectotherms with warmer body temperatures. To my knowledge, my study is the first to use cocoa butter for CORT treatment in an ectothermic tetrapod. I tested Silastic implants in

the field for 2 months, and then in controlled laboratory conditions for 28 days, and I tested cocoa butter injections in the field for 2 months.

In Chapter 2, I tested the effect of chronic CORT elevation on the acute stress responsiveness, immune function, and body condition of free-living painted turtles. For the first time in an ectothermic tetrapod, I used cocoa butter injections of CORT to elevate CORT levels chronically for approximately 3 weeks. At 1 week and 3 weeks post-implantation, I recaptured turtles and determined the acute CORT stress response following a standardized restraint protocol, parasitaemia, H:L ratios, and total WBC counts in treated, sham and control turtles. I also determined body condition 1 week, 3 weeks, and 1 year after treatment.

CHAPTER 1

Cocoa butter injections, but not sealed or perforated Silastic implants, of corticosterone can be used to chronically elevate corticosterone in free-living painted turtles (*Chrysemys picta*)

This chapter formed the basis for the following publication:

Juneau, V., Gilmour, K.M., and Blouin-Demers, G. Cocoa butter injections, but not sealed or perforated Silastic implants, of corticosterone can be used to chronically elevate corticosterone in free-living painted turtles (*Chrysemys picta*). In review.

ABSTRACT

Chronic stress can result in an elevation of circulating levels of glucocorticoid (GC) hormones in vertebrates, which may affect their fitness. To isolate the effect of GCs on stressed organisms, one approach consists in manipulating circulating levels of GCs. We investigated the usefulness of two corticosterone (CORT) administration methods, Silastic implants and cocoa butter injections, in chronically elevating circulating CORT levels in painted turtles (*Chrysemys picta*). First, free-living turtles received subcutaneous Silastic implants for 2 months. We observed no significant difference in baseline CORT levels between 2 doses of CORT and sham-treated turtles. Then, captive turtles received a subcutaneous Silastic implant for 28 days. We observed no effect on baseline CORT levels, hormonal stress response, or body mass, suggesting that sealed and perforated Silastic implants of CORT may not be an effective way to elevate CORT in painted turtles. Second, we tested injections of CORT-laden cocoa butter for the first time in an ectothermic tetrapod. Free-living turtles received an epicoelomic injection of liquid cocoa butter mixed with CORT and were recaptured in the field over 2 months. Despite large inter-individual variation, we found that this injection approach generally kept circulating CORT levels elevated for up to 3 weeks. Achieved CORT concentrations were probably physiologically and ecologically relevant for the species, although concentrations possibly remained elevated longer than would be the case in wild animals. Cocoa butter injections, but not sealed or perforated Silastic implants, can be used in painted turtles to chronically elevate CORT. Further, this represents a promising method for other temperate ectotherms, such as amphibians and reptiles.

INTRODUCTION

When vertebrates face a stressful stimulus, they release hormones that help them cope with and survive the adverse conditions. One key aspect of the vertebrate stress response is the activation of the hypothalamic-pituitary-adrenal (HPA) axis, culminating in glucocorticoid (GC; corticosterone, CORT, in reptiles) release several minutes after initiation of a stressful stimulus (Sapolsky et al., 2000). GCs then induce a variety of behavioural and physiological effects, such as mediating the cardiovascular stress response, increasing circulating glucose concentrations and feeding behaviour, suppressing immunological and inflammatory responses, as well as reproductive behaviour (reviewed in Sapolsky et al., 2000). Collectively, these effects are believed to promote survival of the organism in the short term. Although the effects of an acute stressor generally fade within hours of the initial stimulus, sustained elevated levels of GCs can have negative physiological effects in the long term (Sapolsky et al., 2000), notably on growth, immunity, and reproduction (Guillette et al., 1995; Norris, 2007). Although subject to some debate, the term chronic stress can be defined as multiple, frequent exposure and/or long-term constant exposure to stressors (Cyr and Romero, 2009). Prolonged inclement weather and food deprivation, social interactions, habitat disturbance, and human activities are some examples of chronic stressors. Until recently, another definition was long-term allostatic overload, during which GC levels remain above baseline (i.e., within the range typically associated with acute stress, with type I and II GC receptors bound; Bonier et al., 2009). Boonstra (2013) argued that a stressor should be defined as acute or chronic based on the duration not of the stressor itself, but of its consequences on the physiology of the animal. Knowledge of patterns of GC secretion in wild animals is limited but expanding. A recent review in wild animals, however, uncovered

that chronically stressed animals do not necessarily exhibit a consistent, predictable, endocrine response to chronic stress; the fact that the response changes at all is more important than the direction of the changes (Dickens and Romero, 2013). Ultimately, chronic stressors can impact negatively the overall fitness of organisms and, eventually, their population dynamics (Bonier et al., 2009; Crespi et al., 2013).

In this context, a possible approach to determine the consequences of elevated GCs on the organisms - for instance on stress response, immune function, reproduction, or survival - consists in manipulating circulating levels of GC hormones. Although GC implantation does not represent a perfect mimic of stress, it provides an effective tool to examine a key constituent of the stress response, namely the rise in circulating GCs (Denardo and Licht, 1993). The direct manipulation of GCs eliminates the perceptual component of the stress response, reducing the complexity of stress-related questions (Denardo and Sinervo, 1994a, b). This allows the effect of stress on various fitness indicators, immunity, or reproductive success to be studied independently of any confounding effect of the actual stressor (e.g., habituation) or other related factors (e.g., parasite density or habitat variations).

Several methods have been used to administer GCs chronically to animals, but few validation studies exist for reptiles. Two common methods are Silastic® implants and cocoa butter injections. Silastic implants containing CORT have been used in several reptile and bird species. For example, subcutaneous implants successfully elevated CORT levels for 5 days post-treatment in red-eared slider turtles (*Trachemys scripta elegans*) held in captivity (Cash and Holberton, 1999). Sealed implants have been used intracoelomically to keep plasma CORT levels elevated in side-blotched lizards (*Uta stansburiana*), both in short-term laboratory experiments (Denardo and Licht, 1993; Miles et al., 2007) and in long-term (1.5

months) field studies (Denardo and Sinervo, 1994a, b), although formal validation of the effectiveness of these implants in the long-term appears to be lacking. Subcutaneous CORT Silastic implants elevated CORT levels for at least 3 days in black-legged kittiwakes (*Rissa tridactyla*), which the authors expected to be metabolized within 2–3 weeks after implantation (Kitaysky et al., 2001), and for 2–4 days in tree sparrows (*Spizella arborea*) (Astheimer et al., 2000). Newman and colleagues (2010) found that implants with perforated holes had a higher CORT delivery than sealed implants *in vitro*, but still only elevated baseline levels over those of controls for 2–3 days *in vivo* in a 28-day validation study in song sparrows (*Melospiza melodia*). In birds, Silastic implants with one or both ends open have been used to provide a rapid release over a limited time period (e.g. Goutte et al., 2010), but sealed or perforated implants are thought to provide a slower release and thus are more likely to be useful if the goal is to elevate CORT chronically.

Because cocoa butter is a lipid that melts at ~38°C, but remains solid at colder temperatures, it has been widely used as a vehicle for corticosteroid injections in fish, mostly in cold water. A single intra-peritoneal injection of cortisol-laden cocoa butter can be used to chronically elevate plasma cortisol levels in fish (for a review, see Gamperl et al., 1994). For example, intra-peritoneal injections of cortisol in cocoa butter successfully elevated plasma cortisol levels for 5 weeks in brown trout (*Salmo trutta*) at 12–17°C (Pickering and Duston, 1983). Cortisol cocoa-butter implants were also successfully used in the tropical fish tilapia (*Oreochromis mossambicus*) at 24–27°C to elevate and maintain cortisol at a level characteristic of a stressed fish for 19 days (Foo and Lam, 1993), which suggests that this method could be successfully used in other ectotherms with similarly warm body temperatures.

In this study, our objective was to assess the effectiveness of two CORT administration methods (Silastic implants and cocoa butter injections) at chronically elevating circulating CORT levels in a temperate reptile, the painted turtle (*Chrysemys picta*). To our knowledge, our study is the first to use cocoa butter for CORT treatment in an ectothermic tetrapod. We tested Silastic implants in the field for 2 months, then in controlled laboratory conditions for 28 days, and cocoa butter injections in the field for 2 months.

MATERIALS AND METHODS

Study species and study site

We used the painted turtle as our study species because it is the most abundant local turtle (pers. obs.), as well as one of the most studied and most widely distributed turtles in North America (Ernst and Lovich, 2009). We captured all turtles in Lake Renaud, a small lake located in Gatineau Park (45°36'10''N, 76°01'24''W), Québec, Canada, using a dip net, hoop nets (with air space) baited with sardines, or opportunistically by hand from a canoe. At their first capture, we marked turtles semi-permanently by notching with a file one to four of their marginal scutes in unique combinations. We released all turtles at their capture location at the end of each experiment.

Experiment 1A: Silastic implants in the field

Silastic implants

In summer 2009, we made slow-release CORT implants using medical grade Silastic tubing (o.d. = 1.96 mm, i.d. = 1.47 mm, length = 20 mm; Dow Corning), filled with 20 mg of crystalline CORT (C2505, Sigma Chemicals), and we sealed both ends with Silastic medical

adhesive silicone type A (Dow Corning). We soaked the implants in sterile saline prior to implantation. We assigned adult turtles randomly to one of three treatments: two empty Silastic implants (Sham, $n = 10$), one sealed CORT-filled Silastic implant and one empty implant (1 CORT, $n = 9$), or two sealed CORT-filled Silastic implants (2 CORT, $n = 9$). We used a design with two implants in each turtle to test two doses while keeping constant the number and length of the implants. After applying a local topical anesthetic (cream lidocaine) on the skin, we inserted the implants subcutaneously, one in each thigh, using a syringe-style implanter with a 12-gauge needle (MK10 implanter with N125 needle, Biomark, ID, USA). We sealed the puncture site with surgical glue and allowed the glue to dry thoroughly (up to 20 min) before releasing individuals at their capture location. Over 2 months (67 days), we successfully recaptured and blood-sampled 28 implanted turtles opportunistically in the field.

Blood sampling

On day 0 (before the implantation) and over a 2-month period during the summer, we collected baseline blood samples to determine whether the implants elevated circulating baseline CORT levels over time. We used the moment at which the turtle first noticed the presence of the capturer as the starting time (t = 0 min). Immediately after capture, and no later than t = 10 min (Cash et al., 1997) (mean = 4.20 min, SD = 2.25 min, $n = 94$), we collected a first blood sample (300–500 μL) from the coccygeal vein (Bulté et al., 2006) using pre-heparinized syringes (0.5 cc with 28-gauge needle). We stored blood samples in an ice slurry for up to 8 h, and then we centrifuged them to collect plasma. We snap-froze plasma samples on dry ice then placed them in a freezer at −80°C. Using samples collected within 10 min on day 0, we observed no significant relationship between bleed time and

baseline CORT level (linear regression, $R^2 = 0.075$, $P = 0.16$, $n = 28$), and found no difference between baseline CORT levels from samples collected within and after 5 min (*t*-test, $t_{(26)} = 0.615$, $P = 0.54$). On day 0, baseline CORT levels were not different between the sexes (*t*-test, $t_{(26)} = 0.50$, $P = 0.62$, $n = 5$ females and 23 males); therefore data from males and females were combined in the analyses. On day 0, baseline CORT levels were not different among treatment groups (one-way ANOVA, $F_{(2, 25)} = 0.69$, $P = 0.51$). Following implantation (day 0), turtles were recaptured 1–6 times (mean = 2.1, median = 2) over 2 months (67 days).

Experiment 1B: Silastic implants in the laboratory

Animal collection and husbandry

In August 2010, we captured 28 adult male painted turtles (as described above). We used only adult males to avoid any potential confounding effect of developmental or reproductive stage. We measured and weighed turtles and drove them back the same day to the University of Ottawa. Before starting the experiment, we allowed turtles to acclimate to captivity for 12.8 days on average (± 1.1 days, no difference among treatment groups, $P = 0.99$). We housed turtles individually in vivaria containing water and a basking platform, kept them at the preferred temperature for the species (~23°C, typical in the field, Edwards and Blouin-Demers, 2007) on a natural photoperiod cycle (13 L: 11 D), fed them with earthworms twice a week, and provided them with fresh dark leafy greens ad libitum.

Silastic implants

We made and inserted slow-release CORT implants as described for Experiment 1A, with the exception that each turtle received only one implant instead of two. We assigned turtles randomly to one of four treatments ($n = 7$ in each group): no implant (Control), one

empty Silastic implant (Sham), one sealed CORT-filled Silastic implant (CORT sealed), or one CORT-filled Silastic implant perforated with 8 holes (made with a 25-gauge needle) to increase delivery (CORT holes). We sealed the puncture site with surgical glue and allowed the glue to dry thoroughly (up to 20 min) before placing individuals back into vivaria. At the end of the study, we made a small incision with a scalpel through anesthetized skin to remove the implants, and then sealed the skin with surgical glue.

Blood sampling and animal processing

Prior to implantation on day 0, then on days 2, 4, 7, 14, 21, and 28, we collected baseline blood samples, as described for Experiment 1A (mean = 4.17 min, SD = 2.95 min, n = 196), to determine whether the implants elevated the circulating baseline CORT levels over time. Using samples collected within 10 min on day 0, we observed no relationship between bleed time and baseline CORT level (linear regression, $R^2 = 0.009$, $P = 0.65$, n = 26), and found no difference between baseline CORT levels from samples collected within and after 5 min (t-test, $t_{(22.1)} = -0.412$, $P = 0.68$). On days 0, 7, and 28, we aimed at collecting a stress-induced sample after 30 min (mean = 35.45 min, SD = 3.55 min, n = 84) of restraint in a plastic bin, to determine whether CORT treatment reduced stress responsiveness. On day 0, baseline CORT levels were not different among treatment groups (one-way ANOVA, $F_{(3, 24)} = 1.78$, $P = 0.18$, $n = 28$, Fig. 1-2A), nor were stress-induced levels (one-way Welch ANOVA, $F_{(3, 12.98)} = 0.37$, $P = 0.77$, $n = 28$, Fig. 1-2A). On each sampling day, we weighed the turtles.

Experiment 2: Cocoa butter injections

Cocoa butter implants

In summer 2011, we dissolved crystalline CORT (C2505, Sigma Chemicals) in liquid pure cocoa butter (10 mg CORT / 1 mL butter). We kept this mixture in a warm water bath and injected it epicoelomically (near the pectoral muscle) to CORT-treated individuals (n = 17) using 1-mL syringe and 18-gauge, 38-mm-long needles, at a dose of 10 mg CORT in 1 mL cocoa butter per 1 kg body mass, then we sealed the injection site with surgical glue. Sham-treated individuals (n = 5) received an injection of cocoa butter only, while controls (n = 6) received no injection. Over 2 months, we successfully recaptured and blood-sampled 28 adult turtles opportunistically in the field.

Blood sampling

On day 0 (before the cocoa butter injection) and over a 60-day period during the summer, we collected baseline blood samples, as described for Experiment 1A (mean = 4.89 min, SD = 2.42 min, n = 22), to determine whether the implants elevated the circulating baseline CORT levels over time. Using samples collected within 10 min on day 0, we observed no relationship between bleed time and baseline CORT level (linear regression, R^2 = 0.0002, P = 0.95, n = 22), and found no difference between baseline CORT levels from samples collected within and after 5 min (t-test, $t_{(15.2)}$ = 0.359, P = 0.72). On day 0, we also collected a stress-induced sample, as described for Experiment 1B (mean = 33.91 min, SD = 1.72 min, n = 22). On day 0, baseline CORT levels were not different between the sexes (t-test, $t_{(20)}$ = 0.60, P = 0.55, n = 5 females and 17 males), nor were stress-induced levels (t-test, $t_{(20)}$ = 0.38, P = 0.71), and therefore data from males and females were combined in the analyses. On day 0, baseline CORT levels were not different among treatment groups (one-

way ANOVA, $F_{(2, 19)} = 0.75$, $P = 0.49$, $n = 4$ control, 5 sham, 13 CORT, Fig. 1-3A), nor were stress-induced levels (one-way ANOVA, $F_{(2,19)} = 0.01$, $P = 0.99$, Fig. 1-3A). Following injection (day 0), turtles were recaptured 1–5 times (mean = 2, median = 2) over 60 days.

Corticosterone assays

We determined total CORT concentrations using a competitive enzyme-linked immunosorbent assay (ELISA) (sensitivity 27 pg/mL, Cat. No. 900-097, Assay Designs Inc.). We used the kit as per the manufacturer's instructions. We diluted our samples 5 to 50 times in the provided assay buffer to obtain concentrations within the range of the standard curve. To a known volume of plasma, we added the same volume of the provided steroid displacement reagent (previously diluted 1:100 in assay buffer), let sit 10 min, then added assay buffer to reach the desired dilution. We used SoftMax Pro 4.0 (Molecular Devices) to calculate the concentration of CORT in the samples from the optical density data. Using serial dilutions of turtle plasma samples, we obtained curves that were parallel to standard curves.

We ran all standard curves in triplicate (mean C.V. = 3.3%) and all samples in duplicate (mean C.V. = 11.2%). We determined an intra-assay coefficient of variation of 10.9% by running a randomly chosen sample in two sets of duplicates on a given plate, and an inter-assay variation of 30.0% by running duplicated aliquots from a pool of samples on each plate, and of 20.4% by running duplicates of the same samples on different plates. We kept all samples from a given individual on the same plate, and we had individuals of each treatment groups on each plate we ran. This sample randomization mitigated the potential effect of the high inter-assay variation on our data.

Statistical analyses

We conducted all statistical analyses using the statistical software JMP (versions 5 and 8, SAS). We log-transformed data as needed to satisfy assumptions of normality (tested with Shapiro-Wilk) and homogeneity of variances (tested with Levene's test).

Experiment 1A: Silastic implants in the field

To assess whether CORT treatment elevated baseline CORT levels, we used an ANCOVA of baseline CORT levels (excluding day 0) vs. treatment group and time to determine whether the slopes differed among the groups.

Experiment 1B: Silastic implants in the laboratory

We used a paired *t*-test to determine whether baseline and stress-induced levels differed among individuals on day 0. To determine whether CORT treatment affected baseline CORT levels and stress responsiveness, we analysed baseline and stress-induced CORT levels vs. time and treatment group using two-way ANOVAs with repeated measures. To determine whether treatment affected body condition, we used a *t*-test to ascertain whether the mass lost during the experiment (% difference between days 0 and 28) differed from 0, and a one-way ANOVA to find whether this mass loss differed among groups.

Experiment 2: Cocoa butter injections

We used a paired *t*-test to determine whether baseline and stress-induced levels differed among individuals on day 0. To assess whether CORT treatment elevated baseline CORT levels, we used an ANCOVA of baseline CORT levels (excluding day 0) vs. treatment group and time to determine whether the slopes differed among the groups, then performed linear regressions of baseline CORT levels (excluding day 0) vs. time for each treatment group.

RESULTS AND DISCUSSION

Experiment 1A: Silastic implants in the field

During the experiment, the painted turtles exhibited baseline CORT levels (Table 1-1; Fig. 1-1A) similar to those previously observed in other painted turtles (Keiver et al., 1992) and red-eared sliders (Cash et al., 1997). On day 0, baseline CORT levels for all groups combined (n = 28) were: mean = 5.1 ng/mL; SE = 2.6; median = 0.9; min = 0.1; max = 64.0. In Table 1-1, we report mean (+ SE) and median baseline CORT concentrations on day 0 and for various time periods for each treatment group. It is worth noting that 2 individuals had higher baseline CORT concentrations starting on day 0 (Fig. 1-1), which remained high throughout the experiment. The elevated means at certain time points are mostly driven by these individuals, and are not related to treatment. Therefore, mean values should be interpreted with caution, and median values provide a more accurate representation of central tendency. Over the 2 months of the experiment, baseline CORT levels remained low for all groups (Fig. 1-1A; Table 1-1). Individual profiles (Fig. 1-1B) indicate that CORT values were fairly constant through time for most individuals, with some variability that does not appear to be associated with treatment, and highly variable among individuals. For baseline CORT levels measured on or after day 1 (n = 62 data points from 28 individuals: 25 from 9 individuals with 2-CORT, 15 from 9 individuals with 1-CORT, and 22 from 10 individuals with shams), we found a non-significant group*time interaction (P = 0.34), a non-significant effect of group (P = 0.57), and a non-significant effect of time (P = 0.81). This non-significant interaction term means that the slopes of the relationship did not differ for the three groups. The results suggest that the sealed Silastic implants do not elevate circulating levels of CORT over baseline levels in free-living painted turtles.

Experiment 1B: Silastic implants in the laboratory

On day 0 of the experiment, the male painted turtles had baseline CORT levels (Fig. 1-2A) (mean = 5.2 ng/mL; SE = 1.2; median = 2.8; min = 0.6; max = 25.0; n = 28 for all groups combined) similar to those previously observed in other painted turtles (Keiver et al., 1992) and red-eared sliders (Cash et al., 1997). Turtles exhibited the typical vertebrate stress response, with stress-induced CORT levels higher than baseline levels after 30 min of handling and restraint ($t_{(27)} = 9.65$, $P < 0.001$, $n = 28$, Fig. 1-2A) (mean = 16.5 ng/mL; SE = 3.1; median = 8.2; min = 1.2; max = 59.8; n = 28 for all groups combined). Three individuals (one Control and two CORT holes) had unusually high baseline CORT levels on day 0 (Fig. 1-2A), and had consistently higher levels throughout the experiment. The presence of these three individuals in the analyses does not qualitatively change the conclusions, and there is no particular reason to exclude them, therefore we left them in the data set. We report mean (+ SE) and median baseline (Table 1-2) and stress-induced (Table 1-3) CORT concentrations on day 0 and for various time points for each treatment group. The elevated means at certain time points are driven by these three individuals, and are not related to treatment. Therefore, mean values should be interpreted with caution, and median values (Fig. 1-2B, C) provide a more accurate representation of central tendency. Individual profiles (Fig. 1-2D) indicate that baseline CORT values were somewhat variable within individuals, with some variability that does not appear to be associated with treatment, and highly variable among individuals. Some profiles from each treatment group (including Control and Sham) show a rise at day 2 and/or 4, while CORT levels remain steady or decline in others. Overall, we did not observe a clear effect of treatment group on baseline CORT levels over the duration of the experiment (Fig. 1-2B, D). We detected no time*group interaction ($F_{(18, 54.2)} = 0.99$, $P = 0.49$),

no effect of treatment group ($F_{(3, 24)} = 2.10$, $P = 0.13$), but a significant effect of time ($F_{(6, 19)} = 3.39$, $P = 0.02$). This time effect likely resulted from captivity stress. These results indicate that our Silastic implants of CORT do not elevate circulating baseline CORT levels on days 2–28. For stress-induced levels (Fig. 1-2C), we found no time*group interaction ($F_{(6, 46)} = 1.63$, $P = 0.16$), no effect of treatment group ($F_{(3, 24)} = 1.10$, $P = 0.37$), and no effect of time ($F_{(2, 23)} = 0.67$, $P = 0.52$). Therefore, our Silastic CORT treatment did not affect stress responsiveness in our turtles. Turtles lost an average of 1.45 ± 0.48% of body mass over the 28 days of the experiment (significantly different from 0, $t_{(27)} = 3.06$, $P = 0.005$), however mass loss was the same across the treatment groups ($F_{(3, 24)} = 0.72$, $P = 0.55$).

A visual inspection of the implants after their removal from the animals suggested that the implants with holes released some CORT during the experiment, whereas the sealed implants did not appear to release any CORT. A possible explanation for the lack of a measured increase in circulating CORT levels in the animals with perforated implants is that this CORT was excreted rapidly, preventing any detectable elevation in circulating CORT levels as well as any effects on the acute stress response and body condition. The renal portal system, present in most non-mammalian vertebrates including turtles and thought to collect blood from the lower body and bring a portion of it to the kidneys, could potentially lead to a rapid excretion of drugs injected in the lower body, and thus affect their efficacy. Holz and colleagues (1997a) tested this possibility in red-eared slider turtles and concluded that injection site was unlikely to cause clinically significant effects on renal extraction of drugs through the renal portal system, and that the lower body could be used for drug administration in reptiles. In a companion study (Holz et al., 1997b), they demonstrated, however, that circulation from the hindlimbs flowed mainly to the liver and bypassed the

kidneys, contrary to what was previously thought. Because glucocorticoids are metabolized by target cells or the liver (Norris, 2007), it is possible that part of the CORT we administered was metabolized after a first pass in the liver, reducing its concentration in the blood and its effect on other tissues. Some researchers also leave one end of the Silastic implants open to improve CORT delivery, but this is typically used for short-term rapid release, not for a chronic elevation of CORT. It appears that sealed and perforated Silastic implants cannot be used to chronically elevate circulating CORT levels in painted turtles.

Experiment 2: Cocoa butter injections

On day 0 of the experiment, our painted turtles had baseline CORT levels (mean = 1.1 ng/mL; SE = 0.2; median = 0.6; min = 0.3; max = 4.6; n = 22 for all groups combined) (Fig. 1-3A) similar to those previously observed in other painted turtles (Keiver et al., 1992) and red-eared sliders (Cash et al., 1997), and overlapped with the lower values measured in captive male turtles in Experiment 1B (Fig. 1-2A). Turtles in this field-based experiment also exhibited the typical vertebrate stress response, with stress-induced CORT levels higher than baseline levels after 30 min of handling and restraint ($t_{(21)} = 6.82$, $P < 0.001$, Fig. 1-3A) (mean = 4.1 ng/mL; SE = 0.9; median = 2.3; min = 0.4; max = 15.7; n = 22 for all groups combined). In Table 1-4, we report mean (+ SE) and median baseline CORT concentrations on day 0 and for various time periods for each treatment group.

Over the 2 months of the experiment, baseline CORT levels remained low for control and sham-treated turtles, while they were elevated starting on day 1 and for up to 3 weeks for CORT-treated individuals (Fig. 1-3B). In individual profiles (Fig. 1-3C) of control and sham-treated turtles, CORT levels remained steady or slightly decreased within the first week, and then showed some variability not associated with treatment and within the

baseline range. In CORT-treated individuals, however, there was a rapid or progressive increase in CORT levels over the first 7-10 days, followed by a slower decline around the third week. Despite large inter-individual variation, this observation suggests that the injection of CORT in cocoa butter can be used to elevate circulating levels of CORT over baseline levels in free-living painted turtles for a period of a few weeks. For baseline CORT levels measured on or after day 1 (n = 66 data points from 28 individuals: 14 from 6 controls, 7 from 5 shams, and 45 from 17 CORT-treated individuals), we found a significant group*time interaction ($P < 0.001$), a significant effect of group ($P < 0.001$), and a non-significant effect of time ($P = 0.27$). This significant interaction term means that, as expected, the slopes of the relationship differed for the three groups. To make sure this effect was not due to pseudo-replication, we repeated the analysis by using only one randomly selected point for each individual (n = 6 controls, 5 shams, and 17 CORT-treated individuals), and we found a marginally significant group*time interaction ($P = 0.09$), a significant effect of group ($P < 0.001$), and a non-significant effect of time ($P = 0.32$). As expected, we found a significant negative linear relationship of baseline CORT levels over time for the CORT-treated group ($R^2 = 0.58$, $F_{(1, 43)} = 60.52$, $P < 0.001$, $n = 45$), but not for the control ($R^2 = 0.02$, $F_{(1, 12)} = 0.19$, $P = 0.67$, $n = 14$) or the sham-treated group ($R^2 = 0.18$, $F_{(1, 5)} = 1.09$, $P = 0.35$, $n = 7$).

To the best of our knowledge, there are no published data on patterns of CORT secretion in wild painted turtles that experience natural stressors. Therefore, we need to rely on experimental studies to assess whether our achieved concentrations were relevant in an ecological and physiological context (as opposed to delivering pharmacological doses). Although it does not appear that the pattern is ubiquitous (Dickens and Romero, 2013), in

some situations chronic stress can result in baseline CORT levels elevated into the range of values typical of acute stress (Bonier et al., 2009) for the duration of the chronic stress. Keiver and colleagues (1992) reported that cannulated painted turtles undergoing a 10-h underwater anoxia at 22°C in laboratory conditions had CORT levels in the typical baseline range (0.8 to 4.5 ng/mL in their study) while catecholamines increased greatly. However, when anoxic turtles were allowed to surface and recover, catecholamines rapidly decreased while CORT levels increased 10-fold over controls after 4 h of recovery (mean = 19.0 ng/mL, SD = 9.4, n = 4), were still elevated after 10 h (mean = 15.5 ng/mL, SD = 2.5, n = 4), and slowly decreased until 48 h of recovery, suggesting a role for glucocorticoids in the recovery from anoxic stress. Larocque and colleagues (2012a; 2012b) demonstrated that freshwater commercial fisheries result in bycatch of painted turtles, and that turtles in such submerged nets experience anoxia, which can lead to severe turtle mortality (up to 33%). Therefore, it is likely that turtles recovering from entrapment in fishing nets would be secreting higher CORT levels. To ascertain to what extent painted turtles caught in commercial hoop net fisheries experience such CORT secretion during recovery, we submitted turtles (n = 9) to 7 h of anoxia in submerged hoop nets in Lake Opinicon (eastern Ontario, Canada) (following methods of Larocque et al., 2012b), after which we took turtles out of the nets, allowed them to recover on a platform, and blood-sampled them via venipuncture. When removed from the nets, turtles had typical baseline CORT levels (mean = 3.8 ng/mL, SD = 4.7), which then became elevated after 1 h (mean = 9.6 ng/mL, SD = 12.8, max = 41.9) and 4 h (mean = 9.4 ng/mL, SD = 7.9, max = 23.4) of recovery (V. Juneau & S.M. Larocque, pers. obs.). Although some of our cocoa butter CORT-treated individuals experienced higher levels than those of entrapped turtles, mostly in the first week of

treatment (Fig. 1-3B), a majority (57%) of samples collected from CORT-treated turtles between days 1 and 21 were below the maximum that we measured after 1 h of recovery from anoxia, and 33% below the maximum after 4 h. These results, combined with those of Keiver and colleagues (1992), indicate that most of our cocoa butter CORT-treated turtles experienced CORT levels in the same range as turtles recovering from entrapment in fishing nets, which is probably a relevant stressor for a natural population of freshwater turtles, although our treated turtles may have experienced elevated CORT for longer than wild animals. Moreover, the baseline CORT levels we achieved with our cocoa butter dosage are similar to stress-induced levels measured in free-living painted turtles captured for the first time in Lake Renaud in the summer of 2009 and submitted to a 30-min standardized stress-restraint protocol (mean = 8.5 ng/mL, SD = 17.6, max = 91.4, n = 49; V. Juneau, personal observation). Taken together, these data suggest that the achieved CORT concentrations from our cocoa butter injections were probably physiologically and ecologically relevant for the species (and not pharmacological doses). Chronic stress can result in baseline CORT levels elevated in the range of acute stress (Bonier et al., 2009) for the duration of the chronic stress, at least in some situations. Thus, cocoa butter injections can be used to chronically elevate CORT levels to likely ecologically relevant levels.

CONCLUSIONS

We tested two methods to chronically elevate CORT levels in a temperate reptile, the painted turtle. In the first experiment, we used sealed Silastic implants of CORT in free-living turtles and observed no effect on baseline CORT levels. We then used sealed and perforated Silastic implants in captive turtles and observed no significant difference in baseline CORT levels, hormonal stress response, or body mass among treatment groups. Our

findings suggest that sealed or perforated Silastic implants of CORT do not necessarily function as previously expected and should be used with caution. In the second experiment, we validated injections of CORT-laden cocoa butter for the first time in an ectothermic tetrapod. Despite large inter-individual variation, we found that our CORT dosage generally elevated circulating CORT levels for up to 3 weeks in free-living turtles, at concentrations that were likely physiologically and ecologically relevant for the species, i.e., that were mostly around typical stress-induced levels, or levels experienced by turtles recovering from entrapment in commercial fishing nets. The duration of the CORT elevation, however, is possibly longer than that experienced by wild turtles. We conclude that cocoa butter injections, but not sealed or perforated Silastic implants, of CORT can be successfully used in painted turtles to chronically elevate circulating CORT levels, and represent a promising method for other ectotherms, such as amphibians and other reptiles, living in temperate climates.

Table 1-1. Plasma total baseline corticosterone concentrations ([CORT]) for various time intervals after treatment in free-living adult painted turtles (*Chrysemys picta*) in Experiment 1A. Median, mean, standard error (SE), and sample size (n) are presented. Individuals were randomly assigned to one of three treatment groups: two empty Silastic implants (Sham), one sealed CORT-filled Silastic implant and one empty implant (1 CORT), or two sealed CORT-filled Silastic implants (2 CORT). Only the first sample was included for individuals that were sampled twice within the same time interval (n = 2).

Treatment group	Days post-implant	Baseline [CORT] (ng/mL)			*n*
		Median	Mean	SE	
Sham	0	0.9	7.5	6.3	10
	1 – 4	0.7	0.7	0.2	3
	5 – 10	1.0	1.0	0.1	2
	11 – 20	0.6	0.9	0.4	4
	21 – 30	3.9	15.9	13.1	5
	31 – 40	0.7	0.7	-	1
	41 – 50	1.6	18.7	17.3	4
	51 – 67	0.8	0.8	0.3	2
1 CORT	0	0.4	1.8	1.0	9
	1 – 4	0.3	0.3	-	1
	5 – 10	1.9	1.9	-	1
	11 – 20	3.2	3.2	-	1
	21 – 30	1.7	1.5	0.2	3
	31 – 40	1.4	1.2	0.2	5
	41 – 50	1.1	1.1	0.8	2
	51 – 67	0.6	0.6	-	1
2 CORT	0	0.6	5.8	4.4	9
	1 – 4	0.7	2.8	2.3	3
	5 – 10	15.5	15.5	9.7	2
	11 – 20	3.1	3.1	2.9	2
	21 – 30	1.3	4.7	3.7	5
	31 – 40	0.8	14.8	13.6	6
	41 – 50	1.6	12.3	11.2	3
	51 – 67	1.5	1.7	0.5	4

Table 1-2. Plasma total baseline corticosterone concentrations ([CORT]) at various time points after treatment in captive adult male painted turtles (*Chrysemys picta*) in Experiment 1B. Median, mean, and standard error (SE) are presented. Individuals were randomly assigned to one of four treatment groups ($n = 7$ in each group): no implant (Control), one empty Silastic implant (Sham), one sealed CORT-filled Silastic implant (CORT sealed), or one perforated CORT-filled Silastic implant (CORT holes).

Treatment group	Days post-implant	Baseline [CORT] (ng/mL)		
		Median	Mean	SE
Control	0	3.1	5.2	2.8
	2	2.9	9.7	7.2
	4	3.0	6.3	3.0
	7	2.2	4.9	2.5
	14	2.9	7.3	3.4
	21	3.1	9.5	5.3
	28	2.6	6.2	2.9
Sham	0	2.8	3.8	1.2
	2	5.7	6.2	1.9
	4	4.4	7.3	3.1
	7	3.6	4.6	1.0
	14	5.6	5.5	1.1
	21	4.9	5.1	1.5
	28	3.9	4.1	0.9
CORT sealed	0	2.3	2.5	0.5
	2	3.9	4.5	1.3
	4	3.6	3.9	0.7
	7	2.8	3.3	0.8
	14	2.6	2.6	0.4
	21	3.1	3.3	0.6
	28	2.7	3.3	0.6
CORT holes	0	5.5	9.1	3.3
	2	11.1	29.8	13.9
	4	6.1	13.2	6.8
	7	6.3	11.1	4.2
	14	10.2	10.7	3.1
	21	11.7	15.0	5.8
	28	11.7	11.9	3.6

Table 1-3. Plasma total stress-induced corticosterone concentrations ([CORT]) at various time points after treatment in captive adult male painted turtles (*Chrysemys picta*) in Experiment 1B. Median, mean, and standard error (SE) are presented. Individuals were randomly assigned to one of four treatment groups (n = 7 in each group): no implant (Control), one empty Silastic implant (Sham), one sealed CORT-filled Silastic implant (CORT sealed), or one perforated CORT-filled Silastic implant (CORT holes).

Treatment group	Days post-implant	Stress-induced [CORT] (ng/mL)		
		Median	Mean	SE
Control	0	7.0	15.1	5.6
	7	5.2	14.1	6.9
	28	5.1	22.9	11.0
Sham	0	5.5	15.7	6.6
	7	14.8	23.9	9.3
	28	15.7	20.7	6.5
CORT sealed	0	7.7	10.1	3.0
	7	6.1	6.4	1.1
	28	6.3	7.3	1.3
CORT holes	0	11.7	25.1	8.6
	7	13.8	22.5	6.9
	28	11.2	23.4	10.8

Table 1-4. Plasma total baseline corticosterone concentrations ([CORT]) for various time intervals after treatment in free-living adult painted turtles (*Chrysemys picta*) in Experiment 2. Median, mean, standard error (SE), and sample size (*n*) are presented. Individuals were randomly assigned to one of three treatment groups: without injection (Control), injected with cocoa butter only (Sham), or injected with CORT-laden cocoa butter (CORT). Only the first sample was included for individuals that were sampled twice within the same time interval ($n = 6$).

Treatment group	Days post-implant	Baseline [CORT] (ng/mL)			*n*
		Median	Mean	SE	
Control	0	0.7	0.7	0.2	4
	1 – 4	0.8	0.9	0.2	3
	5 – 10	0.3	0.5	0.2	3
	11 – 20	0.9	0.9	0.6	2
	21 – 30	0.6	0.8	0.3	3
	31 – 40	-	-	-	0
	41 – 50	-	-	-	0
	51 – 60	0.5	0.5	0.2	2
Sham	0	0.4	0.6	0.2	5
	1 – 4	0.3	0.3	-	1
	5 – 10	0.3	0.3	-	1
	11 – 20	-	-	-	0
	21 – 30	0.3	0.8	0.5	4
	31 – 40	1.0	1.0	-	1
	41 – 50	-	-	-	0
	51 – 60	-	-	-	0
CORT	0	0.7	1.3	0.4	13
	1 – 4	86.7	75.0	19.2	6
	5 – 10	27.2	35.7	8.6	14
	11 – 20	27.8	33.2	8.2	8
	21 – 30	8.4	8.4	6.5	2
	31 – 40	3.6	4.4	1.8	4
	41 – 50	1.6	1.6	0.1	2
	51 – 60	3.0	2.8	0.9	4

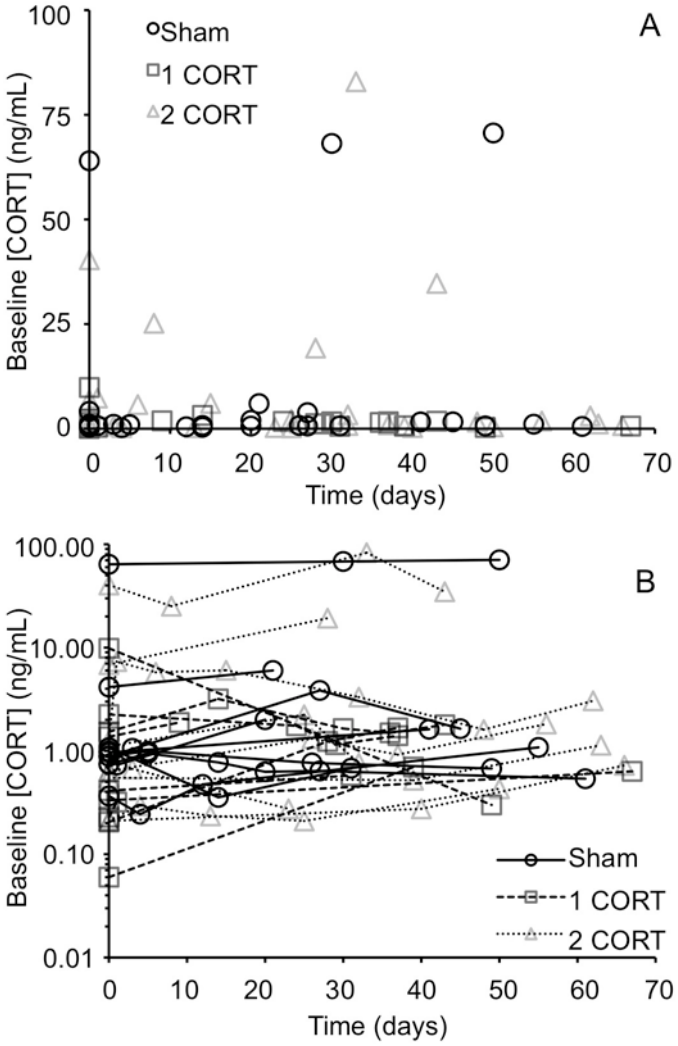


Figure 1-1. Plasma total baseline corticosterone concentrations ([CORT]) as a function of time since treatment in free-living adult painted turtles (*Chrysemys picta*) (90 data points from 28 individuals) in Experiment 1A. Individuals were randomly assigned to one of three treatment groups: two empty Silastic implants (Sham, $n = 10$), one sealed CORT-filled Silastic implant and one empty implant (1 CORT, $n = 9$), or two sealed CORT-filled Silastic implants (2 CORT, $n = 9$). (A) [CORT] values are presented on a linear scale. (B) Individual profiles over time. [CORT] values are presented on a logarithmic scale to allow better visualization of each profile.

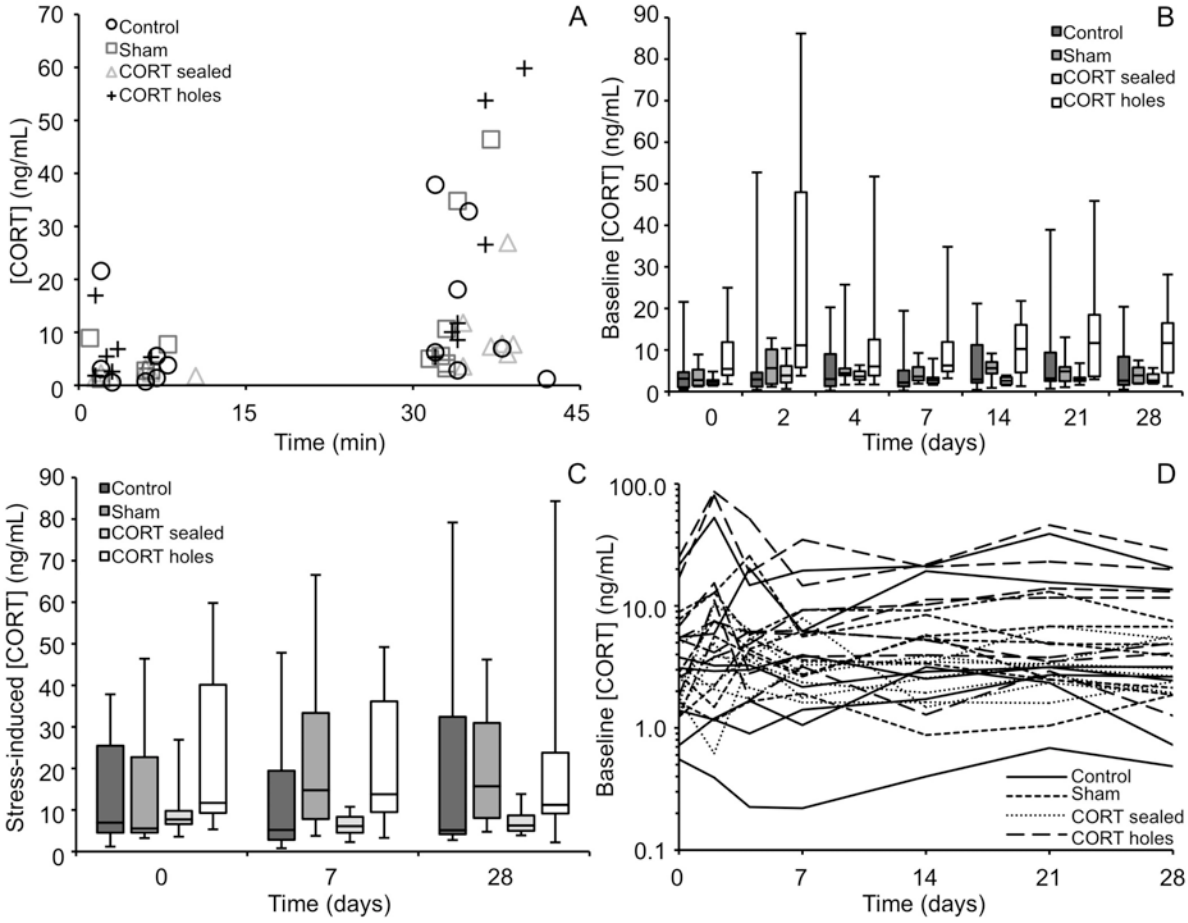


Figure 1-2. Plasma total corticosterone concentrations ([CORT]) from captive adult male painted turtles (*Chrysemys picta*) in Experiment 1B. Individuals were randomly assigned to one of four treatment groups (n = 7 for each group): no implant (Control), one empty Silastic implant (Sham), one sealed CORT-filled Silastic implant (CORT sealed), or one perforated CORT-filled Silastic implant (CORT holes). Baseline samples were collected as quickly as possible after taking the turtle out of its tank, while stress-induced samples were collected following a 30-min restraint. (A) Baseline (scatter on left) and stress-induced (scatter on right) [CORT] on day 0, prior to treatment, as a function of time since first handling the animal (n = 28 pairs). (B) Baseline [CORT] as a function of time since implantation. (C) Stress-induced [CORT] as a function of time since implantation. (D) Individual profiles of baseline [CORT] over time. [CORT] values are presented on a logarithmic scale to allow better visualization of each profile.

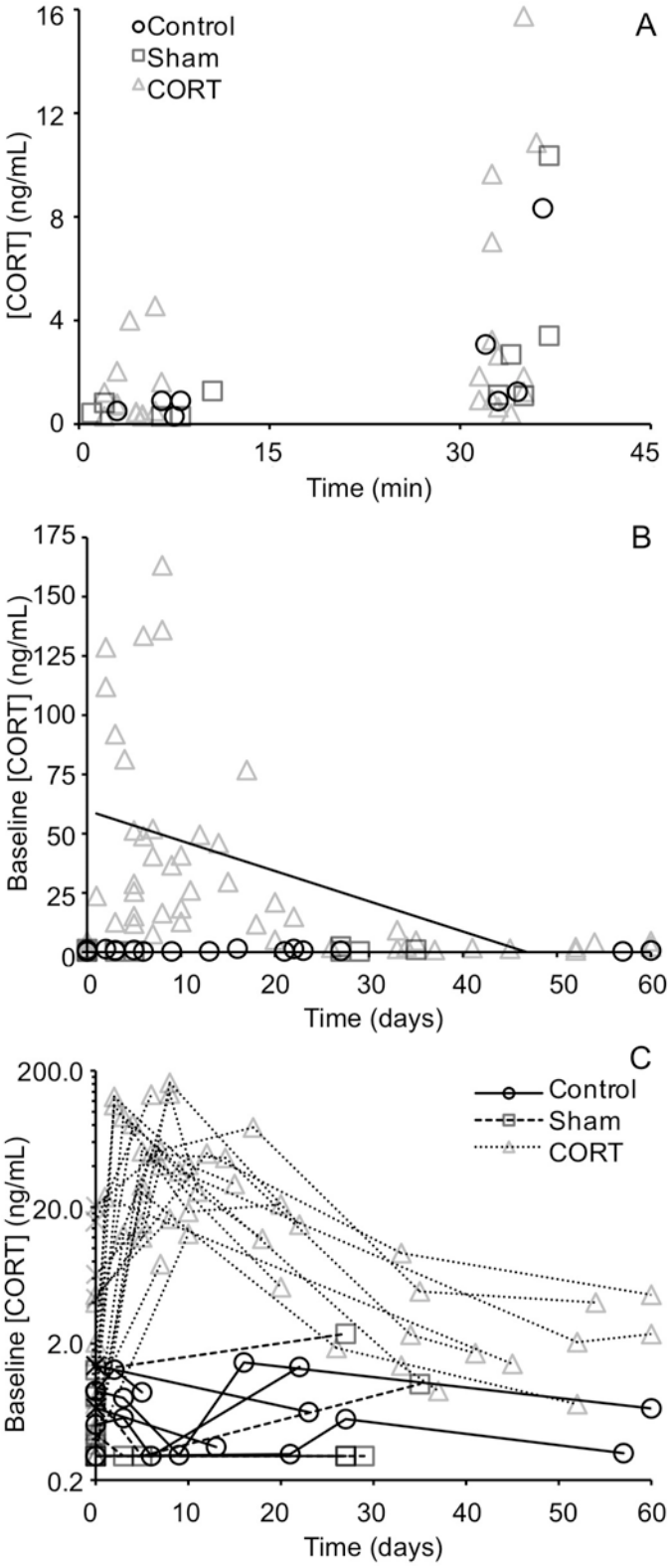

A
Control
Sham
CORT
[CORT] (ng/mL)
16
12
8
4
0
0
15
30
45
Time (min)
B
Baseline [CORT] (ng/mL)
175
150
125
100
75
50
25
0
0
10
20
30
40
50
60
Time (days)
C
Control
Sham
CORT
Baseline [CORT] (ng/mL)
200.0
20.0
2.0
0.2
0
10
20
30
40
50
60
Time (days)

Figure 1-3. Plasma total corticosterone concentrations ([CORT]) from free-living adult painted turtles (*Chrysemys picta*) in Experiment 2. Individuals were randomly assigned to one of three treatment groups: without injection (Control), injected with cocoa butter only (Sham), or injected with CORT-laden cocoa butter (CORT). Baseline samples were collected as quickly as possible after disturbing the turtle, while stress-induced samples were collected following a 30-min restraint. (A) Baseline (scatter on left) and stress-induced (scatter on right) [CORT] on day 0, prior to treatment, as a function of time since first handling the animal (n = 22 pairs). (B) Baseline [CORT] as a function of time since treatment (88 data points from 28 individuals: 6 controls, 5 sham-treated, and 17 CORT-treated). The solid line shows the significant linear regression calculated after day 0 for the CORT-treated group. Regressions were not significant for the other groups. (C) Individual profiles of baseline [CORT] over time. [CORT] values are presented on a logarithmic scale to allow better visualization of each profile. Six baseline samples (n = 2 Control and 4 CORT) collected after 10 min (11-21 min) on day 0 were excluded from other analyses and figures, but are shown (×) to allow presentation of all profiles and still demonstrate increase due to CORT treatment.

CHAPTER 2

Chronic corticosterone elevation does not affect stress response, parasitaemia, heterophil-to-lymphocyte ratios, leukocyte counts, and body condition in free-living painted turtles (*Chrysemys picta*)

This chapter formed the basis for the following publication:

Juneau, V., Gilmour, K.M., and Blouin-Demers, G. Chronic corticosterone elevation does not affect stress response, parasitaemia, heterophil-to-lymphocyte ratios, leukocyte counts, and body condition in free-living painted turtles (*Chrysemys picta*). In review.

ABSTRACT

Chronic stress can result in an elevation of circulating levels of glucocorticoid (GC) hormones in vertebrates, which can affect their stress response, immune function, and fitness. We attempted to isolate the effect of chronic corticosterone (CORT) elevation (~3 weeks) on the acute stress responsiveness, immune function, and body condition of free-living painted turtles (*Chrysemys picta*), using cocoa butter injections of CORT. At 1 week and 3 weeks post-implantation, we measured the acute CORT response following a standardized restraint protocol, parasitaemia, heterophil-to-lymphocyte (H:L) ratios, and total leukocyte (WBC) counts in treated, sham and control turtles. We determined body condition 1 week, 3 weeks, and 1 year after treatment. We did not observe an effect of treatment on hormonal (stress response), immune (parasitaemia, H:L ratios, WBC counts), or body condition metrics of stress. The general lack of effect we observed may be because CORT mediates resource allocation only in the presence of additional immune or energy challenges, or because of the masking effect of several extrinsic factors. Future studies should focus on the effects of CORT in animals facing additional immune or energetic challenges, and include measurements of corticosteroid-binding globulins (CBG) because free, not total, CORT appears to be biologically active.

INTRODUCTION

Vertebrates facing a stressor secrete hormones that aid them survive difficult situations. One essential component of the vertebrate stress response is the activation of the hypothalamic-pituitary-adrenal (HPA) axis, culminating in the release of glucocorticoids (GCs; e.g. corticosterone, CORT, in reptiles) several minutes after the onset of a stressful stimulus (Sapolsky et al., 2000). Various behavioural and physiological effects are induced by GCs: mediation of the cardiovascular stress response, increase in circulating glucose concentrations and feeding behaviour, suppression of immunological and inflammatory responses, and of reproductive behaviour (reviewed in Sapolsky et al., 2000), promoting short-term survival of the organism. Although the effects of an acute stressor are usually short-lived, body condition, growth, immunity, and reproduction can be adversely affected in the long term by prolonged high levels of GCs (Dickens and Romero, 2013; Guillette et al., 1995; Norris, 2007; Sapolsky et al., 2000). Ultimately, overall fitness of organisms and, eventually, their population dynamics can be negatively impacted by chronic stressors (Bonier et al., 2009; Crespi et al., 2013). Boonstra (2013) posited that wild animals will evolve to be chronically stressed if it is adaptive and, if it is not, they will only respond acutely.

Chronic stress, baseline CORT levels, and acute stress responsiveness

Cyr and Romero (2009) defined "chronic stress" as multiple, frequent exposure and/or long-term constant exposure to stressors. Although there is some debate around the term, we retained their definition for the purpose of the present study. Examples of chronic stressors include enduring bad weather and food shortages, social interactions, habitat

disturbance, and human activities. Another definition of chronic stress in use until recently was long-term allostatic overload, throughout which GC levels stay over baseline (i.e., within the range typically associated with acute stress, with type I and II GC receptors bound; Bonier et al., 2009). Johnstone and colleagues (2012b) stated that an animal can recover from chronic stress, which they defined as a state of elevated stress that is prolonged or severe enough that it is fitness reducing (i.e. prolonged distress). Boonstra (2013) suggested that a stressor should be defined as acute or chronic depending on the duration of its consequences on the physiology of the animal, and not of the stressor itself.

Chronic or frequent and repeated stressors can lead to elevated circulating baseline CORT levels in free-living animals (Romero, 2004; Sapolsky et al., 2000). Down-regulation of the stress response to acute stressors has also been associated with exposure to chronic stressors in a number of field studies (Romero, 2004). For example, Homan and colleagues (2003) found that spotted salamanders (*Ambystoma maculatum*) from a disturbed breeding pond (partly surrounded by a recent housing development) had a lower stress responsiveness to capture and handling, compared to individuals in an undisturbed pond. Owen and colleagues (2014) found that free-ranging copperhead snakes (*Agkistrodon contortrix*) captured while in contact with public roads exhibited a lower stress response compared to snakes captured within the forest interior, although they did not differ in baseline CORT levels.

Patterns of GC secretion in wild animals are not well known. The effect of chronic stress on wild animals has been increasingly investigated, but many studies have provided unexpected conclusions. Recently, a review concluded that chronically stressed wild animals do not automatically show a consistent, predictable, endocrine response to chronic stress; the

direction of the changes seems to be less important than the fact that the response changes at all (Dickens and Romero, 2013). Johnstone and colleagues (2012b) suggested measuring multiple stress indicators, such as hormone and leukocyte levels, to reduce the risk of potential confounds, such as trapping stress, in the interpretation of indices of physiological stress. It is typically expected that a higher level of physiological stress should be associated with poorer body condition and higher parasite loads, so measuring other condition indices such as those can supplement the stress data.

Leukocyte profiles as an indicator of stress

Leukocyte profiles, composed of the proportions of each white blood cell (WBC) types, are highly related to GC levels in all vertebrate taxa, in response to either natural stressors or exogenous administration of stress hormones (reviewed by Davis et al., 2008). GCs induce alterations in the redistribution of lymphocytes from the blood to other body compartments, and stimulate an influx of neutrophils (heterophils in birds and reptiles; the primary phagocytic leukocyte) from the bone marrow to the blood. This increases the number and proportion of neutrophils, and decreases lymphocyte numbers (involved in a variety of immunological functions). Therefore, the relative proportion of neutrophils/heterophils to lymphocytes, called the N:L or H:L ratio, can be considered as a composite measure of the stress response, and a high ratio is thought to indicate high GC levels. This approach offers advantages over direct GC measurement, as the initial leukocyte response time varies from hours to days, which allows more time for sampling than for baseline GC levels. This technique is also relatively inexpensive, and requires only a very small blood sample, allowing its use in small animals. Using this tool, Selman and colleagues (2013) found that yellow-blotched sawbacks (*Graptemys flavimaculata*), an imperiled

freshwater turtle, had significantly higher H:L levels at a site disturbed by recreational boating compared to an undisturbed site. Similarly, Johnstone and colleagues (2012a) demonstrated that agile antechinus (*Antechinus agilis*) living in forest fragments had elevated N:L ratios, and experienced greater chronic stress and poorer condition than conspecifics inhabiting unfragmented forest.

Immune function during chronic stress

GCs are generally immunosuppressive under chronic conditions (Sapolsky et al., 2000). The most general effect of GCs is to inhibit the synthesis, the release or the efficacy of cytokines and other mediators promoting immune and inflammatory reactions. GCs also lower circulating levels of most WBC types.

Few studies have documented the physiological effect of chronically elevated levels of CORT on immunosuppression in wild animals. Berger and colleagues (2005), however, directly tested CORT's immunosuppressive role in wild Galápagos marine iguanas (*Amblyrhynchus cristatus*) by either restraining animals or injecting CORT in reproductive males. They found that such experimental elevation of CORT significantly decreased immune activity, measured using the phytohemagglutinin swelling-response (PHA) test, which shows that CORT can induce immunosuppression.

Important physiological and behavioural processes of hosts are known to be strongly impacted by parasites. For example, the fitness of lizards is affected by hemoparasites, which decrease hemoglobin levels, resting oxygen consumption, running speed, and cell regeneration (Oppliger et al., 1996; Oppliger and Clobert, 1997). Due to the heavy demands of maintaining immune function, immunosuppression can happen if energy is rerouted from immune defence to deal with a stressful situation. Therefore, stressed individuals are more

likely to become infected by parasites (Lafferty and Holt, 2003). For example, stress factors (crowding, limited refuges and food resources, simulated predation) had a notable effect on both natural and experimentally-induced parasitaemia in common lizards (*Lacerta vivipara*) (Oppliger et al., 1998). Moreover, increased physiological stress was associated with suppressed immunity (measured as bactericidal ability and cutaneous wound healing) in common side-blotched lizards (*Uta stansburiana*) experiencing variable levels of environmental stress (Lucas and French, 2012).

Objectives

In the present context, the manipulation of circulating levels of GC hormones has been proposed as a potential approach to determine the consequences of elevated GCs on the organisms, for instance on stress response, immune function, reproduction, or survival. CORT implantation, although unable to mimic stress perfectly, does provide an effective tool to look into a main component of the stress response, i.e. the increase in circulating GCs (Denardo and Licht, 1993). Manipulating CORT directly eliminates the perceptual component of the stress response, making stress-related questions simpler to evaluate (Denardo and Sinervo, 1994a, b). Therefore, confounding effects of the actual stressor (e.g., habituation) or other related factors (e.g., parasite density or habitat variations) can be separated from the effect of stress on various fitness indicators, immunity, or reproductive success.

In this study, our objective was to investigate the effect of chronic CORT elevation (as observed in some situations of chronic stress) on the acute stress responsiveness, immune function, and body condition of free-living painted turtles (*Chrysemys picta*). For the first time in an ectothermic tetrapod, we used cocoa butter injections of CORT (which we

previously validated in Chapter 1) to elevate CORT levels chronically for approximately 3 weeks. At 1 week and 3 weeks post-implantation, we measured the acute CORT stress response following a standardized restraint protocol, parasitaemia, H:L ratios, and total WBC counts in treated, sham and control turtles. We also determined body condition 1 week, 3 weeks, and 1 year after treatment.

Hypotheses and predictions

(1) If chronically high CORT levels act to down-regulate CORT release following an acute stressor, which results in an impaired acute stress responsiveness, then we predict that turtles implanted with slow-release CORT will exhibit a decrease in the magnitude of their CORT response to a stressor (i.e. difference between stress-induced and baseline levels), compared to sham-treated and control individuals.

(2) If chronically high CORT levels suppress immune function by affecting mediators of immune and inflammatory reactions, then we predict that turtles implanted with slow-release CORT will have a higher intensity of infection (parasitaemia) by the hemoparasites *Haemogregarina balli* and *Trypanosoma chrysemydis*, compared to sham-treated and control individuals.

(3) If chronically high CORT levels cause an increase in heterophils and a decrease in lymphocytes, resulting in a higher H:L ratio, then we predict that turtles implanted with slow-release CORT will show an increase in the H:L ratio, compared to sham-treated and control individuals.

(4) If chronically high CORT levels act to decrease circulating levels of most leukocytes (white blood cells, WBCs), then we predict that turtles implanted with slow-release

CORT will show a decrease in total WBC counts, compared to sham-treated and control individuals.

(5) If chronically high CORT levels induce lipolysis, then we predict that turtles implanted with slow-release CORT will show a decrease in body condition, compared to sham-treated and control individuals.

MATERIALS AND METHODS

Study species, study site, and animal processing

We used the painted turtle as our study species because it is the most abundant local turtle (pers. obs.), as well as the most studied and most widely distributed turtles in North America (Ernst and Lovich, 2009). During the summer of 2011, we captured 115 adult turtles (63 males and 52 females; mean body mass = 353 g, range: 130-890 g; mean plastron length = 129 mm, range: 88-180 mm) in Lake Renaud, a small lake located in Gatineau Park (45°36'10''N, 76°01'24''W), Québec, Canada, using a dip net, hoop nets (with air space) baited with sardines, opportunistically by hand from a canoe, or on land. At their first capture (day 0), we marked turtles semi-permanently by notching with a file one to four of their marginal scutes in unique combinations. We also painted a unique number on their carapace, allowing the identification of individuals more easily for subsequent blood sampling. We recaptured turtles after approximately 1 week (n = 24, 18 males and 6 females; range: 5-10 days; median = 7 days, 6.5 days for males and 8 days for females), 3 weeks (n = 53, 36 males and 17 females; range: 15-36 days; median = 23 days, 27 days for males and 20 days for females) and 1 year (n = 103, 56 males and 47 females; range: 260-422 days; median = 338 days, 340 days for males and 338 days for females). We chose the 3-week time period

because we were interested in the effect of chronic stress and we found previously that our implants keep circulating CORT levels elevated for up to 3 weeks (see Chapter 1). We included the 1-week time period to determine whether CORT elevation has a shorter-term effect on the variables of interest. Finally, we included the 1-year time period to determine whether a ~3-week CORT elevation has a long-lasting effect on body condition (data for the other variables were not available). We chose to include the particular ranges of days as a trade-off between the precision in our time frame and increased sample size. On each sampling occasion (day 0, and after 1 week, 3 weeks, 1 year), we weighed and measured the turtle, determined its sex using morphological characteristics, and released it at its capture location.

Blood sampling

On day 0 (before the cocoa butter injection), then after 1 week and 3 weeks, we collected a baseline blood sample. We used the moment at which the turtle first noticed the presence of the capturer as the starting time (t = 0 min). Immediately after capture, and no later than t = 10 min (Cash et al., 1997; see also Chapter 1) (mean = 6.0 min, SD = 3.5 min, n = 21), we collected a first blood sample (300–500 μL) from the coccygeal vein (Bulté et al., 2006) using pre-heparinized syringes (0.5 cc with 28-gauge needle). We used a drop from this sample to make a thin blood smear on a microscope slide, that we allowed to air-dry in the field (Bennett, 1970). We aimed at collecting a stress-induced sample after 30 min (mean = 34.2 min, SD = 2.0 min, n = 21) of restraint in a plastic bin, to determine whether CORT treatment reduced stress responsiveness. We stored blood samples in an ice slurry for up to 8 h until we centrifuged them to collect plasma. We snap-froze plasma samples on dry ice then placed them in a freezer at −80°C.

Cocoa butter implants

We dissolved crystalline CORT (C2505, Sigma Chemicals) in liquid pure cocoa butter (10 mg CORT / 1 mL butter). We kept this mixture in a warm water bath and injected it epicoelomically (near the pectoral muscle) into CORT-treated individuals (n = 43, 19 males and 24 females) using 1-mL syringe and 18-gauge, 38-mm-long needles, at a dose of 10 mg CORT in 1 mL cocoa butter per 1 kg body mass, then we sealed the injection site with surgical glue. We previously demonstrated that this method successfully elevates circulating CORT levels in painted turtles for up to 3 weeks (see Chapter 1). Sham-treated individuals (n = 12 males) received an injection of cocoa butter only, while controls (n = 60, 32 males and 28 females) received no injection. Some individuals (n = 6 males) received a second CORT injection in July, approximately 2 months (mean = 59 days, SD = 9 days, range: 50-71 days) after their first CORT injection in May, to mimic longer-term stress. Please note that we only provide the number of individuals that contributed data. Because this experiment was conducted in the field, many more individuals were captured, but never recovered. Opportunistically, in the field, we successfully recaptured 24 turtles after 1 week (4 controls (4 M), 1 sham (1 M), 16 CORT (10 M and 6 F) and 3 CORT-2 (3 M)), 53 turtles after 3 weeks (20 controls (11 M and 9 F), 9 shams (9 M), 19 CORT (11 M and 8 F) and 5 CORT-2 (5 M)), and 103 turtles after 1 year (56 controls (30 M and 26 F), 11 shams (11 M), 31 CORT (10 M and 21 F) and 5 CORT-2 (5 M)).

Blood smear preparation and microscope analyses

We fixed and stained blood smears using SureStain™ Wright-Giemsa in methanol (Fisher Scientific). We dipped slides in the stain for 15 seconds, then in distilled water for 30 seconds; we then rinsed them by dipping the slides in distilled water for a few seconds, then

air-dried the slides. We observed slides using bright field microscopy under a total magnification of 400x, using an Olympus CX41 light microscope. Leeches are one of the most commonly observed parasites of freshwater turtles (Readel et al., 2008). Hematophagus leeches are vectors for hemoparasites such as the coccidian parasites *Haemogregarina* spp. and the hemoflagellates *Trypanosoma* spp. (Mann, 1962; Siddall and Desser, 1991, 2001) and can transmit these parasites between turtles, both intra- and interspecifically (Siddall and Desser, 2001). Therefore, we determined parasitaemia by measuring the number of hemoparasites (*Haemogregarina balli* and *Trypanosoma chrysemydis*, Fig. 2-1) in 10000 erythrocytes (red blood cells, RBCs). We determined the ratio of heterophils to lymphocytes (H:L) by counting their numbers in 100 leukocytes (white blood cells, WBCs). We determined total WBC count by counting the number of WBCs in 4000 RBCs. We identified parasites by comparison with published descriptions (Desser, 1973; Paterson and Desser, 1976; Siddall and Desser, 1991, 1992a, 2001) and WBC types according to Campbell (2004). Two observers, who were blind to treatment, performed all parasite and WBC identification.

Body condition index

To determine body condition for our turtles, we used data from the first capture of each individual between 2009-2012 to determine the equation of linear regression of log body mass vs. log plastron length (PL), separately for males (log mass = -3.23 + 2.73 log PL, $R^2 = 0.95$, $P < 0.0001$, $n = 113$) and females (log mass = -2.92 + 2.59 log PL, $R^2 = 0.96$, $P < 0.0001$, $n = 96$). We used those equations to calculate the predicted body mass from the measured PL of each of our turtles, and then used the following equation to determine the body condition index (BCI) for each turtle at every capture:

$$BCI = [(\text{measured} - \text{predicted mass (g)}) / \text{predicted mass (g)}] + 1$$

Corticosterone assays

In a subset of samples, we determined total CORT concentrations using a competitive enzyme-linked immunosorbent assay (ELISA) (sensitivity 27 pg/mL, Cat. No. 900-097, Assay Designs Inc.). We used the kit according to the manufacturer's instructions. We diluted our samples 5 to 50 times in the provided assay buffer to obtain concentrations within the range of the standard curve. To a known volume of plasma, we added the same volume of the provided steroid displacement reagent (previously diluted 1:100 in assay buffer), let sit 10 min, then added assay buffer to reach the desired dilution. We used SoftMax Pro 4.0 (Molecular Devices) to calculate the concentration of CORT in the samples from the optical density data. Using serial dilutions of turtle plasma samples, we obtained curves that were parallel to standard curves.

We ran all standard curves in triplicate (mean C.V. = 3.3%) and all samples in duplicate (mean C.V. = 11.2%). We determined an intra-assay coefficient of variation of 10.9% by running a randomly chosen sample in two sets of duplicates on a given plate, and an inter-assay variation of 30.0% by running duplicated aliquots from a pool of samples on each plate, and of 16.7% by running duplicates of the same samples on different plates. We kept all samples from a given individual on the same plate, and we had individuals of each treatment group on each plate that we ran. This sample randomization mitigated the potential effect of the high inter-assay variation on our data.

Statistical analyses

We conducted all statistical analyses using the statistical software JMP (version 8, SAS), and used G*Power 3.1.9.2 for power analysis (Faul et al., 2007) . We verified assumptions of normality with Shapiro-Wilk's test and homogeneity of variances with

Levene's test. We calculated the CORT response for each individual at each time period as stress-induced [CORT] / baseline [CORT] on the same day x 100. Given our modest sample sizes, to control for inter-individual variation we calculated the difference in stress response, parasitaemia, H:L ratio, WBC count, and BCI for each individual between day 0 and our time periods of interest (1 week, 3 weeks, and 1 year), and used these differences as our dependent variables in the models below. To determine whether CORT treatment affected those 5 variables, we used a separate generalized linear model for each variable at each time period, with Y = *Treatment group* + *Date on day 0* + *Days post-implant* + *Sex*. We included the last three parameters as covariates to account for their potential effects on the dependent variable. *Treatment group* was Control, Sham (both combined in Ctrl-sham in further analyses, see below), CORT or CORT-2. Day 0 represented the first capture of each individual. We expressed the *Date on day 0* for each individual as a number where 1 corresponds to May 1 (1-week range: day 10-75, median = 18; 3-week range: day 10-82, median = 32; 1-year range: day 10-115, median = 45). *Days post-implant* is the exact number of days between day 0 and our time periods (1-week range: 5-10 days; 3-week range: 15-36 days; 1-year range: 260-422 days). *Sex* is male or female. At 1 week, data were available for 4-6 females for each variable, but all in the CORT group, therefore females were excluded from the analyses. At 3 weeks, stress response data were available for only 2 females, both in the CORT group; therefore these 2 females were excluded from the analyses. We show those excluded female data in Appendix (Fig. A-1).

We first ran the models only with the Control and Sham groups and found no effect of Treatment group (see Appendix, Table A-1); therefore we combined Control and Sham individuals together for further analyses. We also analysed these data using univariate tests,

i.e. ANOVAs testing the effect of *Treatment* for each variable at each time period (except Welch's ANOVAs allowing unequal variances where specified in the table captions), to make sure the results did not differ. As with the full models, we found no difference between the Control and Sham groups (see Appendix, Table A-2); therefore we combined them for further univariate analyses. We then compared the Control-Sham and CORT groups (see Appendix, Table A-3). Conclusions remained the same as with the full models, therefore we report only the full models in the results section.

We repeated the same analyses including the CORT-2 group, therefore comparing the Control-Sham, CORT, and CORT-2 groups, using both the full models and univariate tests. The CORT-2 group had a small sample size (n = 2-5, depending on time periods and variables), and the results remained the same as when testing CORT only so we present them in the Appendix (Tables A-4 and A-5, Fig. A-1).

We tested only one variable (BCI) at 1 year, therefore we used an alpha level of 0.05 and we considered an effect to be significant if $P < 0.05$. Since we tested multiple variables for the 1-week and 3-week time periods, we applied a Bonferroni correction to the alpha level of 0.05 by dividing it by the number of variables tested (5), and we considered an effect to be significant if $P < 0.01$.

RESULTS

Data from variables measured on day 0 are summarized in Table 2-1. In addition to the five variables of interest (stress response, parasitaemia, H:L ratio, WBC count and body condition index (BCI)), we also present data for baseline and stress-induced [CORT], as well as plastron length and body mass, as we used those variables to calculate the CORT stress response and body condition index. We also included data for each hemoparasite species of

interest. The meront and/or gametocyte stages of *Haemogregarina balli* (Fig. 2-1A and B) were observed in parasitized individuals, while *Trypanosoma chrysemydis* (Fig. 2-1C) was observed only once, in a CORT-treated male after 1 week.

Figure 2-2 shows the effects of CORT implantation on painted turtles on the five variables of interest. For stress response, we observed no effect after 1 week, but a small difference between the groups after 3 weeks, as CORT-treated turtles showed no difference from day 0, while Control-sham turtles showed an increased stress response (Fig. 2-2A), but this effect was not statistically significant (Table 2-2). For parasitaemia, the difference from day 0 after 1 week and 3 weeks was 0 parasite / 10000 RBCs for both groups (Fig. 2-2B). For H:L ratio, the difference from day 0 was also around zero for both groups and both time periods (Fig. 2-2C). For WBC count, we observed no effect of group on the difference from day 0 (Fig. 2-2D). Lastly, for BCI, although we observed large inter-individual variation in some groups for all time periods (1 week, 3 weeks and 1 year), the median difference from day 0 remained around zero and there was no effect of treatment (Fig. 2-2E). Overall, we observed some inter-individual variation, but not much difference from day 0, and no effect of CORT treatment after 1 week, 3 weeks or 1 year. Figure 2-2 also shows no effect of sex on the five variables.

These observations are confirmed by the statistical results from our full model analyses, comparing the Control-sham and CORT treatment groups (Table 2-2). For all variables and all time periods, the *Treatment* effect had low R^2 and high P values (except 3-week stress response, with a higher R^2 of 0.39 and small, but non significant, P value). We tested the effect of *Sex* on parasitaemia, H:L ratio, WBC count, and BCI after 3 weeks, and on BCI after 1 year, and we similarly found very small R^2 and high P values. Also, we found

that the effects of *Date on day 0* and *Days post-implant* were mostly very small and always not significant. The non-significant *P* values and low R^2 values for *Date on day 0*, *Days post-implant*, and *Sex* indicate that these variables had no significant effect on the five dependent variables of interest.

DISCUSSION

Patterns of variation pre-treatment

Baseline and stress-induced CORT values measured on day 0 (Table 2-1) were similar to those previously observed in other painted turtles (Keiver et al., 1992) and slightly lower than those observed in red-eared sliders (Cash et al., 1997). We observed large inter-individual variation in CORT levels and stress responses, as shown by large ranges and standard deviations in Table 2-1, but a recent review indicated that individual differences in stress responses were found to be extensive in all vertebrate taxa (Cockrem, 2013). It is possible that the normal inter-individual variation observed here is more extensive than a potential effect of CORT, which therefore could not be detected. Combined with limited power due to our modest sample size for this variable, individual differences in the stress-response system could obscure the small non-significant difference observed at 3 weeks.

Mean and median infection intensities observed in this study (Table 2-1) were quite low, but comparable with those of other studies that assessed blood parasitism in painted turtles. Strohlein and Christensen (1984) reported an intensity of 0.05% (5 in 10000) infected erythrocytes in Murphy's Pond, Kentucky. Siddall and Desser (1992b) found that infections exceeding 1 in 10000 erythrocytes were rare in painted turtles from Wolf Howl Pond, in Algonquin Park, Ontario. The leeches *Placobdella parasitica* and *Placobdella ornata* are the

invertebrate hosts for *Haemogregarina balli* and *Trypanosoma chrysemydis,* as well as the vectors to the chelonian vertebrate hosts (Siddall and Desser, 1992a, b, 2001). Throughout the duration of the study, prevalence and intensity of leech parasitism was generally low (V. Juneau, pers. obs.), which could explain the low intensity of infection by *H. balli* and *T. chrysemydis*. Low intensities of parasite infection made detecting an effect of CORT unlikely.

The mean and median H:L values measured in our painted turtles on day 0 differ from mean values measured in western pond turtles (*Emys marmorata*) from natural (~0.37) and altered (~0.74) habitats (Polo-Cavia et al., 2010), as well as from yellow-blotched sawback turtles (*Graptemys flavimaculata*) at sites disturbed by recreational boating (0.32 for males, 0.31 for females) or not disturbed (0.25 for males, 0.19 for females) (Selman et al., 2013). Our data, however, show large inter-individual variation so our range of observed values encompasses the values in these two other studies.

The low R^2 and high P values that we obtained from our models (Table 2-2) indicate that CORT implantation did not affect the CORT stress response, parasitaemia, H:L ratios, and total WBC counts after 1 week and 3 weeks, nor body condition after 1 week, 3 weeks, and 1 year. Since we previously demonstrated that our CORT treatment elevates circulating CORT levels for up to 3 weeks in painted turtles, at concentrations that were likely physiologically and ecologically relevant for the species (see Chapter 1), the lack of an effect of treatment on hormonal (stress response), immune (parasitaemia, H:L ratios, WBC counts), and body condition metrics of stress was unexpected.

We had low power to detect the effect sizes we observed as being statistically significant, with power values ranging from 0.05 to 0.48, depending on the variables and

time periods (Table 2-2). This is not very informative if the effect sizes we observed are small and not biologically significant. Therefore, we calculated a biologically relevant effect size where possible (using our observed mean and standard deviation values) and determined the power we had to detect it as being statistically significant, given our sample size. For stress response, we had low power (0.16 after 1 week and 0.26 after 3 weeks) to detect a 50% difference between the Control-sham and CORT-treated groups (which would be biologically relevant, but is still smaller than the non-significant effect we observed in our data). Due to the high inter-individual variation we observed, it appears that a larger sample size would be required to properly test our hypothesis for this variable (total $n = 94$ after 1 week and $n = 68$ after 3 weeks, for a power of 0.80). For parasitaemia, although we observed very small effect sizes, we would have been able to detect an increase of 8 or more parasites in 10000 RBCs after 1 week (with a power of 0.88), and of as small as 2 or more parasites in 10000 RBCs after 3 weeks of treatment (with a power of 0.81) as being statistically significant. Such parasite loads are still small; therefore we are confident that our CORT treatment did not affect parasitaemia in an ecologically relevant way. We did not perform such power analyses for H:L ratios or WBC counts because relevant data are too limited to determine what a biologically relevant effect should be. Lastly, after 1 week, and despite our small sample size, we had a power of 0.83 to detect a difference of 15% in body condition, but only 0.49 for 10% and 0.16 for 5%, the latter being more likely to occur in such a short time period. Due to our larger sample sizes, however, we had a power of 0.78 and 0.99 to detect differences in body condition of only 5% and 10%, respectively, between the groups after 3 weeks, and of 0.88 for 5% and 0.99 for 10% after 1 year. We are therefore confident that our CORT treatment did not impact body condition in a biologically relevant way.

Below we discuss recent evidence that cast doubts on the typical predictions in stress studies, the role of CORT as a mediator of resource allocation in the presence of additional immune or energy challenges, the potential role of extrinsic factors, and the importance of measuring corticosteroid-binding globulin (CBG) and free vs. total CORT.

Lack of support for the predictions

Other studies have found no effect of CORT or chronic stress on their variables of interest, or results contrary to initial predictions. For example, in a recent review of the effects of chronic stress on GC function and body weight in wild animals, Dickens and Romero (2013) concluded that, contrary to the general assumption that chronic stress increases measures of GC function, there is no consistent, predictable, endocrine response to chronic stress; the fact that the response changes at all is more important than the direction of the changes. They, however, found that body weight decrease was a consistent response to chronic stress, as most reviewed studies showed a decrease, few showed no change, and none showed an increase (n = 65). Our lack of response on body condition therefore remains contrary to general expectations.

Some studies have reported that H:L ratios are not always correlated to CORT. For example, in male southeastern five-lined skinks (*Plestiodon inexpectatus*), a 1-hr confinement stress resulted in elevated H:L ratios and elevated CORT concentrations, and CORT was positively correlated with the H:L ratio. A confinement of 2 hours, however, did not affect the H:L ratio, although CORT was maximal (Seddon and Klukowski, 2012). In light of these results, the authors concluded that, in reptiles at least, the H:L ratio and plasma CORT do not covary consistently and one measure of stress cannot be used in lieu of the other. Another recent study in garter snakes (*Thamnophis elegans*) observed that CORT

levels and H:L ratios were not correlated within individuals (Sparkman et al., 2014). The authors found, however, that gravid females exhibited an increase in CORT and H:L ratios over time spent in captivity, not only between June in the field and August in captivity, but also between August and October in captivity. Interestingly, CORT showed the largest increase in the first period while the rise in H:L ratios was more pronounced in the second period, which supports the hypothesis that the redistribution of leukocytes is driven by CORT.

CORT as a mediator of resource allocation

According to life-history theory, if animals live in an environment with finite resources, then individuals need to allocate such resources to competing functions, which results in trade-offs between life-history characteristics and/or endocrine, immune or condition metrics. CORT regulates energy balance (Sapolsky et al., 2000) and is thought to mediate allocation of resources to maintain homeostasis (see below for examples). While our CORT treatment chronically increased total circulating GC levels, it did not impose an actual stressor on the animals. Since turtles did not need to behaviourally or otherwise energetically cope with a stressful situation, it is possible that available resources did not have to be diverted from immune defence and body maintenance, which would explain the lack of detected effect of our treatment. It appears that our turtles were robust to high levels of CORT owing to the absence of other considerable energetic or immune challenges.

Other studies have found that the physiological effects of CORT are dependent on the presence of additional challenges. For example, French and colleagues (2006) used the rate of cutaneous wound healing as an integrated measure of stress-sensitive innate immune function, and found that, compared to controls, captive male tree lizards (*Urosaurus ornatus*)

submitted to daily restraint stress healed more slowly and had higher CORT levels. The authors therefore came to the conclusion that stress adversely affects healing and that CORT at least partly mediates the effects of stress. French and colleagues (2007) later tested alternative hypotheses examining the effects of CORT (through implants) on wound healing during reproduction, using female tree lizards. Their hypothesis was that CORT acted either directly, in which case CORT-treated animals should all have suppressed wound healing regardless of diet or energy state, or indirectly, in which case only under energetically limiting conditions would CORT suppress wound healing, to conserve resources. They found that only animals that were CORT-treated as well as on a restricted diet had suppressed healing in pre-reproductive state, whereas in vitellogenic (reproductive) animals, healing was suppressed by CORT treatment regardless of food treatment. CORT would therefore appear to be a mediator of physiological trade-offs between the reproductive and immune systems, with its action apparently depending on energy availability. Similarly, Brooks and Mateo (2013) found that Belding's ground squirrels (*Urocitellus beldingi*) chronically treated with GC for 6 days had lower innate immune function (measured as bacteria killing ability) after, but not before, receiving an immune challenge of lipopolysaccharide. Their results also suggest that chronic stress may not be detrimental to immune function until an individual is challenged with an infection. Johnstone and colleagues (2012b) suggested that the functional reason why H:L is higher in association with stressors is that the physiology of the animal has been affected to acclimate it to an environment with a higher risk of injury. In our study, we attempted to chronically elevate CORT levels, but in the absence of an actual stressor. It is therefore possible that our CORT treatment had no effect because turtles were not

otherwise limited by immune or energetic challenges. Future research should focus on the effects of CORT in animals facing additional challenges.

The potential confounding effect of extrinsic factors

Our study design controlled for many extrinsic factors that could have otherwise influenced CORT levels and immune measurements. By experimentally manipulating CORT levels, we removed the perceptual component of stress to isolate the effects of chronically elevated stress hormones *per se*. Our study animals were all from the same population, therefore presumably from the same genetic background. We captured turtles from the same location, a small lake, therefore they were exposed to the same environmental conditions, including habitat quality, low levels of anthropogenic disturbance and chemical pollutants, predator and parasite pressure, food availability, climate, and thermoregulation opportunities. Moreover, we tried to account for potential effects of sex (*Sex*), seasonality (*Date on day 0*) and exact duration of treatment (*Days post-implant*) by including them as covariates in our models. Our analyses assumed linear relationships between our 5 variables of interest and our continuous covariates *Date on day 0* and *Days post-implant*. While such a linear relationship is the most plausible for the latter, seasonal variations could be non-linear, for example if stress response and immune function are decreased during the nesting season. We did not, however, visually observe obvious non-linear trends in our data. Even if reproductive status of females changed during the experiment, they were excluded from analyses for stress response due to sample size, and we detected no effect of sex on the other variables.

There are other factors that we did not account for in our experiment. Circadian variations in CORT levels are possible and could have added variability to our stress

response data. One could also argue that temperature is a factor that could have influenced our results. First, owing to its effect on metabolic activity in ectotherms, temperature has been suggested as having an effect on CORT secretion, with mixed results from different species. As ectotherms, reptiles show wide variations in body temperature (T_b). A positive relationship between baseline CORT and T_b and/or environmental temperature has been suggested by studies on tuataras (*Sphenodon punctatus*) (Tyrrell and Cree, 1998), marine iguanas (*Amblyrhynchus cristatus*) (Woodley et al., 2003), common geckos (*Hoplodactylus maculatus*) (Cree et al., 2003; Girling and Cree, 1995), and Galápagos tortoises (*Geochelone nigra*) (Schramm et al., 1999), although no such correlation was found in western fence lizards (*Sceloporus occidentalis*) (Dunlap and Wingfield, 1995). Cree and colleagues (2003) suggested that the strong positive correlation between baseline CORT levels (0.5-9.5 ng/mL) and T_b (range: 9-30°C) observed in common geckos reflects a normal increase in metabolic activity and that T_b should always be examined for a possible predictive influence on plasma CORT in reptiles. More recently, Dupoué and colleagues (2013), however, found higher baseline and stress-induced CORT concentrations in Children's pythons (*Antaresia children*) in cold vs. warm conditions. They suggested that high CORT concentrations could help the organism maintain an alert state at suboptimal temperatures, although the mechanism linking increased CORT concentrations to cold temperatures remains unclear.

Since it had been shown that baseline CORT levels can be positively correlated with body temperature (T_b) in some reptiles, we used CORT concentrations measured in the same painted turtle population (V. Juneau, 2009, pers. obs.) to look for such a possible effect, and we found a non-significant negative correlation ($R^2 = 0.08$, $P = 0.10$, $n = 38$) between baseline CORT and T_b (range: 20.7-33.8°C). Therefore, T_b did not seem to have a strong

influence on baseline CORT in our turtles. It is possible that the effect of temperature on CORT secretion is limited when animals experience environmental temperatures within a range close to their preferred temperature (~23°C, Edwards and Blouin-Demers, 2007), as was the case in our analysis.

Another possible effect of temperature would be through higher CORT delivery from our cocoa butter implants in turtles experiencing higher environmental temperatures. We assumed this effect to be negligible overall because turtles were likely exposed to similar variations in environmental temperatures in the long term. Such effect could however become problematic if it is influencing individual values measured on day 0, which we used as the reference to calculate the effect of treatment over time. The inclusion of *Date on day 0* as a covariate in our analyses likely accounted for some of this potential variation.

The importance of CBG and free vs. total CORT

The CORT response to stressors is regulated by plasma corticosteroid-binding globulins (CBG) (reviewed by Breuner and Orchinik, 2002; Romero, 2002). This variable is now measured in an increasing, but still limited, number of stress studies (e.g. Busch et al., 2008; Cyr and Romero, 2007). The exact role of CBG has been debated: it appears that binding of CORT to CBG may serve as a tissue buffer against potentially negative effects of elevated CORT levels, regulate the availability of free hormone to target tissues, alter CORT clearance rates, and modulate CORT actions by altering local CORT concentrations (Breuner and Orchinik, 2002). Malisch and Breuner (2010) proposed that in the plasma, free CORT is the biologically active fraction (available for immediate use by tissues), while the bound fraction is a relevant reservoir for periods of increasing metabolic need (as a source of potential free CORT). More recently, Breuner and colleagues (2013) reviewed studies

providing solid evidence supporting this free hormone hypothesis. They also suggested that, in addition to the typically measured CORT levels, downstream metrics such as immune function or body mass changes should be used to assess the physiological changes in CORT and CBG. Desantis and colleagues (2013) concluded that, in the majority of vertebrate species, high levels of CBG bind ~90% of the GCs, which can act as a circulating reservoir of GCs.

As in the vast majority of ecological studies of stress hormone metrics (Breuner et al., 2013), we measured total, not free, CORT concentrations, nor CBG levels. Although we measured increased total CORT levels in our CORT-treated turtles (see Chapter 1), it is possible that CBG levels also varied and acted as a buffer of high CORT levels, preventing deleterious effects. If most of the exogenous CORT was bound to CBG, and not free, then it is not surprising that we observed no effect of chronically elevated (total) CORT levels on hormonal stress response, immune function, and body condition. As suggested by recent reviews (e.g. Breuner et al., 2013; Crespi et al., 2013), future studies would be more informative if they included measurements of downstream determinants of GC function, such as CBG or receptors action. Measuring CBG levels would allow the calculation of free CORT concentrations, and should improve correlations between CORT levels and life-history or fitness traits.

CONCLUSIONS

We tested the effect of a 3-week chronic CORT elevation on the acute stress responsiveness, immune function, and body condition of free-living painted turtles. We hypothesized that if chronically high CORT levels act to down-regulate CORT release following an acute stressor, suppress immune function by affecting mediators of immune and

inflammatory reactions, cause an increase in heterophils and a decrease in lymphocytes, act to decrease circulating levels of most leukocytes (white blood cells, WBCs), and induce lipolysis, then turtles implanted with slow-release CORT would have a decrease in the magnitude of their stress response, a higher intensity of infection (parasitaemia) by the hemoparasites *Haemogregarina balli* and *Trypanosoma chrysemydis*, an increase in their H:L ratio, a decrease in total WBC counts, and a decrease in body condition, compared to sham-treated and control individuals. At 1 week and 3 weeks post-implantation, we recaptured turtles and measured the acute CORT stress response following a standardized restraint protocol, parasitaemia, heterophil-to-lymphocyte (H:L) ratios, and total WBC counts in treated, sham and control turtles. We also determined body condition 1 week, 3 weeks, and 1 year after treatment. Interestingly, we did not observe an effect of treatment on hormonal (stress response), immune (parasitaemia, H:L ratios, WBC counts), and body condition metrics of stress. We discussed the role of CORT as a mediator of resource allocation in the presence of additional immune or energy challenges, the potential role of extrinsic factors, and the importance of measuring corticosteroid-binding globulin (CBG) and free vs. total CORT. Future studies should focus on the effects of CORT in animals facing additional immune or energetic challenges. The inclusion of measurements of downstream determinants of GC function such as CBG levels to calculate free CORT concentrations is also promising, as it should improve correlations between CORT levels and life-history or fitness traits.

Table 2-1. Distribution data of variables measured on day 0 in adult painted turtles from Gatineau Park, QC, Canada. Minimum (Min), median, maximum (Max), mean ± standard deviation (SD) and sample size (*n*) are presented. Note the use of different units. Indented variables were not used in analyses but are shown for reference purposes.

Variable (units)	Min	Median	Max	Mean ± SD	*n*
Stress response (calculated as S/Bx100)	61	363	1263	491 ± 361	21
Total baseline [CORT] (ng/mL)	0.3	0.5	15.7	1.5 ± 3.3	21
Total stress-induced [CORT] (ng/mL)	0.7	1.9	52.3	5.8 ± 11.3	21
Parasitaemia (no. /10000 RBCs)	0	1	24	2.7 ± 4.5	61
H. balli, meront stage	0	0	22	1.2 ± 3.2	61
H. balli, gametocyte stage	0	0	13	1.5 ± 2.7	61
T. chrysemydis	0	0	0	0 ± 0	61
H:L ratio	0.09	0.68	2.95	0.81 ± 0.58	61
WBC count (no. /4000 RBCs)	84	206	548	231 ± 114	61
Body condition index	0.79	0.98	1.24	0.98 ± 0.08	115
Plastron length (mm)	88	128	180	129 ± 19	115
Body mass (g)	130	325	890	353 ± 141	115

Table 2-2. Statistical results from full model analyses, comparing the Control-sham and CORT treatment groups. R^2 value for the full models and for each parameter, p-value (*P*), F ratio (*F*), degrees of freedom (df), and sample size (*n*) are presented. Sample sizes shown are: Full model = *n* total; Treatment group = *n* Control-sham, *n* CORT; Sex = *n* males, *n* females. The effect of Sex is not available for the 1-week variables and the 3-week stress response, as those data were only analysed for males. For 1 week and 3 weeks, significant differences are bolded after Bonferroni correction within time period ($\alpha = 0.05 / 5 = 0.01$). For the effect of Treatment group, the achieved power to detect the observed effect size, as well as sample size (*n*) required to detect the observed effect as significant with a power of 80% ($\alpha = 0.05$), are presented.

Time period	Variable		R^2	*P*	*F*	df	*n*	Power	Required *n*
1 week	Diff Stress response	Full model	0.62	0.028	4.84	3, 9	13		
		Treatment group	0.02	0.54	0.41	1, 9	5, 8	0.476	25
		Date on day 0	0.21	0.05	4.90	1, 9			
		Days post-implant	0.14	0.10	3.30	1, 9			
	Diff Parasitaemia	Full model	0.36	0.16	2.06	3, 11	15		
		Treatment group	0.11	0.19	1.91	1, 11	5, 10	0.115	181
		Date on day 0	0.06	0.33	1.05	1, 11			
		Days post-implant	0.30	0.043	5.21	1, 11			
	Diff H:L	Full model	0.25	0.36	1.19	3, 11	15		
		Treatment group	0.19	0.13	2.73	1, 11	5, 10	0.162	108
		Date on day 0	0.14	0.19	1.97	1, 11			
		Days post-implant	0.01	0.69	0.17	1, 11			
	Diff WBC count	Full model	0.24	0.37	1.15	3, 11	15		
		Treatment group	0.16	0.16	2.25	1, 11	5, 10	0.079	395
		Date on day 0	0.21	0.11	3.02	1, 11			
		Days post-implant	< 0.01	0.97	< 0.01	1, 11			

Time period	Variable		R^2	*P*	*F*	df	*n*	Power	Required *n*
1 week	Diff BCI	Full model	0.04	0.93	0.14	3, 11	15		
		Treatment group	< 0.01	0.90	0.02	1, 11	5, 10	0.050	53179
		Date on day 0	< 0.01	0.91	0.01	1, 11			
		Days post-implant	0.04	0.53	0.42	1, 11			
3 weeks	Diff Stress response	Full model	0.40	0.08	2.84	3, 13	17		
		Treatment group	0.39	0.012	8.49	1, 13	9, 8	0.371	46
		Date on day 0	0.17	0.08	3.70	1, 13			
		Days post-implant	< 0.01	0.79	0.07	1, 13			
	Diff Parasitaemia	Full model	0.04	0.77	0.45	4, 38	43		
		Treatment group	< 0.01	0.96	< 0.01	1, 38	26, 17	0.067	2183
		Date on day 0	< 0.01	0.65	0.21	1, 38			
		Days post-implant	0.04	0.23	1.50	1, 38			
		Sex	< 0.01	0.90	0.02	1, 38	30, 13		
	Diff H:L	Full model	0.04	0.83	0.37	4, 38	43		
		Treatment group	0.02	0.37	0.83	1, 38	26, 17	0.101	737
		Date on day 0	0.01	0.45	0.57	1, 38			
		Days post-implant	< 0.01	0.96	< 0.01	1, 38			
		Sex	< 0.01	0.55	0.36	1, 38	30, 13		
	Diff WBC count	Full model	0.11	0.32	1.22	4, 38	43		
		Treatment group	0.09	0.06	3.67	1, 38	26, 17	0.472	92
		Date on day 0	< 0.01	0.67	0.19	1, 38			
		Days post-implant	< 0.01	0.78	0.08	1, 38			
		Sex	0.02	0.32	1.00	1, 38	30, 13		

Time period	Variable		R^2	*P*	*F*	df	*n*	Power	Required *n*
3 weeks	Diff BCI	Full model	0.02	0.91	0.25	4, 43	48		
		Treatment group	< 0.01	0.85	0.03	1, 43	29, 19	0.071	1952
		Date on day 0	< 0.01	0.84	0.04	1, 43			
		Days post-implant	0.02	0.41	0.70	1, 43			
		Sex	< 0.01	0.93	0.01	1, 43	31, 17		
1 year	Diff BCI	Full model	0.05	0.34	1.14	4, 93	98		
		Treatment group	< 0.01	0.45	0.57	1, 93	67, 31	0.407	256
		Date on day 0	< 0.01	0.67	0.19	1, 93			
		Days post-implant	< 0.01	0.52	0.43	1, 93			
		Sex	< 0.01	0.74	0.11	1, 93	51, 47		

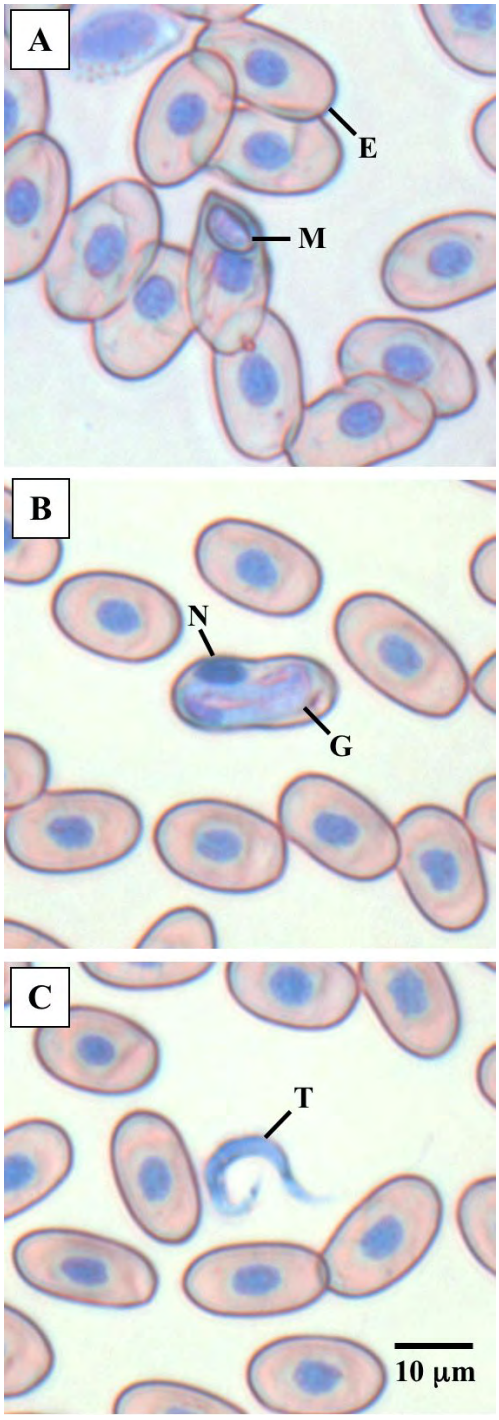
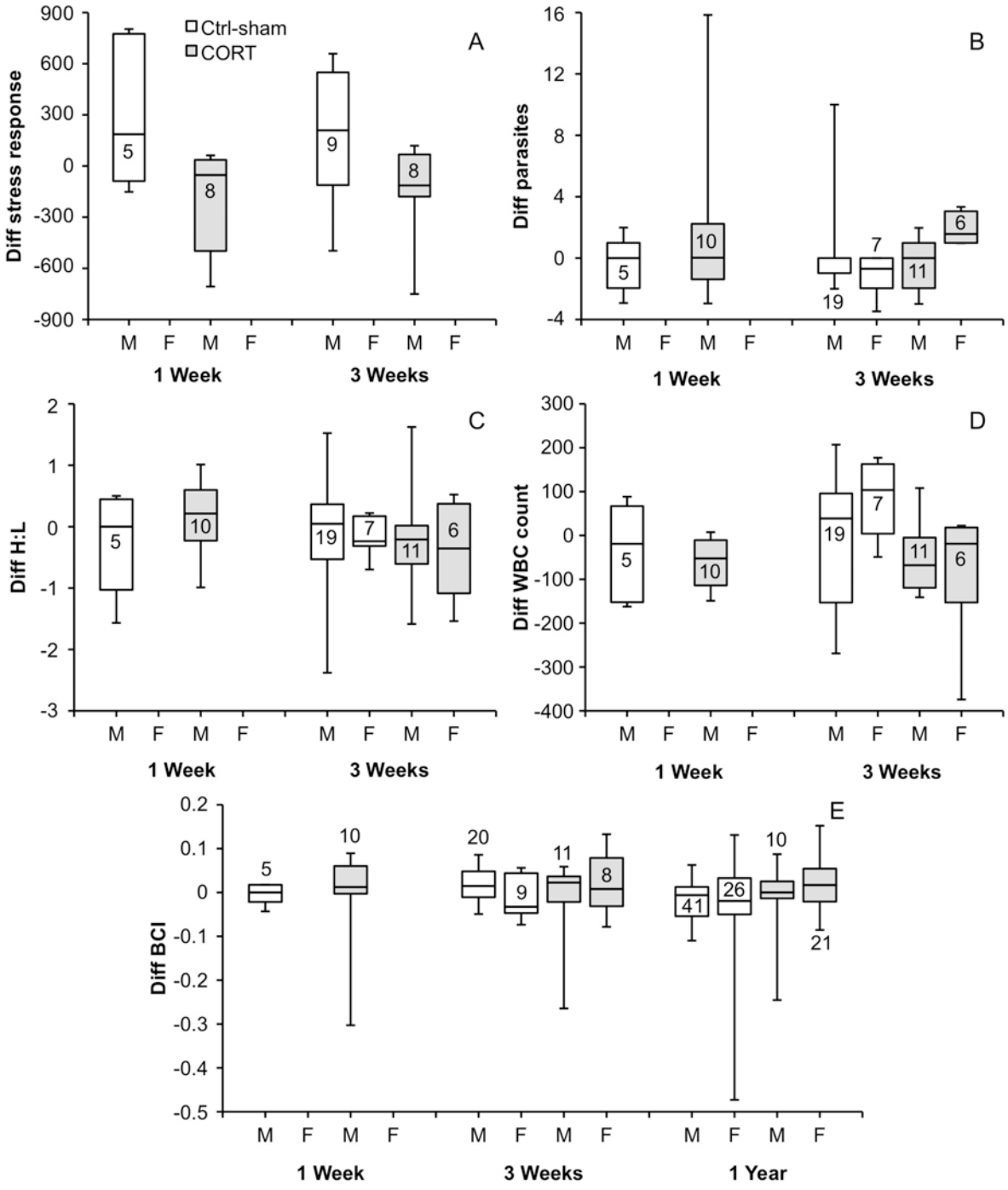


Figure 2-1. Micrographs of hemoparasites observed in the peripheral circulation of painted turtles (*Chrysemys picta*), using bright field microscopy at a total magnification of 400x. (A) The meront, M, stage of *Haemogregarina balli* is found in erythrocytes, E, which are nucleated in reptiles. (B) The nucleus, N, of the host erythrocyte is displaced laterally by a gametocyte, G, of *H. balli*. (C) A free specimen of *Trypanosoma chrysemydis*.


Ctrl-sham
CORT
A
Diff stress response
900
600
300
0
-300
-600
-900
5
8
9
8
M F M F M F M F
1 Week
3 Weeks
B
Diff parasites
16
12
8
4
0
-4
5
10
19
7
11
6
M F M F M F M F
1 Week
3 Weeks
C
Diff H:L
2
1
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-1
-2
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5
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19
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M F M F M F M F
1 Week
3 Weeks
D
Diff WBC count
300
200
100
0
-100
-200
-300
-400
5
10
19
7
11
6
M F M F M F M F
1 Week
3 Weeks
E
Diff BCI
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
5
10
20
9
11
8
41
26
10
21
M F M F M F M F M F M F
1 Week
3 Weeks
1 Year

Figure 2-2. Results for the effects of corticosterone implantation on painted turtles, *Chrysemys picta*, in Gatineau Park, QC, Canada. We analysed and presented the differences between the time periods of interest (1 week, 3 weeks, and 1 year) and day 0, by Treatment group and Sex (male, M, and female, F). Individuals were assigned to one of three treatment groups: without injection (Control), injected with cocoa butter only (Sham), or injected with CORT-laden cocoa butter (CORT, light grey boxes). Statistical analyses revealed no difference between the Control and Sham groups; therefore they were combined for further analyses, as shown here (Ctrl-sham, white boxes). Numbers on bars represent sample size. (A) Difference in stress response. (B) Difference in parasitaemia (*H. balli* and *T. chrysemydis*, no. /10000 RBCs). (C) Difference in H:L ratio. (D) Difference in WBC count (no. /4000 RBCs). (E) Difference in body condition index (BCI).

GENERAL CONCLUSION

In Chapter 1, I aimed to evaluate the effectiveness of two corticosterone (CORT) administration methods at chronically elevating circulating CORT levels in a temperate reptile, the painted turtle (*Chrysemys picta*): Silastic implants and cocoa butter injections. In the first experiment, I observed no effect of sealed Silastic implants of CORT on baseline CORT levels in free-living turtles. Subsequently, I observed no significant difference in baseline CORT levels, hormonal stress response, or body mass among treatment groups, using sealed and perforated Silastic implants in captive turtles. My findings imply that sealed or perforated Silastic implants of CORT do not automatically function as previously expected and should be used with caution. In the second experiment, I assessed injections of CORT-laden cocoa butter for the first time in an ectothermic tetrapod. I observed that my CORT dosage generally raised circulating CORT levels for up to 3 weeks in free-living turtles, at concentrations that were likely physiologically and ecologically relevant for the species (i.e., that were mostly around typical stress-induced levels or levels experienced by turtles recovering from entrapment in commercial fishing nets), despite large inter-individual variation. The duration of the CORT elevation, however, is possibly longer than that experienced by wild turtles. I conclude that cocoa butter injections, but not sealed or perforated Silastic implants, of CORT can be used in painted turtles to chronically elevate circulating CORT levels successfully, and represent a promising method for delivery of CORT in other ectotherms living in temperate climates.

In Chapter 2, I investigated the effect of chronic CORT elevation on the acute stress responsiveness, immune function, and body condition of free-living painted turtles. For the

first time in an ectothermic tetrapod, I used cocoa butter injections of CORT (validated in Chapter 1) to elevate CORT levels chronically for approximately 3 weeks. After 1 week and 3 weeks, I recaptured turtles to determine the acute CORT stress response following a standardized restraint protocol, parasitaemia, heterophil-to-lymphocyte (H:L) ratios, and total WBC counts in treated, sham and control turtles. I also assessed body condition 1 week, 3 weeks, and 1 year after treatment. Unexpectedly, I did not observe an effect of treatment on hormonal (stress response), immune (parasitaemia, H:L ratios, WBC counts), or body condition metrics of stress. I discussed the role of CORT as a mediator of resource allocation in the presence of additional immune or energy challenges, the potential role of extrinsic factors, and the importance of measuring corticosteroid-binding globulin (CBG) and free vs. total CORT. Future studies should concentrate on the effects of CORT in animals faced with additional immune or energetic challenges. Also, correlations between CORT levels and life-history or fitness traits should be improved by the inclusion of measurements of downstream determinants of GC function, such as CBG levels to calculate free CORT concentrations.

APPENDIX

Complementary results for the effects of CORT implantation on painted turtles

Table A-1. Statistical results from full model analyses, comparing the Control and Sham treatment groups. R^2 value for the full models and for each parameter, p-value (*P*), F ratio (*F*), degrees of freedom (df), and sample size (*n*) are presented. Sample sizes shown are: Full model = *n* total; Treatment group = *n* Control, *n* Sham; Sex = *n* males, *n* females. The effect of Sex is not available for the 1-week variables and the 3-week stress response, as those data were only analysed for males. For 1 week and 3 weeks, significant differences are bolded after Bonferroni correction within time period ($\alpha = 0.05 / 5 = 0.01$).

Time period	Variable		R^2	*P*	*F*	df	*n*
1 week	Diff Stress response	Full model	0.89	0.42	2.61	3, 1	5
		Treatment group	0.29	0.36	2.53	1, 1	4, 1
		Date on day 0	0.19	0.41	1.70	1, 1	
		Days post-implant	< 0.01	0.84	0.06	1, 1	
	Diff Parasitaemia	Full model	0.59	0.76	0.48	3, 1	5
		Treatment group	0.58	0.45	1.41	1, 1	4, 1
		Date on day 0	0.56	0.45	1.36	1, 1	
		Days post-implant	0.19	0.62	0.46	1, 1	
	Diff H:L	Full model	0.93	0.34	4.14	3, 1	5
		Treatment group	0.64	0.21	8.61	1, 1	4, 1
		Date on day 0	0.60	0.22	8.00	1, 1	
		Days post-implant	0.57	0.22	7.72	1, 1	
	Diff WBC count	Full model	0.90	0.39	3.08	3, 1	5
		Treatment group	0.60	0.24	6.18	1, 1	4, 1
		Date on day 0	0.50	0.26	5.17	1, 1	
		Days post-implant	0.19	0.39	1.98	1, 1	
	Diff BCI	Full model	0.61	0.74	0.51	3, 1	5
		Treatment group	0.47	0.47	1.18	1, 1	4, 1
		Date on day 0	0.50	0.46	1.28	1, 1	
		Days post-implant	0.52	0.45	1.33	1, 1	

Time period	Variable		R^2	*P*	*F*	df	*n*
3 weeks	Diff Stress response	Full model	0.56	0.22	2.13	3, 5	9
		Treatment group	0.17	0.23	1.88	1, 5	4, 5
		Date on day 0	0.07	0.41	0.82	1, 5	
		Days post-implant	0.05	0.48	0.58	1, 5	
	Diff Parasitaemia	Full model	0.27	0.15	1.90	4, 21	26
		Treatment group	0.12	0.08	3.41	1, 21	17, 9
		Date on day 0	0.03	0.40	0.74	1, 21	
		Days post-implant	< 0.01	0.82	0.05	1, 21	
		Sex	0.21	0.023	6.01	1, 21	19, 7
	Diff H:L	Full model	0.08	0.77	0.45	4, 21	26
		Treatment group	0.02	0.55	0.38	1, 21	17, 9
		Date on day 0	< 0.01	0.86	0.03	1, 21	
		Days post-implant	0.01	0.63	0.24	1, 21	
		Sex	0.02	0.47	0.53	1, 21	19, 7
	Diff WBC count	Full model	0.11	0.64	0.64	4, 21	26
		Treatment group	< 0.01	0.64	0.22	1, 21	17, 9
		Date on day 0	< 0.01	0.86	0.03	1, 21	
		Days post-implant	< 0.01	0.83	0.05	1, 21	
		Sex	0.05	0.31	1.08	1, 21	19, 7
	Diff BCI	Full model	0.30	0.06	2.57	4, 24	29
		Treatment group	0.17	0.023	5.87	1, 24	20, 9
		Date on day 0	0.12	0.06	4.06	1, 24	
		Days post-implant	< 0.01	0.61	0.27	1, 24	
		Sex	0.21	0.012	7.32	1, 24	20, 9
1 yr	Diff BCI	Full model	0.06	0.43	0.96	4, 62	67
		Treatment group	< 0.01	0.67	0.18	1, 62	56, 11
		Date on day 0	< 0.01	0.59	0.29	1, 62	
		Days post-implant	0.01	0.35	0.90	1, 62	
		Sex	< 0.01	0.60	0.28	1, 62	41, 26

Table A-2. Statistical results of univariate analyses comparing the variables of interest between the Control and Sham groups. R^2, p-value (*P*), F ratio (*F*), degrees of freedom (df), and sample size (*n*) are presented. Sample sizes shown are: *n* total (*n* Control, *n* Sham). ANOVA was used for each variable, except Welch's ANOVA allowing unequal variances for 3-week BCI. Data for the 1-week variables and the 3-week stress response are from males only. For 1 week and 3 weeks, significant differences are bolded after Bonferroni correction within time period ($\alpha = 0.05 / 5 = 0.01$).

Time period	Variable	R^2	*P*	*F*	df	*n*
1 week	Diff Stress response	0.39	0.26	1.91	1, 3	5 (4, 1)
	Diff Parasitaemia	0.01	0.85	0.04	1, 3	5 (4, 1)
	Diff H:L	0.24	0.40	0.94	1, 3	5 (4, 1)
	Diff WBC count	0.39	0.26	1.92	1, 3	5 (4, 1)
	Diff BCI	< 0.01	0.95	< 0.01	1, 3	5 (4, 1)
3 weeks	Diff Stress response	0.48	0.039	6.46	1, 7	9 (4, 5)
	Diff Parasitaemia	0.05	0.26	1.34	1, 24	26 (17, 9)
	Diff H:L	0.04	0.31	1.05	1, 24	26 (17, 9)
	Diff WBC count	0.04	0.30	1.11	1, 24	26 (17, 9)
	Diff BCI	< 0.01	0.76	0.10	1, 26.9	29 (20, 9)
1 year	Diff BCI	< 0.01	0.60	0.28	1, 65	67 (56, 11)

Table A-3. Statistical results of univariate analyses comparing the variables of interest between the Control-sham and CORT groups. R^2, p-value (*P*), F ratio (*F*), degrees of freedom (df), and sample size (*n*) are presented. Sample sizes shown are: *n* total (*n* Control-sham, *n* CORT). ANOVA was used for each variable, except Welch's ANOVA allowing unequal variances for 1-week WBC count. Data for the 1-week variables and the 3-week stress response are from males only. For 1 week and 3 weeks, significant differences are bolded after Bonferroni correction within time period ($\alpha = 0.05 / 5 = 0.01$).

Time period	Variable	R^2	*P*	*F*	df	*n*
1 week	Diff Stress response	0.36	0.031	6.11	1, 11	13 (5, 8)
	Diff Parasitaemia	0.04	0.46	0.57	1, 13	15 (5, 10)
	Diff H:L	0.07	0.34	0.99	1, 13	15 (5, 10)
	Diff WBC count	0.02	0.70	0.16	1, 5	15 (5, 10)
	Diff BCI	< 0.01	0.97	< 0.01	1, 13	15 (5, 10)
3 weeks	Diff Stress response	0.19	0.08	3.59	1, 15	17 (9, 8)
	Diff Parasitaemia	< 0.01	0.71	0.15	1, 41	43 (26, 17)
	Diff H:L	0.01	0.51	0.43	1, 41	43 (26, 17)
	Diff WBC count	0.09	0.06	3.83	1, 41	43 (26, 17)
	Diff BCI	< 0.01	0.67	0.18	1, 46	48 (29, 19)
1 year	Diff BCI	0.03	0.11	2.67	1, 96	98 (67, 31)

Table A-4. Statistical results from full model analyses, comparing the Control-sham, CORT, and CORT-2 treatment groups. R^2 value for the full models and for each parameter, p-value (*P*), F ratio (*F*), degrees of freedom (df), and sample size (*n*) are presented. Sample sizes shown are: Full model = *n* total; Treatment group = *n* Control-sham, *n* CORT, *n* CORT-2; Sex = *n* males, *n* females. The effect of Sex is not available for the 1-week variables and the 3-week stress response, as those data were only analysed for males. For 1 week and 3 weeks, significant differences are bolded after Bonferroni correction within time period ($\alpha = 0.05 / 5 = 0.01$).

Time period	Variable		R^2	*P*	*F*	df	*n*
1 week	Diff Stress response	Full model	0.62	0.032	4.12	4, 10	15
		Treatment group	0.06	0.49	0.76	2, 10	5, 8, 2
		Date on day 0	0.21	0.041	5.50	1, 10	
		Days post-implant	0.15	0.08	3.89	1, 10	
	Diff Parasitaemia	Full model	0.41	0.12	2.26	4, 13	18
		Treatment group	0.14	0.24	1.57	2, 13	5, 10, 3
		Date on day 0	0.05	0.32	1.09	1, 13	
		Days post-implant	0.24	0.038	5.35	1, 13	
	Diff H:L	Full model	0.21	0.50	0.89	4, 13	18
		Treatment group	0.17	0.27	1.45	2, 13	5, 10, 3
		Date on day 0	0.13	0.17	2.12	1, 13	
		Days post-implant	< 0.01	0.87	0.03	1, 13	
	Diff WBC count	Full model	0.25	0.40	1.10	4, 13	18
		Treatment group	0.16	0.28	1.41	2, 13	5, 10, 3
		Date on day 0	0.21	0.08	3.64	1, 13	
		Days post-implant	< 0.01	0.90	0.01	1, 13	
	Diff BCI	Full model	0.08	0.87	0.30	4, 13	18
		Treatment group	0.07	0.62	0.50	2, 13	5, 10, 3
		Date on day 0	< 0.01	0.92	0.01	1, 13	
		Days post-implant	0.03	0.55	0.37	1, 13	
3 weeks	Diff Stress response	Full model	0.41	0.09	2.48	4, 14	19
		Treatment group	0.40	0.026	4.81	2, 14	9, 8, 2
		Date on day 0	0.17	0.06	4.07	1, 14	
		Days post-implant	< 0.01	0.78	0.08	1, 14	

Time period	Variable		R^2	P	F	df	n
3 weeks	Diff	Full model	0.01	0.99	0.05	5, 42	48
	Parasitaemia	Treatment group	< 0.01	0.95	0.05	2, 42	26, 17, 5
		Date on day 0	< 0.01	0.97	< 0.01	1, 42	
		Days post-implant	< 0.01	0.87	0.03	1, 42	
		Sex	< 0.01	0.95	< 0.01	1, 42	35, 13
	Diff H:L	Full model	0.04	0.90	0.31	5, 42	48
		Treatment group	0.02	0.60	0.51	2, 42	26, 17, 5
		Date on day 0	0.01	0.47	0.53	1, 42	
		Days post-implant	< 0.01	0.99	< 0.01	1, 42	
		Sex	< 0.01	0.53	0.39	1, 42	35, 13
	Diff WBC	Full model	0.11	0.38	1.09	5, 42	48
	count	Treatment group	0.09	0.14	2.10	2, 42	26, 17, 5
		Date on day 0	< 0.01	0.69	0.17	1, 42	
		Days post-implant	< 0.01	0.86	0.03	1, 42	
		Sex	0.02	0.32	1.02	1, 42	35, 13
	Diff BCI	Full model	0.06	0.73	0.56	5, 47	53
		Treatment group	0.01	0.69	0.37	2, 47	29, 19, 5
		Date on day 0	< 0.01	0.92	0.01	1, 47	
		Days post-implant	< 0.01	0.52	0.41	1, 47	
		Sex	< 0.01	0.96	< 0.01	1, 47	36, 17
1 year	Diff BCI	Full model	0.05	0.38	1.07	5, 97	103
		Treatment group	< 0.01	0.71	0.35	2, 97	67, 31, 5
		Date on day 0	< 0.01	0.67	0.19	1, 97	
		Days post-implant	< 0.01	0.48	0.50	1, 97	
		Sex	< 0.01	0.74	0.11	1, 97	56, 47

Table A-5. Statistical results of univariate analyses comparing the variables of interest between the Control-sham, CORT, and CORT-2 groups. R^2, p-value (*P*), F ratio (*F*), degrees of freedom (df), and sample size (*n*) are presented. Sample sizes shown are: *n* total (*n* Control-sham, *n* CORT, *n* CORT-2). ANOVA was used for each variable, except Welch's ANOVA allowing unequal variances for 1-week WBC count and 3-week parasitaemia. Data for the 1-week variables and the 3-week stress response are from males only. For 1 week and 3 weeks, significant differences are bolded after Bonferroni correction within time period ($\alpha = 0.05 / 5 = 0.01$).

Time period	Variable	R^2	*P*	*F*	df	*n*
1 week	Diff Stress response	0.36	0.07	3.32	2, 12	15 (5, 8, 2)
	Diff Parasitaemia	0.15	0.30	1.32	2, 15	18 (5, 10, 3)
	Diff H:L	0.07	0.56	0.60	2, 15	18 (5, 10, 3)
	Diff WBC count	0.04	0.46	0.87	2, 6.8	18 (5, 10, 3)
	Diff BCI	0.06	0.64	0.46	2, 15	18 (5, 10, 3)
3 weeks	Diff Stress response	0.22	0.14	2.20	2, 16	19 (9, 8, 2)
	Diff Parasitaemia	< 0.01	0.92	0.08	2, 9.9	48 (26, 17, 5)
	Diff H:L	0.01	0.79	0.24	2, 45	48 (26, 17, 5)
	Diff WBC count	0.09	0.13	2.15	2, 45	48 (26, 17, 5)
	Diff BCI	0.05	0.31	1.20	2, 50	53 (29, 19, 5)
1 year	Diff BCI	0.03	0.20	1.66	2, 100	103 (67, 31, 5)

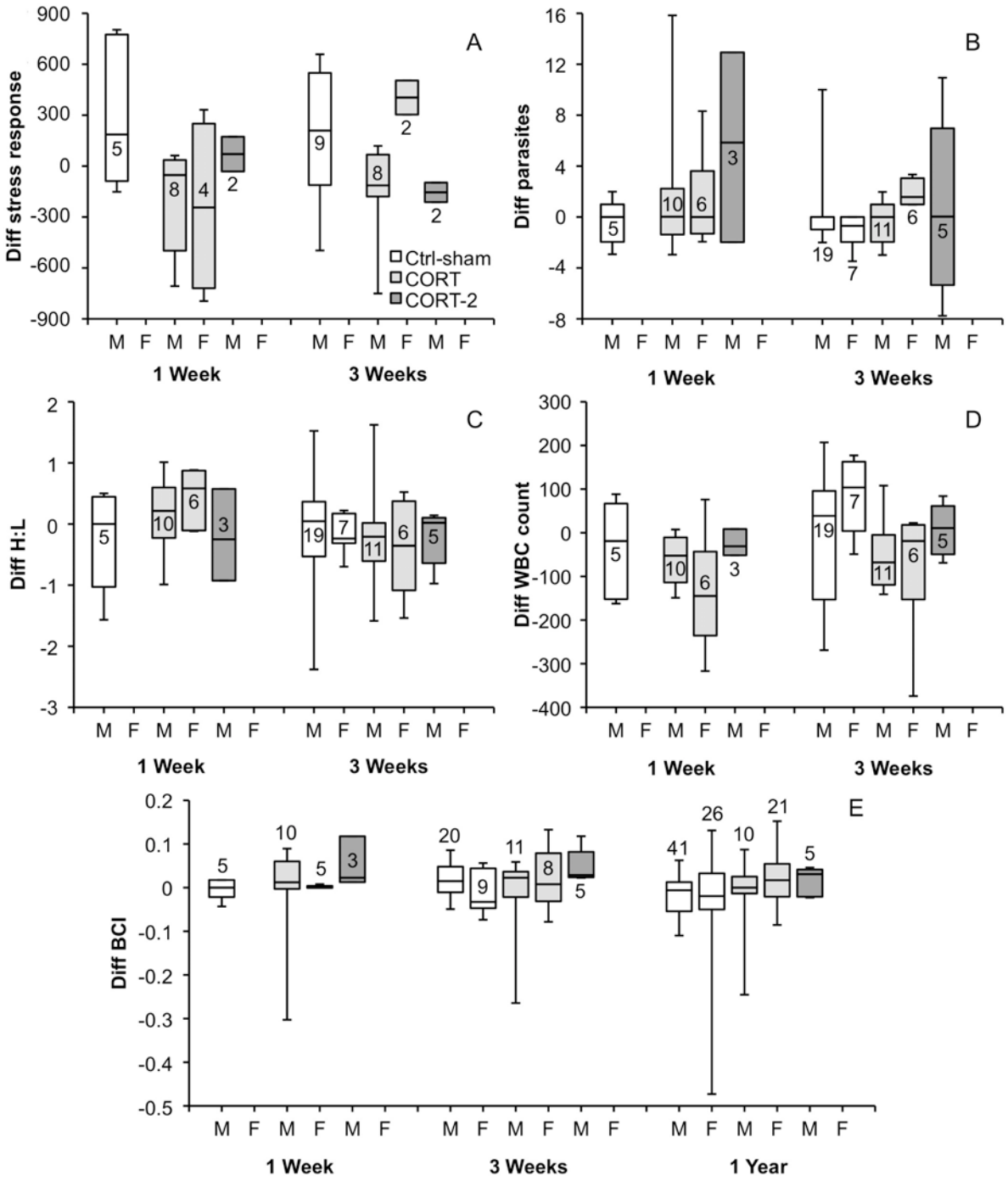
A
Diff stress response
900
600
300
0
-300
-600
-900
Ctrl-sham
CORT
CORT-2
M F M F M F M F M F M F
1 Week
3 Weeks
B
Diff parasites
16
12
8
4
0
-4
-8
C
Diff H:L
2
1
0
-1
-2
-3
D
Diff WBC count
300
200
100
0
-100
-200
-300
-400
E
Diff BCI
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
1 Year

Figure A-1. Results for the effects of corticosterone implantation on painted turtles, *Chrysemys picta*, in Gatineau Park, QC, Canada. We analysed and presented the differences between the time periods of interest (1 week, 3 weeks, and 1 year) and day 0, by Treatment group and Sex (male, M, and female, F). Individuals were assigned to one of four treatment groups: without injection (Control), injected with cocoa butter only (Sham), injected with CORT-laden cocoa butter (CORT, light grey boxes), or re-injected with CORT two months after the first CORT treatment (CORT-2, dark grey boxes). Statistical analyses revealed no difference between the Control and Sham groups; therefore they were combined for further analyses, as shown here (Ctrl-sham, white boxes). Numbers on bars represent sample size. Note that data for CORT females at 1 week for the 5 variables and at 3 weeks for stress response were excluded from the analyses but are shown here. (A) Difference in stress response. (B) Difference in parasitaemia (*H. balli* and *T. chrysemydis*, no. /10000 RBCs). (C) Difference in H:L ratio. (D) Difference in WBC count (no. /4000 RBCs). (E) Difference in body condition index (BCI).

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