Master's thesis

2019

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MSBIO
Submission date: May 2019
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Acknowledgements

This master's thesis was conducted at the Department of Biology at the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway. I would like to thank my supervisor, Associate Professor Clare Stawski, for introducing me to the fascinating world of bats, for making me consider the physiological processes I know and love in a (to me) completely novel context, and for always greeting my initial crisis maximization when faced with a problem with calm assurances that "It'll be fine." I'd also like to thank Jeroen van der Kooij, for his insight into the identification of bats, as well as his knowledge of the system in which I worked. To the entirety of the 2018 SCANDBAT team, thank you for your help during the somewhat hectic colony captures, but perhaps most of all to April McKay, for always being available for a second opinion when I was unsure what I was doing with the bats. Thank you to Karoline Hansen Skåra, for being supportive in so many ways throughout this whole process, and also for amusing me with your fear of things that go bump in the night during otherwise uneventful netting sessions. Finally, a thank you to my family, who are always there to support me in all my ventures.

Trondheim, May 2019

Håvard A. Hald

Cover photo: *Myotis sp.* in the hand after capture. Photo by Rune Sørås, with permission.

Abstract

Physiological stress is known to impact multiple aspects of life for animals in the wild, notably the capacity for growth, reproduction and immunological response. This phenomenon is triggered by adverse environmental or social conditions and is facilitated among other things by an increased secretion of glucocorticoids from the adrenal gland. While many studies have established stress profiles for a multitude of animals, often in the context of human encroachment or other forms of anthropogenic disturbance, few studies have attempted to quantify stress levels in bats. This represents an inadequacy in the current understanding of human-animal interactions, as many bat species can often be found living in close proximity to humans, and frequently use human structures and light sources as roost sites and hunting grounds, respectively. I have thus aimed to establish profiles of stress during the waking season in a species of *Myotis* bats in a rural area of Norway, by measuring the levels of free glucocorticoid (cortisol) in fecal samples by enzyme assay. The results indicate that levels of free glucocorticoids are lower in a pregnant state than in a post-lactating state, or if there is no indication of reproductive state. Further, there is a negative correlation between body mass and free glucocorticoid levels, unless the bat is pregnant, in which case this relationship is reversed and magnified. In addition, mean temperature, but not magnitude of deviation from normal temperature, positively correlates with free glucocorticoid levels. However, these findings were confounded by an uncharacteristically warm and dry season, and they should be verified under normal conditions. I conclude that the high stress levels during critical phases of reproduction observed here are most likely detrimental to the health of both the individual bat and its offspring, and that high stress levels in the long run may negatively impact the survival and reproductive success of the entire population.

Sammendrag

Fysiologisk stress påvirker flere aspekter av tilværelsen for dyr i det fri, spesielt evnen til vekst, reproduksjon og immunrespons. Dette fenomenet utløses av uønskede miljømessige eller sosiale forhold, og fasiliters blant annet av økt sekresjon av glukokortikoider fra binyrene. Selv om studier har etablert stressprofiler for flere arter, ofte i sammenheng med menneskelig innblanding eller andre former for antropogen forstyrrelse, har få studier forsøkt å kvantifisere stressnivået i flaggermus. Dette er utilstrekkelig, da mange flaggermusarter ofte lever i nærheten av mennesker, og brukte ofte menneskelige strukturer og lyskilder som oppholdssted og jaktområder. Jeg har derfor forsøkt å karakterisere stressprofiler i løpet av den aktive sesongen hos en art Myotis-flaggermus i et landsområde i Norge ved å måle nivåene av fritt glukokortikoid (kortisol) i fekale prøver ved enzymanalyse. Resultatene indikerer at nivåene av frie glukokortikoider er lavere ved graviditet enn i post-lakterende tilstander, eller hvis det ikke er noen indikasjon på reproduktivitet. Videre er det en negativ korrelasjon mellom kroppsvekt og frie glukokortikoidnivåer, med mindre flaggermusen er gravid, i hvis tilfelle dette forholdet reverseres og forstørres. I tillegg korrelerer den gjennomsnittlige temperaturen, men ikke størrelsen av avviket fra normal temperatur, med frie glukokortikoidnivåer. Det er imidlertid vanskelig å tolke disse resultatene, da de kan ha blitt forvrengt av en ukarakteristisk varm og tørr sesong, og de burde verifiseres under normale forhold. Jeg konkluderer med at de høye stressnivåene som ble observert her under kritiske faser av reproduksjon mest sannsynlig er skadelige for helsen til både den enkelte flaggermusen og dens avkom, og at høye stressnivåer i det lange løp kan påvirke overlevelse og reproduktiv suksess for hele populasjonen negativt.

List of abbreviations

11B-HSD-2	11β-hydroxysteroid-dehydrogenase
AB	Antibody
AG	Antigen
ACTH	Adrenocorticotropic hormone
ATGL	Adipose triglyceride lipase
BAT	Brown adipose tissue
CAMP	Cyclic adenosine monophosphate
CBG	Corticosteroid-binding globulin
CRH	Corticotropin-releasing hormone
CSF-1	Macrophage colony-stimulating factor
ELISA	Enzyme-linked immunosorbent assay
FGM	Fecal glucocorticoid metabolites
FSH	Follicle-stimulating hormone
GC	Glucocorticoid
GH	Growth hormone
GHIH	Growth hormone inhibiting hormone
GHRH	Growth hormone releasing hormone
GNRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
HPA	Hypophyseal-pituitary-adrenal axis
HPG	Hypophyseal-pituitary-gonadal axis
HSL	Hormone-sensitive lipase
IGF	Insulin-like growth factor
LH	Luteinizing hormone
MR	Mineralocorticoid receptor
MRNA	Messenger RNA
PMCC	Product moment correlation coefficient
PG	Prostaglandin
POMC	Proopiomelanocortin

PVN	Paraventricular nucleus
RANKL	Receptor activator of nuclear factor κβ ligand
TNF-A	Tumor necrosis factor α
TAG	Triacylglyceride
TRH	Thyroid-releasing hormone
TSH	Thyroid-stimulating hormone
UCP-1	Uncoupling protein 1
WAT	White adipose tissue

Contents

1. Introduction

1.1 Bats in the environment and in the context of physiological stress

Bats (order Chiroptera) are the second most prolific group of mammals (after rodents), comprising 1,254 extant species at the time of writing (IUCN, 2019). They are the only group of mammals to have mastered powered, sustained flight, and fill several ecological niches, being represented as frugivores, nectarivores, insectivores, piscivores, (partial) carnivores and, in a few cases, sanguivores (Boles, 1999; Brooke, 1994; Fleming, 1982; Vaughan, 1997). Moreover, being primarily nocturnal allows them to utilize niches that during the day are occupied by diurnal animals. For example, several species of tropical and subtropical plants that only open at night rely at least partially on nectarivore bats for pollination (Bawa, 1990). Further, insectivorous bats are uniquely adapted to nocturnal aerial hunting, being able to use echolocation to navigate and locate prey (though the use of echolocation is not limited to insectivores), and they often share habitats with insectivorous diurnal birds.

Because of their capacity for both pollination and pest control, it is perhaps unsurprising that in addition to potentially being vital for ecosystem stability, bats may also have an economic impact on human agriculture. In conjunction with a global increase in the use of wind turbines (which bats notoriously fly into, for as yet unknown reasons), and increased national spread of the fungal disease white-nose syndrome in the U.S., one study concluded that the complete loss of bats would result in a 3.7 billion USD (422,776,800,000 NOK) loss in annual agricultural income (Boyles et al., 2011). While the total yearly U.S. agricultural revenue in 2018 was 404.1 billion USD, and the projected loss thus constitutes less than 1% of total income, this is still a staggering amount (Statista, 2019). Another study valued pest control by Brazilian free-tailed bats at between 2 and 12% of total income from cotton farms in Texas, showing that there is probably regional variance in benefit (Cleveland et al., 2006).

In addition to the already fascinating use of flight and echolocation, bats also exhibit pronounced use of torpor and hibernation (Ruf & Geiser, 2015; Stawski et al., 2014). This combination of traits provides a fascinating organism to consider in the context of physiological stress; for example, torpor can lead to a buildup of endotoxins, which would be stressful, the arousal process itself can be influenced by stress, the relatively short active season the animals have to perform essential functions, such as weight gain and reproduction, may make them especially vulnerable to exogenous stressors, and the large variability in heart rate associated with flight may make the cardiovascular system susceptible to the effects of excessive stressors. Further, bats are long-lived, meaning they may be inclined towards getting disorders related to chronic stress exposure in the same way humans do. Paired with the facts that bats frequently act as reservoirs for diseases, but seldom get the negative symptoms associated with the disease, and further that they are often found living in close proximity to humans and using human structures as roost sites, this makes research on the stress levels of bats highly relevant in the frame of conservation efforts (Brunet-Rossinni & Austad, 2004; Dobson, 2005; Han et al., 2015).

1.2 Torpor

During torpor, an animal's body temperature is lowered, sometimes drastically. For example, arctic ground squirrels, having a mean normothermic body temperature of 38.5°C, can exhibit torpid temperatures of -2°C, though normal temperature during torpor across animals that utilize it is somewhat higher, at 5°C (Geiser, 2004; Long, Martin, et al., 2005). Most metabolic processes are substantially downregulated in a torpid state, reducing the need to expend energy to survive during unfavorable conditions, but simultaneously placing several demands on the physiological capacity of the animal. For instance, the animal needs to be able to store and, upon arousal, utilize lipids for thermogenesis in a practical fashion. Active arousal is also associated with an increase in metabolic rate well above baseline (see Figure 1), and the animal must also have the capacity to defend itself from reperfusion injuries and the formation of reactive oxygen species associated with arousal (Rouble et al., 2014). However, it is also possible, at least for smaller animals, to use external heat sources to rewarm, thereby requiring far less endogenous energy input. For example, in one study in stripe-faced dunnarts (*Sminthopsis macroura*), it was

found that while arousal using only shivering and non-shivering thermogenesis required an increase in metabolic rate by a factor of 11.6, arousal in the presence of a heat source mimicking what would be available in the wild reduced this number to 3.2 (Geiser & Drury, 2003). Therefore, ambient temperature is important to the energetics of torpor use.

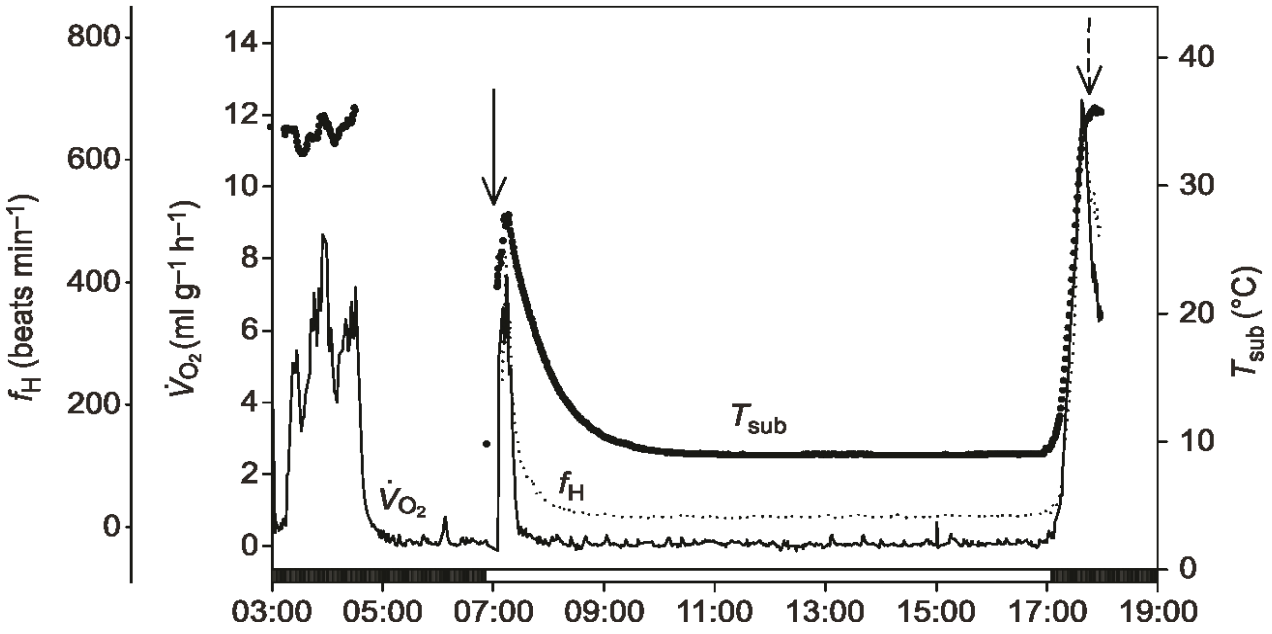

Figure 1: Heart rate (f_H), metabolic rate (expressed as oxygen consuption,V_{O2}) and subcutaneous temperature (T_{sub}) during a torpor-arousal cycle in Gould's long-eared bat (Nyctophilus gouldi) at 10 °C ambient temperature. Solid arrow denotes partial arousal in conjunction with attachment of EKG, dashed arrow denotes active arousal after dark. Note that metabolic rate is highest when exiting torpor. Figure after Currie et al., 2014.

Across bat species, both daily torpor and hibernation, prolonged torpor bouts interspaced with brief arousals during winter, are prevalent (Ruf & Geiser, 2015). While metabolic rates during hibernation may be reduced to just a few percent of values observed during euthermia, there is an energetic cost to rewarming, and arousal can account for three quarters of the energy expenditure of an extended torpor bout (Thomas et al., 1990). Nevertheless, strategic use of torpor and hibernation may still reduce the net energetic cost of survival under adverse conditions, and it has been observed that bats live an average of three times longer than other mammals of comparable size and metabolic rate (Austad & Fischer, 1991). While this is undoubtedly in part due to their ability to fly and thus avoid most potential predators and environmental hazards, it has also been shown that life span is longer in hibernating than non-hibernating species (Wilkinson & South,

2002). While both food-storing and fat-storing strategies are used by mammals for torpor and hibernation, bats are fat-storing, meaning they are dependent on a relative abundance of food during their active season to prepare for the next hibernation period, and also need to be physiologically adapted to utilizing their fat stores optimally.

1.3 Stress and glucocorticoids

Stress is associated with several changes to an organism, both in hormonal balance, nervous activity and behavioral change. Of the hormones involved, there are two main classes, catecholamines, such adrenaline and noradrenaline, and glucocorticoids (GCs), such as corticosterone (the primary GC of reptiles, birds, and some mammalians, perhaps most notably in the common laboratory rat) and cortisol (the primary GC in most mammals). While all of these factors may be interesting to study, the focus of this research is on GCs. The reasons for this are that unlike catecholamine levels and nervous activity, GCs are easy to measure in a non-invasive way in wildlife. Further, both these measures provide data primarily on the stress the animal is experiencing at the time of sampling, which in a wild bat is likely to be heavily influenced by capture and handling. GCs instead provide data on more long-term stress, thus making it more relevant when it comes to establishing baseline values. Finally, GC measurements do not require the use of behavioral assays, which may be difficult to implement in the case of bats.

1.3.1 HPA-axis

Upon introducing an animal to a stressor, be it physical or psychological, the hypothalamic-pituitary-adrenal (HPA) axis is activated. Note that this is not necessarily a wholly reactive process, as stress can also be anticipatory (Monat et al., 1972). Here, corticotropin releasing hormone (CRH) is released from parvicellular neuronal projections from the paraventricular nucleus (PVN) of the hypothalamus into the hypothalamic median eminence (Pariante & Lightman, 2008). From here, CRF may enter the hypothalamo-hypophyseal portal vein system, allowing it to displace to the anterior pituitary via the blood. Acting upon corticotrope cells in the anterior pituitary, CRH stimulates the production of adrenocorticotropic hormone (ACTH) through the cleavage of proopiomelanocortin (POMC) into ACTH and β-lipotropin (the latter of

which is in turn modified into β-endorphin in a second cleavage process) (Guillemin et al., 1977; Lazarus et al., 1976). ACTH, in turn, enters the general circulation and upon arrival at the adrenal gland act on cells in the zona fasciculata of the adrenal cortex to stimulate increased levels of GC release. These GCs elicit a marked physiological response, regulating a variety of systems to promote gluconeogenesis and energy mobilization in face of the current (or impending) crisis, but also act on both the hypothalamus and the anterior pituitary to downregulate secretion of CRH and ACTH. Thus, blood GC concentrations are stabilized, and a disproportionally large stress response is prevented through negative feedback control (see Figure 2) (Keller-Wood & Dallman, 1984).

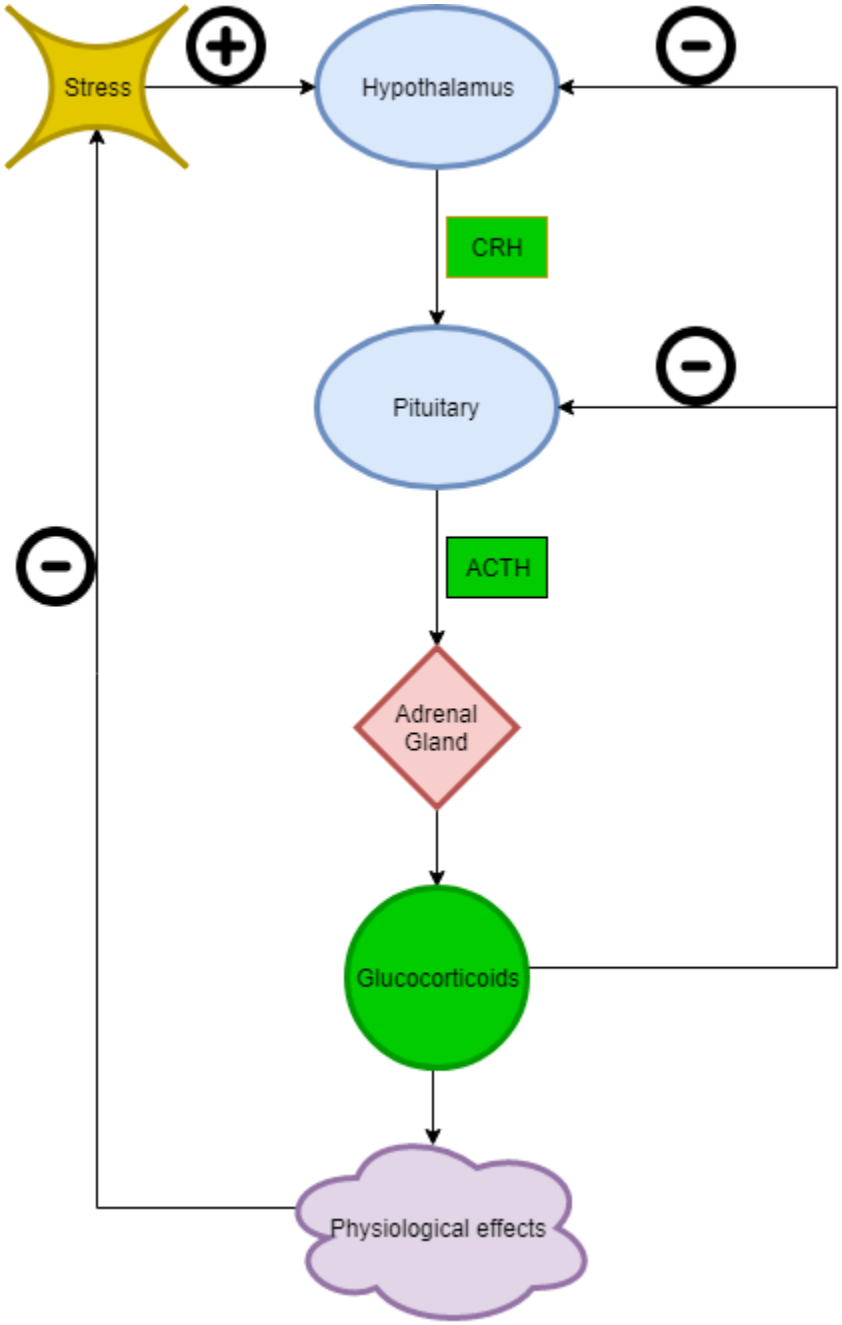

Figure 2: Schematic of the HPA axis. Brain structures denoted in blue, hormones in green. Enhancing and inhibitory effects are denoted by plus and minus signs, respectively.

1.3.2 Physiological effects

1.3.2.1 Pain suppression

The β-lipotropin produced concomitantly with ACTH is further cleaved into β-endorphin. β-endorphin's main role is as an analgesic, accomplished by its affinity to the μ-opioid receptor. Activation of this receptor causes synthesis of cyclic adenosine monophosphate (cAMP) to be inhibited, resulting in reduced signal transduction in spinal neurons associated with pain stimulus, as well as at sites of injury or inflammation (Dalayeun et al., 1993; Loh et al., 1976; Stein et al., 2003). Note that it has also been demonstrated that locally expressed (but not circulating) CRF also mediates opioid release at sites of inflammation, indicating that physiological stress can influence pain perception by several pathways (Schafer et al., 1996). The suppression of pain in stressful situations can be highly advantageous, as it can allow an injured animal to escape a predator or other dangerous situations despite its wounds.

1.3.2.2 Thyroid inhibition

High doses of glucocorticoids have long been known to be associated with reduced pituitary secretion of thyroid stimulating hormone (TSH) (Wilber & Utiger, 1969). The result of this inhibition is a decrease in the production of thyroid hormones, in turn inhibiting the effects these hormones normally have on various metabolic processes, such as their enhancing effect on carbohydrate metabolism through regulation of gene expression, their modulating effect on insulin activity (especially in the liver), and their role in thermogenesis in both white and brown adipose tissue (WAT and BAT, respectively), as well as in skeletal muscle (Mullur et al., 2014).

Early studies indicated that GC-mediated regulation of thyroid activity does not occur at the level of the pituitary, but at some higher center. Later studies have suggested that the mechanism of action involves GC-mediated downregulation of thyroid-releasing hormone (TRH, a hypothalamic hormone inducing pituitary release of TSH in much the same fashion as CRH induces release of ACTH) mRNA in the PVN, though it is unclear whether this effect is direct or indirect (Alkemade et al., 2005).

In the context of an acute stress, a downregulation of thyroid hormone can be adaptive, as it allows for shifting energy usage from long-term growth processes to more immediately relevant muscular action. In the long term, however, lowered thyroid levels would cause fatigue, heart rate reduction, muscle stiffness, or, in more severe cases, myopathy (Fessel, 1968; Golding, 1970).

In the seasonally hibernating little brown bat (*Myotis lucifugus*), it has been observed that the follicular epithelial cells of the thyroid (where thyroid hormone is produced) atrophy substantially during hibernation. While this seems reasonable, given that metabolic activity in this state is lowered significantly, it was also observed that post-arousal return to normal thyroid state could take as much as 80 days (Nunez & Gersohn, 1972). Presumably, the tissue would have reduced capacity for hormone synthesis in this period. It is unclear whether this prolonged regenerative period occurs under natural conditions, or if it was caused by stressful conditions induced by the laboratory setting. If it is the former, it would seem bats should have a reduced capacity for growth after a period of hibernation. If the latter is the case, the implication seems to be that exposure to severe stressors in the period following arousal would greatly reduce the capacity of the bats to upregulate their metabolic processes, which is in turn likely to negatively impact their reproductive output later in the season. If this happens throughout the system to all bats coming out of hibernation, it is likely that the presence of chronic stressors will cause a population decline, or, if certain individuals are more resistant to the stressor in question, a shift in the genetic composition of the population.

1.3.2.3 Effects on growth hormone

Growth hormone (GH), as released from the pituitary in response to growth hormone releasing hormone (GHRH), normally induces local production of somatomedins, such as insulin-like growth-factors (IGF) 1 and 2, which in turn promote cell growth, proliferation and repair (Thakore & Dinan, 1994). However, it has also been demonstrated that GH can act to enhance cell proliferation in the growth plate germinal zone even in the absence of IGF-1, though it is not yet clear if this is caused directly by GH, or if the process is instead mediated by IGF-2. (Le Roith et al., 2001). Further, GH deficiency has been shown to cause not only the expected stunting of growth, but also accumulation of adipose tissue, a condition that can be reversed with

the administration of exogenous GH, indicating that the hormone also has some lipolytic properties (Russell-Jones et al., 1993).

The mechanism of action for an increased GH release seems to be that GCs increase the number of GHRH receptors, though there is no apparent effect on the sensitivity of these receptors by GC. Further, it has been shown that GC injection increases the amount of cAMP second messenger produced by binding of GHRH to its receptor, thereby causing an increase in GH production (Michel et al., 1984; Thakore & Dinan, 1994).

It has long been apparent that with long-term exposure to elevated GC levels, somatic growth tends to be inhibited (Ingle et al., 1938). Given the above enhancing effects of GCs on GH secretion, this might seem counter-intuitive, but *in vivo* studies have determined that the net effect of GC stimulus on growth is dependent on dose and duration (Giustina & Wehrenberg, 1992; Mazziotti & Giustina, 2013). Whereas a short exposure to GCs can, indeed, cause a GH surge, longer exposure instead causes GH levels to drop. It is noteworthy that, in humans, the administration of high doses of GC initially causes an increase in GH secretion. If GC levels remain high, GH secretion is instead decreased (Thakore & Dinan, 1994). However, this does not appear to be the case in rats, where instead, GH secretion is immediately decreased and remains so until GC levels have normalized, thus indicating some species-specificity to this phenomenon (Wehrenberg et al., 1990). As outlined above, the initial GH surge, if present, is caused by an increase in the GH-secretory response to a given amount of GHRH at the level of the pituitary, but simultaneously, GCs also elicit effects at the level of the hypothalamus. In addition to the promoting effects of GHRH, GH secretion can also be inhibited by growth hormone inhibiting hormone (GHIH) by reduction in GH second messenger activity and reduction in intracellular calcium concentration (Borgeat et al., 1974). GCs have been demonstrated to be able to increase the tone of GHIH, thereby resulting in a significant net reduction in amount of GH secreted (Wehrenberg et al., 1990).

What is the adaptive advantage of having a dual and opposed response to the same stimulus? As previously mentioned, the effect of GH is twofold; promotion of growth and lipolysis. In the context of an acute stressor, it is advantageous to have an increase in lipolytic action, as this

ensures heightened levels of circulating nutrients. However, it is also adaptive to temporarily postpone processes that are not essential to immediate survival, such as growth. Hence, we see that the initial upregulation and subsequent downregulation of GH in response to GC exposure is adaptive, provided that the stressor eventually goes away. In the event of the stressor being chronic, the continued reduction in GH levels will eventually inhibit growth through reduced production of growth factors and promote adiposity due to decreased lipolytic activity.

It is unknown whether bats have an initial GH increase in response to transient stressors, or if they go directly to GC-induced GH depression, though it seems reasonable to believe that more chronic stressors will cause an eventual GH decrease, and in turn slow down growth and other GH-dependent processes. This is perhaps most critical for juveniles, as failure to grow normally may interfere with their capacity to prepare for winter hibernation. Inability to successfully hibernate and arouse appropriately is likely to be fatal and would be damaging to population recruitment.

1.3.2.4 Glucose and insulin

Because of the nature of the situations during which GC secretion is enhanced, it is no small wonder that these hormones have a substantial effect on the metabolization pattern of all three classes of macronutrients. In the case of carbohydrates, GCs act through a multitude of transcription-regulatory pathways to inhibit glucose uptake and utilization, and also to inhibit the synthesis of glycogen (Le et al., 2005). Further, GCs have an enhancing effect of the glycogenolytic actions of catecholamines, with the net result of increased hepatic gluconeogenesis and increased circulating glucose levels (Kuo et al., 2013).

Equally important, however, if not more so, is the pronounced effect GCs have on the pancreatic hormone insulin. Insulin mediates glucose uptake and glycogen synthesis in muscle and adipose tissue, but elevated GC levels will inhibit this effect (Ruzzin et al., 2005). The mechanism behind this seems to be that GCs inhibit the action of insulin receptor substrate-1 (IRS-1), a protein essential for signal transmission after insulin binds to its receptor (Morgan et al., 2009). The effect is further exacerbated by GC-mediated inhibition of pancreatic insulin secretion (Pivonello et al., 2010).

In the event of a transient stressor, an increase in gluconeogenesis to facilitate energy availability can be highly adaptive. However, continued hyperglycemia and insulin resistance are likely to be detrimental, and in fact, human studies have found strong correlations between insulin resistance and age-related diseases, such as hypertension, heart disease, and type 2 diabetes (Facchini et al., 2001). This is perhaps particularly interesting for bats, considering their long lifespans and high relative basal blood pressure (Maina, 2000). Further, we would expect it to impair their capacity for lipid storage, which would be detrimental to winter survival.

1.3.2.5 Lipid shift

As discussed above, GCs are able to influence lipid catabolism indirectly, through regulating GH levels. However, both *in vitro* and *in vivo* studies indicate that other pathways associated with lipolysis are also influenced by GC levels. For example, during acute GC exposure, it has been shown that both basal and catecholamine-induced lipolysis is decreased in the presence of insulin, while pre-exposure to GH instead causes GC to increase basal and catecholamine-induced lipolysis (Ottosson et al., 2000). Further, in the presence of GCs, levels of both adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) (enzymes that catalyze the first and second hydrolysis of triacylglycerides (TAGs), respectively) are increased (Slavin et al., 1994; Villena et al., 2004). Thus, it appears GCs are able to increase the turnover rate of lipids, and also modulate the sensitivity to other lipolysis-relevant stimuli. While fewer studies have examined the lipolytic effects of chronic GC exposure in healthy animals, the results seem to indicate that the enhancing effects do not last, at least not on a systemic level, though lipolysis may still be increased in subcutaneous adipose tissue (Gravholt et al., 2002; Johnston et al., 1982; Miyoshi et al., 1988).

The mechanism behind the lack of a long-term change appears to be that as the stressor persists, catecholamine levels fail to remain elevated, and the synergistic effect of these hormones with GC is lost. When combined with GCs effects on glucose balance and the induction of insulin resistance by high levels of circulating fatty acids, this causes a preferential hepatic release of TAGs and an upregulation of lipoprotein lipase, an enzyme that hydrolyzes TAGs in the bloodstream. Additionally, due to differing tissue-specific sensitivity to the various factors

involved, lipolysis is still induced in subcutaneous tissue. The net result of this is high circulating levels of free fatty acids. However, in response to insulin resistance, hyperinsulinemia is induced, resulting in a shift in turnover rates favoring the storage of lipids as TAG in and around internal organs (Macfarlane et al., 2008).

When exposed to an acute transient stressor, it is beneficial for an animal to have an increased level of circulating lipids, as these may be utilized either to replenish energy depleted by the metabolization of circulating glucose, or, to a lesser extent, to directly fuel metabolic processes. When the stressor becomes chronic, however, the net effect is a redistribution of lipid stores in favor of obese conditions. This has, in turn, been shown to impair the capacity of adipose tissue to respond in a normal fashion to post-feeding fluctuations in blood nutrient levels (Frayn, 2002).

When considering the implications GC-induced changes to lipid catabolism can have on bats, it is particularly noteworthy that part of the shift involves an increased storage of BAT, which is essential for thermogenesis in the context of torpor arousal (Shima et al., 1994). However, CGs also have the effect of inhibiting uncoupling protein-1 (UCP-1), which is essential for BAT thermogenesis to actually occur (Soumano et al., 2000). Thus, it seems exposure to chronic stressors would make bats particularly vulnerable during arousal from torpor in cooler conditions, when passive warming is not a viable option. If this results in an inability to arouse, the results may be fatal.

1.3.2.6 Muscle catabolism

In muscle tissue, high exposure to GCs has been demonstrated to cause atrophy of type IIb muscle fibers, but not type I or type IIa fibers (Dekhuijzen et al., 1995). Type IIb fibers are fast, glycolytic, and prone to fatigue during prolonged exercise, and are typically associated with maximum muscle force generation.

In general, proteins can be broken down by two pathways, depending on whether it is an intra- or extracellular compound. If the protein is extracellular (located in the interstitial fluid, not in the digestive tract), the process is achieved by pinocytosis or endocytosis, whereby the proteins in question are transported in a closed vesicle into the cell. Said vesicle then merges with a

lysosome, and the enclosed hydrolytic enzymes degrade the protein. If the protein is intracellular, however, such an indiscriminate approach would damage other cell components. Instead, intracellular proteolysis is typically handled by the ubiquitin-proteasome pathway. Here, a protein designated for degradation is tagged with multiple copies of the ubiquitin protein. The resulting polyubiquitin chain is then recognized by a proteasome complex, which selectively breaks the protein down, leaving ubiquitin to mark other proteins for destruction (Glickman & Ciechanover, 2002).

The mechanism of a GC-induced increase in proteolysis is that GCs alter the methylation state upstream of the gene coding for ubiquitin, resulting in increased transcription rates (though there is no apparent change in mRNA encoding components) (Löfberg et al., 2002; Marinovic et al., 2002). This increases protein breakdown rate. Furthermore, the previously mentioned GC-mediated insulin resistance also means that insulin's promotion of protein synthesis is diminished.

Flight muscle in greater tube-nosed bat (*Murina leucogaster*) contains no type IIb fibers (Kim et al., 2000), and the flight muscle of little brown bats have been found to be composed almost exclusively of high-oxidative types of muscle fibers, with no composition change during hibernation (Armstrong et al., 1977). As such, it seems unlikely that stress will impact flight capabilities by myopathy alone. However, the muscles involved in echolocation are extremely fast acting (Hoh, 2005; Revel, 1962). While it is unknown if this represents a novel isoform of the myosin heavy chain allowing for faster myosin-actin uncoupling rates (and thereby lower muscle refractory period), such isoforms are known to occur in ultrasound-emitting rats, and the potential uses in bat echolocation are readily apparent (Elemans et al., 2011). Unfortunately, it does not appear any studies have been done on the sensitivity of such muscle to elevated GC levels, though stress-related impairment of echolocation could have severe consequences for the capacity of bats to capture prey.

1.3.2.7 Bone remodeling

In bone tissue, high GC is associated with lowered bone mass and increased incidence rate of osteoporosis (Clowes et al., 2001). Biopsy data shows that this is caused by both increases in

bone resorption and reduced bone formation (Carbonare et al., 2001). The continuous remodeling of bone is mediated by two primary cell types: osteoblasts, which form new bone, and osteoclasts, which break it down. Unsurprisingly, given that GC affects both resorption and formation rates, pathways involving both of these cell types are GC targets.

Normally, osteoblasts and osteoclast exist in a partially self-regulating equilibrium, in that osteoblasts produce macrophage colony-stimulating factor (CSF-1) and receptor activator of nuclear factor κβ ligand (RANKL), both of which are required for osteoclast differentiation (Suda et al., 1999). However, in addition to binding to its osteoclast receptor, RANKL may also bond to the "decoy" receptor osteoprotegerin, meaning that even in the presence of CSF-1, osteoclastogenesis does not occur. GCs increase the expression of both CSF-1 and RANKL, while decreasing expression of osteoprotegerin, resulting in a net increase in the number of osteoclasts, thereby potentiating the capacity for bone breakdown (Hofbauer et al., 1999).

When it comes to GC effects on osteoblasts, the consensus on GC effects is more lacking. Some studies have found that GC treatment induces osteoblast differentiation, while others find the opposite (Bellows et al., 1987; Pereira et al., 2001). Regardless, GCs have been found to reduce the capacity of osteoblasts to synthesize the essential bone matrix component type I collagen, presumably by regulation of transcription (Delany et al., 1995). GCs have also been found to directly induce osteoblast apoptosis (O'Brien et al., 2004). Given the net bone loss observed in cases with long-term GC exposure, it seems likely that either osteoblast differentiation is reduced, or that the other effects on both osteoblasts and osteoclasts outweigh the increased bone formation from an increase in osteoblast differentiation.

While few (if any) animals would be expected to deal well with osteoporosis, bats may be particularly vulnerable, as their primary mode of locomotion (flying) is highly dependent on an intact set of wings. Weakening of the fingerbones, and subsequent breaking of these, is likely to have a severe negative impact on the bat's capacity for prey acquisition. We could further theorize that the hyoid bone, which aids in laryngeal movement and is thus important for echolocation, would likely be subject to considerable force during hunting, and therefore

susceptible to damage if it were in an osteoporotic condition. The impairment of echolocation following damage to this bone would further aggravate the issue of prey procurement.

1.3.2.8 Immunosuppression

In medicine, perhaps the most recognized role of GCs is as immunosuppressants, and many anti-inflammatory, anti-allergic and anti-autoimmune drugs utilize these effects with great success, albeit with the potential for side effects related to GC mechanisms already outlined here, such as osteoporosis, myopathy and hyperglycemia, among others (Moghadam-Kia & Werth, 2010; Schleimer, 1993).

However, it has also been observed that, at least in the short term, stress may enhance some aspects of immune function (Dhabhar & McEwen, 1996). This is because catecholamines, which are released during the initial phases of stress, stimulate cells in the spleen and lymph nodes to increase the production of cytokines, leukocytes, and antibodies, and also affects the distribution of these from blood to other cells (Dhabhar, 2009; Madden et al., 1995). However, this effect seems to later be depressed if the stress is chronic in nature (i.e., there is a shift from catecholamine-dominated action to GC-dominated action). Therefore, factors that are influential on the net effect of stress-mediated change include the duration of exposure, the effect on leukocyte distribution, and the timing of GC exposure relative to the time of immune response activation (Dhabhar & McEwen, 1999).

Some of the most important components of the mammalian immune system are the pro-inflammatory transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein-1 (AP-1) and nuclear factor of activated T-cells (NFAT). These are all activated in response to cytokine signaling, free radicals or bacterial or viral antigens. However, GCs downregulate the activity of these transcription factors, resulting in a reduced inflammatory response (Adcock & Caramori, 2001; De Bosscher et al., 2000; De Bosscher et al., 2003). The pathways for this inactivation may go by indirect routes. For example, in the case of NF-κB, GCs actions are not on the transcription factor itself, but instead cause enhanced synthesis of the inhibitory protein complex IκBα, preventing NF-κB from activating target genes (Auphan et al., 1995).

While nuclear transcription alteration is likely to have slow but long-lasting effects, GCs may also impact immunity in a more rapid fashion. It has for example been shown that GC treatment inhibits the activity of macrophages in a dose-dependent fashion in less than 30 minutes (Long, Wang, et al., 2005). Further, rapid GC-mediated changes to calcium influx (calcium being an important second messenger in many pathways) and phosphorylation state of mitogen-activated protein kinases can occur, at least *in vitro* (Qiu et al., 1998; Rider et al., 1996).

In a natural setting, selective enhancement of immune function in response to a stressor can be a good thing, especially if the stressor happens to be of a pathogenic nature. Further, it has been theorized that the subsequent inhibition of immune function serves to "rein in" the initial enhancing effects, so as not to induce an autoimmune state (Sapolsky et al., 2000). However, in prolonged stressful situations, particularly ones that increase exposure to pathogens, a reduced immune response would likely be detrimental. In bats, such a situation may occur in the context of a crowded roost site, and it could be further aggravated if there were other factors that contributed to heightened stress levels, such as pregnancy or maternal care.

1.3.2.9 Effect on HPG axis

The hypothalamic-pituitary-gonadal (HPG) axis is responsible for stimulating synthesis of reproductive hormones in both males and females. Gonadotropin releasing-hormone (GnRH) is released from GnRH neurons scattered throughout the hypothalamus, and after release into the median eminence travel through the hypothalamo-pituitary portal vein system. In the pituitary, GnRH stimulate gonadotropic cells to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These, in turn, migrate through the bloodstream to the gonads, where the final effect depends on the type of gonad in question (see Figure 3). In males, LH stimulates Leydig cells to produce testosterone, while FSH stimulates Sertoli cells to perform spermatogenesis (and production of inhibin, a hormone that elicits negative feedback control on FSH production the pituitary). In females, LH acts on follicular theca cells to promote the production of androgens, which may in turn be converted into estrogens (specifically 17β-estradiol). A surge of LH precedes and triggers ovulation, and the hormone is vital for the maintenance of the early maintenance of the corpus luteum. FSH in females stimulates follicular recruitment and growth,

and also the follicular granulosa cells that convert androgens to estrogens (and produce inhibin). Note that LH and FSH are released in a pulsatile manner, and which hormone is primarily released depends on the amplitude and frequency of the GnRH signal, with LH being secreted preferentially during high amp/high frequency GnRH stimulus, and FSH being secreted preferentially during low amp/low frequency GnRH stimulus (Davies & Norman, 2002).

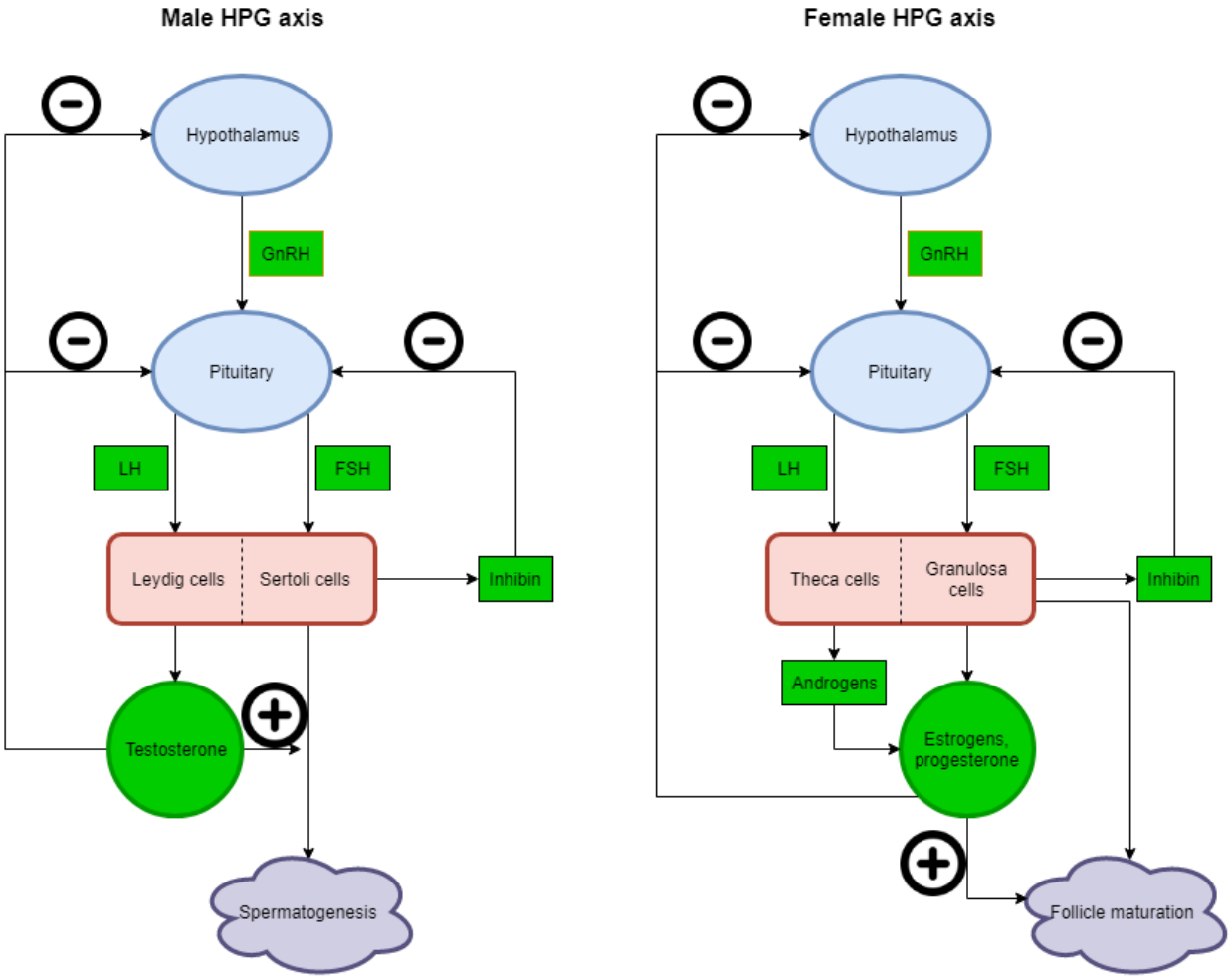

Figure 3: HPG axis in males and females. Brain structures denoted in blue, hormones in green. Gonadal cells denoted in red. Enhancing and inhibitory effects are denoted by plus and minus signs, respectively.

It has long been accepted that stress causes a reduction in both reproductive output and amount of circulating sex hormones (Wingfield & Sapolsky, 2003). Further, it has been shown that GC

administration alone can cause this decrease (Cunningham et al., 1975). Thus, it has been assumed that GCs elicit some inhibitory effect on the HPG axis.

Studies have found that GC inhibits GnRH synthesis in hypothalamic neurons by transcriptional regulation (Chandran et al., 1994). However, it has also been shown that GC can upregulate the expression of GnRH receptors in the pituitary, which should increase sensitivity to this hormone (Kotitschke et al., 2009). *In vitro*, it has been demonstrated that GC treatment causes a decrease in secretion of LH, but an increase in the secretion of FSH (Suter & Schwartz, 1985). It also appears that the magnitude of the LH decrease is sex-dependent, with males being more responsive (Stackpole et al., 2006). However, studies in human males show that, while testosterone levels remain very low during chronic stress, LH levels are normalized (Bernton et al., 1995). Thus, it seems likely that GCs may also have some more direct effects on the gonads. In males, several studies have shown GC-mediated upregulation of Leydig cell apoptosis, which would help explain this observation (Gao et al., 2003; Yazawa et al., 1999). Similar reduction in sex steroid production has been observed in ovarian cells, both by direct LH inhibition and by reduction of FSH-mediated aromatase activity (Hsueh & Erickson, 1978; Michael et al., 1993). However, GC treatment increases FSH-mediated progesterone production, probably through a combination of increased production and reduced metabolization. Furthermore, many of the processes of the ovaries are mediated at least in part by GC fluctuations, specifically by regulation of expression of hydroxysteroid dehydrogenases vital to normal oocyte development (Whirledge & Cidlowski, 2010). Excessive disturbance of this fine balance is likely to be detrimental to follicle maturation, but also to estrogen-induced uterine growth and to implantation (Johnson & Dey, 1980; Velardo et al., 1956). It is also worth noting that estrogen has inhibitory effects on bone resorption, meaning that if LH and FSH balance is sufficiently disturbed by high GC, females (especially post-menopausal) become particularly vulnerable to osteoporosis (D'Amelio et al., 2008).

Of further interest is the fact that GnRH pulsatile activity can be disrupted by the proinflammatory cytokine tumor necrosis factor α (TNF-α) (Yoo et al., 1997). TNF-α is released in large quantities during the early phase of infection, and the inhibitory effect on GnRH pulsatile activity is mediated by brain prostaglandins (PGs) (Harris et al., 2000). However, it has been

shown that the effect of TNF-α is enhanced after an adrenalectomy, and further that administration of GCs in adrenalectomized rats brings the inhibitory effect back to "normal" levels (Matsuwaki et al., 2004; Matsuwaki et al., 2003). As such, it would appear that GCs in fact have a protective effect on the normal function of the HPG axis and on reproductive function. Work by the same group has further indicated that the mechanism of action seems to be that GCs inhibit PG synthesis (Matsuwaki et al., 2006). Further, they have demonstrated that not all species of PG have an inhibitory effect, and also that at least one PG species can either inhibit or enhance LH pulsatility, depending on the presence or absence of a second PG species (Matsuwaki et al., 2017). Evidently, more research is needed on whether GCs specifically target PGs involved in gonadotropin pulsatility.

In a stress context, temporarily reducing reproductive investment in order to promote individual survival is probably adaptive. If, as described above, GCs simultaneously preserve the essential pulsatile gonadotropin activity required for normal oocyte development in females, this is also beneficial. It is not, however, known if this protective effect remains effective (or even active) during chronic stress. Further, as mentioned earlier, it is well established that chronic stress is associated with reduced reproductive output, though isolating this net effect to modulation of the HPG axis would be erroneous, as it is also dependent on stress-related changes to behavior and fetal development.

1.3.2.10 Prenatal stress effects on offspring

As previously noted, stress can have a pronounced effect on reproductive capacity in that the HPA axis can modulate the HPG axis prior to mating or implantation, and has also been demonstrated to increase the incidence rate of spontaneous termination of pregnancy (García-Ispierto et al., 2006). However, it has been shown that should the young survive, maternal stress can also have lifelong consequences for offspring.

Maternal stress is associated with both preterm birth and reduced birth weight (Dole et al., 2003; Rondó et al., 2003). Low birth weight, in turn, is associated with increased adult blood pressure, insulin resistance, and risk of cardiovascular and metabolic disorders (Gennser et al., 1988; Nobili et al., 2008; Phillips et al., 1994; Rich-Edwards et al., 1997). Further, maternal stress is

associated with decreased feedback inhibition of the HPA axis and prolonged elevation of GC levels following stress in offspring (Weinstock, 1997). It has also been demonstrated that maternal treatment with high doses of GC late in pregnancy is sufficient to induce these effects (Levitt et al., 1996; Nyirenda et al., 1998).

The mechanism behind modulation of the HPA axis seems to be that early exposure to GCs reduces fetal expression of the gene coding for the two main glucocorticoid receptors, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), both in the hypothalamus and the hippocampus (Barbazanges et al., 1996; Weinstock et al., 1992). These receptors mediate most GC actions, and reduced expression at the level of the hypothalamus and hippocampus is likely to reduce the capacity for GC-induced negative feedback on hypothalamic CRH secretion. It has also been observed that maternal stress tends to exert a greater effect in females than in males (McCormick et al., 1995). Whether this is the result of the sex-specific variance in whether GR or MR is most affected by maternal stress, or if it is because the extent of GC transport across the placenta is different in male and female fetuses, or if it is a combination of the two remains to be elucidated (Kapoor et al., 2006; Montano et al., 1993).

In a natural setting, the apparent offspring sensitization to stress that occurs during late-pregnancy maternal stress is most likely adaptive, as it represents a way to prepare the offspring for the risks of the environment it is being born into. However, if the magnitude of the stressor is great, or if the signal is not representative of what actually occurs in the environment, the fetus may maladapt, as has been observed in humans undergoing their final trimester during the Dutch hunger winter in 1944/45 (and also, to a lesser extent, in their offspring). Here, there was a sudden and massive decrease in food availability, followed by an equally sudden normalization. Individuals from this cohort that were third trimester fetuses during this period exhibit increased risk of diabetes in adulthood, a trait not observed in the Leningrad cohort the same year, where food availability was equally low, but it remained so after the children were born (Lumey, 1998). The implication of this is that in the long term, stressing of pregnant mothers is likely to cause sensitization and HPA dysregulation in the entire population, resulting in heightened mortality and reduced reproductive output by the mechanisms outlined earlier in this section.

1.3.3 Glucocorticoid transport and elimination

1.3.3.1 Transport

GCs are steroids, and thus have low water solubility. Therefore, in order to be efficiently distributed to tissues, they must transiently bind to transport proteins. In mammals, there are two transport proteins of note in this context; corticosteroid-binding globulin (CBG) and albumin. Albumin is the most abundant blood hormone in vertebrates, serving to maintain colloid blood pressure and transport a variety of blood-borne compounds (Peters Jr, 1985). Its binding capacity for GC is relatively high, though its affinity is low. In contrast, CBG has a high affinity to GCs, but a low capacity. In spite of this, CBG remains the main GC carrier protein, with a study across seven mammalian species finding that the CBG-bound fraction GCs ranged from 67-87%, albumin-bound fraction varied between 7-19%, and the free fraction ranged from 6-14%. Note that in this study, it was determined that in squirrel monkeys, CBG-bound fraction was found to be 0% (Gayrard et al., 1996). While the prevalence of this phenomenon is not fully mapped, one study has also shown that there is no circulating CBG in the blood of vampire bats (*Desmondus rotundus*), but it was present in all the other species of bats in this study, including vesper bats (Kwiecinski et al., 1987). Thus, we presume that the *Myotis* bats in this study most likely utilize CBG as their main GC transporter.

While GC is bound to one of its transporters, it can move freely through the circulation. However, during transient stress, the synthesis of GC overshoots the binding capacity of CBG, resulting in a disproportionate increase in circulating GC (Richard et al., 2010). The consensus has long been that it is biologically active only in its unbound state (known as the “free hormone hypothesis”) (Mendel, 1989). However, this view has been challenged on the basis that albumin has such a low affinity for GCs (i.e., the association/dissociation rate is very high) that the albumin-bound fraction should also, for practical purposes, be considered “free” (Malisch & Breuner, 2010; Tait & Burstein, 1964). This second view is supported by the observation that hepatic GC uptake in humans is approximately three times larger than free plasma GC levels (Levine et al., 2007). Therefore, it seems likely that the low-affinity binding to albumin is

important for the sake of efficacy, as it can continuously associate/dissociate with the excess GC secreted during stress, ensuring that the hormone moves to and affects target tissues more rapidly.

1.3.3.2 Elimination

The primary elimination pathway for GCs is by enzymatic breakdown in the liver, and subsequent excretion through the bile (Taylor, 1971). While this allows most GC metabolites to be taken up across the intestine and passed through the kidneys to the urine, a portion are inevitably eliminated through the feces. Note that it is possible for GCs to be processed in this fashion several times (i.e., liver → digestive tract → liver → digestive tract, etc.), meaning that several species of urinary and FGMs can arise (Palme et al., 2005). In addition to these primary pathways, some circulating free GC may also be excreted through the saliva (though along the way it is converted to inactive cortisone) (Kirschbaum & Hellhammer, 1994; Smith et al., 1996). Some will also be incorporated into hair, feathers or scales, either directly through the blood supply, or through secretions from local glands (Cone, 1996). Further, it has been shown that hair cells may to some extent be capable of their own GC synthesis (Sharpley et al., 2009). As such, the dynamics of hair-related GCs is complex, and to date, no comprehensive overview of the individual contributions of the various inputs on total GC content of hair exists.

1.4 Study objective

The purpose of this study was to examine the variation of GC levels in two species of Norwegian bats over the course of a summer, and to ask the following questions:

1) is there a variation in GC levels over time or between different physiological states,
2) what are the likely causes of this variation,
3) what are the likely consequences of this variation,
4) based on the above, are there specific situations or time periods where bats are more or less vulnerable to additional external stressors, and how may this knowledge help guide conservation efforts.

2. Methods

2.2 Study site

Samples were collected around Nittedal, Akershus, Norway (see Figure 4) from June to August of 2018. This area is a north-south oriented river valley where primary habitat types are mixed with coniferous forest along the valley sides, and farmland at the bottom of the valley. Agricultural plots are often separated by forested corridors, granting bats a mostly sheltered approach to the river, though other sources of drinking water may be found throughout the area.

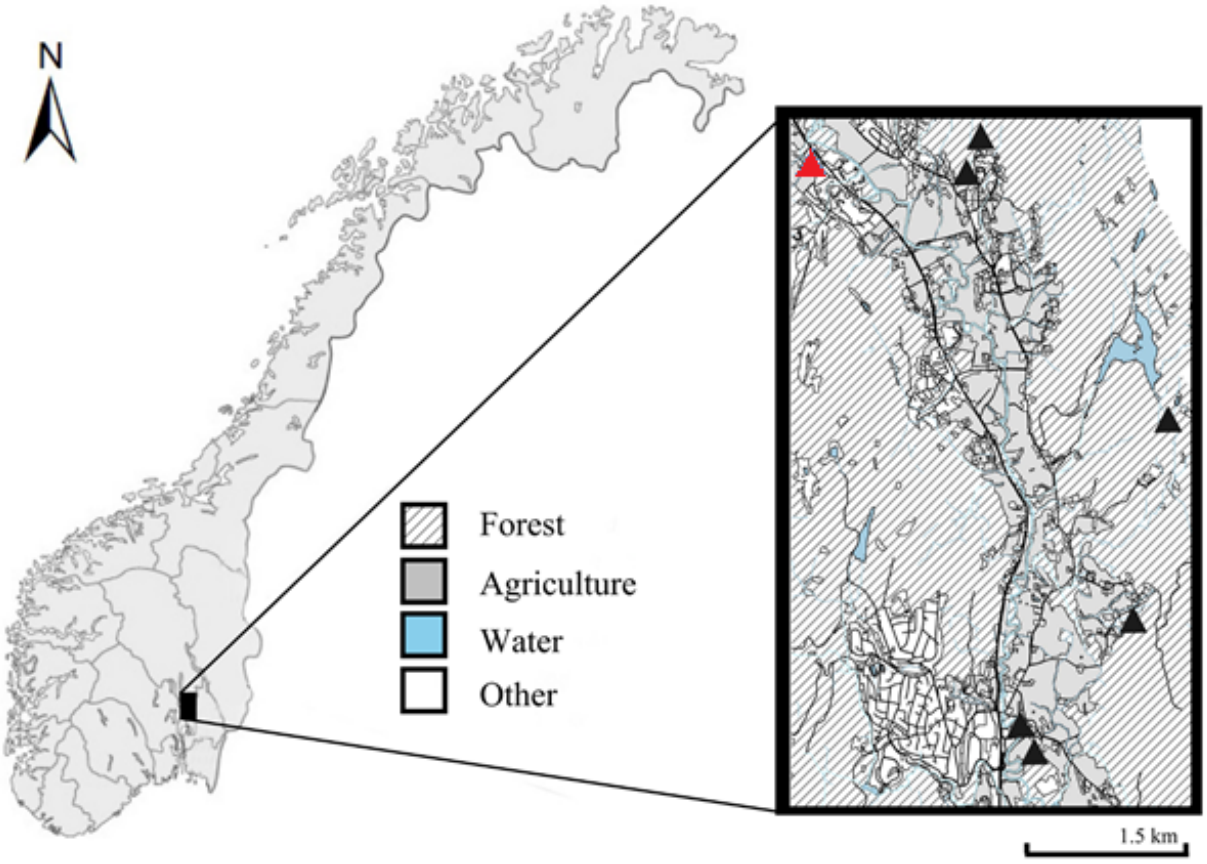

Figure 4: Map of the study area in Nittedal. Capture sites are denoted with black triangles. Red triangle denotes Hakadal målestasjon. Figure by Rune Sørås, with permission.

2.3 Study animal

Whiskered bat (*Myotis mystacinus*) and Brandt's bat (*Myotis brandtii*) are two species of indigenous Norwegian vesper bats, both being insectivores that form maternal colonies in the spring after arousing from hibernation. Mating occurs in fall, and sperm may be stored for several months before fertilization occurs upon arousal (Racey, 1979; Wang et al., 2008). Both species are cited in literature to weigh between 4 g and 8 g (Schober et al., 1997). In the observed data, body mass ranges from 3.8 g to 9.7 g, with a mean of 6.1 g. Forearm length in both species varies between 31 mm and 39 mm. While the species are morphologically very similar (to the point where *M. mystacinus* was only distinguished from *M. brandtii* in 1970), they differ in penis morphology and dentition, with the protocone of P4 in the upper jaw being larger than P3 in Brandt's bat, while the same protocone is smaller than P3 (or absent) in the whiskered bat (see Figure 5). While some later captures were juveniles, only data from adult females are incorporated into this study.

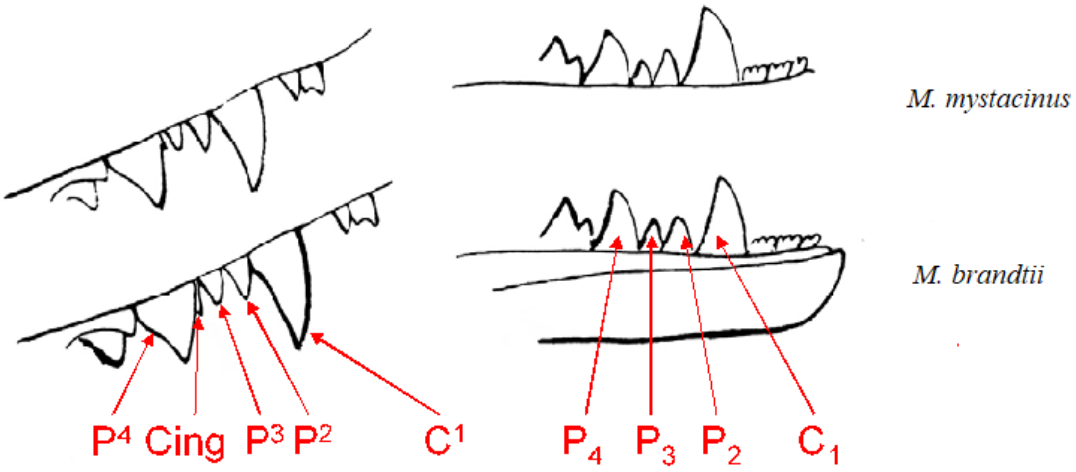

Figure 5: Dentition of M. mystacinus and M. brandtii. P4 protocone denoted as "Cing." Figure modified from Dietz & von Helversen, 2004.

2.4 Capture, sampling and storage

Bats were captured using mist nets, either upon emergence from or return to the roost site, or during foraging. A total of 126 animals were captured during the season, 78 of whom gave samples. Of these, 24 were *M. brandtii*, 44 were *M. mystacinus*, 8 were brown long-eared bats (*Plecotus auritus*), and 2 were northern bats (*Eptesicus nilssonii*). The latter two groups were excluded from this study on account of low sample sizes. Full roost emergence and return captures were accomplished once per month (June-August) for M. mystacinus, for a total of three large capture events, while the same was accomplished only once for *M. brandtii*. The reason for this discrepancy was that maternity roost colonies dispersed to varying degrees over the course of the season, with the effect being most pronounced in *M. brandtii*, to the extent that attempts at large-scale captures near the original site failed. While individuals were captured in the general area and radio-tracked for a different project, new roost sites were unstable, and capture near any of them was not feasible due to the lack of stable emergence and return corridors and out of consideration for the nearby human inhabitants. While the adult female *M. brandtii* samples were analyzed, these data are excluded from this study because no data from later months exists. Thus, only the *M. mystacinus* data are considered here. The numbers of both adult male and juvenile *M. mystacinus* were low (n = 11 and n = 2, respectively) and temporally stratified (adult males were captured primarily in July-August, juveniles only in August), and were therefore also excluded. Of the remaining 31 adult female *M. mystacinus* samples, 4 were sacrificed to establish sample handling methodology and to determine dilution concentrations for enzyme assay. Thus, the final sample size in this study was 27, see Table 1.

Table 1: Capture dates and numbers of this study, including number of bats of different reproductive states in each capture. Number of bats with a given reproductive state captured indicated in separate columns.

Capture date	Total bats captured	Pregnant bats	Post-lactating bats	Bats with no indication of reproductive state
11.06.18	10	10	0	0
13.07.18	1	0	0	1
14.07.18	3	0	0	3
15.07.18	1	0	1	0
16.07.18	8	0	6	2
20.08.18	4	0	4	0

Initially, I attempted to take salivary samples using a Micro•SAL™ Small Animal Saliva Collection Device (Oasis Diagnostics, Vancouver, WA, USA), but while it was possible to modify this device to fit into the bat's mouth, salivary flow proved to be insufficient to obtain the required volume of sample. I would like to stress that this is a constraint due to the size of the bat, not a flaw of the device, and the same procedure might still be feasible with a larger bat. I thus opted to instead measure fecal glucocorticoid metabolites (FGM). Fecal matter was obtained by putting the bats in cotton bags upon capture and leaving them inside for a span of time (ranging from ~10 min to ~2 hours, depending on volume of captures). While this period was not standardized nor adequately recorded, bats were put in bags labeled numerically in ascending order, meaning one could feasibly use bag number as a proxy for holding time. Correlation tests showed that there was no correlation between amount of feces and bag number (cor = 0.2974, p = 0.1319, n = 27) and further that there was no correlation between bag number and obtained cortisol values (cor = 0.2076, p = 0.2989, n = 27). Thus, I assume that holding time had no impact on results. Biometrics, including species, sex, body mass, forearm length, injuries, age (adult or juvenile) parasite loads (fleas, lice, mites of genus *Spinturnix* and other mites quantified separately) and reproductive state (if female) were then taken. Reproductive states were quantified according to criteria detailed in the field guide *Bats of Britain and Europe* (Dietz & Kiefer, 2016). Note that the initial quantification indicated that none of the bats captured in June were pregnant. However, this represents a discrepancy with the observation that almost all bats

captured in August were post-lactating, and also the reproductive strategy of this species. As it can be difficult to establish with certainty that a bat is pregnant without the use of hormonal tests or ultrasound, it is therefore assumed that data obtained in the field may be erroneous, and all captures in June were redefined post-hoc as pregnant.

Any fecal pellets in the bag were preserved in plastic tubes on ice in the field and then stored in a freezer (-23.34°C) at the earliest convenience (typically within a few hours). GC metabolites have previously been demonstrated to be stable at this temperature (Hunt & Wasser, 2003). If the bat did not defecate in the allotted time, it was assumed that it had voided its gastrointestinal tract before capture, and it was released without obtaining a sample. At the end of the field season, samples were transported by car from Nittedal to Trondheim on ice (temperature recorded during transport reached a maximum peak at 14°C, with a mean of 5.49°C across four iButtons (iButton DS1921G, Maxim Integrated Products, Sunnyvale, CA, USA), see Figure 6, where they were again stored in a freezer (-22.43°C) until analyzed.

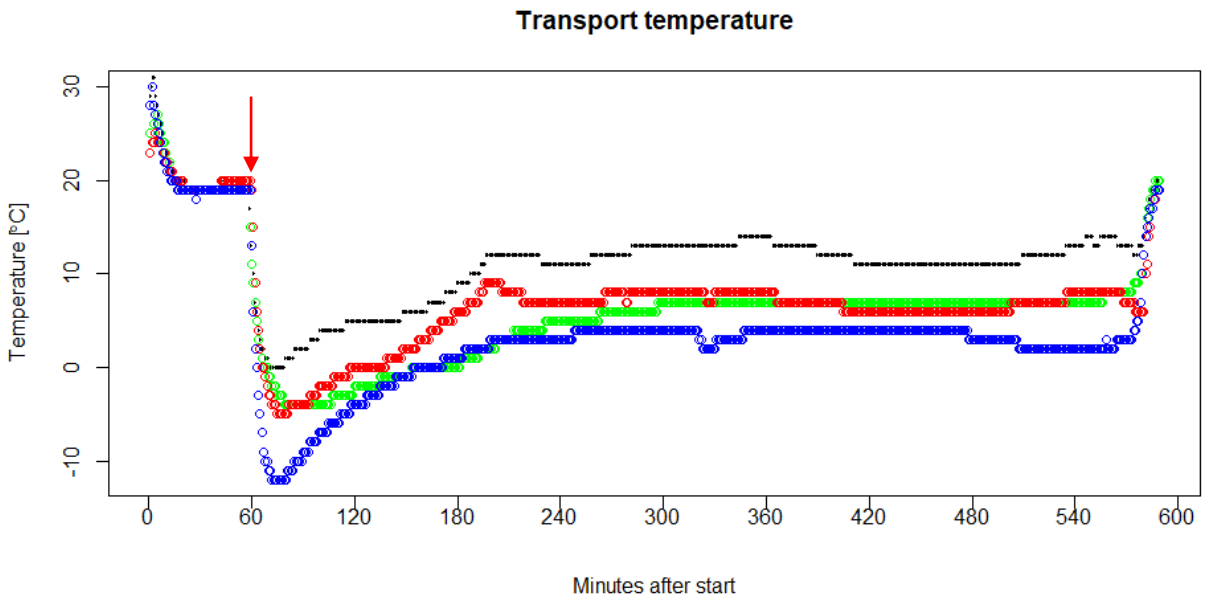

Figure 6: Temperature during transport, as measured by four iButtons at different places in the storage unit (addition of iButtons to unit denoted by red arrow). Final temperature elevation coincides with sample removal and transfer to freezer. iButtons were allowed to calibrate for one hour before being put in the cooler unit.

2.5 Temperature

Transport and storage temperature were measured using iButtons at a sampling frequency of once every 100 seconds, with a resolution of ± 0.5°C. Temperature data were graphed and an average from a stable period was taken. Attempts were made with using the same type of device to measure roost site temperatures throughout the season. However, iButtons hung on the outside of the roost had a tendency to be inadvertently exposed to sunlight (the setup was that the iButton was attached inside a paper cup, which was then suspended upside down, primarily somewhere it would **not** be exposed overmuch to sunlight, but the device proved susceptible to wind-manipulation, and frequently had to be picked out of roof gutters and the like). iButtons placed inside the roost were limited in that they could only be placed very close to the entrance (as the devices need to be recovered to extract data), and the temperatures recorded here were not necessarily representative for the places the bats normally stay at during their inactive hours. Also, the recorded temperature fluctuations were very large, on some days exceeding 70°C, possibly indicating that the iButton was subjected to heat radiation from the sun-exposed roof tiles, I cannot ascertain would be the case for the bats. Because of these shortcomings, temperature data from a nearby weather station (Hakadal målestasjon, red triangle on Figure 4) were used instead (downloaded from eKlima.met.no).

2.6 Competitive enzyme-linked immunosorbent assay (ELISA) principle

The basic principle of an enzyme-linked immunosorbent assay (ELISA) is that the compound of interest (acting as an antigen) is chemically bound to highly specific antibodies. While several types of ELISA tests exist, such as direct, "sandwich" and reverse methods, the methodology used here is the competitive method. Here, a sample containing antibodies (Ab) are mixed with a solution of antigen (Ag_s), and the two bond to one another:

$$Ag_{s(aq)} + Ab_{(aq)} \leftrightarrow Ag_sAb_{(aq)} + Ab_{(aq)}$$

The solution is then added to a well coated in the same antigen (Ag_w), in a fixed number of binding sites. The antigen will competitively bond to antibody in the sample:

$$Ag_sAb_{(aq)} + Ab_{(aq)} + Ag_w \leftrightarrow Ag_sAb_{(aq)} + Ag_wAb$$

Thus, the amount of Ab at any time bound to Ag_w is dependent on the amount of Ab in the sample (note that depending on manufacturer, the situation is sometimes reversed, with antibodies being present in the it and sample antigen being the measured variable). A secondary enzyme-linked antibody is then added, bonding to the Ag_wAb-complex. A mild detergent is then applied, washing away any Ag_sAb (and any free Ag_s or Ab, though if Ab is able to saturate both Ag_s and Ag_w that would mean that the sample is too concentrated and should be diluted before analysis), leaving only Ag_wAb complexes. A substrate is added to convert the enzyme bound to the secondary antibody, so that it either attains a specific color or fluorescent property. This output can then be measured, giving a quantitative output (absorbance or fluorescence) proportional to the extent of binding. The actual amount of compound in the sample can then be determined by comparing obtained values with values from a simultaneously obtained curve based on standards of known concentrations (see Figure 7) (Engvall & Perlmann, 1972).

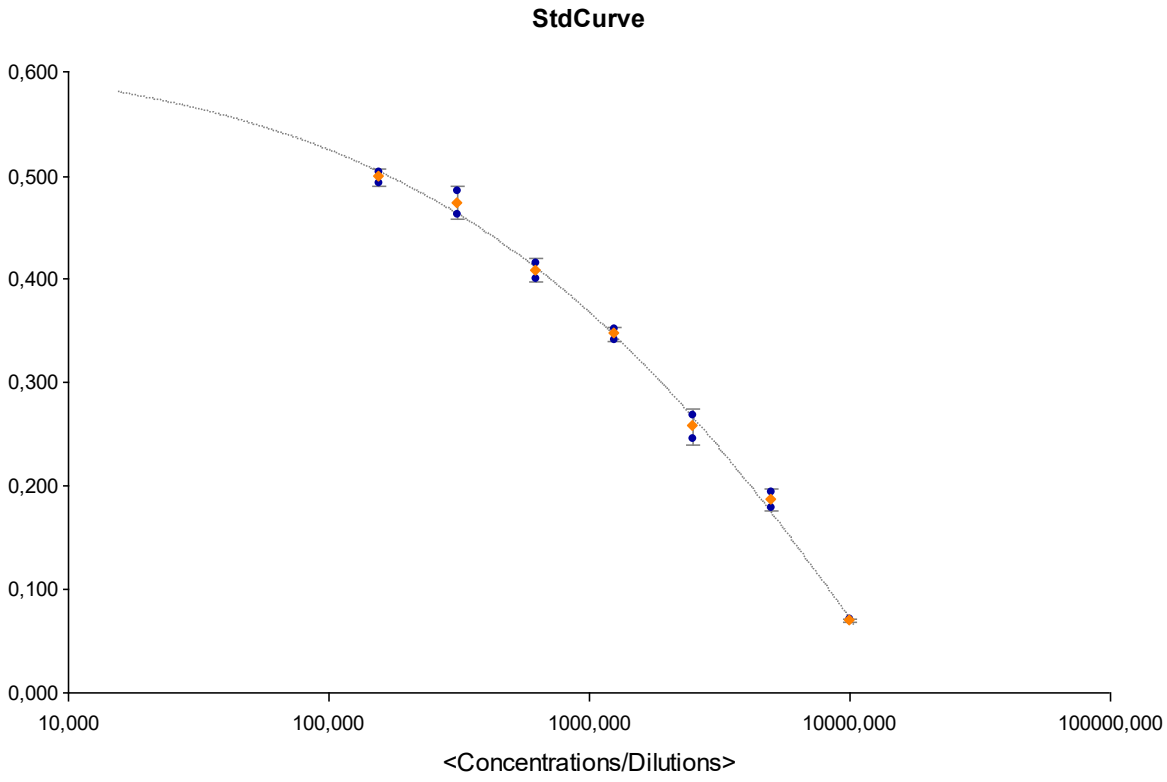

Figure 7: Example of an obtained standard curve. Samples will have an absorbance value (y-axis) corresponding to a cortisol-concentration (x-axis).

2.7 Cortisol ELISA

Fecal GC metabolite concentrations were determined using a commercial ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA). This kit type has previously been validated for use with fecal GC metabolites (Cinque et al., 2017). A fecal sample of 0.05 g was crushed with a set of forceps in order to maximize surface area for extraction. Any samples weighing below 0.05 g were then increased to this weight using distilled water (for samples weighing more than 0.05 g total, fecal matter was removed until weight was within 0.001 g of 0.05 g). Samples were then submerged in 1 ml methanol (82.5%) and incubated for 15 minutes at room temperature on a rotary shaker (Grant-Bio PS-3D Sunflower Mini-Shaker). Then, samples were centrifuged at 3000 rpm for 3 minutes (Eppendorf MiniSpin Plus) before being incubated in a refrigerator (3.74 °C) for 12-14 hours. Upon completion of the incubation period, samples were again centrifuged at 3000 rpm for

3 min, and 200 µl of supernatant was extracted. This supernatant was then diluted in a 1:1 relationship with distilled water and mixed in a centrifuge at 1500 rpm for 3 minutes. This dilution factor was determined experimentally, by testing supernatant:water relationships of 0:1 (undiluted sample), 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 in duplicate for a sacrificial sample, and then comparing these to a simultaneously obtained standard curve. The resulting data showed that a 1:1 dilution would give absorption values close to the center of the curve, and thus a minimal change in sample cortisol levels would be required to be detectable.

This kit uses the basic principle of ELISA by use of a well-bound monoclonal cortisol antibody binding competitively to free cortisol (or cortisol covalently bonded to an alkaline phosphatase) in a sample. Bound fraction was measured by absorption using a plate reader (Cytation 5 Cell Imaging Multi Mode Reader, BioTek Instruments Inc., Winooski, VT, USA) at 405 nm (no wavelength correction) with 8 measurements per data point. Sample concentrations were obtained using a simultaneously generated seven-point standard curve. All samples and standards were run in duplicate.

2.7 Statistics and graphics

All statistical analyses were performed in RStudio version 1.1.456 for Microsoft Windows (R Core Development Team, 2018).

Pearson's product moment coefficient (PMCC) was used to test for variable and parameter correlation, where applicable. A chi-square test was used to determine if date and reproductive state were independent. A paired t-test was performed when comparing 2018 temperatures to normal. The significance level was set to $p < 0.05$. All values are presented as parameter estimates ± standard error.

Because the data contains both fixed and random effects, and because of scarcity of data, a nonlinear mixed effect (NLME) model is presented (created using packages "nlme" (Pinheiro et al., 2018), "lme4" (Bates et al., 2015) and "gamm4" (Wood & Scheipl, 2017)). Initial models

included the explanatory variables reproductive state, parasite load and body mass, as well as interaction effects between these, temperature maxima, minima, means and ranges in the 24-hour period before capture, temperature deviance from the 1961-1990 normal, wind speed in the hour before capture, and precipitation in the last 12 and 24 hours. Date was omitted as an explanatory variable, as the correlation between date and reproductive state (χ^2>25.188 with 10 degrees of freedom, $p < 0.0001$) greatly reduced the predictive power of the model. In addition, whether the bat was emerging from the roost, foraging or returning, where the bat was captured, which bag the bat was held in, capture time, how much the bat defecated, whether the night was clear or overcast, and which of the two analysis kits were used to analyze the sample were included as random variables.

Model selection is based on the Akaike Information Criterion for small sample sizes (AICc; using package “MuMIn” (Barton, 2018)). Models where AICc value was within 2.0 of the model with the lowest AICc-value were considered based on their biological merit. For selected models, explanatory variables were then removed stepwise if their estimates were exceeded by their variance. Graphical presentation of data was done using package “ggplot2” (Wickham, 2016) and, where applicable, with “lubridate” (Grolemund & Wickham, 2011). Hormonal axis figures were made using draw.io version 10.6.7 (Alder & Benson, 2005).

3. Results

3.1 Climatic parameters

In the period May-July 2018, mean temperatures were, on average, 3.1°C above normal across all of Norway, exceeding the previous highest recorded such measure of 1.9°C. In Østlandet specifically, the normal was exceeded by 4.3°C. Simultaneously, national precipitation levels were 26% below normal, and from January to August a total of 1,906 forest and brush fires were registered, with 788 of them occurring in July (Skaland et al., 2019). In Nittedal, mean temperatures reflected national patterns, with a mean elevation over the whole period of 2.69 °C ($p < 0.0001$, $n = 92$), and in July mean temperature was elevated by 5.13°C ($p < 0.0001$, $n = 31$) (see Figure 8). Furthermore, a major forest fire was registered on the 30th of June (red arrow on Figure 8), beginning in the evening and lasting until the evening of July 2nd.

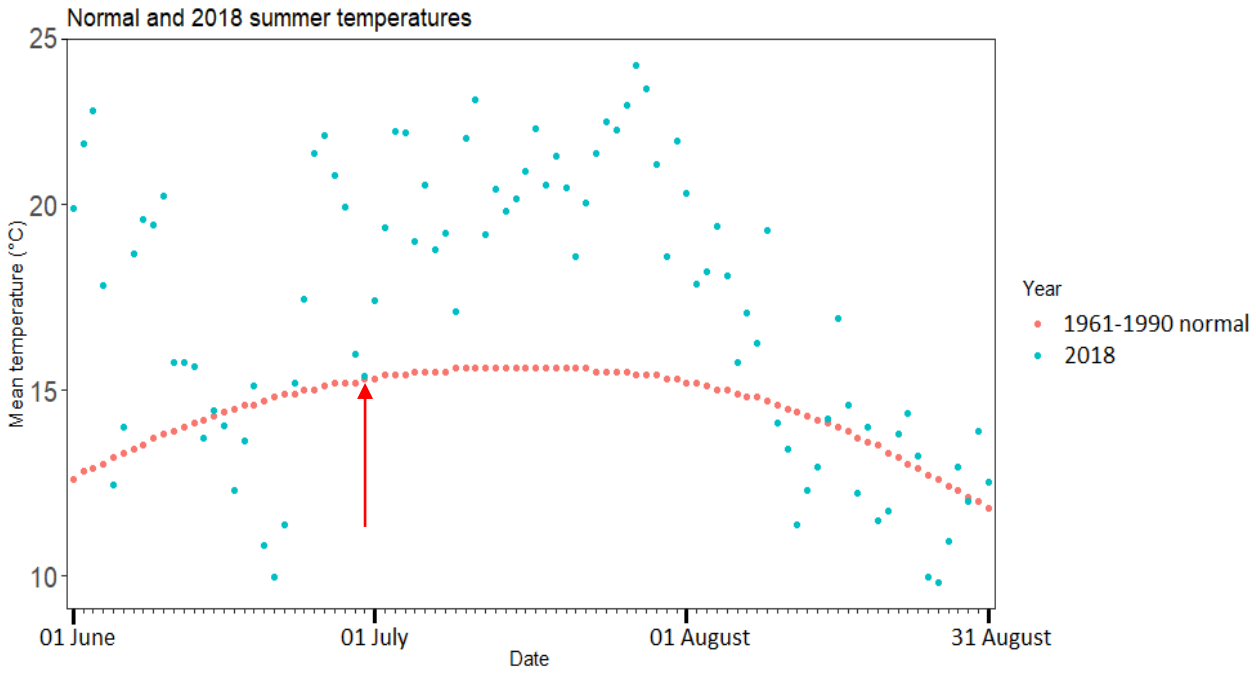

Figure 8:Mean temperature of Nittedal in the period June-August for 2018, as well as normal from Østlandet from 1961-1990. Time of forest fire denoted with red arrow.

3.2 Cortisol measurements

FGM measurements show that there is variation in the amount of free GC (see Figure 9). Several possible statistical models have deltaAICc values below 2 (see Table 2 for a full list). However, all of these models agree that parasite load, wind speed and precipitation have no significant effect on FGM levels.

Table 2: Models used to develop final model. Variables found to have an impact in the various models are: temperature deviation from 1961-1990 normal (Tdev); body mass (BM); mean, maximum, minimum and range in temperature in the past 24 hours (Tmean, Tmax, Tmin and Trange, respectively); reproductive state (R); and an interaction effect between body mas and reproductive state (M:R). Most parsimonious model denoted in red, model considered here denoted in blue

Model	**AICc**	**Delta**
T_{dev} + BM + T_{min} + R + M:R	402.8698	0.0000
T_{dev} + BM + T_{mean} + R + M:R	403.1122	0.2424
T_{dev} + BM + T_{max} + T_{mean} + R + M:R	403.7276	0.8578
T_{dev} + BM + T_{mean} + T_{min} + R + M:R	403.7535	0.8836
T_{dev} + BM + T_{max} + T_{min} + R + M:R	404.2885	1.4186
T_{dev} + BM + R + M:R	404.5979	1.7281
BM + R + M:R	404.6264	1.7566
BM + T_{max} + T_{mean} + R + M:R	404.6501	1.7803
T_{dev} + BM + T_{min} + R + M:R	404.8429	1.9730
T_{dev} + BM + T_{max} + T_{min} + R + M:R	404.8429	1.9730
T_{dev} + BM + T_{max} + T_{min} + R + T_{range} + M:R	404.8429	1.9730
T_{dev} + BM + T_{max} + R + M:R	404.8429	1.9730
Final model	**BM + T_{mean} + R + M:R**	

The most parsimonious of these (denoted in red in Table 2) indicates an influence of body mass, reproductive state and an interaction effect. However, it seems reasonable to expect an effect of high ambient temperatures on glucocorticoid levels. While most of the presented models include deviance from normal temperature, it is also feasible that either the absolute minimum, maximum or mean temperature will have an effect. Because minimum temperatures in this season were still

relatively high, and maximum temperature is partially nested in the temperature deviance, I instead consider the model indicating an effect of body mass, reproductive state, an interaction between body mass and reproductive state, and also mean and deviance from historical mean temperatures (denoted in blue in Table 2). This model was tested according to the criteria outlined earlier, and T_{dev} was eliminated because SE was larger than the parameter estimate, resulting in the final model presented in bold in Table 2.

The resulting model indicates that the intercept for FGM levels are highly variable, but they can be estimated at 3175 ± 2429 pg/ml. Observed values, however, are influenced by body mass, reproductive state and the mean temperature in the 24-hour period before capture. Additionally, mass affects FGM differently according to which reproductive state the bat is in. Note that the "intercept" value describes a bat with no reproductive state and no mass, at 0 °C. Thus, it does not have any biological meaning, but serves as a basis of comparison with other estimates.

The magnitude of body mass impact is that for every gram of added weight, there is a decrease in FGM of 332 ± 236 pg/ml. If the bat is pregnant, however, this effect is modified by an increase of 801 ± 264 pg/ml FGM per gram of added body mass (see Figure 10). This interaction is not significant in a post-lactating or "no indication" state.

Pregnancy alone also has an effect, reducing FGM concentrations by 4471 ± 1998 pg/ml. While this would seem to but the FGM levels of a pregnant bat below 0, one must note that the variance of both the intercept and pregnancy are high, and further that the mass-related increase in FGM when pregnant does compensate somewhat. A post-lactating state is also associated with an FGM decrease relative to intercept, but the variability makes this effect unpredictable.

Temperature in the day before capture is linked to increased levels of FGM. Specifically, a one degree increase in mean temperature in this period results in an increase of FGM by 99 ± 66 pg/ml.

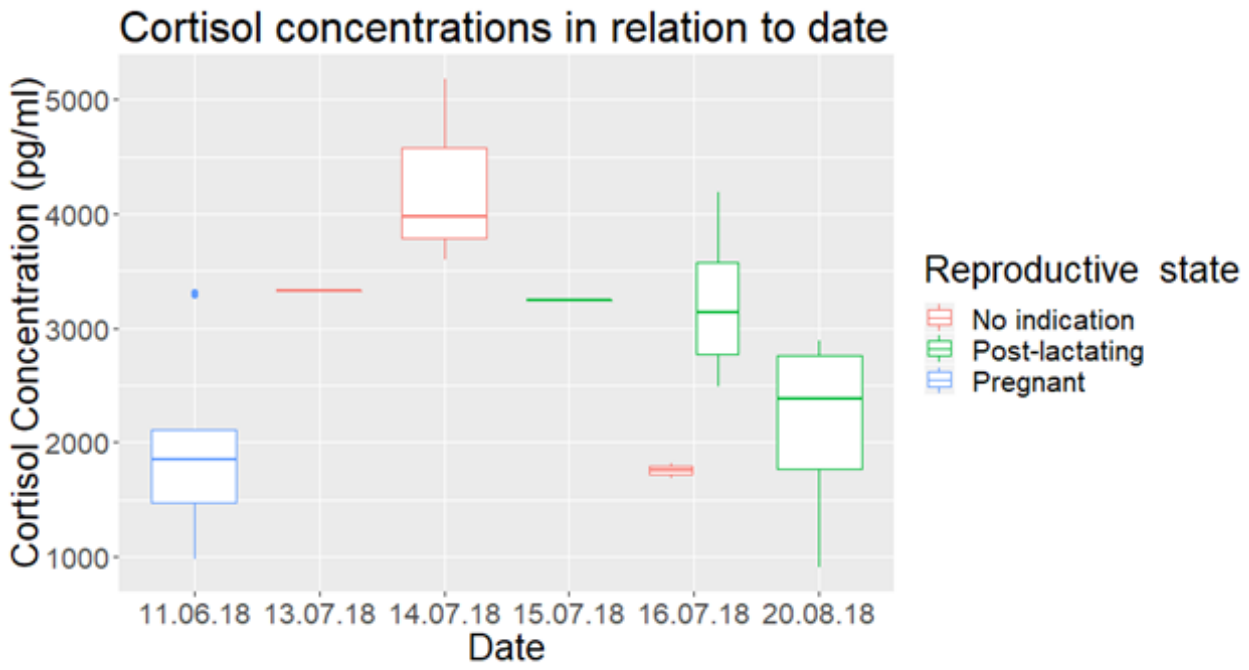

Figure 9: FGM concentrations in M. mystacinus sampled at different dates. Different colors denote reproductive states. Band inside boxes denote mean, whiskers denote interquartile range. Dots denote outliers. Note that several dates from July are represented, but only one from June and August, respectively.

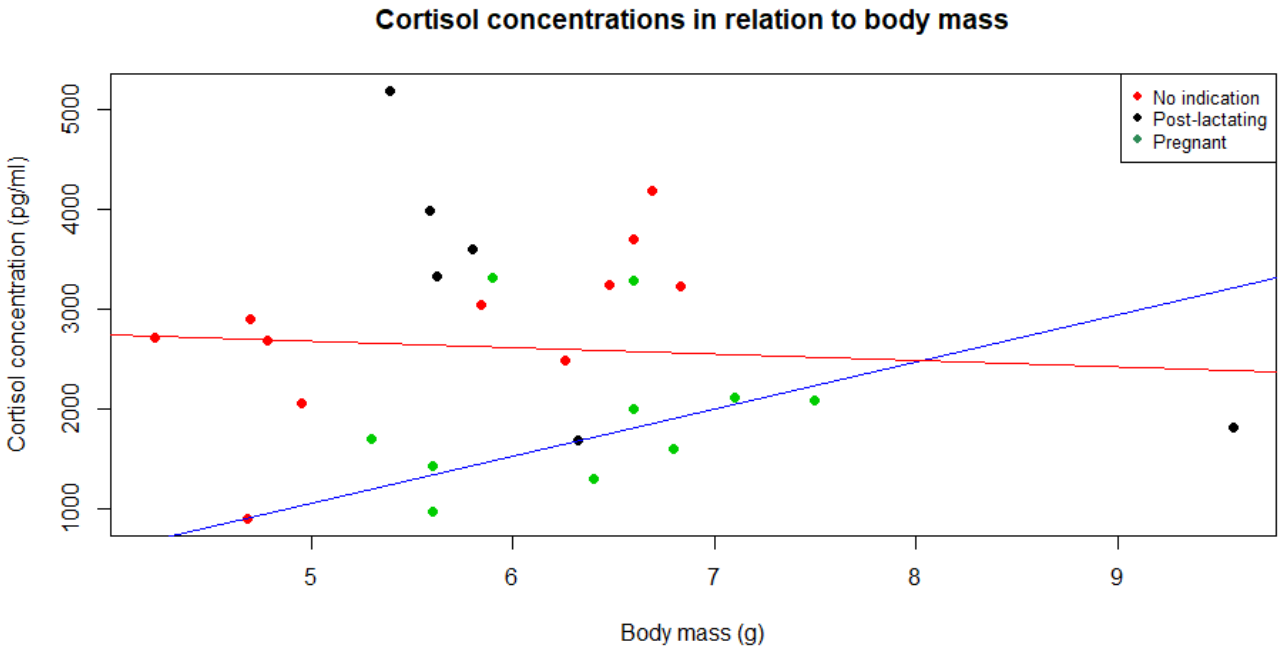

Figure 10: Projected FGM concentration as a function of body mass. Red line corresponds to post-lactating or "no indication" individuals, blue line corresponds to pregnant individuals. Different colors denote different reproductive states.

4. Discussion

4.1 Observations

My results seem to indicate that the reproductive state of the bats is the best predictor for FGM levels. Therefore, it seems likely that animals in a post-lactating state or animals with no indication of reproductive state should be more vulnerable to additional exogenous stressors than pregnant ones. However, there are several caveats to this. Firstly, note again that the "pregnant" state was inferred based on the species' reproductive pattern and observation that most bats captured after the middle of July seemed to have lactated at some point, not on actual observations of pregnancy-related traits. Further, it is impossible to ignore the temporal stratification of the observed data; all early bats are defined as pregnant, while "no indication" and post-lactating states appear in distinct groups later on. Because of this, it is difficult to separate reproductive effects from effects induced by seasonal variables, such as day length, in the present data. In addition, the bats with no reproductive indications were mainly sampled in the period when they should have recently given birth or been close to term (Jeroen van der Kooij, personal communication, March 04, 2019). Thus, it is possible that an observed "no indication" state is actually indicative of the bat having recently prematurely terminated pregnancy or experienced a stillbirth, both of which could influence the observed data. The additional observation that August captures included fewer juveniles than in a normal year seems to suggest that either pups were not carried to term, or that mortality was high after birth. The latter scenario could also influence stress levels of adults, though it is likely that this would occur when the bats were observably lactating, and such animals are underrepresented to the point of exclusion from analysis in this study. A design where more continuous captures are performed throughout the season would be required to elucidate this point. Note that it is also possible that the animals labeled as "no indication" in July may have been erroneously classified, and were in fact lactating, as this may be difficult to discern in an early stage of lactation without invasive methods (Jeroen van der Kooij, personal communication, April 05, 2019). This is supported by the timing of registration compared to the period it is estimated that the bats would have given birth. The author intends, however, to attempt a validation study of this aspect in June of 2019, which should help elucidate the current data set.

Because of the factors described above, and when they occurred, it is entirely possible that values obtained for ostensibly pregnant bats captured in June are closer to "true" baseline levels than data from non-reproductive (or possibly lactating) ones captured in July during exceptionally warm and dry conditions with (potentially) high pup mortality. Note, however, that while data of this nature are scarce in bats, a study of the common fruit-eating bat (*Artibeus jamaicensis*) found that pregnant females had both the highest baseline GC and stress-related GC response when compared to both males and female conspecifics of any other reproductive state (Klose et al., 2006). It is therefore possible that the observed data in the current study in reality gives a picture of non-stressed pregnant bats compared to highly stressed non-pregnant bats, but further studies are required to verify this. Regardless of the underlying cause, however, stress levels in this study seem to be highest in July and August. Because it has been shown that bats respond with heightened stress levels to disturbance of habitat, it is therefore important to conserve both the home range and especially roost sites of these animals in this period (Seltmann et al., 2017). Further, this interval coincides with critical periods for rearing of young. If these animals are stressed, GC-changes could interfere with their ability to build up fat reserves for the upcoming winter, which would adversely affect survival rates. As it seems likely that juveniles would deal even less well than adults with habitat disturbance and roost changes, it is therefore doubly important to avoid disturbances to maternity roosts. Because these roosts are often found in conjunction with buildings, close attention should be paid to local bat populations before construction projects are undertaken in the summer months.

Also of note is the magnitude of temperature effects on FGM levels. While this effect seems small compared to the apparent effect of reproductive state, it is nonetheless noteworthy that an increase in mean temperature of 5.13 °C (the mean increase over normal in July) would increase FGM levels by more than 500 pg/ml. In this study, the mean across all samples was 2611 pg/ml, thus the observed temperature increase would be expected to increase FGM levels by almost 20%. It is, therefore, also possible that increased stress levels due to high temperatures were the *cause* of premature terminations of pregnancy in this season, not the other way around. If temperature-related stress is sufficient to induce near-total reproductive collapse in the population, especially considering the projected increase in temperature flux and heatwave

frequency due to climate change, then bats may be headed for an uncertain fate (Easterling et al., 2000; Schär et al., 2004).

It is further important to note that it appears heavier bats are better suited to dealing with stressors than lighter ones. While this species only has a body mass range of 4 g, the effect is that a very large bat will have FGM levels that are more than 1200 pg/ml lower than those of a very small bat. Therefore, if both of these bats are exposed to the same exogenous stressors (and are influenced by them equally), the smaller bat would be more likely to experience more severe GC-mediated effects. If the stressor is chronic in nature, such as with high mean ambient temperatures, this should mean that large bats would be favored over small ones. However, because of chronic GC-effects on growth and fat deposition, small bats would have difficulties ever becoming large, and juveniles and small adults may have disproportionally lower success rates than larger animals, both in survival and reproductive efforts. It is also interesting that when bats are pregnant, there is a positive relationship between body mass and FGM. It is not known whether this is because being heavier in this state is associated with being more stressed, or if heavier individuals in the pregnant category were further along in their pregnancies than lighter individuals, and therefore had higher circulating GC levels. In either case, it would appear that pregnant females are at special risk when faced with exogenous stressors, as their GC levels are already increasing dur to pregnancy-related mass gain. Not only because of the effects this can have on the individual female, but also because of the effects maternal GCs can have on offspring, it therefore becomes important to minimize stress exposure in these animals. Note that it is possible that this magnitude of impact of body mass is isolated to females of the observed weight range, but more data are needed to establish these relations for males, juveniles and more extreme values of female body mass.

4.2 Implications for future generations

As noted previously, maternal stress can have pronounced effects on offspring. While, as mentioned, recruitment during this season appears to have been lower than normal, some pups were nevertheless born and made it to a flight-capable stage (no data yet exists on winter survival

rates for this cohort). Thus, it becomes relevant to consider what impact the warm and dry summer may have had on these animals.

Per the outline made previously of physiological changes due to elevated GCs, one would expect these pups to exhibit reduced birth weight and lifelong heightened sensitivity to stress. Because of this, they would be especially at risk for hypoglycemia and insulin resistance, and would likely either struggle to gain weight, or, if energy availability allows, have pronounced dysregulation of lipid storage under future stressful conditions. They may also have impaired capacity for thermogenesis following torpor and be more at risk for cardiovascular disease and osteoporosis. Finally, their reproductive output will likely be more sensitive to disturbance by external factors. However, if the new stressor occurs before these bats have had time to normalize after their initial difficult year and subsequent hibernation, for example if another hot and dry summer were to occur in 2019, it seems likely that they will experience a greater energy deficit than other bats going into the season, and they would then have more difficulty catching up to their conspecifics in terms of weight gain and fat storage. The mortality of this cohort would likely be high, reproductive output would be low, and they would probably have more difficulties preparing for the next season of hibernation.

However, here, too there are caveats that need to be addressed. The observant reader may note that in section 1.2.2.10 *Prenatal stress effects on offspring*, it is stated that early exposure to GCs modifies fetal expression of GR and MR. However, this requires exposure to actually take place. In most eutherians, the placenta expresses the enzyme 11β-hydroxysteroid-dehydrogenase (11β-HSD-2), which converts GCs to inactive cortisone, thus affording the fetus some protection from maternal GCs (Benediktsson et al., 1997; Yang, 1997). Unfortunately, no studies exist on the presence or activity level of this enzyme in bats, and thus, it becomes difficult to say to what extent bat fetuses are exposed to maternal GCs (Seckl & Walker, 2001; Tomlinson et al., 2004). Elsewhere, it has been demonstrated that in some species, exposing the placenta to GCs induces placental production of CRH, causing the fetus to produce endogenous GCs, which modulate development and sensitivity of the HPA axis (Challis et al., 2000). Again, data on if, and if so to what extent, this happens in bats is not available. Further, the impact to be had on development of the HPA axis naturally depends on when exactly it is it develops, as seen in studies noted earlier

where GC exposure in the final stages of pregnancy are the ones that have a pronounced effect. However, data on when this occurs in bats is, once more, lacking (Misek, 1989; Reep & Bhatnagar, 2000). If this occurs postnatally, as in rats, it is likely that sensitivity to maternal stress is lowered (Sapolsky & Meaney, 1986). More bat-specific research is required to attain a better understanding of these factors.

While the potential effects on individuals from this cohort are evident, as is the fact that they, being few in number, are likely to have a decreased reproductive output due to other cohorts, it would also be prudent to consider the ramifications if hot and dry summers become more commonplace in the future. It has been observed in several bird species that phenological mismatch between migratory animals and their prey can and does occur as a result of changes in the timing of temperature-related ecological events (Saino et al., 2010). Similar mismatching has been observed in hibernating yellow-bellied marmots (*Marmota flaviventris*) and mountain pygmy possums (*Burramys parvus*), who emerge before normal food sources become readily available (Green, 2010; Inouye et al., 2000). While *M. mystacinus*, unlike several other species of bats, is not known to be obviously migratory, it is worth noting that to date, few winter hibernacula have been found in the study area. If this is indicative of the bats normally undergoing some minor migratory event between arousal from hibernation and settling in in their summer habitat, the potential for such mismatch is present, though further studies are required to discern whether this is the case.

It has been shown that temperature changes are associated with a greater abundance of insects, and also with expansion of species' natural ranges (DeLucia et al., 2008; Walther et al., 2009). However, such expansionist activity is often associated with displacement or eradication of native species (Sujay et al., 2010). Further, it is also apparent that an increase in temperature may accelerate insect development and thus life history (Robinet & Roques, 2010). Therefore, regardless of the presence or absence of a migratory event, if the cue for arousal does not match the appearance of prey species, or if favored prey no longer exists locally, the bats may emerge into a habitat with low food availability. If this problem is compounded by fat stores being lower than normal due to events of the previous season, the survival rates for the population are likely to drop. Additionally, if heat stress is combined with the stress of food shortage, it is likely that

the stress-related problems outlined earlier will only worsen over time. As noted earlier, bats can potentially have large benefits to agriculture, and the prospect of increased pest populations and a simultaneous decline in bat populations should give pause, given that a colony of 300 bats of a size similar to *M. mystacinus* have been estimated to eat 6.3 million insects per year (Whitaker Jr & Clem, 1992).

4.3 Some remarks on methodology

When measuring FGM, the measure one gets is of GCs that have been metabolized and subsequently carried with the digesta. The metabolization process affects the free fraction of GC, though, as discussed earlier, albumin-bound GC may in this context be considered "free." It is therefore worth noting that the measures given here are of a fraction of free GC, obtained over the period since the last voiding of the gastrointestinal tract. As such, they represent a measure of how much GC has been metabolized in the past X units of time, not how much GC, free or otherwise, is in the animal's blood stream at the time of sampling. The magnitude of X is unknown in this study, as is both minimal, maximal and mean digestive passage time. While I have attempted to homogenize the samples as outlined in the methods section, it is possible that a high-speed passage sample may be different in its GC content than a slow-passage sample, even under the same serum GC conditions. In a study on meridional serotine (*Eptesicus isabellinus*), it was found that FGM levels increased after 1.5 hours (Kelm et al., 2016). In the present study, most animals were kept significantly shorter than this, though a few individuals were kept for more. Therefore, it is possible that some samples reflect handling-induced stress, though the lack of correlation between holding time and sample amount should be reiterated here. Kelm et al. also noted in the same study that FGM levels increased with age. However, I have no measure of age in these bats, as this requires longitudinal data that was not available for this system, and thus I could not control for it.

On the note of FGM being a measure of free GC, it has been observed in some species that while GC levels increase during pregnancy, so do CBG levels. However, there is typically a proportionately larger increase in GC, meaning that even if free GC is measured, a pregnancy-related increase should be detectable (Trainer, 2002). Nevertheless, it should be pointed out that it

is possible to have a seasonal variation in CBG independently of reproductive state, and thus the free fraction of serum GC (Breuner & Orchinik, 2001). The presence or extent of such a variation is unknown for this species, and it would go unnoticed by this sampling method.

It is also important to consider external factors and sources of contamination that could influence results. While it has been demonstrated that older samples show increased degradation of FGMs, especially if the sample has been subject to temperature extremes or precipitation, this effect is only apparent after several hours (Millspaugh & Washburn, 2004). Additionally, when stored at room temperature, it has been shown that microbial activity can cause a substantial increase in measured FGM in feces from cows, horses and pigs after 1 h, 4 h and 24 h, respectively (Messmann et al., 1999). These times are probably influenced by the specifics of the digestive systems of the animals involved, with both cattle and horses having high quantities of cellulose-digesting bacteria in their gut. Therefore, it seems likely that the timeframe for pigs is a more appropriate model to use for the bat. As all samples were rapidly put on ice in the field, and subsequently frozen within less than 24 hours, it seems unlikely that either of these factors should have a great impact on the validity of the results, however. A larger source of concern is the fact that GCs are mostly excreted through the urine, and I have no way of knowing whether the bats also urinated while in the bag (though it seems likely), and if so to what extent the fecal matter was contaminated. However, it seems probable that if this is the case for all samples in this data set, to a similar extent, then the data should at least be comparable to each other.

Finally, one must consider the analysis itself. The most relevant aspect here is that due to human error, a deviance in methodology occurred. The kit does not call for the 12-14-hour refrigerated incubation period utilized here, but because this was done in the first batch of samples, it was deemed best to maintain this procedure throughout. During this period, the sample was exposed to a solution of 82.5% methanol, in order to extract cortisol from the feces. However, because long-duration methanol exposure is a preferred method for GC extraction from hair, it is assumed that FGMs, too, will be relatively stable in this solution, especially at lower temperatures (Davenport et al., 2006). Because of the high surface to volume ratio of the crushed fecal matter, it also seems unlikely that the 2-hour difference in exposure time should significantly influence

extraction efficiency in this case. Thus, I assume that this deviation, so long as it is standardized, does not negatively impact the validity of the results.

The kit manufacturer specifies a series of compounds known to interact with the assay, and their relative cross reactivities. Of these, there are three worthy of note that could impact this study. Firstly, there is a 27.68% cross reactivity with corticosterone. In most mammals, cortisol serves as the main GC, with corticosterone taking a secondary role. However, in some species it is the other way around, for example in rodents. For *M. mystacinus*, it is unknown which of these is the "main" stress hormone, and because the kit has some responsiveness to both, I would not be able to detect if I measured the wrong one in this study, though the implication of an elevated corticosterone level would be the same as for cortisol. The kit also has a 4.0% cross reactivity with cortodoxone, a low-potency stress hormone. Again, elevation would probably have the same physiological implications as for cortisol, and thus, not overly important for this study, though it may contribute to variance in the data. Finally, there is a 3.64% cross reactivity with the female sex hormone progesterone. This hormone is particularly associated with maintenance of pregnancy, and it could lead to elevated GC measurements in this state. However, because pregnant animals show the *lowest* GC values in this study, this is likely not a major confound.

5. Conclusions

While I initially set out to establish normal patterns of GC levels over an active season, the decidedly abnormal climatic conditions of the study year mean I cannot do this with certainty. However, my results do seem to indicate a strong reproductive influence on stress, and it is likely that this would still be present in a normal year. Because of this, I would suggest that bats are more vulnerable to exogenous stressors in July and August than they are in June, especially in the period surrounding the birth of young. I would further predict that the cohort recruited this season, (seemingly small though it may be), is likely to have heightened sensitivity to future stressors. Additionally, because the high mean temperature was not a local phenomenon (though the study was conducted in the area where the deviance from normal was most extreme), but rather affected most of Norway, it seems likely that this effect will affect the entire national cohort of bats, to some extent. However, because baseline levels still have not been established, future studies (under normal conditions) are required to ascertain exactly to what extent these bats may have been affected, and also to make further conservational recommendations.

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