INDIVIDUAL VARIATION IN GLUCOCORTICOID LEVELS: A FIELD STUDY OF REPEATABILITY

by

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A thesis submitted to the Graduate Program in Biology in conformity with the requirements for the degree of Master of Science

Queen's University

Kingston, Ontario, Canada

November, 2017

Abstract

We readily appreciate the wide phenotypic diversity that distinguishes animal species; however, even within a population of the same species, individuals can express significant phenotypic variation in their behavior, morphology, and physiology. Natural selection acts on among-individual phenotypic variation, linking an individual's phenotype to its fitness. A necessary first step toward understanding some evolutionary and ecological processes, which are driven by the relative fitness of certain phenotypes over others, is to accurately characterize consistent individual differences in traits that influence survival and/or reproduction. Circulating concentrations of glucocorticoid (GC) hormones are plastic (i.e., capable of changing across a range of temporal scales in response to internal or external conditions) and involved in numerous processes related to fitness. We assessed the repeatability (or, trait consistency) of corticosterone (CORT; the primary avian GC) titers in free-living black-capped chickadees (*Poecile atricapillus*) during the non-breeding season. We also investigated internal and external factors that could explain the degree of repeatability/plasticity we observed. We found that initial CORT concentration had a significant agreement repeatability of 0.284. On average, CORT concentrations were higher during the second capture compared to the first. Furthermore, initial CORT was lower among individuals that experienced lower temperatures and higher wind speeds prior to capture, and among individuals with relatively poorer oxidative balance. Overall, our results suggest that individuals respond plastically to harsh winter conditions, while also maintaining a degree of within-individual consistency that may reflect the influence of a combination of genetic differences among individuals and stability in aspects of their environment. These

findings add to the accumulating evidence that suggests that individual variation in GC concentrations within populations can often reflect short-term, dynamic changes in the environment more so than fixed differences among individuals.

Co-Authorship

Manuscripts resulting from the thesis are outlined below:

Schoenemann K, Montreuil-Spencer C, Bonier F. In prep. Circulating glucocorticoids are repeatable in a wild bird during the non-breeding season. General and Comparative Endocrinology.

Author contributions: KS and CM developed research methods, collected and compiled data, and performed lab analyses; KS performed statistical analyses, and wrote the manuscript; FB conceived the idea, supervised research, provided funding, and edited the manuscript

Acknowledgements

Thank you, Dr. Fran Bonier, for your steady guidance these past 2 years. I am tremendously and eternally grateful for everything you have done to impart a uniquely-focused, scientific competency that will guide me in my professional, as well as personal, life. Thank you, Dr. Bob Montgomerie and Dr. Virginia Walker, for your patience, and for your deliberate advice and criticism that ultimately helped me to understand my topic all the better. Thank you, Chloé Montreuil-Spencer, for making this project possible; I literally could not have done all of this without you. You always challenged me to be better and I am glad for our time together. Thank you, Mathieson Smith, for your friendship and invaluable assistance in the field. Our friendship was a surprise, but a remarkable one, which reminded me that we are never as alone as we think. Thank you, Ivy Schoepf and Amelia Cox, for your kindnesses and thoughtful conversations, be they shared over a meal or a session of R-script troubleshooting. Our discussions sparked new curiosities for my own, as well as foreign, lines of research, and often brought me much comfort and reassurance. Thank you to the entire QUBS staff and to our lab technician, Shannon Smith, for all of the help and support that made this project such a success. Thank you, mom and dad, Drs. Janine Brown and Marty Schoenemann, for ...everything? I am quite sure you already know what all you did (and continue to do!) to help me. Thank you, Baron Lin, for putting up with me; I really do not know how you do it! Finally, I dedicate this thesis to strawberrycream ("sc") for being a source of solace and precious little happinesses through all the moments of darkness.

We acknowledge a Natural Sciences and Engineering Research Council of Canada Discovery Grant awarded to F. Bonier for providing funding for this project.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACTH – *adrenocorticotropic hormone*

CBG – *corticosterone binding globulin*

CORT – *corticosterone*

CRH – *corticotropin-releasing hormone*

EIA – *enzyme-immunoassay*

GC – *glucocorticoid*

GR – *glucocorticoid receptor*

HPA axis – *hypothalamic-pituitary-adrenal axis*

ICC – *intraclass correlation coefficient*

OI – *oxidative index*

R – *trait repeatability in a population*

r – *estimate of trait repeatability in a population*

RIA – *radioimmunoassay*

ROM – *reactive oxygen metabolites*

TAC – *total antioxidant capacity*

Chapter 1: General Introduction

We can readily appreciate the wide phenotypic diversity that distinguishes animal species; yet, even within a single population, individuals can vary significantly in body size and shape (Garland, 1985), behavioral syndrome (Verbeek et al., 1994; reviewed in Wilson, 1998), foraging niche (reviewed in Bolnick et al., 2003), basal metabolic rate (reviewed in Burton et al., 2011), parasite resistance (i.e., sex differences, reviewed in Zuk & McKean, 1996), and competitive ability (Caldow et al., 1999; reviewed in Sutherland & Parker, 1985). Natural selection acts on phenotypic variation at the individual level (Brodie et al., 1995) and, thus, the fitness of a given genotype is linked to the phenotype of the individual (Lande and Arnold, 1983). How and why do individuals express such a staggering degree of phenotypic variation in their behavior, morphology, and physiology?

Much variation among individuals in plastic traits (i.e., those capable of changing in response to internal or external conditions) results from differences in environmental and genetic factors. For example, many plastic traits, such as physiology and behavior, change within an individual in response to challenging conditions (e.g., weather, predation risk, food availability, or disease) that threaten an organism's homeostasis (*sensu* Romero et al., 2009). These changes are, in part, coordinated by the action of the hypothalamo-pituitary-adrenal (HPA) axis (McEwen and Wingfield, 2003). Activation of the HPA axis results in secretion of glucocorticoid (GC) hormones into circulation to regulate processes such as energy metabolism, escape behavior, inflammation, and reproductive investment (Legagneux et al., 2011; Rivier and Rivest, 1991; Schreck 1993; Sternberg, 2006; Vegiopoulos and Herzig, 2007; Webster et al., 2002; Wingfield et al.,

1998). Acute GC secretion enables an organism to respond to and recover from acute challenges, while the modulation of baseline concentrations (i.e., those unrelated to acute challenges) support alterations in responses to predictable daily or seasonal conditions (Romero, 2004; Romero et al., 2009; Sapolsky et al., 2000; Wingfield, 2005). Thus, as a result of environmental factors that stimulate the HPA response and genetic differences among individuals, both the rate of GC production/metabolism, as well as the concentration of circulating GCs, varies considerably within and among individuals (Cockrem, 2013).

Among-individual variation in GC concentrations may reflect broader, phenotypic differences in health, strategy, or performance, which are more or less suited to the situation, and can often predict survival or reproduction (Bonier et al., 2009; Breuner et al., 2008). The variation in GC concentrations among individuals is often used as a metric of relative quality, health, or welfare of individuals or populations (Ellis et al., 2012; Jaatinen et al., 2013; Martínez-Mota et al., 2007; Munshi-South et al., 2008; Wada et al., 2008). The first step toward understanding the causes of individual variation in GC concentrations and the consequences of this variation on fitness-related traits is to recognize and accurately interpret sources of individual variation in HPA activity. Below, I review the basic function of the HPA axis, and summarize three primary sources of variation in an individual's GC phenotype.

The HPA axis

The HPA axis is activated by neural signals originating from the hippocampus and amygdala; at which point, the hypothalamus synthesizes and releases stimulatory peptides to the pituitary. These peptides initiate a cascade of hormone signaling, which ultimately results in the release of GC hormones from adrenal cortical tissues into circulation (Fig 1). The subsequent effects of GCs at their target cells ultimately help the organism to respond to and recover from challenges. At target cells, GCs exert both genomic and non-genomic effects (Norris and Carr, 2013). Genomic effects induce changes that generally take hours to days to be noticeable at the organismal level (Adkins-Regan, 2005), and include both the activation and suppression of gene expression. For instance, GC-receptor complexes can initiate the expression of genes that encode anti-inflammatory proteins, or suppress the synthesis of pro-inflammatory proteins (Stahn and Buttgereit, 2008). It is the expression, or suppression, of these and other genes that is responsible for many of the effects classically associated

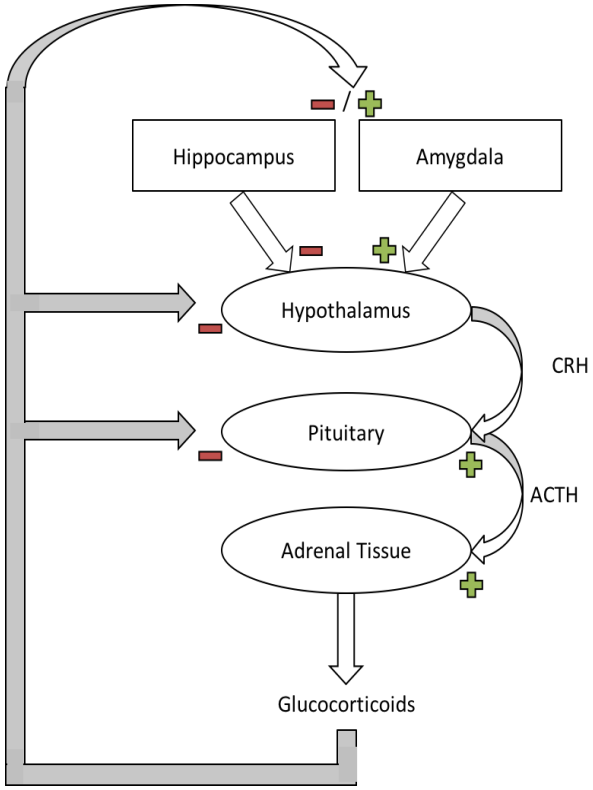

Fig 1. Schematic diagram of hypothalamic-pituitary-adrenal axis. Signals from the hippocampus and amygdala are integrated in the hypothalamus. The hypothalamus releases peptides, such as CRH, which induces the release of ACTH from the pituitary. ACTH signals the production and release of GCs from adrenal tissues. GCs act on GRs to activate negative feedback pathways at the pituitary and hypothalamus, and alter signals of the hippocampus and amygdala. GC, glucocorticoid; GR, glucocorticoid receptor; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone.

with GCs (i.e., energy metabolism, inflammation). Non-genomic effects, by contrast, are evident rapidly, even within minutes of activation (Adkins-Regan, 2005). Many of these effects are mediated by chaperone proteins that dissociate from the GC receptor-protein complexes upon GC binding (Croxtall et al., 2000). Others appear to be the result of GCs binding to membrane receptors (Tasker et al., 2006), or GCs interacting non-specifically with the cell membrane to alter membrane permeability (Buttgereit et al., 2004; Buttgereit and Scheffold, 2002; Dindia et al., 2013).

In order to travel in circulation in the plasma and reach a target cell, hydrophobic GC hormones must first bind to hydrophilic proteins (generally referred to as corticosterone binding globulins, CBGs). In mammals, typically less than 6% of cortisol in circulation is unbound (Lewis et al., 2005). Unbound GCs can be quickly metabolized (i.e., converted to forms with decreased biological activity and increased solubility in water) and excreted; thus, depending on metabolic rate, the half-life of steroid hormones in circulation is on the order of minutes to hours (Adkins-Regan, 2005).

Sources of individual variation in glucocorticoids

Why might individuals differ in circulating concentrations of GCs, or HPA axis responsiveness? Sources of variation in the GC hormone phenotype may be broadly ascribed to environmental, genetic, or methodological influences. Methodological sources of variation affect the measurement of GC concentrations, while genetic and environmental sources of variation generate true differences in GC hormone phenotypes within and among individuals (Fig 2). To illustrate environmental and genetic sources of variation, consider a population of free-living individuals. Each individual receives

different external signals about current challenges, reflecting their particular life experience (e.g., predation attempts, exposure to pathogens, social interactions, food abundance, demands of parental care, etc.). Each individual interprets these signals differently, reflecting their particular behavioral strategy or physiological state (e.g., migratory stage, Ramenofsky et al., 2012; or age, Goutte et al., 2010). Finally, each individual responds to these signals differently, reflecting the sensitivity of the HPA axis, which is shaped by their genotype and environmental conditions, including environment during development (Bolton et al., 2014; Lynn et al., 2010; Weaver et al., 2006). Investigating how these factors generate variation in GC concentrations will help us to understand why individuals differ in their GC phenotypes. Below, I review these three sources of among-individual variation in HPA activity and GC hormone concentrations.

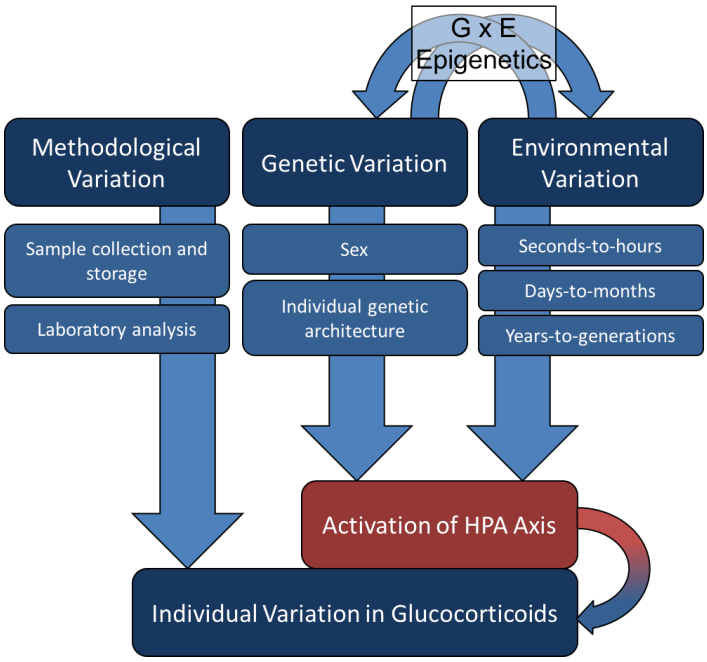

Fig 2. Conceptual diagram of sources of individual variation in glucocorticoids (GCs). Methodological variation in sample collection, storage, and analysis affects measurements of individual GC levels, while genetic and environmental variation impacts the activity of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in variable GC concentrations within and among individuals. As well, epigenetic interactions of genes and environment further modify the effect of variation at these levels on HPA activity and individual GC secretion.

Environmental sources of GC variation

Organisms experience changes in their environment on the scale of seconds to hours, seasons to life history stages, and years to generations. These temporal and spatial changes can differentially affect individuals' HPA activity, including stimulatory and negative feedback responses, to generate among-individual variation in circulating concentrations of GCs.

In response to challenges, organisms usually increase circulating concentrations of GCs, which return to baseline levels after the termination of the stressor. Even witnessing a challenging event (e.g., a predation attempt on a conspecific) can activate an individual's acute GC response, increasing circulating concentrations of total GCs (i.e., CBG-bound and -unbound hormone) (Jones et al., 2015), suggesting that organisms are highly attuned to perceiving and responding to potential threats. Further, the capacity and/or affinity of CBG may decrease during the minutes or hours following a challenge, significantly increasing levels of free GCs (i.e., hormone not bound to CBG) in circulation, absent any additional GC synthesis or secretion (Breuner et al., 2006; Malisch and Breuner, 2010). Thus, baseline concentrations of free or total GCs may frequently be affected by challenges occurring hours before, and be more representative of recent challenges than resting levels (Madliger et al., 2015). Moreover, individuals do not necessarily respond to similar challenges in the same way, reflecting differences in behavioral strategy or physiological state. For example, socially subordinate baboons (*Papio anubis*) exhibit prolonged GC secretion relative to dominants and thus, despite their lower peak levels, they experience greater total GC release (Sapolsky, 1993).

When acute stress responses are not sufficient for coping with gradual or chronic challenges, organisms can adaptively modify HPA activity and circulating GCs over a longer period of time. These challenges may be associated with increases, decreases, or no changes in circulating GCs (e.g., Bonier et al., 2011; Cyr and Romero, 2007; Madliger et al., 2015), depending on the context in which they were measured (Madliger and Love, 2014). In addition, circulating concentrations of GCs can change in maladaptive ways; concentrations may rise above that required for coordinating responses to unpredictable challenges or fall below that required for coping with predictable daily and seasonal challenges (Romero et al., 2009). However, it is not clear when or how the HPA axis will fail, nor is there evidence to support a conserved, predictable GC response to chronic stress across species (Dickens and Romero, 2013). Thus, exposure to acute and chronic challenges can influence individual differences in GCs in significant and complicated ways.

Another source of environmental variation is related to daily and ultradian (i.e., shorter than 24-hour) rhythms in HPA activity organized by abiotic cues and circadian processes. Diel rhythms of baseline and stress-induced GC secretion have been characterized in fish (Ellis et al., 2012 and references therein), birds (Breuner et al., 1999; Carere et al., 2003; Romero and Remage-Healey, 2000), herptiles (Pancak and Taylor, 1983; Tyrrell and Cree, 1998), and mammals (Kramer and Sothern, 2001; Malisch et al., 2008). Peak daily GC secretion typically coincides with the onset of activity, although specific patterns vary across species (Carsia and Harvey, 2000). CBG capacity also fluctuates during the day in many mammals (Fujieda et al., 1982; Hsu and Kuhn, 1988; Meaney et al., 1992) and birds (Lynn et al., 2003), which can concomitantly drive cycles

in free hormone levels. Moreover, alongside the daily peaks and dips, more frequent pulses of secretory and inhibitory signals also drive changes in the HPA axis (Lightman et al 2000; Lightman et al 2008). Ultradian GC cycles have been found in most mammal species investigated (e.g., rhesus monkey *Macaca mulatta*, Carnes et al., 1988; horse *Equus caballus*, Cudd et al., 1995; Syrian hamster *Mesocricetus auratus*, Loudon et al, 1994; Sprague-Dawley rat *Rattus norvegicus*, Windle et al, 1998), but have not been studied in many non-mammalian taxa. Importantly, timing of a stimulus can interact with the phase of an ultradian pulse to augment or diminish the response. For example, individuals that experience an acute stressor which coincides with the rising phase of a GC pulse exhibit a significantly greater GC response compared to those that experience a stressor during the falling phase (Windle et al., 1998). In these ways, daily and ultradian rhythms can drive among-individual variation in GCs.

Seasonal shifts in photoperiod and climate can alter the environment dramatically: affecting food availability, disease transmission, rates of competition and predation, and weather. Many animals will also transition between life-history stages across seasons. Most vertebrates studied cope with these predictable changes by seasonally modulating HPA activity (Ellis et al., 2012; Romero, 2002). As an example of seasonal HPA modulation, consider migration. Many species that breed in seasonal climates migrate to avoid poor conditions during part of the year. In general, GCs increase with migratory activity (Cornelius et al., 2012); although, individuals and species can express a spectrum of migratory behaviors that relate to particular facultative or obligate strategies, which are driven by differential activation of physiological triggers, including the HPA axis (Ramenofsky et al., 2012, and references therein). Thus, GCs can be expected to vary

more among individuals that are compared across different times of the year (e.g., before or during migration), or among those that adopt divergent life history strategies (e.g., in their extent of facultative migration).

The activity of the HPA axis also changes through development. At birth or hatching, many species of mammals, birds, and fish exhibit hypo-responsiveness to acute stressors, which is replaced by more typical HPA responsiveness as an organism matures (Barry et al., 1995; Blas et al., 2006; Haltmeyer et al., 1966; Wada et al., 2007). Additionally, pre-pubertal fish, turtles, and mammals have been shown to have greater responses than adults (Green and McCormick, 2016; Klein and Romeo, 2013; McCormick and Mathews, 2007; Jessop et al., 2005; Pottinger et al., 1995; Barcellos et al., 2014; but see Donaldson and Ulf 1970). Even during adulthood, the activity of the HPA axis continues to change. In general, as mammalian species age, the magnitude of the GC stress response decreases (Hess and Riegle, 1970; Brett et al., 1983; Odio et al 1989; but see van Eeken et al 1992), while responses often become protracted (Sapolsky et al., 1983; van Eekelen et al., 1992), and baseline GC levels tend to increase (Sapolsky 1991; Hassan et al 1999; but see Lightman et al 2000). As well, aged humans and rats demonstrate disrupted circadian rhythms, resulting from diminished peaks/troughs and elevated baseline levels (Lightman et al., 2000; Van Cauter et al., 1996). However, across taxa, changes in the HPA axis with aging vary. For instance, GC stress responses increased with age in a snake and bird species (red-sided garter snake *Thamnophis sirtalis parietalis* Moore et al., 2000; snow petrel *Pagodroma nivea*, Goutte et al., 2010), but decreased in a turtle (green turtle *Chelonia mydas*, Jessop and Hamann, 2005), and

several other bird species (Heidinger et al., 2008; Lendvai et al., 2015; Wilcoxen et al., 2011).

Prior experiences or environmental exposures can prime or direct changes in the HPA axis, either temporarily (i.e., in following stages, seasons, or years; carry-over effects) or permanently (i.e., into adulthood; developmental effects). In this way, individuals' GC concentrations can vary according to the extent to which their experiences differ and the extent to which those experiences affect subsequent HPA activity. Carry-over effects occur whenever an individual's previous experiences explains their current performance, with the caveat that these effects occur between discrete timescales (e.g., across life history stages, social contexts, or experimental treatments; *sensu* O'Connor et al., 2014). Investigations of this process are few, but are becoming more common (Harrison et al., 2011; Norris and Marra, 2007). For example, bluegill sunfish (*Lepomis macrochirus*) exposed to GCs at supraphysiological doses exhibited altered GC responses to heat and food stress 30 days later, even though circulating levels had returned to a baseline that was comparable to controls (McConnachie et al., 2012). Similarly, eastern bluebirds (*Sialia sialis*) that experienced a single acute stressor (capture and handling) had altered HPA responsiveness 7 weeks later (Lynn et al., 2010). In contrast to carry-over effects, which can occur at any stage and are generally temporary, developmental effects impart stable individual differences in a trait (e.g., HPA activity) due to experiences or exposures specifically occurring during pre-natal and early life periods. For instance, mothers that are exposed to a challenge or exogenous doses of GCs during all or even part of their pregnancy produce offspring with exaggerated HPA responses later in life (e.g., in lab rats (*Rattus norvegicus*) and mice (*Mus musculus*);

Valleé et al., 1997; and reviewed in Harris and Seckl, 2011; Welberg and Seckl, 2001). Similarly, offspring that experience physical trauma, toxin exposure, deprivation of maternal contact, or reduced maternal attention during a critical postnatal period exhibit increased HPA responsiveness in adulthood (Meaney, 2001; Meaney et al., 1996). These effects have also been demonstrated in birds (Emmerson and Spencer, 2017; Henriksen et al., 2011; Schoech et al., 2011; Zimmer et al., 2017) and teleost fish (Steenbergen et al., 2011, and references therein). Thus, GC variation can be caused by a number of intrinsic and extrinsic factors, and in particular, an individuals' previous experiences or environmental exposures can (temporarily or permanently) affect how the HPA axis responds to different situations.

Genetic sources of GC variation

Some evidence suggests that a degree of individual variation in circulating levels of GCs and the GC stress response is heritable. Numerous artificial selection studies have revealed moderate to high heritability (0.15–0.33) of stress-induced GCs in Japanese quail *Coturnix japonica* (Satterlee and Johnson, 1988), zebra finch *Taeniopygia guttata* (0.20) (Evans et al., 2006), rainbow trout *Oncorhynchus mykiss* (0.41) (Pottinger and Carrick, 1999), and mice *Mus musculus* (0.28–0.48) (Touma et al., 2008), as well as urinary GCs in pigs *Sus scrofa domesticus* (0.40–0.70) (Kadarmideen and Janss, 2007). Importantly, one of the first heritability estimates of baseline and stress-induced corticosterone in a free-living organism, the barn swallow (*Hirundo rustica*), exposed to natural environmental variation provided similar estimates (0.152 and 0.343, respectively) to those derived from captive studies (Jenkins et al., 2014).

Many vertebrate species (excluding crocodiles, most turtles, some fish, and hermaphroditic species) exhibit genetic sex determination. A pattern of elevated GC responsiveness in females has been repeatedly documented in mammals, particularly in laboratory rats (*Rattus norvegicus*) and humans (Kudielka and Kirschbaum, 2005; reviewed in McCormick and Mathews, 2007; Rhodes and Rubin, 1999). GC levels increase more quickly, achieve a higher peak, and stay elevated for longer periods in female than in male rats, and these sex differences in HPA activity appear at 2 weeks of age (Kant et al., 1983; Kitay, 1961; Rhodes and Rubin, 1999; Yoshimura et al., 2003). Across taxa, however, the picture is less clear. In Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*), males exhibited heightened GC responses to capture, but only during the breeding season (Astheimer et al., 1994), while rainbow trout (*Oncorhynchus mykiss*) exhibited no sex differences in their GC response to confinement (Øverli et al., 2006). Perhaps some of these species-specific patterns in sex differences could be attributable to differences in reproductive strategy or regulation of reproductive hormones. Indeed, male reproductive hormones (i.e., androgens) appear to inhibit HPA activity while female reproductive hormones (i.e., estrogens) enhance it (Bingham and Viau, 2008; Handa et al., 1994; Norman et al., 1992). Thus, there could be a number of additional physiological or behavioral factors that explain how sex is linked to variation between males and females in their GC phenotype.

Changes in the binding affinity, capacity, or concentration of CBGs influence the amount of free GCs in circulation (Breuner and Orchinik, 2002); therefore, genetic differences in genes encoding CBGs, or related structures, can affect individual variation in GCs. For example, three rare, but heritable, variants of human CBG, known as the

Leuven, Lyon, and Null variants, severely reduce CBG binding affinity and concentration (Gagliardi et al., 2010). Furthermore, genetic variation within a region of chromosome 14 that contains genes for CBG and α1-antitrypsin (which inhibits cortisol release from CBG) has been recently shown to account for a small (less than 1% of total variance) but significant portion of inter-individual variation in human plasma cortisol (Bolton et al., 2014). Bolton et al. (2014) contend that this accounting may be a substantial underestimate because of uncontrollable confounding and measurement error, and that these genes may yet contribute a considerable component of human plasma cortisol's previously-estimated 30–60% heritability. As well, evidence from *in vitro* transfection assays and association studies in the pig suggest a role for two mutations of the *Cbg* gene in increasing CBG binding capacity or decreasing affinity for cortisol by as much as 25–70% (Guyonnet-Duperat, 2006). Together, these studies suggest a role of genetic variation in CBGs to affect concentrations of free GCs.

Genetic differences in GC receptors can also affect circulating levels of GCs. Indeed, GC receptors participate in the negative feedback of the HPA axis and thus affect GC release (Cole et al., 1999), while GCs that are bound to receptors are effectively removed from circulation. Several single nucleotide polymorphisms have been identified and functionally characterized at the loci of multiple receptors of GCs. These receptor variants occur at very low to moderate frequencies (3–50%), and have been shown to alter HPA axis regulation differently via changes in baseline, stress response, and/or negative feedback mechanisms (DeRijk and de Kloet, 2008). In addition, alternative splicing at some GC receptor gene exons yields several isoforms that do not bind to GCs and are expressed at much lower concentrations (DuBois et al., 2013). Differences among

individuals in the genetic material encoding GC receptors can therefore impact among-individual variation in circulating GCs.

While GCs play a crucial role in the negative feedback of the HPA axis via receptors of GCs, there are also many non-GC mediated inhibitors of this system (Jessop, 1999), which can influence circulating levels of GCs. For example, endogenous endorphins, such as β-endorphin, exert a tonic inhibition on the HPA axis, primarily acting on neurons in the hypothalamus. The gene *OPRM1* encodes the μ-opioid receptor, which is a primary target of β-endorphin. A common SNP of this receptor gene results in increased receptor affinity and activation potency, but reduced expression on cell membranes (Bond et al., 1998). Numerous studies have investigated the effect of this gene variant on HPA axis activity. Application of a receptor antagonist caused a significantly greater rise in unstressed cortisol, while a social stressor caused a significantly lower cortisol response, among subjects with the variant SNP (Chong et al., 2006; Hernandez-Avila et al., 2003; Wand et al., 2002). A recent study corroborates these results, finding that application of metyrapone, which blocks GC synthesis in the adrenal cortical tissue, resulted in a slower and blunted rise in ACTH among subjects with the receptor variant (Ducat et al., 2013). Inexplicably, however, some studies also report higher baseline cortisol among subjects with the variant SNP (Bart et al., 2006; Hernandez-Avila et al., 2007). The combined results of these studies nevertheless suggest that variant receptors can affect HPA axis activity.

Methodological sources of GC variation

Any measurement of a biological phenomenon will be associated with some level of error. This error may introduce noise into a dataset if it affects all measurements

equally and randomly; however, error may also bias results if it affects the data in a non-random way. Excessive noise in datasets can lead to over-estimation of among-individual variation, or otherwise make it difficult to distinguish individual differences from error. Depending on the question being addressed and logistical limitations of the field or captive environment, investigators may collect a variety of biological samples (e.g., feces, urine, blood, saliva, feathers, or hair) with which to assess concentrations of GCs in their subjects; with each, aspects of collection, storage, and analysis can affect measurement error.

Sample collection procedures may introduce variation into individual measures of GCs. While GCs measured in blood collected within 2–3 minutes of capture often reflect baseline levels (Romero and Reed, 2005), concentrations may rise considerably faster or slower in some species (Small et al., 2017; Tyrrell and Cree, 1998), which can confound among-individual GCs variation with differences in the time that the researcher took to collect the sample after capturing an individual. In a similar way, fecal bacteria and enzymes continue to metabolize steroids after deposition, causing significant increases in measurable metabolites over time until collection and freezing (Khan et al., 2002; Möstl et al., 1999; Washburn and Millspaugh, 2002). Storage procedures, such as use of preservatives or freezing, may also affect GCs in samples. For example, GC levels increased significantly in fecal extractions following just two freeze-thaw cycles (Pappano et al., 2010). Finally, laboratory measurement of hormone levels can introduce variation into measures of GCs. Many hormone assay reagents are prepared in batches (e.g., buffers) or expire (e.g., proteins, catalysts, and tracers). For this reason, analyzing samples from the same individual at different times (i.e., with different reagent batches or

sample storage times) can introduce variation. Even putatively identical assay reagents can generate quantitatively different estimates of GC levels if used in different systems (i.e., enzyme-immunoassays or radioimmunoassays) (Brown et al., 2010) or if different companies produced them (Wasser et al., 2000). These examples illustrate the importance of consistency in sample collection and analysis techniques to reduce or control variation. However, with the advent of commercial kit-based assays, the possibility exists that the rigor of assay validation and control monitoring may decline (Buchanan and Goldsmith, 2004) and result in unaccounted measurement error being attributed to individual variation (Fanson et al., 2017).

Repeatability of glucocorticoids

One reason why we might fail to find consistent patterns in relationships between among-individual variation in GCs and, for example, fitness parameters (Bonier et al., 2009; Breuner et al., 2008; Crespi et al., 2013) is because we lack information about how within-individual variation in GCs (i.e., plasticity) influences hormone levels or their relationship to other traits (Bonier et al., 2009). Failure to acknowledge, measure, or control for within-individual variation of GC titers might diminish our ability to detect biologically significant patterns in GC secretion (Bonier and Martin, 2016). Estimating the repeatability (i.e., consistency over time or across contexts) of GC titers is one technique for avoiding this pitfall.

Like other sample statistics, such as a mean or standard deviation, repeatability estimates (r) approximate a population parameter (i.e., the 'true' repeatability, R) (Sokal and Rohlf, 1995). The most common statistic used in recent literature to estimate

repeatability is the intraclass correlation coefficient (ICC), which approximates repeatability as the proportion of total phenotypic variation that is attributable to the individual (Sokal and Rohlf 1995; Nakagawa and Schielzeth, 2010). Statistical repeatability (i.e., the ICC) may be low because of high within-individual variation, high measurement error, low among-individual variation, or a combination of all three.

The repeatability of GCs within individuals can be used to determine whether inferences made about GC measures may be generalized beyond providing information about the individuals at the time of sampling (e.g., Bosson et al., 2009; Harris et al., 2016; Wada et al., 2008). Moreover, repeatability itself may reflect the ability or strategy of an individual to cope with a challenge and, thus, is worthy of study in its own right (Careau et al., 2014; Roche et al., 2016). Finally, estimates of repeatability can approximate the upper limit of heritability of individual variation and, thereby, the extent to which natural selection can shape a trait (Falconer and Mackay 1996; but see Dohm, 2002). Perhaps in recognition of these points, many studies have estimated the repeatability of GC measures (e.g., Cook et al., 2012; Narayan et al., 2013; Romero and Reed, 2008; Wada et al., 2008). A meta-analysis of these repeatability estimates indicates low to moderate repeatability of GCs, however, there is a lot of unexplained variation among taxa in the magnitude of these estimates (Schoenemann and Bonier, in prep).

Spatial, temporal, and genetic factors like those described above can exert distinct, and sometimes contrasting, effects on GC variation within and among individuals (Busch and Hayward, 2009; Dantzer et al., 2014; Madliger and Love, 2014), which makes it difficult to determine how these factors, in combination, influence the plasticity or repeatability of GCs within individuals.

The black-capped chickadee

The black-capped chickadee (*Poecile atricapillus*) is a small (10–14 g), non-migratory songbird found throughout most of Canada and the United States. They demonstrate a variety of behavioral and physiological strategies that help them live year-round in seasonal forest habitats. During winter, these strategies include cavity roosting to conserve heat, caching food and storing fat subcutaneously to provide auxiliary energy resources, as well as entering hypothermia and diminishing metabolic rate to minimize energy use (Foote et al., 2010). These strategies are particularly important in northern populations, where individuals may face very short days and extremely cold temperatures. Yet, although chickadees can live as long as 12 yr in captivity, their average lifespan in the wild is just 2.5 yr (Smith, 1991); and starvation during winter could be a major source of mortality (Desrochers et al., 1988). Thus, phenotypic variation among overwintering individuals may have striking implications for survival and reproduction, with GCs likely helping to mediate coping strategies.

Research goal

The primary goal of the study reported in Chapter 2 is to investigate sources of variation in GC concentrations, which affect trait repeatability, in order to inform our interpretation of among-individual GC variation. We measured circulating GCs free-living black-capped chickadees on two sampling occasions to estimate the repeatability of baseline concentrations. Additionally, we analyzed relationships between GC concentrations and several environmental and physiological variables to investigate the influence of plasticity on among-individual variation in GCs.

Chapter 2: Circulating glucocorticoids are repeatable in a wild bird during the non-breeding season

Introduction

Natural selection links an individual's phenotype and fitness (Brodie et al., 1995). The first step toward understanding the fitness consequences of individual phenotypic variation is to accurately characterize individual differences in traits that influence survival and/or reproduction (Roche et al., 2016). However, many fitness-related traits are plastic; that is, they are capable of changing across a range of temporal scales in response to internal or external conditions (DeWitt and Scheiner 2004; reviewed in Wolak et al., 2012).

How can we characterize an individual with respect to a labile trait? Estimating the 'broad sense repeatability' of a trait is one approach to measuring its consistency (Biro and Stamps, 2015), while estimating the 'narrow sense repeatability' of a trait attempts to quantify the proportion of variation in a trait of interest in a given population that is due to differences among individuals (Lessells and Boag, 1987). Estimating repeatability in these two ways can greatly aid in interpreting measures of individual phenotype. Inferences made about observable differences among individuals in labile traits are meaningful only insofar as measures thereof represent the phenotype in a way that is appropriate to the question. The degree to which a trait is plastic/repeatable within an individual may itself be an individual characteristic (Careau et al., 2014; Roche et al., 2016). Repeatability can approximate the upper limit of heritability of a trait (Boake, 1989; Dohm, 2002; Hayes and Jenkins, 1997). In these ways, estimates of repeatability

can inform our understanding of the causes of individual variation in plastic traits and the consequences of this variation on fitness-related traits.

Variation in circulating concentrations of glucocorticoid (GC) hormones can have important fitness consequences because GCs are involved in numerous processes related to survival and reproduction, such as energy metabolism, escape behavior, inflammation, and reproductive investment (Legagneux et al., 2011; Rivier and Rivest, 1991; Schreck 1993; Sternberg, 2006; Vegiopoulos and Herzig, 2007; Webster et al., 2002; Wingfield et al., 1998). GC levels can vary greatly both within and among individuals (Cockrem, 2013). Variation within an individual can drive physiological and behavioral responses to challenges that threaten their survival or reproduction (Sapolsky et al., 2000), while the variation among individuals is often used as a metric of relative quality, health, or welfare (Ellis et al., 2012; Jaatinen et al., 2013; Martínez-Mota et al., 2007; Munshi-South et al., 2008; Wada et al., 2008). However, researchers studying GCs, especially those using difficult-to-catch free-living species, sometimes assume that a single measurement of individuals' hormone concentrations captures a relevant biological signal. Yet, the relationship between GCs and fitness metrics is not always present nor consistent in its direction, suggesting that this assumption may not be valid and that individuals should be sampled on multiple occasions (Angelier et al., 2010; Bonier et al., 2009; Breuner et al., 2008; Crespi et al., 2013).

Perhaps in recognition of this point, the repeatability of GC concentrations has been estimated dozens of times (e.g., Cook et al., 2012; Narayan et al., 2013; Romero and Reed, 2008; Wada et al., 2008). While the majority of evidence indicates low to moderate repeatability, there is considerable unexplained variation in the magnitude of these

estimates (Schoenemann and Bonier, in prep). To understand why this is the case, it helps to recall what a repeatability estimate represents. In the biological literature, and in this thesis, r typically refers to narrow-sense (rather than broad-sense) repeatability (Nakagawa and Schielzeth, 2010), following the equation: $r = V_{ind}/(V_{ind} + V_e)$, where V_{ind} is the phenotypic variance (i.e., the amount of variation in hormone concentrations) among individuals and V_e is the phenotypic variance within individuals (Lessells and Boag, 1987; Nakagawa and Schielzeth, 2010). Therefore, factors that influence either within-individual variation, among-individual variation, or measurement error will also affect estimates of trait repeatability. Spatial, temporal, and genetic factors (e.g., time of day, weather, life history stage, age, and sex) can exert distinct, even contrasting, effects on GC variation within and among individuals (Busch and Hayward, 2009; Dantzer et al., 2014; Madliger and Love, 2014), which makes it difficult to determine how these factors, in combination, influence the plasticity or repeatability of GCs within individuals.

We sought to 1) assess the repeatability of corticosterone concentrations (hereafter CORT; the primary avian glucocorticoid) in a free-living bird, black capped chickadee (*Poecile atricapillus*), during the non-breeding season; 2) investigate which internal and external factors predict CORT levels; and 3) examine how those factors influence the repeatability/plasticity of CORT.

Methods

All animal procedures were conducted with approval of the Queen's University Animal Care Committee and the Canadian Wildlife Service.

Study System

Black-capped chickadees (*Poecile atricapillus*; hereafter chickadees) are year-round residents in southeastern Ontario. In the winter months, chickadees form flocks of 3 to 12 individuals that re-associate between years (Schubert et al., 2007). Individuals within these flocks exhibit a linear dominance hierarchy, where males are dominant to females and older birds are dominant to juveniles (Otter et al., 1999; Ratcliffe et al., 2007; Smith, 1991). The territory size of these flocks generally range from 6.8 ha to 14.6 ha (Hartzler, 1970; Odum, 1942; Smith, 1984).

Studying the repeatability of endocrine traits in chickadees during the winter (i.e., non-breeding season) allowed us to minimize variation among individuals due to life history stage or environment. If we had sampled, for example, during the breeding season, when individuals shift asynchronously from egg-laying to brood-rearing phases, we could conflate among-individual differences with differences due to individuals occupying different breeding stages. Furthermore, winter sampling allowed us to capture multiple individuals from the same flock at one sampling location, potentially reducing individual variation in environmental conditions. Thus, winter sampling increased our ability to detect trait repeatability.

Field Methods

Beginning in December 2015, we searched the properties surrounding Queen's University Biological Station (near Chaffey's Lock, Ontario; 44°34'N, 76°19'W) for flocks of overwintering chickadees. Once a flock was located, we tracked individuals to estimate the location and extent of the flock's territory. In total, we identified 14 sites

where we observed chickadee flocking activity. These sites were on average 6 km apart to minimize flock territory overlap (range = 0.33–15.7 km). Once we located a flock's territory, we hung bird feeders filled with sunflower seed in an area of relatively high activity and easy accessibility. We replenished feeders up to two times per month prior to trapping, and as needed once trapping began.

During late January and February, we set up walk-in style 'Potter' traps on platforms on 3 m poles within 10 m of the previously-hung feeders. These traps had either 2 or 4 chambers. We baited the traps with sunflower seed in a locked-open position at least 1 day before trapping (mean = 7 d, range = 1–14 d). Setting the traps with seed prior to trapping gave the birds time to locate and habituate to entering and feeding from them.

All trapping occurred after sunrise (between 0730 and 1300) to minimize the influence of diel rhythms in CORT secretion (Breuner et al., 1999). On the day of trapping, we removed the hanging bird feeders and re-baited and activated the walk-in traps. We generally monitored the traps for 4 hrs, except in 11 of 32 trapping sessions, when extremely poor weather (temperatures below -25 °C or heavy precipitation) or overlapping trapping schedules prevented completion of the full trapping session. We trapped a second time at the same location at least 6 days after the initial capture period (mean = 7.8 d, range = 6–15 d). We captured a total of 144 individuals and collected a second blood sample from 53 of them.

We started a stopwatch the instant a bird was caught in the trap to record time between capture and blood sample collection and time in hand. On average, birds spent 11.3 minutes in hand (range = 5–21.2 min).

We collected approximately 70 μL of blood into heparinized capillary tubes through puncture of the brachial vein with a 26-gauge needle. We attempted to collect these samples within 3 minutes (Romero and Reed, 2005) to minimize the effect of capture on circulating GC concentrations (mean time to sample collection = 2.5 minutes, range = 1.05–4.17 min). We kept samples cool and centrifuged them at approximately 5000 x g for 10 minutes to separate plasma from red blood cells within 10 hours of collection. We then stored plasma and red blood cell samples separately at -20 ºC.

We fitted each bird with 3 leg bands: 1 plastic color band (Avinet, Dryden, NY), 1 plastic color band with a passive integrated transponder (IB Technology, Item Number EM4102, Buckinghamshire, UK), and 1 uniquely-numbered aluminum Canadian Wildlife Services band.

We measured body mass with a Pesola spring scale (±0.5 g), tarsus length with a manual caliper (±0.1 mm), and flattened wing length with a wing ruler (±0.5 mm). We estimated the fat stores of each bird by scoring subcutaneous fat in the furculum on a scale from 0 (no fat) to 5 (bulging fat) (Krementz and Pendleton, 1990). We calculated a scaled-mass index (SMI) for each individual using body mass and wing length and following the Thorpe-Lleonart equation recommended by Peig and Green (2009).

To assess weather conditions over the duration of our study, we obtained daily temperature, wind speed, and liquid (i.e., rain and melted snow) precipitation readings from a weather station located an average of 5.63 km (range = 1.02–8.67 km) from our trapping sites (N44° 33.871' W76° 19.502'; Hoskin Scientific: Satlink-E18, Edmonton, Alberta). This station recorded weather parameters every 15 minutes and we summarized these data over two periods of time prior to each individual's capture. Specifically, we

compiled the mean air temperature and mean wind speed experienced by each individual during 4 h and 2 d before capture, and total liquid precipitation (i.e., rain and melted snow) that fell during the 2 d before capture.

Lab Methods

We determined the sex of each individual using molecular sexing techniques. We extracted DNA from red blood cells using a DNeasy Blood and Tissue kit (Qiagen Inc., Toronto, Ontario) according to supplier instructions. We used primers P2 and P8 to amplify two variants of the chromo-helicase-DNA-binding (CHD) gene, CHD-W and CHD-Z, which are located on the W and Z chromosome, respectively (Griffiths et al., 1998). While the exonic (coding) regions of these genes are highly conserved, the intronic (non-coding) regions are not, and thus their lengths usually differ between the two genes (Griffiths et al., 1998). We separated the amplified gene product on a 2.5% agarose gel stained with Hydra Green Advanced DNA stain (DGel Sciences, Fisher Scientific, Edmonton, Alberta) and run at 110 volts for 45 minutes. Because the W chromosome is unique to females, while the Z chromosome is common to both sexes, amplification of the CHD genes allowed differentiation between homogametic (ZZ, single band = male) and heterogametic (ZW, two bands = female) individuals (Griffiths et al., 1998). For quality control, we amplified and ran the DNA of a known female with every amplification and gel separation procedure.

We quantified total CORT concentrations in plasma using an enzyme-immunossay (EIA, Cayman Chemical, Ann Arbor, MI, Item Number 501320). This assay was previously validated for use in chickadees in our laboratory (Burns and Bonier,

2015). We analyzed all samples from an individual on the same plate. We diluted each sample 1:16 in assay buffer using 7.5 µL plasma, and assayed the dilution in duplicate. We followed manufacturer instructions, except that we incubated the plate for 2 hours at room temperature, rather than overnight at 4° C. The detection limit of the assay is approximately 0.03 ng/mL, and no samples fell below this threshold. Within-assay coefficients of variation determined through assay of a standard plasma pool at the beginning and end of each plate (four replicates per plate) ranged from 4.9–22.1% (mean 13.0%, n = 8 plates). Among-assay variation across all eight assay plates was 19.9%.

To assess oxidative balance, we quantified concentrations of reactive oxygen metabolites (ROM) and the total antioxidant capacity (TAC) of plasma. The chemical processes involved in aerobic metabolism produce ROM as a by-product, which are also known as free radicals. At high levels, ROM can damage proteins, lipids, and DNA, disrupt signaling pathways, and ultimately contribute to senescence (Finkel and Holbrook, 2000). ROM are neutralized by antioxidants, which animals largely obtain from their diet (Halvorsen et al., 2002; Lobo et al., 2010).

To measure ROM, we used the d-ROMs kit (Diacron International, Grosseto, Italy), following a similar protocol as described in Haussmann et al. (2012) that we optimized for chickadee plasma. We ran all samples collected from an individual on the same plate. This assay measures levels of hydroperoxides, which are byproducts of the peroxidation process by which ROM inflict damage on lipids, proteins, and DNA (Hulbert et al., 2007). We assayed 2 µL of undiluted plasma in duplicate. To each duplicate, we added 202 µL of a 2:200 µL ratio mixture of N,N-diethyl-p-phenylenediamine chromogen reagent and 0.01 M acidic acetate buffer solution. The

hydroperoxides reacted with the chromogen reagent to produce a color change in the sample proportional to their concentration. Immediately after adding the reagent mixture, we began to read the absorbance of sample color at 505 nm using a spectrophotometer (Molecular Devices, Spectramax Plus, CA, USA). The spectrophotometer read the absorbance every minute for 30 mins while incubating the plates at 37° C. We calculated ROM concentrations (mmol/L of H_2O_2) from absorbance values using a manufacturer-supplied conversion formula. Within-assay coefficients of variation between duplicate standards of pooled plasma ranged from 13.7 to 65.1% (n = 4 plates). Among-assay variation based on standards of pooled plasma was 33.2%. Recognizing the high degree of within and among assay variation, we are cautious about interpreting these data and discuss how this error may have affected our findings.

To measure TAC, we used the OXY-absorbent test kit (Diacron International, Grosseto, Italy), again following a similar protocol as described in Haussmann et al. (2012) that we optimized for chickadee plasma. We ran all samples from an individual on the same plate. This assay measures the plasma's ability to neutralize an oxidizing agent, hypochlorous acid (HClO). We diluted 5 µL of plasma in 95 µL of doubly distilled H_2O and assayed 2 µL of diluted plasma in duplicate. To each duplicate, we added 200 µL of HClO. We mixed the samples at room temperature on a plate shaker at 250 rpm for 2 minutes, and then incubated them at room temperature for an additional 8 minutes. Following incubation, we added 2 µL of an alkyl-substituted aromatic amine (A-NH_2) chromogen reagent, which is oxidized by the HClO in the sample and thereby changes color to a degree that is inversely proportional to the plasma's antioxidant power. Immediately after adding the chromogen reagent, we began to read the absorbance of this

color change at 505 nm (Molecular Devices, Spectramax Plus). The spectrophotometer read the absorbance every minute for 25 minutes. We calculated TAC (mmol/L of HClO neutralized) from absorbance values using a manufacturer-supplied conversion formula. Within-assay coefficients of variation between duplicate standards of pooled plasma ranged from 0.009 to 0.05% (n = 4 plates). Among-assay variation based on standards of pooled plasma was 10.28%. Due to systematic differences in TAC values across plates, we standardized these data by subtracting the plate average from each sample value and then adding the grand average to the difference. We used these values in all analyses.

We combined ROM and TAC values to calculate an oxidative index (OI) that reflects an individual's oxidative balance, which depends on both the concentration of oxidants (ROM) and capacity of antioxidants (TAC) (Vassalle et al., 2008). To do so, we first calculated standardized ROM and TAC values according to the equation, $x - \frac{\bar{x}}{sd}$, where x is the parameter value, $\bar{x}$ is the mean parameter value, and sd is the standard deviation of the parameter. The OI is the difference between the standardized ROM and TAC values (Vassalle et al., 2008). Thus, OI should reflect differences among individuals in oxidative damage. A higher oxidative index indicates an individual with a less favorable oxidative balance, with a standardized concentration of ROM greater than that of TAC.

Statistical Analyses

CORT concentrations are typically described as reflecting a baseline level when blood samples are collected within 2–3 min of capture (Romero and Reed, 2005). While most of our samples were collected within this time window (n=174/195, 89.2%), we

found a significant relationship between sample time and log-transformed CORT (lm: $\beta = 0.007$, $p < 0.0001$; Supplementary Fig 1). To determine when a significant rise in CORT levels occurred in our samples, we conducted change point analysis on the logarithm of CORT, similar to methods used in Romero and Reed (2005) and Small et al. (2017). We used the *cpt.mean* function from the 'changepoint' package (version: 2.2.2) to analyze only the first sample collected from each individual, which avoids including non-independent data. Our analyses indicated that CORT levels in our population sample were significantly increased at 159 secs (2:39 min) after capture ($p < 0.0001$). Therefore, we included sample time as a control variable in all analyses and retained samples collected after 159 seconds in our analyses (n = 75, 40.3%). Hereafter, we refer to all CORT measurements as initial, rather than baseline, CORT.

We modeled raw CORT using generalized linear mixed effects models (GLMMs) with a Gaussian error distribution and a log link function. Modeling untransformed CORT data with a log link allowed us to estimate the relationship between predictors and the response on the raw data scale for greater ease of data interpretation. We scaled numeric predictors included in the GLMM to have a mean of 0 and a variance of 1, which allowed us to compare the relative effect size of predictors on different measurement scales. We performed analyses using data from all individuals, and separately for males and females, to determine if CORT repeatability differs between the sexes.

To assess the repeatability of CORT (goal 1), we calculated agreement, consistency, and adjusted repeatabilities using the *rpt* function of the 'rptR' package (version: 0.9.2) by coding GLMMs with individual identify as the random effect and confounding factors as the fixed effects. Agreement repeatability refers to the absolute

similarity of values between measurements (McGraw and Wong 1996). Consistency repeatability, by contrast, describes the similarity of values after controlling for differences in means between repeated measurements. For example, if all individuals tend to have higher GCs at the second sampling point than at the first, consistency repeatability will reflect repeatability after controlling for this change. Similarly, adjusted repeatability controls for the effect of any variables that might differ between individuals at the time of sampling and also influence CORT (Nakagawa and Schielzeth, 2010). To investigate which factors influence estimates of repeatability (goal 2), we statistically controlled for relevant variables (i.e., variables identified as predictors of CORT in the selection process described below) in our adjusted repeatability calculations. In all repeatability calculations, we only included data from individuals caught twice. We generated confidence intervals around the repeatability estimates based on the distribution of estimates from 1,000 parametric bootstraps, and p-values based on 1,000 permutation tests (Nakagawa and Schielzeth, 2010).

Finally, to examine how internal or external factors influence the plasticity/repeatability of CORT (goal 3), we used an information theoretic approach to select predictors from a suite of candidate variables. In these analyses, we included data from all individuals, whether they were caught once or twice. We coded two global mixed effect models with individual identity as the random effect. These models differed in which weather variables were included as fixed effects, but included all the same non-environmental variables (see Table 1). We used the *dredge* function from the 'MuMIn' package (version: 1.15.6) to rank models, which included all combinations of the candidate variables, using Akaike's information criterion corrected for small sample

sizes (AICc). We eliminated variables that did not occur in the top models (delta AICc < 2) of both GLMM 1 and 2 (Supplementary Table 1). We ranked models with

Table 1. Variables included (indicated with a check mark) as fixed effects in two initial and one final generalized mixed effect models (GLMM) explaining variation in corticosterone concentrations in free-living chickadees. Global GLMM 1 and 2 were evaluated using an information theoretic approach (see text) to select variables for inclusion in adjusted repeatability and regression analyses; these selected variables appear in the "Final GLMM" column.

Candidate Variable	***Global GLMM 1*** [a]	***Global GLMM 2*** [a]	***Final GLMM*** [a]
capture time	✓	✓	✓
sample time	✓	✓	✓
fat score	✓	✓	
scaled-mass index	✓	✓	✓
oxidative index	✓	✓	✓
initial or recapture	✓	✓	✓
average temperature during 2 d prior to capture	✓		
average wind speed during 2 d prior to capture	✓		✓
total liquid precipitation during 2 d prior to capture	✓		✓
average temperature during 4 h prior to capture		✓	✓
average wind speed during 4 h prior to capture		✓	

[a] The random effect for all models is individual identity.

environmental variables averaged over 4 hr before capture separately from those averaged over 2 d before capture to 1) avoid collinearity among predictors in our global models, and 2) investigate whether environmental conditions within 4 hr or 2 d explained more variation in CORT. When environmental variables from both 4 hr and 2 d before capture were retained in the top models following this procedure, we ranked the AICc of models with either the 4 hr average, 2 d average, or neither (Supplementary Table 2). We confirmed the robustness of our selections by repeating this process on reduced datasets, which respectively excluded CORT data from samples with >20% coefficient of variation between duplicates (n = 18/195, 9.2%) or birds that subsequently died after sampling (n = 6/195, 3.1%). No fixed effects identified in the first analysis were omitted from the top models of these reduced datasets. See Table 1 for a complete list of selected variables.

Results

Below, we report all repeatabilities as: mean [95% CI] (p-value). We detected moderately low repeatability in initial CORT concentrations within individuals during the non-breeding season (goal 1; Fig 3; Supplementary Fig 2). Overall, we estimated a significant agreement repeatability of 0.284; in other words, individual identity accounted for 28.4% of total phenotypic variation (r_{agr} = 0.284 [0.028, 0.516] (p = 0.022), n = 53 individuals). We found qualitatively similar answers when we analyzed male and female repeatability separately, although confidence intervals for these repeatability estimates included zero, likely due to reduced sample size (male r_{agr} = 0.268 [0.0, 0.54] (p = 0.055), n = 34; female r_{agr} = 0.331 [0.0, 0.68] (p = 0.078), n = 19).

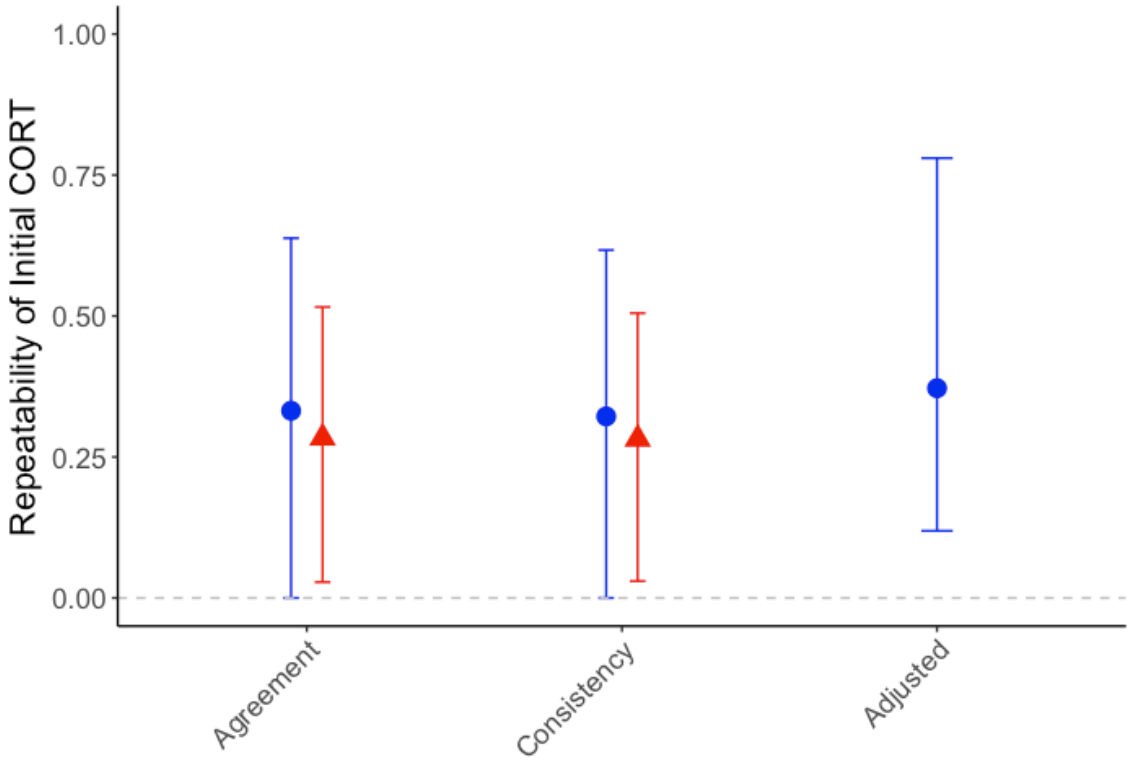

Fig 3. Repeatability estimates of initial corticosterone (CORT) concentrations. Points represent mean estimates, and bars represent 95% confidence intervals. CORT titers were measured in free-living black-capped chickadees at two time points during the non-breeding season. Three analyses of repeatability are shown: agreement [i.e., narrow sense repeatability], consistency [i.e., correcting for mean differences between sampling events], and adjusted [i.e., correcting for differences in variables that explain variation in CORT]. Due to decreased sample size for the adjusted repeatability calculations, re-analyses of agreement and consistency repeatability using the same, smaller dataset are included to aid in interpretation (blue circles, n=26; red triangles, n=53).

When estimating consistency repeatability, we detected a strong effect of initial capture on CORT. CORT increased by an average of 11.6% across all individuals (6.9% in females, and 12.6% in males) between the first and second capture (Fig 4; lmm: n = 117 observations, $\beta = 0.477$, $t = 6.226$, $p < 0.0001$). A post-hoc linear regression to explore possible causes of this effect revealed that this change in CORT was not related to time in hand (lm: n = 52, $\beta < 0.0001$, $t = 0.286$, $p = 0.776$) during the first capture. Even after controlling for mean differences in CORT between captures, we estimated a consistency repeatability similar in magnitude to agreement repeatability ($r_{con} = 0.282$

[0.03, 0.505] (p = 0.029), n = 53). We found qualitatively similar answers when we analyzed male and female repeatability separately, although, again, confidence intervals for these repeatability estimates included zero, likely due to reduced sample size (male r_{con} = 0.258 [0.0, 0.56] (p = 0.085), n = 34; female r_{con} = 0.322 [0.0, 0.69] (p = 0.107), n = 19).

Using an information theoretic approach, we identified several factors relevant to explaining variation in CORT (goal 2). When adjusted for these confounding factors, individual identity accounted for 37.2% of total phenotypic variation (r_{adj} = 0.372 [0.119, 0.780] (p = 0.204), n = 26). The decrease in sample size from agreement to adjusted repeatabilities is the result of missing data from some individuals for some of the factors we investigated. Furthermore, due to small sample size, we did not have sufficient power to calculate adjusted repeatability for males or females separately.

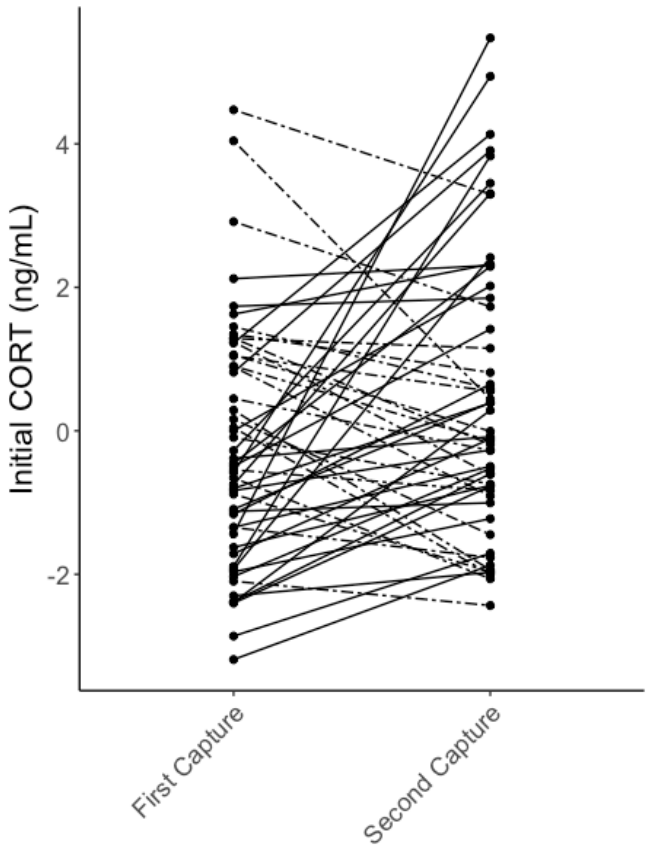

Fig 4. Within-individual change in corticosterone (CORT) titers between captures. Initial CORT is plotted as the residuals of the linear regression of CORT on sample time, to correct for differences in time between capture and blood sample collection. CORT was measured twice in non-breeding black-capped chickadees. Each line connects samples from one individual, and line type indicates direction of change (solid increases, and dashed decreases).

Finally, in addition to relationships with sample time and initial vs. recapture (described above),

we found several environmental and physiological variables that also predicted variation in CORT (goal 3; Table 2). Importantly, all predictor variables were standardized prior to regression analysis and, therefore, the effect size of these variables (i.e., βs) reflects the relative magnitude of their association with CORT.

We found that chickadees exposed to lower temperatures during the 4 hr prior to capture and higher wind speeds 2 d prior to capture had lower initial CORT levels (n = 112, temperature: $\beta = 0.132$, $t = 2.38$, $p = 0.017$; wind: $\beta = -0.189$, $t = -5.35$, $p < 0.0001$). The magnitudes of these relationships were between one-third and one-half that of the relationship between CORT and the time between capture and sample collection (n = 112, $\beta = 0.360$, $t = 9.71$, $p < 0.00001$). Given that capture and handling can induce large increases in CORT titers within 3 minutes (e.g., in this study; see Small et al., 2017), these results support the interpretation that temperature and wind speed are also relatively strongly associated with variation in CORT. In addition, individuals with a higher oxidative index had elevated CORT relative to individuals with lower oxidative index (n = 112, $\beta = 0.132$, $t = 4.45$, $p < 0.0001$); and the magnitude of this effect was very similar to that of temperature prior to capture. Lastly, although inclusion of

Table 2. Results from a generalized mixed effect model[1] explaining variation in initial corticosterone concentrations, which were measured twice in non-breeding black-capped chickadees[2].

Variable[3]	***Std β***	***SE***	***t-value***	***p-value***
(Intercept)	0.944	0.077	12.23	<0.00001
avgWind.2d	-0.189	0.035	-5.35	**<0.00001**
totPrecip.2d	0.056	0.050	1.12	0.262
avgTemp.4hr	0.132	0.055	2.38	**0.017**
SMI	-0.052	0.054	-0.97	0.334
OI	0.132	0.030	4.45	**<0.00001**
capture.time	-0.073	0.039	-1.89	0.058
sample.time	0.360	0.037	9.71	**<0.00001**
initial.recap	0.278	0.070	3.96	**<0.0001**

[1]The random effect is individual identity.
[2]N = 86 individuals, 112 observations.
[3]The fixed effects were selected via an information theoretic selection procedure.
Abbreviations: SMI, scaled-mass index; OI, oxidative index

scaled-mass index, capture time, and total precipitation prior to capture improved the fit of our global model (and hence, they were retained in the analysis), these factors did not significantly predict variation in CORT (Table 2). Notably, the effect of capture time (i.e., time of day when the individual was trapped) approached significance, with CORT tending to decrease between 0730 and 1300, although the magnitude of this effect was relatively minor ($n = 112$, $\beta = -0.073$, $t = -1.89$, $p = 0.058$).

Discussion

In our study of free-living black-capped chickadees during the non-breeding season, we found 1) moderately low, but significant, repeatable differences in initial CORT concentrations among individuals; 2) increases in mean CORT titers between the first and second sampling occasions; and 3) significant relationships between CORT and weather and physiological variables. Overall, individual identity accounted for 28.4% of total individual variation in CORT ($r_{agr} = 0.284$). CORT concentrations were lower in individuals that experienced higher wind speeds and lower temperatures, and higher in individuals with higher oxidative index (i.e., greater ROM relative to antioxidant capacity).

On the one hand, we should expect individuals in a population to express relatively consistent (i.e., repeatable) levels of circulating GCs because of genetic differences among individuals, as well as stability in aspects of their physical and social environment during that period. Our low repeatability estimates may reflect the relatively minor degree to which genetic differences explain variation in CORT; this interpretation aligns well with other studies that also report low estimates of the heritability of CORT

(e.g., baseline: 0.152, stress-induced: 0.343; Jenkins et al., 2014). Furthermore, we attempted to minimize environmental variation between sample times. We measured chickadees during the winter (i.e., their non-breeding season) to minimize individual variation caused by differences in reproductive status or energetic demands of parenthood (Bonier et al., 2011). Social interactions among wintering chickadees should have been relatively consistent over the timespan of our study because flock membership and dominance status are stable features of these winter flocks (Desrochers, 1989; Desrochers and Hannon, 1989; Hartzler, 1970; Ratcliffe et al., 2007; but see Smith and Van Buskirk, 1988). Chickadees can cache food within their territory in preparation for winter and then access these stores weeks later (Hitchcock and Sherry, 1990; Sherry, 1984), which can provide an auxiliary food source when other foraging opportunities are limited. Therefore, our finding of consistent individual differences in CORT, a highly plastic trait, supports the interpretation that some variation in CORT may be due to genetic differences among individuals, and that individuals experienced relatively similar environments between our two sampling periods.

Although statistically significant, our estimates of CORT repeatability were still considerably lower than repeatability estimates of many other plastic traits, including mating behavior, habitat selection, and metabolic rate, all of which have estimated repeatabilities greater than 0.50 (Bell et al., 2009; Nespolo and Franco, 2007). Thus, initial CORT concentrations in our population appear to demonstrate a greater degree of plasticity than these traits. Individuals adaptively alter CORT concentrations, reducing their repeatability, in order to respond to energetic challenges, such the acute stress of capture (Lynn et al., 2010). We found that average CORT was significantly higher upon

recapture, possibly indicating that individuals were still responding to the initial capture when they were recaptured up to 10 days later. However, the low agreement repeatability estimate is not due solely to population-level changes between captures. If this were the case, consistency repeatability (adjusted for mean differences in CORT) should be higher than agreement repeatability; however, consistency repeatability ($r_{con} = 0.282$) was similar to agreement repeatability ($r_{agr} = 0.284$). The similarity of agreement and consistency repeatability estimates is likely the result of many individuals exhibiting increased CORT upon recapture, while others had CORT concentrations that decreased or stayed relatively the same (see Fig 4). Thus, the low repeatability of CORT in our population indicates a degree of within-individual trait plasticity, but which cannot be completely explained by the effect of initial capture.

Another explanation for the low repeatability detected here is that individuals were caught under varying weather conditions. During our study, air temperatures varied from -30°C to +9°C (mean = -5.51°C ± 0.14 SE), and wind speeds varied from 0 to 15 m/s (mean = 3.29 m/s ± 0.04 SE). Given the limitations of field sampling, we could not sample all individuals in the population instantaneously, therefore, some birds experienced relatively mild weather (i.e., relatively higher temperature and lower wind speed) in the days or hours before capture, while others experienced harsh weather (i.e., snowstorms, high winds) before capture. Moreover, this difference also occurred within individuals between sampling events. Coping with winter weather and/or severe storms can be energetically costly, and concentrations of GCs have been shown to increase in non-breeding birds under these conditions (e.g., De Bruijn and Romero, 2013; Rogers et al., 1993; Smith et al., 1994).

By contrast, in our population, individuals that experienced lower air temperatures during the 4 hours prior to capture and higher wind speeds during the 2 days prior to capture had lower initial CORT concentrations. Perhaps cold, windy weather is *less* energetically challenging than warmer, calmer weather. Chickadees seasonally elevate maximal thermogenic capacity and standard metabolic rate during the winter (and to a degree that is greater than in other passerines) in order to tolerate cold stress (Cooper and Swanson, 1994). Chickadees can also produce enough metabolic heat during foraging to substitute shivering thermogenesis (Cooper and Sonsthagen, 2007). Therefore, chickadees that have acclimatized to winter conditions may produce excess heat during warmer and calmer weather, and may expend more energy altering their behavior or physiology to dissipate excess heat during these conditions. This effect may be exaggerated in the chickadee, compared to species that migrate or overwinter in milder climates, which could explain why our results contrast with studies of other birds (i.e., de Bruijn and Romero, 2013; Rogers et al., 1993; Smith et al., 1994). However, Cooper and Sonsthagen (2007) also found that chickadees produced significantly less heat at higher ambient temperatures, which suggests that (despite acclimatization) chickadees retain the ability to plastically modulate metabolic heat production under warm conditions.

Alternatively, perhaps cold, windy weather *is* energetically challenging, and lower initial CORT concentrations represent an adaptive response (Boonstra, 2004). Chickadees must forage to accumulate enough fat to survive periods of extreme cold and inactivity (e.g., at night or during poor weather) (Houston and McNamara, 1993). Great tits (*Parus major*), a close relative of the chickadee, used daily temperatures as a cue for how much fat to store, with lower temperatures associated with more fat storage (Gosler, 2002).

Thus, chickadees exposed to cold may alter behavioral or physiological strategies to maximize fat utilization (Houston and McNamara, 1993), including reducing metabolic rate and inducing facultative hypothermia (Chaplin, 1974), as well as altering behavioral foraging strategies (Grubb Jr., 1975), all to minimize energy expenditure. However, individuals may need to fast during periods of severe weather, food scarcity, or at night; the suppression of the HPA axis may enable individuals to do so without compromising long-term health (Boonstra, 2004). GC concentrations remain low during the early phases of fasting, when glycogen and fat stores supply the animal's energy; only once these fats stores are depleted do GCs increase rapidly to mobilize proteins (Cherel et al., 1988a, 1988b). Thus, chickadees exposed to periods of challenging weather (i.e., cold and windy) may lower circulating CORT concentrations while fasting to protect muscle tissues vital for effective foraging and predator escape in the future. Chickadees are residential species that have adapted to survive a northern winter climate, and one component of this strategy may be the plastic modulation of circulating GCs according to weather conditions.

Additionally, differences among individuals' physiological state, reflected in a metric of oxidative balance, might be related to the large within-individual changes in CORT and low repeatability observed. At high levels, ROM can damage proteins, lipids, and DNA, disrupt signaling pathways, and ultimately contribute to senescence (Finkel and Holbrook, 2000). ROM are neutralized by antioxidants, which animals largely obtain from their diet (Halvorsen et al., 2002; Lobo et al., 2010). The oxidative index values we report here represent the difference between standardized concentrations of ROM and TAC, with a high oxidative index indicating more ROM relative to antioxidant capacity.

In our population, individuals with a high oxidative index had higher initial CORT. Oxidative damage can increase with food consumption, energy expenditure, and metabolic rate (Costantini, 2008; Masoro, 2000; Metcalfe and Alonso-Alvarez, 2010; but see Beamonte-Barrientos and Verhulst, 2013), and CORT plays a large role in stimulating locomotor, foraging, and food-caching behaviors (Dallman et al., 2004; Landys et al., 2006; Pravosudov, 2003). Together, relatively high oxidative index and initial CORT might indicate that an individual is adopting a strategy of active fat-accumulation and foraging, rather than passive fasting. While we found no relationship between CORT and subcutaneous fat score or body condition (SMI), it is possible that these individuals do not store resources over the long term; in which case, we might expect no relationship between these factors. After controlling for differences among individuals in oxidative balance and weather conditions experienced prior to capture, the estimate of GC repeatability increased, while within- and among-individual variance decreased, indicating that variation in these factors contributed to the plasticity of initial CORT concentrations.

Methodological error associated with the d-ROMs assay may have affected the accuracy of our measures of oxidative balance. While measurements that are imprecise, but accurate, could obscure real relationships between oxidative balance and other variables, imprecise and inaccurate measures can generate misleading or spurious patterns. To assess the accuracy of our measurements, we need to further validate these laboratory methods in our study system. Biological validation, involving the physical or chemical application and/or neutralization of oxidative damage in several individuals, and periodic sample collection before and after such tests, to evaluate whether the

measurements of oxidative balance in those samples match what would be biologically expected (e.g., as in the validation of fecal GC metabolites, Touma and Palme, 2005), was beyond the scope of this study. Thus, until we thoroughly validate these methods, we must be cautious in interpreting our oxidative index data.

Our results suggest that internal and external factors are associated with adaptive, plastic changes in circulating GCs and that, despite these plastic changes, initial CORT concentrations are significantly repeatable in a free-living species during the non-breeding season. The extent to which natural selection can shape a genetic trait depends on how well the phenotype reflects individual differences. Thus, the environmental causes and organismal consequences of individual variation in HPA activity represent some of the proximate drivers of evolutionary and ecological processes. Our findings contribute to an understanding of sources of individual variation in GC concentrations, and thereby improve our ability to interpret the relative fitness of individual endocrine phenotypes and the extent to which these adaptive, plastic responses may be heritable.

Chapter 3: General Discussion

The study of the causes and consequences of individual variation in plastic traits, such as GC hormones, can enrich our understanding of evolution, species persistence, and community ecology (Madliger and Love, 2016; Roche et al., 2016; Williams, 2008). However, spatial, temporal, and genetic factors exert distinct, and sometimes contrasting, effects on GC variation within and among individuals (Busch and Hayward, 2009; Dantzer et al., 2014; Madliger and Love, 2014). Failure to acknowledge, measure, or control sources of within-individual variation of GC titers might diminish our ability to detect biologically significant patterns in GC secretion (Bonier and Martin, 2016). Estimating the repeatability (i.e., consistency over time or across contexts) of GC titers is one technique for avoiding this pitfall. In the study reported in Chapter 2 of this thesis, we measured circulating GCs in free-living black-capped chickadees (*Poecile atricapillus*) at two time points, to investigate environmental/physiological conditions that predict individual variation in initial CORT, and to estimate the repeatability of initial CORT concentrations.

Our findings advance our understanding of the repeatability of glucocorticoid concentrations in free-living species. While initial CORT concentrations were significantly repeatable during the non-breeding season, internal and external factors were associated with plastic changes in circulating GCs that ultimately lead to a moderately-low repeatability estimate. This plasticity may reflect chickadees' adaptive physiological strategy to cope with winter conditions. More broadly, the idea that GCs exhibit adaptive plasticity may be relevant to explaining among-individual variation in GCs in other species. Repeatability is a characteristic of a given population, which varies

across and within taxonomic groups (Schoenemann and Bonier, in prep), while plasticity is a characteristic of an individual, which varies among individuals (Lendvai et al., 2014). The causes and consequences of variable levels of GC repeatability among groups, or trait plasticity among individuals, as they relate to a population or individual's adaptive strategy warrants further investigation to understand their implications for evolutionary and ecological processes.

Our study also calls attention to the benefits of estimating the repeatability of plastic traits, which can inform study design and enrich interpretations of individual phenotypic variation. Whether a population exhibits low repeatability due to large within-individual variability or due to among-individual differences in trait consistency has different implications for the collection and analysis of data. In the former case, a single measure of individual differences will be most accurate when collected from all individuals as close to the same time as possible, whereas, in the latter case, a single measure of individual differences will not likely capture consistent individual differences. Low repeatability can also negatively bias the degree to which traits correlate with each other when measured (Garamszegi et al., 2012), and necessitate increasing sample size to detect real relationships. However, if individuals are unknowingly sampled in different physical or social environments, or if some individuals differ in personality-related strategies, then the relationship between their hormone levels and environmental factors/traits of interest may differ from the population-level response in unexpected ways (Roche et al., 2016). Importantly, this effect could obscure biologically-relevant patterns, regardless of sample size. For example, Bonier et al. (2011) found that, while there was no relationship between brood size and baseline CORT among female tree

swallows (*Tachycineta bicolor*), a significant increase in baseline CORT was observed within individuals following experimental increase in brood size. Additionally, olive flounder (*Paralichthys olivaceus*) with bold behavioral phenotypes responded physiologically to an acute stress in a manner opposite that of shy types, and these divergent responses were repeatable (Rupia et al., 2016). In both of these cases, failure to measure within-individual responses, or to recognize among-individual variation in the direction of those responses, would have obscured detection of the effects of the energetic challenge (i.e., brood size, acute stress) at the population level.

Finally, the potential for methodological variation in laboratory assays to introduce variation in GC titers supports the utility of estimating the repeatability of traits measured with assays. All techniques for hormone measurement can introduce error to hormone measurements (Buchanan and Goldsmith, 2004), and the importance of rigorous validation, as well as advice for establishing best practices, has been discussed at length in multiple reviews (Hodges et al., 2010; Sheriff et al., 2011). Due to the large extent of inter-laboratory variation in measurements of absolute steroid hormone concentrations (Bókony et al., 2009; Fanson et al., 2017; Feswick et al., 2014; Ganswindt et al., 2012), across-study comparisons of absolute values of individuals' GC titers are valid only if the studies used identical hormone assay protocols. Estimates of the repeatability of GC concentrations (measured using a single, validated protocol), by contrast, provide a metric of HPA activity that is unaffected by among-laboratory differences in the absolute measurement of hormone titers and, thus, can be compared across studies. Indeed, repeatability estimates could even detect methodological sources of individual variation,

if they are found to be much lower than biologically expected (i.e., if GC titers are found to vary more with laboratory variables than ecological ones).

Altogether, this work contributes meaningful insights to further our understanding of evolutionary and ecological processes driven by individual variation in endocrine phenotypes. We encourage additional research to follow up on the lines of inquiry discussed, and invite research programs to incorporate repeatability estimation into their active investigations in the ways mentioned here, as well as to explore new applications of this versatile tool.

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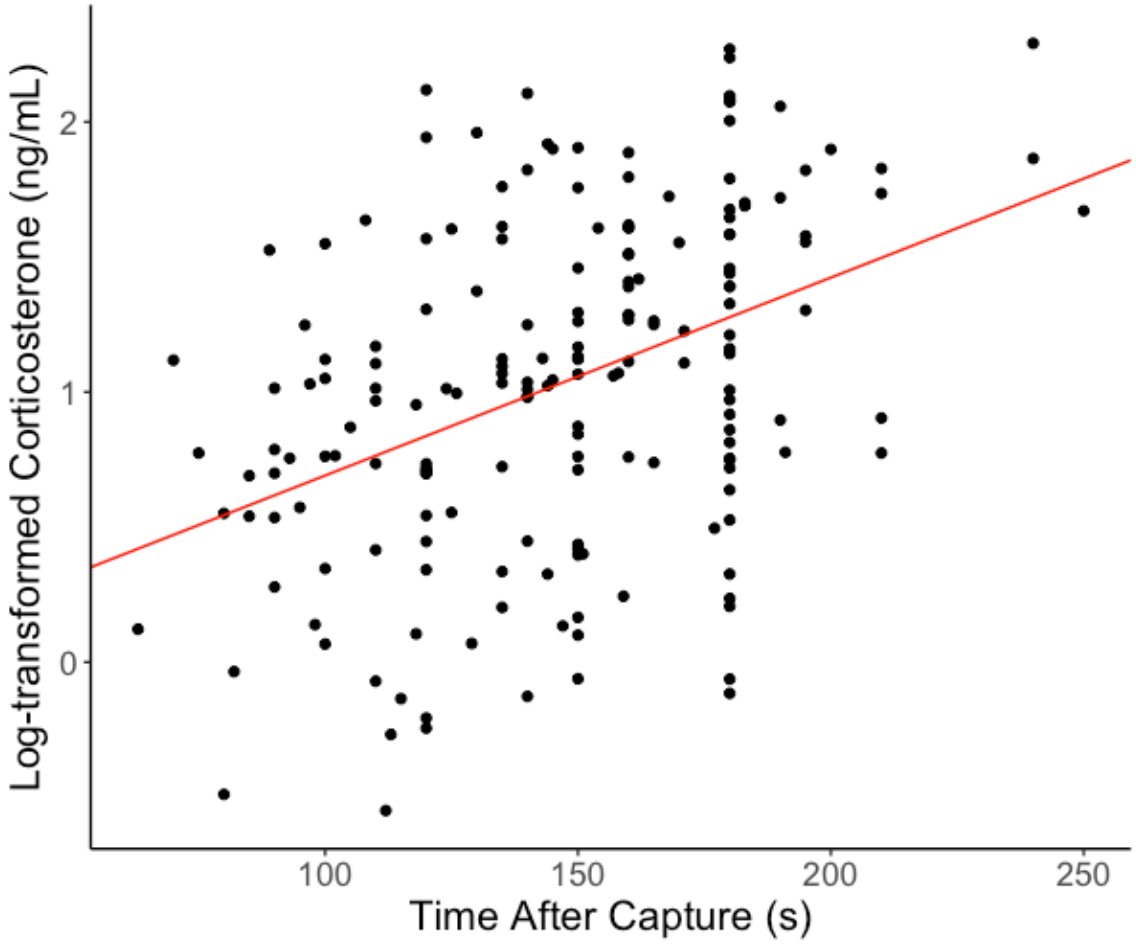
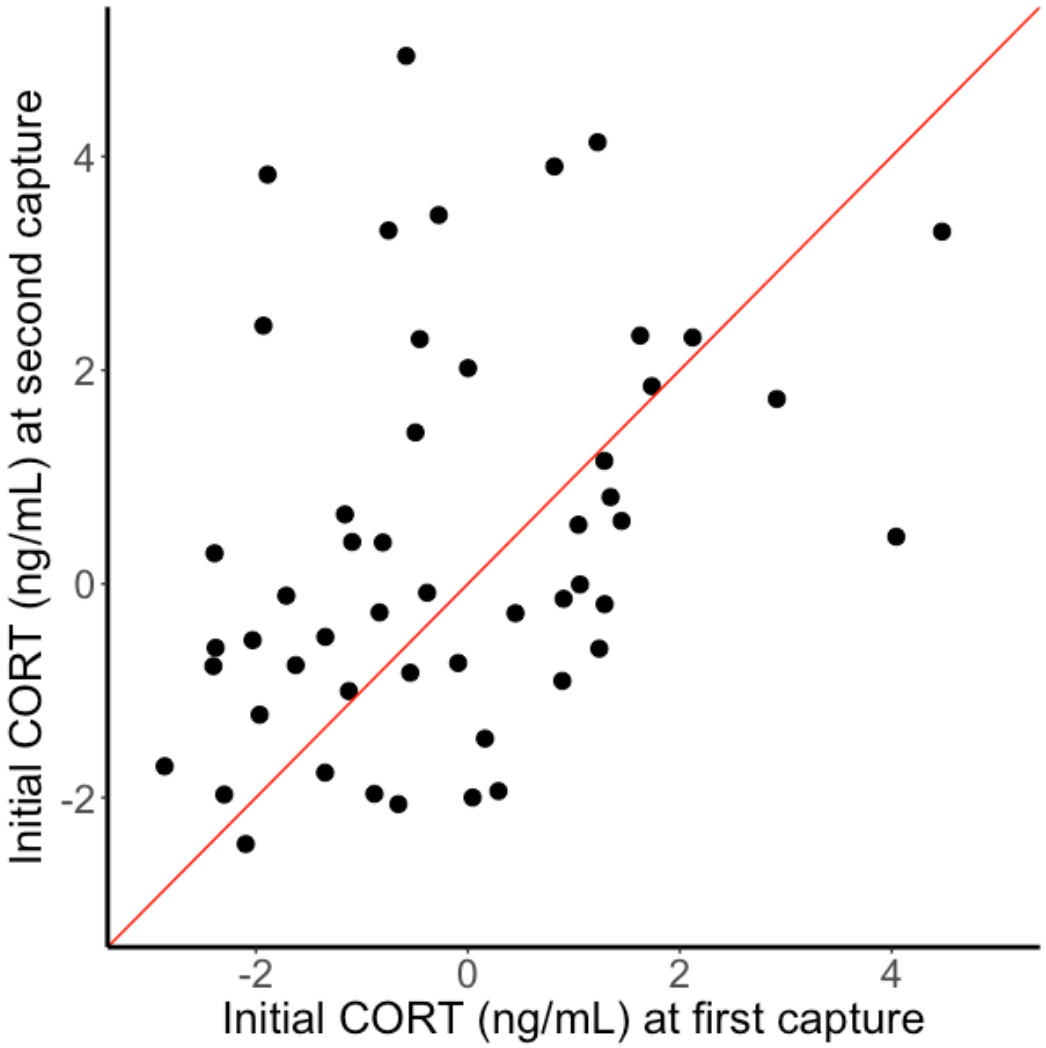
Appendix



Supplementary Fig 1. Log-transformed initial corticosterone concentrations (ng/mL) increase with time between capture and blood sample collection in free-ranging black-capped chickadees captured twice during the non-breeding season (lm: $\beta = 0.007$, $p < 0.0001$).



Supplementary Fig 2. Initial corticosterone (CORT) concentrations during the first capture predict CORT during a second capture (agreement repeatability = 0.284, 95% CI:0.028, 0.516, p = 0.022, n = 53). We measured CORT in free-ranging black-capped chickadees twice during the winter. Here, CORT is plotted as the residuals of the linear model, CORT~sample time, to correct for differences in the time between capture and blood sample collection. Each point represents one individual. The red line (slope = 1; intercept = 0) illustrates where points would fall if CORT were identical between captures (i.e., repeatability of 1).

Supplementary Table 1. Rankings of GLMMs[1] explaining variation in initial CORT concentrations that were measured twice in wild, non-breeding chickadees[2]. Models were constructed from re-combinations of two global models, Model 1 and Model 2. Variables that did not appear in the top models[3] (ΔAICc[4] < 2) of both sets of variables were eliminated from later regression and adjusted repeatability analyses.

Model 1 – Variable Selection

Avg Temp 2 d	Avg Wind 2 d	Sample Time	Fat Score	Oxidative Index	Scaled-Mass Index	Capture Time	Initial or Recapture	Total Precip 2 d	DF	-2ln*L*[5]	△AICc	weight
+	+	+		+		+	+		9	-190.38	0.00	0.07
	+	+		+	+		+		8	-191.65	0.17	0.07
	+	+		+	+		+	+	9	-190.54	0.32	0.06
+	+	+		+			+		8	-191.78	0.44	0.06
	+	+		+		+	+	+	9	-190.63	0.51	0.06
	+	+		+	+	+	+		9	-190.71	0.65	0.05
	+	+		+			+	+	8	-191.91	0.70	0.05
+	+	+		+	+		+		9	-190.75	0.74	0.05
	+	+		+		+	+		8	-191.94	0.75	0.05
	+	+		+	+	+	+	+	10	-189.74	1.13	0.04
+	+	+		+	+	+	+		10	-189.79	1.23	0.04
	+	+	+	+	+		+		9	-191.00	1.25	0.04
	+	+		+			+		7	-193.46	1.48	0.03
	+	+	+	+			+		8	-192.50	1.87	0.03
	+	+	+	+			+	+	9	-191.34	1.92	0.03
	+	+	+	+	+		+	+	10	-190.15	1.96	0.03
+	+	+	+	+			+		9	-191.41	2.06	0.03
+	+	+		+		+	+	+	10	-190.25	2.16	0.02

Model 2 – Variable Selection

Avg Temp 4 hr	Avg Wind 4 hr	Sample Time	Fat Score	Oxidative Index	Scaled-Mass Index	Capture Time	Initial or Recapture	DF	-2ln*L*[5]	△AICc	weight
		+		+	+		+	7	-201.60	0.00	0.17
	+	+		+	+		+	8	-200.85	0.81	0.12
+		+		+	+		+	8	-201.22	1.55	0.08
		+		+	+	+	+	8	-201.40	1.92	0.07
		+	+	+	+		+	8	-201.48	2.07	0.06
		+		+			+	6	-204.04	2.59	0.05

[1]The random effect for all models was individual identity.
[2]N=112 observations, 86 individuals
[3]Models were ranked by Akaike's Information Criterion adjusted for small sample bias (AICc).
[4]ΔAICc refers to the differences between the difference between the AICc of a given model and the best-fit model.
[5]-2ln*L* refers to the maximized log-likelihood.

Supplementary Table 2. Rankings of GLMMs[1] explaining variation in initial CORT concentrations that were measured twice in wild, non-breeding chickadees[2]. Models were coded to include different weather variables, with either the 4 hr average, 2 d average, or neither. Variables that appear in the single top model[3] were selected for inclusion in regression and adjusted repeatability analyses.

Avg Temp 2d	**Avg Temp 4h**	**Avg Wind 2d**	Avg Wind 4h	**Sample Time**	**Oxidative Index**	**Scaled-Mass Index**	**Capture Time**	**Initial or Recapture**	**Total Precip 2d**	DF	-2ln*L*[4]	△AICc[5]
	+	+		+	+	+	+	+	+	11	-187.18	0.00
+		+		+	+	+	+	+	+	11	-189.60	4.84
+			+	+	+	+	+	+	+	11	-192.56	10.75
				+	+	+	+	+	+	9	-200.85	22.47
	+		+	+	+	+	+	+	+	11	-200.47	26.57

[1]The random effect for all models was individual identity.
[2]N=112 observations, 86 individuals
[3]Models were ranked by Akaike's Information Criterion adjusted for small sample bias (AICc).
[4]-2ln*L* refers to the maximized log-likelihood.
[5]ΔAICc refers to the differences between the difference between the AICc of a given model and the best-fit model.