7-11-2015

Corticosterone in Feathers as a Biomarker: Biological Relevance, Considerations and Cautions

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Corticosterone in Feathers as a Biomarker: Biological Relevance, Considerations and Cautions

By

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A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

2015

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9 April 2015

DECLARATION OF CO-AUTHORSHIP

I hereby declare that this thesis incorporates material that is the result of joint research. All 3 of my data chapters are co-authored with my supervisor, Dr. Oliver Love, and chapters 3 and 4 are co-authored with my collaborator, Ms. Christine Madliger. In all chapters, the primary ideas, contributions, experimental designs, data analysis, and interpretation are those of the author. Dr. Love has provided important guidance and feedback in all phases of the project, as well as the funding, materials, and equipment required to complete it. Ms. Madliger has shared in field data collection while conducting her own doctoral research on a shared tree swallow colony and has provided important feedback in the chapters in which she is acknowledged. She further contributed to the design of the experiments in chapter 3, as well as assisted in laboratory work. The manipulation conducted in chapter 4 was originally designed by Ms. Madliger and Dr. Love for their own research and was later adapted to include my own questions. Finally, Ms. Madliger provided the circulating corticosterone data used in some of the analyses in chapter 4.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-authors to include the above materials in my thesis. I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work completed during my registration as graduate student at the University of Windsor.

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ABSTRACT

The measurement of stress through glucocorticoid levels in feathers has been proposed as a key physiological tool useful to the investigation of mechanistic linkages of ecological and conservation problems. However, a number of details of the method are not well-understood, limiting the current interpretation and applications of this tool. Here we investigate the pattern and repeatability of corticosterone levels in naturally-grown feathers, assess the long-term stability of these levels and their resistance to external change, and evaluate their ability to respond to a long-term stressor during breeding using feathers from a wild population of tree swallows (*Tachycineta bicolor*). Our results indicate gaps in the current understanding of feather corticosterone and provide important guidance on the future measurement of stress in feathers and its use in assessing natural and anthropogenic impacts in the wild.

ACKNOWLEDGEMENTS

First, I would like to thank my supervisor, Oliver Love, for deciding I was worth the time and effort to turn into a scientist, and for persevering despite all further evidence to the contrary. You have allowed me continual freedom and resources to explore and learn, and have expressed no worse than mild frustration whenever I have learned an expensive lesson or disobeyed a direct order to chase a new idea. I have deeply enjoyed my time in your lab and appreciate the amount of work you put into your students. I also thank my committee members Aaron Fisk and Dennis Higgs for their time and input into this project.

I would also like to thank Peter Marier for helping during the busiest field season and for his willingness to weigh vials or wash and chop feathers in all environmental conditions. Without your help I would still be in the lab chasing small bits of feather. Additionally, the rest of the Love lab: Holly Hennin, Christie Macdonald, Sarah Guindre-Parker, Sarah Baldo, Pauline Capelle, and Graham Sorenson, have continually set a high standard for me to work towards and allowed me to learn through their projects. That, in addition to the copious amount of treats we have consumed together, will always be appreciated. I also thank Meghan Vankosky for keeping me supplied in treats and for acting as a statistical resource that was never more than a text away.

Thank you as well to Rick Ludkin and Nancy Furber for your passion and openness in educating about birds. Rick, while I do not thank you for suggesting we sit under camouflage sheets in June, I appreciate the amount of time you have spent teaching me the finer points of bird banding and for being the other half of our clearly inferior bleed team during the field season. Nancy, your support and assistance both in the field and out has been tremendously helpful. To the rest of the Ruthven family: Marilynn Havelka, Natalie Campbell, Caitlin Cannon, Sandy Turner, and the Lower Grand River Land Trust Board, thank you for making our field site a second home. Additionally, thank you to the Grand River Conservation Authority and Habitat Haldimand for access to Taquanyah Conservation Area. Also, thank you to David Hussell for his early insights on how to conduct studies using tree swallows and David Lamble for his insights and nestboxes.

I would also like to thank my family, especially my parents, Cliff and Susan Harris, for always encouraging and supporting me. I also appreciate your willingness to care for Darwin for the months I spent chasing birds. Thank you to Darwin for your "help" in the field and for making sure I left the lab and went for a walk every day. Also, thank you to Timber for shaking up these last few months and making it both more and less stressful to complete this project.

Finally, to my wife, Christine, I could never have completed this project, and likely would never have even started, without your continued and unwavering support. I have deeply enjoyed learning everything I could about ecology, stress, birds, and conservation both from you and with you. Your passion for science and life is inspiring, and I look forward to following you into many more mosquito-infested sloughs in search of critters.

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CHAPTER 1 – GENERAL INTRODUCTION

CONSERVATION PHYSIOLOGY

The fields of ecological and conservation physiology have sought to develop and apply a variety of physiological tools to assess environmental health from the perspective of the individual (Stevenson et al., 2005). These fields use measures of metabolism, immune function, nutrition, and endocrine responses to move beyond the description of patterns to an understanding of mechanistic causes (Ricklefs & Wikelski, 2002). By using an integrative approach to the study of the behaviour and physiology of individuals, it is possible to examine the mechanistic connections between an organism's environment and fitness (Wikelski & Cooke, 2006) and determine downstream effects of environmental variation at the population level (Cooke & O'Connor, 2010). Importantly, the rapid response time of physiological measures to environmental change means that they have the potential to provide predictive capacity for impact assessment in real time, long before census data indicates population decline (Busch & Hayward, 2009). In particular, measures of biological stress (an individual's effort to maintain homeostasis by matching regulatory capacity with changing environmental demands) are some of the best positioned for these uses (McEwen & Wingfield, 2010; Koolhaas et al., 2011). For example, in populations of Galápagos marine iguanas (*Amblyrhynchus cristatus*), elevated stress hormone levels predicted reduced survival both during El Niño famine events (Romero & Wikelski, 2001) and in response to oil contamination after a nearby spill (Wikelski et al., 2002), indicating that physiological measures may be predictive of fitness outcomes during environmental change. As such, a mechanistic approach enables researchers to determine the full extent and potential impact of environmental stressors, making the development of mitigation measures more biologically realistic (Carey, 2005).

STRESS HORMONES

Stress hormones, also known as glucocorticoids (GCs), are a key physiological tool to indicate mechanism throughout ecology to help assess the health and state of populations as they cope with and respond to change (Busch & Hayward, 2009). Circulating GCs are one of the strongest measures of stress as they act to maintain energy

balance under both typical and challenging conditions, and they accurately reflect activation of the hypothalamic-pituitary-adrenal axis (McEwen & Wingfield, 2003; Landys et al., 2006). Represented by corticosterone in birds, reptiles, and amphibians and as cortisol in mammals and fish, the role of GCs can be best understood as being concentration-dependent (Busch & Hayward, 2009). At low or baseline levels, GCs are important in regulating energy balance by activating high-affinity mineralocorticoid receptors and helping to control acquisition, deposition, and mobilization of energy (Busch & Hayward, 2009). Baseline GC levels represent an individual's attempt to maintain homeostasis through all of the predictable challenges (energetic, behavioural, and preparatory needs) that it will face in a given day or season (Romero et al., 2009). At higher concentrations, GCs begin binding to low-affinity glucocorticoid receptors and their role shifts to what is often called the acute stress response (Landys et al., 2006). This acute response helps an individual focus on reacting to and coping with unexpected or challenging conditions at the expense of long-term activities such as growth or reproduction (Busch & Hayward, 2009; Romero et al., 2009). However, long-term elevation of GCs can lead to a variety of pathologies such as immune suppression, hypertension, and protein catabolism and is therefore termed chronic stress (Romero et al., 2009; McEwen & Wingfield, 2010).

GLUCOCORTICOIDS AS BIOMARKERS

Determining how GC levels ultimately relate to fitness is a critical end requirement for their use as a conservation tool and biomarker since this knowledge is key to using physiological responses to predict mechanistic and biologically-relevant changes in demographics (Cooke & O'Connor, 2010; Madliger & Love, 2014). However, our current understanding of the relationship between GCs and fitness is far from straightforward (Bonier et al., 2009). Since it is currently impossible to monitor the GC levels of an individual continuously, a key variable in the fitness relationship is how GCs are sampled from the organism of interest (Mormède et al., 2007). While GCs have traditionally been measured in blood, this method can be difficult, invasive, and limited in its scope (Sheriff et al., 2011). As baseline GC levels increase within minutes by the act of sampling (Romero & Reed, 2005), blood sampling can have adverse impacts on

sensitive or at-risk species, and samples give only instantaneous measures. Given these limitations, researchers have recently begun refining the measurement of GCs in alternative sample media like saliva, urine, feces, and, most recently, outer integuments such as hair and feathers (Sheriff et al., 2011; Table 1.1). Ideally, alternative sampling techniques should be less invasive and must provide similar or greater information to that provided by blood. In addition, the mechanism by which GCs originate in the alternative sampling media is also of particular importance, since unless correlated with circulating GC levels, alternative measures cannot be interpreted as representing stress as it is currently understood from circulating levels.

MEASUREMENT OF GLUCOCORTICOIDS IN KERATINIZED INTEGUMENTS

Sampling GCs in outer integument tissue currently represents an extremely promising but poorly understood method (Sheriff et al., 2011). The primary benefit of sampling hair or feathers is that their slow growth rate suggests it is possible to evaluate GC levels over a much longer time period than the rapid turnover of blood or feces (Bortolotti et al., 2009). This could allow for an unprecedented ability to monitor an organism's cumulative stress load over weeks or months, a measure that could be invaluable since it can bypass the complexities introduced by the daily, seasonal, and short-term stressor variation in circulating GC levels (Bortolotti et al., 2009), simplifying the evaluation of chronic stress. As a result, the measurement of GC levels in integuments has been applied to such diverse integuments as baleen (Hunt et al., 2014), snake skin (Berkvens et al., 2013), and turtle claw (Baxter-Gilbert et al., 2014), though the technique is most often applied to samples of hair (Gow et al., 2010; Stalder & Kirschbaum, 2012) and feathers (Bortolotti et al., 2008; Fairhurst et al., 2013). The study of GCs in hair is more advanced than other integuments since researchers have used these measures to indirectly detect steroid variation in laboratory, domestic, and wild mammals as well as humans for over a decade (Macbeth et al., 2010). Indeed, measures of cortisol in hair have been applied not only to studies in ecological physiology and conservation (Koren, 2002), but also in human and animal studies in fields such as psychology (Groeneveld et al., 2013), healthcare (D'Anna-Hernandez et al., 2011), veterinary science (Carlitz et al., 2014), and sports medicine (Raul et al., 2004). Although a lack of knowledge transfer

between the disparate fields has slowed their use as a biomarker in ecological applications, it is generally accepted that GC levels in hair are a combination of both internal and external sources, thereby reflecting the individual's previous and current GC exposure (Gow et al., 2010; Meyer & Novak, 2012).

FEATHERS AND MOULT

Unlike hair, which is grown and replaced continuously (Sachs, 1995), the growth and replacement of feathers occurs at fixed intervals (most often annually) determined by the moult strategy for a particular species (Howell, 2010). Moult is a necessary process as feathers are essential to a bird's ability to thermoregulate, communicate, and fly and are worn significantly by time and use (Howell, 2010). The most important feathers are the primary and secondary feathers of the wing and the rectrix feathers of the tail, as they provide much of the bird's flight surface (collectively called flight feathers; Howell, 2010; Figures 1.1 & 1.2). Although a number of variations exist due to life-history demands, most species moult flight feathers symmetrically between wings, beginning with the innermost primaries outward to the wing tip, from the outer secondaries in to the centre, and from the centre of the tail outwards (Howell 2010; Figure 1.1). Due to their higher spatial density, smaller size, and large number, body feathers are generally moulted by region rather than in a defined order (Howell, 2010). Since large gaps in feathers can impair the bird's performance, the initiation of moult in each feather tract is timed to minimize compromises in function (Howell, 2010). While some species undergo multiple moults within a given cycle, moults are identified by the plumage (a set of feathers creating the general appearance of the bird) that they create, with all adult birds undergoing a prebasic moult which results in a complete basic plumage and defines the base moult cycle (Howell et al., 2003). Additionally, feathers which are lost between moult are generally replaced, but replacement feathers may be of lower quality and are replaced in the following moult (Grubb, 2006; Howell, 2010).

Feathers themselves are complex β-keratin structures composed of a branching hierarchy from rachis, to barbs, to barbules, with barbules possessing small hooks, or barbicels, allowing them to interlock (Maderson et al., 2009; Figure 1.3). Near the base of the feather, the rachis becomes the tubular calamus which anchors the feather in the

follicle (Stettenheim, 2000). These basic elements can be altered in size, characteristic, composition, or arrangement to produce the large variety of feather colours, shapes, and functions (Yu et al., 2004; Badyaev & Landeen, 2007). Feathers are grown from follicles in the skin as a sheet of keratinocytes wrapped in a concentric layer around a vascularized pulp and encased in a sheath (Stettenheim, 1972; Figure 1.4). All 3 layers of the growing feather are pushed up and out of the follicle by new cells produced at the base, and feather cells pattern, differentiate, and keratinize as they move upward (Maderson et al., 2009). Once beyond the skin, the pulp recedes, ending vascularization and leaving pulp caps (Stettenheim, 2000). The fully grown, keratinized, dehydrated, inert feather tissues are then deployed by the physical removal of the sheath and hardened pulp caps (Stettenheim, 1972).

MEASUREMENT OF GLUCOCORTICOIDS IN FEATHERS

The ease and convenience of collection of feather samples has led to quick, wide-spread adoption of their use in the study of GCs. The method was developed as a natural progression from the measurement of toxins and environmental contaminants in feathers using knowledge from the practice of measuring GCs in hair (Bortolotti et al. 2008; Bortolotti, 2010). However, although this origin provided a basic set of methodologies for the measurement GCs, it did not offer a broad framework of what variation in GCs represents, since the mechanisms of deposition are not well-understood for most non-structural analytes that can be detected in feathers (e.g., Hobson, 2008; García-Fernández et al., 2013). Moreover, unlike studies focusing on contaminants or other targeted molecules where simple absence/presence of the analyte in the sample is sufficient to draw conclusions, studies of feather GCs are concerned primarily with comparative levels of the targeted molecule (Bortolotti, 2010). This added complexity and quantitative requirement necessitates a broader understanding of how the hormone enters and is held in the feather, as it is crucial to the proper interpretation of feather GC levels.

It is currently believed that corticosterone (CORT), the primary avian GC, enters the feather during the vascularized portion of feather growth according to circulating levels at that time (Bortolotti et al., 2008; Jenni-Eiermann et al., 2015). This model of deposition suggests that feather CORT levels should reflect average circulating levels

throughout the period of feather growth (Bortolotti et al., 2009). Since a long period of CORT integration should result in less sensitivity to acute stress responses, but a greater ability to detect chronic stress, feather CORT should represent an ideal tool to assess the intrinsic state, health, or disturbance of an individual (Cook, 2012). This dependence on the average implies that several baseline measures of circulating CORT levels over feather growth should correlate well with feather CORT levels; however, results to date have been mixed (Bortolotti et al., 2008; Lattin et al., 2011; Fairhurst et al., 2013; Jenni-Eiermann et al., 2015). Instead, feather CORT levels appear better correlated to experimentally elevated or stress-induced circulating levels after a standardized stressor (Bortolotti et al., 2008; Lattin et al., 2011; Fairhurst et al., 2013; Jenni-Eiermann et al., 2015). Additionally, although feather CORT is currently assumed to be fixed at the end of vascularization and therefore stable throughout the life of a feather (Bortolotti et al., 2009), other analytes in feathers have already demonstrated the capacity to alter their levels externally after growth (11 heavy metals: Dauwe et al., 2003; Jaspers et al., 2004; polyhalogenated compounds: García-Fernández et al., 2013) and several recent feather CORT studies have suggested external changes as a possible reason for their results (Lattin et al., 2011; Jenni-Eiermann et al., 2015). Together, these issues hinder the proper interpretation and utility of the technique.

RESEARCH OBJECTIVES

Overall, there is a current need for investigation into the primary gaps in our understanding of feather CORT: 1) the mechanism of deposition; 2) the stability of CORT levels; and 3) how levels of CORT in feathers relate to circulating levels of GCs and/or fitness. In addition, the important caveats and contexts of the method need to be determined to establish stronger guidelines and protocols for the use of the tool from sampling to interpretation with the goal of increasing our ability to compare and reproduce future feather CORT studies. To address these gaps, we investigate the pattern and repeatability of CORT levels in naturally-grown feathers (Chapter 2), assess the long-term stability of these levels and their resistance to external change (Chapter 3), and evaluate their ability to respond to a long-term stressor during breeding (Chapter 4) using feathers from a wild population of tree swallows (*Tachycineta bicolor*). Offering answers

to these issues will provide considerations for the appropriate interpretation and biological relevance of feather CORT to help ensure this alternative GC sampling method is a reliable and robust physiological biomarker of stress.

STUDY SYSTEM

Tree swallows are an iridescent blue passerine that are ideal for validations of feather CORT as they are a cavity-nesting model avian species (Jones, 2003) that breeds readily in nestboxes throughout northern and central North America. A diurnal migrant, they undergo prebasic moult from July to November (Stutchbury and Rohwer, 1990; Howell, 2010) while migrating to the wintering grounds in southern United States, Mexico, the Caribbean, and Central America. They return to the breeding grounds by April (Hussell, 2003). Because they are highly philopatric to their breeding grounds (Winkler et al., 2004) and are tolerant to human intrusion and manipulation (Jones, 2003), they have been widely studied and a variety of important life history details, such as moult timing and order (Stutchbury & Rohwer, 1990), are well understood. As a result, they are frequently used to assess impacts of habitat change, anthropogenic disturbance, and habitat reclamation (Ghilain & Bélisle, 2008; Harms et al., 2010; Custer, 2011; Paquette et al., 2013; Cruz-Martinez et al., 2015). Additionally, tree swallows are part of the aerial insectivore guild, a group of birds specializing in eating flying insects, that has experienced sharp declines over the last 50 years (Nebel et al., 2010). As the most well-studied and a still abundant member of this group, an established physiological biomarker in this species would not only be useful in the assessment of disturbance, but also in the conservation of aerial insectivores. The tree swallows used in this study bred in a nestbox colony at Ruthven Park National Historic Site (42°58’N, 79°52’W) and Taquanyah Conservation Area (42°59’N, 79°54’W) in Haldimand County, Ontario, Canada. The site’s 175 nestboxes have been scientifically monitored daily throughout the breeding period (late April to early July) since 2010.

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FIGURES

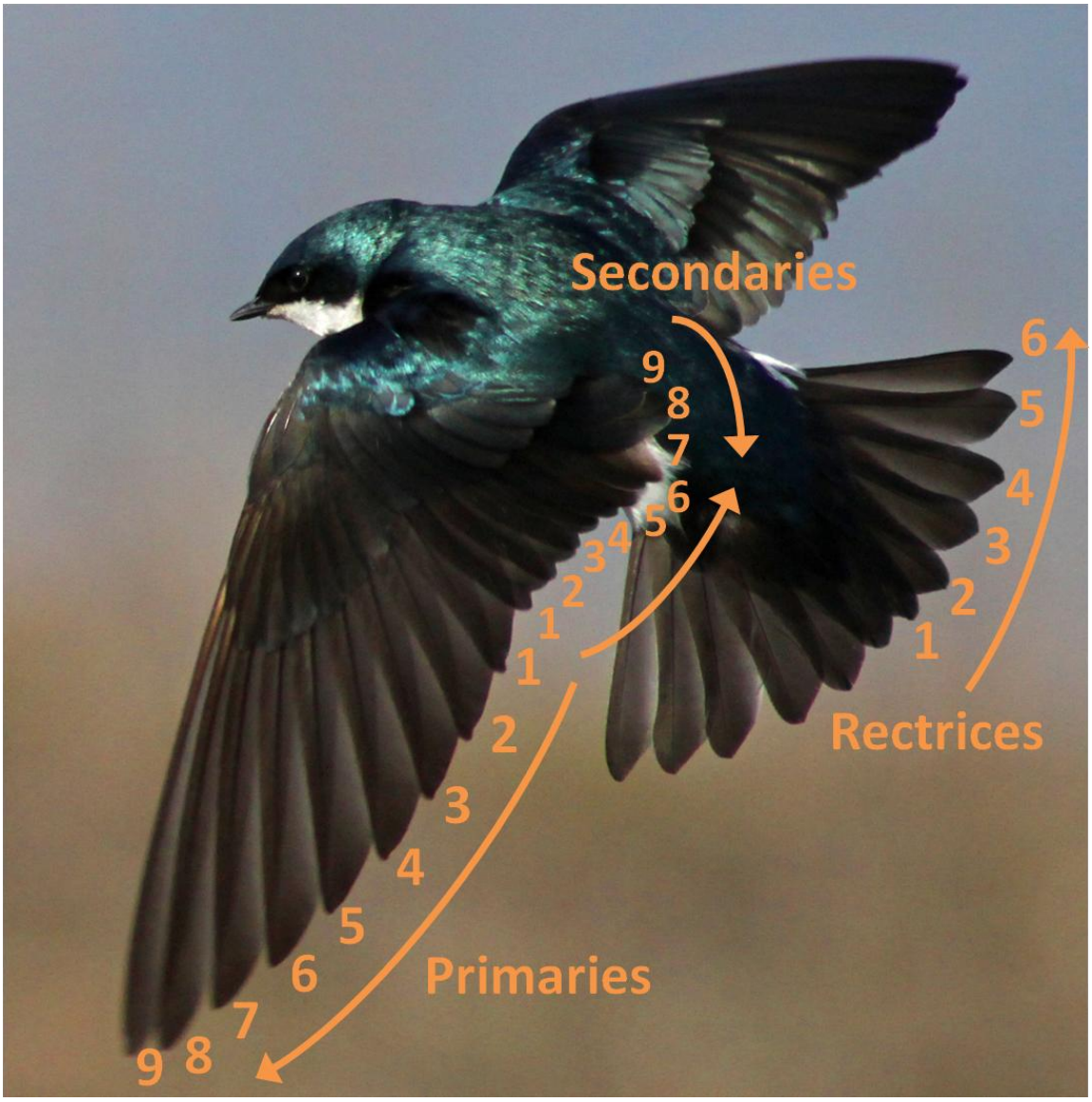

Figure 1.1 – Tree swallow (*Tachycineta bicolor*) with all 3 types of flight feathers numbered. Order and direction of normal feather moult shown by arrows. Primary feathers are numbered and moulted from the centre of the wing outward. Secondary feathers are numbered from the centre of the wing inward and 1-6 are moulted sequentially. Secondaries 8,9, and 7 are moulted in that order independently of 1-6. Rectrices are numbered and moulted from the centre of the tail outward. (Photo modified from Tachycineta Bicolor by Bear Golden Retriever, 3 April 2010, Creative Commons Attribution 2.0 Generic license via Wikimedia Commons).

Figure 1.2 – Full complement of flight feathers from the right side of a tree swallow.

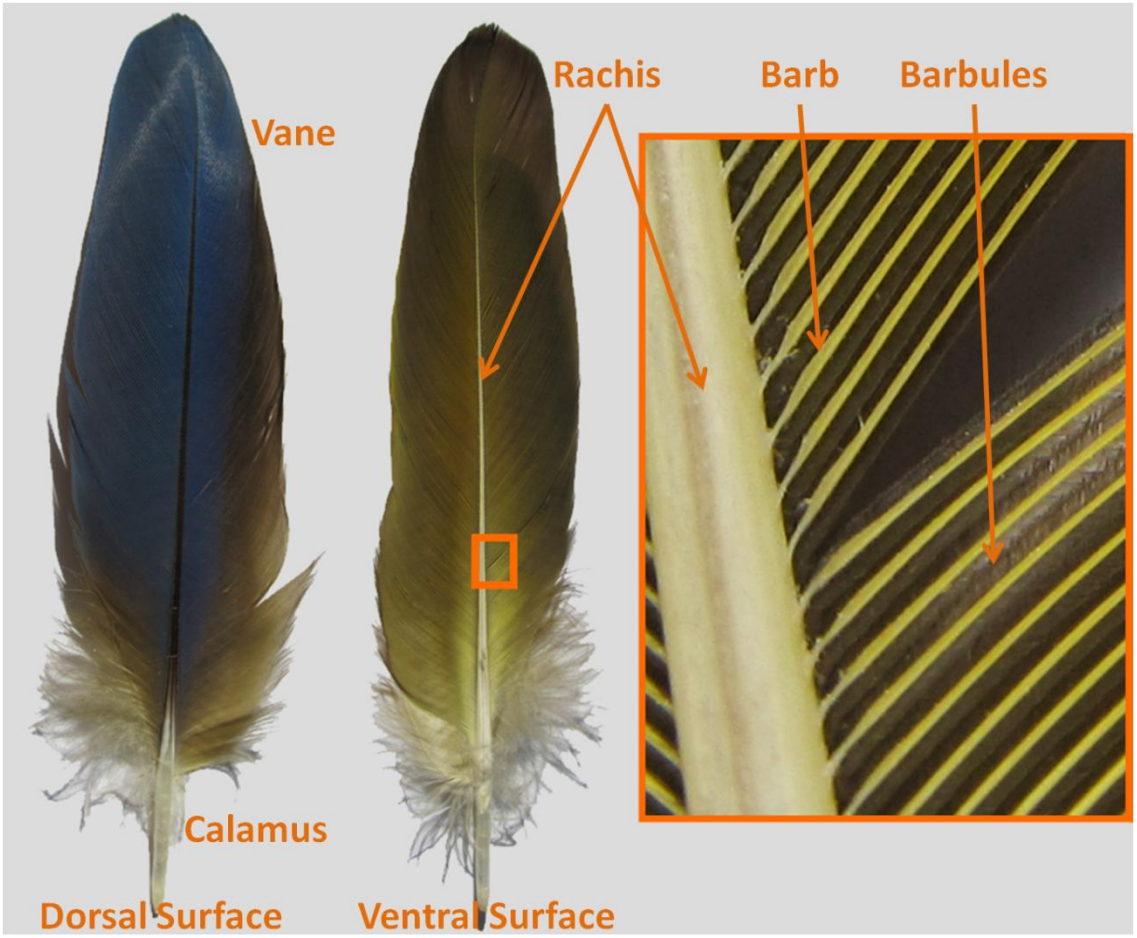

Figure 1.3 – Dorsal and ventral surface of a blue-and-yellow macaw (*Ara ararauna*) feather with zoomed cut-out of a portion of the ventral surface illustrating the branching structure of the feather from rachis, to barb, to barbule. The calamus anchors the feather in the follicle while the rachis acts as the support shaft for the vanes, which are composed of barbs and barbules.

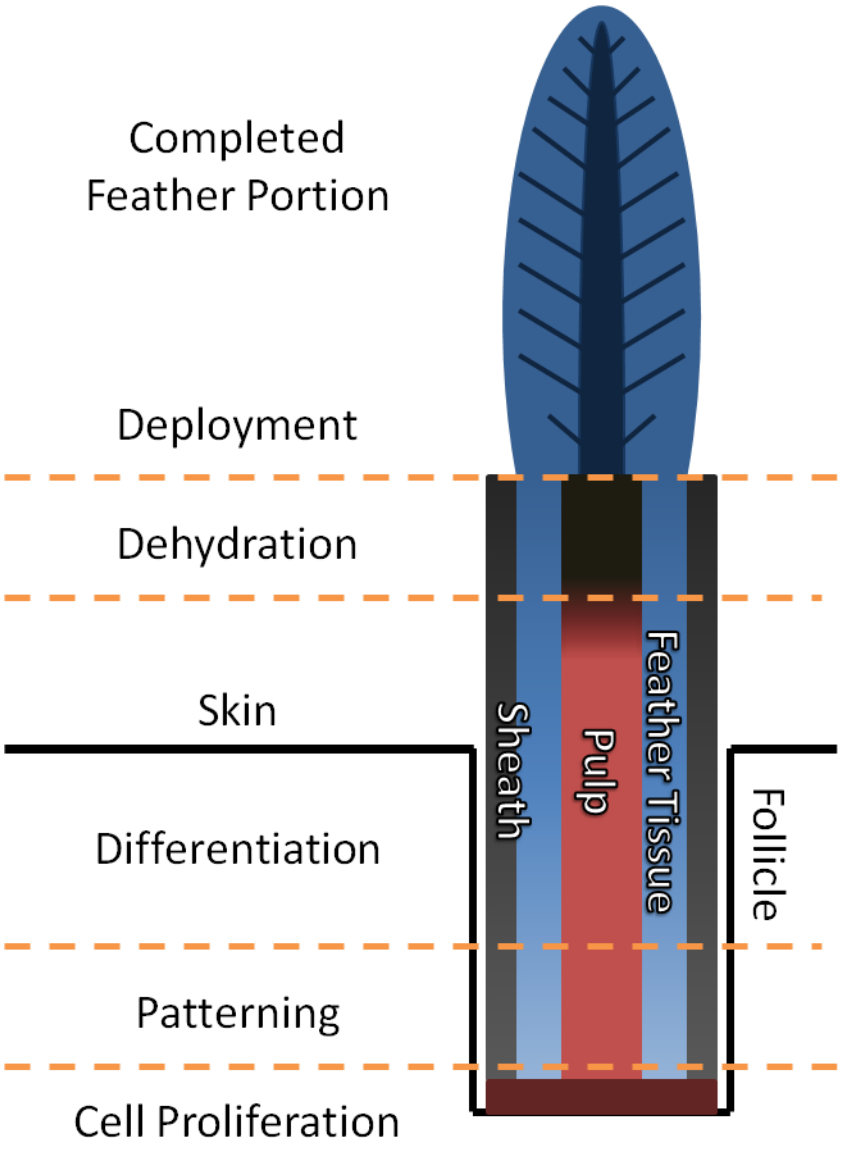

Figure 1.4 – Longitudinal cross-section of a simplified and idealized feather at mid-growth. Cells proliferate at the base of the feather follicle pushing previously grown feather cells upwards (Maderson et al., 2009). The cells pattern and differentiate as they move upward through the follicle, forming an inner vascularized pulp, surrounding feather tissue, and an outer sheath (Stettenheim, 2000). Once beyond the skin, feather tissues are completed and the pulp recedes leaving a pulp cap as its remnant (Lin et al., 2006). All tissues dehydrate and the outer sheath and pulp caps are removed by friction and preening, deploying completed feather tissues (Stettenheim, 1972).

TABLES

Table 1.1 – Key features of common glucocorticoid sampling methods (adapted from Sheriff 2011).

	Sampling Method			
	Blood	**Saliva**	**Excreta**	**Integument**
State of GC	Free (active) and bound to proteins (inactive)	Free GCs from plasma, salivary glands can convert to inactive form	Metabolites from free plasma GCs	Unknown, assumed free plasma GCs
Time Period	Instantaneous	Near instantaneous (Correlates with free plasma GCs)	Hours	Weeks/Months
Invasiveness	Medium to High	Low	None to Low	None to Medium
Training Requirements	High	Low	Low	Low
Collection Difficulty	High	Medium	Low	Low
Considerations	Stress response within 2-3 minutes of disturbance, peaking within 10-60 minutes. Levels are composite of endogenous cycles and long-term and immediate prior experience.	Difficult to collect in the field. More useful in captive settings where animal can be trained to accept sampling. Shows stress response similar to blood.	Requires metabolism studies to understand sampling period. Urine and feces can be mixed in cloaca prior to excretion in birds and reptiles.	Method of hormone incorporation unknown. Sampling period not well understood. Levels likely not fixed throughout life of integument.

CHAPTER 2 – TEMPORAL OVERLAP AND REPEATABILITY OF FEATHER CORTICOSTERONE LEVELS: PRACTICAL CONSIDERATIONS FOR USE AS A BIOMARKER

INTRODUCTION

The use of physiological measures as biomarkers of environmental change and disturbance in species of conservation importance has been proposed to be a powerful tool for conservation practitioners (Cooke et al., 2013). To be effective in this capacity, potential measures need to be consistent and reliable indicators of condition or intrinsic state (Madliger & Love, 2014). Stress, as measured by glucocorticoid (GC) activity, has been proposed as one such biomarker due to the role of GCs in both daily energy balance and in response to acutely stressful events (Landys et al., 2006; McEwen & Wingfield, 2010; Dantzer et al., 2014). However, measuring GCs in circulation can be difficult, invasive, and limiting, concerns which are especially undesirable in a metric directed towards species of conservation concern (Sheriff et al., 2011). As a result, a number of alternative, less invasive sampling media have been proposed and tested (i.e., fecal, saliva, keratin integuments; Sheriff et al., 2011) of which hormone extraction from feathers is a promising, but currently less understood method (Bortolotti et al., 2008).

The currently proposed model of GC feather deposition involves entrapment of corticosterone (CORT), the primary avian GC, as it circulates in the vascularized section of the feather pulp which supplies nutrients and other resources to the surrounding structures during feather growth (Bortolotti et al., 2008). This process takes place in a growing feather between the area of cell proliferation at the base of the feather follicle and the area of pulp recession preceding feather deployment (Maderson et al., 2009; Figure 2.1); circulating CORT levels can be reflected throughout this blood quill (Jenni-Eiermann et al., 2015). Once pulp caps are formed, this section of the feather is no longer vascularized and CORT entrapped within the feather is assumed to be held securely until sampling and analysis of the fully grown feather (Bortolotti et al., 2009).

The longer time of integration of integument CORT when compared to other measures such as blood or feces should result in this measure being less sensitive to

short-term perturbations or concentration changes since the CORT level from a full feather is expected to represent the average circulating level during the entire period of feather growth (i.e., a period of weeks rather than minutes or hours; Bortolotti et al., 2009). Since moult occurs in a defined sequence at fixed and predictable intervals, with multiple feather tracts re-growing simultaneously during heavy periods of moult, the analysis of feathers grown concurrently and sequentially offers a method of testing the reliability of feather CORT to reflect stress exposure. For example, the longer time integration and inherent insensitivity of the average CORT level over the time of growth further suggests that feathers which overlap in growth time, but are found at different locations on the bird, should also show strong agreement in levels since they share the same circulating levels and therefore deposition opportunities. If this understanding of CORT deposition into feathers is correct, a chronic environmental stressor experienced by a bird should translate into high CORT levels in all feathers grown at the same time. This property is necessary in order for feather CORT to be interpreted as a relevant and robust indicator of past exposure to chronic elevated stress levels.

To date, multiple studies have shown that feather CORT most often relates to measures of circulating levels after a standardized stressor rather than those measured at baseline levels (Bortolotti et al., 2008; Lattin et al., 2011; Fairhurst et al., 2013; Jenni-Eiermann et al., 2015). Though this apparent deposition bias towards stress-induced levels may be due to the difference in magnitude between baseline and acute levels (Fairhurst et al., 2013), it nevertheless renders the interpretation of feather CORT as an average of circulating levels during feather growth problematic. In addition, the short duration of the acute increase in CORT during the stress response in relation to the duration of feather growth suggests that feathers which overlap significantly, but not completely, in growth may have very different exposures in the face of stressful events. Therefore, it is currently unclear to what extent feather CORT can be expected to be consistent throughout the naturally-grown feathers of an individual in the wild. Consequently, the evaluation of assumed consistency is important to the selection of which feather to sample, as well as the final interpretation of measured levels. It should also be noted that while feather CORT should be consistent across feathers, it is not required to have equal levels in absolute terms, as differences in size, shape, colour, and

structure could result in different levels per unit of length. Also, the recently described mass-dependency of the extraction (Lattin et al., 2011) suggests that hormone levels measured from feathers of very different sizes are not directly comparable due to differences in extraction efficiency that cannot be overcome by the addition of more solvent (Berk & Breuner, 2015). As a result, when comparing different feathers, feather CORT levels should instead have similar levels in relative terms.

Here we investigate patterns of feather CORT levels across feather groups and assess the symmetry and consistency of CORT levels in wild adult tree swallow (*Tachycineta bicolor*) feathers grown during natural moult. Under the assumption that feather CORT is a consistent and therefore reliable biomarker of stress, we predicted that: 1) Different feather types should differ in absolute CORT levels on a per-length basis due to differences in size, structure, and extraction efficiency; 2) The same flight feather on both sides of the bird should have the same CORT level since these feathers are moulted symmetrically; 3) Different types of feathers (i.e., flight, contour) should differ in absolute levels, but should have the same relative levels if they were moulted at the same time (i.e., an individual with high relative wing feather CORT should also have high body and tail feather CORT if they were moulted at the same time).

METHODS

Feather Collection

Feathers were obtained from tree swallows in a system of nestboxes at Ruthven Park National Historic Site (42°58'N, 79°52'W) and Taquanyah Conservation Area (42°59'N, 79°54'W) in Haldimand County, Ontario, Canada. Feathers were collected from adult individuals that died naturally during the 2010-2013 breeding seasons for reasons such as starvation, vehicle collision, and conflict with invasive house sparrows (*Passer domesticus*). Birds were found within 24 hours of death and whole feathers were collected if they were not visibly contaminated due to the manner of death and stored at -80°C until assay. Birds and feathers were collected under Environment Canada/Canadian Wildlife Service Scientific Permit CA0266.

Tree swallows were selected for this validation because they are a free-living model species (Jones, 2003) that undergoes prebasic moult during migration from July to November and a limited spring moult of chin feathers in some individuals (Stutchbury and Rohwer, 1990). The species' wide distribution and resilience to study has led to their extensive use in ecological applications such as impact assessment, where physiological biomarkers would be useful tools (Ghilain & Bélisle, 2008; Harms et al., 2010; Custer, 2011; Paquette et al., 2013; Cruz-Martinez et al., 2015). Finally, tree swallow flight feathers are uniformly dark, preventing confounding effects of pigment differences when comparing feather CORT levels (Jenni-Eiermann et al., 2015).

Feather Preparation and Hormone Assay

To remove surface contaminants before analysis, intact feathers were washed by immersion and swirling in a 50mL falcon tube filled with a dilute (1%) soap and ultrapure water solution for 30 seconds (Bortolotti et al., 2008). They were then rinsed using ultrapure water to remove all soap solution and allowed to air-dry overnight. The calamus was removed from the feather using a razor blade, the remaining feather length was measured with calipers, and feathers were minced into fine (<1mm) pieces using scissors. Feather pieces were collected in a weighed glass scintillation vial and the vial was weighed a second time to determine the mass of the feather available to be extracted. CORT was extracted from the minced feathers according to the protocol outlined in Bortolotti et al. (2008) using 10mL of HPLC grade methanol. Samples were sonicated for 30 minutes and then placed in a 50℃ water bath overnight. Feather pieces were removed from the hormone extract by vacuum filtration, after which the methanol was evaporated in a fume hood. Samples were reconstituted using assay buffer and assayed using Enzo Life Sciences Corticosterone Enzyme Immunoassay (ADI-901-097). Assayed samples showed an intra and inter-assay coefficient of variation of 4.22% and 13.78%, respectively. Feather CORT levels were expressed per length of feather analyzed, as this measure is commonly used and thought to reflect incorporation rates during feather growth (Bortolotti et al., 2009).

Statistical Analysis

Patterns across Feather Groups

To examine patterns of feather CORT distribution across different feather types, we first compared mean CORT levels obtained from 4 feather regions: primary and secondary feathers of the wing, rectrix feathers from the tail, and body feathers from the back. Feather group means for 12 individuals were calculated using levels from a representative selection of feathers for each feather group to avoid the need to assay every feather on every bird. The mean for primaries is composed of levels from primaries P2, P4, P6, and P8 from the right side of an individual. Similarly, the mean for secondaries is composed of levels from right secondaries S1, S2, S4, and S8, while the mean for rectrices was composed of levels from right rectrices R1, R3, and R5. Back feathers were extracted and assayed as 5 pooled feathers due to their small size and as such the level obtained from the assay already represents the mean level. Since all 4 feather groups are from the same 12 individuals and the data could not be normalized across groups using transformations, groups were compared using a Friedman test blocked for individual identity (Friedman, 1937). This analysis was repeated using the weight per unit of length of the feathers in place of CORT levels to examine differences in density across feather types and groups were again compared using a Friedman test blocked for individual identity. Analyses were completed using R 3.1.3 (R Development Core Team, 2015) and post-hoc comparisons were performed using a Wilcoxon-Nemenyi-McDonald-Thompson test (Galili, 2010).

Feather Corticosterone Symmetry

Left- and right-side flight feathers across all 3 flight feather groups were compared to assess the degree to which feathers moulted symmetrically contain the same amount of CORT. To minimize the effect of pseudoreplication, 6 representative feathers were chosen from all flight feathers: primaries P2 and P6, secondaries S2 and S4, and rectrices R1 and R5 from both sides were assayed in 8 birds. CORT levels in the feathers were compared using linear regression including all 48 feather pairs. The CORT levels of both left and right feathers were normal without transformation.

Consistency of Feather Corticosterone Levels

Finally, to assess the consistency of the information provided by feather CORT, the repeatability of 6 different feathers, expected to be grown naturally at overlapping or similar times in moult, was evaluated using feathers from 16 birds according to Lessells and Boag (1987). The feathers were chosen to coincide with a heavy period of moult (i.e., a large degree of temporal overlap across several feather types) to allow for a large number of comparisons. Primaries P4 and P5, secondary S1 and S8, rectrix R1, and back feathers were used as they are all moulted at overlapping or similar times (Stutchbury & Rohwer, 1990). Since absolute levels of different feathers are not directly comparable due to differences in extraction efficiency across different masses (Lattin et al., 2011), as well as differences in feather size, structure, growth rate, and possible CORT-holding capacity, levels were standardized by dividing by the mean CORT level of that feather type. This allows for the evaluation of the consistency of the signal across feathers relative to those of conspecifics, as an individual with higher relative circulating CORT levels is also expected to have higher relative feather CORT levels in all feathers grown during that time, though none of these levels are directly comparable to each other. These relative feather CORT levels were log-transformed to achieve normality. The ranked repeatability of these same feather CORT levels was also assessed to further determine the within-individual consistency of feather CORT. All analyses were conducted in JMP 10.

RESULTS

Patterns across Feather Groups

The four feather types showed significant differences in feather CORT levels (Friedman test: $\chi^2(3)=30$, $P<0.0001$; Figure 2.2A) and post-hoc analysis indicated that, on a per length basis, primary feathers contained more feather CORT than secondary and back feathers, while back feathers contained less feather CORT than primaries and rectrices. The four feather types in the same samples also showed significant differences in weight per unit of length (Friedman test: $\chi^2(3)=32.5$, $P<0.0001$; Figure 2.2B) and post-hoc analysis indicated that given the same length of feather, primary feathers are heavier than secondary and rectrices, and back feathers are lighter than all flight feathers.

Symmetry and Consistency of Feather Corticosterone Levels

The linear regression between the sides of the matched feather pairs did not reach significance (P=0.0506, R^2=0.0805; Figure 2.3) and the coefficient of determination for the model was low, indicating that feathers moulted at the same time do not have the same feather CORT level.

Repeatability of feather CORT levels across various feathers moulted at the same time was low (calculated repeatability statistic of r=0.188; $F_{(15,80)}$= 2.385, P=0.0067; Figure 2.4), as the variation across feathers within individuals is larger than the variation between individuals. Post-hoc analysis indicated that only the lowest 4 birds were significantly different from the highest bird, while the remaining 11 birds could not be categorized as either high or low due to within-individual variation. When assessed by rank, the calculated repeatability statistic was found to be even lower (r=0.152, $F_{(15,80)}$=2.075, P=0.0196; Figure 2.5).

DISCUSSION

Patterns across Feather Groups

In general, we found that larger, heavier feathers held more CORT per unit length, indicating that primary feathers held more CORT than secondary and back feathers, while back feathers held less CORT than primary and rectrix feathers. This result is in accordance with our predictions given that longer feathers are not only heavier, but heavier per unit length across feather types (also discussed in Bortolotti, 2010), allowing larger feathers to entrap more CORT under the same exposure. Similarly, Patterson et al. (2015) found that feather CORT on a per length basis was positively related to feather mass in 10th primaries and primary coverts of Caspian tern (*Hydroprogne caspia*) chicks and suggested that reductions in feather densities due to food limitation may reduce feather CORT concentrations. Considering that feathers at opposite extremes of size differed greatly in mass and may therefore have exhibited some mass-dependency in their extraction (Lattin et al., 2011), it is further possible that the differences between the groups may be larger than those shown here, as any mass-dependency experienced would have reduced levels of the largest feathers relative to the smallest. These results suggest

that studies using feather CORT levels should consider the effect of total feather volume available for deposition, and that length may not always be an adequate proxy for this in some comparisons. For example, the current model of deposition does not account for differences in the total amount of keratin that different feather types possess, suggesting that CORT levels should only vary stochastically throughout the length of the feather according to varying circulating levels (Bortolotti et al., 2009). However, this expected pattern requires: 1) the smaller and lighter distal tip of the feather to hold more CORT per unit of keratin than the wider, thicker, and heavier feather midsection; 2) the rachis to hold the same amount of CORT throughout its length regardless of its proximal to distal taper; and 3) the feather vane to hold the same amount of CORT as the rachis despite its lower volume and mass of keratin. Our results instead suggest that keratin volume should be considered when assessing these patterns and caution the interpretation of comparative levels of sections of a feather when those sections differ markedly in volume and structure.

Symmetry and Consistency of Feather Corticosterone Levels

Matching left and right feathers from 6 representative feather pairs across all flight feathers did not contain the same feather CORT levels. In addition, calculated repeatability values for 6 feathers across different regions that overlap in moult timing were low for both relative feather CORT levels (19%) and ranked levels (15%), indicating that there is much larger variation in feather CORT levels within individuals than between (Lessells & Boag, 1987; Boake, 1989). These results together suggest that, at least in some species, naturally-grown feathers collected long after moult may not reflect the stress status of an individual consistently and that the analysis of multiple feathers may give conflicting results. Although it should theoretically improve consistency, the longer period of GC integration in feathers compared to other media (i.e., plasma, fecal, etc.) does not appear to improve our ability to characterize an individual's stress phenotype. These results are similar to those of other studies that have investigated the repeatability of CORT levels of more than 1 feather from the same individual. For example, CORT levels of different contour feathers of red-winged blackbirds (*Agelaius phoeniceus*) from the same individuals were not significantly

different, but were also not correlated due to high within-individual variation (Kennedy et al., 2013). Similarly, on a per mass basis, CORT levels of house finch (*Haemorhous mexicanus*) tail and breast feathers were not significantly different and though significantly correlated, showed a repeatability of 43% (Lendvai et al., 2013). In addition, feather CORT levels were not repeatable within individuals across years in common eiders (*Somateria mollissima*) or snow geese (*Chen caerulescens*; Legagneux et al., 2013), and showed a repeatability of 40% in yellow warblers (*Setophaga petechia*) after controlling for a year effect (23% repeatability before controlling for the year effect; Grunst et al., 2014).

Potential Causes of Increased Intra-individual Variation

Taken together, these results suggest that different feathers, even when grown at the same time during moult, may not contain as similar levels of CORT as predicted by the current model of deposition. Therefore, feather CORT is either not always a straightforward record of circulating CORT levels during feather growth, or levels are not fixed throughout the life of the feather. As discussed earlier, some of the within-individual variation in feather CORT levels may be the result of an overrepresentation of stress-induced levels experienced during feather growth (Bortolotti et al., 2009; Fairhurst et al., 2013). Because stress-induced secretion of GCs is much higher than baseline, although relatively short-lived in comparison to feather replacement, feathers that overlap but differ partially in growth period may have very different CORT exposure profiles if stress-induced levels are differentially deposited. However, this scenario does not explain the observed lack of correlation in left and right paired feathers. Similarly, as the feathers used in this study were grown naturally in adult tree swallows, differences in moult order, timing, and growth rate are likely to increase variation similar to the higher within-individual differences observed in stable isotope levels of adult birds when compared to the synchronous moult of nestlings (Carravieri et al., 2014). Moreover, this added variation is in addition to the lack of correlation between left and right paired feathers, and the same sources of variation should be expected in many studies of feather CORT in wild birds which undergo moult during inaccessible times. This high within-individual

variation therefore represents a barrier to the many potential applications of measuring CORT levels in naturally-grown feathers as a biomarker of stress in adult birds.

As large circulating levels of CORT are harmful to protein formation, elevated CORT levels during feather growth can have profound negative effects on feather structure that can be maintained throughout the remainder of integument growth (Romero et al., 2005; Peters et al., 2011; Jenni-Eiermann et al., 2015). Since feathers are necessary for thermoregulation and flight, it follows that birds must minimize CORT-based reductions in feather quality (Jovani & Blas, 2004; Romero et al., 2005). Despite down-regulation of the hormone during moult (Romero, 2002) and the importance of growing high quality feathers, fault bars (small visible lines caused by structural errors from abnormal feather growth) occur with some frequency (Jovani & Diaz-Real, 2012), and it has been suggested that stress-induced fault bars should be differentially allocated across feathers to minimize their impacts (Jovani & Blas, 2004). Since both flight and contour feather tracts are moulted at the same time in many species, differential allocation cannot be accomplished solely through modification of the level of down-regulation, suggesting that there may be further mechanisms to prevent CORT from affecting feather growth in key areas. Additionally, as above, stress-induced changes in feather density may change the feather's ability to reflect CORT levels (Patterson et al., 2015), which may account for the low repeatability of feather CORT.

A second possibility is that initial feather CORT concentrations following moult may be repeatable, but that levels did not remain static between moult and the point at which feathers were collected. It has been shown that GCs in hair can be reduced by washing and weathering following their deposition (D'Anna-Hernandez et al 2011; Hamel et al. 2011), and there is evidence that preparatory washes before assay can reduce feather CORT levels (Bortolotti et al., 2008; Jenni-Eiermann et al., 2015). Indeed, external changes in feather CORT concentrations have been proposed as explanations of discordant results in other studies (Lattin et al., 2011; Jenni-Eiermann et al., 2015). External changes could result in a lack of repeatability, and in the lack of agreement between left and right feathers, since different feathers could have different levels of

exposure both within and between feather types due to placement, function, structure, and preening behaviour.

In conclusion, feather CORT levels were found to differ across feather types due to differences in feather density. Left and right paired, symmetrically moulted feathers did not contain the same CORT levels and the repeatability of different feathers which overlapped temporally during moult was low. These combined results caution against the use of naturally-grown feathers as a reliable indicator of circulating CORT phenotype and suggest that future work is needed to examine the mechanisms of deposition, external effects, permanence of signal, and responses to known stressors in the wild before feather CORT can be used effectively as a tool for conservation and ecological applications.

ACKNOWLEDGMENTS

We thank Ruthven Park National Historic Site for lodging and access to their study site and nestboxes. We also thank the Grand River Conservation Authority and Habitat Haldimand for access to their study site and nestboxes. We thank Peter Marier and Christine Madliger for their assistance processing feather samples. OPL was supported by NSERC operating, Canada Foundation for Innovation (CFI), and Canada Research Chair (CRC) funding.

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FIGURES

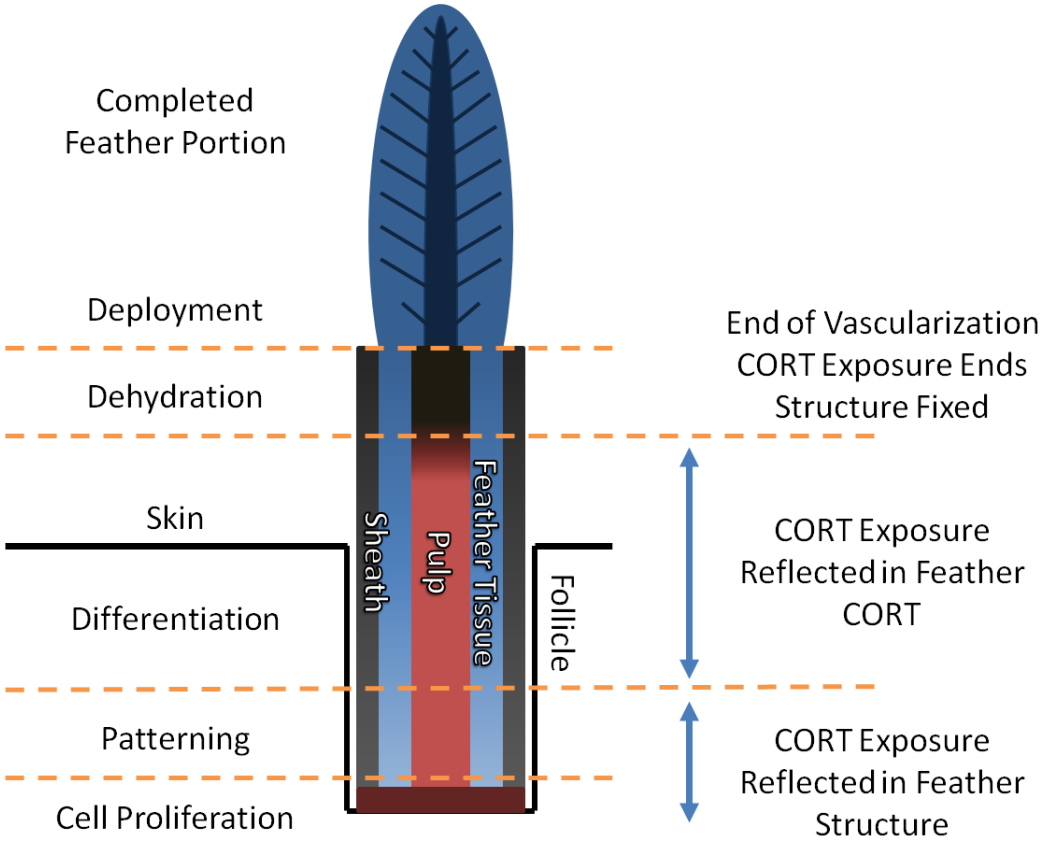

Figure 2.1 – Diagram representing our current understanding of corticosterone deposition into the longitudinal cross-section of a simplified and idealized feather at mid-growth. Cells proliferate at the base of the feather follicle pushing previously grown feather cells upwards (Maderson et al., 2009). The cells pattern and differentiate as they move upward through the follicle, forming an inner vascularized dermal core, surrounding feather tissue, and an outer sheath (Stettenheim, 2000). Once beyond the skin, feather tissues are completed and the dermal core recedes leaving a pulp cap as its remnant (Lin et al., 2006). All tissues dehydrate and the outer sheath and pulp caps are removed by friction and preening, deploying completed feather tissues (Stettenheim, 1972). Corticosterone exposure during early feather growth results in changes to feather structure due to interference with protein production, while exposure later in development is entrapped within feather tissues and thus reflected in feather corticosterone levels (Jenni-Eiermann et al., 2015). Corticosterone exposure ends with the completion of vascularization (Bortolotti et al., 2008).

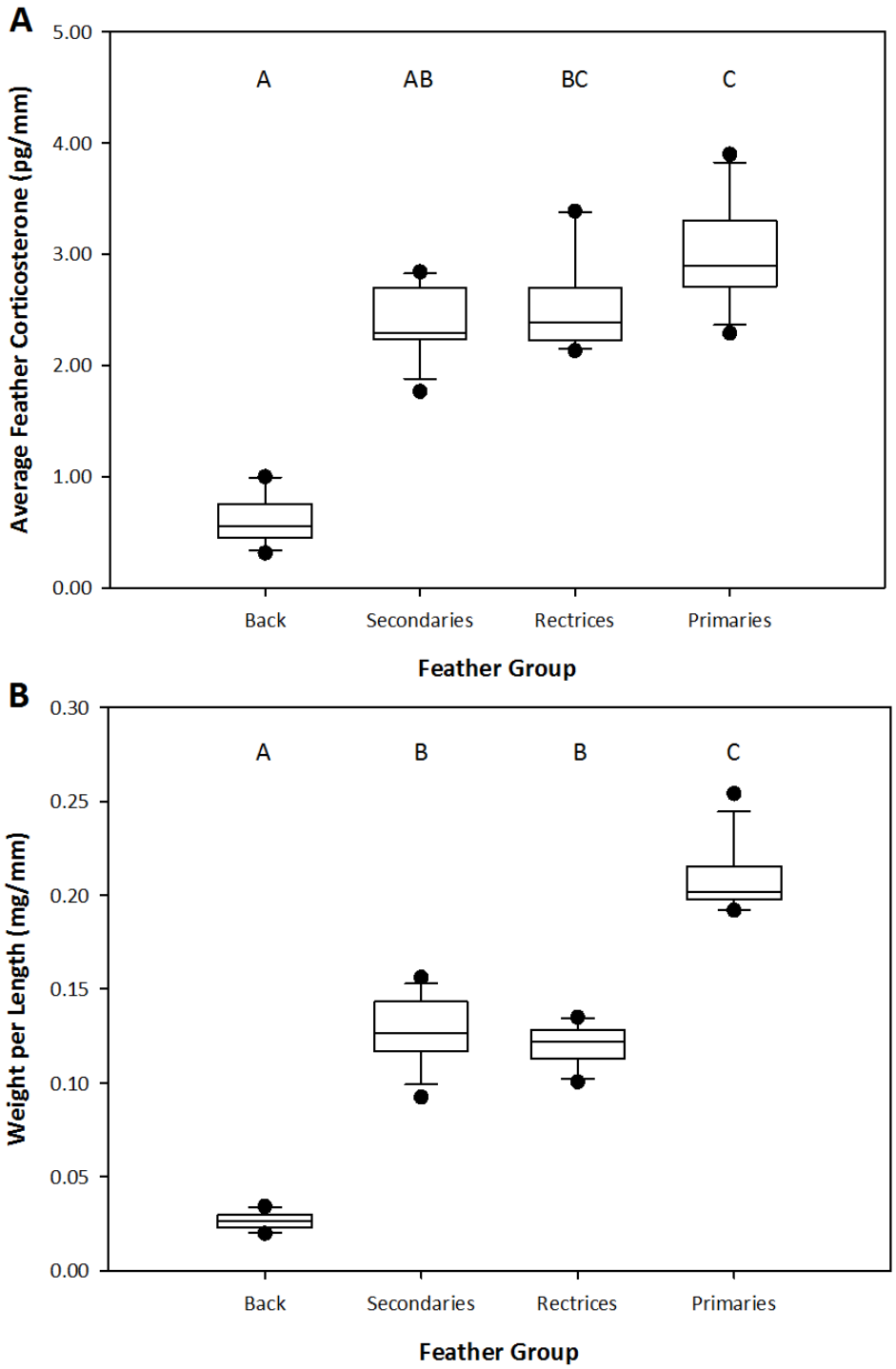

Figure 2.2 – A) Box plot of average corticosterone level across different types of feathers in 12 individuals. Letters denote which feather groups are significantly different by Friedman test blocked for individual identity. B) Box plot of the average feather weight per unit length for different types of feathers in 12 individuals. Letters denote which feather groups are significantly different by Friedman test blocked for individual identity.

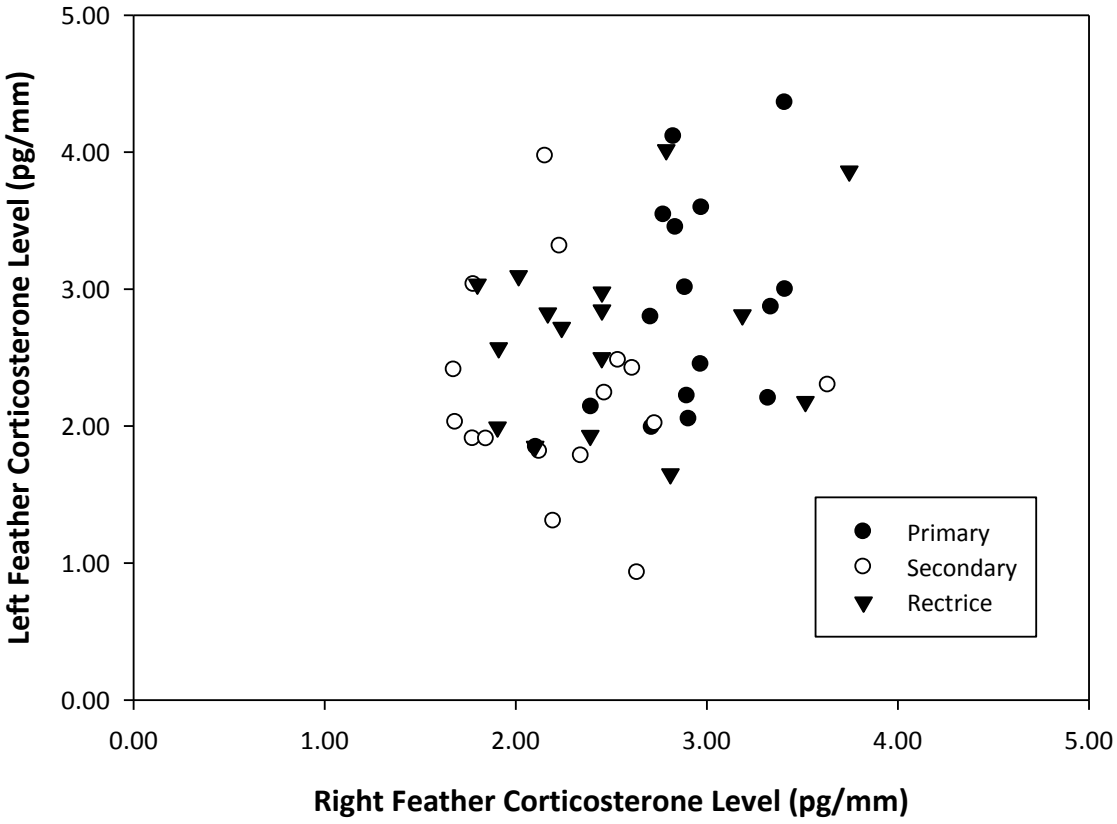

Figure 2.3 – Linear regression of right and left feather corticosterone levels in 2 representative feathers of 3 feather groups in 8 birds (N=48).

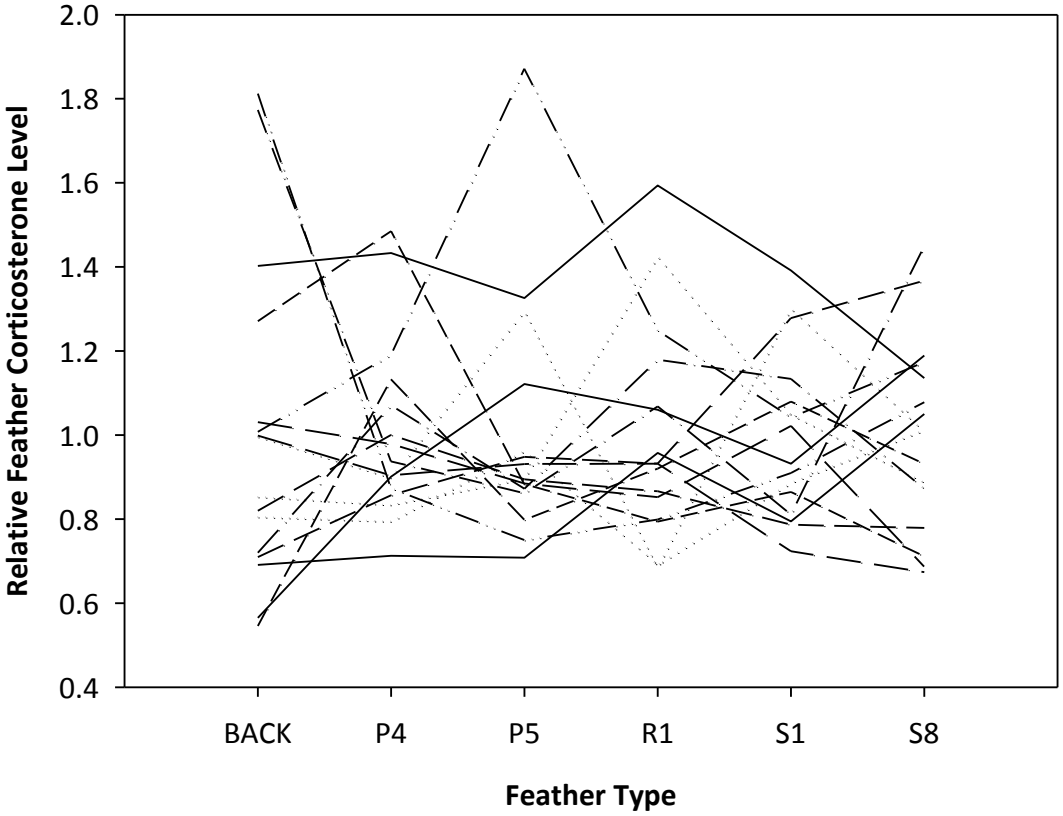

Figure 2.4 – Feather corticosterone levels across different feather types grown at a similar time during moult. To improve comparisons between feathers of different sizes, feather corticosterone levels have been relativized (see methods). Each line represents levels from 5 pooled back feathers, primaries P4 and P5, rectrix R1, secondary S1, and tertial S8 from an individual bird. Under perfect repeatability, individual lines would be horizontal, each with a different intercept.

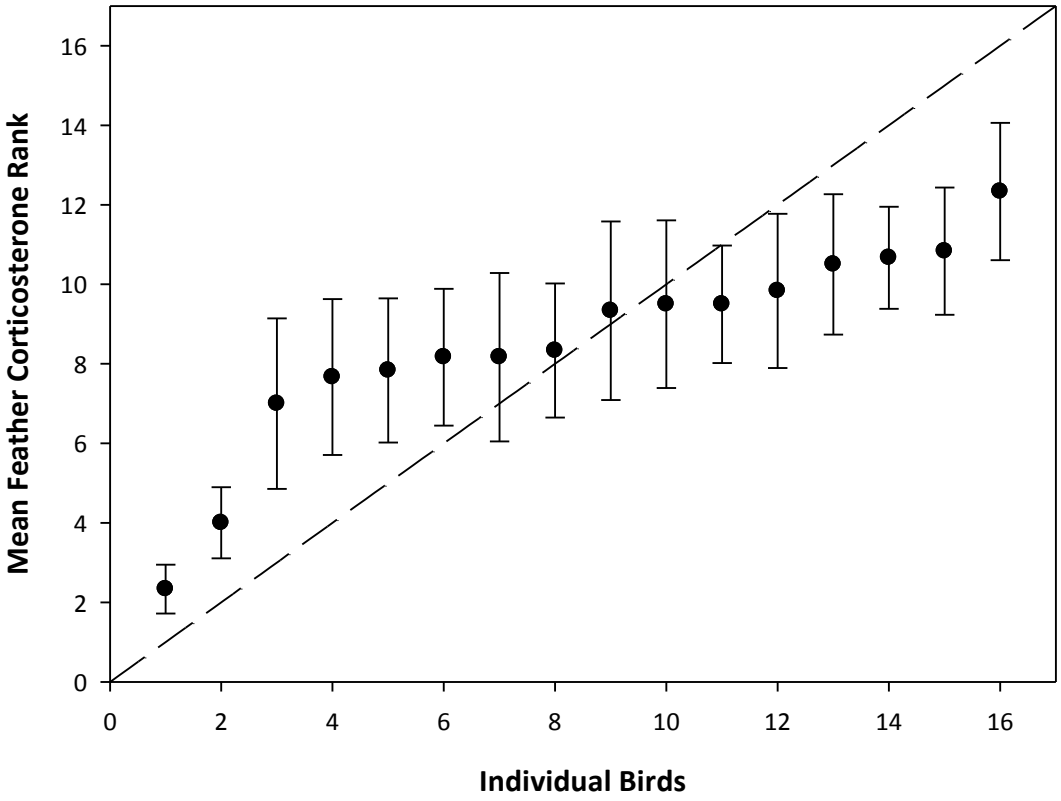

Figure 2.5 – Ranked repeatability of feather corticosterone levels of 16 individuals across six feather types moulted during similar time periods. Points represent mean feather corticosterone rank of the individual using measures from primaries P4 and P5, rectrix R1, secondary S1, tertial S8, and 5 pooled back feathers. Error bars represent 1 SEM and the dotted line represents perfect repeatability.

CHAPTER 3 – FEATHER CORTICOSTERONE LEVELS ARE NOT FIXED

INTRODUCTION

Measurement of glucocorticoids (GCs) in outer integuments, especially feathers, has recently gained attention as a powerful tool for its ability to quantify the energy balance and overall stress status in wildlife (Sheriff et al., 2011). This is in large part due to the reduced invasiveness and ease of integumentary collection, as well as the potential to sample a specific and longer time period than that of blood samples (Bortolotti et al., 2008). Feather corticosterone (CORT) is currently thought to be a long-term integrated measure of GC activity during feather growth (Bortolotti et al., 2008); however, there is currently limited understanding of how CORT enters a feather, where or how it resides, and to what extent it remains fixed over the life of the feather (Lattin et al., 2011).

Similar to those of feathers, GC levels in hair are currently thought to be a measure of long-term hypothalamic-pituitary-adrenal activity (Gow et al., 2010; Meyer & Novak, 2012; Stalder & Kirschbaum, 2012). GC levels in hair are believed to be a combination of internal and external sources of incorporation, and though internal incorporation during growth is assumed to be the primary source, the addition and depletion of those levels via external mechanisms has recently been demonstrated (Stalder & Kirschbaum, 2012). For example, repeated washing or weathering can reduce cortisol levels proportionally to the number of washes (Kirschbaum et al. 2009; Hamel et al. 2011), and GCs in sweat can attach to hair externally and persist despite surface washing (Russell et al., 2013).

Though the feathers and skin of birds possess a number of important differences (Maderson et al., 2009), the study of GCs in both keratinized outer-integuments share a number of similar early findings that suggest enough common features exist to similarly question the stability of CORT in feathers. A first potential issue was raised by studies indicating that CORT may be incorporated externally in addition to the assumed internal mechanism (Lattin et al., 2011; Jenni-Eiermann et al., 2015). A second concern was noted in the paper that introduced the measurement of CORT in feathers where Bortolotti et al. (2008) demonstrated that a hexane wash before final measurement reduced feather

CORT levels. Finally, a subsequent study also found that preparatory washes can reduce feather CORT levels (Jenni-Eiermann et al., 2015). Although the long-term stability of feather CORT in storage and museum specimens or heat treatment has been shown (Bortolotti et al., 2009), the stability of CORT in feathers under real-world use and exposure has not been assessed. Experimental validations of this stability are necessary for the proper interpretation of feather CORT levels, particularly in carry-over studies using feathers grown during natural moult that are either collected long after growth or from species with very long moult intervals. To address this gap, we conducted experiments similar in aim to those conducted in hair, to evaluate, using symmetrically moulted feathers, whether CORT can be incorporated into a feather externally through a CORT soak and whether CORT can be removed from a feather externally using water washes.

METHODS

Feather Collection

Feathers were obtained from tree swallows (*Tachycineta bicolour*) during the breeding season in or near a set of nestboxes at Ruthven Park National Historic Site and Taquanyah Conservation Area in Haldimand County, Ontario, Canada. Feathers used in the analysis were collected from birds that died during the 2010-2013 breeding seasons for reasons such as starvation, vehicle collision, and conflict with house sparrows (*Passer domesticus*). Birds were found within 24 hours of death and feathers were collected if they were not visibly contaminated due to the manner of death and stored at -80°C until assay. Birds and feathers were collected under Environment Canada/Canadian Wildlife Service Scientific Permit CA0266.

Stability Experiments

To test absorption and depletion, left and right side matched primary and secondary feather pairs from 8 individuals were used, with one feather acting as a treatment and the other as a control. Sets of feather pairs of the same feather type were randomly assigned to each experimental group (Table 3.1) and we further randomly chose which feather (right or left) was used as the control in each pair. Before the

experiment, all of the feather types we used were assessed using paired feathers from a different set of 8 individuals and found to be not significantly different ($P>0.05$) by paired t-test.

External Deposition of Corticosterone

To evaluate the ability of feathers to incorporate CORT externally, sets of 8 feather pairs were subjected to no treatment, a CORT soak treatment, or a sham soak treatment. Treatment CORT-soaked feathers were immersed for 24 hours in a 4ng/mL solution of CORT (200ng/mL CORT in ethanol standard added to ultrapure water). This concentration represents the average circulating baseline CORT found in this population of tree swallows during the breeding season (Madliger unpublished). The sham soak treatment was prepared identically except that the ethanol contained no CORT, and the control group received no treatment.

To remove any excess soak solution and ensure that only CORT which was retained in the feather was measured, feathers were washed before extraction using a hexane wash which has been previously shown to remove surface contaminants as well as partially deplete feather CORT levels (Bortolotti et al. 2008 appendix S1). The hexane wash consisted of immersion and swirling of the feather in a 50mL falcon tube filled with HPLC grade hexane for 30 seconds and allowed to dry overnight on paper towel. Both feathers in a pair (treatment and control) were subjected to the same wash before being minced for extraction.

External Depletion of Corticosterone

To evaluate the ability of feathers to deplete CORT externally through repeated environmental weathering, 2 sets of 8 feather pairs were subjected to 25 water wash treatments. Both feathers in the first group were only rinsed briefly with ultrapure water before treatment, while the second group was hexane-washed as above before treatment. After washing, the treatment feather alone was then subjected to repeated (25 times) 1-minute immersion and swirling in ultrapure water in a 50mL falcon tube.

Hormone Quantification

After feathers were allowed to dry overnight, we removed the calamus and the remaining feather length was measured with calipers and extracted according to the protocol given in Bortolotti et al. (2008). Feathers were minced into fine pieces using scissors in a weighed glass scintillation vial. Hormone was extracted using 10mL of HPLC grade methanol and samples were sonicated for 30 minutes and placed in a 50°C water bath overnight. We removed feather pieces from the extract by vacuum filtration, after which the methanol was evaporated in a fume hood. We then reconstituted samples using assay buffer and assayed using Enzo Life Sciences Corticosterone Enzyme Immunoassay (ADI-901-097). Assayed samples showed an intra and inter-assay coefficient of variation of 3.98% and 14.41%, respectively.

Statistical Analysis

The effect of each treatment was assessed using paired t-tests between control and treatment feathers within a given group. Analyses were performed using JMP10 and paired differences were normally distributed requiring no transformation. A control feather in the sham-soaked, hexane-washed group had a feather CORT level more than 3 standard deviations from the mean so that feather pair was excluded as an outlier.

RESULTS

The CORT soak increased feather CORT levels significantly and these increased levels persisted despite the standard hexane preparation wash ($t(7)=13.0062$, $P<0.0001$; Figure 3.1). Feather CORT levels were not different in the hexane-washed control group in which both feathers were only hexane-washed ($t(7)=-2.2550$, $P=0.0588$; Figure 3.1), and levels were not changed by the sham soak ($t(6)=0.2897$, $P=0.7818$; Figure 3.1). Feather CORT levels were significantly depleted by 25 water washes in both unwashed feathers ($t(7)=-5.2251$, $P=0.0012$; Figure 3.2) and hexane-washed feathers ($t(7)=-2.8407$, $P=0.0250$; Figure 3.2).

DISCUSSION

When intact feathers were soaked in a solution of CORT with a concentration corresponding to that of average tree swallow baseline plasma levels, feather CORT

levels were increased. This increase persisted despite post-soak washing with hexane, a solvent which removes surface contaminants and likely extracts some internal CORT from the feathers. Similarly, feather CORT levels were decreased when intact feathers were washed with water 25 times regardless of whether the feather was left untreated or hexane-washed before water exposure. These results suggest that feather CORT may not be a fixed measure of stress activity that is maintained throughout the life of a feather. They further suggest that external deposition could be at least a partial mechanism by which CORT is normally deposited into feathers over and above that which is deposited during feather growth. External deposition of analytes of interest into feathers is not unprecedented, as results from toxicological studies of feathers have shown that birds after heavy metal exposure had higher levels of most heavy metals in both re-grown and original feathers as compared to levels before exposure (Dauwe et al., 2003; Jaspers et al., 2004). Also, results similar to those of this study have been shown for hair cortisol levels (see introduction), and external input and removal are likely to be somewhat true of all keratinized outer-integuments. However, the relative importance of external changes to each may be different both between and within an integument type as species differences have already been shown in hair (Stalder & Kirschbaum, 2012).

While avian skin does not contain sweat or sebaceous glands, the skin as a whole is highly lipogenic, and is best considered a holocrine secretory unit (Menon & Menon 2000). As a result, avian skin has a number of adaptations to allow dermal evaporative cooling, and both uropygial (preen gland) and skin secretions are deposited on feathers in detectable amounts in various levels across species (Wrench et al. 1980). Together the secretions work to keep feathers in good condition and able to repel water, have antibacterial and anti-mycotic properties, and have odorant and pheromone properties (Stettenheim, 2000). While CORT has not been detected in uropygial oil, hydrocarbon wax esters may be important in preventing or facilitating hormone diffusion in grown feathers (Lattin et al., 2011). In addition, this layer could play some role in holding CORT, as lipophilic contaminants have been shown to originate and accumulate in preen oil (Van den Brink, 1997; Jaspers et al., 2007) which would impact the interpretation of measured levels. Finally, since feathers provide the outer barrier between organism and environment and birds spend a significant amount of time preening, it is likely that

feathers come into external contact with CORT in saliva, blood, and perhaps feces during their normal use. The relative importance of the inclusion of any metabolized forms of GCs would depend on the specificity of the method of hormone measurement.

Another possible source of external deposition is that of a proposed peripheral stress-axis in avian skin. The existence of this local production of GCs has been demonstrated in the follicles and skin of mammals (Ito et al., 2005; Slominski et al., 2008) and recently suggested in birds (Koren et al., 2012; Jenni-Eiermann et al., 2015). Indeed, local production of GCs was proposed as a possible explanation for why levels of cortisol comparable to corticosterone were detected in feathers of house sparrows, despite plasma possessing extremely low or non-detectable levels (Koren et al., 2012). This potential mechanism is bolstered by the fact that avian skin has also been shown to possess GC receptors (Lattin et al., 2012) and the ability to metabolize GCs (Bortolotti et al., 2008).

The long-term stability of CORT in feathers may differ between feather types due to altered structure and in different body locations due to differences in use, care, wear, and weathering. It also likely depends on species-specific differences in feather structure, moult strategy, and life history. For example, iridescent plumage has reduced waterproofing due to the structural differences necessary to produce the colouration (Eliason & Shawkey, 2011) thereby potentially changing the ability for hormone to access or be removed from the feather externally. Stability differences between pigments may therefore play a role in the conflicting results of studies of feather CORT levels and plumage colouration (Kennedy et al., 2013; Lendvai et al., 2013; Jenni-Eiermann et al., 2015).

Overall, our results suggest feathers are able to take up exogenous CORT and that existing levels can be depleted. Future studies are needed to determine whether different species, feather types, pigmentation, or environmental exposure can influence the stability of CORT levels. In general, the effect of external changes should be considered in studies of feather CORT and levels should not be considered a fixed trait in feathers collected long after moult without further validation.

ACKNOWLEDGEMENTS

We thank Ruthven Park National Historic Site for lodging and access to their study site and nestboxes. We also thank the Grand River Conservation Authority and Habitat Haldimand for access to their study site and nestboxes. We thank Peter Marier for his assistance processing feather samples. CLM and OPL were supported by NSERC postgraduate and operating grants, respectively. Canada Foundation for Innovation (CFI) and Canada Research Chair (CRC) funding to OPL also supported this work.

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FIGURES

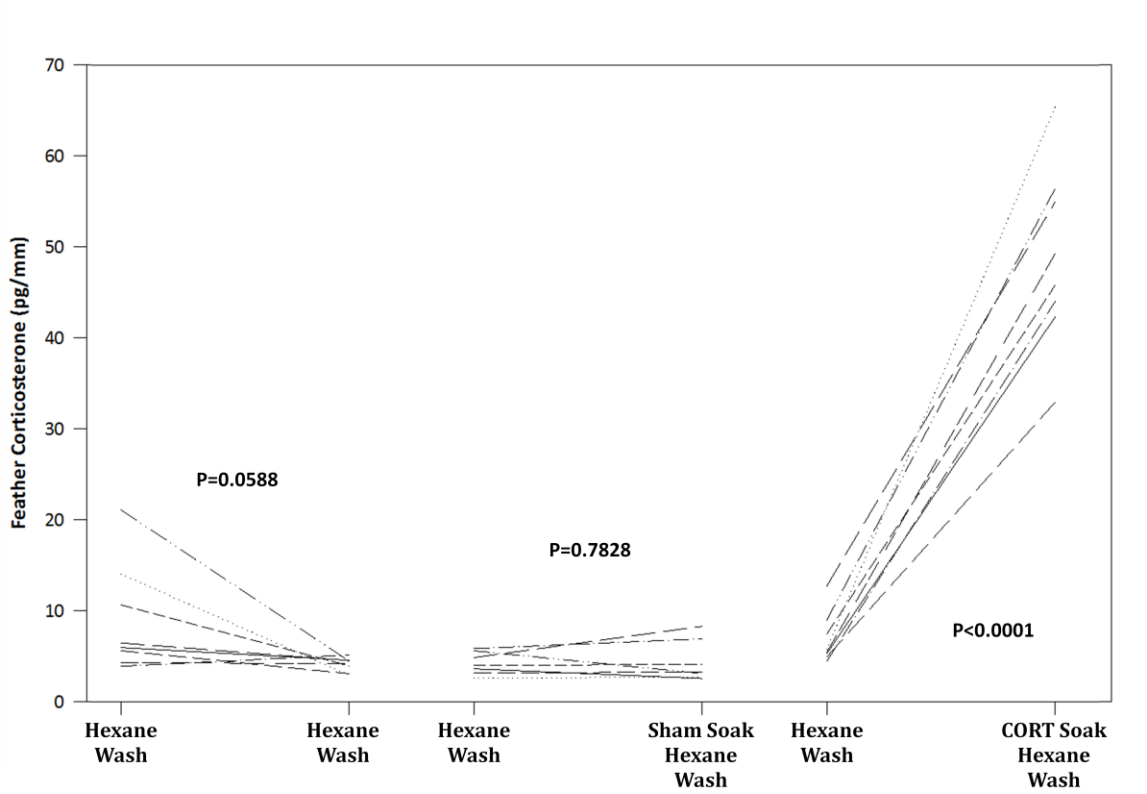

Figure 3.1 – Effect of corticosterone soak treatment on feather levels. Each line represents an individual left and right (randomized) feather pair from the same bird, and each group is composed of the same feather pair from different birds (N=8,7,8, respectively). The left end of each line acts as a control, while the right acts as a treatment. All feathers were subjected to the same post-treatment hexane wash. P values are provided from paired t-test comparisons.

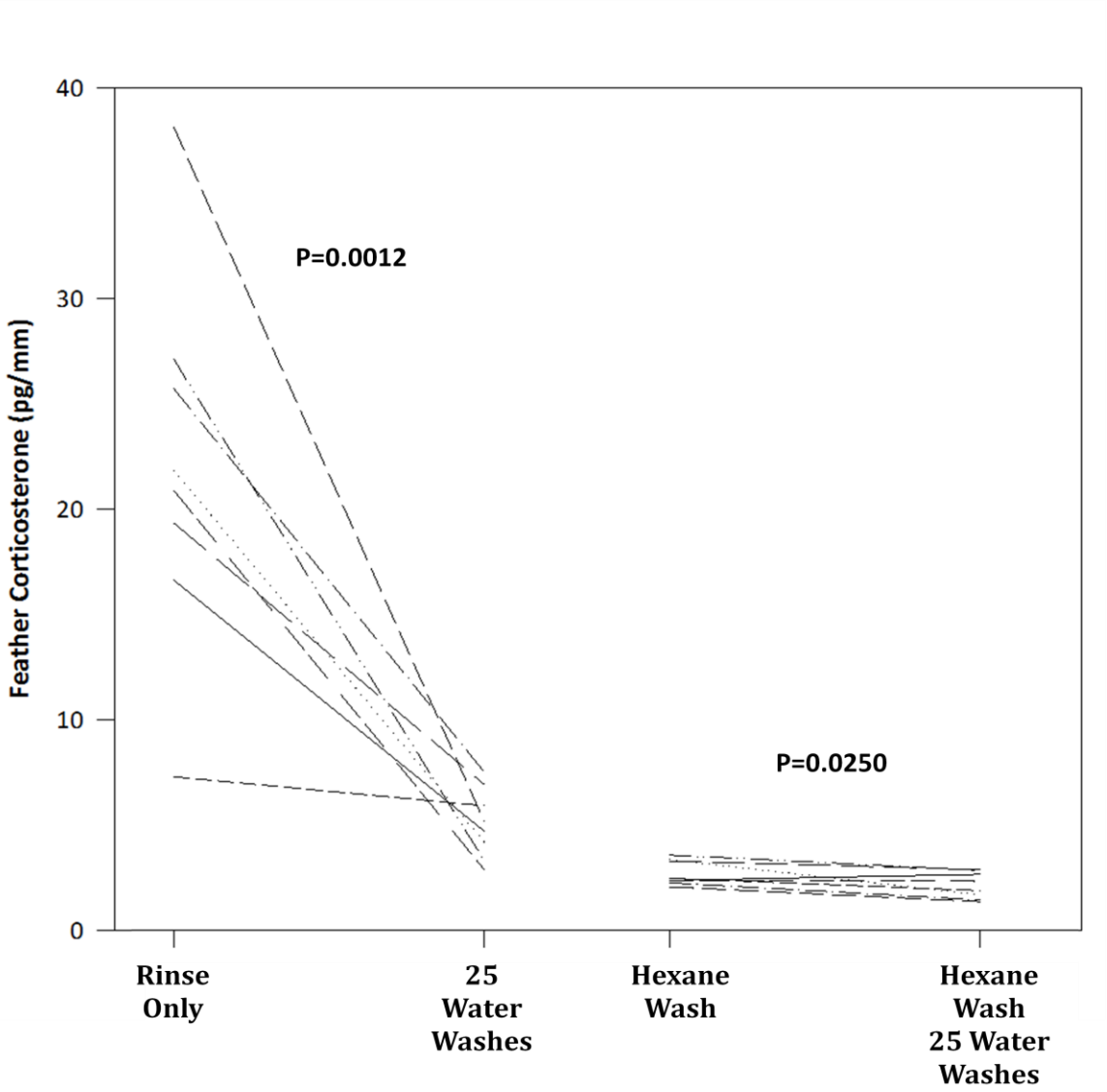

Figure 3.2 – Effect of 25 repeated 1 minute water washes on feather corticosterone levels in unwashed and hexane-washed feathers. Each line represents an individual left and right (randomized) feather pair and each group is composed of the same feather pair from 8 different birds. The left end of each line acts as a control, while the right acts as a treatment. P values are provided from paired t-test comparisons.

TABLES

Table 3.1 – Feather pairs used for each experimental group.

Treatment	Preparation	Feather Type
Control	Hexane Wash	Primary 4
Sham Soak	Hexane Wash	Secondary 1
CORT Soak	Hexane Wash	Primary 5
25 Washes	Rinse	Primary 2
25 Washes	Hexane Wash	Secondary 2

CHAPTER 4 – EVALUATING FEATHER CORTICOSTERONE AS A BIOMARKER FOR DETECTING BIOLOGICALLY RELEVANT STRESSFUL EVENTS IN THE WILD

INTRODUCTION

The fields of ecological and conservation physiology seek to develop and use physiological biomarkers of health and environmental quality as tools to provide context and mechanism across a variety of applications and taxa (Stevenson et al., 2005). To be relevant for conservation purposes, physiological biomarkers must be sensitive enough to accurately reflect an individual's capacity to manage daily challenges more rapidly than traditional demographics, while also providing the mechanistic capacity to causally predict how organisms will respond to future increases in these challenges (Cooke & O'Connor, 2010). Although a number of physiological traits have been proposed and studied, the concept and measurements of 'stress' are some of the most heavily investigated because of their ability to incorporate the balance between an organism's energy resources and demands (McEwen & Wingfield, 2010; Dantzer et al., 2014; Madliger & Love, 2014). In particular, the measurement of glucocorticoid hormones has long-been proposed as an important tool for assessing the current state of individuals and populations given their role in regulating homeostasis (Landys et al., 2006; Busch & Hayward, 2009).

The method and medium of glucocorticoid (GC) sampling also has a large influence on their use and interpretation (Sheriff et al., 2011). For example, the standard quantification of circulating baseline levels of GCs in blood is often difficult to obtain and interpret in free-living species since levels respond rapidly to the capture and handling required to collect samples, and as such are further subject to the organism's experiences in the hours prior to capture (Romero & Reed, 2005). To remedy this, a number of alternative sampling media have been developed (e.g., fecal, salivary), of which sampling in keratinized outer integuments (i.e., hair and feathers) is a promising and potentially attractive, although poorly understood, emerging technique (Sheriff et al., 2011). Since GC levels in feathers are thought to primarily be the result of deposition

during feather growth, it has been proposed that feather GCs should reflect circulating levels over a period of weeks (Bortolotti et al., 2008). If correct, feather GCs could represent a cumulative measure of the challenges faced over this time period, which would make them valuable biomarkers of exposure to chronic environmental stressors (Bortolotti et al., 2008). In addition, since a relatively large amount of material can be obtained from feathers and individually identifiable flight and covert feathers make consistent, standardized sampling possible, they provide potential advantages compared with other sampling media. Finally, since feathers are normally only moulted at strict periods and are only vascularized during growth (Howell et al., 2004), if deposition is limited to this vascularized period then feather-based measures of corticosterone (CORT) could provide a lasting measure of GC activity of a target period for the life of the integument.

To date, studies suggest that CORT levels found in feathers replaced after plucking reflect the significantly elevated circulating levels caused by experimental exogenous CORT supplementation during feather growth (Lattin et al., 2011; Fairhurst et al., 2013; Jenni-Eiermann et al., 2015). These elevated levels can appear in already grown sections of feather (Lattin et al., 2011), possibly because the vascularized section of feather extends beyond the skin (Maderson et al., 2009; Jenni-Eiermann et al., 2015). However, as researchers often look for this signal only where they think it will show up and omit feather sections where it is not expected, the certainty of the above assertion of deposition remains inconclusive. In addition, manipulated CORT levels are often elevated beyond stress-induced levels and are enough to cause changes in feather structure (Bortolotti et al., 2009a; DesRochers et al., 2009; Jenni-Eiermann et al., 2015), confounding understanding of how these levels may relate to normal deposition and feather capacity. Indeed, feather CORT does not appear to reflect the relatively small daily variation in baseline circulating CORT levels (Bortolotti et al., 2008; Fairhurst et al., 2013). This may be due to the instantaneous nature of circulating measures from blood sampling; however, experiments utilizing stressors to increase endogenous CORT do not correlate well either (Bortolotti et al., 2009b; Fairhurst et al., 2011; Hõrak et al., 2013; Gow & Wiebe, 2014a; Lattin et al., 2014; Cruz-Martinez et al., 2015; Patterson et

al., 2015). Instead, it may be that only large, prolonged increases in CORT levels are sufficient to be deposited accurately naturally.

Overall, it is currently unclear to what extent feather CORT levels of feathers grown during natural moult reflect the stress status of a wild bird. To address this, we assessed whether feather CORT represents a relevant biomarker of environmental and reproductive stress in wild tree swallows (*Tachycineta bicolor*) in two ways. First, we examined whether individual state and reproductive investment could predict feather CORT levels in subsequently moulted feathers and whether those feather CORT levels could predict future survival and reproductive success. Second, through a manipulation of increased flight cost during breeding, we experimentally investigated whether a biologically-relevant increase in stress level would be reflected in naturally-grown feathers, and whether those levels could, in turn, predict future success. If feather CORT is indeed a robust and reliable measure of stress, we would predict that integument hormone levels reflect individual state and reproductive investment immediately preceding moult and indicate future capacity to survive and invest in offspring. Further, after a manipulation which raised circulating CORT, increased body mass loss over reproduction, and decreased survival to the following year in our study (Madliger unpublished), as well as in other studies in this species (Winkler & Allen, 1995; Ardia & Clotfelter, 2006; Patterson et al., 2011), feather CORT levels should reflect a manipulated female's diminished state, increased costs, and reduced capacity for future fitness.

METHODS

Study System

Fieldwork was carried out on a nestbox-breeding population of tree swallows at Ruthven Park National Historic Site and Taquanyah Conservation Area in southern Ontario, Canada. The site's 175 nestboxes have been scientifically monitored daily through the breeding period (late April to early July) since 2010. We weighed and numbered all eggs on the day of laying, recorded incubation length and nestling hatch date, weighed nestlings at 6 and 12 days post-hatching (i.e., linear phase of post-natal growth: Quinney et al., 1986; McCarty, 2001), banded nestlings with a federal aluminum band and obtained morphological measurements at day 12 post-hatching. We also

determined fledging date and success (number of nestlings successfully leaving the nest). In addition, we trapped all adult females in their nestboxes to band (Canadian Wildlife Service Permit 10808), weigh and obtain blood samples from the brachial vein for baseline circulating levels of CORT at 10-12 days into incubation and again at day 12-14 of nestling-rearing.

Tree swallows are an ideal candidate for feather CORT biomarker validations as they are a well-studied free-living model species (Jones, 2003), meaning that important life history factors such as moult timing and order are understood (Stutchbury & Rohwer, 1990). Both prebasic moult and migration occur soon after the breeding season, from July to November (Stutchbury & Rohwer, 1990). Also, their flight feathers are relatively uniform in colour, reducing any confounding effects of pigment differences on feather CORT capacity or affinity (Jenni-Eiermann et al., 2015). In addition, they are highly philopatric (Winkler et al., 2004) and respond well to human intrusion and manipulation (Jones, 2003). Finally, their widespread distribution and willingness to breed in nestboxes in a variety of habitat types has led to their frequent use in assessing impacts of habitat change, anthropogenic disturbance, and habitat reclamation where the principles and tools of ecological and conservation physiology aspire to assist (Ghilain & Bélisle, 2008; Harms et al., 2010; Custer, 2011; Paquette et al., 2013; Cruz-Martinez et al., 2015).

Manipulation of Stress and Feather Collection

In 2011, we conducted a primary feather clipping manipulation (previously validated in this and other species: Winkler & Allen, 1995; Ardia & Clotfelter, 2006; Love & Williams, 2008) designed to increase energetic workload and therefore baseline plasma CORT levels throughout the nestling-rearing stage. All experimental methods were approved by the University of Windsor's Animal Care Committee (AUPP#10-10) and the Canadian Wildlife Service (Permit CA0266). Treatment birds (N= 36) had every other primary feather (P8,6,4,2) clipped using scissors at the level of the coverts (Winkler & Allen, 1995; Ardia & Clotfelter, 2006) immediately after being blood sampled and processed just prior to the nestling-rearing period (i.e., at 10-12 days of incubation). As feathers were clipped rather than plucked, they were not replaced immediately and birds remained handicapped until moult. Control birds (N= 41) were blood sampled, handled

and processed as above, leaving all primary feathers intact. Treatment females were paired with control females to ensure spatial, temporal, and reproductive investment balance across the manipulation. Second year females (i.e., first-time breeders as identified by plumage colouration; Hussell, 1983) were excluded from the clipping manipulation due to low sample size.

In the following breeding season (2012), we again collected a feather sample from returning birds to assess whether the manipulation of stress in 2011 affected subsequent CORT levels in feathers grown after the breeding season. As the goal in 2012 was to sample rather than induce a handicap, we clipped only the right 2nd primary feather at the level of the coverts following blood sampling. Since tree swallows moult primary feathers from primary 1 outwards (Stutchbury & Rohwer, 1990), the 2nd primary represents the first feather from the manipulation to be replaced. To facilitate the maximum number of comparisons between treatments, feather samples were taken from returning birds from both the clipped and control groups. All feathers were stored in brown paper envelopes in a sealed container until lab processing.

Feather Preparation and Hormone Analysis

To remove surface contaminants (Bortolotti et al., 2008), feathers were washed before analysis by immersion and swirling in a 50mL falcon tube filled with a dilute (1%) soap solution made from Dawn™ and ultrapure water for 30 seconds. We then rinsed feathers briefly with ultrapure water. After feathers were allowed to dry overnight, the length of the feather sample was measured with calipers and extracted according to the protocol outlined in Bortolotti et al. (2008). To summarize, feathers were minced into fine pieces using scissors in a weighed glass scintillation vial. Hormone was extracted using 10mL of HPLC grade methanol. Samples were sonicated for 30 minutes and then placed in a 50℃ water bath overnight. We separated feather pieces from the extract by vacuum filtration, after which the methanol was evaporated. We reconstituted samples using kit-provided assay buffer and assayed samples using a previously-optimized commercial ELISA (Enzo Life Sciences CORT Enzyme Immunoassay - ADI-901-097). Intra- and inter-assay variation was 6.52% and 10.66% respectively, and all samples were

of a similar mass to minimize any potential issues of mass-dependency of the extraction (Lattin et al., 2011).

Statistical Analyses

Previous Investment as a Predictor of Feather Corticosterone

We first investigated potential sources of inter-individual variation in feather CORT levels in unmanipulated birds. We used a general linear mixed-effects model with measures of individual female state and reproductive effort from the previous year immediately prior to moult to predict current year feather CORT levels (i.e., those moulted after the previous breeding season and collected during the current reproductive attempt). Individuals were included in this analysis only if state and reproductive data for these birds was available from both the previous and current year, as well as feather CORT levels in the current year (N=29). Since this first goal was to illuminate correlative relationships in unmanipulated birds, individuals returning in 2012 from the clipping manipulation in 2011 were excluded to avoid any confounding effects. The percent change in female body mass from late incubation to the 12th day of nestling-rearing, baseline circulating plasma CORT levels at peak nestling provisioning (log transformed), clutch initiation date (expressed as days since May 1st and square-root transformed), and total brood mass at day 12 of nestling rearing were used as measures of individual state and reproductive effort, since they represent energy invested in self-maintenance, GC function before moult, reproductive timing, and energy invested in the clutch, respectively. Feather CORT was log transformed and all other variables were transformed to normality where appropriate. Year was included in the model as a random factor. The least significant variables were removed from the model in a backwards stepwise manner (alpha=0.05). All analyses were performed in JMP 10.

Feather Corticosterone as a Predictor of Future Success

To investigate whether feather CORT levels were indicative of future capacity or carry-over effects, we evaluated its ability to predict future reproductive success and survival using separate generalized linear models. As above, individuals were only included in these analyses if they were not manipulated (N=18), as clipped individuals may have had their current and future success altered. All individuals used were sampled

in 2012 so year was not included in the models. As a historical record of CORT activity cannot directly affect success, it is likely to have a large number of subtle mechanisms beyond the sample size and scope of this study. We therefore focused on ultimate measures of success to limit comparisons. We did not include covariates from the current year (i.e., lay date, clutch size, etc.) as our sample size was more suited to detecting a relationship between feather CORT and success rather than the mechanisms by which it occurs, and, given their temporal distance, those covariates may themselves be affected by feather CORT. The relationship between feather CORT measured in the current year and the number of nestlings fledged in that year was assessed using a generalized linear model with a Poisson distribution and log link function. The relationship between feather CORT measured in the current year and survival to the next year was assessed using a generalized linear model with a binomial distribution and logit link function. Survival was defined as the recapture of the individual in the next 2 years subsequent to sampling. As tree swallows are highly philopatric (Winkler et al., 2004) and our study sites represent the largest concentration of nestboxes across a broad spatial area, this local return rate provides a proxy for survival.

Effort as a Predictor of Feather Corticosterone under Energetic Challenge

To assess the impact of the feather clipping manipulation on feather CORT, we compared feather CORT levels in the feathers of clipped birds from 2011 and those of both returning clipped and control birds in 2012 using separate t-tests. Since it was not possible to collect feathers from control individuals in the manipulation year (2011) without compromising their ability to act as controls, a fully balanced repeated-measures approach was not possible. We first tested for inter-annual consistency in feather CORT levels by comparing CORT levels in feathers from clipped females in 2011 to those of returned control birds in 2012 (n=36, 14, respectively). This interpretation is relevant because the manipulation was balanced by lay date and clutch size between the clipped and control group throughout the breeding season, meaning that clipped birds were as similar as possible to controls until the moment of the handicap. We then tested whether the experimental manipulation increased feather CORT levels by comparing feather CORT levels of feathers collected from clipped individuals in 2011 with those of females returning from the clipping manipulation in 2012. We conducted this analysis at 2 scales

by comparing across the 2 years in the entire group (n=36, 10), as well as within-individuals using a paired comparison (n=10). Finally, to determine if manipulated individuals could be differentiated from controls using feather CORT as a biomarker of exposure of stress during reproduction, feather CORT levels of returned clipped females were compared with those of returned control birds (n=10, 14, respectively). Feather CORT values were log transformed to ensure normality and all groups showed equal variance as indicated by a Levene's test.

Feather Corticosterone as a Predictor of Success under Energetic Challenge

To assess whether CORT deposited into feathers following a significant reproductive stressor predicted future reproductive investment or survival, we used generalized linear models to investigate the effects of the 2011 manipulation on both the number of nestlings fledged in 2012 and subsequent adult survival (N=36). In this case, survival was defined as recapture of the same individual in the next 3 years subsequent to sampling.

RESULTS

Previous Investment as a Predictor of Feather Corticosterone

None of the chosen variables representing individual female state, reproductive investment or effort from the previous breeding season predicted feather CORT levels upon return the next year (Table 4.1). Baseline CORT levels, lay date, total brood weight, and the change in female body mass over breeding were not significant predictors of feather CORT levels. In addition, none of the variables achieved significance through stepwise removal.

Feather Corticosterone as a Predictor of Future Success

Under normal, unmanipulated conditions, CORT levels of feathers collected in the current year from female tree swallows did not predict the number of nestlings fledged in the current breeding season (N=18, DF=1, χ^2=1.0051, P=0.3161). In addition, CORT levels of feathers collected in the current year did not predict survival to the following breeding seasons (N=18, DF=1, χ^2=0.4278, P=0.5131).

Effort as a Predictor of Feather Corticosterone under Energetic Challenge

Overall, we found no significant difference between the CORT levels of feathers taken for the clipping manipulation in 2011 and the feathers of returning control birds in 2012 ($t(48)=1.9496$, $P=0.0571$; Figure 4.1), indicating that feather CORT levels did not differ between years. In addition, the feather clipping manipulation during reproduction in 2011 did not increase the CORT levels of subsequently grown feathers collected in 2012; there was no significant difference between feather CORT levels of feathers from birds clipped in 2011 and feathers from those same birds returning in 2012 both as a group ($t(44)=1.6830$, $P=0.1086$; Figure 4.1) or within-individual pairwise ($t(9)=0.2049$, $P=0.8422$; Figure 4.2). Importantly, birds which had undergone the manipulation in 2011 could not be differentiated from the rest of the population by feather CORT alone as there was no significant difference between feather CORT levels from returning clipped birds and those of returning control birds in 2012 ($t(22)=0.1054$, $P=0.9170$; Figure 4.1).

Feather Corticosterone as a Predictor of Success under Energetic Challenge

The CORT levels of feathers collected in the current year from manipulated females failed to predict the number of offspring fledged in the manipulation season ($N=36$, $DF=1$, $\chi^2=0.2687$, $P=0.6042$). In contrast to results from reproductive success and the previous investigation of unmanipulated females, feather CORT levels of feather-clipped birds in 2011 significantly predicted survival of the manipulation year ($N=36$, $DF=1$, $\chi^2=4.7028$, $P=0.0301$, $e\beta=4.084$; Figure 4.3), indicating that manipulated birds with higher feather CORT levels had a higher chance of survival.

DISCUSSION

Feather Corticosterone after Natural Moult

The current understanding of feather CORT deposition hypothesizes that CORT levels in feathers represent an integrative measure of circulating levels over the period of feather growth (Bortolotti et al., 2008). Further, it is thought that these levels remain relatively fixed after the end of vascularization, meaning that researchers can reliably measure CORT in feathers collected any time before the following moult (Bortolotti et

al., 2009a). It therefore follows that individual birds undergoing chronic elevated stress immediately prior to moult, or those still working to mitigate carry-over costs at the time of moult, should exhibit elevated CORT levels in subsequently grown feathers. Despite this reasoning, circulating CORT levels in unmanipulated tree swallows measured during the peak of the nestling provisioning stage and female mass loss from incubation to peak provisioning did not predict the CORT levels of subsequently moulted feathers. Moreover, neither the timing of reproduction nor the degree of reproductive effort appeared to impact the CORT levels of subsequently moulted feathers in these birds. Similarly, feather CORT deposited in the feather during the previous moult did not predict reproductive success or survival in the current year in unmanipulated females.

Feather Corticosterone after Energetic Challenge before Molt

Our feather clipping manipulation, designed to increase energetic workload during an already energetically-demanding life-history stage (i.e., nestling provisioning), successfully increased both circulating CORT levels, female body mass loss, and decreased total brood mass (Madliger unpublished). In addition, as feathers were clipped rather than plucked and the feather used for hormone quantification (primary 2) would have been the first of the clipped feathers to be replaced in the normal moult order (Stutchbury & Rohwer, 1990), this handicap should have persisted in these aerial insectivores throughout the growth of that feather. Despite this, there was no change to CORT levels in subsequently moulted feathers collected from birds returning the following year. Furthermore, a within-female analysis in feather clipped females failed to detect an increase in feather CORT across years; females undergoing the manipulation the year before could not be distinguished from returning control birds by feather CORT levels (despite fledging the same number of offspring the year before), and we could not detect any significant inter-annual variability in feather CORT in the general population. As with unmanipulated females, feather CORT levels also did not predict reproductive success after a significant manipulation of workload during reproduction. In particular, the significant relationship between feather CORT and survival for the feather-clipped females alone suggests that feather CORT levels may only be useful in predicting susceptibility to mortality after a large-scale change or challenge rather than small-scale

environmental variability. Interestingly, and somewhat counter-intuitively (i.e., see Koren et al., 2011), higher feather CORT predicted a higher probability of survival following the challenge. These results suggest that while feather CORT might be used to forecast which individuals will be best able to weather a serious challenge, feather CORT in feathers grown during natural moult does not appear to always respond to elevated stress as expected by the current understanding of deposition.

Sensitivity of Feather Corticosterone in Moulted Feathers

While our feather clipping manipulation caused significant changes in female body mass and circulating CORT, it did not cause increased rates of nest abandonment and females raised a similar number of, albeit smaller, offspring. As such, it is difficult to suggest that our manipulation was not harsh enough to reach the sensitivity required to alter feather CORT as a more drastic manipulation may have reduced fledging success or caused abandonment of reproduction. This lack of response at this level of disturbance suggests that the sensitivity threshold of feather CORT may limit its relevance as a biologically-relevant biomarker as it would be unable to indicate stress or disturbance with more sensitivity than demographics. In addition, as a group, feather CORT levels should have been higher in birds returning from manipulation if for no reason other than birds that survived generally had higher feather CORT levels. It is possible that with a larger sample size the mean of the distribution could have been increased because low feather CORT birds were more susceptible to death and fewer returned from the manipulation, removing them from the distribution. However, in this scenario, the increased average feather CORT levels would have been a result of selection instead of a response to chronic stress, and those higher-level individuals would incorrectly be considered to be of lower individual quality despite their survival in the face of a large unexpected perturbation.

A potential reason for why feather CORT did not reflect individual state and reproductive effort in unmanipulated birds or change after a large perturbation could be due to differences between natural moult and induced feather replacement (the primary source for validation of this technique to date; e.g., Bortolotti et al., 2008; Lattin et al., 2011; Jenni-Eiermann et al., 2015). Because moult is an energetically demanding period

involving the regeneration of a number of tissues and partially compromises flight and thermoregulation (Howell, 2010), it is thought that the observed decrease in both baseline and acute levels of circulating CORT in comparison to other life history stages is a result of down-regulation of CORT release in order to minimize the amount of time required to re-grow quality feathers (Romero, 2002; Strochlic & Romero, 2008). In addition, while exogenous CORT causes slower feather growth rates, it appears to do so differently in natural compared with induced moult (Romero et al., 2005). In contrast, physical and psychological stress can result in much lower increases in circulating CORT levels due to the down-regulation of the hypothalamic-pituitary-adrenal (stress) axis, and only the nutritional stress of food deprivation has been shown to slow feather re-growth rate (Strochlic & Romero, 2008). We would therefore expect that the moult characteristics and feather CORT levels of the feather clipped birds should not be different from the controls if clipped birds were still able to find sufficient food for proper feather growth while also suppressing their elevated circulating CORT levels. This possible explanation is particularly relevant to our study as we were unable to observe moult and are therefore unable to account for potential modifications of timing or rate of moult in clipped individuals. However, the same compensatory changes would likely occur in any similar use of this technique, meaning that feather CORT would not be useful as a sole biomarker of exposure to stress.

A further possibility is that feather CORT levels under free-living conditions are not as stable over the long-term as we currently assume they are (as shown in Chapter 3). If feather CORT levels are not stable in the feather once it has been grown, even if feather CORT levels were increased appropriately at moult in the feather clipped group, the potential increase and the differences between clipped and control females may not persist until sampling during the following breeding season. A related issue would be if feather CORT levels are responsive to changes in stress physiology but the differences caused in the feathers are not great enough to be detected without large sample sizes due to the variation introduced by feather wear, washing, extraction, and assaying. If the effect of these issues was species-specific due to differences in feather structure, pigmentation, and environmental exposure, it would induce even further variation in interpreting feather CORT levels. Finally, feather CORT relationships may also depend

on the feather sampled as feathers from other body regions may have different signals or differ in environmental and preening exposure.

Conclusion

We found that feather CORT levels of tree swallow feathers grown during natural moult did not 1) reflect past breeding experience, 2) predict reproductive output, or 3) respond to a significant manipulation of flight effort during the height of reproduction. Furthermore, while high feather CORT levels predicted survival, they did so only in the group undergoing the manipulation. These combined results join the growing number of studies reporting that high feather CORT levels can be positively (Kouwenberg et al., 2013; Sild et al., 2014) or negatively (Koren et al., 2011; Gow & Wiebe, 2014b) related to proxies of individual state or fitness, or that they do (Crossin et al., 2013) or do not (Bourgeon et al., 2014) represent carry-over effects. Taken together, this growing body of results suggests that feather CORT may be no less context-dependent than measures of circulating levels (Madliger & Love, 2014). These results further suggest that the current understanding of deposition and stability of CORT in naturally-grown feathers is inadequate to allow the type of interpretation necessary for use as a biomarker. Without a stronger understanding of how feather CORT relates to circulating CORT and stress, interpretation of high feather CORT as negative is problematic, as even in circulating levels, a "higher is worse" interpretation may not be enough (Dantzer et al., 2014; Madliger & Love, 2014). Overall, our results caution the use of feather CORT as a simplistic indicator of exposure to chronic stress in feathers of free-living birds when those feathers were grown during moult.

ACKNOWLEDGEMENTS

We thank Ruthven Park National Historic Site for lodging and access to their study site and nestboxes. We also thank the Grand River Conservation Authority and Habitat Haldimand for access to their study site and nestboxes. We thank Peter Marier for his assistance processing feather samples, and Rick Ludkin, Peter Marier, and Nancy Furber for their help with field collection of samples and data. CLM and OPL were supported by NSERC postgraduate and operating grants, respectively. Canada

Foundation for Innovation (CFI) and Canada Research Chair (CRC) funding to OPL also supported this work.

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FIGURES

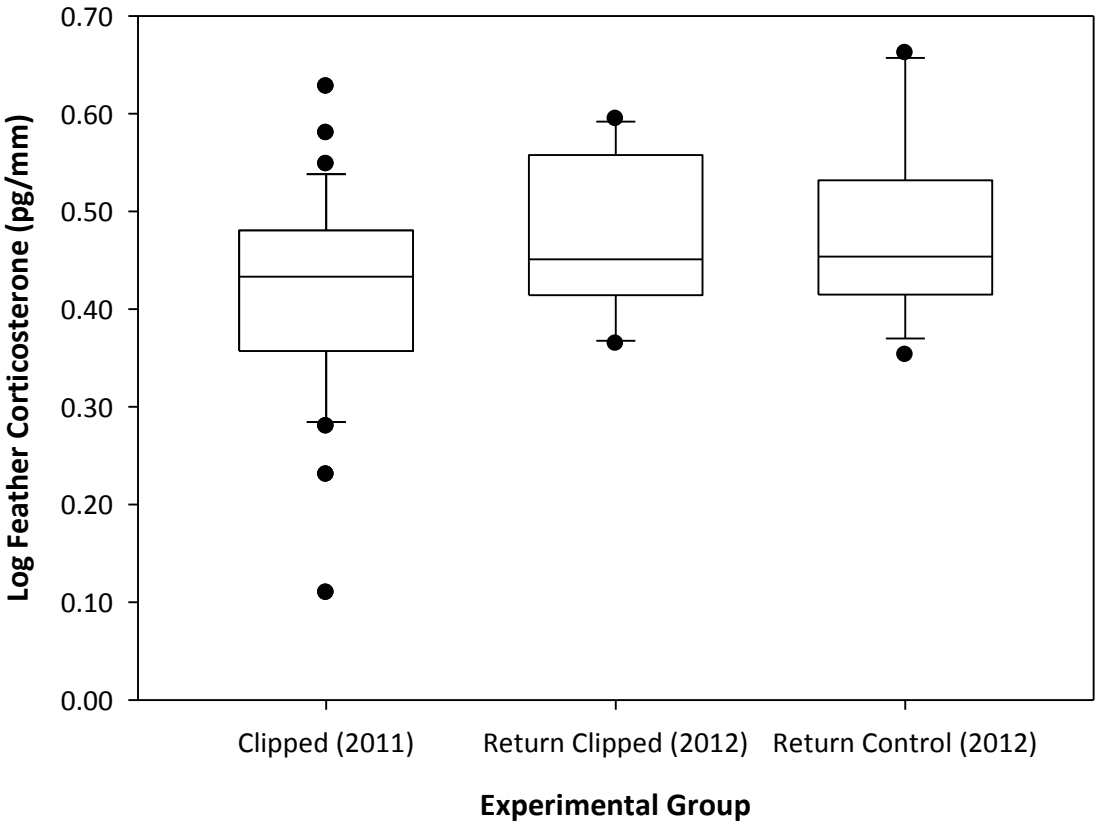

Figure 4.1 – Box plot showing feather corticosterone levels in birds before clipping (N=36) and after their return the following year (N=10), as well as levels in control birds (not manipulated) (N=14) after their return the following year.

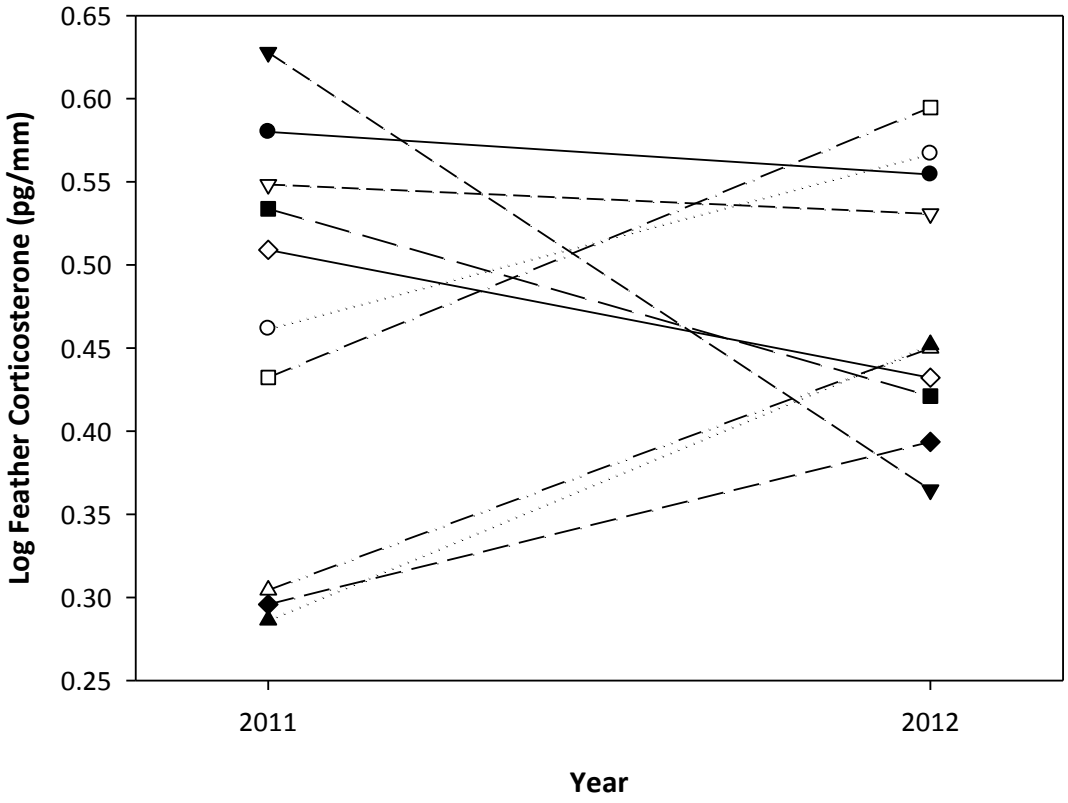

Figure 4.2 – Line graph displaying individual feather corticosterone response to the clipping manipulation (N=10). Levels in 2011 are hormone levels from clipped feathers while 2012 levels are those of feathers re-grown during moult following the manipulation.

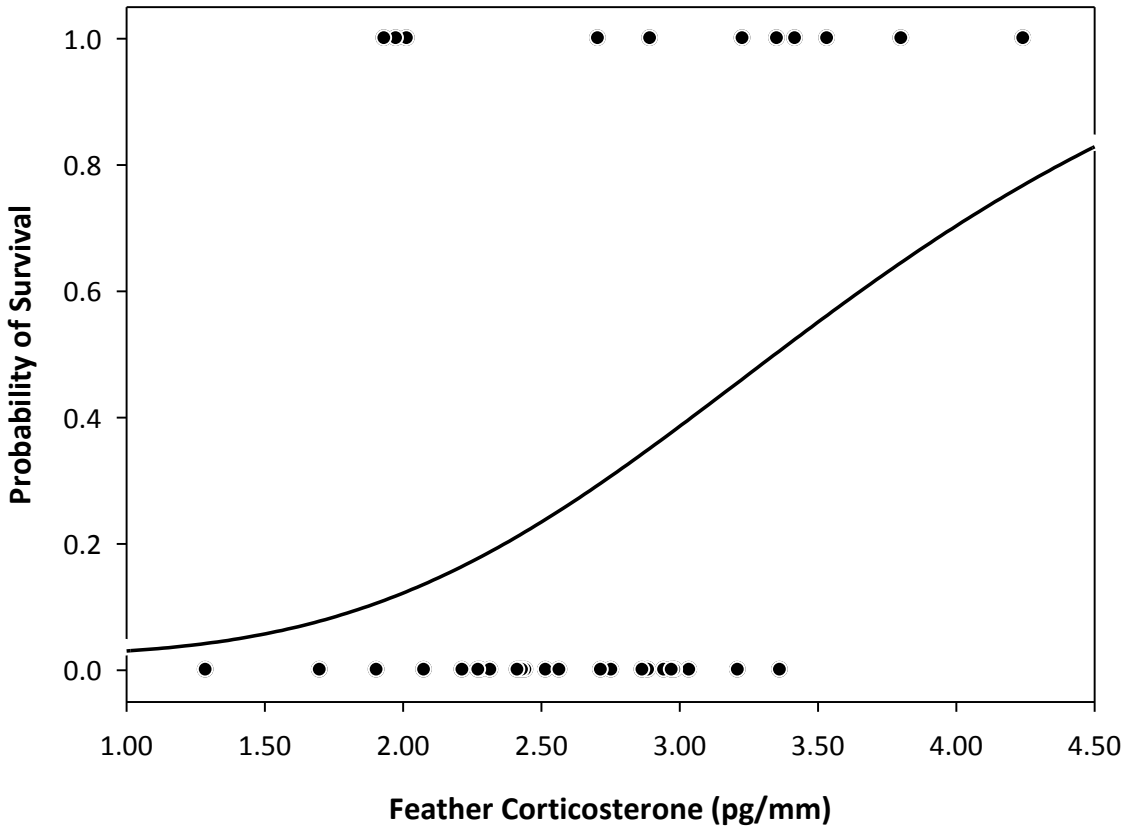

Figure 4.3 – Logistic fit of survival to future breeding seasons by current feather corticosterone level (N=36, DF=1, χ^2=4.7028, P=0.0301, e^{β}=4.084). The relationship shows probability of survival after clipping of primary flight feathers.

TABLES

Table 4.1 – Global model summary for general linear mixed-effects model between current year feather corticosterone levels and previous year measures of circulating corticosterone levels, reproductive effort, and timing (N=29).

Previous Year Variable	β	SE	F Ratio	P
Baseline Corticosterone Level At Provisioning	-0.0265	0.0730	0.13	0.72
Percent Change in Female Body Mass	0.0051	0.0058	0.75	0.39
Total Brood Mass	-0.0001	0.0010	0.01	0.91
Clutch Initiation Date	0.0115	0.0157	0.54	0.47

CHAPTER 5 – GENERAL DISCUSSION

SUMMARY OF FINDINGS

Taken together, we found that feather corticosterone (CORT) levels did not appear to accurately reflect the stress status of wild tree swallows (*Tachycineta bicolor*) when feathers were collected long after moult. Our results indicate that the capacity to reflect circulating CORT levels depends on the amount of keratin available for deposition, but that even in relative or ranked terms, feather CORT levels are not very repeatable within an individual even in feathers grown during an overlapping period of moult. This result is perhaps less surprising given that our analysis of symmetrically grown, paired left and right feathers also showed intra-individual variation in all flight feathers was high enough that levels in the pairs were not comparable. This lack of agreement may have been the result of external changes in feather CORT level after completion of feather growth, as the results of our soaking and washing experiments show that feathers can incorporate externally available CORT into the feather, and that repeated washing and weathering of feathers during their normal use can deplete levels. This suggests that feather CORT levels should not be considered fixed throughout the life of the feather and that their ability to reflect stress status may diminish over time. Additionally, if the repeatability results are due to external changes to feather CORT, then this suggests that the external incorporation and depletion of CORT levels may be different in different feathers.

Another possible explanation for the lack of repeatability in feather CORT levels within an individual comes from our study of concentrations in both normal and manipulated wild tree swallows. We found that CORT levels in a primary feather from a normal adult female tree swallow did not reflect individual reproductive effort immediately preceding moult, nor did it predict future reproductive success or survival in the year when the feather was collected. Additionally, feather CORT levels did not change in response to a feather clipping manipulation which increased flight and foraging effort, despite the manipulation's effect on the state, circulating CORT, and therefore stress of the clipped female. While feather CORT levels were able to predict the survival of manipulated females, they did so counter-intuitively in that individuals with high

feather CORT had higher odds of survival. While these results may also be the result of a lack of stability in CORT levels under practical feather uses in the wild, they also suggest that, at least for some species, feather CORT may not be the straightforward record of glucocorticoid (GC) activity that it has been proposed to be.

Additionally, it is also possible that the high within-individual variation in feather CORT in comparison to that between individuals could explain the lack of ability for feather CORT to reflect past effort or predict future success in our study as results may be different if multiple feathers or mean levels of multiple feathers were used instead. However, without a better understanding of deposition and the causes of this high variation, it is currently impossible to say which feather or combination of feathers would give a more accurate record of circulating CORT phenotype. Also, given the importance of flight feathers, it is difficult to justify the collection of multiple feathers from a living, wild bird especially in sensitive species or situations where the loss of multiple flight feathers could be expected to confound results in the remainder of the study. Clipped feathers do not regrow until moulted, and while plucked feathers are usually replaced, wing feathers are anchored deeply and should not be plucked without anaesthetic and pain control and may cause the feather follicle to close (Gentle & Hunter, 1991; Katzner et al., 2012; Delnatte et al., 2014), all undesirable features in a "less invasive" alternative measure of stress. While tail feathers and less vital feathers can be plucked without causing significant harm, it is difficult to say when a body feather was moulted or detect if it has been replaced since moult, which may make it difficult to know the contexts necessary for the proper interpretation of CORT levels.

Finally, questions remain about the sensitivity of feather CORT as a biomarker under biologically-relevant GC secretion changes that are likely to occur with disturbance. Given the lack of response of feather CORT to the feather clipping manipulation, it appears that changes in feather CORT due to chronic stressors are not large enough to be detected. While feather CORT does respond when circulating CORT levels are modified by implant or injection during feather growth (Lattin et al., 2011; Fairhurst et al., 2013; Hõrak et al., 2013; Jenni-Eiermann et al., 2015), the lack of response to applied stressors is problematic (Bortolotti et al., 2009a; Fairhurst et al.,

2011; Hõrak et al., 2013; Gow & Wiebe, 2014a; Lattin et al., 2014; Cruz-Martinez et al., 2015; Patterson et al., 2015). Further, though feather CORT predicted female survival of the clipping manipulation, it did so counter to the current understanding of GC function and the expected pattern of feather CORT in that females with higher feather CORT had higher probability of survival.

PRIORITIES FOR FUTURE STUDY

Due to the unknown mechanism of deposition, questions remain about the nature and state of CORT in feathers. Measuring hormone levels using a variety of assay methods and antibodies has provided divergent results (Lattin et al., 2011; Harris unpublished data), and hormone levels are much lower than those found in blood, leading to questions of whether the hormone in feathers is not only free CORT but a more complex mixture including metabolites (Lattin et al., 2011). If so, it is possible that different detection methods are measuring different components of the GC levels in feathers and as such important portions of the measure of stress may be omitted by some detection methods. Indeed, analysis of human hair samples using high performance liquid chromatography-ion spray mass spectrometry found 10 different corticosteroids (Cirimele et al., 2000), and a study in house sparrows (*Passer domesticus*) unexpectedly detected cortisol in addition to corticosterone (Koren et al., 2012). Also, a better understanding of the types of GCs found in feathers would assist in determining all of the sources of GC exposure and their relative importance, and could lead to new applications of the technique.

The minute levels of hormone found in feathers raises questions of sensitivity and the ability for feather CORT to reflect small differences in circulating CORT between individuals. This is especially true in the face of all of the possible sources of variation in the growth and use of the feather by the bird, and the preparation and hormone extraction by the researcher. This variation could mask important differences and make them impossible to detect in applications of this method unless the effect of these sources can be better taken into account. While these contexts are difficult to know when studying feather CORT because the long period of growth increases the time over which context

matters, the interpretation of all GC measures requires considerable information and context if the goal is to relate the trait back to fitness (Madliger & Love, 2014).

Further assessment of the stability of CORT in feathers under any expected environmental conditions in a variety of species is necessary to establishing the permanence of the stress signal. While other studies have shown that the shipment and long-term storage of feathers does not alter feather CORT (Bortolotti et al., 2009b) and we have shown that feather CORT levels in tree swallow wing feathers can be depleted by a combined 25 minutes of vigorous water exposure, the effect and importance of long-term real-world exposure to water, abrasives, sunlight, extreme temperatures, and feather mites on feather CORT levels is unknown. Additionally, these effects may interact with feather age due to wear or with seasonal changes in preen oil or skin secretions (Bhattacharyya & Chowdhury, 1995; Soini et al., 2006). For example, the effects of wear may be especially important in species such as European starlings (*Sturnus vulgaris*) and snow buntings (*Plectrophenax nivalis*) which undergo seasonal plumage changes due to the abrasion of pigmented feather tips rather than moult (Howell, 2010). However, the effect of feather age will be difficult to test experimentally in a rigorous manner due to differences in exposure between wild individuals. Also, the high within-individual variation in feather CORT levels shown here suggests selecting an unworn or unexposed control feather to compare treatment feathers against may be difficult.

Finally, feather growth rate and faults can themselves be used as a measure of stress (Grubb, 2006) and the impact of feather quality on feather CORT levels is unclear, especially given our results that more dense feathers hold more CORT. As both growth rate and feather quality can be expected to differ between stressed and unstressed individuals (Bortolotti et al. 2002) and high circulating CORT levels during feather growth can result in reductions in feather quality (Romero et al., 2005; Strochlic & Romero, 2008), it is difficult to determine if lower quality feathers caused by higher circulating CORT levels should be expected to possess high CORT levels due to the high amounts of CORT available for deposition (Bortolotti et al., 2009b) or if the total amount of CORT able to be held should be reduced by the reduction in feather density (Patterson, 2015). This relationship is further complicated by the findings that CORT-induced

structural changes occur lower in the forming feather while CORT deposition occurs higher (Jenni-Eiermann et al., 2015). An understanding of this apparent trade-off is important as it will occur to some extent in all feathers. However, it may be difficult to investigate due to the unsolved mass-dependency of the extraction, as feather sections containing faults would inherently have lower mass and therefore provide more CORT per weight extracted, masking the differences.

THE WAY FORWARD

Clearly, a better model of deposition is needed for feather CORT to be considered useful as a biomarker of stress in the wild. To develop one, a greater understanding of the state of the hormone, where it resides within the feather, and how it got there is required. This line of inquiry may also lead to interesting future work on the role of skin and sebaceous secretions in birds. Because of uncertainty in what measures of GC activity feather CORT correlates with, it is unclear whether feather CORT levels reflect the total or average CORT exposure over growth, if hormone is only deposited when CORT reaches or exceeds a threshold value, or if deposition occurs at higher rates at higher concentrations. In addition, different mechanisms of deposition may be more or less important at different concentrations and changes in levels after growth due to external depletion and deposition may overvalue more recent levels, especially elevated ones.

It is therefore necessary to begin considering the broader literature surrounding the study of feathers and investigating what insights this can provide into how this unique sample media may affect measured GCs. Findings from studies of feather growth, structure, coloration, and function have the potential to have an important impact on the interpretation of hormone levels. There is also a significant potential to gain from a careful comparison of the similarities between the measurement of GCs in feathers and other keratinized structures. As a result, considering the technique of measuring feather CORT as simply one part of a broader effort to measure GCs in keratinized structures greatly increases the field of available information and will allow the tool to develop much faster since our understanding of what it represents and the limitations of its use, though by no means complete, is much more advanced. Simply replicating or

investigating some of the key findings of hair cortisol studies to compare and contrast the results has the potential to elevate the use of integument GCs in a shorter amount of time.

In contrast to the results in naturally-grown feathers collected long after moult, feather CORT levels of replacement feathers plucked just before the period of interest and collected immediately after regrowth seem to generally behave more predictably (Bortolotti et al., 2008; Lattin et al., 2011; Gow & Wiebe, 2014b; Jenni-Eiermann et al., 2015).The deposition of CORT in naturally-grown feathers in wild birds may differ significantly from those of replacement feathers as moult is a complicated life history stage with a number of physiological regenerative activities (Murphy, 1996), suppressed GC activity (Romero, 2002), and altered behaviors due to reduced flight performance (Cyr et al., 2008; Cornelius et al., 2011). Replacement feathers are simpler to interpret, as details such as growth timing and rate are observed, environmental exposure is limited to a short period of use, and the effects of the life history stage of interest can be studied without the confounds of moult. However, future studies using feathers grown during target times should also measure the plucked feather and other original feathers that remained on the bird throughout this target time. These values can then be compared to allow the internal and external components of feather CORT and the effect of weathering to be evaluated. This will additionally provide important guidance in the correct sample preparation steps (e.g. washes) and their effect on interpretations. While limiting the use of feather CORT to replacement and nestling feathers decreases the total applications of the technique, the CORT concentrations in naturally-grown feathers collected long after moult from wild birds should not be viewed as a long-term integrative measure of GC activity and their use as a biomarker of stress should be cautioned.

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