USING FEATHER CORTICOSTERONE TO ASSESS THE EFFECTS OF NON-BREEDING SEASON CONDITIONS ON BREEDING OF ATLANTIC PUFFINS (*FRATERCULA ARCTICA*) AND RHINOCEROS AUKLETS (*CERORHINICA MONOCERATA*)

by

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ABSTRACT

In order to fully understand factors that affect animals during distinct parts of their annual cycle, it is important to consider that processes acting in one season may carry over to influence an individual's success in the following season. Measuring conditions over multiple seasons and life history stages allows carry over effects to be identified and places an individual's current condition into a broader context. Corticosterone levels measured in blood reflect hypothalmic-pituitary-adrenal (HPA) activity in birds in response to challenges that threaten homeostasis. Circulating corticosterone is integrated into growing feathers and can provide physiological information about birds during times when they are unavailable for blood sampling. Here, we used a commercially-available enzyme immunoassay kit to measure corticosterone in alcid feathers, demonstrated the benefits of acetonitrile/hexane purification of samples, and showed that blood and feather corticosterone are biologically-meaningful, albeit non-identical, measures. We used our enzyme-immunoassay and purification method in tandem with stable isotope analysis to measure corticosterone and stable isotopes in feathers and blood collected from rhinoceros auklets *Cerorhinca monocerata* nesting on three widely-dispersed colonies during years with different oceanographic conditions. We found that individuals from different colonies could be distinguished by their δ15N and δ13C stable isotope values

during, but not prior to the breeding season, and that corticosterone levels were consistent with this pattern. Furthermore, we found that rhinoceros auklets had significantly lower corticosterone levels in a year and on a colony assumed to have less favourable feeding conditions, which is opposite to results for other taxa. In a relative of the rhinoceros auklet, the Atlantic puffin *Fratercula arctica*, we found that egg mass increased in relation to female corticosterone and $\delta^{15}N$ values in feathers grown in the months prior to breeding, indicating that physiological state of females prior to the breeding season can influence egg mass. In contrast, we found that pre-breeding corticosterone and $\delta^{15}N$ values of rhinoceros auklet females were not correlated with egg mass or egg protein levels (pilot study). Overall our results support our hypotheses that corticosterone levels vary with environmental conditions and that differences in corticosterone levels during pre-breeding correlate with egg size in the subsequent season (breeding). However, our results also indicate that interpreting these relationships requires careful consideration of ecological and physiological characteristics of the individual or taxa in question.

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CHAPTER 1 – INTRODUCTION AND OVERVIEW

1.1 WHAT ARE THE FUNCTIONS OF GLUCOCORTICOID HORMONES?

Neuroendocrine mediators of 'stress', which include the hypothalamo-pituitary-adrenal (HPA) axis, glucocorticoid hormones, catecholamines (e.g., epinephrine) and their various hormone receptors (e.g., Carr & Summers 2002; Greenberg 2002; Greenberg et al. 2002), have been measured in a number of taxa in an attempt to understand how organisms respond to change. Glucocorticoid hormones, in particular, have been widely studied to explore how environmental variables influence organisms' behavioural decisions. In concert with catecholamines and central nervous system peptides, glucocorticoids coordinate complex physiological and behavioural responses to challenges (Wingfield and Romero, 2000). However, the extent to which organisms respond hormonally to environmental challenges differs among and within species. This has led to confusion regarding the function and role of glucocorticoids.

As outlined in a review by Landys et al. (2006), the functions of glucocorticoids include: (1) stimulating gluconeogenesis, (2) mobilizing amino acids, (3) breaking down fat stores, (4) helping to maintain water and salt balance, (5) stimulating immune response, and (5) regulating behaviours that control energy intake, such as locomotion, appetite and foraging. Hence, in moderate amounts, glucocorticoids are essential for energy metabolism and the maintenance of homeostasis. In contrast, prolonged exposure to high concentrations of glucocorticoid hormones may suppress immune function (e.g.,

French et al. 2006; Munck & Holbrook 1984) and reproduction (Wingfield & Romero 2000), reduce cognitive function (Kitaysky et al. 2003), affect development of young (Caldij et al. 2001), and can cause death of neurons (Uno et al. 1989; Sapolsky 1992) and individuals (e.g., Summers & Greenberg 1994). These negative effects of glucocorticoids have perpetuated the idea that all increases in glucocorticoid levels are pathological and maladaptive (i.e., reduce lifetime reproductive success; McEwen 2002). For over a decade, there have been significant efforts to redefine elevated glucocorticoid hormones as a beneficial response to changes experienced by organisms (see Wingfield et al. 1998; Wingfield & Ramenofsky 1997; Wingfield & Kitaysky 2002; Doody et al. 2008). A broader view of the role of glucocorticoids is well summarized by Bonier et al. (2009) as: (1) the “corticosterone-fitness hypothesis,” which states that individuals with higher glucocorticoid levels have lower fitness and/or are experiencing adverse environmental conditions, and (2) the “corticosterone-adaption hypothesis,” which states that individuals able to mount an increase in glucocorticoid levels, in order to meet metabolic demands, have greater fitness.

Wingfield and Kitaysky (2002) argue that organisms continually face internal and external changes and that some of these changes are predictable (e.g., seasonal cycles), while others are unpredictable (e.g., sudden changes in social status or food resources, disease, increased predator numbers). Short-lived, unpredictable events induce neuroendocrine processes, such as the release of glucocorticoid hormones, which alter the organism's physiology and

behaviour and cause it to adopt the Emergency Life History Stage (ELHS). In this case, “life history stage” is defined as one of several phenotypic stages that occur through an animals lifetime to optimize the animal's lifetime fitness (e.g., for birds, non-breeding stage, breeding stage, pre-basic moult stage; Jacobs & Wingfield 2000; Wingfield & Kitaysky 2002). As defined in a detailed review by Wingfield and Kitaysky (2002), the ELHS helps an organism avoid unhealthy situations, such as prolonged exposure to high glucocorticoid levels, by redirecting behaviour from a normal life history stage to engaging in behaviours like increased foraging and irruptive-type migration. As well, an ELHS may promote enhanced restfulness at night, elevated gluconeogenesis, and recovery once the perturbation passes (Wingfield & Kitaysky 2002).

Building upon the idea of ELHS (Wingfield & Kitaysky 2002) and other models that attempt to describe the causes and effects of elevations in glucocorticoids (such as the Allostatic Model; McEwen & Wingfield 2003a), Romero et al. (2009) developed the Reactive Scope Model to identify both beneficial and pathological fluctuations in glucocorticoid hormones. The Reactive Scope Model uses various “physiological mediators” as its measurable variables. Similarly to those defined by McEwen & Wingfield (2003a), these mediators may include actions and secretions of the HPA axis (glucocorticoids, etc.), cardiovascular changes, immune system mediators, central nervous system changes and behavioural actions. The Reactive Scope Model describes the levels of a given mediator at a given time using four homeostatic ranges: (1)

Predictive Homeostasis, which encompasses mediator levels that fall within predictable circadian and seasonal ranges, (2) Reactive Homeostasis, which encompasses mediator levels that allow the organism to cope with an unpredictable event (i.e., traditional stress response; emergency life history stage), (3) Homeostatic Failure, which refers to mediator levels that fall below the Predictive Homeostasis range and represents the point at which mediator levels are insufficient to sustain basic biological function, and (4) Homeostatic Overload, which refers to mediator levels that rise above the Reactive Homeostasis range and represents the point at which the mediator itself becomes pathological. In this case, the term “pathological” is defined as causing physical illness in or harm to an organism. Romero et al. (2009) assume that the threshold at which Homeostatic Failure occurs remains constant over time. In contrast, the threshold for Homeostatic Overload (i.e., the point at which a mediator itself causes illness) does not appear to follow any circadian or seasonal rhythms, and the level of this threshold is known to be unique for different taxa and individuals (Romero et. al. 2009), and is likely different for the same individual at different times. The mediator levels that fall between these two thresholds (for Homeostatic Overload and for Homeostatic Failure) are known collectively as an organism's “normal reactive scope”, the concept from which the Reactive Scope Model derives its name.

The Reactive Scope Model accounts for the costs, like energy consumption or lost opportunities, that result from maintaining mediators within

the Reactive Homeostasis range, and which may have implications for whether the mediator response being modelled improves fitness. Romero et al. (2009) describe these costs as “wear and tear.” They define wear and tear as the gradual reduction in coping ability that is experienced by organisms when their mediator levels remain in Reactive Homeostasis for a long period of time. The concept is represented graphically as a decrease in the threshold that mediator levels must reach in order to cause an organism to enter Homeostatic Overload. Homeostatic Overload can occur in two ways: (1) as a result of a reduction in the threshold required to enter Homeostatic Overload, or (2) as an increase in mediator levels. It is important to distinguish that wear and tear itself is not a pathological state, but instead, it represents an organism's increased vulnerability to entering into a state where mediators cause illness and potentially become maladaptive (i.e., Homeostatic Overload). Most often, once a challenge has ceased, the Homeostatic Overload threshold returns to its previous level. However, in extreme cases, prolonged elevation of mediators may cause neurons to die (Sapolsky 1992), which may result in a permanent reduction in the threshold of Homeostatic Overload.

The Reactive Scope Model provides an excellent framework for understanding why a change in mediator concentration for one organism may have different consequences for that organism as compared to a similar mediator level change in a conspecific. As well, by defining stress according to its mediators and introducing the concept of Homeostatic Overload, the Reactive

Scope Model is the first to provide a clear concept of exactly what it means for stress to be pathological. For example, using traditional views on stress, Moberg (2000) states that the prevention of ovulation caused by high glucocorticoid levels is an example of a pathological effect of stress. Evaluating this example using the Reactive Scope Model results in the alternative interpretation that this elevation of glucocorticoids is not pathological. Specifically, the elevated glucocorticoid level reflects a challenge faced by the individual, and it is this challenge that may cause a pathological or maladaptive situation for the individual, not the glucocorticoid response itself. This distinction is important because it allows for a broader exploration of the ultimate causes of stress. In this case, unless the glucocorticoids themselves are causing harm (e.g., killing neurons, causing immunosuppression, and excessive consumption of energy stores), we interpret that the individual is in Reactive Homeostasis and that the high glucocorticoid response may improve fitness because it causes the animal to avoid becoming pregnant during conditions that are not ideal for its own survival or that of its offspring.

Another example is the finding that nesting adult common murres *Uria aalge* had higher baseline glucocorticoid concentrations in a year when the peak spawning time of capelin, their main prey species, did not coincide with chick hatching (Doody et al. 2008). In the late capelin year (“mismatch year”), breeding adults with higher glucocorticoid levels also showed above average rates of chick provisioning (Doody et al. 2008). Through the lens of the Reactive Scope

Model, it is most intuitive to infer that the increased challenge of finding food in the absence of capelin (e.g., longer foraging trips, lower prey quality), or perhaps increased begging from hungry chicks, caused an increase in glucocorticoid levels from the Predictive Homeostasis range to the Reactive Homeostasis range. This increase in glucocorticoids subsequently caused an increase in foraging effort, as implied by the increase in chick provisioning behaviour. Since the birds with higher glucocorticoid levels during the mismatch year also experienced lower mass loss than birds with lower glucocorticoid levels (Doody et al. 2008), the necessary increase in foraging effort likely fell within the high glucocorticoid birds' normal reactive scope. That is, the birds with higher glucocorticoid levels did not experience Homeostatic Overload because their elevated glucocorticoid levels did not cause pathologically high levels of fat or protein breakdown. In contrast, the greater mass loss found for the lower glucocorticoid birds suggests that the birds in this category were less able to cope with the wear and tear arising from low food availability. This may have lowered the threshold at which they began to use stored energy and may eventually have caused them to enter Homeostatic Overload.

As is evident from these examples, determining whether increases in stress responses are pathological requires consideration of many different factors, which illustrates the need for an integrated approach that considers the context in which glucocorticoid fluctuations occur. The term 'context' is used throughout this thesis to indicate the particular set of internal and external

circumstances that may affect an organism's ability or strategy to cope with challenges. Internal factors, which include age, stage of the annual cycle, body condition and health status (e.g., parasite load, injury), are considered in relation to species differences in life history. External factors include changes in weather, food availability and predation risk. One of the central aims of this thesis is to consider glucocorticoid fluctuations over a range of contexts, encompassing various seasonal activities, inter-seasonal transitions, environmental conditions, species, and time periods. As well, the experiments in this thesis consider glucocorticoid fluctuations on both individual and population levels.

1.2 HOW DO GLUCOCORTICOIDS AFFECT AVIAN REPRODUCTION?

Corticosterone (CORT), the main glucocorticoid hormone in birds, plays a significant role in regulating circulating glucose levels, and is important for the maintenance of energy homeostasis during predictable seasonal activities (McEwan & Wingfield 2003), such as reproduction (Wingfield & Sapolsky 2003; Goutte et al. 2010), and unpredictable events, such as extreme weather (Wingfield & Kitaysky 2002) and variation in food availability (Kitaysky et al. 1999, 2007). Specifically, changes in CORT levels are thought to help birds mediate trade-offs between reproduction and survival by influencing factors such as foraging behaviour (Astheimer et al.1992), chick provisioning (Doody et al. 2008; Bonier et al. 2011), and reproductive investment decisions (Love et al.

2004; Bokony et al. 2009; Bonier et al. 2011). Although countless studies have found correlations between CORT fluctuations and various measures of reproduction and survival, a recent review by Crespi et al. (2013) highlights that the direction/nature of these relationships is often inconsistent among studies. Particularly, Crespi et al. (2013) conclude that the effects of CORT fluctuations on resource allocation trade-offs and expression of life-history traits depend heavily on the ecological and evolutionary context in which they occur. Therefore, these authors call for studies that measure the effects of CORT on multiple life-history traits over a range of environmental conditions over time.

1.2.1 Carry-over Effects

The inconsistency in the relationships found between CORT and reproduction/survival may, at least in part, be due to the fact that most studies encompass only a small part of a bird's annual cycle, often only the breeding season. The study of carry-over effects is an emerging field that attempts to consider more of the annual cycle by examining how processes acting in one season can influence an individual's success in the following season (Harrison et al. 2011). For example, timing of arrival on the breeding colony and breeding success have been linked to the quality of wintering habitat prior to breeding (Norris et al. 2004), and pre-breeding body condition has been linked reproductive success in the subsequent breeding season (Bety et al. 2003).

As it is often challenging to quantify physiological state of migratory

individuals outside of the breeding season, the recent development of techniques that allow CORT to be measured in tissues grown outside the breeding season have led to exciting advances in the study of carry-over effects. Particularly, in a study on northern common eiders *Somateria mollissima borealis*, Harms et al. (2015) found that CORT levels measured just after breeding (in feathers, see Section 1.3) were related to colony arrival date and body condition in the subsequent breeding season, and were negatively related to reproductive success and likelihood to survive a cholera outbreak. In contrast, Bourgeon et al. (2014) found that non-breeding season CORT levels of great skuas *Stercorarius skua* were not related to breeding traits in the subsequent breeding season. These results illustrate that measuring CORT outside the breeding season may help to identify the potential for carry-over effects and provide new insight into the relationship between CORT and avian reproduction. Hence, identifying and interpreting carry-over effects is a central aim of this thesis.

1.3 MEASURING CORT IN FEATHERS: A RAPIDLY DEVELOPING TOOL FOR CORT RESEARCH

Measuring CORT over a range of time and/or conditions is challenging due to the practical constraints of blood sampling, the most common procedure for measuring both baseline and stress-induced CORT levels. Baseline levels

are defined as circulating CORT levels during predictable events (McEwan & Wingfield 2003), while stress-induced levels are defined as circulating CORT levels during unpredictable challenges (real or perceived; Wingfield et al. 1998). Each blood sample requires a bird to be caught and have blood drawn manually with a needle and syringe/capillary tube, which is largely unfeasible during some parts of the seasonal cycle, such as during offshore non-breeding periods or migration. To measure baseline CORT levels, blood samples often need to be taken within three minutes of contact (Romero & Reed 2005), which is not always possible for all birds in a sample (e.g., birds in deep burrows that require multiple attempts to extract). Stress-induced CORT levels often require birds to be restrained for fifteen minutes or more, which requires a safe and supervised area for birds to be restrained. A further complication is that each blood sample represents a 'snapshot' of a bird's circulating CORT levels at the time of sampling, which means that multiple samples are needed to represent CORT over an extended period.

Due to its practical limitations, blood sampling is ill-suited for measuring CORT fluctuations over long periods and/or over the course of an animal's annual cycle. Therefore, researchers interested in CORT (and other glucocorticoids) over inter-seasonal periods, or during times when animals are inaccessible, have turned to measuring CORT in other tissues, such as hair (Koren et al. 2002, Pereg et al. 2012), blubber (Mansour et al. 2002) and feathers (Bortolloti et al. 2008, 2009a, 2009b; Koren et al. 2012; Lattin et al.

2011; Fairhurst et al. 2013a, 2013b). In birds, CORT is incorporated into growing feathers such that feather CORT levels are correlated with CORT circulating in blood during feather growth (Bortolloti et al. 2008, 2009a). Hence, CORT levels measured in feathers have the potential to represent an integrated value of CORT levels experienced by the bird over the time that the feather (or feather segment) was grown (Bortolloti et al. 2008, 2009a). For seabirds that moult and grow new feathers during periods when they are inaccessible for blood sampling (during their offshore non-breeding season), feather CORT provides a tool to examine the role of CORT during previously unstudied parts of the life-cycle, and to identify potential carry-over effects.

Since 2008, when the first feather CORT study was published by Bortolotti et al. there have been a number of articles published that either test feather CORT methods and/or relate feather CORT levels to life-history traits. These studies have found correlations between feather CORT and body condition of chicks (Harms et al. 2010), microclimate provided by nest-boxes (Fairhurst et al. 2012a), environmental enrichment (Fairhurst et al. 2011), survival of adult birds (Koren et al. 2012), current and subsequent breeding success (Crossin et al. 2013), carbon stable isotopes (Fairhurst et al. 2013b), the degree to which testosterone and parasites influence sexual ornamentation (Bortolotti et al. 2009b), carotenoid-based colouration of adult feathers (Kennedy et al. 2013; Lendvai et al. 2013) and carotenoid-based colouration of chick integument (legs and cere; Martinez-Padilla et al. 2013). Bortolotti et al. (2009b), Lattin et al.

(2011), Koren et al. (2012) and Fairhurst et al. (2013a) have reported results that help to validate feather CORT techniques and identify inconsistencies among results, which highlight gaps in the current understanding of how CORT is deposited in feathers and how feather CORT is related to circulating CORT levels.

Bortolotti et al. (2008) found that CORT levels in feathers were correlated with stress-induced (after 60min of restraint), but not baseline, plasma CORT levels in red-legged partridges *Alectoris rufus*. The authors argue that the lack of correlation between baseline plasma and feather CORT levels is not unexpected because baseline CORT fluctuations are likely to be over-shadowed by stress-induced CORT fluctuations as CORT is deposited in feathers. Furthermore, they argue that the correlation between stress-induced plasma CORT and feather CORT may not be clear in studies of wild birds that are experiencing a range of challenges. Instead, they conclude that feather CORT represents an integrated measure of hypothalmic-pituitary-adrenal activity that incorporates both the amplitude and duration of CORT secretion. In a follow up study, Bortolotti et al. (2009a) further support this conclusion by demonstrating that CORT in feathers is stable over time, that CORT is deposited in a time-dependant (pg/mm) way, and that punctuated increases in CORT are reflected in feather sections and are related to fault bars in feathers.

Lattin et al. (2011) further tested the link between feather and plasma CORT by artificially increasing CORT levels via implants. They found that

increased plasma CORT was clearly reflected in overall feather CORT levels, but that CORT in different sections of feathers did not always correspond with plasma CORT level at the time of section growth. Lattin et al. (2011) concede that this discrepancy may be the result of abnormally high levels of CORT produced by implants, but they suggest that their result may indicate deposition of CORT via an external source, such as preen oil. However, this explanation is unlikely because, similarly to Bortolotti et al. (2008), Lattin et al. (2011) were unable to detect CORT in preen oil. Lattin et al. (2011) do, however, make a convincing argument that CORT concentration (pg CORT/mg feather) decreases for increasing feather mass (mg), especially for samples with small mass. To account for this potentially confounding relationship, they recommend avoiding samples with mass under 20mg, comparing CORT using feathers of similar mass, or including mass as a covariate in analysis. Overall, Lattin et al. (2011) call for more research into how CORT is deposited into feathers, and suggest that measuring CORT in feather sections, as opposed to whole feathers, may be problematic.

Horak et al. (2013) and Fairhurst et al. (2013a) have attempted to clarify the relationship between circulating CORT and feather CORT levels by artificially enhancing (with corticosterone-releasing implants or injection to elicit an immune response) and/or inhibiting (dexamethasone-releasing implants or injection) CORT secretion. Horak et al. (2013) found that feathers of adult greenfinches *Carduelis chloris* grown after injection with dexamethasone had lower CORT, as

predicted. However, contrary to predictions, feather CORT did not correlate with two haematological indices of stress that reflect immune response. In explanation, Horak et al. (2013) suggest that immune response may not have been severe enough to increase feather CORT levels, and they highlight that there are also several studies of plasma CORT in which a correlation between CORT and immune response is not evident. Fairhurst et al. (2013a) found a positive relationship between plasma CORT levels and feather CORT levels of tree swallow *Tachycineta bicolor* nestlings, but only when plasma CORT levels were at their highest and most variable between treatments (CORT, CORT + Dexamethasone, Dexamethasone, Control, Handled-Only). However, feather CORT levels of the same nestlings were negatively correlated with nestling size and whether or not the nestling fledged. Since plasma CORT represents a 'snapshot' of CORT at the time of sampling, while feather CORT represents the summation of these snapshots over days to weeks, Fairhurst et al. (2013a) conclude that only snapshots of plasma CORT elevations that are particularly high or occur over longer periods will be evident in the total CORT value measured in feathers. Therefore, it is unlikely that a single plasma CORT sample will correlate with the total CORT value measured in a feather, unless the plasma CORT elevation is significant in amplitude and duration, such as was found for tree swallow nestlings (Fairhurst et al. 2013a). Hence, the authors suggest that feather CORT and plasma CORT should be recognized as different values.

As the field of feather CORT continues to evolve, many researchers echo

the opinion that more research is needed to support the hypothesis that CORT is passively integrated into feathers as they are grown. Specifically, more information is needed about the mechanisms of CORT deposition, so that researchers can avoid or quantify factors that may interfere with this process. This point is illustrated by recent findings suggest that nutritional stress may affect CORT deposition in feathers of some species (Patterson et al. 2014), but not others (Will et al. 2014).

1.4 WHAT DO WE KNOW ABOUT FEATHER CORT, ENVIRONMENTAL CONDITIONS AND LIFE HISTORY, AND WHAT STILL NEEDS TO BE ADDRESSED?

To date, research on feather CORT has established that, despite the need for further research into the mechanisms of CORT deposition in feathers, feather CORT can provide important information regarding HPA activity over a longer time frame than plasma CORT. Results have also shown that it is a promising tool for answering questions regarding avian reproduction and survival. For example, in the initial feather CORT study, Bortolotti et al. (2008) found a positive relationship between female feather CORT and clutch size in the previous year, and they found that feather CORT levels were lower while breeding than prior to breeding, which is consistent with previous findings for plasma CORT. More recent studies have measured feather CORT in a number of different species

and over a variety of contexts. Crossin et al. (2013) found that successfully breeding giant petrel females *Macronectes spp* had higher feather CORT levels (feathers grown while breeding) than unsuccessful breeders, and if successful breeders delayed moulting before winter migration, they were much more likely to defer breeding the subsequent year. Additionally, negative relationships have been found between feather CORT (feathers grown while breeding) and body condition of female tree swallow nestlings (Harms et al. 2010) and between feather CORT (feathers grown outside the breeding season) and subsequent survival in adult house sparrows *Passer domesticus* (Koren et al. 2012). Feather CORT has also shown negative relationships with brightness (Kennedy et al. 2013) and hue (Lendvai et al. 2013; Martinez-Padilla et al. 2013) of carotenoid-based plumage and integument ornaments. The above studies suggest that measuring feather CORT during and prior to the breeding season represents a promising avenue for gaining a deeper understanding of avian reproduction and survival. However, variable results of previous studies indicate that feather CORT values must be interpreted cautiously.

Another area in which measuring feather CORT shows potential is in helping to quantify the effects of environmental conditions on populations of birds. As is evident from the studies cited in previous paragraphs, the majority of feather CORT studies to date examine CORT relationships on an individual level rather than on a population level. However, Legagneux et al. (2013) found that feather CORT levels of common eiders *Somateria mollissima* varied with annual

differences in environmental temperature, rather than with differences in individual quality. As well, Satterthwaite et al. (2012) found that black-legged kittiwakes *Rissa tridactyla* from two distinct regions showed opposite relationships between CORT levels and environmental metrics that indicate warming conditions. The results of these studies suggest that differences in environmental conditions experienced by avian populations can be reflected by variation in feather CORT levels.

Although identifying relationships between feather CORT and various measures of quality and/or environment at the individual and/or population level is generally straightforward, interpreting these relationships can be challenging. As mentioned in previous sections, inconsistency in CORT results among studies indicates that CORT relationships are heavily context-based, and can be influenced by a host of interacting factors. Emerging evidence suggests that some of these factors occur outside of the breeding season (the period when CORT levels are traditionally measured in many birds). Therefore, measuring CORT in feathers grown during the non-breeding season or on migration may provide insight on carry-over effects and potentially explain contradicting results. As well, considering that CORT relationships may be interpreted differently depending on whether they are viewed at an individual level or population level may also help to disentangle seemingly contradictory effects. For example, for a given individual, CORT may be positively or negatively related to environmental conditions (or show no relationship) depending on the severity of the

environmental conditions and the physiological condition of the individual. An individual in good condition (i.e., within Normal Homeostatic Range; Romero et al. 2009) may have a beneficial increase in CORT levels which allows it to get away from or cope with very poor environmental conditions, while an individual in poor condition (Homeostatic Overload; Romero et al. 2009) may have a pathological increase in CORT levels in response to poor environmental conditions. On a population level, these two individuals would be counted together within an overall positive relationship between CORT and environmental conditions, despite the fact that their elevated CORT levels indicate two opposite biological responses.

1.4.1 Stable Isotope Analysis and CORT

One approach to quantifying the spatial and dietary circumstances of birds (and other animals) is the use of stable isotope analysis (SIA). In this technique, relative abundance of isotopes (e.g., $^{15}N/^{14}N$) are measured and the ratio is compared to the ratio of $^{15}N/^{14}N$ isotopes in a standard substance. Stable isotope values are then presented in delta notation (δ) as parts per thousand ($^{0}/_{00}$) using the equation:

$$\delta^{15}N = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where R is the ratio of $^{15}N/^{14}N$ and $R_{standard}$ for ^{15}N is atmospheric N_2 (AIR).
In avian studies, the most common isotopes are stable nitrogen isotopes (δ15N),

which indicate the relative trophic level at which a bird has been feeding, and stable carbon isotopes (δ13C), which characterize its foraging habitat. δ15N does not give the absolute abundance of each prey species in a diet, but rather provides a measure of relative contribution of prey types of different trophic levels. δ13C values indicate the relative contribution of C_3 (most plants) and C_4 (crops, such as corn) plants in diets of terrestrial animals, and the relative contribution of offshore and inshore prey in diets of animals in some marine environments (Cherel and Hobson, 2007).

Although there have been studies that measure both CORT and SIA in blood of the same individuals (e.g., Barger & Kitaysky 2011; Beaulieu et al. 2010), very few studies incorporate analysis of both CORT and SIA in feathers (Fairhurst et al. 2013b). The combination of these techniques has the potential to provide valuable information about how birds respond physiologically to local differences in habitat and food sources during periods when birds are unavailable for blood sampling or observation. Specifically, measuring stable isotopes in a bird's feather provides a measure of local environment for that individual and measuring CORT in a feather from the same bird provides insight into the individual's physiological response to that local environment. For example, in the Dupont's lark *Chersophilus duponti*, feather CORT levels were negatively correlated feather carbon stable isotope values, which varied with the degree of agricultural land use in the lark habitat (Fairhurst et al. 2013b). As well, in Leach's Storm Petrels *Oceanodroma leucorhoa*, feather CORT levels were

negatively correlated with feather nitrogen stable isotope values, indicating that birds feeding on higher trophic level prey had lower CORT levels (Fairhurst et al. 2015).

Measuring stable isotopes in feathers also has the potential to provide context when testing relationships between CORT and other variables. Specifically, Bourgeon et al. (2014) used feather stable isotope values to identify wintering grounds of great female skuas in order to help test their hypothesis that winter (feather) CORT levels carry-over to affect breeding phenology. Due to the complementary nature of the information provided by stable isotope and CORT values, both SIA and CORT analysis are used in most of the experiments in this thesis.

1.4.2 Feather CORT Techniques

In addition to differences in interpretation of CORT results, there is also variation in the techniques for measuring feather CORT. To date, the majority of studies have measured feather CORT using a radioimmunoassay (RIA), which has been validated and tested by Bortolotti et al. (2008, 2009a), Lattin et al. (2011) and Fairhurst et al. (2013a). Koren et al. (2012) also validated liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for measuring CORT in feathers, but thus far, this method has not been widely used. Both of these methods have advantages and disadvantages. Unlike RIA, the LC-MS/MS method allows for multiple steroid hormones to be quantified

simultaneously and avoids the issue of antibody cross-reactivity (Koren et al. 2012). Furthermore, using their LC-MS/MS method, Koren et al. (2012) identified the presence of a significant amount of cortisol in feathers, in addition to corticosterone. This surprising result suggests that measuring multiple related hormones simultaneously may be important to fully understand the dynamics of CORT in feathers. When measuring CORT with RIA, a degree of antibody cross-reactivity is expected (e.g., some molecules with similar structures may be incorrectly identified as the target molecule; Ismail et al. 2002), but cross-reactivity is quantified and minimized to generally accepted levels. The absence of cross-reactivity in the LC-MS/MS is naturally superior to any degree of cross-reactivity in RIA. Arguably, RIA requires less specialized equipment and lower levels of technical expertise to carry out. For this reason, and because it has been widely used for plasma CORT research for many years, RIA remains the most commonly used method for feather CORT analysis.

Enzyme-immunoassay (EIA) is a competitive-binding assay and works in a similar manner to RIA, except that it uses enzymes to identify CORT molecules, rather than radioisotopes. EIA has been extensively used to measure plasma CORT, but to date, EIA has not been validated for measuring CORT in feathers. A significant practical disadvantage of RIA is that it requires the use of radioactive materials, which require extra permits to obtain and handle, and the disposal of which can be difficult (Sheriff et al. 2011). As well, it requires equipment used solely for the RIA (gamma counter). In contrast, EIA uses a

more versatile piece of equipment, a microplate reader (Sherrif et al. 2011) and avoids the hazards and inconveniences of working with radioactivity.

1.5 RESEARCH APPROACH

The planning, fieldwork, lab work, analysis, and conclusions that form the content of this thesis have spanned the time from the first feather CORT article in 2008 to the present day. Therefore, this document has developed and evolved in tandem with the field of feather CORT research, which since 2008, has seen regular publication of pivotal information about feather CORT measurement and/or interpretation. Following the field of feather CORT from its natal stages has been a twisting journey that has led over mountains of fabulous excitement and through valleys of crushing frustration. Since this journey is still on-going and the field of feather CORT research is still developing, the final version of my thesis includes both research regarding a method for measuring CORT in feathers, and research that applies this method to questions in physiological and behavioural ecology.

1.5.1 Study Species and Study Areas

The research in this thesis focuses on two closely-related alcid seabird species of the subfamily Fraterculini: the diurnal Atlantic puffin *Fratercula arctica* and the nocturnal, Pacific Ocean dwelling rhinoceros auklet *Cerorhinca*

monocerata. Both are monogamous, bi-parental seabirds that spend the majority of their lives at sea, coming to land only for a few months each year to lay eggs and raise nestlings. Both species nest in island colonies, returning to the same island each year to raise a single chick. Both species dive for small fish prey to feed themselves and their young, but will feed on a wide variety of other prey when fish are scarce. Research on Atlantic puffins was carried out on Gull Island in the Witless Bay Ecological Reserve, Newfoundland (47°16′N, 52°46′W; Figure 1.1). Research on rhinoceros auklets was carried out on: Triangle Island in the Anne Vallee Ecological Reserve (50°52′N, 129°05′W), Lucy Islands (54°18'N, 130°37'W), Pine Island (50°58′N, 127°41′W) and S'Gang Gwaay (52°05′N, 131°13′W), all of which are off the coast of British Columbia (Figure 1.2).

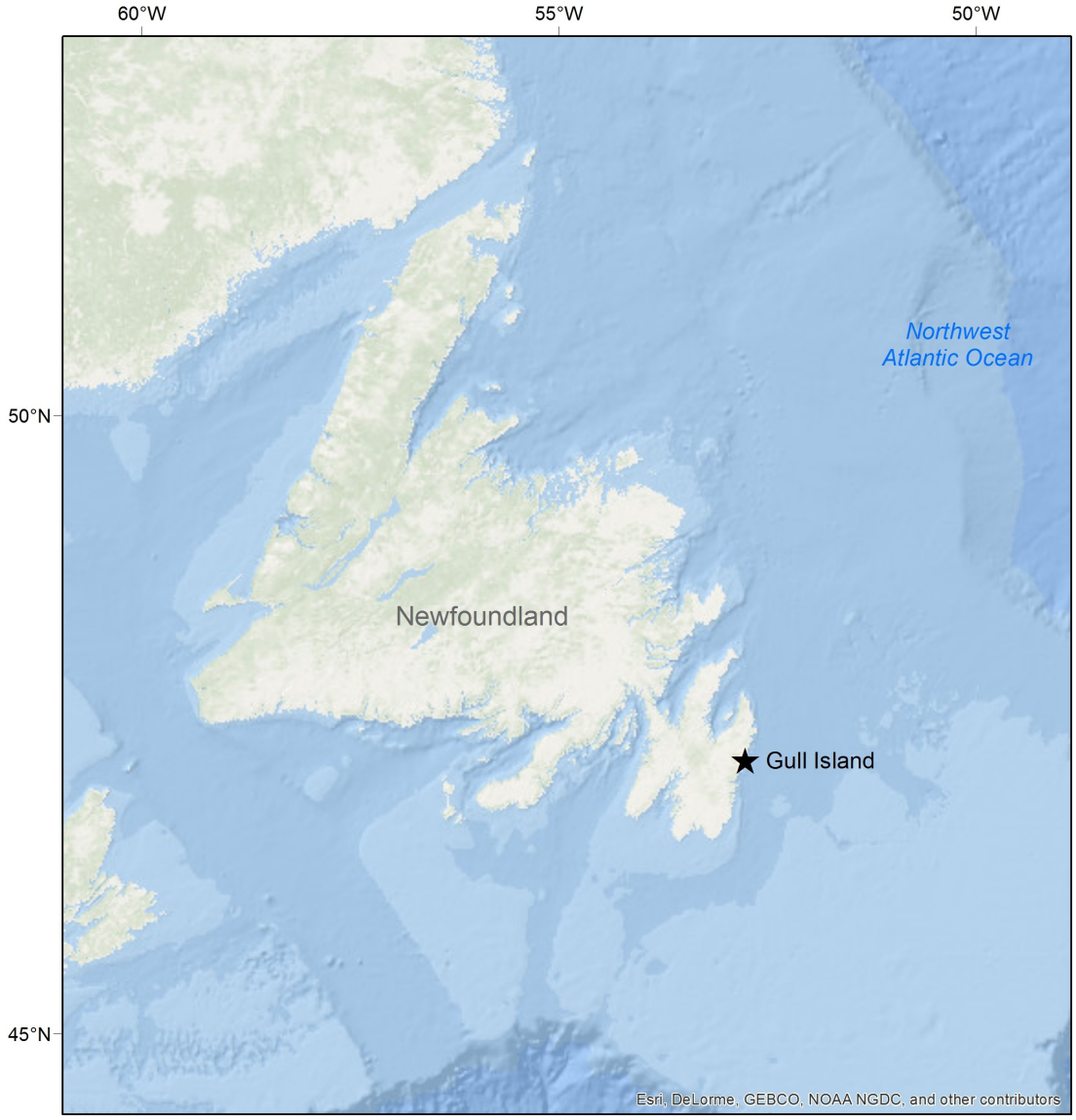

Figure 1.1. Location of Atlantic puffin breeding colony (Gull Island, Witless Bay Ecological Reserve, Newfoundland, Canada)

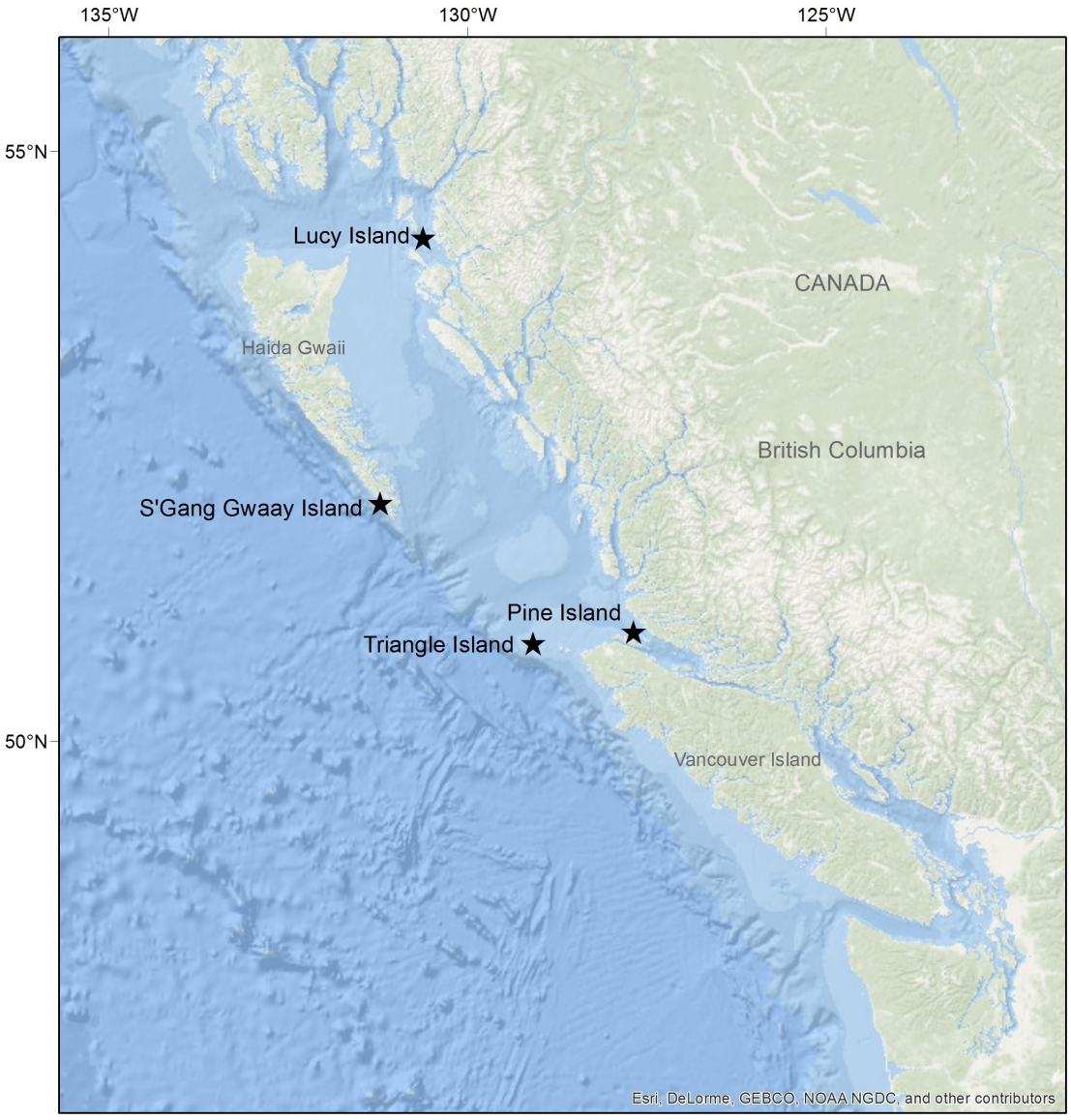

Figure 1.2. Location of rhinoceros auklet breeding colonies (Lucy, Pine, S'Gang Gwaay and Triangle Islands, British Columbia, Canada)

1.6 AIMS AND ORGANIZATION OF THESIS

The aim of the research presented in this thesis is to assess the following hypotheses:

1. Enzyme-immunoassay is a valid way to measure corticosterone in alcid feathers.
2. Corticosterone levels experienced by alcid populations while breeding (measured in blood) and prior to breeding (measured in feathers) reflect environmental conditions.
3. Corticosterone levels experienced by alcids prior to breeding (measured in feathers) explain variation in egg mass during the subsequent breeding season.

This thesis is organized into six chapters: Introduction (Chapter 1), four manuscript-style data chapters (Chapters 2-5) and Conclusion (Chapter 6). In accordance with the guidelines of Memorial University, data chapters are presented as self-contained manuscripts that are in the format in which they will be (Chapter 3, 5) or are (Chapter 2, 4) published. Further details about each data chapter are given in the following paragraphs.

Chapter 2 is devoted to assessing the first hypothesis listed above, the support of which is essential for exploration of the remaining two hypotheses (Kouwenberg et al. in press). In Chapter 2, I report the results of a series of

experiments (parallelism, recovery) that support the use of a Cayman Chemical Company EIA kit for measuring CORT in alcid feathers. Although I had previously used RIA kits to measure CORT in blood, upon consulting with several experienced CORT researchers, I chose to pursue EIA. My rationale for using EIA was that it eliminated the difficulties of working with radioisotopes, and that the EIA kit in question had higher sensitivity and lower cross-reactivity than RIA kits I had used previously. Using EIA proved to be a considerable undertaking; eventually leading to the adaptation of a purification step originally used for removing interfering components in blubber samples (Mansour et al. 2002). This step removed interfering components from my feather samples, allowing for accurate quantification of CORT levels by the EIA.

Chapter 3 outlines a population-level study that spans three-years and three rhinoceros auklet colonies in the north Pacific Ocean to test the hypothesis that CORT levels during and prior to breeding vary with environmental conditions. This study represents a significant amount of fieldwork to collect blood samples, feather samples and breeding data, and a significant amount of lab work to measure blood CORT, feather CORT, and carbon and nitrogen stable isotopes. The results of this chapter provide background information about the relationship between CORT and environmental conditions for the subsequent two chapters.

Chapter 4 is, to my knowledge, the first feather CORT study using EIA in the published literature (Kouwenberg et al. 2013). The research in this chapter

tests for a relationship between egg mass and non-breeding season CORT levels and non-breeding season stable isotope values in female Atlantic puffins. This study was initially intended to happen on two seabird colonies in the north Atlantic in the same year, but the presence of (and interaction with!) a polar bear at the second colony reduced the planned one-month field season to just one day (mostly spent sitting on the roof of the cabin with a gun). Therefore, this study was completed at only one colony.

In Chapter 5, I report a study similar to that in Chapter 4, but this study was carried out on rhinoceros auklets on Triangle Island, BC. In addition to testing the relationship between CORT prior to breeding and egg mass, this study also features a pilot experiment examining egg components in relation to feather CORT.

1.7 CO-AUTHORSHIP STATEMENT

This thesis is the result of a Ph.D. project in the Cognitive and Behavioural Ecology Programme at Memorial University of Newfoundland. Principle financial and logistical support was facilitated by Ph.D. supervisors, Anne. E. Storey (NSERC Discovery Grant) and Dr. J. Mark Hipfner (Environment Canada). Substantial logistical support for fieldwork was also provided by numerous field assistants, the Canadian Coast Guard, and Dr. Sabina I. Wilhelm and Dr. Gregory J. Robertson of Environment Canada. Laboratory bench space and

equipment were provided by Dr. Donald W. McKay, Dr. Jules J. E. Dore, Dr. Carolyn Walsh, Dr. William A. Montevecchi, and the Memorial University GaP Lab (Dr. Elizabeth A. Perry, Lidan Tao). Research questions for this thesis and plans for data collection were developed by A.-L. Kouwenberg in collaboration with AES and JMH. Feather CORT laboratory procedures were developed by ALK in collaboration with DWM, with advice from Tracy A. Marchant. Additional contributions to this thesis were made as follows:

i) design and identification of the research proposal:

ALK, AES, JMH, GJR, DWM

ii) practical aspects of the research:

Collection of field data (blood, feather, egg and prey samples, measurements of eggs and birds and observations) was primarily coordinated by JMH in British Columbia, and by ALK and AES in Newfoundland. ALK, with the assistance of AES, Michelle G. Fitzsimmons, Megan E. Rector and field assistants (see Acknowledgements), collected data on Gull Island. JMH, with the assistance of Glen Keddie, Moira Lemon and field assistants, collected data on Lucy, Pine and S'Gang Gwaay Islands. ALK, JMH, Kristin Charleton, Marjorie C. Sorensen and field assistants collected data on Triangle Island.

Tissue and blood samples were prepared for stable isotope analysis by ALK, with assistance from Morag Ryan, Kylie Goodyear and Meghan Donovan.

Genetic sexing of birds via blood samples was done by ALK and MGF, under the guidance of Dr. Elizabeth A. Perry and Dr. Edward S. Yaskowiak. Yolk protein analysis was done by Ms. Kathy Clow in the lab of Dr. John T. (Sean) Brosnan. Determination of CORT levels in blood via radioimmunoassay was primarily done by ALK, MER and MGF, with assistance from DWM, Morag G. Ryan and Lesley M. Doody. Determination of CORT levels via enzyme immunoassay in feathers was done by ALK, with technical assistance and expert advice from DWM.

iii) data analyses:

All statistical analyses were performed by ALK, with brilliant advice, mental-health support, and life-changing R code solutions from Paul M. Regular, Laura McFarlane-Tranquilla, Alejandro D. Buren, GJR, AES and JMH.

iv) manuscript preparation:

As the principal author, ALK wrote all text, interpreted all results, prepared all tables and figures and took all photographs presented within this thesis. Thesis chapters were greatly improved by revisions and suggestions provided by: AES, JMH and GJR (All Chapters), DWM (Chapter 2 and 4), three anonymous reviewers (Chapter 2), Stephan J. Schoech, Daniel Hanley and two anonymous reviewers (Chapter 4). Relevant co-authors are identified at the beginning of each thesis chapter.

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CHAPTER 2 – MEASURING CORTICOSTERONE IN FEATHERS USING AN ACETONITRILE/HEXANE EXTRACTION AND ENZYME IMMUNOASSAY: FEATHER CORTICOSTERONE LEVELS OF FOOD-SUPPLEMENTED ATLANTIC PUFFIN CHICKS.[#]

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2.1 SUMMARY

Glucocorticoid levels measured in the blood of animals reflect hypothalamic-pituitary-adrenal (HPA) activity in response to predictable and unpredictable changes. In birds, circulating corticosterone is incorporated into growing feathers and provides an integrated measure of HPA activity over the period of feather growth. Measuring corticosterone in feathers can provide insight into the physiological state of birds during times when they are unavailable for blood sampling (e.g., during migratory or non-breeding periods of the annual cycle). Building upon studies that used radioimmunoassay or liquid chromatography, we used a commercially available enzyme immunoassay kit to measure corticosterone in feathers of nestling Atlantic Puffins (*Fratercula arctica*) on Gull Island, Newfoundland, Canada, in 2012, and demonstrate the benefits of sample preparation via acetonitrile/hexane purification. We used this method to measure corticosterone in feathers of Atlantic Puffin chicks that experienced differences in mass gain due to a supplementary feeding study. We found a positive relationship between feather corticosterone and mass gain, and a negative relationship between feather corticosterone and pre-treatment body condition. Because feathers were growing prior to and during the supplementary feeding period, our results suggest that corticosterone levels in feathers were influenced by nutritional status. Our results also suggest that extracting seabird

feather samples with acetonitrile/hexane (in addition to methanol) prior to measuring corticosterone with enzyme immunoassay is beneficial, and, as reported in previous studies, blood and feather corticosterone values reflect different measures.

2.2 INTRODUCTION

Vertebrates face predictable (e.g., seasonal cycles) and unpredictable changes (e.g., sudden changes in social status or food resources, disease) that may activate their hypothalamic-pituitary-adrenal (HPA) axis causing the release of glucocorticoid hormones into the bloodstream (Wingfield & Kitaysky 2002). Measuring variation in blood glucocorticoid levels among populations and individuals can help show how vertebrates negotiate challenges, such as the trade-off between reproduction and survival (Romero et al. 2000, Rector et al. 2012). In some cases, glucocorticoid release may cause an animal to engage in a suite of behavioural (e.g., movement away from a threat) and physiological (e.g., energy mobilization) changes that help to re-establish homeostasis (Emergency Life-History Stage; Wingfield & Kitaysky 2002), but, in other cases, elevated blood glucocorticoid levels can be detrimental, reducing an animal's immune function (Munck et al. 1984; French et al. 2006), ability to survive (Summers & Greenberg 1994), or ability to reproduce (Moberg 2000). The

Reactive Scope Model (Romero et al. 2009) is one of several models examining the pathological and beneficial fluctuations in glucocorticoids. This model views physiological mediators of stress (e.g., glucocorticoid) in the context of four homeostatic ranges (Predictive, Reactive, Overload, and Failure). More specifically, Romero et al. (2009) suggested that glucocorticoid elevations within an animal's "normal reactive scope" can be beneficial, or at least not detrimental. The concept that glucocorticoid elevations can be both beneficial and detrimental to fitness is also summarized in Bonier et al.'s (2009) examination of the "corticosterone-fitness hypothesis." This hypothesis predicts that individuals with higher glucocorticoid levels have lower fitness, but Bonier et al. (2009) extended the original hypothesis to predict positive relationships between corticosterone (hereafter, CORT) and fitness in some cases ("corticosterone-adaption hypothesis"). Obtaining blood glucocorticoid levels in free-ranging animals typically requires researchers to capture animals and collect blood samples. For animals that spend most of their time offshore, such as seabirds, blood sampling outside the short terrestrial breeding period is largely unfeasible. In addition, blood samples provide an instantaneous 'snapshot' of an animal's glucocorticoid level at the time of sampling rather than an integrated measure of glucocorticoid exposure over a longer period (days to weeks). These limitations have led researchers to develop methods for measuring steroid hormones in other tissues, such as hair (Koren et al. 2002,

Pereg et al. 2012), blubber (Mansour et al. 2002), and feathers (Bortolotti et al. 2008, 2009a, b, Fairhurst et al. 2011, Lattin et al. 2011, Koren et al. 2012, Fairhurst et al. 2013a, Will et al. 2014). In birds, CORT is incorporated into growing feathers so that CORT levels in feathers can be correlated with baseline or stress-induced CORT titers in plasma during feather growth (Bortolotti et al. 2008, Fairhurst et al. 2013a). Subsequent studies have shown that feather CORT is related to many biological and ecological factors, providing new insight into many research questions, including those that are well-studied (e.g., ornamentation; Kennedy et al. 2013; Lendvai et al. 2013) and those that are emerging (e.g., carry-over effects; Kouwenberg et al. 2013; Legagneux et al. 2013). To date, feather CORT has been related to body condition of chicks (Harms et al. 2010), microclimates in nest boxes (Fairhurst et al. 2012), environmental enrichment (Fairhurst et al. 2011), survival of adult birds (Koren et al. 2012), current and subsequent breeding success (Crossin et al. 2013), carbon stable isotope values (Fairhurst et al. 2013b), the degree to which testosterone and parasites influence sexual ornamentation (Bortolotti et al. 2009b), carotenoid-based coloration of adult feathers (Kennedy et al. 2013, Lendvai et al. 2013, Fairhurst et al. 2014), and carotenoid-based coloration of chick integument (Martinez-Padilla et al. 2013). In previous studies, researchers have validated and used several techniques to measure CORT in feathers, including radioimmunoassay (RIA; Bortolotti et al. 2008, 2009a, b, Lattin et al.

2011, Fairhurst et al. 2013a, Horak et al. 2013, Will et al. 2014), liquid chromatography coupled to tandem mass spectrometry (LCMS/MS; Koren et al. 2012), and enzyme immunoassay (EIA; Kouwenberg et al. 2013, Bourgeon et al. 2014). In this study, we use a commercially available EIA kit to measure CORT in Atlantic Puffin (*Fratercula arctica*) feathers. We used EIA because it requires fewer regulatory permits than RIA and requires a microplate reader, which is a more versatile piece of equipment than the devices to quantify radioactivity needed for RIA (Sheriff et al. 2011). We tested the hypothesis that sample extraction with acetonitrile and hexane (in addition to methanol extraction) prior to EIA results in less interference in CORT measurement from non-CORT compounds. Because CORT is relatively polar compared to many other lipids, we expected that subjecting methanol extracts of feathers to a two-phase extraction system consisting of a moderately polar (acetonitrile) layer and a nonpolar (hexane) layer would allow us to partition CORT from less polar lipids (e.g., tryglycerides) found in the original methanol extract. Additionally, we measured the percent recovery of exogenous CORT added to feather extracts prior to acetonitrile/hexane purification, and assessed whether a curve produced by serially diluted feather extracts was parallel to the standard curve (i.e., assessed assay 'parallelism'). In addition to the above assessments, we also compared CORT levels in the blood and feathers of puffin chicks to determine if feather and blood CORT levels are correlated and if they vary with chick body

condition and mass gain. Chicks were used for this experiment because: (1) they were part of a supplementary feeding study, allowing us to compare blood and feather CORT of chicks experiencing different levels of food intake, (2) there was overlap between the supplementary feeding period and the growth of the feathers in which we measured CORT, and (3) feather growth occurred at a time when chicks were available for both feather and blood sampling (breeding season). We hypothesized that differences in CORT levels of chicks would be reflected in feathers grown during the treatment period and in blood sampled at the end of the treatment period. Recent findings suggest that plasma CORT elevations need to be significant and sustained to be reflected in measurable feather CORT (Fairhurst et al. 2013a). Therefore, we predicted that if the effects of supplementary feeding caused significant changes in chick mass gain and these changes were reflected in both blood and feather CORT levels, then blood and feather CORT levels would be significantly correlated. We expected that measuring body condition and mass gain would provide insight into what blood and feather CORT levels reflect biologically, particularly if chick feather and blood CORT are not correlated at most points (e.g., Fairhurst et al. 2013a). Previous studies have shown that CORT can have positive and negative effects on the fitness of seabird chicks. For example, in Black-legged Kittiwake chicks (*Rissa tridactyla*), plasma CORT elevation was associated with increased food intake, begging, and aggression, but also with less efficient

growth and reduced cognitive abilities (Kitaysky et al. 2003). Kitaysky et al. (2005) found that plasma CORT levels of Tufted Puffin (*Fratercula cirrhata*) chicks were not elevated in response to moderate food deprivation, and also that plasma CORT levels of Tufted Puffin chicks were significantly and positively correlated to their level of daily energy intake and to endogenous fat reserves. Because chicks in our study were food-supplemented instead of deprived, we predicted that feather CORT levels of puffin chicks would be positively related to mass gained during the treatment period, and that chick blood CORT levels would be positively related to chick body condition at the end of the treatment period

2.3 METHODS

2.3.1 Feather collection and preparation

Feathers were collected from Atlantic Puffin (hereafter, puffin) chicks on Gull Island, Witless Bay Ecological Reserve, Newfoundland, Canada (47 ° 16' N, 52 ° 46' W) in 2012. Three to five back feathers were plucked from each chick, but only one feather (*N*= 18 chicks) was analyzed per chick. Before analysis, the afterfeather (downy barbs at the base of the feather) and residual down were removed from each feather, and a Kimwipe was used to remove any observable feather dander or dirt from the vane. The calamus was removed

from each feather, then the feather was weighed (± 1 mg) on an analytic balance and its length measured (± 1 mm) using a ruler. Only feathers of a similar size and type were compared in each experiment so that analyses were not compromised by feather mass (Lattin et al. 2011, Patterson et al. 2014). Mean feather mass was 2.9 ± 0.3 (SD) mg (range = 2.4–3.4 mg) for chick back feathers. Feathers for each experiment were assayed on a single 96 well plate. Samples were randomized within each plate. All statistical analyses in this paper were performed using R.2.15.2 software (R Core Team 2012). Results were considered significant at $p < 0.05$.

2.3.2 Methanol CORT extraction: general procedure

Similar to Mansour et al. (2002), all glassware was silanized using 1% v/v dichloromethylsilane [CH_3SiHCl_2] in toluene [$C_6H_5CH_3$], rinsed twice with methanol, and air-dried. Silanization prevents CORT from adhering to glassware and was shown to significantly reduce losses of CORT during the extraction process (C. Baker, unpubl. data). The methanol CORT extraction procedure was modified from Bortolotti et al. (2008). One feather per chick was placed in a 10 ml glass scintillation vial and minced into small bits (< 3 mm) using scissors. Using a serological pipette, 3 ml of HPLC-grade methanol (Thermo Fisher Scientific, Waltham, MA) was added to each vial. Vials were capped and sealed with Parafilm M (Bemis Company, Oshkosh, WI), and were placed in a

degassed sonicating water bath (Branson Ultrasonics, Danbury, CT) at room temperature for 30 min. Vials were then placed in a 50 °C shaking water bath (Grant Instruments, Cambridge, UK) for 15 h. After incubation, the contents of each vial were poured into a sintered glass Buchner funnel lined with filter paper (Whatman GF/B, 2.4- cm circles) and vacuum filtered into a 15-ml borosilicate glass centrifuge tube (Kimble Chase Life Science and Research Products, Vineland, NJ). Each vial was rinsed twice with 2.5 ml of methanol, and these rinse solutions were also filtered and collected. Tubes containing filtrate were placed in an N-Evap dryer (Organomation, Berlin, MA) and the filtrate was evaporated under a steady stream of nitrogen while the tubes were partially immersed in a 50 °C water bath.

2.3.3 Acetonitrile/Hexane Purification: general procedure

After methanol extraction, we adapted (for CORT) the acetonitrile/hexane purification described by Mansour et al. (2002) where the more polar steroid hormones partition into the acetonitrile layer and the less polar lipids (e.g., triacylglycerols) partition into the hexane layer. Each evaporated sample was reconstituted in 3 ml of 95% nanograde hexane (Mallinckrodt Baker Inc., Phillipsburg, NJ) and then agitated 12X using a vortex mixer. Following this reconstitution, 3 ml of HPLC-grade acetonitrile (Thermo Fisher Scientific) were added and the hexane mixture was agitated another 12 times. Samples were

then centrifuged at 1250 × *g* for 15 min to facilitate separation of the acetonitrile and hexane layers. After centrifugation, the hexane (top) layer was collected from each sample by aspiration using a glass Pasteur pipette and transferred to a clean tube, resulting in one tube with hexane and one tube with acetonitrile. Starting with these two tubes, the extraction was repeated to improve the separation of CORT from unwanted non-polar lipids. That is, 3 ml of fresh acetonitrile was added to the tube containing the hexane fraction, and 3 ml of fresh hexane was added to the tube that contained the acetonitrile fraction. Then, sample mixing and centrifugation were repeated. Hexane layers were removed from both tubes by aspiration with a glass Pasteur pipette and discarded, and the acetonitrile layers were then combined in the newer of the two tubes. Tubes were placed in the N-Evap device as described above and the acetonitrile was evaporated under a stream of nitrogen. The residue in each tube was reconstituted in 400 l EIA buffer (Cayman Chemical Company, Ann Arbor, MI), and this solution was arbitrarily assigned the dilution of 1:1.

2.3.4 Experiment 1: Demonstrating parallelism and measuring recovery

CORT in extracted feather samples was measured using commercially available CORT EIA kits (Cayman Chemical Company) with a manufacturer reported detection limit of 35 pg/ml and low cross-reactivity for a variety of common steroids and CORT metabolites (all < 0.31% crossreactivity, except for 11-

deoxycorticosterone and 11-dehydrocorticosterone at 7% and 11%, respectively). To measure parallelism, one back feather from each of three puffin chicks was used. Each feather was first extracted with methanol and then with acetonitrile/hexane as described above. Each resulting sample was serially diluted, and these diluted samples were assayed in duplicate on the same EIA plate (Plate 1). The slope of the relationship between feather CORT (pg/ml) and relative binding (%B/Bo) for each dilution and for a set of standard CORT samples were compared using analysis of covariance (ANCOVA). Feather CORT values were log transformed to allow for linear analysis. In an additional experiment on the same plate (Plate 1), back feathers from three puffin chicks were extracted with methanol and each sample was divided into twin 3.5 ml samples. One twin was spiked with 1000 pg of CORT and the other was not. Both twins were then subjected to acetonitrile/hexane purification as described above. The spiked and unspiked twin samples were dissolved in 200 µl of EIA buffer and then assayed in duplicate.

2.3.5 Experiment 2: Testing the acetonitrile/hexane purification step

To test the need for the acetonitrile/hexane purification step, one back feather from each of 18 puffin chicks was extracted with methanol as described earlier. After the vacuum-filtration step, each sample was brought to a vortex 20 times after which 3.5 ml of methanol-feather solution was pipetted from the original

tube into a new tube. A second volume of 3.5 ml was pipetted into another new tube, resulting in twin samples for each feather. Methanol was evaporated as described above. One of the twins for each feather was subjected to the acetonitrile/hexane purification step described above before being reconstituted in 200 µl EIA buffer. The other twin for each sample was reconstituted in 200 µl EIA buffer without undergoing any further extraction. Both twins were assayed in duplicate within the same EIA plate (Plate 2). Means of twin samples (one purified and one non-purified) were compared using a paired *t*test. In addition, %CVs of duplicates of purified samples were compared to %CVs of duplicates of non-purified samples using a paired *t*-test.

2.3.6 Experiment 3: Comparing CORT in blood and feathers

Blood samples were collected within 3 min of capture to ensure baseline levels (Romero & Reed 2005) from the 18 chicks for which feather CORT was measured in Experiment 1. Prior to feather and blood collection, the 18 chicks were part of a 13-d supplementary feeding experiment. In addition to normal feeding by their parents, half of the chicks received supplementary food from researchers. For the food-supplemented group (*N*= 9 chicks), we placed two capelin (*Mallotus vallosus*) in each burrow per day, whereas for the non-supplemented group (N= 9 chicks) we reached into burrows, but did not leave capelin. Supplementary feeding began when chicks

were 17-d-old and ended when chicks were 30 d old. Timing of the supplementary feeding period was selected to coincide with the chicks' linear growth phase (i.e., before the beginning of prefledge mass loss that begins 6 to 10 d before fledging; Myrberget 1962, Harris 1976). All chicks in our study fledged at least 10 d after the end of the treatment period. Chick mass and tarsus length measurements were taken on the first day and last day of the feeding period to quantify pre-treatment and post-treatment body condition and mass gained by each treatment group. Body condition (or size-corrected mass) was calculated by dividing chick mass (g) by chick tarsus length (mm). Because tarsus length increases with chick age (Cook & Hamer 1997) and body mass varies with nutritional condition (Baillie & Jones 2004), dividing mass by tarsus length standardized mass among chicks of different ages. Blood and feather samples were collected at the end of the supplementary feeding period, when chicks were 30 d old. In alcids, feathers begin to develop a few days after hatching (Gaston 1985) and full pre-juvenile moult is complete by age 30 d (Lowther et al. 2002), so the period of feather growth encompassed the treatment period as well as the period from hatch to the start of treatment. All chicks in our study had well-developed back feathers at the time of feather sampling (30 d). Approximately 1 ml of blood was taken from the brachial vein of each chick and dispensed in drops onto a filter paper blood spot card (Whatman) so that blood soaked through the card. Blood cards were

dried for at least 24 h and a 3.2-mm punch was used to punch 24 spots from each sample card (Doody et al. 2008, Rector et al. 2012). Spots were analyzed in duplicate (12 spots for each duplicate) using a COAT-A-COUNT Rat Corticosterone radioimmunoassay kit (TKRC1, InterMedico, Markham, Ontario, Canada) as validated and described by Doody et al. (2008) and Rector et al. (2012). Intra-assay CV was 5.87, similar to %CVs reported in Rector et al. (2012). Blood spot CORT values resulting from the assay were converted using the equation described and validated in Rector et al. (2012). Converted values were reported in ng CORT/ml blood and were compared to feather CORT values for each chick. DNA was extracted from dried blood from blood cards using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), and chick sex was determined using a CHD (chromodomain helicase DNA) based molecular method (Fridolfsson & Ellegren 1999). Highly conserved primers (2550F and 2718R) were used. Females were characterized by two fragments (CHD1W and CHD1Z) and males by one fragment (CHD1Z) following polymerase chain reactions and agarose electrophoresis. The effects of treatment on both mass gain and post-treatment body condition were determined using separate ANOVAs: chick mass gain = treatment + ε (error), and chick post-treatment body condition = treatment + ε. Correlation between blood and feather CORT levels was tested using Pearson *r*. Separate linear regressions were used to test the effects of mass gained during the treatment

period, chick body condition prior to the start of the treatment period, and chick body condition at the end of the treatment period on feather CORT and on blood CORT levels (feather CORT = chick mass gain + chick pre-treatment body condition + chick post-treatment body condition + ε and blood CORT = chick mass gain + chick pre-treatment body condition + chick post-treatment body condition + ε). Interactions among independent variables in relation to feather CORT were examined using ANCOVA, but were not significant and are not discussed further (blood CORT x mass gain: $F_{1,7} = 0.3$, $p = 0.58$, blood CORT x sex: $F_{1,7} = 0.3$, $p = 0.58$, blood CORT x chick age: $F_{1,7} = 0.7$, $p = 0.44$, mass gain x sex = $F_{1,7} = 0.2$, $p = 0.66$, mass gain x chick age: $F_{1,7} = 0.01$, $p = 0.91$, and sex x chick age: $F_{1,7} = 0.2$, $p = 0.64$).

2.4 RESULTS

2.4.1 Experiment 1: Demonstrating parallelism and measuring recovery

The slope of the line constructed from the CORT standards provided in the Cayman Chemical Company did not differ from the slope of the line produced by serially diluted feather CORT samples (Figure 2.1). Both standard and serially diluted samples had negative slopes ($F_{1,6} = 2003.1$, $p < 0.001$) and the serially diluted line was significantly higher than the standard CORT line ($F_{3,6} = 34.5$, $p < 0.001$), but the lines did not cross (no interaction between CORT concentration

and sample type; $F_{3,6} = 0.3$, $p = 0.84$). Also, samples spiked with 1000 pg exogenous CORT before acetonitrile/hexane purification showed 87% recovery of CORT.

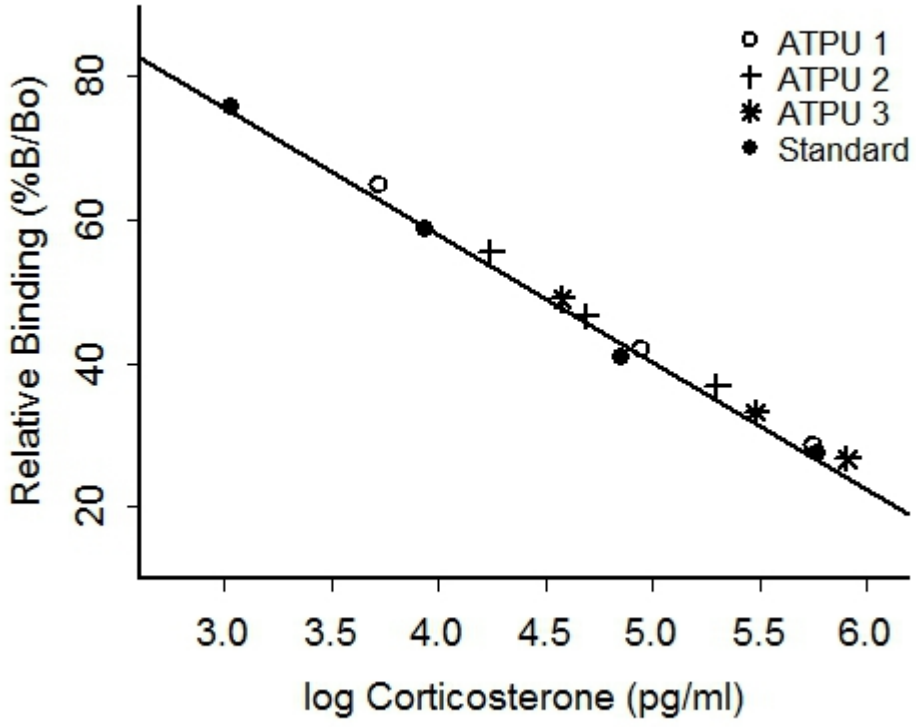

Figure 2.1 Relative binding (%B/Bo) plotted against log CORT concentrations (pg/ml) showing parallelism of CORT standards and serially diluted feather CORT samples from three different Atlantic Puffins.

2.4.2 Experiment 2: Testing the acetonitrile/hexane purification step

When twin chick feather samples were compared (one twin extracted with both methanol and acetonitrile/hexane and the other twin extracted with methanol only), mean CORT values were significantly lower for the twin extracted with both methanol and acetonitrile/hexane (mean = 329.35 pg/ml) than for the twin extracted with methanol only (mean = 642.28 pg/ml; paired $t_{17} = -7.5$, $p < 0.001$). Furthermore, duplicates of the acetonitrile/hexane-purified samples were not significantly different from each other (paired $t_{17} = -0.6$, $p = 0.53$), whereas the duplicates of the methanol-only extracted samples were significantly different (paired $t_{17} = 3.4$, $p = 0.004$). Duplicates of acetonitrile/hexane-purified samples had a mean %CV of 8.06%, significantly lower than the mean %CV of 16.32% for duplicates of methanol-only extracted samples (paired $t_{16} = -3.0$, $p < 0.001$).

2.4.3 Experiment 4: Comparing CORT levels in blood and feathers

Food-supplemented chicks gained more mass ($F_{1,16} = 5.3$, $p = 0.035$) than non-supplemented chicks. However, there was no difference in either the pre-treatment ($F_{1,16} = 0.1$, $p = 0.76$) or post-treatment ($F_{1,15} = 2.3$, $p = 0.15$) body condition of food-supplemented and non-supplemented chicks. Furthermore, pre- and post-treatment body conditions were positively correlated across all chicks ($F_{1,15} = 16.9$, $p = 0.001$). Because there was also no direct effect of

supplementary feeding on either feather CORT ($F_{1,16}$ = 1.2, p = 0.29) or blood CORT ($F_{1,16}$ = 2.7, p = 0.12), the two treatment groups were combined in subsequent analyses. Feather and blood CORT levels were not significantly correlated (Pearson r = –0.33, t_{13} = –1.3, p = 0.23). The lack of correlation between feather and blood CORT is consistent with the results of multiple linear regressions comparing feather CORT and blood CORT to measures of chick mass gain and body condition (Table 2.1). Specifically, we found that CORT levels of chick feathers were positively related to chick mass gain (Figure 2.2) and negatively related to pre-treatment chick body condition (Figure 2.3), whereas chick blood CORT levels were not. Furthermore, neither post-treatment chick blood CORT nor chick feather CORT was related to post-treatment chick body condition. Additionally, we found no relationship between feather sample mass and feather CORT values (R^2 = 0.01, t_{16} = 0.4, P= 0.73), supporting our assumption that feather CORT results were not affected by differences in feather mass among chicks.

Table 2.1 Results of linear regressions comparing feather CORT and blood CORT of Puffin chicks to (1) mass gained during the treatment period, (2) pre-treatment body condition (mass in grams/ tarsus in mm), (3) and post-treatment body condition. Statistically significant ($p < 0.05$) results are marked with an asterisk (*).

	Feather CORT				**Blood CORT**			
	$F_{1, 16}$	t_{16}	P	R^2	$F_{1, 13}$	t_{13}	P	R^2
Mass Gain	4.865	2.206	0.042*	0.23	0.788	0.888	0.391	0.06
Pre-Treatment Body Condition	11.660	-3.415	0.004*	0.42	0.790	0.889	0.390	0.06
Post-Treatment Body Condition	1.307	-1.143	0.270	0.08	1.194	1.093	0.294	0.08

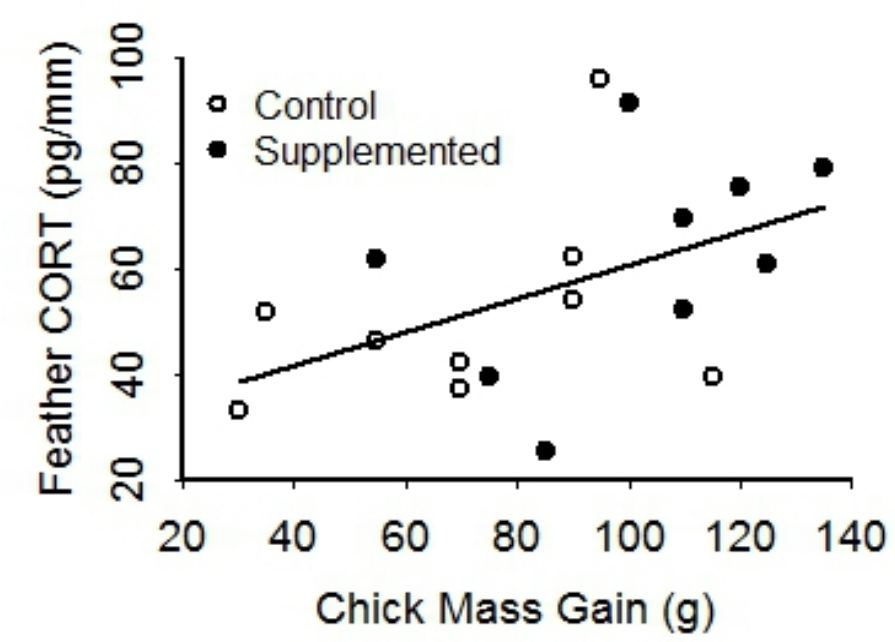

Figure 2.2 Positive linear relationship between mass gained by Puffin chicks during the supplementary feeding period and CORT levels in feathers grown by Puffin chicks during the same period.

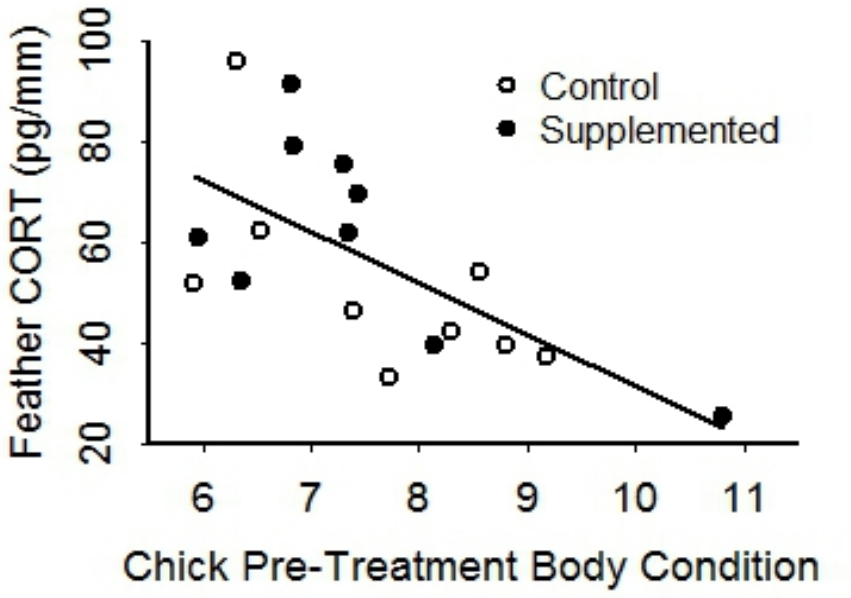

Figure 2.3 Negative linear relationship between Puffin chicks' body condition (g body mass / mm tarsus length) before the start of the supplementary feeding period and feather CORT levels.

2.5 DISCUSSION

Using EIA to measure CORT in the feathers of puffin chicks, we found that %CVs between duplicates of samples subjected to acetonitrile/hexane purification were significantly lower than %CVs between duplicates of samples extracted only in methanol. This reduction in variability suggests that compounds that interfere with assay binding were removed by acetonitrile-

hexane purification. Being relatively polar compared to other lipids, CORT dissolves in the acetonitrile fraction (which was retained for EIA), whereas the least polar lipids (e.g., tryglycerides) readily dissolve in the highly nonpolar hexane fraction (which was discarded). We also found that serial dilutions of samples extracted with both methanol and acetonitrile/hexane produced a curve that was parallel to the standard curve. An absence of parallelism would indicate the presence of interfering compounds (Miller & Levinson 1996). Although parallelism has been shown for feather samples extracted with only methanol and serially diluted and assayed with EIA (Bourgeon et al. 2014), the reduced variation between duplicates of our acetonitrile/hexane purified samples (compared to methanol-only) suggests that the purification procedure affects the accuracy of EIA on some level. For twin samples from the same feather, the mean measured CORT value was lower for samples extracted with both methanol and acetonitrile/hexane than samples extracted with methanol only. Because a high percentage of CORT was recovered when we added exogenous CORT to feather samples before acetonitrile/hexane purification, we suggest that lower CORT values for acetonitrile/hexane purified samples was not due to a major loss of CORT during the purification procedure. For each sample, we re-extracted both the hexane and acetonitrile fractions that resulted from the first round of extraction, so we are confident that CORT levels in discarded hexane fractions were minimal. Furthermore,

Mansour et al. (2002) tested the acetonitrile/hexane purification procedure on progesterone samples using the same laboratory and equipment we used, and found (using thin-layer chromatography) that <1% of the hormone was present in the hexane fraction. Taken together, our results indicate that prior to measuring CORT in puffin feathers with EIA, extracting feathers with acetonitrile/hexane in addition to methanol extraction is beneficial. We found that CORT levels in chick feathers and CORT levels in chick blood were not significantly correlated, and Will et al. (2014) reported similar results. This result is consistent with our finding that feather CORT levels, but not blood CORT levels, were related to chick mass gain. Because there was an overlap between feather growth and mass gain during the supplementary feeding period and CORT is integrated into feathers as they are grown (Bortolotti et al. 2008, 2009a), we expected that the CORT level of feathers would reflect differences in mass gain. Conversely, we expected blood CORT levels to reflect conditions at the time of blood sampling (post-treatment) rather than the period of supplementary feeding and mass gain. A more appropriate comparison of blood CORT with feathers would be the mean of continuous blood samples throughout the treatment and feather growth periods, but this was not feasible for wild chicks living in deep burrows due to the potentially confounding effects of chick capture, handling, and blood sampling on chick CORT levels (Romero & Reed 2005). Fairhurst et al. (2013a) took multiple blood samples (on

days 7, 9, and 11 posthatching) after experimentally altering CORT levels of nestling Tree Swallows (*Tachycineta bicolor*), but found that blood and feather CORT levels were significantly and positively related only on day 9 when CORT levels were highest. These results suggest that plasma CORT levels must be significant and sustained to be reflected as measurable CORT in feathers (Fairhurst et al. 2013a). Because feathers of chicks in our study began developing prior to the start of supplementary feeding, the negative relationship between feather CORT levels and pre-treatment body condition suggests that the pre-treatment nutritional state of chicks affected their CORT levels. This is consistent with the results of previous studies where, under natural conditions, chicks in poor body condition had higher CORT levels than well-fed chicks (Kitaysky et al. 1999, Saino et al. 2003, Walker et al. 2005, Sears & Hatch 2008, Patterson et al. 2014, Will et al. 2014, but see Kitaysky et al. 2005). Although results reported by Kitaysky et al. (2005) suggest that suppression of the CORT response may be beneficial for puffins during times of low food intake, our results were more consistent with those reported for Rhinocerous Auklet (*Cerorhinca monocerata*) chicks, with higher feather CORT levels in chicks with low food intake (due to restricted diets; Will et al. 2014). Assuming that the poorer pre-treatment body condition of chicks in our study was due to low parental feeding rates, elevated CORT levels of hungry chicks may function to promote increased begging. Therefore, greater mass gain in chicks with high

feather CORT may reflect more intense begging by chicks with lower pre-treatment body condition. Higher CORT levels were associated with increased foraging effort in adult seabirds (Doody et al. 2008, Crossin et al. 2012) and the only way for a chick in a single-chick brood to “forage” (increase food intake) is to beg more from their parents. In a previous study, we found that the nutritional status (manipulated by supplementary feeding) of Atlantic Puffin chicks affected the type and frequency of begging calls produced (Rector et al. 2014). The connection between higher CORT levels and begging is also supported by the observation that chicks given CORT implants begged more than controls (Kitaysky et al. 2001, 2003, Loiseau et al. 2008). Contrary to our prediction, blood CORT measured at the end of the treatment period was not related to post-treatment body condition, suggesting that chicks with poor pre-treatment body condition no longer had elevated CORT levels by the time blood samples were taken (i.e., higher feather CORT reflected in feathers were mostly driven by elevations in feather CORT before or early in the treatment period rather than the end). However, in the larger sample of which chicks in our study were a small subset, there was a positive relationship between blood CORT and post-treatment body condition (M. Fitzsimmons, pers. comm.), suggesting that the lack of relationship in our study may have been due to our smaller sample size. In summary, the results of our experiments: (1) suggest that it is beneficial to extract seabird feather samples with acetonitrile/hexane (in addition to

methanol) prior to measuring CORT with EIA, (2) support previous findings that blood and feather CORT values reflect different measures, and (3) suggest that CORT levels in Atlantic Puffin chick feathers are influenced by nutritional status. Further study of CORT levels in feathers of developing puffin chicks could provide greater insight into the causes and effects of CORT fluctuations in response to food supplementation or deprivation.

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CHAPTER 3 – CORTICOSTERONE LEVELS IN FEATHERS AND BLOOD OF RHINOCEROS AUKLETS *CERORHINCA MONOCERATA* REFLECT VARIATION IN ENVIRONMENTAL CONDITIONS.

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3.1 SUMMARY

In order to fully understand factors that affect animals during their annual cycle, it is important to measure conditions over multiple seasons and life history stages. Measuring corticosterone, the main glucocorticoid in birds, in blood and feathers can allow for inter-seasonal comparisons because these measures potentially reflect corticosterone at the time of sampling and during feather growth, respectively. Here, we tested the hypothesis that corticosterone levels reflect spatial and temporal variation in environmental conditions by measuring corticosterone levels in feathers (pre-breeding) and blood (breeding) of rhinoceros auklets *Cerorhinca monocerata* (auklets) nesting on three widely-dispersed colonies during years with different oceanographic conditions. We also measured the condition of chicks at each colony in one year to test whether corticosterone levels vary with breeding success. We found that auklets could be distinguished by their δ15N and δ13C stable isotope values during, but not prior to, the breeding season. This indicates that the ranges of auklets from different colonies did not overlap while breeding, but did overlap during the offshore pre-breeding period, which is consistent with evidence that auklets from these colonies are genetically similar. Consistent with stable isotope results, corticosterone levels were different among colonies during the breeding season (blood collected in summer), but not prior to breeding (feathers grown during

Feb-March). Specifically, blood CORT levels were higher for auklets that bred on a colony with chlorophyll-a concentrations that indicated good feeding conditions than for auklets that bred on a colony with indications of poor feeding conditions. Among years, we found that feather CORT levels were significantly higher in 2009 (favourable cold-water pre-breeding conditions) than in 2010 (unfavourable warm-water El Niño pre-breeding conditions), while 2011 (intermediate pre-breeding conditions) was not different from either. Although chick condition was lower at one of the study colonies, there was no difference in breeding season CORT levels among colonies, suggesting that CORT did not vary with reproductive success. Taken together, our results support our hypothesis that corticosterone levels prior to and during the breeding season reflect differences in environmental conditions among colonies and years. However, the implications of this relationship appear to depend heavily on context, and thus, require careful interpretation.

3.2 INTRODUCTION

Glucocorticoid hormones play a complex role in mediating how vertebrates invest in life-history traits and how they negotiate transitions between life-history stages. In a recent review, Crespi et al. (2013) specify that relationships between glucocorticoids and life-history traits have been highly

inconsistent across species. No consistent patterns have emerged despite strong evidence that glucocorticoids regulate circulating glucose levels, and thus, are essential for the maintenance of energy homeostasis during predictable seasonal activities (e.g., reproduction; McEwen & Wingfield 2003) and unpredictable events that threaten survival (e.g., extreme weather; Wingfield & Kitaysky 2002). The specific effects of glucocorticoid fluctuations on resource allocation trade-offs and expression of life-history traits depend heavily on the ecological and evolutionary context of the organism in question (Crespi et al. 2013). For example, even within a single group of birds, corticosterone (the main glucocorticoid in birds, CORT) levels of individual birds were positively related to reproductive success and adult survival during chick provisioning, but negatively related to the same factors in the same birds during incubation (Bonier et. al., 2009). Additionally, a negative relationship between CORT levels and food abundance was found in the Common murre *Uria aalge* (Doody et al. 2008, Kitaysky et al. 2007, 2010), but not in the closely-related Atlantic puffin *Fratercula arctica* (Rector et al. 2012). This difference may be related to differences between the length of these species' chick-rearing periods, and/or the timing and severity of prey variation experienced by subjects in a given study. Therefore, in order to understand how glucocorticoids mediate life-history traits and transitions it is essential to measure their effects over different life stages and over environmental conditions that vary to a degree that is relevant

for the species in question (Crespi et al. 2013).

Recent work has shown that CORT circulating in the blood is incorporated into growing feathers, which provides the potential to integrate measures of CORT fluctuations experienced by moulting birds (Bortolotti et al. 2008, 2009; Horak et al. 2013). Unlike blood, which reflects baseline or stress-induced CORT levels at the time of sampling, feathers provide a value for CORT that is integrated over the time of feather growth. Therefore, feather CORT can help to explain processes happening over longer time scales, and during periods when birds are inaccessible for blood sampling. For example, during a year with relatively low food availability, feather CORT levels of female Atlantic puffins were positively related to egg mass (Kouwenberg et al. 2013), which suggests that conditions experienced by seabirds while feathers are grown (during the non-breeding season) may have consequences during the following breeding season.

To date, the relationship between variation in feather CORT and variation in environmental conditions has not been well studied. However, the existence of this relationship is supported by Legagneux et al. (2013), who found that feather CORT levels of common eiders *Somateria mollissima* varied with annual differences in environmental temperature, rather than with differences in individual quality. Here, we tested the hypothesis that CORT levels reflect spatial and temporal variation in environmental conditions by measuring CORT

levels in feathers (2009 – 2011) and blood (2010 and 2011) of rhinoceros auklets *Cerorhinca monocerata* (hereafter, auklets) nesting on three widely-dispersed colonies in British Columbia: Lucy Island along the North Coast, Pine Island along the Central Coast, and S'Gang Gwaay in the Haida Gwaii archipelago. In our study, feather samples represent an integrated value of CORT levels over the pre-breeding season (February-March, when auklets undergo a partial pre-alternate moult), and blood samples represent CORT levels at a single point during the chick-rearing period (July). Microsatellite analysis has shown that the auklets from the Lucy, Pine and S'Gang Gwaay colonies are genetically similar (Abbott et al. 2014), and Friesen et al. (2007) suggest that such genetic similarity is most likely to occur in populations with overlapping distributions during their non-breeding season. While breeding, auklets are central-place foragers (Orians & Pearson 1979) and range only within tens of km of their respective colonies (McFarlane-Tranquilla et al. 2005). Therefore, we assumed that auklets from the three colonies experienced similar environmental conditions prior to breeding, but different conditions during the chick-rearing period, which led us to predict that CORT levels would be similar among colonies prior to breeding (in feathers), but not during the chick-rearing period (in blood).

To validate the assumption that environmental conditions experienced by birds from the three colonies were similar prior to breeding, but different during

chick rearing, we compared stable isotope values in their feathers and blood, respectively. Stable nitrogen isotopes (δ15N) indicate the relative trophic level at which a bird has been feeding, while stable carbon isotopes (δ13C) characterize its foraging habitat (i.e., inshore habitats tend to have lower δ13C values than offshore habitats). Analyzing δ15N and δ13C in combination allows for segregation of groups of seabirds according to habitat use or ecological niche (Jaeger et al. 2009; Cherel et al. 2013). We will consider our assumption validated if auklets from the three colonies cannot be distinguished by δ15N and δ13C values in their feathers (pre-breeding), but can be by their blood δ15N and δ13C values (chick rearing). We accounted for regional differences in isotopic baselines by comparing δ15N and δ13C values in the birds' blood against stable isotope values in a common prey species, the Pacific sandlance, collected at the three colonies.

Our three-year study spanned a period of dramatic variation in environmental conditions in the Northeast Pacific Ocean (DFO 2010, 2011, 2012; see Table 3.1 in Methods). In 2009, the auklet pre-breeding period (February-March) featured unusually cold water ocean conditions associated with a La Niña event that began in 2008. Cold-water conditions persisted until June 2009, but by the auklet chick-rearing period (July-August) ocean conditions were warm enough to trigger the strongest El Niño event of the century. Warm-water El Niño conditions persisted through the 2010 pre-

breeding period until April 2010, when cold-water La Niña conditions returned and persisted through the entire breeding season of 2010, and the pre-breeding and breeding seasons of 2011. Rhinoceros auklets feed across a range of trophic levels, from zooplankton to forage fish (Hipfner & Galbraith 2013). As cold-water (higher latitude) zooplankton species tend to be more lipid-rich and larger than warm water (lower-latitude) species (Benson & Lee 1975; Mackas et al. 2007), and cold-water species are more abundant when ocean waters are cooler across our study region (Crawford & Irvine 2010, 2011, 2012), we assumed that feeding conditions prior to breeding were better for auklets in 2009 than 2010, and intermediate in 2011. Previous studies have linked unfavourable environmental conditions (Romero 2004) and unfavourable feeding conditions (Kitaysky et al. 2007, Doody et al. 2008) with elevated blood CORT, so we predicted that feather (pre-breeding) CORT levels in auklets would be higher in 2010 than in 2009, and intermediate in 2011.

When assessing annual oceanographic conditions for the breeding season, we also considered ocean cholorophyll data (downloaded from the NASA Earth Observations website: http://neo.sci.gsfc.nasa.gov/view.php?datasetId=MY1DMM_CHLORA; see Table 3.2 in Methods). Rhinoceros auklets bred more successfully on Triangle Island (51°52′N, 129°05′W), another auklet colony in the north Pacific, in years in which surface chlorophyll-a concentrations first exceeded 2 mg/ml^3 in waters within a 15 km radius of the

island in the first two weeks of April (Borsad et al. 2011). On Triangle Island, strong spring bloom that persists through late May increases the recruitment of young-of-year Pacific sandlance and increases the abundance of this small forage fish in the diets of rhinoceros auklet chicks (Borstad et al. 2011). On Pine and Lucy (and Triangle) islands, ocean productivity has been linked with Pacific sandlance *Ammodytes hexapterus* abundance in rhinoceros auklet chick diets (Bertram and Kaiser 1993). Pacific sandlance is known to be an important prey item for auklets on Lucy, Pine and S'Gang Gwaay, because the birds breed more successfully in years when nestlings receive large amounts of this prey type in their diets (Bertram et al. 1991; Bertram et al. 2002; Hedd et al. 2006; Borstad et al. 2011).

In order to test the hypothesis that CORT levels in auklets are related to breeding success, we measured the condition of chicks at each colony in 2011. We expected that sandlance availability would be similar among colonies in 2011 because spring chlorophyll concentration reached 2 mg/ml^3 at around the same time at all colonies in this year. As individual seabirds that have higher provisioning rates under challenging conditions have been found to have higher blood CORT levels than lower provisioners (Doody et al. 2008), we predicted that individuals from colonies with chicks in better condition would have higher blood CORT.

3.3 METHODS

3.3.1 Locations of Study Colonies

Experiments were carried out on auklets nesting on Lucy (54°17'N, 130°37'W), Pine (50°35′N, 127°26′W) and S'Gang Gwaay (52°05′N, 131°13′W) islands off the coast of British Columbia, Canada (Figure 3.1). Lucy and Pine are both situated along the mainland coast, well up on the continental shelf, while S'Gang Gwaay is further offshore, closer to the shelf break, along the west coast of Haida Gwaii. The shelf break is the area of transition between continental shelf and the deeper water of the ocean basin, an area that is associated with zooplankton aggregation and known to influence seabird foraging ecology by affecting prey aggregation (e.g., Russell et al. 1999).

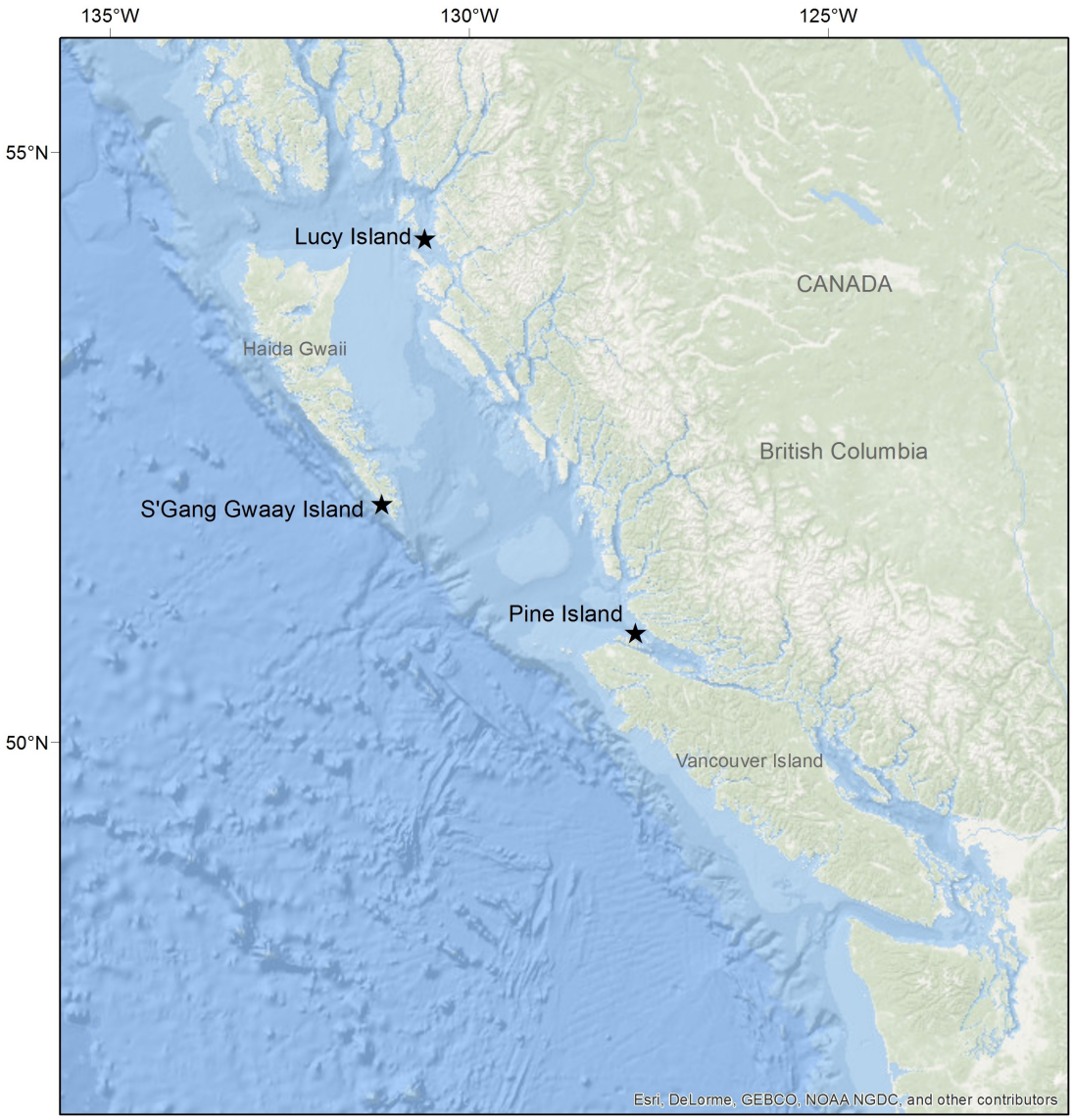

Figure 3.1 Location of Lucy, Pine and S'Gang Gwaay Islands

3.3.2 Oceanographic Conditions

Based on information reported in the annual State of the Pacific Ocean reports (DFO 2009, 2010, 2011) and as detailed in the Introduction, pre-breeding ocean conditions were generally moderate in 2011 (cold-water conditions), and more extreme in 2009 (exceptionally cold-water conditions) and 2010 (warm-water conditions; Table 3.1).

During chick rearing, chlorophyll data were used to determine the timing and strength of the spring phytoplankton bloom (downloaded from the NASA Earth Observations website: http://neo.sci.gsfc.nasa.gov/view.php?datasetId=MY1DMM_CHLORA). In 2010, chlorophyll concentration reached 2 mg/ml^3 by April within 15km of Lucy and Pine, but never reached 2 mg/ml^3 near S'Gang Gwaay (Table 3.2). In 2011, chlorophyll concentration within 15km of Lucy, Pine and S'Gang Gwaay reached 2 mg/ml^3 in May.

Table 3.1 Ocean conditions prior to breeding and during the breeding season in 2009, 2010 and 2011 (DFO 2009, 2010, 2011) and predicted CORT levels of adult rhinoceros auklets on Lucy, Pine and S'Gang Gwaay during the same periods.

	Ocean Conditions		Predicted adult auklet CORT levels	
	Pre-breeding	Breeding	Pre-breeding (feather)	Breeding (blood)
2009	Exceptionally cold ocean, strong La Niña	Warming ocean, start of strong El Niño	Lowest	No data
2010	Warm ocean, strong El Niño	Cooling ocean, start of La Niña	Highest	Higher than 2011
2011	Cold ocean, La Niña	Cold ocean, La Niña	Intermediate	Lower than 2010

Table 3.2 Mean monthly chlorophyll concentrations (mg/ml^3) recorded by NASA Earth Observations (http://neo.sci.gsfc.nasa.gov) which indicate the spring phytoplanktom bloom intensity for the ocean waters within 15km of Lucy, Pine and S'Gang Gwaay in April and May of 2010 and 2011. The chlorophyll concentration reached 2 mg/ml^3 in April 2010 on Lucy and Pine, but never reached 2 mg/ml^3 on S'Gang Gwaay in 2010. All colonies reached 2 mg/ml^3 in May in 2011.

	Chlorophyll concentration mg/ml^3				Predicted breeding auklet CORT levels	
	2010		2011		2010	2011
Colony	April	May	April	May		
Lucy	5.77	9.86	0.85	4.41	Intermediate	Lower than S'Gang Gwaay
Pine	8.50	18.55	0.94	4.95	Lowest	Lower than S'Gang Gwaay
S'Gang Gwaay	0.96	1.14	0.54	2.09	Highest	Higher than Lucy and Pine

3.3.3 Corticosterone Analysis

We measured CORT in a single breast feather from each of 144 auklets (72 females, 72 males) from Lucy, Pine and S'Gang Gwaay islands in 2009, 2010 and 2011 (N = 16 auklets for each island in each year). Although auklets moult most of their breast feathers during a pre-basic moult that occurs from August to January, they also undergo a partial pre-alternate moult from February to March that includes some breast feathers (Pyle 2008; Howell 2010). Therefore, similarly to other studies measuring rhinoceros auklet breast feathers (Sorensen et al. 2009; Carle 2014), we assumed that breast feathers showing minimal wear were grown in February-March, and thus reflected the auklet pre-breeding period. As breast feathers were collected by a number of people and assessment of wear can be subjective, we acknowledge that some feathers analyzed may have been grown post-breeding (August-January). However, to mitigate this problem, ~5 feathers were collected from each auklet and only one person chose (based on feather condition) the feathers to be analyzed. We measured CORT in blood of 164 auklets (68 females, 63 males, 33 unknown sex) from Lucy, Pine and S'Gang Gwaay islands in 2010 (N = 30, 29, and 29) and 2011 (N = 30, 30, and 16). Both feather CORT and blood CORT were measured in 90 of the aforementioned individuals. We removed the calamus from each breast feather and measured feather length to the nearest millimetre. Whole feathers were minced, incubated in methanol, filtered, purified using

acetonitrile and hexane, and assayed using an Enzyme Immunoassay kit (Cayman Chemical Company, Ann Arbor, USA) as described in Kouwenberg et al. (2013; in press). The intra-assay coefficient of variation (CV) calculated from duplicate absorbance values was 2.75 %. Samples were counter-balanced by colony and year over four EIA plates and the inter-assay CV between a known quantity of CORT assayed in each plate was 12.57 %. As suggested by Bortolotti et al. (2009), we report CORT values in pg/mm. Breast feathers used in our study were all of similar length (mean = 35 mm, range = 29 mm – 40 mm) and mass (mean = 4.04 mg, range = 2.41 mg – 5.11 mg) such that analyses were not likely to be compromised by differences in feather mass (Lattin *et al*. 2011).

For blood CORT analysis, approximately 1ml of blood was taken from the brachial vein of each adult auklet within 3 min of capture to ensure baseline levels (Romero & Reed 2005) and dispensed in drops onto a paper blood spot card (Whatman), ensuring that blood soaked through the filter paper card. Blood cards were dried for at least 24 hours and a 3.2 mm punch was used to punch 24 spots from each sample card. Spots were analyzed in duplicate (12 spots for each duplicate) using COAT-A-COUNT Rat Corticosterone radioimmunoassay kits (Cat. # TKRC1, InterMedico, Markham, Ontario) as validated and described by Doody et al. (2008) and Rector et al. (2012). Resulting blood spot CORT values were converted using the equation described and validated in Rector et

al. (2012), and were reported as ng CORT / ml blood. Blood spots from 2010 and 2011 were assayed with different kits, which had intra-assay CVs of 8.5% and 6.3%, respectively, which are similar to %CVs reported by Rector et al. (2012). Variation between assays (2010 and 2011) was quantified using by including a standardized blood spot sample (same pooled murre sample as described in Rector et al. 2012). In the 2010 assay, CORT level of the standardized sample was very similar to previous CORT levels reported for the same sample in different assays (see Rector et al. 2012 for more detail on previous assays). However, in 2011, CORT levels of the standardized sample was higher than average, so CORT concentrations for 2011 were reduced by 15% (the same adjustment for a previous RIA assay with a high standardized sample reading, as described in Rector et al. 2012 and Doody et al. 2008). The difference between the standardized samples among kits is likely due to kits being manufactured in different lots.

3.3.4 Stable Isotope Analysis

We measured δ15N and δ13C values in a single breast feather from 64 female auklets from Lucy, Pine and S'Gang Gwaay colonies in 2009 (N = 8 on Lucy, N = 7 on Pine, N = 6 on S'Gang Gwaay), 2010 (N = 8, 8, 6) and 2011 (N = 8, 8, 7). We also measured δ15N and δ13C values in blood of females (40 of the same females as were used for feather δ15N and δ13C analysis, plus 12

additional females) on Lucy, Pine and S'Gang Gwaay in 2010 (N = 10, 17, 10) and 2011 (N = 11, 9, 5).

Fish samples were collected from auklets on Lucy, Pine and S'Gang Gwaay in 2010. Auklets returning to the colony with food for their chicks were startled and/or caught with a large, dip-net in order collect the fish they were carrying in their bills. Fish in each food load were identified to genus or species. The fork lengths of Pacific sandlance were measured using a ruler (± 1 mm), and whole fish were stored in 70% ethanol before being analyzed for δ15N and δ13C.

Whole breast feathers and a small piece of muscle from each fish were used for δ15N and δ13C analysis. Feathers and muscle pieces were placed in individual vials and soaked in 2:1 chloroform:methanol solution for 24 hours and then decanted. Feathers were air-dried and minced with scissors. Muscle samples were dried in an oven and ground with mortar and pestle. Approximately one milligram of each sample was weighed and placed in an individual tin capsule. Relative abundance of $^{15}N/^{14}N$ and $^{12}C/^{13}C$ were measured at the Stable Isotope Facility of the University of California, Davis. Stable isotope values are presented in delta notation (δ) as parts per thousand ($^{0}/_{00}$) using the equation:

$$\delta^{15}N \text{ (or } \delta^{13}C) = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where R is the ratio of $^{15}N/^{14}N$ and $R_{standard}$ for ^{15}N is atmospheric N_2 (AIR), or R is the ratio of $^{12}C/^{13}C$ and $R_{standard}$ for ^{13}C is PeeDee Belemnite. Measurement error was estimated to be ± 0.09 $^{0}/_{00}$ for $\delta^{15}N$ and ± 0.17 $^{0}/_{00}$ for $\delta^{13}C$ based on within-run replicate measurements of nylon (mean = -9.77 $^{0}/_{00}$) and glutamic acid (mean = -4.26 $^{0}/_{00}$) laboratory standards (2 standards for 12 unknowns). Feather stable isotope values were corrected by the discrimination factors suggested by Cherel et al. 2005: subtracting +0 $^{0}/_{00}$ from feather δ13C values and +4.2 $^{0}/_{00}$ from feather δ15N values. Blood stable isotope values were corrected by subtracting +0 $^{0}/_{00}$ from blood δ13C values and +3.49 $^{0}/_{00}$ from blood δ15N values, based on the discrimination factors of Sears et al. (2009). Prey muscle stable isotope values were converted to whole fish values by subtracting +0.32 $^{0}/_{00}$ from prey δ13C values and +0.86 $^{0}/_{00}$ from prey δ15N values (Cherel et al. 2005).

3.3.5 Chick Condition

Due to logistical constraints (i.e., widely separated colonies visited by our field crews only once per season), it was not possible to ensure that mass was measured in chicks of exactly the same ages. Therefore, we followed the methods of a previous study that compared performance of auklet chicks among different colonies using mass and wing chord (Bertram et al. 2002). We measured mass and wing chord of chicks on Lucy (N = 12), Pine (N = 15) and

S'Gang Gwaay (N = 8) in 2011. Chicks were removed from burrows, placed in a cloth bag, and weighed using a Pesola scale. Wing chord was measured using a wing ruler.

3.3.6 Statistical Analysis

RStudio 0.97.551 (RStudio Inc., 2012) and R version 3.0.0 (The R Foundation for Statistical Computing, 2013) were used for all data analysis.

3.3.6.1 Feather CORT

We used the generalized least squares (GLS) function to analyze the following model: Feather CORT ~ Colony + Year + Lot + ε. Since feather CORT was measured on EIA plates that were manufactured in two different lots, we used the 'VarIdent' term to formulate a variance structure that implements different variances for Lot 1 and Lot 2 (Zuur et al. 2009). We used analysis of variance (ANOVA) to first examine full models containing all interaction terms, but dropped non-significant terms one by one. Interaction terms were not significant, and thus, were not included in the final model in order to improve statistical power.

3.3.6.2 Blood CORT

To test for differences in blood CORT among colonies and years, we built

the following linear model: Blood CORT ~ Colony + Year + Colony * Year + ε. We analyzed this model using ANOVA.

3.3.6.3 δ15N and δ13C

We used multivariate analysis of variance (MANOVA, Wilks' Lambda test) to determine whether auklets from the three colonies (Lucy, Pine and S'Gang Gwaay) could be distinguished by their δ15N and δ13C values. We ran separate MANOVAs for each year for feather (2009, 2010 and 2011), blood (2010 and 2011) and sandlance muscle (2010) δ15N and δ13C values. Tukey post-hoc tests were used to examine differences. Stable isotope data were available for females only, but data from auklets on Triangle Island (Kouwenberg, unpublished data) indicated no significant affect of sex on feather δ15N ($F_{1, 38}$ = 1.108, p = 0.299), feather δ13C ($F_{1, 38}$ = 0.729, p = 0.399), blood δ15N ($F_{1, 38}$ = 1.852, p = 0.186) or blood δ13C ($F_{1, 38}$ = 0.310, p = 0.582).

3.3.6.4 Chick Condition

Following the methods of Bertram et al. (2002), we used analysis of covariance (ANCOVA) to compare the slopes of the relationships between wing chord and mass for chicks on the three different islands in 2011 (log chick mass ~ log wing chord * colony). Since the slopes between wing chord and mass were similar among colonies in 2011 ($F_{2,30}$ = 0.757, p = 0.478), we divided log

mass by log wing chord to create a single term called chick condition. Chick mass and chick wing chord were log-log transformed due to their curve-linear relationship. We then tested the effect of colony and year on chick condition using ANOVA. Tukey post-hoc tests were used to examine differences.

3.4 RESULTS

3.4.1 Can colonies be distinguished by their blood or feather δ15N and δ13C values?

As expected, female auklets that bred on Pine, Lucy and S'Gang Gwaay could not be distinguished by their feather (pre-breeding) δ15N and δ13C values in all years: 2009 (Wilks' lambda = 0.875, $F_{2,18}$ = 0.589, p = 0.673), 2010 (Wilks' lambda = 0.744, $F_{2,19}$ = 1.433, p = 0.243), or 2011 (Wilks' lambda = 0.681, $F_{2,20}$ = 2.010, p = 0.113). Also as expected, female auklets from the three colonies were easily distinguished by their blood (breeding season) δ15N and δ13C values in both 2010 (Wilks' lambda = 0.301, $F_{2,78}$ = 31.624, p < 0.001) and 2011 (Wilks' lambda = 0.321, $F_{2,73}$ = 27.526, p < 0.001). Additionally, muscle samples from Pacific sandlance (auklet prey samples collected in 2010) from different colonies could be distinguished by their δ15N and δ13C values (Wilks' lambda = 0.303, $F_{2,9}$ = 4.798, p = 0.038), suggesting different isotopic baselines among colonies. Consistent with blood stable isotopes, Pacific sandlance

collected on S'Gang Gwaay had lower (δ13C values than Pacific sandlance collected from Lucy (Tukey pairwise comparison: $t_9 = 2.968$, $p = 0.038$). Mean and SEM δ15N and δ13C values for blood, feather and sandlance samples are presented in Table 3.3.

Table 3.3 Mean feather and blood nitrogen (δ15N) and carbon (δ13C) stable isotope values of female rhinoceros auklets and Pacific sandlance prey samples from Lucy, Pine and S'Gang Gwaay colonies. Mean stable isotope values are reported for 2009, 2010 and 2011 (feathers), 2010 and 2011 (blood), and 2010 (Pacific sandlance). Mean feather (pre-breeding) stable isotope values were corrected by the discrimination factors suggested by Cherel et al. 2005: subtracting $+0^{0}/_{00}$ from feather δ13C values and $+4.2\ ^{0}/_{00}$ from feather δ15N values. Mean blood stable isotope values were corrected by subtracting $+0\ ^{0}/_{00}$ from blood δ13C values and $+3.49\ ^{0}/_{00}$ from blood δ15N values, based on the discrimination factors of Sears et al. (2009). Rhinoceros auklets from different colonies could be distinguished by their blood (breeding) stable isotope values, but not by their feather isotope values. Pacific sandlance δ13C values were significantly lower on S'Gang Gwaay than Lucy, and intermediate on Pine.

		Feather ‰ ±SEM		Blood ‰ ±SEM		Sandlance ‰ ±SEM	
Colony	Year	δ13C	δ15N	δ13C	δ15N	δ13C	δ15N
Lucy	2009	-17.84 ±0.28	12.70 ±0.54				
	2010	-17.61 ±0.18	12.55 ±0.29	-18.28 ±0.12	11.73 ±0.21	-17.00 ±0.19	12.98 ±0.46
	2011	-17.05 ±0.33	12.55 ±0.47	-18.69 ±0.13	11.68 ±0.12		
Pine	2009	-17.53 ±0.36	13.13 ±0.54				
	2010	-17.76 ±0.30	13.17 ±0.26	-17.71 ±0.07	12.17 ±0.03	-17.38 ±0.10	12.05 ±0.12
	2011	-17.20 ±0.57	13.18 ±0.42	-18.28 ±0.22	11.61 ±0.06		
S'Gang Gwaay	2009	-17.80 ±0.18	11.95 ±0.66				
	2010	-17.80 ±0.40	12.04 ±0.49	-19.04 ±0.25	12.10 ±0.10	-18.79 ±0.74	12.50 ±0.30
	2011	-17.81 ±0.31	13.74 ±0.26	-19.03 ±0.19	11.91 ±0.04		

3.4.2 Does feather (pre-breeding) CORT vary among colonies and years?

Consistent with stable isotope results, there was no effect of colony on feather CORT levels ($F_{2, 138}$ = 1.685, p = 0.189; Figure 3.2). As expected, given

the wide oceanographic variation (DFO 2009, 2010, 2011), there was a significant effect of year ($F_{2, 138} = 3.636$, $p = 0.029$) on auklet feather CORT levels. There was also the expected effect of lot (EIA plates were manufactured in two different lots; $F_{1, 138} = 237.380$, $p < 0.001$), which was accounted for by the addition of the 'VarIdent' term in the gls model. Feather CORT levels were significantly higher in 2009 (exceptionally cold-water pre-breeding conditions) than in 2010 (warm-water El Niño pre-breeding conditions; Tukey pairwise comparison: $t_{138} = 2.680$, $p = 0.022$), but feather CORT in 2011 (intermediate conditions) was not different from the other two years. Thus, the direction of the CORT level difference among years was opposite to our prediction that CORT levels would be higher in years with less favourable conditions.

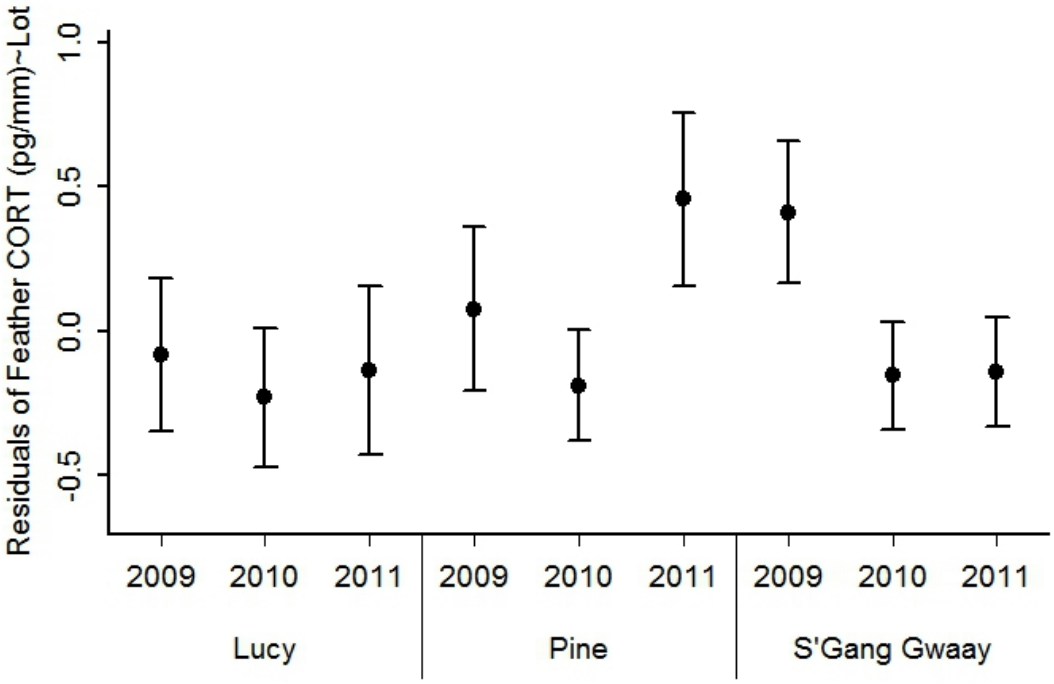

Figure 3.2 Feather CORT levels (pg/mm) for rhinoceros auklets on Lucy, Pine and S'Gang Gwaay in 2009, 2010 and 2011. In order to account for differences in variance due to lot, feather CORT values are presented as standardized residuals of the generalized least squares model: Feather CORT~Lot. Standard errors are plotted for each mean.

3.4.3 Does blood (breeding) CORT vary among colonies and years?

As predicted, there was a significant effect of colony ($F_{2, 159} = 4.131$, $p = 0.018$; Figure 3.3) on auklet blood CORT levels. Blood CORT was significantly

higher for auklets that bred on Lucy than for auklets that bred on S'Gang Gwaay (Tukey pairwise comparison: $t_{161} = 2.661$, $p = 0.023$), but CORT levels of Pine auklets did not differ from auklets on either Lucy or S'Gang Gwaay. Although the lower blood CORT levels of auklets in S'Gang Gwaay appear to be driven mostly by 2010 values (Figure 3.2), contrary to prediction, there was no statistically significant effect of year on auklet blood CORT levels ($F_{1, 159} = 1.432$, $p = 0.233$) or interaction between year and colony ($F_{2, 159} = 2.206$, $p = 0.114$).

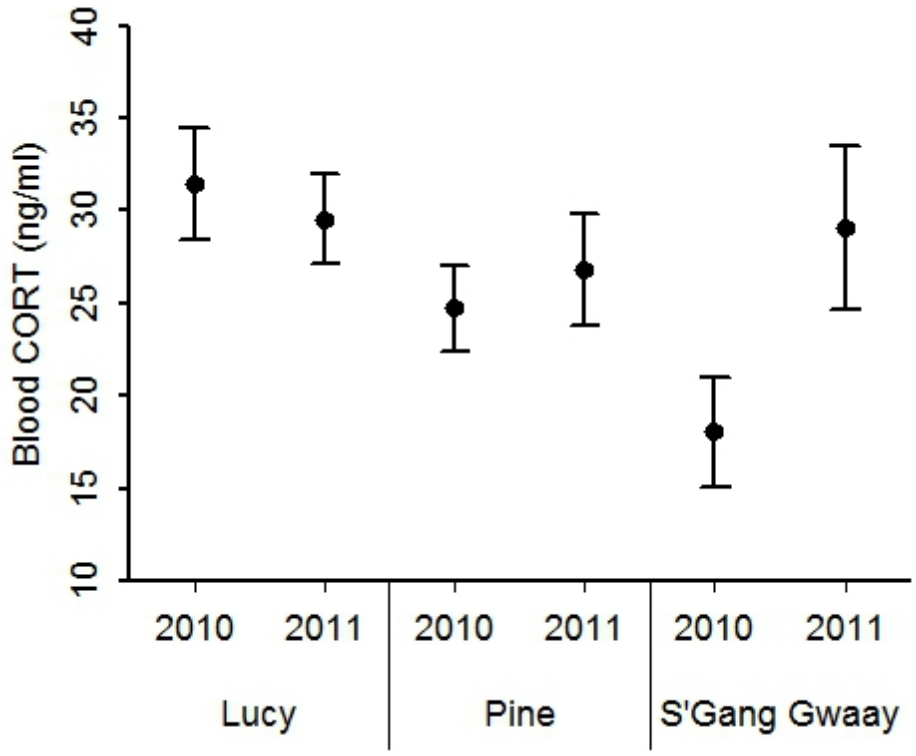

Figure 3.3 Blood CORT levels (ng/ml) for rhinoceros auklets on Lucy, Pine and S'Gang Gwaay in 2010 and 2011. Blood CORT levels of auklets on S'Gang Gwaay were significantly lower than blood CORT levels of auklets on Lucy. There was no significant year effect or interaction between years. Standard errors are plotted for each mean.

3.4.4 Does chick condition vary between colonies and is it consistent with variation in blood CORT?

There was a significant difference in chick condition among colonies in 2011 (ANOVA, $F_{2,33} = 6.636$, $p = 0.004$; Figure 3.4). Chick condition was lower on S'Gang Gwaay than Lucy (Tukey pairwise comparison: $t_{33} = 3.636$, $p = 0.003$), but neither Lucy nor S'Gang Gwaay were different from Pine. This finding is consistent with overall blood CORT levels of auklets in both years combined, which were significantly lower on S'Gang Gwaay than Lucy. However, when 2011 was analyzed separately (since chick condition data was available for 2011 alone), there was no significant difference in blood CORT among colonies ($F_{2, 73} = 0.254$, $p = 0.776$).

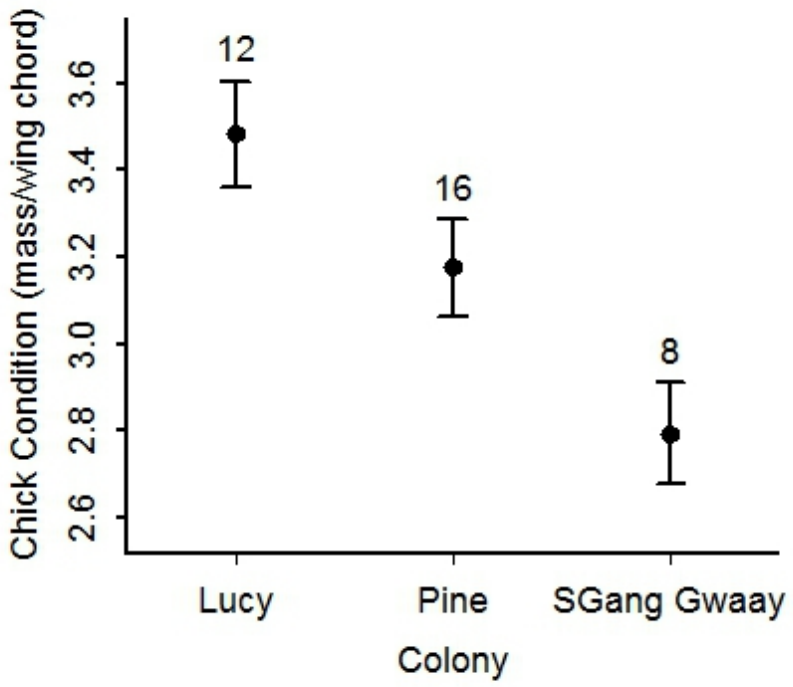

Figure 3.4 Body condition (chick mass (g) divided by chick wing chord (mm)) for rhinoceros auklet chicks on Lucy, Pine and S'Gang Gwaay in 2011. Standard errors are plotted for each mean and sample sizes are indicate above error bars.

3.5 DISCUSSION

Prior to breeding, auklets from different colonies could not be

distinguished by their (feather) δ15N and δ13C values, but while breeding, auklets from different colonies could be distinguished by their (blood) δ15N and δ13C values. Considering genetic similarity among auklets from Lucy, Pine and S'Gang Gwaay (Abbott et al. 2014), and previous studies that link seasonal differences in foraging habitats with patterns in isotopic composition of seabird tissues (Cherel et al. 2000; Cherel and Hobson 2007; Cherel et al. 2013), our stable isotope results validate our assumption that auklets from different colonies were not segregated during the non-breeding season, despite spending the breeding season on distinct, isotopically-segregated colonies (islands). Furthermore, since δ15N and δ13C isotopes define an individual's isotopic niche by reflecting trophic position and foraging habitat, respectively (Newsome et al. 2007; Jaeger et al. 2010), isotopic segregation of auklets by blood suggests that conditions were not identical for auklets from different colonies during the breeding period.

For Pacific sandlance collected from the three colonies during the breeding season, δ13C values were significantly lower on S'Gang Gwaay compared to Lucy and Pine. This difference in δ13C values in prey was matched in auklet blood, with ^{13}C being depleted in blood collected at S'Gang Gwaay, situated very close to the continental shelf break off the west coast of Haida Gwaii, compared to blood collected at Lucy and Pine, two colonies situated well up on the continental shelf and just off the mainland coast of British

Columbia.

Our finding that auklet CORT levels showed the same pattern of variation as isotopic data (different among colonies during the breeding season, i.e., in blood; but not prior to breeding, i.e., in feathers) supports our hypothesis that CORT levels vary with environmental conditions. Based on the late timing of spring phytoplankton bloom (as determined by chlorophyll-a concentrations; Borstad et al. 2012) near S'Gang Gwaay as compared to Lucy and Pine, we expected feeding conditions to be worse, and thus CORT levels to be higher, on S'Gang Gwaay than on Lucy and Pine during the breeding season. We found that CORT was significantly different between Lucy and S'Gang Gwaay, but the direction of the difference was opposite to prediction: auklets breeding on S'Gang Gwaay had lower blood CORT levels than auklets breeding on Lucy. As rhinoceros auklets are generalist foragers (Davies et al. 2009) and fish species that compose their diet samples tend to vary by colony and year (Bertram & Kaiser 1993; Bertram et al. 2002), lower CORT on S'Gang Gwaay may indicate that auklets buffered against assumed lower sandlance availability to feed on more available prey species, rather than mount a CORT response to forage for scarce sandlance. Blood CORT has been found to vary with differing foraging strategies in other species and taxa. For example, Harding et al. (2013) found that thick-billed murres *Uria lomvia* breeding on two colonies closer to a productive shelf-break had higher CORT levels than murres breeding on a more

oceanic colony, far from the shelf-break. Murres from the higher CORT colonies invested extra energy to forage on or near the shelf break, whereas murres from the lower CORT colony invested little energy and foraged in oceanic waters near the colony. As well, Romero and Wikelski (2001) found that, in a year with El Niño-induced famine conditions at six island populations, CORT levels of Galapagos marine iguanas *Amblyrhynchus cristatus* were high on all but one island, a difference that was attributed to the low CORT island being in a location that allowed iguanas to access an alternative nutrient source. Such local influences on food availability, in addition to the effect of spring bloom differences, may have affected food availability at colonies in our study, but further study is needed to determine if these local influences exist.

Our finding that blood CORT levels were lower on S'Gang Gwaay than Lucy may also suggest that auklet CORT levels are positively associated with food availability in certain contexts. This conclusion is supported by our finding that auklet feather CORT levels were significantly lower during the warm-water El Niño pre-breeding conditions of 2010 than during the exceptionally cold-water pre-breeding conditions in 2009. Conditions were presumed to be more favourable for food availability in 2009 than 2010, and both feather CORT levels and oceanographic conditions were intermediate in 2011. Lower CORT during less-favourable conditions was found previously for one colony of adult Atlantic puffins, a close relative of the rhinoceros auklet, in one year, but over a multi-

year dataset, Atlantic puffins showed no relationship between feeding conditions and CORT (Rector et al. 2012). For another seabird, the black-legged kittiwake *Rissa tridactyla*, researchers have consistently found a negative correlation between blood CORT and local food availability (Kitaysky et al. 2007, 2010), and thus, researchers consider lower CORT to be a proxy for greater food availability (Kitaysky et al. 2010; Satterthwaite et al. 2012). For example, Satterthwaite et al. (2012) found that kittiwakes from two distinct regions showed opposite relationships between CORT levels and environmental metrics that indicate warming conditions, and from this assumed that the lower CORT region had higher food availability and the higher CORT region had lower food availability. Our result that CORT levels were lower in auklets experiencing poorer feeding conditions suggests that the assumption made for kittiwakes (described above) cannot be made for rhinoceros auklets.

The above differences among studies may reflect species or population differences, or they may reflect differences in the degree of “bad” conditions experienced. It is possible that CORT levels decrease under fairly drastic conditions when parents decrease their foraging effort and provisioning rates to the point of decreased reproductive success. In contrast, CORT levels may increase under moderately bad conditions where parents can increase foraging effort enough to successfully fledge their chicks.

Although our results support the hypothesis that CORT varies with

environmental conditions, our hypothesis that CORT varies with reproductive success was not well supported. In 2011, a year of moderate environmental conditions and the only year for which chick condition data was available, body condition of chicks was lower on S'Gang Gwaay than Lucy, but adult blood CORT levels were not different among colonies. This finding runs contrary to our prediction, based on findings for Atlantic puffins (Rector et al. 2012), that adult blood CORT levels would be positively related to chick condition. Since elevated CORT levels are associated with increased provisioning or parental effort in birds (Love et al. 2004; Doody et al. 2008; Bonier et al. 2011; Crossin et al. 2012), our finding that blood CORT was not different among colonies suggests that parents' provisioning effort was not different among colonies. Lower chick condition on S'Gang Gwaay is consistent with the assumption that food availability and/or quality was lower on S'Gang Gwaay than Lucy (as predicted by spring bloom, see earlier discussion), but studies measuring adults and chicks throughout the breeding season are needed to fully characterize potential relationships between reproduction and CORT. Though challenging, measuring CORT levels over different seasons (pre-breeding and breeding), locations (colonies) and years provides a more comprehensive understanding of how CORT levels reflect the environmental factors that influence seabirds throughout their annual cycle.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4 – CORTICOSTERONE AND STABLE ISOTOPES IN FEATHERS PREDICT EGG SIZE IN ATLANTIC PUFFINS[#]

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4.1 SUMMARY

Examining factors that operate outside the breeding season may provide new insight into life-history traits such as egg size, in which individual variation has not been fully explained. We measured corticosterone (CORT) levels and $\delta^{15}N$ values (trophic level) in feathers grown several months before egg-laying to test the hypothesis that a female's physiological state and feeding behaviour prior to the breeding season can influence egg mass in Atlantic puffins *Fratercula arctica*. As predicted, egg mass increased with both CORT and $\delta^{15}N$ values in feathers, suggesting that the ability of female puffins to meet the nutritional costs of egg production is related to CORT promoting increased foraging effort during moult and to consumption of a higher trophic-level diet.

4.2 INTRODUCTION

The fitness consequences of egg size have been documented for mothers and offspring in many taxa (Sinervo *et al*. 1992, Einum & Fleming 1999, Kaplan & Phillips 2006). In birds, there is an advantage to offspring that hatch from a larger egg (Krist 2011), yet egg size varies greatly among individual females with no satisfactory explanation (Christians 2002). In his meta-analysis,

Christians (2002) found that repeatability of avian egg size generally exceeds 0.6, higher than for clutch size or laying date, and that single factors such as female age, body size and body mass generally account for less than 20% of the intraspecific variation in egg size. Recent studies support this notion, with female age and body condition either explaining a small to moderate amount of variation in egg size (Johnson *et al.* 2006, Beamonte-Barrientos *et al.* 2010), or having no apparent effect (Potti 2008, Svagelj & Quintana 2011). Within-season factors, such as food availability and temperature also generally account for less than 15% of egg size variation in birds (Christians 2002).

Bernardo (1996) suggested that egg size may be determined by many interacting factors, and depend largely on the ecological context in which the egg is produced. Given the longstanding inability to adequately account for intraspecific variation in avian egg size, researchers have recently begun to consider how behavioural factors operating outside the breeding season might be involved (Sorensen *et al.* 2009), and Williams (2005) has called for more rigorous studies of the physiological basis of the intraspecific variation. In Macaroni penguins *Eudyptes chrysolophus*, for example, physiological processes underlying egg formation, which determine 'reproductive readiness', begin while females are migrating to nesting areas, such that females that lay shortly after returning to the colony produce clutches with greater size variance than females that spend more time at the colony before laying (Crossin *et al.*

2010).

Here, we test the hypothesis that physiological and behavioural factors operating prior to the breeding season influence the size of the single egg laid by a common North Atlantic seabird, the Atlantic puffin *Fratercula arctica* (hereafter, puffin). To do this, we measured δ^{15}N values, which gauge the relative trophic level of feeding, and levels of the steroid hormone corticosterone (CORT), in wing feathers grown several months prior to breeding. CORT levels in avian blood fluctuate in response to environmental challenges (Wingfield & Kitaysky 2002), food availability (Kitaysky *et al.* 1999, 2007), and reproduction (Wingfield & Sapolsky 2003, Goutte *et al.* 2010). However, it is not possible to collect blood from puffins outside of the breeding season. Fortunately, CORT circulating in the blood is incorporated into growing feathers, such that CORT levels in feathers are correlated with circulating levels during moult (Bortolotti *et al.* 2008, 2009). We therefore predicted that δ^{15}N values and CORT levels in primary feathers would be correlated with egg mass. We also included laying date as a covariate because egg size declines with laying date in puffins (Harris 1980) and other seabirds that lay single-egg clutches (Birkhead & Nettleship 1982). In 2010, the abundance of Capelin *Mallotus villosus*, an important prey species for puffins in the northwest Atlantic (Nettleship 1972), was extremely low during the winter and early spring (Department of Fisheries and Ocean Canada 2011); at this time of year, puffins are distributed from the Newfoundland Grand

Bank to the Scotian Shelf in the Atlantic Ocean (Hedd *et al*. 2010).

4.3 METHODS

We worked on Gull Island, Newfoundland, Canada (47º16' N, 52º46' W) in 2010. Puffins were monitored from early May to determine date of egg laying and fresh egg mass. After hatching, the female parent in 12 nesting burrows was caught by hand or with a burrow noose. Primary feather six (p6), which is grown between January and April during the end of the prebasic moult (Pyle 2008), was collected from each adult, as was a small blood sample for genetic sexing. A DNeasy Blood and Tissue kit was used for DNA extraction (QIAGEN, Hilden, Germany). Individuals were sexed using highly conserved primers (2550F and 2718R) and a Chromodomain Helicase DNA-based method (Fridolfsson & Ellegren 1999). Capelin (N = 7) were also collected at this time.

The top 2-3 mm of each p6 feather and a small piece of muscle from each fish were used for stable nitrogen isotope analysis. Feather tips and muscle pieces were placed in individual vials and soaked in 2:1 chloroform:methanol solution for 24 hours and then decanted. Feather tips were air-dried and minced with scissors. Muscle samples were dried in an oven and ground with mortar and pestle. Approximately one milligram of each sample was weighed and placed in an individual tin capsule. Relative abundance of $^{15}N/^{14}N$

was measured at the Stable Isotope Facility of the University of California, Davis. Stable isotope values are presented in delta notation (δ) as parts per thousand ($^{0}/_{00}$) using the equation:

$$\delta^{15}N = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where R is the ratio of $^{15}N/^{14}N$ and $R_{standard}$ for ^{15}N is atmospheric N_2 (AIR). Measurement error was estimated to be $\pm$ 0.12$^{0}/_{00}$ based on within-run replicate measurements of nylon (mean = -9.77$^{0}/_{00}$) and glutamic acid (mean = -4.26$^{0}/_{00}$) laboratory standards (2 standards for 12 unknowns). Since whole fish are depleted in $\delta^{15}N$ compared to fish muscle alone (Cherel *et al.* 2005), Capelin values were corrected by -0.9 $\pm$ 0.1$^{0}/_{00}$. The discrimination factor of Cherel *et al.* (2005) for fish-eating seabirds (+4.2 $\pm$ 0.7$^{0}/_{00}$) was applied to correct for diet-to-feather fractionation.

The remaining part of each feather was used for determination of CORT levels. The calamus was removed and feather length was measured to the nearest millimeter. The feather was minced with scissors into a vial and immersed in 5 ml methanol for 15 hours (modified from Bortolotti *et al.* 2008). The solution was vacuum-filtered through filter paper (Whatman GF/B, 2.4 cm circles) and the resulting filtrate was evaporated under nitrogen gas. To reduce interference from lipids, each sample was subjected to a series of acetonitrile-

hexane extractions, where CORT is partitioned into the acetonitrile (Mansour *et al.* 2002). Earlier, we had found that extracting lipids from samples increased the consistency of CORT measurements (A. Kouwenberg unpubl. data). The purified residue was dissolved in 200 µl of EIA buffer solution (Cayman Chemical Company, Ann Arbor, USA). CORT levels were measured in duplicate using an enzyme immunoassay kit (EIA, Cayman Chemical Company), which is highly sensitive (detection limit: 35 pg/ml) and has low cross-reactivity with non-CORT compounds. As well, the standard curve produced by this EIA was found to run parallel to a curve formed by CORT values of serially-diluted puffin feather samples (A. Kouwenberg unpubl. data). Before being assayed, all samples were diluted with EIA buffer resulting in values that were between 20 and 80% binding, the optimal detection range for the EIA. The intra-assay coefficient of variation (CV) calculated from duplicate absorbance values was 3.13%. There is no inter-assay CV to report because all samples were run within the same assay. CORT values were converted to pg/mm using feather length measurements (see Bortolotti *et al.* 2009). All assayed feathers were of similar length (mean = 74.83 mm, range = 68mm – 83mm) and mass (mean = 53.74 mg, range = 46.89 mg – 61.85 mg) such that analyses were not compromised by feather mass differences (Lattin *et al*. 2011). Because CORT extraction and assay techniques reflect the extractable, immunoreactive CORT in feathers, rather than absolute biological levels, we limited our analysis to identifying

relative differences in CORT levels among feathers extracted identically and measured on a single assay plate.

We expected egg size to decline with laying date (Harris 1980), so were interested in whether effects of $\delta^{15}N$ and CORT, alone or in combination with laying date, would be additive. Five candidate models were developed to explain variation in egg size: (1) null model (intercept-only model), (2) laying date, (3) laying date + $\delta^{15}N$, (4) laying date + CORT, and (5) laying date + $\delta^{15}N$ + CORT. All models within each candidate set were ranked using Akaike's Information Criterion corrected for small sample size (AIC_c), based on the difference between each model's AIC_c and the lowest AIC_c from among the candidate set. These methods identify a single most parsimonious model (ΔAIC_c = 0.0), plus others receiving strong support (ΔAIC_c scores ≤ 4.0; Burnham & Anderson 2002). The AIC_w measures the weight of evidence in favour of a particular model on a scale from 0 to 1, given the data and candidate model set.

4.4 RESULTS

The full model including laying date, $\delta^{15}N$ and CORT offered the most parsimonious explanation for intraspecific variation in egg mass (mean mass ± SEM = 68.75 ± 3.72 g, range = 63 g – 75 g) in puffins (Table 4.1). This model received 95% of the model weight and had very strong explanatory power (R^2 =

0.82); no other model received strong support. Egg mass declined with laying date (parameter estimate in the full model = -0.67 g per day, 95% confidence limits: -0.95 to -0.39), but increased with both $\delta^{15}N$ values (1.26 g egg mass per $^{0}/_{00}$ of $\delta^{15}N$, 95% confidence limits: 0.49 to 2.03) and CORT levels (0.11 g egg mass per pg/mm of CORT, 95% confidence limits: 0.04 to 0.18) measured in primary feathers grown during the pre-breeding period (Figure 4.1). Based on the regression line (Figure 4.1 (b)), egg mass peaked as $\delta^{15}N$ values approached that for a diet consisting of 100% Capelin (mean ± SD = 11.96 ± 0.22$^{0}/_{00}$).

Table 4.1 Regression models considered for predicting variation in egg mass of Atlantic puffins. Models were assessed with Akaike's information criterion for small sample size (AICc; Burnham & Anderson 2002).

Model	R^2	**K**	**LIKAIC**	**ΔAICc**	**AICw**
Lay date + $\delta^{15}N$ + CORT	0.82	5	1	0	0.95
Lay date + CORT	0.57	4	0.02	7.7	0.02
Lay date + $\delta^{15}N$	0.58	4	0.03	7.35	0.02
Lay date	0.37	3	0.01	9.61	0.01
Null		2	0	13.31	0

K, number of estimable parameters; AICw, model weight.

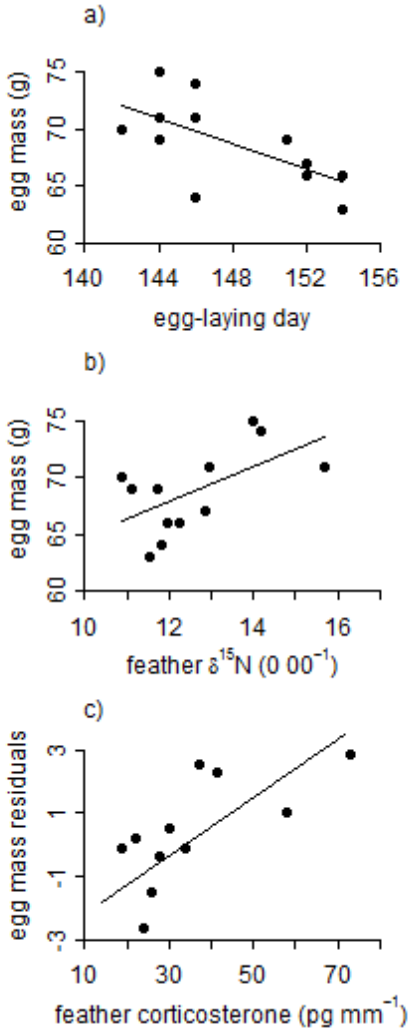

Figure 4.1 (a) Relationship between egg mass (g) and egg-laying date (day of year). (b) Relationship between egg mass (g) and stable nitrogen isotopes (corrected by a discrimination factor for fish-eating seabirds) measured in female Atlantic puffin p6 feathers ($\delta^{15}N$, $^{0}/_{00}$). (c) Relationship between corticosterone in female Atlantic puffin p6 feathers (CORT, pg/mm) and the

residual variation arising from the multiple linear regression of egg mass (g) against egg-laying date and feather stable nitrogen isotopes measured in female Atlantic puffin p6 feathers ($\delta^{15}N$, $^{0}/_{00}$). Solid lines in (a), (b) and (c) depict linear regressions.

4.5 DISCUSSION

As expected (Harris 1980), egg mass declined with laying date in Atlantic puffins, but increased with both $\delta^{15}N$ values (trophic level) and CORT levels measured in primary feathers grown several months before eggs were laid. These results support the hypothesis that behavioural and physiological factors operating prior to the breeding season influence egg size in this species. It appears therefore, that a puffin's ability to meet the considerable nutritional demands of egg production (Williams 2005) may be heightened both by elevating total CORT levels and by consuming a high trophic level diet prior to breeding. In birds, larger eggs produce larger hatchlings, which are more likely to survive periods of low food-availability (Parsons 1970), and in auks, nestlings from larger eggs develop wing feathers more quickly (Hipfner & Gaston 1999, Hipfner 2000). Thus, all else being equal, females should benefit by producing large eggs.

Egg mass increased with trophic level in puffins, peaking amongst

females whose $\delta^{15}N$ values approached those expected from a diet consisting exclusively of Capelin. In contrast, egg mass declined with trophic level in Cassin's auklets *Ptychoramphus aleuticus* (Sorensen *et al.* 2009), but whereas the mouth parts of auklets are adapted for feeding on zooplankton, the mouth parts of puffins are adapted for feeding on both zooplankton and fish (Bédard 1969). However, we caution that $\delta^{15}N$ values measured in feathers can also be affected by the degree to which a diet meets a consumer's amino acid needs, by the efficiency of protein deposition and by the amount of time spent fasting (Wolf *et al.* 2009). Hence, available discrimination factors may not fully reflect the dynamics of fractionation between the tissues of predator and prey (Hobson 2011). Determining trophic level on a finer scale may require analysis of individual amino acids that show constant isotopic variation with trophic level (Hobson 2011).

Moult is known to be a nutritionally demanding process for birds, requiring large amounts of protein (Murphy & King 1992) and an increase of up to 111% of basal metabolic rate (Lindström *et al*. 1993, Hoye & Buttemer 2011). Birds that experience food restriction during moult tend to grow weaker feathers and show abnormal patterns of feather regrowth (Strochlic & Romero 2008, DesRochers *et al.* 2009). Elevated plasma CORT levels are often associated with increased foraging effort in birds, and may help individuals meet environmental challenges (Astheimer *et al.* 1992, Kitaysky *et al.* 2001, Angelier

et al. 2007, Doody *et al.* 2008). With feeding conditions so poor (Department of Fisheries and Oceans 2011), a female puffin that increased CORT levels might have worked harder to obtain the nutrients required by wing-propelled divers to grow high-quality feathers. These high-quality feathers would facilitate maximum foraging efficiency later, during the breeding season. Although artificially elevating circulating CORT using implants has been found to reduce feather quality, CORT elevated through natural sources (psychological stress) affects feather quality only if birds are also food-restricted (Strochlic & Romero 2008, DesRochers *et al.* 2009). Therefore, greater nutrient intake due to increased CORT levels may have had a net positive effect on feather quality. In addition, the production of both eggs and feathers can be limited by availability of sulphur-containing amino acids (Murphy & King 1992, Murphy 1994), so efficient foraging during moult might create a store of these and other amino acids that might later be used in egg production (Kendall *et al* 1973, Houston *et al*. 1995a, 1995b).

Based on the definition of Harrison *et al.* (2011), our results are consistent with a role for carry-over effects in influencing egg size in puffins: under poor feeding conditions, differences among individuals in the trophic level at which they fed and in their CORT levels while they moulted their primary feathers (a clearly-defined transition period) closely predicted inter-individual variation in egg size in the following breeding season. Although our study

spanned just one transition period, and was correlational in nature, our results suggest that it is necessary to consider the entire annual cycle to understand variation during each stage of the cycle. Harrison *et al.* (2011) have called for experimental approaches that might better determine whether relationships such as we found are causal.

We conclude that measurement of CORT in feathers may provide valuable information about birds during stages of the annual cycle when they are inaccessible for sampling. Feathers provide an integrated value of CORT over a longer term, as opposed to the shorter-term 'snapshot' CORT value attained by assaying blood. Further, feathers do not require sampling to be completed in less than three minutes, as from blood (Romero & Reed 2005), and are better suited than plasma for storage in remote field settings. However, like Lattin *et al*. (2011), we believe there is need for further study of the physiology of integration of CORT into feathers if the technique is to provide results that can be fully interpreted.

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CHAPTER 5 – CORTICOSTERONE AND STABLE ISOTOPES IN FEATHERS DO NOT PREDICT EGG SIZE IN RHINOCEROS AUKLETS *CERORHINCA MONOCERATA*

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5.1 SUMMARY

Egg mass of Atlantic puffins has been found to vary with female pre-breeding corticosterone (CORT) levels and δ^{15}N values (trophic level), which suggests that conditions experienced by female puffins in the months prior to breeding play a role in subsequent reproductive effort. Based on this finding, we predicted that feather CORT levels and feather δ^{15}N values would be positively related to egg mass, yolk mass and yolk protein levels of a Pacific-dwelling, nocturnal relative of the Atlantic puffin, the rhinoceros auklet *Cerorhinca monocerata*. Although our sample size was small, we found a negative relationship between yolk mass and CORT levels in female rhinoceros auklet feathers, and no relationships between feather CORT levels or feather δ^{15}N values and egg mass or yolk protein levels. These results indicate that further study, particularly of yolk mass, is needed to fully understand potential carry-over effects on eggs.

5.2 INTRODUCTION

Increasing evidence suggests that carry-over effects, in which processes acting in one season influence an individual's success in the following season, can account for variation in fitness-related traits that cannot be explained by more immediate factors (Harrison et al. 2011). In particular, recent studies

suggest that diet and conditions experienced prior to arrival on the nesting colony are related to egg size of Cassin's auklets *Ptychoramphus aleuticus* (Sorensen et al. 2009), Macaroni Penguins *Eudyptes chrysolophus* (Crossin et al. 2010), and Atlantic puffins *Fratercula arctica* (Kouwenberg et al. 2013). Here, we examine potential carry-over effects on mass and composition of eggs of the Rhinoceros auklet *Cerorhinca monocerata* (hereafter, auklets), a close relative of the Atlantic puffin (hereafter, puffins). Both species nest in colonies during the breeding season, but puffins spend the non-breeding season offshore in the Atlantic ocean, whereas auklets spend it offshore in the Pacific ocean. We predicted that corticosterone (CORT) levels and stable nitrogen (δ^{15}N) values in feathers of female auklets would be positively correlated with egg mass, as was found for puffins (Kouwenberg et al. 2013). CORT is an avian glucocorticoid hormone that helps birds respond to predictable and unpredictable events during their annual cycle, such as reproduction (McEwen & Wingfield 2003; Crossin et al. 2013), severe weather (Wingfield & Kitaysky 2002), and changes in food availability (Kitaysky et al. 1999, 2007). Circulating CORT levels during feather growth have been found to correlate with CORT levels measured in feathers (Bortolotti et al. 2008, 2009), and thus, feathers can provide an integrated value of CORT levels during the pre-breeding period of seabirds, including auklets. Additionally, δ^{15}N values measured in feathers indicate the relative trophic level

at which a bird has been feeding during feather growth.

Numerous studies have found that egg mass is a good measure of egg macronutrient quality (see reviews by Williams 1994 and Hill 1995) and that dry components of yolk and albument are correlated with egg mass (e.g., St. Clair 1996; Kennamer et al. 1997; Flint & Grand 1999). However, it has also been found that some variation in egg mass is explained by egg water content (Ricklefs 1977; Ricklefs & Montevecchi 1979; Montevecchi et al. 1983), which suggests that measuring egg components (e.g., yolk size, protein content) provides a more precise measure of egg quality. Therefore, we measured egg components in this study. Specifically, because egg production may be protein-limited (Reynolds et al. 2003) and yolk mass is reflective of nutrient content of eggs (Williams 1994), we measured protein content and mass of yolks.

Producing a higher quality egg has the potential to be nutritionally and energetically demanding (see review by Williams 2005), and auks related to auklets (puffins and Razorbill, *Alca torda*) derive nutrients for their eggs, in part, from stored sources (i.e., acquired prior to breeding colony arrival; Bond & Diamond 2011). As well, a study of the consequences of relaying in auklets showed that rhinoceros auklets tend to invest more in their first (not taken by researchers) egg than in their relaid egg (Hipfner et al. 2004), suggesting that egg quality may be affected by nutrients acquired in the months before breeding.

Therefore, we hypothesized that diet of and conditions experienced by female auklets during the pre-breeding period (February-March) would be correlated with egg composition. Specifically, we carried out a pilot study to test the prediction that mass and protein content of yolks of auklet eggs would be positively related to feather CORT levels and to feather δ^{15}N values, which may be associated with foraging effort and diet quality in the months prior to breeding.

5.3 METHODS

We collected feather and egg samples from auklets nesting on Triangle Island, British Columbia (50° 52'N, 129° 05'W), which is part of the Scott Islands Group located 45km north-west of Vancouver Island. All samples were collected in May 2010, during the auklet egg-laying period. Stable isotopes (δ^{15}N values) and corticosterone were measured in breast feathers. Although rhinoceros auklets moult most of their breast feathers during a pre-basic moult that occurs from August to January, they also undergo a partial pre-alternate moult from February to March that includes some breast feathers (Pyle 2008; Howell 2010). Therefore, similarly to other studies measuring rhinoceros auklet breast feathers (Sorensen et al. 2009; Carle 2014), we assumed that grey-tipped breast feathers showing minimal wear were grown in February-March, and thus reflected the

auklet pre-breeding period.

5.3.1 Egg Mass Analysis

Grey-tipped breast feathers that appeared to be freshly grown were collected from 16 female auklets. Feather CORT and feather δ^{15}N analyses were performed for all 16 auklets as described in Kouwenberg et al. (2013) and Kouwenberg (in press), except that a whole breast feather was used for each δ^{15}N and CORT analysis, rather than pieces of the same feather. The intra-assay coefficient of variation (CV) for duplicate absorbance values was 6.30%. There was no inter-assay %CV because feather samples were all measured on a single assay plate. Eggs of sampled auklets were weighed (N = 16) to the nearest g with a pesola scale within one day of laying and most were replaced in the burrows. To test whether egg mass varied with δ^{15}N values and/or CORT levels in feathers of female auklets, we developed a series of models and used Akaike's Information Criterion corrected for small sample size (AIC_c) to identify the simplest model ($\Delta AIC_c = 0.0$), plus others receiving strong support (ΔAIC_c scores ≤ 4.0; Burnham & Anderson 2002). Candidate models also included laying date because, as for puffins (Kouwenberg et al. 2013), we expected egg mass to decline with laying date.

5.3.2 Egg Yolk Analysis

For yolk analysis, eggs were collected from 7 of the 16 female auklets described in the previous section (5.3.1). Whole yolk was successfully separated from albumen for 6 of the 7 fresh eggs and measured to the nearest g on an electric scale. A small sample of yolk was taken from all eggs (N = 7) and stored in 70% ethanol before analysis for protein content at Memorial University of Newfoundland. To determine protein content, lipids were removed from yolk samples and remaining protein was dissolved in 0.1 N NaOH and analyzed using the Buiret method (as in Hipfner et al. 2003). After confirming that our data met assumptions for parametric analysis, we used linear regression to test for relationships between feather values (δ^{15}N and CORT) and egg components (mass and protein content). RStudio 0.97.551 (RStudio Inc., 2012) and R version 3.0.0 (The R Foundation for Statistical Computing, 2013) were used for all data analysis.

5.4 RESULTS

5.4.1 Egg Mass Analysis

With 57% of the model weight, the model with lay date only provided the most parsimonious explanation for variation in egg mass of female auklets

(79.02 ± 0.89 g, range = 73 – 88) on Triangle Island (Table 5.1). Other models (Lay date + δ^{15}N, Null, and Lay date + CORT) received marginal support of 11-12% of model weight. As predicted, egg mass declined with date of laying (parameter estimate from top model = -0.50 g egg mass per day). Egg mass did not change significantly in relation to CORT level (0.04 g egg mass per pg/mm of CORT) or δ^{15}N values (0.81 g egg mass per $^{0}/_{00}$). Although our sample size was small (N = 16), power analysis revealed that it should have been possible to detect a relationship between egg mass and CORT of the same magnitude as was found for Atlantic puffins (Kouwenberg et al. 2013). Using the standard error of the parameter estimate for auklet egg mass and CORT (SEM = 0.05) and the parameter estimate for puffins (0.11 g egg mass per pg/mm CORT; Kouwenberg et al. 2013) produced $t_{16} = 2.2$, $p < 0.05$.

Table 5.1 Regression models considered for predicting variation in egg mass of rhinoceros auklets. Models were assessed with Akaike's information criterion for small sample size (AICc; Burnham & Anderson 2002).

Model	R^2	K	LIKAIC	ΔAICc	AICw
Lay date	0.32	3	1	0	0.57
Lay date + δ^{15}N	0.34	4	0.21	3.14	0.12
Null		2	0.20	3.19	0.12
Lay date + CORT	0.37	4	0.19	3.34	0.11
δ^{15}N	0.05	3	0.06	5.53	0.04
CORT	0.01	3	0.05	6.08	0.03
Lay date + δ^{15}N + CORT	0.38	5	0.04	6.64	0.02

K, number of estimable parameters; AICw, model weight.

5.4.2 Egg Content Analysis

Results of the pilot study showed a negative relationship between yolk mass and female feather CORT ($F_{1, 4}$ = 8.09, p = 0.047, N = 6, Figure 5.1), but no relationship between yolk mass and female feather δ^{15}N ($F_{1, 4}$ = 4.53, p = 0.101, N = 6). There was no relationship between yolk protein content and female feather CORT ($F_{1, 5}$ = 0.81, p = 0.410, N = 7) or female feather δ^{15}N ($F_{1, 5}$ = 0.02, p = 0.885, N = 7).

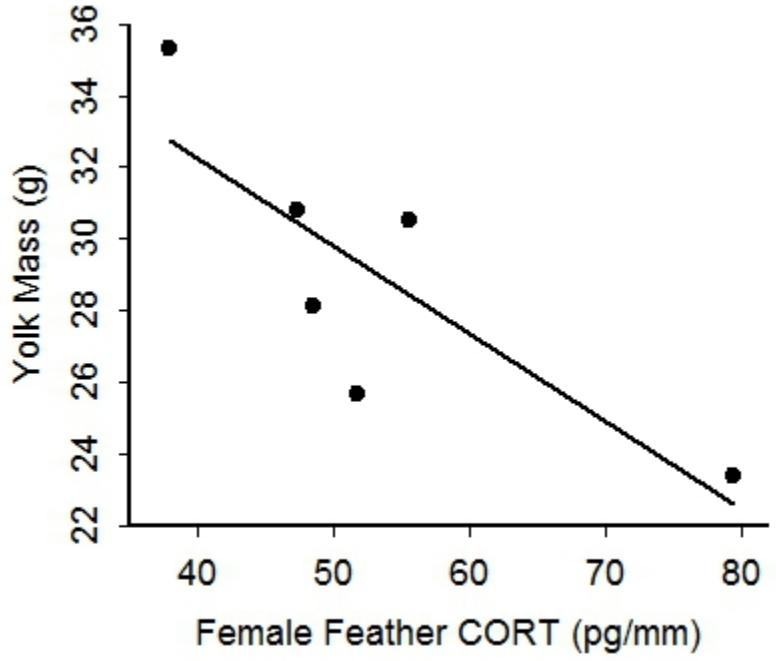

Figure 5.1 Mass of egg yolk (g) plotted against feather corticosterone (pg/mm) for female auklets breeding on Triangle Island in 2010. Least-squares regression line is shown.

5.5 DISCUSSION

Contrary to our hypothesis and previous findings for puffins, neither feather CORT nor feather $\delta^{15}N$ were strong predictors of egg mass in auklets.

However, auklets did show the expected negative relationship between lay date and egg mass. We suggest that pre-breeding conditions for auklets may not have been challenging enough to cause detectable differences in CORT and δ^{15}N of auklets laying larger or smaller eggs. Specifically, the timing of hatching for auklets on Triangle Island in 2010 was close to long-term averages (Crawford & Irvine 2011), despite an El Niño event in 2010 that affected the homogeneity of copopod species consumed by Pacific sand lance *Ammodytes hexapterus* (Hipfner & Galbraith 2013), a forage fish that influences auklet reproductive success (Borstad et al. 2010). In contrast, the puffins in the study by Kouwenberg et al. (2013) experienced low availability of an important prey species (Capelin *Mallotus villosus*) and poor chick growth rates in 2010, relative to other years in a ten-year data set (Rector et al. 2012).

It is also possible that the lack of relationship between egg mass and feather CORT and/or δ^{15}N may be related to potential variation in the time of growth of auklet breast feathers. Although we attempted to select only newly-grown (i.e., grown February-March) breast feathers, it is possibile that we selected some breast feathers that were grown during pre-basic moult (i.e., grown August-January; post-breeding). As our sample size was not very large, this extra variation may have masked relationships between pre-breeding signals and egg mass.

The significant negative relationship between female auklet feather CORT and yolk mass was opposite to our prediction. This result is preliminary, due to the small sample size, but it suggests that auklets were able to acquire nutrients for yolk production without drastically raising CORT to stimulate foraging. Alternatively, as high circulating CORT levels are associated with inhibition of yolk precursor production (Salvante & Williams 2003), it is possible that increases in CORT to stimulate foraging may have a net negative affect on yolk mass. The results of our preliminary auklet egg component analysis suggest that the relationship between feather CORT and yolk mass should be a focus for further research.

Taken together with the results of Kouwenberg et al. (2013), the results of the current study suggest that relationships between measures of pre-breeding conditions and egg mass may vary among puffin species, and that such relationships may only be detectable when puffins are experiencing challenging conditions. Specifically, egg mass and feather CORT/δ^{15}N may be positively correlated only when feeding conditions are poor and high quality prey require more effort to obtain. Overall, the results highlight the need for further study of larger samples of both species over multiple years and conditions in order to better detect correlations between CORT and δ^{15}N in feathers and egg mass/components, and to better understand what pre-breeding CORT and δ^{15}N

values reflect ecologically and how that might cause variation in egg mass/components.

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CHAPTER 6 – CONCLUSIONS AND SYNTHESIS

In this thesis, enzyme-immunoassay (EIA) was used to measure corticosterone (CORT) in feathers to test if CORT varies with spatial and temporal differences in environmental conditions, and in order to identify potential carry-over effects in alcid seabirds. The main strengths of this thesis are:

1. It contains, to my knowledge, the first article in the published literature that uses EIA to measure CORT in feathers.
2. It provides information valuable to other researchers who wish to use EIA and acetonitrile/hexane purification to measure CORT in feathers.
3. It uses a multi-season, multi-colony, multi-year dataset to provide support for the hypothesis that CORT varies with environmental conditions.
4. It provides some of the first evidence that CORT measured in feathers reflects conditions prior to breeding and can identify reproductive carry-over effects.

In addition to the main contributions listed above, the results of Chapters 3, 4 and 5 this thesis also provide support for previous suggestions (Fairhurst et al. 2013b; Bourgeon et al. 2014) that measuring feather stable isotope and feather CORT in tandem can help us to better understand relationships between

habitat/diet and physiology in birds. Analysis of stable isotopes in feathers provides an individual-based measure of habitat/diet and analysis of CORT in feathers provides an individual-based measure of physiological state. Therefore, when considered together, these metrics provided more precise, individual-based information, which allowed us to better test for relationships between environmental niche and CORT levels, and relationships between pre-breeding physiological and feeding conditions and subsequent breeding effort (i.e., carry-over effects).

The results of the experiments described in the chapters of this thesis are best summarized in terms of the hypotheses that were outlined in the thesis introduction. Therefore, the hypotheses are discussed individually in the sections below.

6.1 ENZYME-IMMUNOASSAY IS A VALID WAY TO MEASURE CORTICOSTERONE IN ALCID FEATHERS.

The results of experiments in Chapter 2 support the hypothesis that the Cayman Chemical Company EIA kit used in tandem with acetonitrile/hexane purification is a valid option for measuring CORT in the feathers of alcids. Moreover, our results suggest that subjecting feather samples to an

acetonitrile/hexane extraction procedure (in addition to methanol extraction) before EIA reduces variation between sample duplicates and provides a more reliable measure of feather CORT than methanol extraction and EIA alone. This finding led us to extract feather samples with acetonitrile/hexane before analysis with EIA in all experiments described in this thesis. As well, because we did not validate a set of standardized control feather extracts to measure inter-assay variation, we restricted all experiments to within-plate comparisons of feather CORT. We recommend both acetonitrile/hexane extraction and within-plate comparison as part of the standard procedure for using EIA to measure CORT in feathers.

Similar to Fairhurst et al. (2013a) and Will et al. (2014), we found that feather and blood CORT values were not correlated and do not represent identical measures. Specifically, we found that blood CORT values were not related to mass gain or body condition. In contrast, we found a negative relationship between feather corticosterone levels and pre-treatment body condition and a positive relationship between feather corticosterone levels and mass gain for Atlantic puffin chicks subject to a supplementary feeding experiment. As feathers were grown prior to and during the treatment period, our results suggest that corticosterone levels in feathers were influenced by nutritional status.

6.2 CORTICOSTERONE LEVELS EXPERIENCED BY ALCIDS WHILE BREEDING (MEASURED IN BLOOD) AND PRIOR TO BREEDING (MEASURED IN FEATHERS) REFLECT ENVIRONMENTAL CONDITIONS.

As reported in Chapter 3, we tested the hypothesis that CORT levels reflect variation in environmental conditions prior to and during the breeding season by measuring CORT levels and stable isotope values in both feathers (pre-breeding) and blood (breeding) of rhinoceros auklets nesting on three widely-dispersed colonies in British Columbia during years that spanned a broad range of oceanographic conditions in the north Pacific ocean. Our finding that rhinoceros auklets could be distinguished by their δ15N and δ13C values while breeding, but not prior to breeding was consistent with our assumption that auklets from different colonies were separated while breeding, but were not segregated during the pre-breeding season. Therefore, our finding that CORT levels were consistent with this pattern (different during the breeding season, but not prior to breeding) supports our hypothesis that CORT levels reflect differences in environmental conditions. However, we found that differences in blood CORT among colonies and years were opposite to predictions based on the spring chlorophyll bloom (Borstad et al. 2012) and ocean temperatures/events (Crawford & Irvine 2012): rhinoceros auklets had

significantly lower feather CORT levels in a year and on a colony assumed to have less favourable feeding conditions. Findings for other taxa generally show a negative relationship between food availability and CORT levels (Harding et al. 2013; Kitaysky et al. 2010; Romero & Wikelski 2001). Taken together, our results support our hypothesis that CORT varies with environmental conditions, but they also illustrate that the direction of this relationship may vary among taxa and contexts.

6.3 CORTICOSTERONE LEVELS EXPERIENCED BY ALCIDS PRIOR TO BREEDING (MEASURED IN FEATHERS) EXPLAIN VARIATION IN EGG MASS DURING THE SUBSEQUENT BREEDING SEASON

To determine if conditions experienced prior to breeding are correlated with subsequent reproductive effort, we measured both feather CORT levels and egg mass of female Atlantic puffins (Chapter 4) and rhinoceros auklets (Chapter 5). In Atlantic puffins, CORT levels and $\delta^{15}N$ values (trophic level) in feathers grown several months before egg-laying were positively correlated with egg mass during the subsequent breeding season, suggesting that the ability of female puffins to meet the nutritional costs of egg production is related to CORT promoting increased foraging effort during moult and to consumption of a higher

trophic-level diet. In contrast, in rhinoceros auklets, a close relative of the Atlantic puffin, pre-breeding (feather) CORT levels and $\delta^{15}N$ values were not related to egg mass, which suggests that the presence of a relationship between pre-breeding CORT and egg mass depends upon specific conditions and varies with differences in habitat and/or species. Our results indicate that conditions, such as availability of preferred prey, may influence the relationship between pre-breeding CORT and egg mass, and thus we suggest further study over varying conditions and species is needed to better understand this inter-seasonal relationship.

6.4 FUTURE DIRECTIONS

The results of this thesis indicate that EIA is valid for measuring CORT in feathers, and provide evidence that CORT levels reflect variation in environmental conditions and may carry-over between seasons. These findings provide a foundation for future studies, especially since the measurement of CORT in feathers is still a relatively new tool and many questions remain regarding the role of CORT in helping animals to mediate life-history trade-offs and transitions throughout the annual cycle. Follow-up experiments to those described in the preceding chapters could include: 1) validating EIA for other

non-alcid bird species, 2) testing ways to make the EIA and acetonitrile/hexane procedure less work intensive, 3) measuring CORT of individual rhinoceros auklets (and other puffin species) on colonies with quantified differences in food-availability and environmental conditions in order to determine the conditions under which puffin/auklet CORT levels are positively or negatively related to food availability, and 4) measuring reproductive performance (eggs and chicks) and feather CORT of the same birds over multiple seasons, years and colonies in order to better understand within- and between-season interactions among environmental conditions, reproductive success, and CORT.

As was outlined in the introduction of this thesis, the field of feather CORT research has evolved significantly over the past few years. One significant and persisting gap is the lack of detailed understanding of physiological and biochemical mechanisms by which CORT is deposited into feathers, and how this process may be influenced by other hormones or physiological processes. Considering that many researchers who are interested in using feather CORT are behavioural ecologists rather than endocrinologists or biochemists, this gap is not surprising; however, research in this area is an important 'next step' as more researchers begin to use feather CORT to interpret ecological and behavioural processes, particularly over long time scales. Although the basic principle that CORT is integrated into feathers as they are grown is well

supported (Bortolloti et al. 2008, 2009a), the results of this thesis, along with others (Fairhurst et al. 2013a; Lattin et al. 2011) have shown that questions remain about the nature of the relationship between circulating CORT and feather CORT and findings are often contradictory. A more detailed understanding of the process of CORT integration into feathers and how this integration relates to circulating CORT levels will allow for more precise interpretation of the biological and ecological implications of CORT level changes and/or differences. Overall, measuring CORT in feathers has the potential to be a powerful tool for answering long-standing questions of bird behaviour and ecology. However, as with any evolving technique, we must continue to develop our understanding of the biological meaning of values obtained in the laboratory. Furthermore, as suggested by Crespi et al. (2013), we must recognize that the implications of CORT level changes often depend on the ecological and evolutionary context in which they occur.

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