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Endogenous regulation of seasonal energetic phenotypes: investigating the hormonal mechanisms of fat gain and muscle growth across avian life-history stages in two Arctic birds

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By

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DECLARATION OF CO-AUTHORSHIP

I. Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is the result of joint research, as follows: I am the only author of Chapters 1 and 4, and I am the primary author on Chapters 2 and 3. Further, my thesis is co-authored by my co-supervisors, Dr. Oliver Love (Chapter 2 and 3) and Dr. François Vézina (Chapter 3), and my collaborators, Dr. Holly Hennin, Mr. Christopher Harris (Chapters 2 and 3), and Audrey Le Pogam (Chapter 3). In all cases, the key ideas, primary contributions, experimental designs, data analysis, interpretation, and writing were performed by the author, and contributions of co-authors was primarily on experimental design, lab work, editing, and funding. The exact contribution of each co-author is as stated: Dr. Love has provided guidance, insightful feedback, and funding throughout the entire duration of this degree; Dr. Vézina has contributed to housing and maintenance of my study species, as well as feedback on Chapter 3; Dr. Hennin aided in data collection and has provided feedback for all chapters; Mr. Harris provided logistical support in the laboratory and feedback on Chapter 2 and 3; and Ms. Le Pogam collected ample data.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

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ABSTRACT

Animals living or breeding in highly seasonal temperate and polar ecosystems require stored energy (i.e. lipids and protein) in the form of somatic reserves to prepare for predictable energetically demanding stages within their annual cycle, such as hibernation, migration, reproduction, or overwintering. However, the physiological mechanisms underlying fat and muscle gain in free-living vertebrates are not fully known. Nonetheless, research in mammals and poultry have identified a number of energy-regulating hormones that mediate metabolic (peripheral) and behavioural (central) effects on lipid and protein stores. Here I extensively reviewed the mechanistic advances on energy-regulating hormones in birds, and then integrate concepts from mammalian studies to design a conceptual framework for field-testing in avian systems. To test this, I then used a comparative approach to examine the temporal and stage-related variation in circulating levels of fat- (baseline corticosterone) and muscle-promoting (testosterone and insulin-like growth factor-1 or IGF-1) hormones before spring migration in captive male snow buntings (*Plectrophenax nivalis*) and breeding in free-living female common eiders (*Somateria mollissima*). Baseline corticosterone did not appear to signal for fat deposition in pre-migratory buntings, while slight and rapid elevations in eiders may stimulate fattening and may fuel reproduction, respectively. Elevated testosterone in buntings may mediate skeletal muscle growth as the variation temporally matched muscle scores, where elevations in female eiders are potentially more important for breeding behaviours. In both species, the down-regulation of IGF-1 may represent a fat-sparing action, where the up-regulation may be for visceral organ remodeling. My results demonstrate potential for hormone pleiotropy on complex phenotypes, and my thesis collectively highlights the value of examining naturally circulating hormone levels as foundational information on phenotypic changes across a broad range of birds with stages in highly seasonal ecosystems.

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GLOSSARY OF TERMS

Although terms and definitions may be used differently in other scientific literature, we define terms here specifically for this thesis document:

***Energetic gain*:** a broad categorization of energy assimilation and conversion into nutrient stores (i.e. carbohydrate, protein, and lipid storage), causing an overall positive energy balance in the organism.

***Energetic readiness*:** an ecological-based term of adaptive energetic gain that occurs during periods, or life-history (sub)stages, of long-term somatic energy storage associated with hyperphagia, fattening, and muscle gain.

***Homeostatic mechanisms*:** short-term, acute regulation of physiological processes that maintain energy balance through corrective responses from external or internal perturbations, including hormonal responses to energy deviation from seasonal levels.

***Rheostatic mechanisms*:** long-term control of homeostasis, such that temporal or seasonal shifts in the underlying mechanisms allow organisms to reach threshold levels of energetic storage. Rheostasis is therefore the physiological processes that regulate energetic readiness.

***Energy-regulating hormones*:** used synonymously with “metabolic hormones”; circulating hormones that regulate the increase or decrease of energy through both genomic and non-genomic pathways.

***Lipogenesis*:** the development of lipid accumulation (hypertrophy in mature adipocytes), in addition to the growth and division (proliferation) and conversion of pre-adipocytes into mature adipocytes (differentiation) in white adipose tissue.

***De novo lipogenesis*:** conversion of non-lipid macromolecules (i.e. carbohydrates and amino acids) into fatty acids within the liver.

***Myogenesis*:** a combination of myofiber (muscle cell) hypertrophy from protein synthesis and myosatellite cells’ proliferation and differentiation in adult skeletal muscle.

***Hypertrophy*:** increase in cell size, in the case of energetic stores referring to lipid (adipose) and protein (skeletal muscle) stores allows for greater intracellular storage of oxidative fuels.

***Hyperplasia*:** increase in cell number; typically from the conversion (differentiation) of precursor cells to active/mature storage cells

CHAPTER 1 – GENERAL INTRODUCTION

ENVIRONMENTAL SIGNALS OF SEASONAL CHANGES IN PHENOTYPES

Many organisms living in highly seasonal temperate and polar ecosystems exhibit adaptive phenotypic changes within the annual life cycle (Wingfield 2005; Piersma & van Gils 2011). From a proximate point of view, the phenology of these phenotypic changes are thought to be regulated by an endogenous clock that controls circannual rhythms (i.e. annual patterns of phenotypic change; Gwinner 2003; Visser et al. 2010; Gwinner 2012). The central elements of this endogenous clock (i.e. circadian clock genes located in the suprachiasmatic nucleus of the hypothalamus; Kumar et al. 2010) are entrained by the temporal changes in environmental information, including photoperiod as the primary predictive cue (or daylength; Dawson et al. 2001; Helm et al. 2013) and other secondary modifiers, such as temperature, weather, food availability, and social status (Wikelski et al. 2008). Furthermore, unpredictable perturbations in environmental conditions (i.e. weather) can be stressful in that it may lead to direct disruption of life-history stages and cause facultative responses in individuals beyond those predictable changes (Wingfield & Ramenofsky 2011). Nonetheless, photosensitive organisms rely on photic information to synchronize the appropriate timing of circannual rhythms, despite being considered “free-running” in constant photoperiod conditions (Gwinner 2012). In free-living animals, photoperiod and circannual rhythms together orchestrate changes in the physiology and behaviours that regulate phenotypic responses, such as colouration for camouflage (Ducrest et al. 2008), social behaviours for reproduction (Soares et al. 2010), thermoregulatory capacity for cold winters (Swanson 2010), and fat storage for migration (Ramenofsky 1990). These seasonal phenotypic responses have been broadly categorized into distinct life-history stages to more easily examine the underlying intra- and inter-individual variation in physiological and behavioural traits within stage- and species-specific contexts (Piersma & van Gils 2011; Williams 2012).

From an ultimate point of view, phenotypic changes in response to environmental variation prepare organisms for demands of upcoming life-history stages, with many corresponding to energetic demands across a sequence of consecutively ordered or even overlapping stages within the life cycle (Wingfield 2008). To meet the requirements of these energetically demanding stages such as migration (Ramenofsky & Wingfield 2007), hibernation or estivation (Geiser & England 2010), reproduction (Stephens et al. 2009), and overwintering (Giacomini & Shuter 2013), many organisms rely on energetic storage as an adaptive strategy to draw on nutrient (fuel) reserves. Further, individuals of many species must budget their total energy content by allocating food resources towards flexible adjustments in body composition (Guglielmo & Williams 2003; Hurst & Conover 2003; Arnold et al. 2006). Not surprisingly then, seasonal changes in food intake and rate of energy assimilation causes increases in body weight (Bairlein & Gwinner 1994; Loudon 1994). Taken together, the precise timing and execution for acquiring and storing energy is predicted to have important implications on performance traits during periods of expected high energy expenditure (Mrosovsky & Powley 1977; Moghadam et al. 2015).

ENERGETIC READINESS: AN ECOLOGICAL PERSPECTIVE OF SEASONAL ENERGY STORAGE

Across vertebrates, at the cellular level, the universal metabolic currency molecule is adenosine triphosphate (ATP) produced from all three major oxidative fuel sources (carbohydrates, lipids, and protein), with the majority of long-term energy production from stored lipids (Weber 2001). Expanding to the whole-organism scale, excess accumulation of these fuel sources in peripheral tissue (i.e. white adipose tissue, skeletal muscle, and liver) can be readily hydrolyzed and mobilized to provide energy to functionally active tissue (Jenni et al. 1998; Humphries et al. 2003). Previous studies often categorize the gain in stored energy into only specific stages (i.e. migratory fattening; Cornelius et al. 2013). To encompass both molecular and whole-organism scales, I have developed the term *energetic readiness* for this thesis, which describes a

preparative period of long-term, somatic energy storage acquired specifically to fuel metabolic processes during energetically demanding periods.

In using this term, I emphasize the articulation of long-term energy storage, as some species regulate within-day changes in body composition in response to local environmental conditions. For instance, a manipulative study on European starlings (*Sturnus vulgaris*) given an unpredictable food supply and high wind exposure had significantly higher fat mass at dusk than individuals with a predictable food supply and low wind conditions (Cuthill et al. 2000). Additionally, since glycogen (carbohydrate) stores in the liver and skeletal muscle do not provide a long-lasting energy source (Weber & Haman 2004), lipid stores in white adipose tissue is typically the primary source of long-term energy, followed by protein sources from skeletal muscle tissue and visceral organs that can act as reserve fuel when stored lipids are depleted past a critical threshold (Guglielmo 2010; McCue 2010). Another implicit component of the term energetic readiness within an ecological and evolutionary framework is the adaptive value of seasonal energetic storage to optimize organismal performance during a period of high energy demand rather than acting solely as a survival strategy (Rogers 2015; Higginson et al. 2016). Altogether, energetic readiness captures the ecological context of energetic storage within a life-history stage framework, and I frequently revisit and employ this term throughout this thesis.

Vertebrates living in seasonal environments demonstrate energetic readiness across a wide variety of life-history stages. For example, some teleost fish acquire pre-winter lipid stores to endure adverse overwintering conditions in temperate and polar regions (Atlantic silverside, *Menidia menidia*; Schultz & Conover 1997; Atlantic salmon, *Salmo salar*; Berg et al. 2009; brown trout, *Salmo trutta* Berg et al. 2011). In addition, both short- and long-lived domesticated (i.e. Siberian hamsters, *Phodopus sungorus*; Bartness & Wade 1985; Soay sheep, *Ovis aries*; Lincoln et al. 2001) and free-living mammals (i.e. ungulates, Parker et al. 2009; rodents, Nunes et al. 2006; Li & Wang 2005) increase body mass and adiposity to prepare for energetic costs during breeding or wintering. More dramatic examples which involve the rapid accumulation of

stored lipids, such as transoceanic migrations of *New World* warblers (i.e. Blackpoll warbler, *Dendroica striata*; Davis 2001; Connecticut warbler, *Oporornis agilis*; McKinnon et al. 2017), the winter sleep of brown bears (*Ursus arctos arctos*; Hissa et al. 1998), and the upstream migration of anadromous Atlantic salmon (*Salmo salar*; Jonsson et al. 1997), strongly support a widespread role for energetic readiness. Unfortunately, knowledge of the underlying physiological and molecular mechanisms that regulate these anabolic processes in ecologically relevant species still remains incomplete, especially in non-mammalian species. Nonetheless, integrating molecular, physiological, and ecological techniques has the potential to better define the linkages between endogenous control systems and changes in whole-organismal energetic state (Hahn et al. 2015).

HORMONES AS PHYSIOLOGICAL REGULATORS OF SEASONAL PHENOTYPES

Environmental endocrinologists have identified hormones as an important endogenous link between seasonal changes in environment and adaptive phenotypic change at various life-history stages (Dawson 2008). Moreover, hormones have been extensively studied for their downstream effects *via* activation of various signaling pathways that affect seasonal phenotypes (Israel 2009). For instance, species breeding at mid to high latitudes often experience gonadal maturation from increasing photoperiod associated with increases in central-acting gonadotropin releasing-hormone and circulating levels of luteinizing hormone, which are both important hormones in the hypothalamic-pituitary-gonadal axis for initiating reproductive activities (Hahn et al. 2004; Dawson 2015). Glucocorticoids are another well-studied hormone shown to exhibit seasonal fluctuations at baseline levels that differ according to the degree of energetic demand, typically with the highest levels during breeding in vertebrates (Romero 2002; Romero et al. 2017). Additionally, thyroid hormones have been recently implicated in the central regulation of seasonal timekeeping

mechanisms that may further initiate a cascade of peripheral signals leading to stage-related phenotypes (Wood & Loudon 2014).

While less often appreciated in realm of ecology and evolution, hormones can also act as potent agents of energy balance and metabolism at both the central and peripheral levels (Murphy & Bloom 2006; Scanes & Braun 2013; Chapter 2). These proximate mechanisms of endocrine responses suggest that seasonal regulation of hormone-mediated responses may play a pivotal role in promoting lipid deposition and protein anabolism for energetic readiness (Hahn & Denlinger 2007; Ramenofsky 2011). Despite the increasing application of environmental endocrinology to assess responses of individuals (Williams 2008) and populations in their environments (Bradshaw 2007), it still remains unknown whether energy-regulating hormones are robust regulators of energetic readiness in free-living vertebrates.

STUDY SYSTEMS

To investigate some of these candidate energy-regulating hormones, I chose two Arctic breeding birds, snow bunting (*Plectrophenax nivalis*) and common eider (*Somateria mollissima*), with their annual life cycles being in highly seasonal environments. Importantly, these species act as models of energetic readiness in which both birds require large accumulation of fat stores and skeletal muscle growth to fuel spring migration in snow buntings (Vincent & Bedard 1976; Ramenofsky 2011) and reproduction in common eiders (Sénéchal et al. 2011; Hennin et al. 2015). Despite the reasons for energetic storage in each species being different, I examined potentially common physiological mechanisms underlying energetic readiness at two different stages in species with different life-histories (i.e. lifespan and organization of annual life cycle). Furthermore, in designing my research hypotheses, I predicted the rudimentary physiological mechanisms should be conserved across birds, and depending on results that support either common or divergent mechanisms between species, it would provide insight into the importance of ecological contexts (i.e. species- and stage-related differences) on hormone levels.

Snow buntings. Snow buntings are medium-sized (~31-46 g) cold-adapted songbirds that winter in temperate-circumpolar regions and breed across the circumpolar Arctic (Montgomerie & Lyon 2011). In the wild, these birds are granivorous, ground-level foragers, and feed on mainly seeds (i.e. weeds, grasses, sedges, and grains) during the majority of the annual cycle, shifting to invertebrates (i.e. beetles and fly larvae) during breeding (Montgomerie & Lyon 2011). Snow buntings overwinter throughout northern United States and southern Canada (Macdonald et al. 2012). Similar to other Arctic-breeding passerines, males (mean mass = 40 g; mean wind chord = 110 mm) are larger bodied than females (mean mass = 37 g; mean wing chord = 104 mm; Montgomerie & Lyon 2011). This sexual dimorphism is thought to influence latitudinal differences in non-breeding sites between sexes, as current information in snow buntings supports the *body size hypothesis* that states larger body sizes correspond to a greater thermoregulatory capacity, which allows males to spend the winter in colder, more northern wintering sites relative to females (Macdonald et al. 2016; Laplante 2017).

The wintering population from which the male birds in this study originated spend the winter along the St. Lawrence-Southwestern Ontario corridor (Macdonald et al. 2012; Laplante 2017). During the early spring (April-May), free-living buntings in this population migrate up the St. Lawrence seaway to make a minimum 700-1000 km single flight across the Labrador sea from northern Newfoundland or Labrador to the southern tip of Greenland (Macdonald et al. 2012). This migratory feat makes our study species a strong model for examining the mechanisms underlying changes in energetic storage since prior to crossing this large ecological barrier birds must gain between 15-25 g of body fat and flight muscle growth (i.e. males are between 45-60 g in body mass; O. Love, unpubl. data). These rapid, large scale changes in body composition are seen in other migratory birds flying across physical barriers (i.e. ocean or deserts), such as garden warblers (*Sylvia borin*; Bauchinger et al. 2005), red knots (*Calidris*

canutus; Vézina et al. 2011), and American redstarts (*Setophaga ruticilla*; Smith & Moore 2003).

After arrival on breeding grounds in Western Greenland (May-June), it is presumed that males establish territories and actively defend them in an environment with cold and unpredictable conditions. Contrary to previous findings that males arrive on breeding ground much earlier than females (Meltofte 1983), McKinnon et al. (2016) used modern tracking techniques to determine that only early and older males appear to arrive approximately one week before females, indicating differences in spring arrival dates between sexes are closer temporally than previously thought. However, these birds are from the East Bay breeding population, and modern tracking techniques have not been applied to free-living individuals in the Greenland breeding population. Nonetheless, once females arrive and breeding pairs are formed, snow buntings continue to be socially monogamous throughout the breeding season (June-July), with males assisting in mate provisioning during female incubation and nest building, as well as bi-parental care during chick rearing (Lyon & Montgomerie 1985; Lyon et al. 1987).

Common eiders. Common eiders (*Somateria mollissima*) are long-lived (~10 years on average; Coulson 1984) seaducks that form large colonial aggregates in circumpolar marine environments throughout their annual life cycle (Goudie et al. 2000). These birds dive for benthic food items in open marine habitats to obtain benthic invertebrate prey bivalves (*Hiatella arctica* and *Serripes* species), amphipods (*Gammarus* species), and gasteropods (*Acmea testudinalis*; Heath et al. 2007; Sénéchal et al. 2011). Before sea ice fully develops, the northern population of eiders in the Canadian Arctic (*S. mollissima borealis*) forage in polynyas, open water surrounded by sea ice, along the coasts of southwestern Greenland (Boertmann et al. 2004) and northern Newfoundland and Labrador during the non-breeding period (Mosbech et al. 2006). During spring migration, eiders travel as winter-established pairs or form breeding pairs during the trip to breeding grounds in eastern Canada and western Greenland. Females are reproductively mature around 2-3 years old (Milne 1974), and demonstrate a high

degree of natal philopatry (Reed 1975; Mosbech et al. 2006). Common eiders are sexually dimorphic in colouration, and important to this research, in energetic state throughout the breeding season.

Common eiders are known to be mixed strategy (capital-income) breeders that rely on endogenous fat stores and muscle hypertrophy (capital breeding) along with accrued resources from foraging on Arctic breeding grounds (income breeding; Sénéchal et al. 2011). Capital breeding birds mainly invest in fat stores and skeletal muscle hypertrophy on wintering grounds, during migration, or on breeding grounds to later mobilize lipids and proteins from fuel depots and endogenous tissue to maximize the chance of reproductive success (Klaassen 2003; Sandberg & Moore 1996). On the other hand, income breeding birds acquire energy on the breeding grounds and directly shuttle these nutrients towards egg formation and self-maintenance during reproductive stages (Klaassen et al. 2006). To meet the energetic demands of reproduction, pre-breeding females spend a greater amount of time diving for food and increased feeding intensity relative to the annual average (Christensen 2000; Guillemette 2001). Moreover, females rapidly gain lipid stores after arriving on the breeding grounds for both (i) egg development and growth, and (ii) long (~25-27 days) fasting period during egg incubation (Bottitta et al. 2003; Korschgen 1977; Rigou & Guillemette 2010). During incubation, females only leave the nest infrequently and for very short periods of time to drink because egg predation is common in this species and nest vigilance can reduce the risk of predation (Korschgen 1977; Criscuolo et al. 2000), supporting the need for body reserves for reproduction (Bolduc & Guillemette 2003). In addition, the rate of fattening on the breeding grounds depends on female body condition at arrival and affects the delay before laying (pre-laying period between arrival date and first lay date; Hennin et al. 2016), ultimately affecting the timing of reproduction (Descamps et al. 2011; Love et al. 2010) and fecundity (Hennin et al. 2017, under review). Nevertheless, all females that are committed to breeding must experience some degree of lipid deposition and muscle growth to fuel reproduction, making this a strong model for studying the mechanisms of energetic readiness (Sénéchal et al. 2011).

THESIS OBJECTIVES

My thesis research aims to develop a mechanistic framework to examine the phenology of endocrine responses as potential mediators of energetic readiness during key life-history stages in birds. The overall goals of my exploratory research are to (1) provide a review of the current avian literature with regards to the most likely hormonal regulators of energetic storage based upon what is currently known from birds and mammals, and (2) examine the coarse- and fine-scale temporal relationships between candidate hormones and changes in energetic readiness within two representative species. Specifically, in **Chapter 2**, I use a comprehensive review of domesticated, captive, and free-living birds to assess the role that hormones play in regulating fat storage and skeletal muscle growth in birds, supplementing unknown mechanistic information with biomedical and agricultural research in mammals. In **Chapter 3**, I then assess a number of these pathways by examining the temporal variation in plasma energy-regulating hormones in two Arctic-breeding birds (snow bunting and common eider) that each require significant stores of lipids and protein for two different life-history stages: migration and breeding, respectively. Where possible, I augment these temporal patterns with correlational analyses between hormones and the energetic phenotypes of fat and muscle gain. Finally, in **Chapter 4**, I summarize and discuss the major findings and implication of this thesis, and importantly highlight some gaps of knowledge in endocrine regulation of energetic readiness in birds. Overall, my thesis collectively offers foundational information on likely endogenous control mechanisms not only in these two Arctic-breeding species, but potentially across a broad range of birds that require energetic readiness in highly seasonal ecosystems.

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CHAPTER 2 – HORMONAL CONTROL MECHANISMS OF ENERGETIC STORES ACROSS AVIAN LIFE-HISTORY STAGES: LESSONS FROM MAMMALIAN AND POULTRY SYSTEMS

GENERAL OVERVIEW

All organisms living in seasonal environments must organize their physiological, behavioural, and morphological activities into various life-history stages to maximize survival and reproductive success (Visser et al. 2010). For vertebrate species in highly seasonal ecosystems (i.e. temperate or polar; Wingfield et al. 2004; Ebling & Barrett 2008), the temporal separation of discrete stages reflects varying degrees of energetic demand, prompting adaptive changes in physiological state across the annual cycle (Hazlerigg & Loudon 2008). Physiological responses to these demands often involve some form of stage-related *energetic gain* (i.e. lipid and protein stores), to prepare for energetically demanding periods such as reproduction (birds: Dawson et al. 2001; Houston et al. 2007; fishes: Chemineau et al. 2007; Mcbride et al. 2015; mammals: Lincoln et al. 2006, Barrett et al. 2007), long-distance migration (birds: Jenni et al. 1998; Ramenofsky & Wingfield 2007; fish: van Ginneken 2005; mammals: Owen et al. 2017), or prolonged hibernation or estivation (mammals: Geiser & England 2010; Florant & Healy 2012; Williams et al. 2014; anurans: Secor 2005; Mantle et al. 2009).

Although nearly all vertebrate taxa rely on energy stores to fuel metabolically demanding stages, endothermic mammals and birds that reproduce and over-winter at temperate/polar latitudes are prime models for examining the mechanisms underlying seasonal change in body composition, primarily due to their fixed (predictable) periodicities of pronounced changes in energy stores (Lindstedt & Boyce 1985; Boonstra 2004; Ebling 2015) and high degree of phenotypic flexibility in energetic traits (Piersma & van Gils 2011). In

*This chapter is the result of joint research with C. Harris, H.L. Hennin, and O.P. Love.

addition, the ecological literature on mammalian and avian systems offers the most evidence-based research on *energetic readiness*, long-term somatic energy storage prior to an energetically demanding life-history stage, through studies that incorporate a combination of life-history variation, energetic physiology, and endocrine regulation (Reeder & Kramer 2005; Williams 2012). Furthermore, mammals and birds share similar anabolic effects of fat deposition (Odum & Perkinson 1951; King & Farner 1965; Bartness & Wade 1985; Bartness et al. 2002) and skeletal muscle growth (Bauchinger & Biebach 2001; Bauchinger & Biebach 2005a; Glass 2005), as a direct result of the ingestion and storage of excess nutrients into peripheral somatic tissue. From a broad mechanistic point of view, regulatory systems of energy balance are mediated through (1) *homeostatic mechanisms* (i.e. maintaining positive energy balance; Cone 1999; Boswell 2010) and (2) *rheostatic mechanisms* (i.e. a set-point system to obtain threshold levels of energy; Mrosovsky & Powley 1977; Mrosovsky 1990). Current empirical evidence primarily focuses on the homeostatic mechanisms for short-term increases in food intake and energy stores, whereas the rheostatic mechanisms driving energetic readiness are relatively unknown, but remain an important topic of physiological research in ecology (discussed in Cornelius et al. 2013).

Neuroendocrine and endocrine signals are strong candidate mechanisms underlying energetic readiness, as hormones coordinate rapid transitions between life-history stages (Wingfield 2008) and translate environmental information processed in the central nervous system (CNS) to peripheral organs and other somatic tissue that regulate energy balance (Murphy & Bloom 2006). Endocrine secretions also demonstrate (i) circannual rhythms that are synchronized by photoperiodic timekeeping mechanisms in the CNS (Gwinner 1996; Wikelski et al. 2008), (ii) anabolic effects that promote lipid and protein storage as the most common long-term energy reserves in free-living vertebrates (Weber 2001), and (iii) co-evolution of tightly linked endocrine signals to induce complex life-history phenotypes (Ketterson & Nolan 1999; Higginson et al. 2016). Currently, mammalian studies predominate the field of energetic endocrinology

with extensive research on the human obesity epidemic (Guyenet & Schwartz 2012), despite the known importance of the predictable acquisition of energy stores for energetically demanding life-history stages in birds, primarily for migration (Jenni et al. 1998; Guglielmo 2010) and reproduction (Drent et al. 2007; Varpe et al. 2009). For these reasons, it is important to assess (1) our current knowledge of the role of *energy-regulating hormones* (hormones with central and/or peripheral effects on metabolism), as mediators of energetic balance in avian systems, and (2) whether novel information from mammalian studies can be integrated and tested within avian systems.

Here we review endocrine traits known or assumed to play a mechanistic role in driving energetic readiness in avian systems by building on a conceptual framework from mammalian systems, with the aim of encouraging future research on energy-regulating mechanisms in both captive and free-living birds. First, we broadly review the anabolic mechanisms of lipid and protein synthesis, the two primary forms of energetic storage for migration and breeding in avian species. We then outline the avian-based studies that directly and indirectly measure endocrine responses as signals of energetic storage. Finally, we discuss potential future opportunities and constraints associated with trait-based approaches in examining complex endogenous pathways. The main goal of this review is to provide avian ecophysiologists with a tangible roadmap for field-testing hormonal mechanisms of energetic readiness within free-living systems.

ENERGETIC STORAGE IN BIRDS

A. Why Fat and Muscle? An Avian Life Cycle Perspective

Birds vary in their energetic demands across life-history stages, and species with more stages (i.e. migratory vs. non-migratory species) often show a greater degree of phenotypic flexibility in energy acquisition and storage across seasons (Piersma & Drent 2003; Wingfield 2008). Similar to mammals, birds make use of the three types of biomolecules (carbohydrates, protein, and lipids) that can be oxidized for energy (Jenni et al. 1998). However, relative to similar sized mammals, birds rely little on stored carbohydrates (i.e., glycogen in the liver and

skeletal muscle) due to their exceedingly high blood glucose levels (hyperglycemia; Braun & Sweazea 2008). Instead, birds living in seasonal environments rely on stored lipids and protein, and show designated periods of fattening and muscular growth associated with increasing daylength before migration and reproduction (Ramenofsky 2011). Indeed, many migratory passerines and shorebirds have seasonally controlled periods of hyperphagia to deposit large lipid stores (King & Farner 1965; Ramenofsky 1990; Ramenofsky et al. 1999) and flight muscle growth (Piersma 1990; Lindström & Piersma 1993) before migration from wintering to breeding grounds. Similarly, some breeding birds are reliant in part on capital stores of lipid and protein accumulation for investment in reproductive stages such as egg production or incubation (Drent et al. 2007; Varpe et al. 2009). As such, we first examine the physiological mechanisms of lipid and protein storage in birds, using principles from mammalian systems where information is currently lacking in the avian literature.

B. Lipid Anabolism: From Food to Fat Storage

Fat storage is a highly conserved survival strategy in almost all organisms given the biochemical advantage of using lipid molecules to generate energy compared to either carbohydrates or protein (~8 to 10 times more energy per gram of wet mass), providing clear thermodynamic benefits for the high metabolic activities of birds (Jenni et al. 1998). Triglycerides (TRIGs or triacylglycerols) are the most abundant lipid molecules and the main component of fat storage in all vertebrates (Coleman & Lee 2004). TRIGs are produced when food items are ingested, digested, and then absorbed in the small intestines as hydrolyzed free fatty acids (FFAs; the energy-yielding component of TRIG) and glycerol (backbone of TRIG that produces some minor energy) that are then shuttled to the liver *via* lipoprotein transporters (protomicrons) in the hepatic portal system (reviewed in Williams & Buck 2010). Once in the liver, lipogenesis, the assembly of neutral lipids for energy storage, occurs through (1) dietary (exogenous) intake of lipids and (2) *de novo* (endogenous) synthesis from carbohydrate-derivatives (i.e. acetyl coenzyme-A) converted into lipid molecules (Hermier 1997; Klasing

1998). After assembly, hepatic TRIG molecules are packaged with lipoproteins to create very low-density lipoproteins (VLDLs) that are released into circulation until enzymatically degraded into FFAs and glycerol by membrane-bound lipoprotein lipase at white adipocytes, and then re-assembled inside the cell (Chong et al. 2007; purple box in Figure 2.2). In adipocytes, previous studies have identified that TRIG molecules are sequestered in subcellular compartments called lipid droplets (Holm 2003; Ducharme & Bickel 2008).

There are two ways in which lipogenic signals directed at lipid storage can alter adipocytes: hyperplasia (an increase in cell number) and hypertrophy (an increase in cell size; Jo et al. 2009). In adult vertebrates, including birds, hyperplasia is the cellular response of adipogenesis in which pre-adipocyte (progenitor) cells are recruited and differentiated into immature adipocytes until the signal for maturation occurs when TRIGs begin to fill the lipid droplets, while hypertrophy concurrently expands the storage capacity of lipid droplets in adipocytes (Hausman et al. 2001; Buyse & Decuypere 2015). Eventually, the nutritional status of lipid content increases with over-eating until mature adipocytes reach a genetically determined threshold level of TRIG storage that signals the proliferation of precursor cells and differentiation of pre-adipocytes (Rosen & Spiegelman 2014). Further, free-living birds and mammals in seasonal environments purposely overeat (hyperphagia) and switch to lipid (i.e. grains) and carbohydrate (i.e. fruits) rich diets to build excessive adipose stores (Pond 1992; Bairlein 2002; McWilliams et al. 2004; Pierce & McWilliams 2005), despite the signaling mechanisms for seasonal hyperphagia and subsequent fattening are still not fully known. Overall, ecologists have sought to underpin the underlying physiological signals that promote adaptive fat accumulation for periods of high energy demand (see next section).

C. Protein Anabolism: From Food to Skeletal Muscle Growth

Adult skeletal muscles are highly flexible tissues with a cellular capacity for dynamic re-modelling *via* muscular hypertrophy and atrophy (Hood et al. 2006). The anabolic growth of skeletal muscle occurs from (1) intracellular protein

synthesis and (2) proliferation and differentiation of myosatellite cells (i.e. precursors to skeletal muscle cells). Protein synthesis in skeletal muscle cells is the result of the downstream signaling cascade of the phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase beta-1 (S6K1) pathway (the multi-protein transcriptional regulators of cell growth) from receptor-mediated signals at the cell membrane (Glass 2003; Schiaffino & Mammucari 2011). This up-regulation of protein synthesis increases muscle cell size (hypertrophy), resulting in greater contractile force and muscle mass (Sandri 2008). Proliferation and differentiation of myosatellite cells occur through adult myogenesis, where a local population of quiescent myosatellite cells act as pool of progenitors when growth factors (i.e. IGF-1) signal for new formation of mature muscle cells (Le Grand & Rudnicki 2007; Smith et al. 2013). Additionally, muscle hypertrophy can also be induced by external inputs such as exercise or nutrition (i.e. increases in amino acid uptake), as shown in studies of exercise physiology in birds (Vézina et al. 2007; Swanson et al. 2010) and mammals (Glass 2005; purple box in Figure 2.2).

Unlike lipid storage, skeletal muscle size is largely related to the functional demand of muscle use rather than long-term energy storage (Li et al. 2014). However, in wild birds, protein sources in skeletal muscle cells can be broken down in extreme cases of long-term fasting or nutritional depletion (“Phase III” fasting; Cherel et al. 1988; Jenni et al. 2000). For example, during costly incubation or migratory periods when lipid stores are insufficient or nearly exhausted, plasma uric acid concentrations, a metabolic marker of protein catabolism, are elevated during Phase III fasting, indicating that nitrogen-based amino acids (i.e. protein-based stores) are utilized to produce last-resort energy (Cherel et al. 1988; Battley et al. 2001; Schwilch et al. 2002). Although protein is not stored in inert fuel depots, dissection of breast muscle proteins and mass in long-distance migrants demonstrate significant decreases in skeletal muscle in post-flight individuals (Piersma et al. 1999; Bauchinger & Biebach 2001, 2005a; Bauchinger et al. 2005b). Moreover, in many penguin species, protein accounts for 20 to 40% of energy utilization when fasting during breeding, with minimal

contribution from glycogen stores (Groscolas 1990; Groscolas & Robin 2001). In summary, catabolism of skeletal muscle tissue seems to provide an important "reserve" of alternative energy after the depletion of fat store in birds.

ENERGY-REGULATING HORMONES IN BIRDS

A. Hormonal Regulation of Fat Storage

Baseline Glucocorticoids

Glucocorticoids (i.e. cortisol: mammals and fish; corticosterone: birds, reptiles, and amphibians), are steroid hormones that are secreted from the adrenal cortex in the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis in vertebrates (Sapolsky et al. 2000; Wingfield et al. 2001). Specifically, environmental stimuli induces the hypothalamus to release corticotropin releasing factor, which then stimulates the pituitary to secrete adrenocorticotropic hormone (ACTH), in turn stimulating the secretion of glucocorticoids from either the adrenal or inter-renal glands. Glucocorticoid secretion is traditionally thought to occur at two distinct levels, either at baseline levels to manage daily and seasonal energy balance (Romero 2002), or at stress-induced (challenging) levels to trigger responses to external perturbations from an acute stressor (McEwen & Wingfield 2003). More recently, Williams (2012) argues that glucocorticoid levels should be considered a continuous, phenotypic trait to evaluate its regulation of metabolic processes. Nonetheless, glucocorticoid-mediated effects are executed through cytosolic steroid-binding receptors: (i) high-affinity mineralocorticoid receptors that regulates baseline levels and (ii) low-affinity glucocorticoid receptors that are only bound at saturated, stress-induced levels (Nieuwenhuizen & Rutters 2008). In birds, circulating glucocorticoids bind to the carrier protein, corticosteroid-binding globulin (CBG, known as transcortin in mammals), increasing the half-life of the hormone (Malisch et al. 2008), until dissociation at peripheral effector tissues (i.e. metabolic tissues: hepatocytes, adipocytes, skeletal muscle cells) with a direct effect on the regulation of carbohydrate metabolism (Landys et al. 2006).

In addition to effects on internal energetic stores, components of the HPA/I axis can also have effects on food intake. For example, in response to an

environmental stressor, the hypothalamus produces corticotropin releasing factor (CRF), inhibiting neuropeptide Y neurons, resulting in the reduction of appetite and foraging behaviours (Nieuwenhuizen & Rutters 2008). However, once the CRF has initiated the HPA hormone cascade and glucocorticoids are released, NPY neurons are then stimulated to promote foraging behaviours (Cahill et al. 2013). The relationship between circulating glucocorticoids and food intake has been demonstrated in both correlative and causal studies. Correlative work has shown that the diel cycle of avian baseline glucocorticoid concentrations that peak before beginning daytime activities (Breuner et al. 1999), lending to the assumption that naturally higher levels induces locomotion and potentially drives foraging behaviours (Astheimer et al. 1992; Holberton et al. 2008). Recent research has further corroborated this correlation by linking corticosterone levels and migratory restlessness in birds at stopover sites during spring migration (Eikenaar et al. 2014; Eikenaar 2017). Building on this, manipulative studies using receptor antagonists or adrenalectomy-based experiments indicate that relationship between glucocorticoids and increased food intake is mediated through glucocorticoid receptors (Dallman et al. 1993; Landys et al. 2004). Further experimental studies examining the appetite control systems are needed to distinguish which receptor for glucocorticoids, if any, regulates hyperphagia, or whether it merely has a permissive action on food intake in birds.

Independent of its influence on appetitive and foraging behaviour, elevations of baseline glucocorticoids have been shown to mediate lipid deposition in numerous captive and free-living avian species (Table 1). In birds, seasonal elevations of baseline glucocorticoids correspond to energetic readiness and fattening (Holberton et al. 1996), and may be comparable to the excess adipose tissue and hyperlipidemia seen in mammals with chronic elevations of baseline glucocorticoids (i.e. Cushing's syndrome; Peeke & Chrousos 1995). For example, in domestic poultry species, hepatic lipogenesis is amplified when individuals are administered dexamethasone, a glucocorticoid receptor agonist, and lipid uptake is also increased *via* enhanced lipoprotein lipase expression in white adipose tissue when corticosterone treatments are

combined with up-regulated enzymes (i.e. malic enzyme, fatty acid synthase; reviewed in Scanes 2008). Likewise, in biomedical mammalian models, chronic glucocorticoid elevations antagonize the liberation of lipids to promote lipogenesis and lipid accumulation (Peckett et al. 2011). Further, dexamethasone has been shown to up-regulate NPY proteins and receptors, increasing abdominal adipogenesis of cultured mammalian adipocytes (Kuo et al. 2007). Although the molecular mechanisms of glucocorticoid-induced lipogenesis is still not well understood, research on wild birds show a consistent, positive link between chronically elevated baseline glucocorticoids and increased adiposity (Landys et al. 2006; orange and purple boxes in Figure 2.2). Emerging ecological studies using a life-history stage framework suggest that elevated baseline glucocorticoids can induce fattening in both pre-migratory (i.e. Holberton 1999; Holberton et al. 2007) and pre-breeding birds (i.e. Hennin et al. 2015; Lamarre et al. 2017). Overall, appreciating the contexts of the relative levels of elevation and a species' life-history will be key in determining the effects of elevated baseline glucocorticoids in regulating of fat metabolism, and should be considered in greater detail the future studies of free-living birds (Crespi et al. 2013).

Insulin

In mammals, insulin is a peptide hormone secreted from pancreatic β-cells that is released after food intake to mediate glucose uptake *via* GLUT4 transporters into various tissues, including adipose tissue (Woods et al. 1998), and simultaneously increase glycogenesis (i.e. glycogen storage) and lipogenesis in the liver (Pessin & Saltiel 2000). In contrast, avian insulin is present in lower circulating levels during a hyperglycemic state (Braun & Sweazea 2008), and appears relatively insensitive to insulin receptors and downstream targets of the lipogenic signaling cascade in adipose tissue, raising the question of whether birds are in a perpetual insulin-resistant state (Dupont et al. 2012). Rather than acting alone, it is possible that low insulin levels in birds must be modulated by glucocorticoids to promote adiposity. For instance, in domestic chickens, chronic dexamethasone treatment induces increased plasma insulin (Yuan et al. 2008) and together

these hormones promote hepatic lipogenesis *via* up-regulation of lipogenic enzymes (Cai et al. 2011). Furthermore, both insulin and glucocorticoids have been shown to amplify lipoprotein lipase activity and TRIG storage through the Akt intracellular growth pathway in adipocytes (Tomlinson et al. 2010; Geer et al. 2015), indicating that it may play an important role in active fattening (orange and purple boxes in Figure 2.2). Although the temporal patterns of plasma insulin are poorly studied in birds, studies in golden-mantled squirrels (*Spermophilus lateralis*) indicate that simultaneous increases in insulin and baseline glucocorticoids result in a concomitant increase in body mass before the onset of hibernation (Boswell et al. 1994). Nonetheless, regardless of taxa we still know very little about the temporal variation of plasma insulin across life-history stages, and future studies are particularly warranted to validate the interactive role that insulin and glucocorticoids may play in energetic storage in free-living animals.

Ghrelin

Mammalian ghrelin is a peptide hormone produced in the gastrointestinal tract and released in the fasted state (i.e. hunger signal) with well known metabolic roles in the central regulation of appetite and energy balance (Müller et al. 2015). Avian ghrelin, a 26-amino acid polypeptide mainly secreted from X/A-like cells in the mucosal layer of proventriculus (located between the crop and gizzard in most birds), has been poorly studied to date. In vertebrates ghrelin exists in two forms: (i) acylated ghrelin and (ii) des-acyl ghrelin (Kojima et al. 2001, 2005). Acylated ghrelin most commonly contains an *n*-octanoyl (acyl) side chain on the serine-3 residue that is added by membrane-bound ghrelin *O*-acyltransferase at target cells (Gahete et al. 2010) and binds to growth hormone secretagogue receptor on various tissues (Asakawa 2005). Des-acyl ghrelin, originally labelled as an inactive prohormone, is circulated in higher levels and has numerous antagonistic effects on the acylated form, including central inhibition of orexigenic (hunger-inducing) effects (Delhanty et al. 2015). Currently, most molecular research in vertebrates focuses on acylated ghrelin as the only known gut-

derived hormone to drive hunger responses in the fasted state in vertebrates (Kaiya et al. 2008).

In mammals, injections of acylated ghrelin activates receptors located in the arcuate nucleus (ARC) of the hypothalamus to co-express orexigenic neuropeptides (i.e. NPY and AgRP), resulting in the activation of other hunger-controlling regions of the hypothalamus (Nakazato et al. 2001; López et al. 2008) to increase food intake and adiposity (Tschöp et al. 2000). At a molecular scale, peripheral acylated ghrelin promotes carbohydrate metabolism and reduces fat oxidation *via* AMP-activated protein kinase (AMPK) signaling pathway in hepatocytes and adipocytes (Meier & Gressner 2004; Ueno et al. 2005; Xue & Kahn 2006), as well as stimulates TRIG synthesis through up-regulation of fatty acid synthase and malic enzyme (Perez-Tilve et al. 2011). Moreover, many studies on the broad temporal and stage-related patterns of acylated ghrelin in seasonal mammals suggest that circulating levels are elevated prior to entering energetically challenging life-history stages such as periods of food shortage over winter (Nieminen et al. 2002; Fuglei et al. 2004; Mustonen et al. 2005) and hibernation (Healy et al. 2010, 2011). Importantly, peripheral injection of physiologically relevant acylated ghrelin increases food intake and body weight, regardless of season (Florant & Healy 2012).

In contrast, acylated ghrelin seems to be an anorexigenic (hunger-suppressing) hormone in birds (Kaiya et al. 2013a; Table 1). Initially documented by Furuse and colleagues (2001) as decreasing food intake in neonatal chicks (*Gallus gallus domesticus*), exogenous administration of ghrelin in domestic juvenile chicks now provides support for both anorexigenic and lipolytic roles in birds (Kaiya et al. 2013a). Saito et al. (2005) hypothesized that ghrelin-induced suppression of food intake is likely mediated by corticotropin releasing factor (CRF) family peptides, which inhibit NPY and AgRP (orexigenic) neurons in the brain, and may therefore interact with the HPA axis. Importantly, Shousha and colleagues (2005, 2015) reported that peripheral injections of acylated ghrelin in adult Japanese quail (*Coturnix coturnix japonica*) show dose-dependent effects, with low to intermediate doses (0.5 and 1 nmol/200 μL) increasing food intake,

and high doses (3 nmol/200 μL) decreasing food intake and respiratory quotient. Indeed, Aghdam Shahryar and Lotfi (2016) found that a ghrelin antagonist ([D-Lys^3]-GHRP-6) decreased food intake in broiler chicks in a dose-dependent manner and decreased plasma glucose concentrations with no changes in plasma TRIG, similar to effects of lipid conservation found in mammals (Asakawa et al. 2003). It is important to note, however, that these dosages are alarmingly high, at a magnitude of ~100,000 times the circulating concentration under even fasted conditions, despite still being one of the lowest administrative doses (calculations see "Further Considerations" section). Presently, we know of only one study in broiler chicks that directly tested the effect of acylated ghrelin on lipid metabolism, with single (1 nmol/100 μL), intravenous administration resulting in the down-regulation of fatty acid synthase and up-regulation of lipogenic transcription factors (PPARγ and SREBP-1) mRNA levels in the liver (Buyse et al. 2009). Further, in free-living garden warblers (*Sylvia borin*) during migratory stopover, individuals with remaining lipid reserves were shown to have significantly higher plasma acylated ghrelin levels compared to those with depleted stores (Goymann et al. 2017), suggesting similar results as ghrelin-induced lipolysis previously shown in young chicks (Geelissen et al. 2006). In congruance with mammals, plasma acylated ghrelin is elevated in fasting conditions, demonstrating its conserved role as a hunger signal in birds (Kaiya et al. 2013b). Taken together, the metabolic function of avian acylated ghrelin does not have unanimous support, and although mammalian ecophysiological studies have identified ghrelin as a modulator of adipose stores, the intriguing anti-lipogenic effects of avian acylated ghrelin should be investigated (orange, blue, and purple boxes in Figure 2.2), ideally beginning with examining ghrelin profiles across life-history stages.

Triiodothyronine

In birds, thyroid hormones (L-thyroxine (T_4) and triiodo-L-thyronine (T_3)) regulate many important physiological processes such as pre-natal development and juvenile growth (McNabb 2006), reproductive processes (Chastel et al. 2003),

thermogenesis (Vézina et al. 2009), and metabolism (Hulbert 2000). The hypothalamic-pituitary-thyroidal axis begins with thyrotropin-releasing hormone (TRH) being released from the hypothalamus which stimulates the release of thyrotropin-stimulating hormone (TSH) from the anterior pituitary gland, which then activates hormone secretion into systemic circulation from the thyroid gland (reviewed in Zoeller et al. 2007). Specialized enzymes, deiodinases type I (D1) and II (D2), are responsible for extra-thyroidal conversion of T_4 to T_3 in circulation and tissue through the removal of an iodine molecule from the outer ring structure of thyroid hormones (Zoeller et al. 2007). Binding proteins, such as transthyretin and albumin in birds, carry the thyroid hormones to target cells until bound to intracellular nuclear receptors (α- and β-isoforms; McNabb 2007). Importantly, T_3 has a greater binding affinity than T_4 to receptors, and is therefore the primary regulator of basal metabolic rate in birds (Kim 2008; Elliott et al. 2013; Welcker et al. 2013).

There is still little currently known, however, on the role of thyroid hormones with regards to metabolic effects on stored energy in birds. Studies of laboratory rats have revealed that T_3 has direct metabolic effects on adipose tissue (Oppenheimer et al. 1991), as thyroid-inhibited and dose-dependent T_3 replacement led to greater use of fat stores (oxidation) in “hyperthyroid” compared to greater fatty acid uptake and lipoprotein lipase activity (fat deposition) in “hypothyroid” individuals (Klieverik et al. 2009). Conversely, in mammalian hepatocytes, low levels of T_3 can trigger lipogenesis through the cross-talk between thyroid receptors and liver X receptor (an upstream protein linked to the transcriptional up-regulation of key lipogenic enzymes such as malic enzyme and fatty acid synthase; Sinha et al. 2014), and similar broad lipogenic effects have been found with short-term elevations of administered T_3 in birds (Goodridge et al. 1989; McNabb 2007). However, chronically elevated levels of T_3, whether endogenous or exogenous, causes lipolysis after prolonged exposure in humans and rodents (reviewed in Mullur et al. 2014). Recent mammalian research has identified a T_3-mediated lipolytic mechanism of TRIG in adipose tissue through lipophagy, an intracellular process where TRIG-filled lipid

droplets are trafficked to lysosomes for degradation in which the FFAs can be released into the blood or efficiently used for energy *via* β-oxidation in nearby mitochondria (Sinha et al. 2012; Liu & Czaja 2013). Previous avian studies further support this hypothesis in that (i) prolonged elevations of T_3 result in lipolysis in white adipose tissue (Rosebrough & McMurtry 2000, 2003), and (ii) genetically lean lineages of chickens have higher circulating levels of T_3 than fat lineages (Leclercq et al. 1988). However, the underlying mechanisms of T_3-mediated switches between lipogenic (anabolic) and lipolytic (catabolic) states is currently unclear in vertebrates (Sinha et al. 2014). For instance, administering supplemental T_3 to hypothyroidal birds restores the lipogenic capacity, where, as individual with normal functioning thyroids, T_3 administration decreased plasma TRIG, demonstrating a dose-dependent effect of T_3 on lipid metabolism (Rosebrough et al. 2006). Future experimental studies that compare natural and manipulated hormone levels with different fat phenotypes may help to explain the differences in HPT axis regulation on lipid metabolism. Similar to baseline glucocorticoids, if seasonally elevated levels of plasma T_3 are considered to be equivalent to chronic HPT function, then it is likely that low levels of T_3 suppress lipolysis for energetic readiness (orange and purple boxes in Figure 2.2). Indeed, Wilsterman and colleagues (2015) found that circulating T_3 levels are below the annual average during a period of intense fattening before winter hibernation in free-living Arctic ground squirrels (*Urocitellus parryii*).

In contrast to mammalian work, numerous studies have suggested that avian species display relatively higher levels of T_3 during periods of energetic preparation. For intance, T_3 has been shown to increase prior to spring migration (Canada goose, *Branta canadensis*; John & George 1978; rosy pastor, *Sturnus roseus*; Pathak & Chandola 1984), and individuals with either thyroidectomy or receptor blocking demonstrated reduced pre-migratory fattening and migratory restlessness (Pathak & Chandola 1982a; Pant & Chandola-Saklani 1993). Furthermore, administering either T_3 or T_4 to thryoid-blocked individuals temporally restored fat and muscle profiles of wild-caught, captive white-crowned sparrows, with T_4 exhibiting a greater recovery of energetic profiles (Pérez et al.

2016). Cumulatively, these results suggest that extrathyroidal conversion of T_4 to T_3 during a rapid transition to a longer photoperiod may promote lipogenesis in migratory birds. Much less, unfortunately, is known about T_3 regulating lipid metabolism in pre-breeding birds, despite fat gain in capital and mix-strategy breeders.

B. Hormonal Regulation of Skeletal Muscle Growth

Growth Hormone and Insulin-like Growth Factor-1

Across vertebrate taxa, growth hormone (GH) and insulin-like growth factor-1 (IGF-1) are both anabolic peptide hormones produced by the somatotrophic axis that regulate fundamental processes such as pre- and post-natal somatic growth (i.e. bone growth, skeletal muscle growth, organ development, skin formation; Giustina et al. 2008; Liu & LeRoith 1999), aging and longevity (Bartke 2005), and systemic metabolism (Renaville et al. 2002). Beginning with input in the hypothalamus, growth hormone-releasing hormone (GHRH) is released from the hypothalamus to reach somatotrophs (specialized cells in the anterior pituitary) that secrete GH into circulation, acting *via* (i) GH receptors on peripheral tissues such as skeletal muscle, white adipose tissue, bone, and lymphatic tissue, or (ii) receptors in liver cells to secrete IGF-1 into systemic circulation (Bartke et al. 2013). Notably, liver-derived IGF-1 in the plasma is dependent on GH levels, while tissue-specific IGF-1 is thought to be secreted independently of GH action (LeRoith et al. 2001; Lupu et al. 2001). In mammals, GH and IGF-1 are well known for their metabolic effects on adult skeletal muscle tissue, and their regulation of muscle size and mass through the membrane-bound IGF-1 receptor (Breier 1999; Velloso 2008). Moreover, IGF-1 receptors activate the PI3K/Akt/mTOR/S6K1 (see section 1B on “Protein Anabolism”) and MAPK second messenger pathways that increases the rate of protein synthesis, new myonuclei formation, and differentiation of myosatellite cells (Otto & Patel 2010).

Currently, very little *recent* GH research has been conducted in birds, and the research that has been conducted has often reported opposite effects on skeletal muscle compared to other vertebrates (i.e. mammals, LeRoith et al.

2001; fishes, Fuentes et al. 2013). In fact, most studies in which GH has been exogenously administrated report either no change or a decrease in skeletal (breast) muscle in broiler chicks (Table 1). For instance, in cultured myosatellite cells from chickens, exogenous GH was shown to result in an up-regulation of GH receptor mRNA expression, thereby inhibiting cell differentiation into mature skeletal muscle cells (Halevy et al. 1996). Additionally, Vasilatos-Younken et al. (2000) found an indirect effect of GH administration on increased production of T_3 through the down-regulation of T_3-degrading deiodinase type III, generating a net protein loss and therefore decrease in breast muscle mass in chickens. These complex interactions between somatotrophic and HPT axes on whole-organismal metabolism may explain the decrease in skeletal muscle in chickens with supplementary GH (orange and purple boxes in Figure 2.3).

Research on avian IGF-1, primarily from studies on neonatal chicks and cultured cells, indicates its role as a key signal for muscle growth through hypertrophy and myosatellite cell proliferation and differentiation in birds (Table 1; orange and purple boxes in Figure 2.3). Moreover, these pro-proliferative effects of IGF-1 are attenuated with PI3K and Akt inhibitors, identifying a conserved intracellular mechanism of IGF-1 signaling in skeletal muscle cells in avian species (Yu et al. 2015). Moreover, exogenous administration of IGF-1 in avian species has been shown to increase the rate of protein synthesis (Conlon & Kita 2002), inhibit protein catabolism in skeletal muscle cells (Tomas et al. 1998), and induce differentiation in muscle progenitor cells (Duclos et al. 1991; Buyse & Decuypere 1999). Although both liver- and muscle-produced IGF-1 interact with muscle cell receptors, the relative contribution of each towards muscle growth remains unknown (Velloso 2008).

Even less is currently known about the seasonal regulation of plasma IGF-1 in birds, especially in terms of IGF-1 as an anabolic driver of skeletal muscle growth. Price and colleagues (2011) found that flight muscle IGF-1 mRNA expression in white-throated sparrows (*Zonotrichia albicollis*) was significantly higher in spring migrants compared to wintering, indicating that production of local IGF-1 may be important for protein synthesis in skeletal muscle tissue. In

polar environments with highly constrained breeding seasons, Adélie penguins chicks (*Pygoscelis adeliae*) expressed high IGF-1 mRNA in pectoral muscle during a rapid growth period, indicating that it plays an important role in the development of an enhanced muscle phenotype (Degletagne et al. 2013). Finally, Lodjak et al. (2014, 2016) recently showed a causal link between plasma IGF-1 elevation and increased growth rates in nestlings of free-living great tits (*Parus major*) and pied flycatchers (*Ficedula hypoleuca*). Overall, there is an underwhelming amount of avian-based information on circulating IGF-1 levels across life-history stages, which opens avenues for investigation of IGF-1 effects on skeletal muscle growth and fitness-related traits such as growth rate, brood size, and lifespan (Dantzer & Swanson 2012).

Testosterone

Testosterone, the primary mediator of male traits in vertebrates (Hau 2007; but see Ketterson et al. 2005 for testosterone's role in female traits), is an anabolic androgen that regulates primary (gonad production and spermatogenesis; Garamszegi et al. 2005) and secondary sexual characteristics such as sexual and aggressive behaviours (i.e. courtship, singing, and territorial defense), brain development, suppression of immune system, and anabolic effects on bone and skeletal muscle tissue (Wingfield et al. 1990; Hau 2007). The hypothalamic-pituitary-gonadal (HPG) axis starts with external stimuli activating the hypothalamus to release gonadotropin releasing hormone, which in turn stimulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary, where LH then binds to receptors on (i) Leydig cells in the testes in males and (ii) granulosa cells in the ovaries in females to secrete testosterone, along with some extra-genital production in the adrenal cortex (Handa & Weiser 2014). Metabolic literature on anabolic androgenic steroids have been well studied in sports medicine for performance enhancing effects of lean muscle growth and decreased adipose mass in humans (Evans 2004). Androgen-mediated anabolic effects on skeletal muscle growth occur through androgen receptors in the nuclei of adult skeletal muscle

cells, myosatellite cells, and motor neurons (Dubois et al. 2012) to produce regulatory growth factors (i.e. IGF-1, PI3K and Akt kinases) that synthesize contractile proteins (Kim et al. 2009). In addition, mammalian testosterone has been shown to induce proliferation of myosatellite cells to increase the number of progenitor cells recruited for muscular growth (Sinha-Hikim et al. 2004, 2006).

Surprisingly, despite these strong links between testosterone and muscle growth in mammals, very few empirical studies directly examine levels of testosterone on skeletal muscle tissue in avian species (Table 2.1). Nonetheless, breed-specific differences in mean plasma testosterone revealed higher levels in broiler chickens artificially selected for higher meat production and larger breast muscles compared to layer chickens selected for egg production (Ho et al. 2011). Likewise, administration of mesterolone, a commonly administered anabolic androgenic steroid, increased the number of myosatellite cells in chicken breast muscle (Allouh & Aldirawi 2012). Similarly, elevated yolk testosterone increased musculus complexus size in hatchling red-winged blackbirds (*Agelaius phoeniceus*), a dorsal neck muscle for hatching and begging behaviours, and injections of testosterone antagonist (flutamide) caused a significant decrease in musculus complexus mass (Lipar & Ketterson 2000). Altogether, these studies suggest that testosterone may act as a potent muscle-promoting signal to induce skeletal muscle growth in birds (orange and purple boxes in Figure 2.3).

Seasonally elevated levels of circulating testosterone have generally been linked to breeding phenology in birds (Wingfield et al. 1990). Previous research has shown that testosterone secretion increases in migratory males to advance development of testes and energetic condition in anticipation of pre-breeding competition for territories and mates after arrival on breeding grounds (Wingfield 1984; Morton et al. 1990; Holberton & Dufty 2005). Indeed, the effect of exogenous testosterone administration on migratory and breeding preparation in male dark-eyed juncos (*Junco hyemalis*), has demonstrated that testosterone-treated individuals reach peak migratory condition four days before control individuals (Tonra et al. 2011a), and early arrival males had higher plasma testosterone compared to later arriving male American redstarts (*Setophaga*

ruticilla, Tonra et al. 2011b; supporting results shown in gray catbirds, *Dumetella carolinensis*; Owen et al. 2014). Further, male redstarts with experimentally elevated testosterone departed earlier for breeding grounds, supporting the hypothesis that testosterone initiates migratory readiness (Tonra et al. 2013). Likewise, Ramenofsky and Németh (2014) found that testosterone implants led to a temporary increase of muscle growth in a captive population of wild-caught white-crowned sparrows during the two-week implant period. Altogether, testosterone appears to be an important modulator of both spring migration and breeding phenotypes in which the two life-history stages are not mutually exclusive and presumably both require testosterone for skeletal muscle growth (Table 1). Although testosterone in females has shown to be important for breeding behaviours and offspring phenotype (Groothuis & Schwabl 2008), we currently do not know whether elevated levels in females serve an alternative biological function to increase skeletal muscle tissue for reproductive stages.

C. Additional Candidates as Energy-Regulating Hormones

Prolactin

Prolactin, a peptide hormone secreted from the anterior pituitary when activated by vasoactive intestinal polypeptide from the hypothalamus, is important for generating and maintaining parental care in birds, as elevations occur during the onset of laying and continue after the parental phase (reviewed in Angelier & Chastel 2009a). Earlier avian studies suggested that prolactin might play a role in increasing food intake (Hnasko & Buntin 1993) and fattening (Goodridge & Ball 1967; Meier et al. 1971; Bartov et al. 1980). However, long-term manipulative studies now appear to indicate that peak prolactin levels do not temporally coincide with pre-migratory period of fattening in dark-eyed juncos and white-crowned sparrows (Schwabl et al. 1988; Holberton et al. 2008). The temporal lag of elevations in prolactin after pre-migratory hyperphagia and energetic preparation may be due to known increases in prolactin during laying, incubation, and chick rearing (Angelier & Chastel 2009a). Furthermore, more recent interest in the mechanistic interaction between glucocorticoids and prolactin in parents

has suggested these hormones balance the competing demands of offspring care, future reproductive investment, and self-maintenance (Angelier et al. 2009b; Miller et al. 2009; Spée et al. 2010; Ouyang et al. 2013). Taken together, these confirmed actions during reproductive stages based on work from both domestic and wild birds supports prolactin's primary role as a "parental hormone". Thus, it seems unlikely that prolactin, along with vasoactive intestinal polypeptide, carries any major importance in building fat stores during pre-migration or pre-breeding considering that circulating prolactin levels reach peak concentrations after the conclusion of the pre-laying interval in various species (Williams & Sharp 1993; Angelier et al. 2016; Riou et al. 2010).

Leptin

With the upswing of biomedical research focused on the obesity epidemic in humans, leptin, an adipose-derived hormone that signals satiety (or fullness) in the hypothalamus to decrease food intake, has been perhaps the most well studied hormone on energy balance and metabolism in mammals (reviewed in Friedman & Halaas 1998). In mammals, leptin is a potent suppressor of feeding through up-regulating *POMC* and down-regulating *NPY/AgRP* gene expression (Friedman 2009). In birds, on the other hand, progression on leptin biology has been slow since the controversial characterization of the leptin gene about 20 years ago, which was initially difficult to clone with only ~30% conserved amino acid sequence and a high degree of GC base pairing in the coding region (outlined in Boswell & Dunn 2015). Additionally, in contrast to mammalian leptin, many differences in avian leptin-like proteins have been identified: (a) low/undetectable circulating levels in the plasma (Hen et al. 2008); (b) a widespread tissue expression with low levels in white adipose tissue (Huang et al. 2014; Friedman-Einat et al. 2014); (c) the possibility of loss of leptin gene in poultry genomes from domestication (Friedman-Einat & Seroussi 2014); and (d) may play a role that is independent from energy regulation (Millar 2014). Even with these discrepancies and considering the isolation of an avian leptin receptor (Horev et al. 2000), a number of studies have administered exogenous

mammalian leptin to examine changes in food intake and energetic state in birds (Denbow et al. 2000; Alonso-Alvarez et al. 2007; Cersale et al. 2011; Zajac et al. 2011). However, the uncertainty behind the isolation and biological function of leptin-like protein remains controversial, prompting many more questions about its potential role in energy balance in birds (Boswell & Dunn 2015).

Cholecystokinin

Cholecystokinin (CCK) is a gastrointestinal peptide hormone secreted from the small intestines in birds (Jonson et al. 2000), and similar to other vertebrates, both exogenous peripheral and central administration of CCK decreases food intake in domestic chickens (Denbow & Myers 1982; Furuse et al. 2000; Tachibana et al. 2012). Avian CCK receptors are located in the vagal afferent neurons to produce sensations of satiety, along with simultaneous suppression of gastric emptying and digestive enzyme secretion from the pancreas and liver (Covasa & Forbes 1994). Interestingly, Dunn and colleagues (2013) propose that animal domestication in high-growth selected strains of poultry are resistant to a single intraperitoneal injection of CCK (10 μg/kg of body weight) through lowered expression of CCK receptor in the brain and intestines in growing domestic chicks. Considering the hormonal effects in wild-caught, captive birds, white-crowned sparrows also ingest less food in CCK-treated birds in a dose-dependent fashion (Richardson et al. 1993). However, there is little to no ecological information on the temporal patterns and possible circannual control of CCK in relation to life-history stages or seasonal events, making it an intriguing candidate for an expected down-regulation during energetic readiness.

Peptide YY

Conlon and O'Harte (1992) were the first to isolate and sequence chicken peptide YY (PYY), a brain-gut hormone that has been strongly linked to the inhibition of food intake *via* Y_2 receptors on NPY neurons in the arcuate nucleus of the hypothalamus in mammals (Batterham et al. 2002; Chelikani et al. 2006; McGowan & Bloom 2004). In addition, PYY acts as key satiety signal in rodents

and humans *via* elevated plasma circulating levels after feeding (Stadlbauer et al. 2013). Despite earlier isolation and characterization of chicken PYY, there is limited available research on any potential effects of avian PYY on energy balance in birds, particularly wild species (Honda et al. 2017). Recent research has identified that elevated mRNA expression in the small intestines, specifically in the jejunum, and intravenous infusion of chicken PYY suppresses food intake in male broiler chicks (Aoki et al. 2017). Further investigative studies need to be conducted to augment these findings before implementing this potential satiety signal in energetic balance within a life-history context.

Glucagon-like Peptides

Glucagon-like peptide 1 and 2 (GLP1 and GLP2, respectively) are intestinal hormones that regulate nutritional homeostasis, as well as stimulate the proliferation of pancreatic β-cells and serves as an anti-apoptotic intracellular signal of intestinal epithelium in mammals (Brubaker & Drucker 2004). In mammals, GLP1 is released in response to the ingestion of nutrients, and it plays a primary role in promoting insulin secretion and inhibiting glucagon release by the pancreas, suppressing gastrointestinal motility, and reducing food intake (reviewed by Holst 2007). In chickens, intraperitoneal administration of GLP1 has been shown to inhibit food intake in Japanese quail (Shousha et al. 2007), where the anorexigenic effect may be mediated through the CRF system in the hypothalamus (Tachibana et al. 2006). With regards to energetics, GLP1 receptor mRNA expression is highest in abdominal adipose tissue, proposing a role in lipid metabolism of chickens, despite fasting and re-feeding did not change the plasma GLP1 levels (Richards & McMurtry 2008).

GLP2, derived from the same precursor peptide as GLP1, is important for normal growth and physiological function of the muscosal layer of the small intestines in mammals (Janssen et al. 2013). Furthermore, GLP2 mRNA expression is highest in the brain and gut of chickens, where it has been proposed to function as a nutrient-sensing hormone (Honda 2016). Further, administration of GLP2 significantly decreased food intake in broiler chicks by

crossing the blood brain barrier or median eminence in the arcuate nucleus of the hypothalamus, similar to other centrally acting hormones (Honda et al. 2015a; Honda et al. 2015b). Overall, more studies are needed to determine a possible physiological role of GLPs on energy balance in free-living avian systems.

D. Predicting Patterns of Energy-Regulating Hormones: An Ecological Context

Based on our empirical summary of hormones promoting fat deposition or skeletal muscle growth in birds (Table 2.1), we can, at the very least, make basic predictions about expected up- or down-regulation of these hormones during energetic preparation for migration or breeding (summarized in Table 2.2). Further, we present a simple conceptual diagram that depicts the expected temporal patterns of candidate energy-regulating hormones with regards to four generic stages: (i) reference (i.e. wintering); (ii) energetic gain (i.e. pre-migration or pre-breeding); (iii) energetic use/expenditure (i.e. migration or breeding); and (iv) reference (i.e. post-breeding; Figure 2.1). More specifically, in consideration of the candidate energy-regulating hormones mentioned in sub-sections A and B, we would predict that birds will exhibit elevated levels of baseline corticosterone, insulin, and T_3, and suppressed levels of acylated ghrelin for fattening, and we also expect that there would be suppressed levels of GH and elevated levels of testosterone and IGF-1 for muscle hypertrophy (Figure 2.1). In addition, it is critical that we holistically integrate the currently known endogenous pathways to better visualize and interpret how seasonal changes in physiology (i.e. endocrine, neural, metabolic) lead to whole-organism phenotypes of fat deposition (Figure 2.2) and skeletal muscle growth in birds (Figure 2.3). An underlying assumption of these predictions is that all birds living in seasonal environments require the same endogenous hardware for periods of fattening and/or muscle growth, and if true, then population-level variation in hormone regulation is most likely context (i.e. stage and species) dependent. Nonetheless, we urge ecologists to embark on correlative and experimental studies using this conceptual framework to test

the causal links between candidate energy-regulating hormones and energetic storage in free-living seasonal birds.

MISSING INFORMATION AND FUTURE DIRECTIONS

A. Complexities Arising from Hormone Manipulations: Case Example with Acylated Ghrelin

Hormone manipulation experiments are instrumental for determining causal relationships between hormone-mediated traits (i.e. behaviour and physiology) and phenotypic changes (i.e. energetic state; referred to as "phenotypic engineering" by Ketterson & Nolan 1992; Ketterson & Nolan 1999). Research on avian energy-regulating hormones have readily harnessed this powerful technique to identify key mediators of energy balance in poultry (Scanes 2008) and in some wild species (Table 1). Although there has always been a clear advantage to using a manipulative approach to studying the evolutionary role of hormones (Ketterson & Nolan 1999), we discuss some of the drawbacks and limitations associated with hormone manipulation. Here, we use acylated ghrelin as a case example because of its recently appreciated influence on avian energetic state (Goymann et al. 2017), with hopes of future consideration for experimental designs.

First, dose of the hormone is an important consideration, especially to ensure that the elevations of the hormone are within a biologically relevant range. *In vivo* synthetic hormone administration rapidly elevates circulating levels in the plasma, and doses beyond the natural, physiologically relevant range are termed "pharmacological" (mentioned in Zera 2007b). For example, despite acylated ghrelin being classified as a potent anorexigenic hormone in chickens (see Table 1), most hormone manipulation experiments use pharmacological doses that are significantly higher in concentration than detected naturally in the plasma, even under fasting conditions when concentrations are predicated to be the highest (Kaiya et al. 2013a). As previously mentioned, Shousha et al. (2005) injected one of the lowest known concentrations of acylated ghrelin (7.8 μg/mL; calculated using molecular weight from Phoenix Pharmaceuticals Inc.), and even this

concentration is much higher than physiological natural plasma levels under fasted conditions (~50 pg/mL), indicating a high degree of receptor sensitivity. Moreover, alternative (non-directed) effects resulting from extremely high levels can make the results of *in vivo* experimental manipulations difficult to interpret (Zera et al. 2007b). Although speculative, in the case of pharmacological doses of ghrelin, it may be possible that the anorexigenic effect in birds is caused by the instability and rapid conversion of plasma acylated ghrelin into des-acyl ghrelin, a proposed antagonist through an ghrelin-independent receptor that decreases food intake in mammals and birds (Delhanty et al. 2012, 2014; Goymann et al. 2017). Altogether, we recommend that physiologically relevant doses of hormones should be carefully used to acquire an accurate interpretation on the effects of low or high levels on phenotypic expression.

Further, the choice of administering single, multiple (i.e. chronic), or pulsatile (i.e. small bursts over a period) doses of exogenous hormones are an important, yet rarely accounted for, consideration since the duration of administration can affect negative feedback loops in vertebrates and can potentially induce down-regulation of endogenous hormones or a period of hormonal resistance (Fusani 2008; Dantzer et al. 2016). For instance, most poultry studies have used single dose administration of acylated ghrelin (Kaiya et al. 2013b), despite evidence for diel patterns of pulsatile secretion in fasted human subjects (Natalucci et al. 2005); however, the circadian rhythm of ghrelin has not been investigated in birds. Similarly, careful consideration must be given to the mode of delivery in live animals (i.e. silastic tubes, pellets, peripheral and central injections, osmotic mini-pumps, and food/water) that can cause variability in the degree of disruption in hormone profiles within the natural circadian rhythm (Dantzer et al. 2016; Sopinka et al. 2015). Future research on the daily rhythm of acylated ghrelin may be helpful to identify the best method of administration for manipulation experiments in free-living birds.

Arguably, the most difficult issue to control with hormone manipulations is the pleiotropic nature of hormonal pathways in coordinating complex life-history phenotypes (Zera 2007a). As a result, it is challenging to discern whether it is the

manipulated hormone, another hormone indirectly affected from manipulation, or perhaps an interaction of both that regulate the phenotype (Mcglothlin & Ketterson 2008). For example, Ocłoń & Pietras (2011) found that ghrelin-elicited reduction of food intake was attenuated with astressin, a CRF antagonist, and enhanced with RU486, a glucocorticoid antagonist, suggesting that peripheral acylated ghrelin mediates changes in HPA axis activity that, together, affect feeding. To examine the effect of individual variation in hormones on metabolic phenotypes of free-living birds, a potential solution is to include correlative data or life-history stage variation in natural hormone levels or cite relevant studies that present these data (Williams 2012). Should little or no empirical evidence be available for a given hormone, we argue that correlative temporal studies should be conducted to provide foundational information on non-manipulated circulating levels and phenotype of interest before executing manipulation experiments.

B. Implications for Comparing Domestic and Free-Living Birds

Within birds, domestic poultry are often considered the model organisms frequently used to examine the hormonal regulation of energy balance since the aim of the field is to examine mechanisms that regulate the quality of protein production (Scanes 2008). For many reasons, current research on the influence of energy-regulating hormones on energy metabolism have largely been restricted to domestic poultry (see Table 1). Domesticated species offer many inherent logistical advantages for hormone analyses and experiments, such as the ability to collect large plasma volumes (analysing multiple hormonal traits from a single sample), repeated sampling of an individual, measure of variables before and after manipulation in an individual, and a controlled environment (Cheng 2010). Not surprisingly, there are problems associated with translating and scaling the relevance of findings in selected domestic stocks up to free-living populations of birds.

Artificial selection, the human intervention of genetic improvement for traits that maximize high-quality food in livestock and crops, is the evolutionary principle that has driven poultry breeding (Yamasaki et al. 2007). Although

desired traits of meat and egg yield are molded through artificial selection, genetic modification impinges on the natural behavioural, physiological, and morphological characteristics of free-living birds (Andersson 2001; Cheng 2010). For example, wild red jungle fowl produce an average of 4 to 6 eggs per year compared to layer (domestic) hens that produce on average 300 eggs per year (described in Cheng 2010). Moreover, poultry domestication through thousands of generations have selected for very different energetic demands and hormonal secretion patterns, especially considering domestic stocks have access to *ad libitum* food and water and constant environmental (i.e. lighting) regimes in captivity (Cheng 2010; Kaiya et al. 2013a). Importantly, traditional poultry studies that examine hormones are primarily interested in growth-related metrics associated with different strains in postnatal chicks (Scanes 2008). However, there are well-documented differences between the circulating levels of hormones during postnatal development in young birds compared to adult birds (i.e. GH levels; Harvey 2013), despite most poultry studies examining the energy-regulating hormones in developing chicks (see Table 1). Although it is appealing to suggest that all birds require the same mechanistic hardware to facilitate systematic energy balance (Richards & Proszkowiec-Weglarz 2007), we should be weary to equate hormonal mechanisms between domestic and free-living birds, as well as between young and adult birds. Instead, as mammalian systems have provided a mechanistic basis for poultry studies (Richards 2003), domestic birds may be viable predictive models, rather than being surrogates, for field-testing of hormonal regulation on energetic phenotypes in wild birds.

C. Transcriptomics in Avian Endocrinology and Metabolism

Transcriptomics, the use of high-throughput, genome-wide tools (i.e. DNA microarrays and RNA sequencing) to analyze mRNA levels as a functional measure of gene expression (discussed in Porter 2015) is a rapidly emerging field in avian physiology and endocrinology. Recently, Mello & Lovell (2017) reviewed how avian genomics, particularly comparative analyses of endocrine and metabolic transcriptional profiles, can scan thousands of genes

simultaneously to assess expression of endocrine-related genes. For example, corticosterone treatment on embryonic pituitary cells in chickens induced hundreds of genes compared to control cells, displaying the large-scale capability for mapping mRNA networks from cDNA-based microarrays (Jenkins et al. 2013). A recent study focusing on divergent growth patterns of skeletal muscle tissue in chicken strains discovered that genes with higher mRNA levels were linked to myosatellite cell proliferation in chicks raised for meat production (broiler) compared to those raised for egg-laying (layer; Zheng et al. 2009).

Gene expression results from avian breast muscle has pinpointed key enzymes in lipid and protein metabolism, leading to the identification of genetic markers involved in muscle hypertrophy in domestic chickens (Cui et al. 2012; Sibut et al. 2011) and king penguins (Teulier et al. 2012). Moreover, in an attempt to unravel the underlying mechanisms of excess fat in broiler chickens, Wang and colleagues (2007) discovered that enhanced mRNA expression of lipoprotein lipase, fatty acid binding protein, thyroid-hormone responsive protein, and leptin receptor in high-fat selected lines. Additionally, next-generation RNA sequencing is a progressively feasible and affordable technique to identify candidate genes without genomic resources selected *a priori* (see Ozsolak & Milos 2011 for a thorough review of RNA sequencing). Researchers now have access to the full transcriptomes of model avian species (i.e. chicken, Hubbard et al. 2005; zebra finch; Balakrishnan et al. 2012), and researchers are now generating transcriptomes of free-living, temperate/polar species as well (dark-eyed junco; Peterson et al. 2012) to test gene regulation within an ecological context. Transcriptomics is a powerful tool to assess how gene regulation integrates the cellular expression of numerous endocrine, metabolic, and neural traits in free-living birds, which may provide important insight into the regulation of traits and genes critical in mediating energetic readiness.

D. Long-Term Endocrine Studies as Powerful, but Rarely Applied Tools

Using a seasonal or life-history stage approach to assess organismal traits, environmental endocrinologists can measure the seasonal rhythms of hormone

concentrations, receptor densities, and endocrine-regulatory proteins to quantitatively assess the degree of energetic challenge (Jacobs & Wingfield 2000; Romero et al. 2017). However, long-term endocrine studies are considered rare, despite possessing strong scientific value to produce new or support pre-existing evolutionary theories in ecology (Mills et al. 2015).

In avian systems, “long-term endocrine studies” on captive or wild-caught birds involve the collection of blood samples for hormone analyses spanning from every week (fine scale) to every month (coarse scale) in at least one year. Unfortunately, whether on captive or wild-caught birds, long-term endocrine studies in birds require: (i) objectives that are both broad and detailed enough to justify the significant investment in time and effort; (ii) a model study system that can withstand repeated sampling of multiple individuals over time, yet be applicable to a diversity of species; (iii) consistent funding to span multiple goals that make up the larger project objective, (iv) a breadth and depth of researcher expertise for the analysis and interpretation of multiple metrics, and (v) a consistent and repeatable experimental design.

First, employing research objectives and questions provides a starting point for experimental design and proper selection of long-term study system (Lindenmayer & Likens 2009). Among some of the most frequently studied captive systems are highly seasonal, temperate and Arctic songbirds because they are: (a) able to survive in outdoor aviaries at northern latitudes year-round, (b) small-bodied birds, allowing for a greater sample size per aviary, (c) species with pronounced hormonal rhythms across seasons, and (d) conveniently caught and held in close proximity to an university or research facilities, such as the dark-eyed junco (i.e. Ho et al. 2017; Ramenofsky et al. 1999), white-crowned sparrow (i.e. Breuner et al. 1999; Schwabl & Farner 1989), white-throated sparrow (i.e. Spinney et al. 2006), and European starling (i.e. Romero & Remage-Healey 2000). Moreover, long-term studies require substantially more funding compared to short-term studies and also require sustainable research facilities and logistical support, high-quality and long-lasting equipment, as well as extensive training from the principal investigator. We advise that one sampler

commits to population monitoring and data collection (i.e. avoid sampler bias), unless logistically impossible to do so without assistance. Not surprisingly, long-term, endocrine-based studies are necessary to both examine and identify hormone variation across life-history stages, while controlling for additional environmental contexts. However, they demand a tremendous amount of logistical organization, financial costs, and team effort, and as such, the temporal patterns of many energy-regulating hormones (i.e. IGF, ghrelin, T_3) are still unknown.

CONCLUDING REMARKS

Endocrine responses regulate short-term energy balance through homeostatic mechanisms and seasonal energetic state through rheostatic mechanisms in mammals and birds. In this review, the focus has been on identifying the most likely regulators of muscle hypertrophy and fat accumulation during periods of energetic preparation, principally before migration and breeding, in free-living birds. Biomedical and poultry studies have provided a useful framework for identifying hormonal regulators of energy storage in order to test these presumably adaptive traits within the annual life cycle of free-living birds. Moving forward, continued application of integration of various sub-disciplines, including genetics, behavioural ecology, ecophysiology, and neuroendocrinology, has the potential to provide a more complete understanding of the endogenous mechanisms that govern energetic phenotypes. With the emerging tools of modern transcriptomics combined with the manipulation of environmental variables during long-term endocrine studies, ecologists can begin to causally determine how and why the molecular mechanisms central to seasonal energy storage impact organismal performance and ultimately fitness.

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TABLES

Table 2.1: Summary of studies on experimentally administered and naturally circulating levels of energy-regulating hormones with anabolic effects of fattening or muscle growth in avian species. This table includes studies that detect plasma hormones or gene regulation of hormone mRNA transcripts. Study design (SD), indicated as D = domestic captivity, C = wild-caught captivity, or W = wild/free-living; sex is abbreviated as NS = not stated, F = females, or M = males; stage or sub-stage = tested life-history stage or "-" = experimental manipulation with no specific stage tested; relative level (RL) with arrows indicating (i) up- or down-regulation of hormones for stage-related studies, and (ii) increase/decrease of energetic variable for manipulative studies.

Hormone	Species	SD	Sex, Age	Stage or Substage(s)	RL	Energetic Variable	Support Fat or Muscle Gain?	Notes	Reference
Baseline Corticosterone (CORT)	Broiler chicks *Gallus gallus domesticus*	D	NS, chick	-	↑	Abdominal Fat Pad, Liver Fat	Yes	6 daily injections of CORT induced fat mass gain	Bartov (1982)
	Broiler chicks *Gallus gallus domesticus*	D	F, adult	-	↑	Abdominal Fat Pad, Body Mass	Yes	Two injections of CORT increases abdominal fatness	Buyse et al. (1987)
	Dark-eyed junco *Junco hyemalis*	C	M, adult	-	↑	Body mass, fat score	Yes	Increase in fat score, but no change in lipoprotein lipase after 4 days	Gray et al. (1990)
	Gray catbird *Dumetella carolinensis*	W	Both, adult	Pre-migration Spring migration	↓ ↑	Fat score	Yes	Small sample size	Holberton et al. (1996)
	White-crowned sparrows *Zonotrichia leucophrys gambelii*	C	B, adult	Spring migration Breeding Autumn Migration Winter	↓ ↑ ↓ ↓	Body mass, fat score	Yes	Temporal correlation categorized into stages	Romero et al. (1997)

Baseline CORT	Yellow-rumped warbler *Dendroica coronata*	C	B, adult	Pre-migration (spring)	↑	Body mass, fat score	Yes	Strong temporal correlation	Holberton (1999)
	Red knot *Calidris canutus*	C	B, adult	Pre-migration Spring migration	↓ ↑	Body mass	Yes	Strong temporal correlation	Piersma et al. (2000)
	Semipalmated sandpiper *Calidris pusilla*	W	B, adult	Spring migration	-	Fat scores	No	Weak, non-significant correlation between fat and baseline CORT	Mizrahi et al. (2001)
	Bar-tailed godwits *Limosa lapponica*	W	M, adult	Start of refueling End of refueling	↓ ↑	Body mass	Yes	Sampled at stopover sites and breeding grounds	Landys-Ciannelli et al. (2002)
	Hermit thrush *Catharus guttatus*	W	ND, adult	Autumn migration	↑	Energetic condition	Yes	Low energetic condition = high levels	Long & Holberton (2004)
	Broiler chicks *Gallus gallus domesticus*	D	NS, chick	-	↑	Abdominal fat	Yes	Large dose (30 mg CORT/kg of diet) of corticosterone increases abdominal fat stores	Lin et al. (2006)
	Dark-eyed junco *Junco hyemalis*	C	B, adult	-	↑	Body mass, fat score	Yes	Manipulation with dexamethasone; no specific stage	Holberton et al. (2007)
	Dark-eyed junco *Junco hyemalis*	C	B, adult	Spring migration	↑	Body mass, fat score	Yes	Altered photoperiod to mimic short to long days	Holberton et al. (2008)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↑	Abdominal fat pad, liver weight, TRIG	Yes	Exogenous CORT (dose 30 mg/kg diet) increased body fat; TRIG also increased	Jiang et al. (2008)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↑	Abdominal and sub-cutaneous fat, TRIG, VLDL, LPL	Yes	CORT (30 mg/kg diet) increases insulin and multiple lipid metabolites and body fat stores	Yuan et al. (2008)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↑	Abdominal, cervical,	Yes	Various metabolites and body fat stores were	Cai et al. (2009)

Baseline CORT						and thigh fat, VLDL, TRIG, FAS, ME, ACC		increased with dexamethasone (1 mg/mL, daily for 7 days)	
	Northern wheatears *Oenanthe oenanthe* and *O. leucorhoa*	W	B, adult	Spring migration	↑	Body mass, fat score	Yes	Fuel load (i.e. fat score) was negatively correlated with baseline CORT	Eikenaar et al. (2013)
	Common yellowthroat *Geothlypis trichas*	C	B, adult	Autumn migration	No change	-	No	No seasonal change detected in baseline levels	Wagner et al. (2014)
	Broiler chicks *Gallus gallus domesticus*	D	ND, chick	-	↑	Fat storage	Yes	Experimental activation of AMPK-NPY pathway *via* dexamethasone	Liu et al. (2014)
	Red knot *Calidris canutus*	C	B, adult	Arrival Breeding	↑ ↓	Body mass	No No	Patterns in months of June, July, August	Reneerkens et al. (2015)
	Common eider *Somateria mollissima*	W	F, adult	Pre-breeding	↑	Body mass	Yes	Pre-breeding energetics in relation to baseline levels	Hennin et al. (2015)
	Common blackbird *Turdus merula*	W	B, adult	Spring migration Autumn migration	↓ ↑	Fat score	No	Compared migrants to residents	Eikenaar et al. (2015)
	Dark-eyed junco *Junco hyemalis*	W	B, adult	Spring migration	↓	Fat score	Yes	Compared migrants to residents	Bauer et al. (2015)
	Common eider *Somateria mollissima*	W	F, adult	Pre-breeding	↑	Body mass, VLDL	Yes	VLDL increased with increases in baseline CORT	Hennin et al. (2015)
	White-winged scoter *Melanitta fusca deglandi*	C	B, adult	-	↑	Body mass	Yes	Manipulation experiment; no specific stage	Hennin et al. (2016)
	Peregrine falcon *Falco peregrinus tundrius*	W	F, adult	Pre-breeding	↑	Triglycerides, scaled mass	Yes	Increased baseline CORT levels from arrival to end of follicle growth	Lamarre et al. (2017)

Baseline CORT	Northern wheatears *Oenanthe oenanthe* and *O. leucorhoa*	D	B, adult	-	No change	Fuel load	No	Blood samples at 7 mins were taken as baseline CORT levels	Eikenaar (2017)
Insulin	Domestic chicken *Gallus gallus domesticus*	D	B, adult	-	↓	FFAs	Yes	Glucose administration (fed state) leads to a decrease in FFAs	Langslow et al. (1970)
	Domestic chicks *Gallus gallus domesticus*	D	B, chick	-	↑	Fatty Acid Synthase Expression	Yes	Large amount of insulin needed to simulate increases in enzyme	Goodridge (1973)
	Domestic goose *Anser domesticus*	D	B, adult	-	↑	FFAs	No	Intravenous injection of bovine insulin causes increase in plasma FFAs	Nir & Levy (1973)
	Domestic chicken *Gallus gallus domesticus*	D	NS, adult	-	↑	Lipoprotein Lipase	Yes	*In vitro* addition of insulin increase liver lipogenic enzyme activity	Borron et al. (1979)
	Domestic duck *Anus platyrhynchos domesticus*	D	NS, adult	-	↓	FFAs	Yes	Low-dose insulin stimulates FFA uptake by liver	Gross & Mialhe (1984)
	European Starlings *Sturnus vulgaris*	C	NS, adult	-	No change	TRIG	No	Insulin failed to increase plasma TRIG	Remage-Healey & Romero (2001)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↑	Malic enzyme and FAS	Yes	*De novo* lipogenesis with dexamethasone and insulin	Cai et al. (2011)
Acylated Ghrelin	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↓	Food intake	No	ICV administration of ghrelin significantly decreased food intake	Furuse et al. (2001)
	Japanese quail *Coturnix coturnix japonica*	D	M, adult	-	↑	Food intake	Yes	Peripheral injection increased food intake, but decreased with ICV	Shousha et al. (2005)

Acylated Ghrelin	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↓	Food intake	No	Peripheral injection decreased short-term (~1 hour) food intake	Geelissen et al. (2006)
	Layer chicks *Gallus gallus domesticus*	D	M, chick	-	↓	Food intake	No	Ghrelin increases with fasting (hunger signal), but not food intake	Kaiya et al. (2007)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↓	Fatty acid synthase expression	No	Lowered mRNA levels in liver suggesting decreased lipogenesis	Buyse et al. (2009)
	6 domestic duck *Anus platyrhynchos domesticus* 9 domestic chicken *Gallus gallus domesticus*	D	B, chick	-	↑	Fat reserves	Yes	High mRNA for ghrelin and ghrelin receptor genes in subcutaneous fat of higher-fat breeds with peripheral inject.	Nie et al. (2009)
	Broiler chick *Gallus gallus domesticus*	D	NS, chick	-	↓	Food intake	No	Peripheral ghrelin injection suppresses food intake	Ocłoń & Pietras (2011)
	Domestic geese *Anser anser domesticus*	D	NS, chick	-	-	Breast muscle	No	Peripheral ghrelin did not increase food intake, but high dose increased breast muscle	Aghdam Shahryar & Lotfi (2015)
	Japanese quail *Coturnix coturnix japonica*	D	M, adult	-	↓	Respiratory quotient	No	Peripheral ghrelin injection switches to fat or protein oxidation	Shousha et al. (2015)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↑	TRIG	Yes	Ghrelin antagonist decreases plasma TRIG	Aghdam Shahryar & Lotfi (2016)
	Garden warbler *Sylvia borin*	W	B, adult	Spring migration	↑	Fat score	No	Birds with fat score > 0 had elevated ghrelin	Goymann et al. (2017)

Acylated Ghrelin	Wild turkey *Meleagridis gallopova*	D	NS, chick	-	↑	TRIG	No	Ghrelin injection decreased plasma TRIG; may be dose-dependent	Aghdam Shahryar & Lotfi (2017)
Triiodo-thyronine (T_3)	Canada goose *Branta canadensis*	W	B, adult	Pre-migration Spring migration Breeding	↑ ↓ ↓	None	Yes	T_4 to T_3 conversion from longer photoperiod prepare for reproduction	John & George (1978)
	Domestic chicks *Gallus gallus domesticus*	D	B, chick	-	↓	Abdominal fat	No	T_3 administration causes a decrease in abdominal fat stores; lean line had greater plasma T_3 than fat line	Leclercq et al. (1988)
	Domestic chicks *Gallus gallus domesticus*	D	M, chick	-	↑	*De novo* lipogenesis	Yes	T_3 stimulates hepatic lipogenesis through the up-regulation of malic enzyme	Goodridge et al. (1989)
	Redheaded bunting *Emberiza bruniceps*	C	M, adult	Wintering Pre-migration Spring migration Breeding	↓ ↑ ↓ ↓	Body mass	Yes	Increase in T_3/T_4 ratio during pre-migration	Pathak & Chandola (1982a)
	Redheaded bunting *Emberiza bruniceps*	C	M, adult	Wintering Pre-migration Spring migration	↓ ↑ ↓	None	Yes	High T_4 to T_3 conversion during pre-migration; inhibition of conversion prevented fattening	Pathak & Chandola (1982b)
	Rosy pastor *Sturnus roeus*	C	M, adult	Wintering Pre-migration Migration Breeding	↓ ↑ ↓ ↓	Body mass	Yes	Small sample size (n = 8); T_3 and body weight both increase at pre-migration in the spring	Pathak & Chandola (1984)
	Broiler chicks *Gallus gallus domesticus*	D	M, chicks	-	↑	Free fatty acids, TRIG	Yes	Dietary T_3 increases plasma lipid content	Rosebrough et al. (1992)

T_3	Redheaded bunting *Emberiza bruniceps*	D	M, adult	Spring migration	↑	Fat scores	Yes	Thyroidectomized birds (no fat) with peripheral T_4 restores fat stores; inhibition of T_4 to T_3 conversion decreases fat	Pant & Chandola-Saklani (1993)
	Spotted munia *Lonchura punctulata*	D	F, adult	Spring moult Breeding Wintering	↓ ↑ ↓	Fat score	Yes	T_3 and fat scores concomitantly increase throughout breeding; T_4 profile is lowest in breeding	Pant & Chandola-Saklani (1995)
	Broiler chicks *Gallus gallus domesticus*	D	M, chicks	-	↓	*In vitro* lipogenesis and TRIG	No	No change from basal with dietary T_3 on *in vitro* lipogenesis, but decreased plasma TRIG	Rosebrough & McMurtry (2000)
	Northern cardinal *Cardinalis cardinalis*	W	NS, adult	Wintering	-	Dry lipid mass	No	No correlation between thyroid hormones and fat	Burger & Denver (2002)
	House sparrow *Passer domesticus*	W	B, adult	Pre-breeding Breeding	↓ ↑	Body mass	No	T_3 is positively correlated to BMR, but is not correlated with body mass	Chastel et al. (2003)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↑	*In vitro* lipogenesis, ME	Yes	T_3 diet restored *in vitro* lipogenesis and ME expression, but prolonged treatment decrease lipogenesis	Rosebrough & McMurtry (2003)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↓	*In vitro* lipogenesis, ME	No	Methimazole decreases lipogenesis and ME in a dose-dependent manner	Rosebrough et al. (2006)
	Northern shovelers *Anas clypeata*	C	B, adult	Wintering Pre-alternate Moult Spring migration	↓ ↓ ↑	Fat score	Yes	Significant increase in T_3 during pre-migratory fattening before spring migration	Elarabany et al. (2012)

T_3				Post-migration	↓				
	White-crowned sparrow *Zonotrichia leucophrys gambelii*	C	M, adult	Pre-migration (Spring)	↑	Fat score, body mass	Yes	Chemical thyroid inhibition dropped fat score, while T_3 replacement only partially restored fat profile	Pérez et al. (2016)
Growth Hormone (GH)	Broiler chicks *Gallus gallus domesticus*	M	F, chick	-	No Change	Breast muscle	No	Subcutaneous injection for two weeks (two doses) did not significantly alter muscle	Buonomo & Baile (1988)
	Broiler chicks *Gallus gallus domesticus*	M	F, chick	-	No Change	Breast muscle	No	Protein % in body muscle was not significantly altered with pulsatile infusions of GH	Vasilatos-Younken et al. (1988)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↓	Breast muscle	No	Daily, single dose injections of GH for 14 days decreased total body protein	Cogburn et al. (1989)
	Domestic chicken *Gallus gallus domesticus*	D	M, adult	-	No Change	Breast muscle	No	Protein % in body muscle was not significantly altered with pulsatile infusions of GH	Scanes et al. (1990)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↓	Breast muscle	No	Pulsatile infusions for 21 days led to slightly decreased body protein	Moellers & Cogburn (1995)
	Domestic chicken *Gallus gallus domesticus*	D	M, adult	-	↓	Satellite cells	No	GH inhibited progenitor cell differentiation *in vitro via* muscle-specific genes	Halevy et al. (1996)

GH	Domestic chicken *Gallus gallus domesticus*	D	M, adult	-	No Change	Breast muscle	No	Chicken GH had no affect on breast muscle	Radecki et al. (1997)
	Broiler chicks *Gallus gallus domesticus*	D	F, chick	-	↑	Breast muscle	No	Intravenous GH implants did not increase IGF-1 and decreased skeletal muscle growth; T_3-influenced	Vasilatos-Younken et al. (2000)
Insulin-like Growth Factor-1 (IGF-1)	Domestic chicken *Gallus gallus domesticus*	D	M, adult	-	↑	Satellite cell proliferation	Yes	Satellite cell proliferation occurred through IGF-1 receptor in cultured cells	Duclos et al. (1991)
	Domestic chicken *Gallus gallus domesticus*	D	M, adult	-	↑	Amino acid uptake and protein synthesis	Yes	Cultured satellite cells exposed to IGF-1 had quicker amino acid transport *in vitro*	Duclos et al. (1993)
	Domestic chicken *Gallus gallus domesticus*	D	M, egg	-	↑	Satellite and myoblast proliferation /differentia-tion	Yes	IGF-1 promotes satellite and myoblast cell proliferation and differentiation in embryonic stage	McFarland et al. (1993)
	Domestic chicken *Gallus gallus domesticus*	D	M, adult	-	↓	Muscle protein breakdown	Yes	*N*-methylhistidine, protein metabolite, excretion was decreased in IGF-1-treated birds	Tomas et al. (1998)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↑	3-methyl-histidine	No	IGF-1 infusion causes increase in 3-methylhistidine indicating protein breakdown	Czerwinski et al. (1998)

IGF-1	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↑	Body weight	Yes	Circulating IGF-1 is greater in high-growth rate relative to low-growth rate birds	Beccavin et al. (2001)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↑	Protein synthesis in breast muscle	Yes	Protein synthesis rate is increased with IGF-1	Conlon & Kita (2002)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↑	Muscle IGF-1 mRNA	Yes	Muscle IGF-1 mRNA is up-regulated during post-hatch growth	Guernec et al. (2003)
	Broiler chicks *Gallus gallus domesticus*	D	M, chick	-	↑	Breast muscle	Yes	*IGF-1* gene polymorphism increased plasma IGF-1 and breast muscle weight	Zhou et al. (2005)
	White-throated sparrows *Zonotrichia albicollis*	W	B, adult	Short day Long day	↓ ↑	Dry breast muscle	Yes	IGF-1 mRNA expression higher in long photoperiod	Price et al. (2011)
	Domestic duck *Anas platyrhynchos domestica*	D	NS, egg & chick	-	↑	Breast and leg muscles	Yes	IGF-1 treated eggs had greater muscle mass in breast and legs post-hatch	Liu et al. (2012)
	Adélie penguins *Pygoscelis adeliae*	W	B, chick	Breeding	↑	Muscle IGF-1 mRNA	Yes	Muscle IGF-1 mRNA is elevated during post-hatch growth	Degletagne et al. (2013)
	Broiler chicken *Gallus gallus domesticus*	D	B, chick	-	↑	Myofibers size and number	Yes	Exogenous IGF-1 induces myogenesis from myoblasts *in vivo*	Yu et al. (2015)
	Broiler chicken *Gallus gallus domesticus*	D	M, chick	-	↑	Muscle IGF-1 mRNA	Yes	Skeletal muscle IGF-1 mRNA is down-regulated as chicks grow older	Saneyasu et al. (2016)
Testosterone	Red-winged blackbird *Agelaius phoeniceus*	W	B, chick	Breeding	↑	Musculus complexus	Yes	Testosterone injected into egg yolk increased complexus mass	Lipar & Ketterson (2000)

Testosterone	Broiler chickens *Gallus gallus domesticus*	D	M, chick	-	↓	Breast muscle	No	Castrated birds significantly gained breast muscle mass and weight gain over time	Chen et al. (2010)
	Dark-eyed junco *Junco hyemalis*	C	B, adult	Pre-migration (Spring)	↑	Body mass and fat	Yes	Peripheral administration of testosterone advances body and fat mass gain	Tonra et al. (2011)
	American redstarts *Setophaga ruticilla*	C	M, adult	Pre-migration (Spring)	↑	Muscle score	Yes	Testosterone implants increases breast muscle; advance in departure date	Tonra et al. (2013)
	Broiler chickens *Gallus gallus domesticus*	D	F, chick	-	↑	Satellite cell number	Yes	Mesterolone (testosterone agonist) increases satellite cell and myonuclei number in breast muscle	Allouh & Aldirawi (2012)
	White-crowned sparrow *Zonotrichia leucophrys gambelii*	C	M, adult	Pre-migration (Spring)	↑	Muscle score	Yes	Testosterone implants increase pectoralis muscle	Ramenofsky & Németh (2014)
	Eurasian Skylark *Alauda arvensis* Asian Short-toed Lark *Calandrella cheleensis*	C	B, adult	Spring migration Breeding	↓ ↑	Body mass	Yes	Plasma testosterone levels increases during migration with highest levels during the onset of breeding	Zhao et al. (2017)

Table 2.2: General summary of the expected effects of energy-regulating hormones on peripheral tissue during periods of energetic storage in birds breeding in seasonal environments based on the synthesis of studies in Table 1. Direction of arrows indicate up- (↑) or down- (↓) regulation during energetic readiness (i.e. pre-migration or pre-breeding), and horizontal dash (—) represents no effect. Asterisks indicate hormones that have multiple (or more than a few) studies on temporal patterns across life-history stage in ecology.

Hormones	Effector Tissue	Effect with Slight Elevation	Effect with High or Prolonged Elevation	Levels during Energetic Storage
Baseline Corticosterone*	White adipose tissue	Anabolic	Catabolic	↑
Insulin	White adipose tissue	Unknown	Anabolic	↑↑
Acylated Ghrelin	White adipose tissue	Unknown	Catabolic	↓
T_3*	White adipose tissue	Anabolic	Catabolic	↓
GH	Skeletal muscle	Unknown	Catabolic/No Effect	↓ or —
IGF-1	Skeletal muscle	Anabolic	Anabolic	↑↑
Testosterone*	Skeletal muscle	Anabolic	Anabolic	↑↑

FIGURES

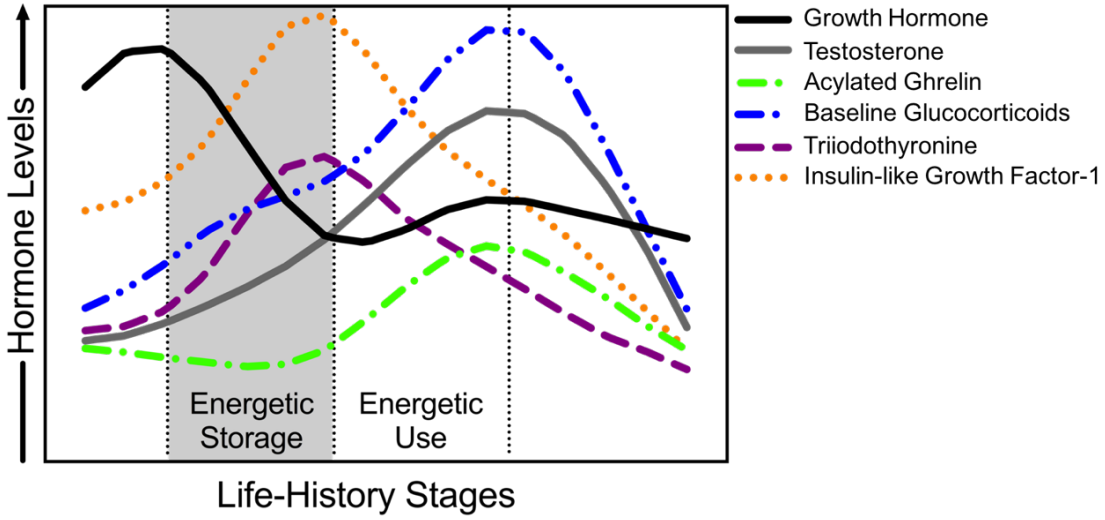

Figure 2.1: General predictive model outlining the expected temporal variation in candidate anabolic energy-regulating hormones during periods of energetic storage (fat accumulation and skeletal muscle growth; shaded in grey) before a period of predictable, high energy expenditure (migration or breeding) in avian systems. Areas outside of the dotted boxes are only used for reference.

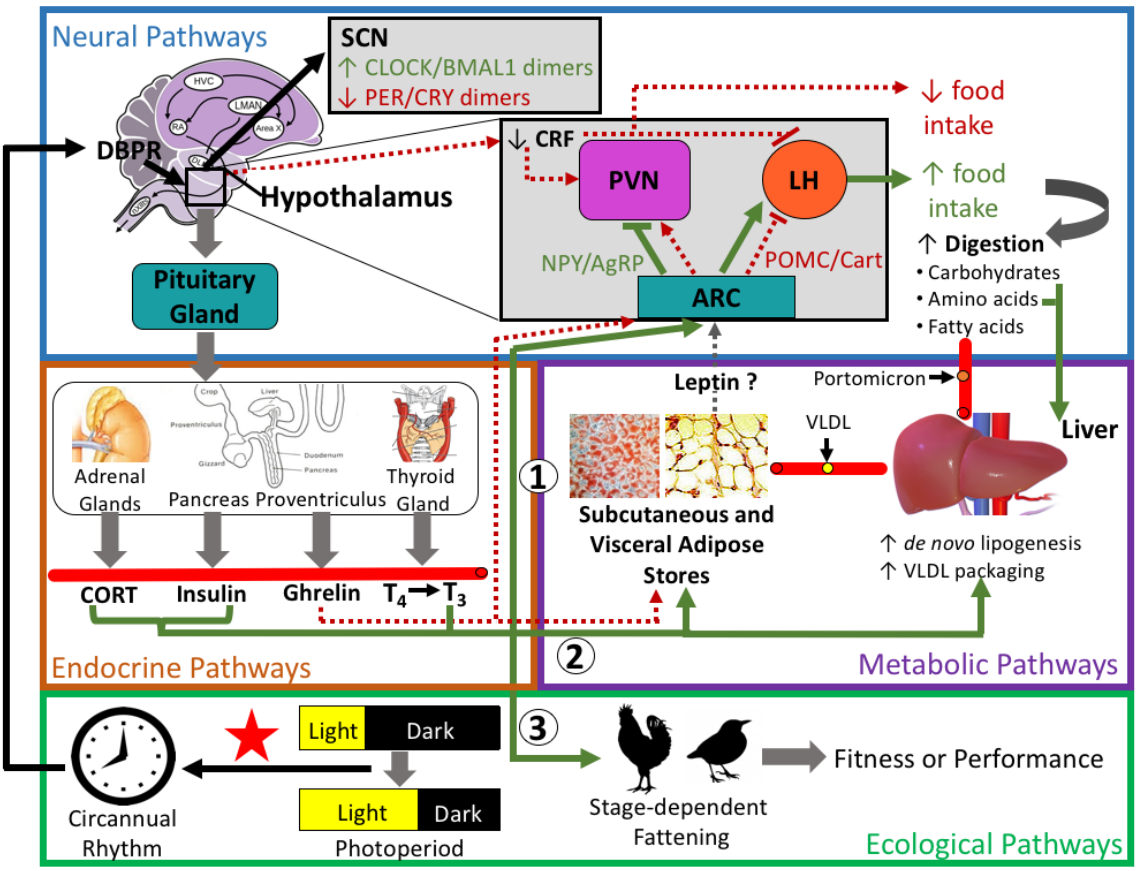

Figure 2.2: Conceptual illustration of anabolic mechanisms that integrate neural, endocrine, metabolic, and ecological pathways within a holistic framework of **seasonal fattening** in avian systems. Molecular pathways in the hypothalamus (neural) are initiated by changes in photoperiods (ecological), regulating the expression of clock genes and proteins (i.e. increase in CLOCK/BMAL1 heterodimers) in the SCN involved in central control of neuroendocrine axes. Releasing hormones from the hypothalamus activates the pituitary gland to secrete intermediate hormones, which leads to secretion of various energy-regulating hormones (endocrine). Baseline glucocorticoids, ghrelin, insulin, and T_3 act through three interrelated bodily pathways: (1) Neural pathway: hormones reach ARC and stimulate orexigenic neurons, NPY and AgRP, that synapse at the LH to drive hunger signaling and increase food intake, (2) Metabolic pathway: ingested food rich in lipid- and carbohydrate-based macronutrients are delivered to the liver, where TRIG and other lipids are shuttled in VLDL to adipose tissue. Circulating energy-regulating hormones can directly activate lipogenesis at the liver or directly bind to adipocytes, increasing fat reserves in subcutaneous and visceral adipose tissue. (3) Ecological pathway: stage-dependent fattening is driven by proposed endocrine mechanisms to meet energetic challenges associated with costly stages (i.e. migration and breeding). *Green arrows represent anabolic pathways, dotted-red arrows indicate catabolic pathways (down-regulated), grey arrows are used generically, question mark indicates unknown role in birds, and **red star depicts starting point**. [Abbrevs: DBPR =

deep brain photoreceptors, SCN = suprachiasmatic nucleus, ARC = arcuate nucleus, PVN = paraventricular nucleus, LH = lateral hypothalamus, CORT = corticosterone, VLDL = very low-density lipoproteins, NPY = neuropeptide Y, AgRP= agouti-related protein, POMC = pro-opiomelanocortin, Cart = cocaine- and amphetamine-regulated transcript, CRF = corticotropin releasing factor, T_4 = thyroxine, T_3 = Triiodothyronine]. Google photos retrieved with the tools option "labeled for reuse with modification". Based on figures from a variety of mammalian and avian sources (Woods et al. 1998; Boswell 2005; Murphy & Bloom 2006; Chong et al. 2007; Williams & Buck 2010).

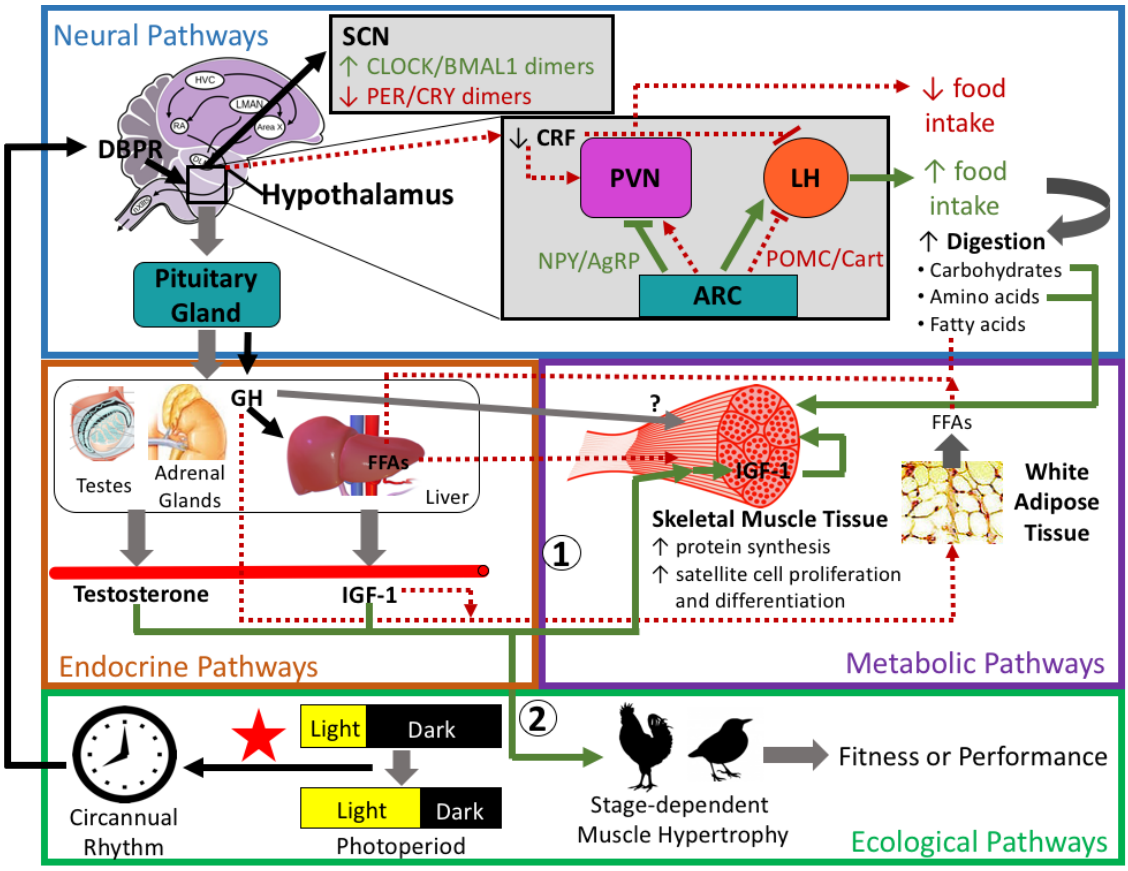

Figure 2.3: Conceptual illustration of anabolic mechanisms that integrate neural, endocrine, metabolic, and ecological pathways within a holistic framework of **seasonal skeletal muscle growth** in avian systems. Similar to fattening, molecular pathways in the hypothalamus (neural) are initiated by changes in photoperiod (ecological), regulating the expression of clock genes and proteins (i.e. increase in CLOCK/BMAL1 heterodimers) in the SCN involved in central control of neuroendocrine axes. Releasing hormones from the hypothalamus activate the pituitary gland to secrete intermediate hormones, which lead to secretion of various energy-regulating hormones (endocrine). GH, IGF-1 and testosterone act in two pathways: (1) Metabolic pathway: ingested amino acids are delivered to the skeletal muscle tissue along with increased amino acid transporter expression on muscle cells. Circulating energy-regulating hormones can directly activate satellite cell differentiation and protein synthesis. In addition, high levels of GH causes the production and release of FFAs from white adipose tissue that can be used for energy in muscle tissue via β-oxidation. (2) Ecological pathway: stage-dependent muscle hypertrophy is driven by proposed endocrine mechanisms to meet energetic challenges associated with costly stages (i.e. migration and breeding). *Green arrows represent anabolic pathways, dotted-red arrows indicate catabolic pathways (down-regulated), grey arrows are used generically, question marks indicate unknown roles in birds, and **red star depicts starting point**. [Abbrevs: DBPR = deep brain photoreceptors, SCN = suprachiasmatic nucleus, ARC = arcuate nucleus, PVN = paraventricular

nucleus, LH = lateral hypothalamus, CRF = corticotropin-releasing factor, FFAs = free fatty acids, NPY = neuropeptide Y, AgRP= agouti-related protein, POMC = pro-opiomelanocortin, Cart = cocaine- and amphetamine-regulated transcript, GH = growth hormone, IGF-1 = insulin-like growth factor 1]. Google photos retrieved with the tools option “labeled for reuse with modification”. Based on figures from a variety of mammalian and avian sources (Buyse & Ducuypere 1999; Herbst & Bhasin 2004; Velloso 2008).

CHAPTER 3 – TEMPORAL INVESTIGATION OF HORMONES REGULATING FAT AND MUSCLE GAIN PRIOR TO ENERGETICALLY DEMANDING STAGES IN TWO ARCTIC BIRDS

INTRODUCTION

Seasonal organisms demonstrate remarkable phenotypic flexibility in morphology, physiology, and behaviour across various stages in their life cycle to maximize fitness and/or performance (Piersma & Drent 2003). Individuals living in highly seasonal environments must be adapted to interpret environmental cues from the change in seasons, translate this external information to adjust phenotypes, and organize these responses into distinct stages (Paul et al. 2008; Wingfield 2008; Visser et al. 2010). Often in these seasonal environments (i.e. temperate or circumpolar), the change in photoperiod is a powerful environmental cue that induces seasonal change in energetic and metabolic physiology (Hazlerigg & Wagner 2006; Lincoln et al. 2006; Wikelski et al. 2008), and results in changes in body composition which reflects predictable shifts in energy demand within the annual schedule (Piersma & Gils 2011). Energetic readiness, a period of resource accrual and nutrient deposition (i.e. fat and protein stores) to prepare for an energetically demanding life-history stage, is an adaptive strategy to provide reliable fuels before costly periods of prolonged food shortage, such as migration (McWilliams et al. 2004), hibernation (Humphries et al. 2003), or breeding (Varpe et al. 2009).

Birds living in highly seasonal environments have long been considered relevant models for examining energetic readiness, given that they exhibit extreme phenotypes of internally stored lipids and protein in white adipose tissue, skeletal muscle, and visceral organs (Lindström & Piersma 1993; Bauchinger & McWilliams 2010), in preparation for reproduction (Meijer & Drent 1999) and

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*This chapter is the result of joint research with F. Vézina, A. Le Pogam, C. Harris, H.L. Hennin, and O.P. Love.

migration (Jenni et al. 1998). In particular, the flexible remodeling of adipose tissue and skeletal muscle at these life-history stages indicate a marked physiological shift in lipid and protein metabolism (Jenni-Eiermann & Jenni 1991; Dietz et al. 1999; Bauchinger & Biebach 2005a). Birds accumulate excess energy stores by converting dietary carbohydrates, protein, and lipids into fat and muscle through various physiological and behavioural processes such as hyperphagia (i.e. rapid increase in food intake), increase in assimilation efficiency, and shifts in diet selection (Bairlein 1987; Bairlein 2002; Davies & Deviche 2014). Although the physiology and behaviours of lipid and protein storage across avian life-history stages have been well-characterized (McWilliams et al. 2004), the endogenous signaling mechanisms that translate the seasonal cues into predictable phenotypic responses of fat deposition and skeletal muscle growth remain incomplete (Ramenofsky 2011). Nonetheless, energy-regulating hormones were originally identified as candidate signaling molecules in mammals (Cahill et al. 2013), providing a strong framework for studies in avian systems (see Table 2.1 in Chapter 2 for full details).

Energy-regulating hormones are proposed regulators of seasonal changes in energetic state for a few reasons, including (i) fat and protein metabolism that affect somatic energy stores (Scanes 2008), (ii) daily and seasonal variation in circulating (endocrine) and local (tissue) levels (Jacobs & Wingfield 2000), and (iii) interactions with neural and metabolic pathways involved in energy balance (Boswell 2005; Richards & Proszkowiec-Weglarz 2007). Candidate energy-regulating hormones, such as baseline glucocorticoids, acylated ghrelin, insulin, and triiodo-L-thyronine, have both central and peripheral effects to regulate food intake and fat metabolism in poultry (outlined in Scanes 2008 and Boswell & Dunn 2017). Glucocorticoids, such as corticosterone (the primary avian glucocorticoid) and cortisol, are the most studied hormones on daily and seasonal energetic processes in birds, as baseline corticosterone is often elevated during life-history stages of high energy demand (Romero 2002; Landys et al. 2006; Crespi et al. 2013; Romero et al. 2017). More specifically, increases in circulating levels of baseline corticosterone have shown to induce foraging

behaviours (Astheimer et al. 1992; Angelier et al. 2007; Angelier et al. 2008; Kitaysky et al. 2010; Crossin et al. 2012), food intake (Dallman et al. 1993; Landys et al. 2006), and fat deposition (Holberton 1999; Long & Holberton 2004; Holberton et al. 2007, 2008; Hennin et al. 2015, 2016; Lamarre et al. 2017).

Considering the importance of selection for rapid growth and development of meat in the poultry industry, two prospective muscle-promoting hormones worth investigating with regards to changes in muscle mass are testosterone and insulin-like growth factor-1 (IGF-1; Scanes 2009; Fuxjager et al. 2012). Testosterone, classically known for reproductive and behavioural functions in birds (Hau 2007), has also experimentally been shown to be a potent anabolic steroid that increases breast muscle mass in migratory passerines (Tonra et al. 2011b, 2013; Ramenofsky & Németh 2014). Likewise, IGF-1 is the downstream hormone of the somatotropic (growth hormone/IGF-1) axis secreted from the liver which increases cellular growth through protein synthesis *via* the PI3K/Akt signaling pathway in various tissue (i.e. reproductive, digestive, bone, and muscle tissues; McMurtry et al. 1997), including its proliferative effects on somatic tissue development in juvenile birds (Lodjak et al. 2014; Lodjak et al. 2016) and on skeletal muscle hypertrophy in adults (Buyse & Decuypere 1999; Velloso 2008). Despite the roles that these two anabolic agents might play in energetic readiness, few empirical studies have examined these hormonal mechanisms in free-living systems, and even fewer are temporally represented over a relevant period of expected responses.

Here, we examine temporal patterns of candidate endocrine markers, measured before, during, and after periods of energetic readiness for energetically demanding life-history stages in two Arctic-breeding species, male snow bunting (*Plectrophenax nivalis*) and female common eider (*Somateria mollissima*). Energetic preparation is important to fuel stages centred around a short, constrained polar breeding season in Arctic environments (Love et al. 2010). Snow buntings gain body mass during pre-migratory preparation for a long, direct migration in the spring (Vincent & Bedard 1976), presumably from accumulation of fat depots and enlarged flight muscles (Piersma et al. 1999;

Bauchinger et al. 2005a; Hua et al. 2013). Female common eiders must also rapidly store enough fat and protein during the pre-breeding period to initiate egg production (Hennin et al. 2015) and fuel a long fast during the incubation period (Groscolas & Robin 2001). To identify signaling mechanisms of physiological responses for energetic storage, we focused on the hormonal profile of (i) the fat-promoting hormone, baseline corticosterone, and (ii) the muscle-promoting hormones, testosterone and IGF-1. Interestingly, to the best of our knowledge, this the first study to explore the temporal patterns of circulating IGF-1 levels in any adult bird (although see Price et al. (2011) for IGF-1 mRNA levels during short and long days). During periods of energetic preparation in both species, we expected to see an elevation in baseline corticosterone to mediate lipid deposition in white adipose tissue and elevations in testosterone and IGF-1 to mediate protein synthesis in skeletal muscle.

METHODS

A. Study Systems and Stages of Energetic Preparation

Snow buntings. Wild, male adult snow buntings (n = 25) were captured locally from a wintering population at Rimouski, Québec, Canada (48°26'N, 68°31'W) using walk-in ground traps that were baited with crushed corn in November 2013 and October 2014. Each bird was given a numbered metal band and a unique, four-colour combination of plastic bands for individual identification (Banding Permit #: 10704). All birds were transported to Université du Québec à Rimouski (UQAR) in small temporary cages and were held in outdoor aviaries (6.1 x 4.6 m). Captive individuals were exposed to natural ambient environmental conditions (i.e. sunlight and wind), but were partially sheltered from heavy precipitation. Birds were given fresh, *ad libitum* food and water each day (i.e. commercial mixed-seed diet: white millet, cracked corn, black oil sunflower seeds).

Previous migratory research suggests that this wintering population of buntings breeds in western Greenland (Macdonald et al. 2012). During pre-migratory preparation on wintering grounds in the late winter, excess fat and

muscle tissues are thought to be developed for a few reasons: (i) direct, non-stop flapping flight over the Labrador Sea to breeding grounds in western Greenland during the early spring (Macdonald et al. 2012); (ii) arrival in cold, unpredictable Arctic climate in the spring may require energy reserve and muscular capacity for shivering heat production (Montgomerie & Lyons 2011; Vézina et al. 2012); and (iii) remaining lipid and protein may fuel carry-over effects from migration into breeding activities (Sandberg 1996). Males were chosen, in particular, because they are expected to experience the harshest environmental conditions associated with earlier arrival than females on the breeding grounds to establish and defend territories (McKinnon et al. 2016).

Data on energetic metrics and hormones were temporally represented across weeks, and then further grouped into "stages" (wintering, pre-migration, migration, and post-migration) for analyses using the annual cycle outlined in Montgomerie & Lyon (2011). Standard morphometric measurements (body mass, wing chord, tarsus length, head-and-bill length) were collected after original capture, and body mass, blood samples, and energetic metrics were recorded bi-weekly for all individuals from January to October 2015. Although we present data for multiple life-history stages (i.e. wintering, pre-migration, spring migration, breeding, pre-basic moult, fall migration) for reference, the primary focus was on the dynamics of energy-regulating hormones and energetic responses during the pre-migration stage.

Common eiders. Data on free-living, adult female common eiders (n = 1,264) were collected from Mitivik Island (64°02'N, 81°47'W) in the East Bay Bird Sanctuary, Nunavut, Canada from 2006 to 2016. This population represents the densest breeding colony (~9,000 pairs on 800 x 400 m island) of common eiders in northern Canada (Hennin et al. 2015). Females were caught following migration from wintering grounds (coasts of Newfoundland and Greenland; Mosbech et al. 2006) using large flight nets from mid-June to early-July (range: June 10 to July 8 across ten years), where capture date was used as a reliable proxy of arrival date for this particular colony (Descamps et al. 2011). After

capture, blood samples were collected within 3 minutes from the tarsal vein to obtain baseline physiological measures (see “Blood Sampling”), and then body mass (in grams, g) and tarsus length (in millimeters, mm) were measured. Each female received an alpha-numeric darvic metal band and an UV-degradable monofilament for individual identification (Banding Permit #10650). Released hens were monitored with spotting scopes from a concealed location to assess nest location, lay date, clutch size, and hatchling success. When uncertain, or if undiscernible, of a female’s lay date, nest visits (2 to 3 per season) were conducted to discover the clutch size and the number of days into incubation using the egg candling technique.

Captured females that were not detected nesting on the island, and were less than 2000 g in mass (the threshold mass at which females often initiate laying; Sénéchal et al. 2011) were considered "non-breeding" females. We assigned females to breeding stages based on the number of days from when she was caught and sampled to when she began laying. Hens that were caught prior to laying were considered pre-breeding birds, and were further divided into three categories centred around initiation of follicle recruitment: "arrival", "pre-recruiting", and "rapid follicular growth" (RFG). We considered a female to be an "arrival" bird if she was at further than 15 days away from laying at the time of capture, "pre-recruiting" (i.e. not yet recruiting follicles) if she was captured 8 to 15 days prior to laying, and "RFG" (i.e. quickly growing follicles) if she was captured 7 to 1 day before the first egg. These stages were determined based on (1) previously published estimates of the RFG period for common eiders (6 days plus an additional 28 hours for shell formation and laying; Watson et al. 1993; Robertson 1995a; Robertson 1995b), (2) the known shifts in female reproductive physiology (i.e. production of yolk-targeted lipoproteins in RFG; Salvante et al. 2007), and (3) trends of physiological traits in response to changing energetic demands (Hennin et al. 2015). Females that were caught while laying (either seen laying in the colony or at candling nest contained a fresh egg) were considered "laying" hens, and those caught while incubating (at candling nest that was aged 4 days or older and contains no fresh eggs) were considered to be

"incubating". Arrival, laying, incubation, and non-breeding stages were provided as important references to the pre-breeding period (pre-recruiting and RFG) in which females are preparing for reproductive investment.

B. Energetic Metrics

Snow buntings. We obtained pectoralis (flight) muscle score (scale of 0 to 3: 0 = emaciated, sternum sharp and 3 = fully rounded; following Bairlein 1995) and body mass (in grams, g) from sampled individuals following blood sampling (see below). Muscle size was scored prior to body mass to prevent size-related bias associated with an observation. Total fat content (in g) was quantified using quantitative magnetic resonance (QMR; EchoMRI^{TM} Body Composition Scanner). QMR is a non-invasive, fast, and accurate method for repeated measures, and this technique has been validated in numerous small flying animals, mostly passerines and bats, with very high accuracy (Guglielmo et al. 2011; McWilliams & Whitman 2013).

Common eiders. We measured body mass (in grams, g) using a spring scale following blood sampling. In this particular colony, uncorrected body mass performs similarly (<1% difference in variation) to size-corrected body mass (Descamps et al. 2010), and body mass of pre-breeding females is highly correlated with abdominal fat mass (Descamps et al. 2011). Moreover, very low-density lipoprotein levels (VLDL), a lipoprotein carrier with attached lipid molecules including triglycerides (TRIG) for fat deposition to adipose tissues, are low and high in lighter and heavier females, respectively (Hennin et al. 2015). We therefore used body mass as a proxy of fat mass during pre-breeding in female eiders. Although we were not able to obtain data on pre-breeding muscle mass, Parker & Holm (1990) found pre-laying pectoral muscle mass was significantly greater than post-laying in a Svalbard population of common eiders. Additionally, we present energetic data collected from 2002 to 2004, showing that breast muscle mass significantly decreases from RFG through incubation in the East Bay population (J. Bêty, unpubl. data used with permission; Figure 3.2). Finally,

Jamieson and colleagues (2006) analyzed carcasses of non-breeding eiders in Greenland and showed that winter breast muscle mass (157.2 ± 2.1 g) is much lower than pre-breeding levels found by Parker & Holm (1990) and Sénéchal et al. (2011). Collectively, these studies are conducive evidence of muscle gain from winter to pre-breeding that may be important for fueling reproductive investment in female eiders. Additionally, we analyzed changes in corrected (i.e. removal of food items) digestive tract mass and total (i.e. all reproductive tissues: ovaries, oviduct, follicles) reproductive tract mass across breeding stages.

C. Blood Sampling

In snow buntings, blood samples were collected in heparinized 75 μL microcapillary tubes (less than 1% of total blood volume) using a sterile 26 G hypodermic needle for venipuncture at the brachial (wing) vein. We only included individuals for analyses that were blood sampled within 4 minutes of capture for baseline corticosterone (average time ± SEM: 3.39 minutes ± 0.052; Romero & Reed 2005; Wingfield 1982) and within 8 minutes for testosterone (3.85 minutes ± 0.087) and IGF-1 (3.83 minutes ± 0.088; Lodjak et al. 2014). Duration of blood collection and sample order (i.e. the order of blood sample collected in a given day) were assumed to have no influence on models because hormone concentrations did not significantly correlate with duration of blood collection (baseline corticosterone: R^2 = 0.0007, n = 152, p = 0.74; testosterone: R^2 = 0.015, n = 131, p = 0.15; IGF-1: R^2 = 0.008, n = 127, p = 0.33) nor with sample order (baseline corticosterone: R^2 = 0.001, n = 121, p = 0.60; IGF-1: R^2 = 0.016, n = 127, p = 0.15), except for testosterone (R^2 = 0.043, n = 131, p = 0.0178). To control for a potential effect of time of day for testosterone, sample order was therefore included in models as a random effect. Plasma samples were collected between 8:30 and 11:30 a.m. to control for diel rhythm in hormone variation (i.e. Romero & Healey 2000). All manipulations on birds were approved by the Animal Care Committees of UQAR and Environment and Climate Change Canada – Canadian Wildlife Service (CPA-54-13-130).

Blood samples were collected in female eiders from the tarsal (leg) vein using a heparinized 23 G needle attached to a 1 mL syringe. Blood was collected within 3 minutes of capture to acquire baseline corticosterone levels (Romero & Reed 2005). Blood samples were collected throughout the day, however, previous research at this colony indicates that there is no diel variation in physiological traits, making all samples comparable and useable (Steenwag et al. 2015). In both species, whole blood was stored at 4°C until centrifuged at 10,000 rpm for 10 minutes to separate plasma and hematocrit, and then was stored separately at -80°C until further analyses. To analyze all three hormones in an individual sample, only samples with plasma volume of > 50 μL were selected. All techniques used on eiders followed the regulations and received permission from the Animal Care Committees of Environment and Climate Change Canada (EC-PN-15-026) and University of Windsor (AUPP 11-06).

D. Hormone Extractions and Assays

Plasma was extracted for the steroid hormones (corticosterone in buntings and testosterone in both species) using a liquid-liquid extraction technique with dichloromethane (CH_2Cl_2, Sigma-Aldrich Canada, Oakville, Ontario, Canada) to partition the steroid of interest in plasma (20 and 10 μL for baseline corticosterone and testosterone, respectively) into the organic phase, excluding other polar molecules (i.e. peptide hormones, carrier proteins, immunoglobulins; modified from Wingfield & Farner 1975). Extracted steroid samples were reconstituted in 1.5% of kit-supplied steroid displacement buffer for assays (Guindre-Parker et al. 2013; Baldo et al. 2015). For the peptide hormone (IGF-1), plasma samples (25 μL) were subjected to an acid extraction with 25 μL of 0.2 M HCl to dissociate IGF-1 from binding proteins (modified from Lodjak et al. 2014).

Plasma baseline corticosterone levels were analyzed using a commercially available enzyme-linked immunosorbent assay (ELISA; Enzo Life Sciences Inc., Farmingdale, NY, USA; ADI-901-097) based on a competitive binding principle and previously validated for birds run in triplicate at a dilution of 1:20 (Hennin et al. 2015; Hennin et al. 2016). Each assay plate was run with kit-

provided standard curve through serial dilutions (200,000 pg/mL corticosterone standard), and an internal control. The plate was read using a spectrophotometer at 405 nm (Biotek Synergy H1 Hybrid Microplate Reader, Winooski, VT, USA). The mean intra- and inter-assay coefficient of variation for plates with bunting samples were 6.46% and 6.73%, respectively, and the intra- and inter-assay coefficients of variation for plates with eider samples were 9.17% and 9.23%, respectively.

Plasma testosterone levels were quantified using an ELISA (Cayman Chemical Company, Ann Arbor, MI, USA; #582701) that was previously validated and optimized for snow buntings at a dilution of 1:10 (Guindre-Parker et al. 2013; Baldo et al. 2015), using the same dilution for eiders. Each plate was run with a kit-provided, serially diluted standard curve (5,000 pg/mL), and an internal control. Standards were run in duplicate, and samples and controls were run in triplicate, and read at 412 nm (Biotek Synergy, VT, USA). Mean intra- and inter-assay coefficient of variation across plates were 7.62% and 6.26%, respectively.

Lastly, kit-supplied neutralization buffer was added to the extracted plasma IGF-1 samples and then vortexed before loading to a multi-species ELISA (Eagle BioSciences, Nashua, NH, USA; IGF31-K01). Dilution solution for the standard curve was prepared with kit-provided 0.01 M HCl, separately purchased 1 mg/mL bovine serum albumin, and 1:20 dilution with ultrapure water. Standards were created through serial dilution of separately purchased chicken IGF-1 standard peptide (20,000 pg/mL; GroPep Chicken IGF-1 Peptide, HU020), and laying chicken plasma (Sigma-Aldrich, Oakville, Ontario, Canada) mixed with diluted standard was used as a control. Standards, samples, and controls were all run in duplicate, and read at 450 nm (Biotek Synergy, VT, USA). Mean intra- and inter-assay coefficients of variation for all plates were 3.40% and 4.13%, respectively.

E. Statistical Analyses

To explore the temporal and stage-specific differences in energetic metrics and hormones, we categorized data into specific life-history stages across time in

both species. We first examined gross differences in energetic variables in each species before and after energetic preparation using paired *t*-tests (buntings) and a two-sample *t*-test (eiders). We then used linear mixed effects models (LMMs) with a standard least squares approach to examine hormonal changes across life-history stages within each species. For snow buntings, body mass was included as a fixed effect to control for size-related differences in hormone secretion. We included individual (to control for repeated sampling of birds across stages) and sample order (for testosterone only, see explanation in "Blood Sampling") as random effects. Daylength was not included as a fixed effect in these models in order to prevent a potential confounding effect on stage-related differences. In common eiders, body mass and relative arrival date (i.e. arrival date of individual relative to the intra-annual mean date of arrival; Hennin et al. 2015) were included as fixed effects in all models, again to control for arrival body size and timing of arrival on hormone secretion. Year was added in all models as a random effect. We used Tukey-Kramer HSD *post-hoc* tests of multiple, pairwise comparisons to determine which stages were significantly different for both species.

In addition, we used general linear models (GLMs) with simple linear regressions to identify potential correlations between change in energetic metrics and change in hormone concentrations during pre-migratory energetic gain in buntings. Further, time interval (i.e. number of days between high and low calculated values) was included to account for time period effects. To meet the assumption of normality in all of the above models, hormone data were transformed (snow bunting: $\log_{10}$ transformation for baseline corticosterone and testosterone, and reciprocal root transformation for IGF-1; common eider: all $\log_{10}$ transformations). Back-transformations were performed and presented for easier visual interpretation (McDonald 2014).

Finally, GLMs with standard least squares and Tukey-Kramer HSD *post-hoc* tests were used to assess the changes in endogenous tissue across breeding stages using previous dissection data on somatic tissue in East Bay breeding females. Date of collected sample was included as a covariate to

control for time-related effects. All data met the assumptions of normality (Shapiro-Wilk tests) and homogeneity of variances (Levene's tests), and all values are presented as mean ± SEM unless stated otherwise. We reported interaction terms as significant if alpha < 0.05. All analyses were performed in JMP version 13.0.0 statistical software (SAS Institute Inc., Cary, NC, USA).

RESULTS

A. Energetic Metrics across Life-History Stages

Snow Buntings. Total fat mass was significantly greater at the end of the pre-migratory preparation compared to the late winter (paired *t*-test: $n = 16$, $t_{(15)} = 26.0$, $p < 0.0001$; Table 3.1 & Figure 3.1A). Muscle scores increased significantly from late winter to the beginning of breeding (paired *t*-test: $n = 11$, $t_{(10)} = 7.02$, $p < 0.0001$; Table 3.1 & Figure 3.1B), as well as from late winter (Week 22: 1.25 ± 0.13) to the end of pre-migratory period (Week 28: 2.25 ± 0.13; paired *t*-test: $n = 16$, $t_{(15)} = 7.74$, $p < 0.0001$).

Common eiders. Body mass increased significantly between arrival and mid-stage rapid follicular growth (RFG; two-sample *t*-test: $n = 34$, $t_{(58.2)} = 4.63$, $p < 0.0001$; Table 3.1 & Figure 3.1C). Further, RFG females have significantly greater abdominal fat mass (49.5 ± 2.87 g) compared to incubating hens (20.0 ± 1.73 g; $F_{(3,99)} = 25.3$, $p < 0.0001$; Figure 3.2A), and laying individuals have significantly greater breast muscle mass (349.4 ± 9.13 g) than those in incubation (312.0 ± 5.32 g; $F_{(3,100)} = 6.05$, $p = 0.0002$; date: $p = 0.013$; Figure 3.2B). Additionally, laying hens showed a significantly greater total reproductive tract mass relative to both the RFG stage and the incubation stage ($F_{(4,98)} = 8.60$, $p < 0.0001$; Figure 3.2C). Laying females demonstrated significantly greater corrected digestive tract masses than the incubation stage, yet RFG hens did not differ significantly from the laying stage ($F_{(4,98)} = 40.0$, $p < 0.0001$; date: $p = 0.028$; Figure 3.2D).

B. Change in the Fat-Promoting Hormone across Life-History Stages

Snow buntings. Coarse-scale patterns suggest that baseline corticosterone has only a weak positive relationship with pre-migratory fattening (Figure 3.3B). Baseline corticosterone levels varied significantly between life-history stages ($F_{(3,137.7)}$ = 3.10, p = 0.023, *n* = 25; Table 3.2); however, *post-hoc* comparisons revealed no differences between groups (Table 3.2).

Common eiders. Fine-scale temporal patterns showed a rapid increase in baseline corticosterone with the onset of follicle recruitment and growth (Figure 3.3D; Hennin et al. 2015). Baseline corticosterone during the RFG stage was significantly higher than in the pre-recruiting and arrival stages ($F_{(18,1216)}$ = 2.90, p < 0.0001, *n* = 1,238; relative arrival date and body mass: p < 0.0001; Table 3.3).

C. Changes in Muscle-Promoting Hormones across Life-History Stages

Snow buntings. Migration levels of testosterone were significantly higher than the wintering and pre-migration stages ($F_{(3,118.1)}$ = 8.84, p < 0.0001, *n* = 25; Table 3.2), with the lowest levels in the winter (week 20: 9.20 ± 1.12 pg/mL, *n* = 16) that elevate and reach peak levels at the beginning of breeding (week 35: 87.4 ± 27.7 pg/mL, *n* = 10; Figure 3.4B). Likewise, pre-migratory levels of IGF-1 were significantly lower than spring migration ($F_{(3,108.3)}$ = 10.9, p < 0.0001, *n* = 25; Table 3.2), as plasma IGF-1 levels in pre-migration (week 26: 30.1 ± 1.92 ng/mL, *n* = 8) elevate until the highest levels are obtained at the beginning of breeding (week 37: 274.6 ± 107.9 ng/mL, *n* = 11; Figure 3.4C).

Common eiders. The patterns for testosterone and IGF-1 appeared to show fine-scale temporal changes throughout the pre-breeding period (Figure 3.4E & F). Testosterone levels decrease from arrival (95.7 ± 22.5 pg/mL, *n* = 7) into pre-recruiting (i.e. 13 days before laying: 80.7 ± 15.1 pg/mL, *n* = 6) and then rapidly increases in RFG (i.e. 5 days before laying: 131.2 ± 35.0 pg/mL, *n* = 6; Figure 3.4E). Similarly, IGF-1 levels are highest from arrival (52.0 ± 4.38 ng/mL, *n* = 7) into early pre-recruiting (i.e. 13 days before laying: 52.0 ± 5.01 ng/mL, *n* = 6) until

a drop 11 days before laying (38.5 ± 2.41 ng/mL, n = 6) and then increases in RFG (i.e. 7 days before laying: 47.4 ± 5.63 ng/mL, n = 6; Figure 3.4F). Nonetheless, testosterone levels did not differ significantly across breeding stages ($F_{(5,58)}$ = 1.11, p = 0.365, n = 69; Table 3.3). Likewise, IGF-1 levels did not differ significantly across stages ($F_{(5, 58.2)}$ = 1.22, p = 0.310, n = 69; Table 3.3).

D. Changes in Hormones Predicting Changes in Energetic Metrics in Snow Buntings

Within-individual changes in baseline corticosterone between the beginning and end of energetic gain showed a significant negative correlation with the change in total fat mass across the same period (R^2 = 0.378, $F_{(2,12)}$ = 3.94, p = 0.046, n = 16; Figure 3.5A). However, the trend between the change in hormones and change in muscle score was non-significant for testosterone (R^2 = 0.376, $F_{(2,10)}$ = 3.02, p = 0.095; Figure 3.5B) and for IGF-1 (R^2 = 0.434, $F_{(2,10)}$ = 3.83, p = 0.058; Figure 3.5C).

DISCUSSION

Given the logistical difficulty in obtaining long-term physiological data in migratory bird populations, research investigating the links between hormones and energy storage have largely been restricted to experiments on model species (i.e. domestic poultry; Scanes 2008), or observational data within a single life-history stage (Ramenofsky 2011), making temporal data very rare and valuable to investigate the mechanisms underlying seasonal adjustments in somatic energy stores. As such, our goal was to examine the seasonal change in candidate energy-regulating hormones that may mediate fattening and skeletal muscle growth to fuel spring migration and reproduction in captive male snow buntings and free-living female common eiders, respectively. In our temporal assessment of the long-term (buntings) and within-season (eiders) changes in multiple physiological traits, we found that both Arctic-breeding birds showed a large degree of stage- and species-related variation in baseline corticosterone (fat-promoting hormone), and testosterone and IGF-1 (muscle-promoting hormones).

More precisely, pre-migratory snow buntings showed only small increases in baseline levels of corticosterone, while pre-breeding common eiders initially showed a small increase in corticosterone during the pre-recruiting stage, followed by a rapid increase in corticosterone with the onset of the rapid follicular growth (RFG) stage. As predicted, snow buntings displayed increases in plasma testosterone, but unexpectedly not in IGF-1 levels during pre-migration, and both exhibited significantly higher levels during the migration stage. Conversely, female eider testosterone and IGF-1 levels appear high at arrival on the breeding grounds following spring migration, only to drop slightly during pre-recruiting, and then show an apparent elevation during the RFG stage.

A. Temporal Patterns of Fat Accumulation and Skeletal Muscle Gain

Snow bunting. As expected, captive male buntings carried little fat in the late winter (3.40 ± 0.48 g) and then showed a period of pronounced fattening over approximately 5 weeks (Figure 3.1A), peaking prior to spring migration (15.9 ± 0.48 g; Table 3.1) in general accordance with data from other migrant songbirds (i.e. white-throated sparrow *Zonotrichia albicollis*, Odum & Perkinson Jr. 1951; white-crowned sparrow *Zonotrichia leucophrys*, King & Farner 1959; red crossbill *Loxia curvirostra*, Cornelius & Hahn 2012; dark-eyed junco *Junco hyemalis*, Ho et al. 2017). Throughout what would have been migration, birds showed a plateau in fat mass that persisted into the first few weeks of the breeding period. This plateau could be explained by the *spring condition hypothesis* that states migrants will conserve energy for arrival on breeding grounds with unpredictable conditions in weather, food availability, and habitat quality (Morrison et al. 2007; Anteau & Afton 2009), which is supported empirically by recent migratory tracking studies on both male and female snow buntings arriving on breeding grounds with some remaining fat reserves (Macdonald et al. 2012; McKinnon et al. 2016). Alternatively, some of the stored fat could be an artefact of captivity through the lack of endurance flight to catabolize fat stores (see discussions of Vincent & Bedard 1976; Aubin et al. 1986). Following the migration stage, male buntings lost fat stores (Figure 3.1A), likely timed to match a period of

accelerated lipid breakdown that occurs during breeding when males are highly active, defending mates and breeding territories (Guindre-Parker et al. 2013; F. Vézina, unpubl. data).

Similar to the rapid gain in fat mass over pre-migration, we found a strong increase in muscle score from late winter (1.25 ± 0.13) to the end of pre-migration (2.25 ± 0.13) in male buntings (Figure 3.1B). Muscular hypertrophy of breast muscle continued until reaching peak values during what would be early breeding, likely due to sustained use of flight muscles for pre-breeding activities such as territory establishment and defense, as well as thermoregulation in the cold, stochastic environment (Montgomerie & Lyon 2011; Swanson & Vézina 2015). Interestingly, decreases in muscle score occurred at the end of the breeding stage, indicating a phase of negative energy balance (Jenni & Jenni-Eiermann 1996; Bauchinger & Biebach 1998). Even in captivity, this evidence for decreasing muscle size is presumably from endogenous (circannual) rhythms controlling these phenotypic changes that correspond to shifts in the annual life cycle, as seasonal patterns still persist with constant photoperiod in red knots (*Calidris canutus*; Piersma et al. 2008; Vézina et al. 2011). However, unlike the lipid catabolism seen when entering pre-alternate moult and fall migration, muscle scores remained higher than those observed during the winter, which is assumed to preserve flight muscles for fall migration back to wintering grounds.

Common eiders. Pre-breeding hens showed expected increases in body mass from arrival (2103.5 ± 33.5 g) up to the rapid follicular growth (RFG) stage (5 days before laying: 2291.2 ± 22.8 g), as birds fattened to fuel both egg production and an incubation stage dominated by fasting (Hennin et al. 2015; Table 3.1). The small decrease in mass in the later portion of RFG, possibly due to the energetic demand of follicle development (Vézina et al. 2002; Hennin et al. 2015), was followed by a sharp and predictable decline through egg laying and incubation (Figure 3.1C). Indeed, in breeding females, there was a significant drop in abdominal fat mass from the RFG stage to the incubation stage, presumably from the oxidation of lipid stores (Figure 3.2A; Sénéchal et al. 2011),

following which female eiders must catabolize muscle protein as a last-resort energy source to extreme fasting during incubation (Criscuolo et al. 2000; Bolduc & Guillemette 2003). Finally, non-breeding females were lighter (2,071.4 ± 8.52 g, *n* = 782; ~1,700 g reported in Sénéchal et al. 2011), as it has been found that females in poor body condition after arrival may forgo reproduction for that year (Legagneux et al. 2016).

Although we expected to see larger breast muscles after arriving to the breeding grounds to help support the energetic costs of heavy wing loading during migratory flight (Guillemette & Ouellet 2005; Ouellet et al. 2008), an analysis of data collected from the East Bay breeding colony indicated that breast muscle mass peaked during laying and was then degraded by the incubation stage (Figure 3.2B). These results suggest that some of the incubating hens may have entered phase III fasting, which is characterized by an increased rate of protein catabolism for 'emergency' energy *via* gluconeogenesis (Cherel et al. 1988). Therefore, female eiders could have increased their protein reserves before and during laying in anticipation of the loss of skeletal muscle tissue during incubation (Cherel et al. 1988).

B. Temporal and Stage-Related Changes in Baseline Corticosterone

Snow buntings. We predicted an elevation in baseline corticosterone would be associated with increases in stored lipids during pre-migration based on previous results in other temperate songbirds (i.e. yellow-rumped warbler *Setophaga coronata*, Holberton 1999; hermit thrush *Catharus guttatus*, Long & Holberton 2004; dark-eyed junco, Holberton et al. 2007), given the reported links between baseline corticosterone and locomotory related to foraging behaviour (Astheimer et al. 1992; Lõhmus et al. 2006; Angelier et al. 2007; Kitaysky et al. 2010). In snow buntings, despite the highest levels of baseline corticosterone being seen during migration, we did not detect significant increases from wintering to either pre-migration nor spring migration stages (Table 3.2 & Figure 3.3B). Moreover, while we found that individual changes in baseline corticosterone were correlated

with changes in fat mass during a period of energetic gain, this relationship was surprisingly negative (Figure 3.5A).

To offer an evolutionary explanation for elevated baseline corticosterone levels during spring migration and breeding, the *preparative hypothesis* posits that seasonal elevation of baseline secretion may prime individuals for periods of predictable energetic demand (Love et al. 2014), or frequent exposure to multiple stressors (Romero et al. 2017). Previous studies support these findings where elevated levels of baseline corticosterone are positively correlated with abundant fat stores or replenished loads after refueling at stopover sites (Piersma et al. 2000; Landys-Ciannelli et al. 2002; Lõhmus et al. 2003; Falsone et al. 2009; Beck et al. 2016). At a mechanistic level, increases in either baseline or stress-induced corticosterone have shown to activate low-affinity glucocorticoid receptors on white adipose tissue to mobilize lipids for strenuous bouts of flight (Landys et al. 2004a, 2006). Accordingly, Eikenaar et al. (2013, 2014a) found that fuel deposition rate and food intake were negatively correlated with baseline corticosterone in northern wheatears (*Oenanthe oenanthe*), inferring that birds with a slower rate of lipid deposition had already greater fat stores and thus were ready to depart on migration. Our results are consistent with these findings in that we found individuals with greater change in fat mass had less of a change in baseline corticosterone, indicating that individuals with decreasing levels of baseline corticosterone during a period of energetic gain deposited more fat (Figure 3.5A), instead of enhancing lipogenesis with elevated levels as predicted (i.e. Holberton et al. 2007). Moreover, Ramenofsky & Wingfield (2016) outline the importance elevated levels of baseline corticosterone to induce migratory restlessness (a measure of migratory hyperactivity) during spring migration, and in fact, higher levels of baseline corticosterone in migratory individuals was a strong predictor of migratory restlessness (Landys et al. 2004b; Eikenaar et al. 2014b, 2017). Altogether, baseline corticosterone may therefore act permissively to activate other endogenous signals to promote fattening (see "Hormonal Regulation of Fat Storage in Birds" in Chapter 2).

A potential methodological issue inherently associated with our snow bunting system may be an artefact of captivity that dampens the seasonal rhythm of baseline corticosterone levels, and thereby does not accurately reflect natural variation of hypothalamic-pituitary-adrenal (HPA) activity within the annual cycle (discussed in Romero et al. 2017). For example, Romero & Wingfield (1999) compared baseline corticosterone levels between wild and captive populations of white-crowned sparrows across various life-history stages, finding that captive individuals had identical concentrations throughout the year with markedly suppressed levels during spring migration and breeding relative to free-living birds. Conversely, in European starlings (*Sturnus vulgaris*), activity of the HPA axis in individuals held under semi-natural outdoor housing conditions mimicked those of free-living birds relative to altered physiology of birds in enriched indoor conditions (Dickens & Bentley 2014). Although our population of snow buntings are wild-caught and adult birds were exposed to outdoor ambient conditions, long-term captivity (~1-2 years) may impact the physiological and behavioural responses *via* confinement-induced stressors, such as social abnormalities, reduced movement, and altered feeding strategies (Morgan & Tromborg 2007). Nevertheless, if elevated levels of baseline corticosterone played a pivotal role in fat gain, then snow buntings with dampened levels of baseline corticosterone would not be expected to undergo seasonal fattening, yet these birds clearly both accumulated and depleted large fat stores in captivity (Figure 3.1A). In summary, our results do not strongly support a role for lipogenesis, and suggest only a weak pattern of elevated circulating baseline corticosterone for energetic readiness during pre-migration in snow buntings.

Common eiders. Free-living female eiders exhibited consistent, fine-scale temporal (days) fluctuations in natural baseline corticosterone concentrations across the breeding season over multiple years (Figure 3.3D). Hennin and colleagues (2015) originally reported a positive correlation between baseline corticosterone levels and body mass in pre-breeding females, and later affirmed the causal effects of baseline corticosterone on increases in body mass through

a physiologically relevant corticosterone-pellet manipulation in white-winged scoters (*Melanitta deglandi*; Hennin et al. 2016). Recently, Hennin and colleagues also found that experimentally elevated baseline corticosterone in female eiders resulted in earlier laying dates, presumably through an increase in the rate of fattening during the pre-recruiting stage (Hennin et al. unpubl. data). After including three additional years of data collection (2006 to 2016), we confirm previous patterns of elevated baseline corticosterone (Hennin et al. 2015). We also found that pre-breeding levels of baseline corticosterone increased from arrival (5.04 ± 1.02 ng/mL, n = 41) to the pre-recruiting stage (6.94 ± 0.55 ng/mL, n = 175) and peaked during the RFG stage (17.09 ± 2.42 ng/mL, n = 200) immediately prior to laying, presumably to achieve optimal levels for reproductive investment (Love et al. 2014; Ouyang et al. 2013).

Importantly, although the increase in baseline corticosterone from arrival to pre-recruiting is small (~1.9 ng/mL), we argue that it may be biologically relevant for a few reasons. First, as glucocorticoids are highly potent steroid hormones, endocrine tissues are designed to secrete the minimum concentration to elicit the hormone-mediated response to avoid potential costs associated with hormone pleiotropy (Sapolsky et al. 2000). Second, tissue-specific responses of glucocorticoids may be amplified with temporal or stage-related changes in receptor densities (Landys et al. 2006), corticosteroid binding globulins (Malisch & Breuner 2010), 11β-hydroxysteroid dehydrogenases (i.e. conversion of inactive 11-keto steroids to active glucocorticoids; Seckl et al. 2004), and transcriptional cofactors at the corticosterone-receptor complex (Crespi et al. 2013). Moreover, although previous studies suggest that only glucocorticoid receptors (GRs) mediate downstream effects on white adipose tissue (see Landys et al. 2006), a more recent review highlighted the activation of high-affinity mineralocorticoid receptors (MRs) with baseline corticosterone levels causing adipocyte differentiation and up-regulation of lipogenic enzymes (i.e. lipoprotein lipase, peroxisome proliferator-activated receptor γ) in mammals (Marzolla et al. 2012). As such, even only slight elevations in baseline corticosterone may be triggering lipogenic pathways through MRs without reaching levels to activate GRs. Finally,

phylogenetic differences in HPA responsiveness axis exist in part to regulate species- and stage-specific energetic processes dependent on the “allostatic load” concept (i.e. total energy demands from unpredictable and predictable environmental changes; McEwen & Wingfield 2003, 2010), where glucocorticoids are therefore considered putative regulators of energy homeostasis during predictable life-history transitions associated with higher allostatic load (Crespi et al. 2013; Schultner et al. 2013). Under this framework, a seemingly minor increase in baseline corticosterone levels may be enough to support allostatic load through lipid stores. Overall, and in support of the findings in Hennin et al. (2015, 2016), elevated baseline corticosterone appears to play a role in lipogenesis in pre-breeding female eiders, and further experimentation needs to explore whether this effect is direct, permissive (indirect), or both in this species.

C. Temporal and Stage-Related Changes in Testosterone

Snow buntings. Given the anabolic role of androgens in vertebrates (Wingfield et al. 2001), we predicted that snow buntings would show elevations of circulating testosterone to build skeletal muscles for spring migration. Indeed, previous experimental manipulation studies showed increases in breast muscle score during energetic preparation before spring migration in both American redstarts (*Setophaga ruticilla*; Tonra et al. 2013) and white-crowned sparrows (Ramenofsky & Németh 2014). Patterns of plasma testosterone in our captive male buntings loosely matched their mean muscle scores with an initial, gradual increase from late winter to early breeding, followed by a significant elevation comparing winter levels to migration (Figure 3.4B; Table 3.2). Further, although non-significant, testosterone levels are almost doubled from winter (9.33 ± 0.74 pg/mL) to pre-migration (18.45 ± 3.37 pg/mL), indicating a whole-magnitude elevation in testosterone secretion may induce muscle hypertrophy. It is important to note that dramatic increases in testosterone secretion during migration and early breeding is assumed to be caused by testes growth for breeding behaviours and reproduction (Wingfield et al. 2001; Bauchinger et al. 2007; Vézina et al. 2010). In addition, we also found a non-significant, positive

correlation between changes in breast muscle score and testosterone levels from late winter to the end of the migration stage into early breeding (Figure 3.5B).

There are a few reasons that may explain why snow buntings show dose- and stage-dependent elevations in testosterone. First, similar to corticosterone, testosterone is a potent steroid hormone with multiple (i.e., pleiotropic) physiological and ecological costs associated with elevated levels (i.e. suppressed immunity, susceptible to injury and predation, decreased parental care; reviewed in Wingfield et al. 2001). Thus, testosterone is presumably under tight regulation to avoid the costs of prolonged elevated levels, except during the breeding season with obvious benefits for reproductive functions and behaviours (Hau 2007). Second, captivity has consistently been shown to have a suppressive effect on absolute androgen secretion relative to free-living birds, despite overall seasonal patterns of circulating testosterone generally remaining intact (Calisi & Bentley 2009; and references therein). Additionally, seasonal regulation of testosterone regulatory proteins, such as androgen receptors, downstream intracellular signals (i.e. parvalbumin and IGF-1; Fuxjager et al. 2012), and corticosteroid-binding globulin (i.e. transporter of steroid hormones in birds; Breuner & Orchinik 2002), all likely play important roles in the physiological flexibility of skeletal muscle. Finally, by only marginally elevating levels of testosterone, pre-migratory birds that are simultaneously building fat and muscle may avoid androgen-induced lipolytic effects on adipocytes such as decreased lipid uptake, down-regulation of lipoprotein lipase, and increased lipolytic β-adrenergic receptors (De Pergola 2000). Considering these biological implications on testosterone levels, we argue that even a small increase in circulating testosterone may be sufficient to induce rapid muscle growth.

Following pre-migration, individuals sampled during the migration stage exhibit dramatically higher levels of testosterone (59.17 ± 13.5 pg/mL), which is assumed to be the photoperiod-induced gonadal recrudescence in the spring (i.e. annual testes growth; Morton et al. 1990; Wingfield & Kenagy 1991). The seasonal testes growth of male songbirds is associated with significant increases in the secretion of testosterone that prompts migratory readiness to transition into

breeding phenology (Tonra et al. 2011a, 2011b). Testosterone in male buntings reached peak levels (87.42 ± 27.7 ng/mL) during the first week of the breeding stage which paralleled with peak muscle scores, and includes a pre-breeding period with aggressive behaviours and territorial defense (Balthazart 1983; Wingfield et al. 1990; Wingfield & Hahn 1994). Testosterone levels dropped substantially by the end of the breeding stage (6.36 ± 1.23 ng/mL), which corresponds to significantly lower levels during post-hatch parental care in this species (Romero et al. 1998) and a simultaneous decrease in muscle score (Weeks 37-40; Figure 3.1B). Beyond the well-characterized elevation in testosterone levels during breeding, our results indicate a correlative link between breast muscle score and plasma testosterone levels during the pre-migration and migration stages, however, manipulative experiments could determine whether a causal relationship exists between testosterone and avian skeletal muscle phenotypes.

Common eiders. Fine-scale temporal patterns of testosterone levels in female eiders depicted day-to-day changes with a high degree of variation in pre-breeding females (Figure 3.4E). Females arriving at East Bay immediately after migration had lower testosterone levels (95.7 ± 22.5 pg/mL, n = 7) than the mean levels throughout all pre-breeding (109.3 ± 9.52 pg/mL, n = 54), and then rapidly increased testosterone levels approximately 15 days before laying (146.7 ± 37.9 pg/mL, n = 5). Although our sample size is low, this surge in plasma testosterone may be important socially for females even before energetic preparation takes place to perhaps display intra-specific aggression toward other conspecifics in order to quickly establish a nesting site or defend foraging patches (Christensen 2000; Sandell 2007). Indeed, a meta-analysis across 56 species and ten avian orders reported that colonial-nesting females have higher testosterone levels than solitary nesters (Møller et al. 2005), and in this population, pre-breeding females engage in territorial (nest-guarding) disputes on the densely aggregated breeding ground (H. Hennin & O. Love, pers. comm.).

Unexpectedly, we found a drop in testosterone levels during the pre-recruiting stage (13 and 11 days before laying: 80.7 ± 15.1 pg/mL, *n* = 6 and 81.6 ± 19.2 pg/mL, *n* = 6), before levels remained consistently high during the RFG stage. A possible reason for the reduction in plasma testosterone levels may aid in fat conservation, *via* inhibition of lipolysis, to engage in a period of hyperphagia and lipid accumulation (De Pergola 2000). However, it is presently difficult to speculate whether this slight reduction is in any way significant for promoting fat deposition for a few reasons: (i) our results, to our knowledge, represent the first study to measure testosterone in female common eiders; (ii) there is a lack of "reference" testosterone levels in the winter and post-breeding stage; and (iii) caution should be taken in comparing absolute levels of hormones across studies due to practical differences in detection techniques (i.e. Romero 2002). Nonetheless, with the knowledge that maximal abdominal fat stores at the RFG stage precede maximal breast muscle mass at laying (Figure 3.2A & B), muscle growth would appear to temporally overlap with fat accumulation, though it may be that majority of muscle growth occurs when testosterone levels are elevated during the RFG stage. Although it would be very hard to obtain without invasive measures, future information on daily changes in breast muscle would be highly valuable to propose a direct, albeit correlational, relationship with the pre-breeding testosterone profile, ideally followed by manipulation experiments.

During the RFG stage, elevation in female testosterone may stimulate aggressive, nest-guarding behaviours in anticipation of laying (see argument above). After the completion of follicle development and ovulation, testosterone levels decreased rapidly into the laying stage (56.7 ± 11.9 pg/mL, *n* = 6) and dropped to their lowest values during incubation (26.7 ± 3.36, *n* = 3; Figure 3.4E). Suppressed levels of testosterone should theoretically serve two mechanistic functions during an energetically costly incubation: (1) fat-sparing through decreased lipid catabolism (De Pergola 2000); and (2) reduce aggressive behaviours (Sandell 2007). Taken together, energetic conservation of fat and protein stores is essential during a long (~25-27 days) incubation period with no recess opportunities for feeding (Criscuolo et al. 2000; Bottitta et al. 2003).

Overall, we found that breeding testosterone levels are notably lower than pre-breeding levels, and whether this is evolutionarily driven by the social instability of colonial breeding (Langmore et al. 2002), the physiological energy demands of reproduction (Sénéchal et al. 2011), or both, androgen-induced muscle hypertrophy is a plausible mechanism that deserves further research in female eiders. Finally, testosterone levels did not differ significantly between breeding stages (Table 3.3), though we suspect the lack of stage-related change in hormone levels is likely attributed to broadly grouping together traits that exhibit day-to-day fluctuations (Figure 3.4E; see general discussion).

D. Temporal and Stage-Related Changes in IGF-1

Snow buntings. To the best of our knowledge, our results are the first to examine the temporal patterns of circulating IGF-1 levels across life-history stages in any adult bird. We predicted that circulating IGF-1 would increase during pre-migration, serving as an anabolic hormone to induce muscle hypertrophy. Contrary to our predictions, we did not find strong support for a concomitant increase in IGF-1 levels and mean muscle scores during pre-migration, primarily because (1) there was an overall temporal lag with IGF-1 levels rising slightly at the end of pre-migration after the completion of an appreciable portion of muscle hypertrophy (Figure 3.4A & C), as well as (2) significantly lower levels of IGF-1 in the pre-migration stage compared to the migration and breeding stages (Table 3.2). Interestingly, IGF-1 levels decrease from the wintering stage (week 20: 79.1 ± 17.2 ng/mL, n = 16) to mid pre-migration (week 26: 30.1 ± 1.9 ng/mL, n = 8), reaching unexpectedly low levels during a period of increasing muscle scores (Figure 3.4A & C). We propose an alternative function for the relatively lower levels of IGF-1 during the pre-migration stage: the suppression of IGF-1-mediated fat catabolism. First, although the mechanism remains to be confirmed in birds (Scanes 2009), the mammalian GH/IGF-1 pathway plays a role in lipid mobilization through the activation of hormone-sensitive lipase in mature adipocytes to degrade TRIG into free fatty acids, possibly through stimulation of growth hormone and IGF-1 receptors that cross-talk with proximally located β-

adrenergic receptors (reviewed in Vijayakumar et al. 2010). Furthermore, Hansen et al. (2013) discussed how long-lived mutants (longer lifespan) in *Drosophila* had a greater degree of fat storage (decreased lipolysis), which may be regulated by lower circulating levels of IGF-1. Despite the underlying molecular mechanisms of IGF-1 on fat metabolism remaining enigmatic, and especially so in birds, we propose that low plasma IGF-1 levels, or perhaps down-regulated IGF-1 signaling, during pre-migration may contribute to seasonal fat phenotype in snow buntings. Given that circulating IGF-1 has anabolic effects on various peripheral tissues (LeRoith 1997; Chapter 2), it appears that our observed stage-related differences in IGF-1 levels likely causes pleiotropic effects. Nonetheless, lowered levels of IGF-1 may provide only a maintenance (anti-atrophic) role to preserve muscle during the pre-migration stage (Figure 3.4F). In this view, it could be that local (autocrine/paracrine) IGF-1 actually drives skeletal muscle growth from other hormones or growth signaling molecules rather than liver-derived IGF-1 in circulation (Price et al. 2011).

We did not see an increase in IGF-1 levels until half-way through the migration stage, with consistent hormone secretion through the first few weeks of the breeding stage (Figure 3.4C). Previous experimental research in both mammals and birds have shown that the IGF-1 signaling pathway influences the physiology of the mammalian male reproductive system by increasing the rate of steroidogenesis, initiating spermatogenesis in Sertoli cells, and promoting mitogenic growth of Leydig cells in testes (Onagbesan & Peddie 1995; Baker et al. 1996; Weinzimer & Cohen 1999; Colón et al. 2007). Additionally, though poorly studied, some emerging evidence suggests that elevated levels of IGF-1 across vertebrates has indirect effects on reproductive investment, embryonic development and postnatal growth (i.e. reptiles: Sparkman et al. 2009; fishes: Reinecke 2010; mammals: Swanson et al. 2014; birds: Lodjak et al. 2016). Another potential reason for heightened levels of IGF-1 during the migration and early breeding stages may be for the dynamic re-building of visceral organs before free-living snow buntings from the same population as our captive birds must endure a long (700-1000 km), single flight across the Labrador sea

(Macdonald et al. 2012). Previous studies found that post-flight dissection data showed reduced tissue masses (i.e. digestive tract organs, liver, kidneys, skeletal muscles) relative to pre-flight data when crossing large ecological barriers in migratory songbirds and shorebirds (Battley et al. 2000; Schwilch et al. 2002; Bauchinger & McWilliams 2010). Consequently, increasing levels of IGF-1 may represent a strong proliferative signal for reproductive tissue during the migration stage and visceral organs after arrival on breeding grounds in male buntings, and future studies on the effects of IGF-1 treatment on migratory buntings may provide some insight on whether this is a candidate signal for post-migratory tissue remodeling. Finally, we found elevated levels of IGF-1 during fall migration (Figure 3.4C), which may compensate for the suppressed testosterone levels from gonadal regression to maintain (i.e. avoid protein-wasting) flight musculature for departure to the wintering ground, as suppressed IGF-1 signaling has shown to result in muscle atrophy in rodents and humans (reviewed by Perrini et al. 2010).

Common eiders. Similar to the IGF-1 secretion patterns in buntings, we predicted that IGF-1 levels would be elevated throughout the pre-breeding period in female common eiders. Like testosterone, we found that pre-breeding female displayed fine-scale temporal changes (days) in plasma IGF-1 levels (Figure 3.4F). Interestingly, circulating levels of IGF-1 were elevated at the post-migration arrival stage and remained so approximately 4 days into the pre-recruiting stage (Figure 3.4F). Considering that individuals reduce the masses of digestive organs during spring migration (Biebach 1996; Piersma & Gill Jr. 1998; Piersma et al. 1999; Battley et al. 2000; Bauchinger et al. 2005b), pre-breeding females may have to rapidly re-build their digestive tract before a period of high nutritional demand associated with hyperphagia (Guillemette 2001) and energy (fat and protein) storage (Sénéchal et al. 2011). Moreover, mammalian studies have shown that IGF-1 stimulates the growth of the intestinal mucosal layer, gastrointestinal organs (i.e. stomach, small and large intestine), and smooth muscle of the digestive tract (Burrin et al. 1996; Kuemmerle et al. 2004; Steeb et

al. 1994; Wang et al. 1997). Despite the vast empirical evidence that demonstrates the phenotypic flexibility of digestive tissues in migratory birds (reviewed in McWilliams & Karasov 2001), the underlying mechanisms regulating large-scale turnover of digestive organs in birds is unknown, and thus, IGF-1 deserves further attention as a prospective hormonal signal to increase the digestive tract after migration.

After this phase, mean IGF-1 levels dropped from 13 (52.0 ± 5.01 ng/mL, n = 6) to 11 (38.5 ± 2.41 ng/mL, n = 6) days before laying, which may indicate a 4-day phase of functionally down-regulated IGF-1 prior to the onset of the RFG stage (Figure 3.4F). Since the primary fuel source for females are stored TRIG in adipose tissue, low IGF-1 levels may reduce the lipolytic action similar to that in pre-migratory buntings, thereby allowing for increased fat accumulation. Transitioning into the RFG stage, individuals exhibited elevations of plasma IGF-1 levels corresponding to a period of anabolism of female reproductive machinery. For example, Vézina & Williams (2003) reported that almost two-thirds of oviduct growth occurs within a short 3-day window prior to egg laying, while degradation happens just as quickly before release of the final egg in European starlings, as the production and maintenance of the avian oviduct has shown to have high metabolic costs (Chappell et al. 1999; Vézina & Williams 2003; Williams & Ames 2004).

Indeed, plasma IGF-1 has been directly linked to increases in the rate of estradiol secretion (Demeestere et al. 2004), follicular development in the ovaries (Velazquez et al. 2008), and the central activation of gonadotropin-releasing hormone neurons through tissue-specific IGF-1 receptors in mammals (Daftary & Gore 2005). Recently, exogenous growth hormone has been implicated as an anti-apoptotic signal through reduced expression of capase-3 enzyme (a signal of programmed cell death) in the layer chicken oviduct (Hrabia et al. 2013, 2014), further reinforcing the importance of GH/IGF-1 axis on the growth of female reproductive organs. Furthermore, in knowing that estradiol induces hypertrophy of ovaries and oviduct in female birds (Farner & Wingfield 1980), future studies should examine how the seasonal regulation of plasma IGF-1 levels may

influence estradiol secretion on the development of reproductive organs, especially as this would be advantageous for female eiders with a temporally and energetically constrained breeding season in the Arctic (Love et al. 2010).

Finally, we discovered very low levels of plasma IGF-1 during laying and incubation (Figure 3.4F), which matches the atrophy of the total reproductive tract mass during the laying stage (Figure 3.2C). Similar to testosterone, although we did not find compelling evidence for a correlative linkage between IGF-1 and muscle growth during the pre-recruiting stage, it may be that skeletal muscle growth happens during the RFG stage when circulating IGF-1 is elevated, similar to testosterone. Given the reported tight relationship between these two hormonal pathways in mammals (reviewed by Bartke et al. 2013), it may not be surprising if both testosterone and IGF-1 increase skeletal muscle growth closer to reproduction. It should be noted that we did not detect significant differences between breeding stages (Table 3.3), which is possibly due to binning data into broad groups (similar to testosterone; see general discussion). On a final note, future studies should aim to investigate the potential anabolic effect of elevated IGF-1 levels on visceral organs (i.e. digestive tract) and reproductive tissue during breeding stages.

CONCLUSIONS

Our comparative approach assessed temporal and stage-related changes of energy-regulating hormone with regards to their anabolic regulation of energy (i.e. fat and muscle) storage prior to spring migration in male snow buntings and breeding in female common eiders. We observed inter-specific differences in the temporal variation of both fat- (baseline corticosterone) and muscle-promoting (testosterone and IGF-1) hormones. However, qualitative temporal patterns of all hormones in both species showed lower concentrations during periods of energetic preparation relative to migration (buntings) or breeding stages (eiders), possibly suggesting optimal hormone levels to promote fat storage *via* reduced lipolysis. We found that baseline corticosterone did not appear to be an important signal for fat deposition in buntings, where contrastingly in eiders, slight elevation

may mediate pre-breeding fattening followed by a rapid elevation for priming reproductive investment. Male buntings may be sensitive to testosterone-induced muscle growth, yet interpretation of testosterone levels in female eiders appears to be more convoluted. Plasma IGF-1 in both buntings and eiders may be an important modulator of reproductive tissue and visceral organs rather than stage-dependent muscle growth. Future experimental research should tease apart these relationships between energy-regulating hormones and life-history phenotypes to advance the underlying mechanisms that control the performance traits of fat and muscle stores.

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TABLES

Table 3.1: Changes in energetic metrics at their lowest and highest points with statistical summaries in birds for captive snow buntings (SNBU) and free-living common eiders (COEI).

Energetic Metric	Species	Lowest	Highest	Statistical Summary
Total Fat Mass (g)	SNBU	3.40±0.48	15.9±0.48	n = 16, $t_{(15)}$ = 26.0, p < 0.0001
Week		22	28	
Muscle Score	SNBU	1.45±0.20	2.91±0.20	n = 11, $t_{(10)}$ = 7.02, p < 0.0001
Week		22	37	
Body Mass (g)	COEI	2103.5±33.5	2291.2±22.8	$t_{(58.2)}$ = 4.63, p < 0.0001
n		34	34	
P-L Interval		16+	5	

*t statistic subscript indicates the degrees of freedom of the model. Reported results are considered significant at $p < 0.05$. Abbrev.: P-L Interval = pre-laying interval.

Table 3.2: Statistical summary of linear mixed models of hormonal variation across various life-history stages in captive male snow buntings in 2015.

Hormone	Wintering	Pre-migration	Migration	Post-migration	Statistical Summary
log Base CORT	0.81 ± 0.04^{a} *49*	0.91 ± 0.05^{a} *36*	0.94 ± 0.05^{a} *31*	0.80 ± 0.05^{a} *34*	$F_{(3,137.7)} = 3.10$, $p = 0.023$
log T	0.92 ± 0.08^{a} *26*	1.11 ± 0.08^{ab} *30*	1.50 ± 0.07^{c} *35*	1.29 ± 0.07^{bc} *40*	$F_{(3,118.1)} = 8.84$, $p < 0.0001$
Recip. Rt IGF-1	-0.020 ± 0.002^{ab} *26*	-0.024 ± 0.002^{a} *30*	-0.016 ± 0.002^{bc} *34*	-0.013 ± 0.002^{c} *38*	$F_{(3,\ 108.3)} = 10.8$, $p < 0.0001$

*F statistic subscripts represent degrees of freedom of the model (former) and degrees of freedom of the error (latter). Values are presented as means ± standard error, and italicized numbers indicate the sample size. Different superscript, lower-case letters between groups represent significance ($p < 0.05$). Abbrevs.: Base CORT = baseline corticosterone, T = testosterone, IGF-1 = insulin-like growth factor-1, Recip. Rt = reciprocal root transformation ($-1/\sqrt{x}$, where x = value).

Table 3.3: Statistical summary of linear mixed models of hormonal variation across breeding stages in free-living female common eiders from 2006-2016 (baseline corticosterone; 2010-2016 for testosterone and insulin-like growth factor-1).

Hormone	Arrival	PR	RFG	Laying	Incubation	NB	Statistical Summary
log Base CORT	0.43±0.08[a] *41*	0.61±0.04[a] *174*	0.93±0.04[b] *200*	0.94±0.07[ab] *48*	0.86±0.18[ab] *8*	0.74±0.02[a] *769*	$F_{(5, 1229)} = 7.66$, $p < 0.0001$
log T	1.88±0.10 *7*	1.97±0.06 *23*	1.96±0.06 *24*	1.70±0.11 *6*	1.41±0.12 *3*	1.64±0.12 *6*	$F_{(5, 58.0)} = 1.11$, $p = 0.365$
log IGF-1	1.71±0.05 *7*	1.64±0.03 *23*	1.65±0.03 *24*	1.55±0.05 *6*	1.51±0.06 *3*	1.62±0.05 *6*	$F_{(5, 58.2)} = 1.22$, $p = 0.310$

*F statistic subscripts represent degrees of freedom of the model (former) and degrees of freedom of the error (latter). Values are presented as means ± standard error, and italicized numbers indicate the sample size. Different superscript, lower-case letters between groups represent significance ($p < 0.05$). Abbrevs.: Base CORT = baseline corticosterone, T = testosterone, IGF-1 = insulin-like growth factor-1, PR = pre-recruiting, RFG = rapid follicular growth, NB = non-breeding.

FIGURES

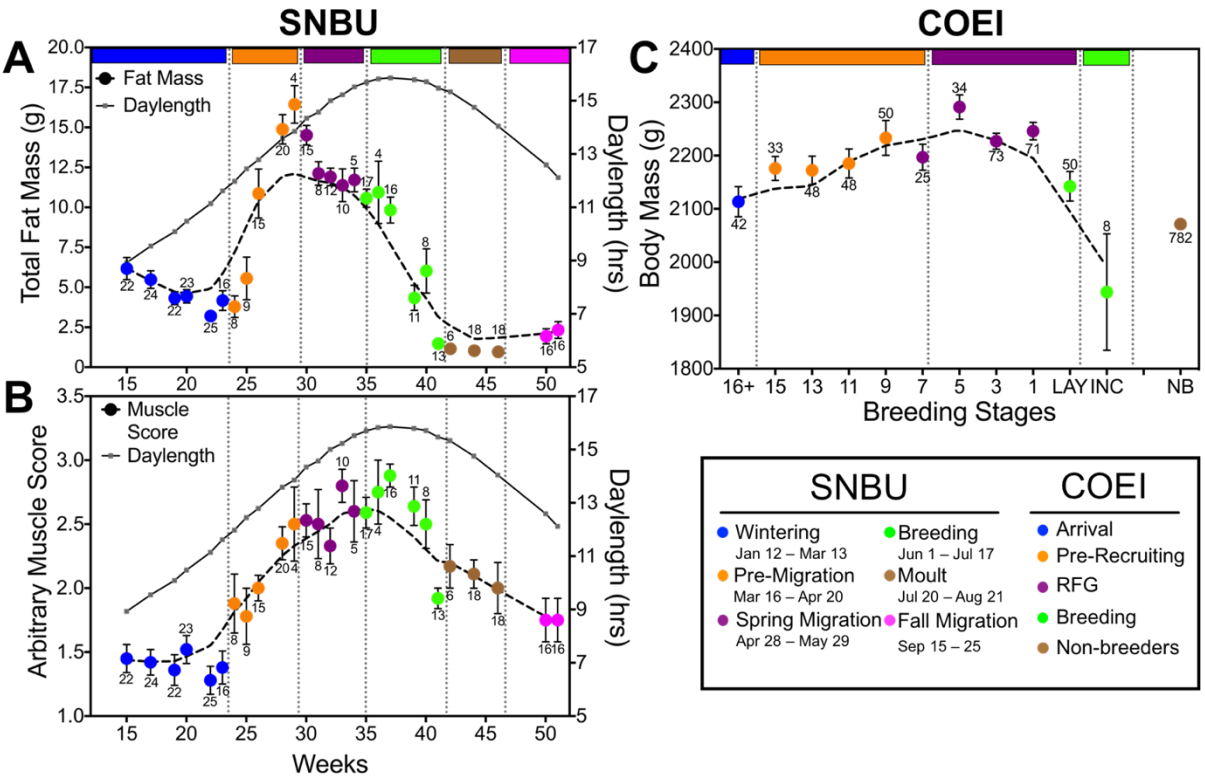

Figure 3.1: Temporal patterns of energetic metrics for (**A**) total fat mass, (**B**) muscle score across the year (2015) in male snow buntings (*n* = 25), and (**C**) body mass across the breeding seasons (2006 to 2016) in female common eiders (*n* = 1,264). Dots represent the means ± SEM, values indicate sample sizes, and dotted grey lines separate life-history stages.

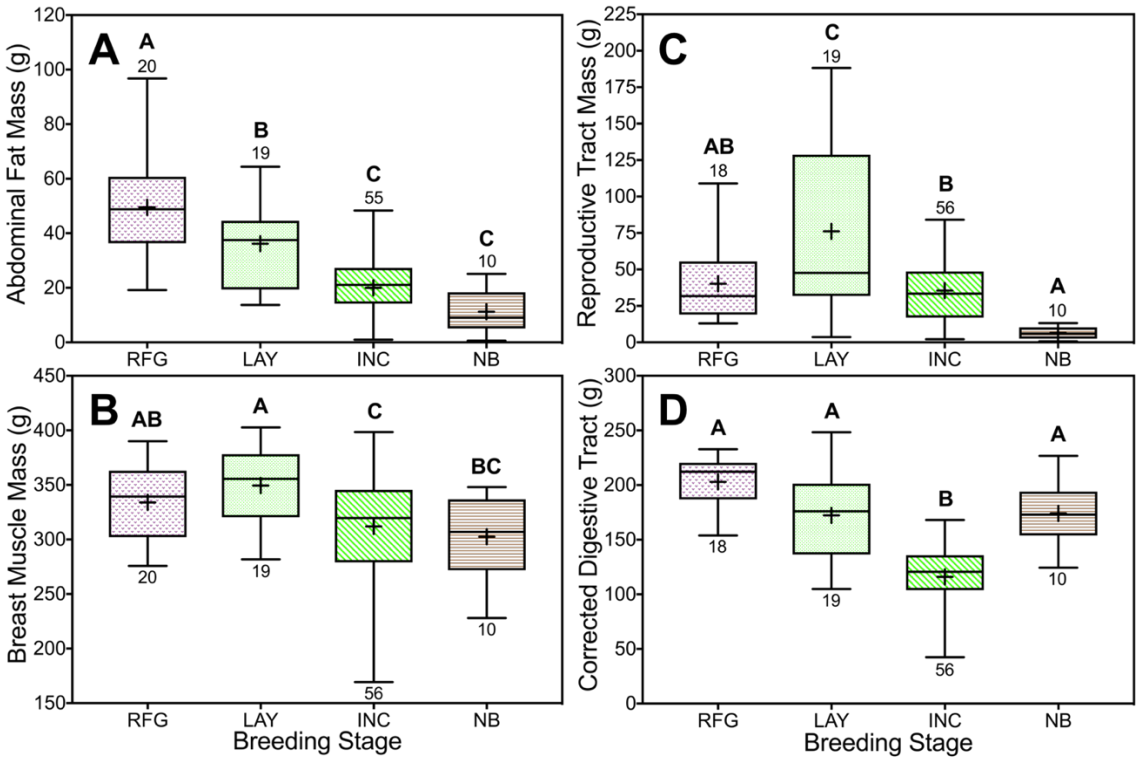

Figure 3.2: Tissue and organ dynamics of breeding female common eiders from the East Bay colony from 2002 to 2004 (J. Bêty, unpubl. data used with permission). Middle lines indicate median tissue masses, "+" depicts the mean, the boxes represent interquartile ranges between the first and third quartiles, and the whiskers extend to the minimum and maximum values. Abbrev.: RFG = rapid follicular growth, LAY = laying, INC = incubation, and NB = non-breeding. Numbers indicate sample size, and different letters represent significant differences between groups ($p < 0.05$).

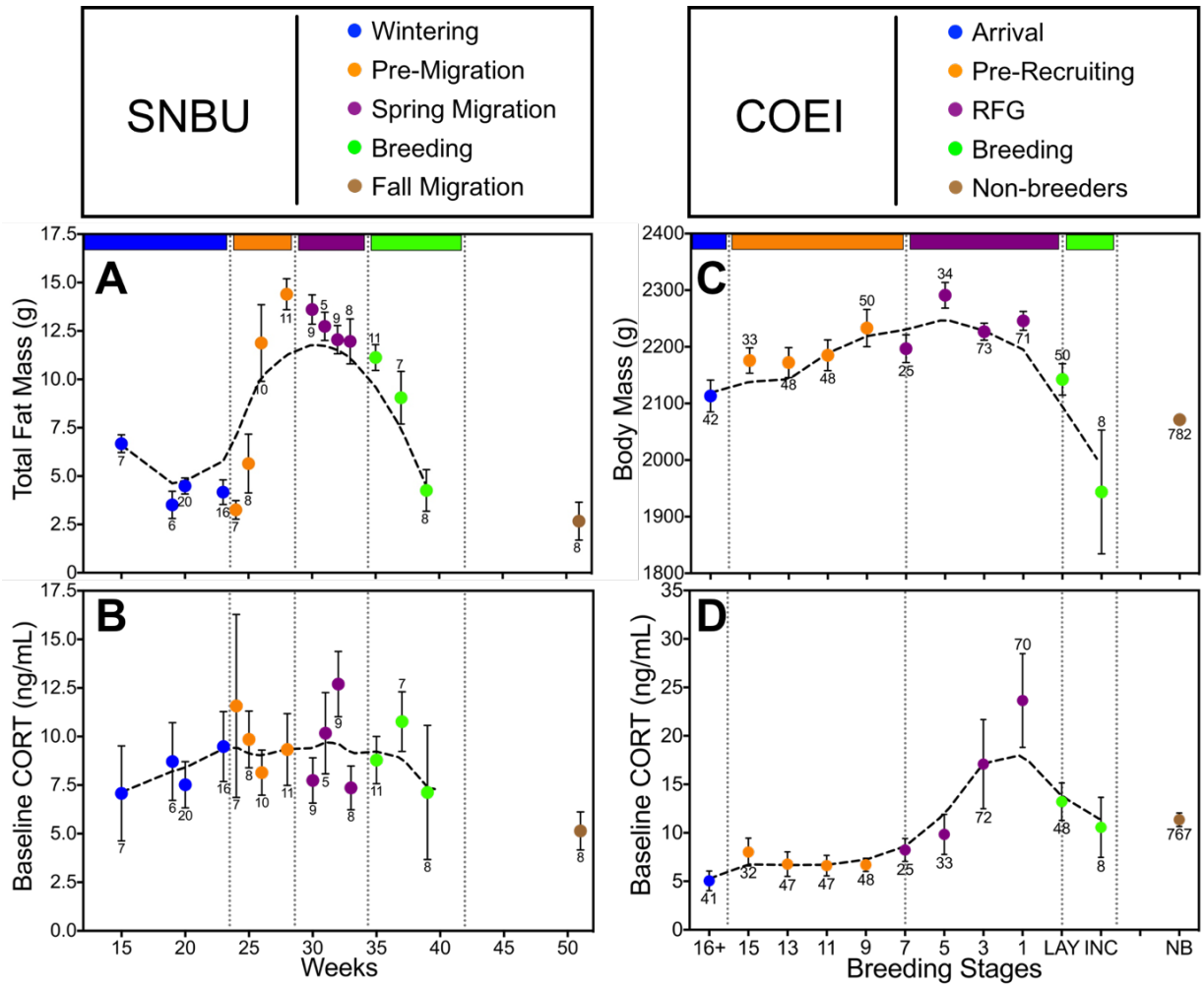

Figure 3.3: Temporal patterns of (**A**) total fat mass in a subset of male snow buntings across the year in 2015 (n = 25), and (**C**) body mass in all female common eiders across breading seasons from 2006 to 2016 (n = 1,264) with the lipogenic hormone, baseline corticosterone (buntings: n = 25; eiders: n = 1,238; **B** and **D**). Dots represent the means ± SEM, values indicate sample sizes, and dotted grey lines separate life-history stages.

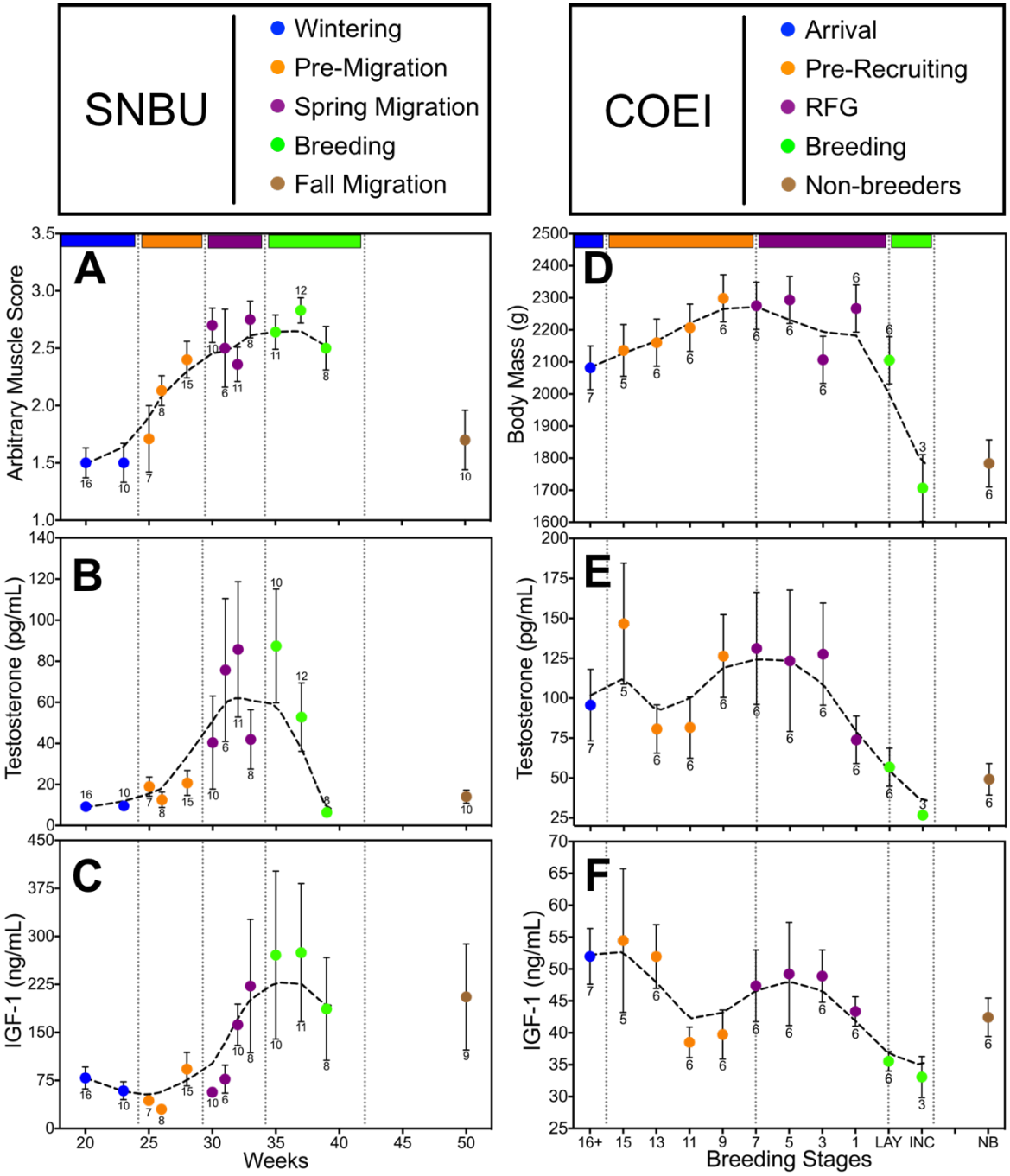

Figure 3.4: Temporal patterns of (**A**) total fat mass in a subset of male snow buntings across the year in 2015 (*n* = 25), and (**D**) body mass in a subset of female common eiders across breading seasons from 2010 to 2016 (*n* = 69) with the muscle-promoting hormones, testosterone (**B** and **E**) and IGF-1 (**C** and **F**). Dots represent the means ± SEM, values indicate sample sizes, and dotted grey lines separate life-history stages.

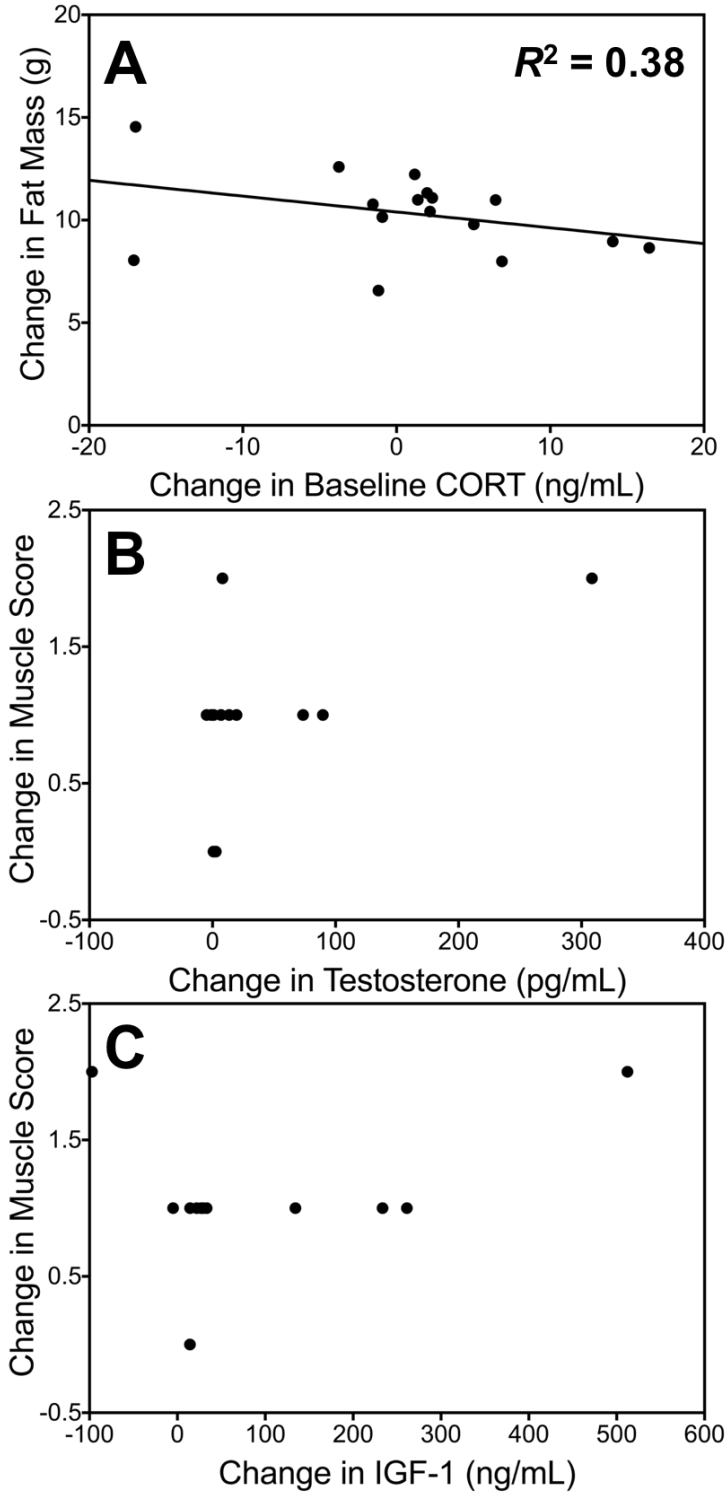

Figure 3.5: Linear regressions that shows the relationship between (**A**) changes in fat mass and baseline corticosterone (*n* = 16), and between changes in muscle score and changes in (**B**) testosterone (*n* = 13) and (**C**) IGF-1 (*n* = 13) during a period of energetic gain. Each point represents an individual, and line indicates a significant trend ($p < 0.05$).

CHAPTER 4 – GENERAL DISCUSSION AND CONCLUSIONS

SUMMARY OF FINDINGS

Circulating hormones that regulate energetic processes in vertebrates have traditionally been investigated in biomedical (Murphy & Bloom 2006), agriculture (Hocquette et al. 1998), aquaculture (MacKenzie et al. 1998), and poultry research (Scanes 2009). Within the past two decades, environmental physiologists have begun to adopt and integrate principles from these studies to explore the hormonal (molecular) control of energy metabolism (Boswell 2005), and hence, the temporal variation in these hormone (Wingfield 2008). However, there is still a lack of ecological research that examines energy-regulating hormones within a life-history stage framework in free-living birds (Williams 2012a).

To address these issues, I highlighted the mechanistic advances on the hormonal regulation of energetic phenotypes in avian ecology, and used mammalian studies to further illustrate key mechanisms in unstudied or poorly studied areas in birds (Chapter 2). Taking a descriptive and correlative approach to examine some of these candidate hormones, I explored the temporal variation of fat- (baseline corticosterone) and muscle-promoting (testosterone and IGF-1) hormones in snow buntings (*Plectrophenax nivalis*) and common eiders (*Somateria mollissima*), two Arctic-breeding birds that are excellent models of extreme energetic readiness across two different life-history stages. My results indicate that plasma energy-regulating hormone levels reflect stage- and species-dependent patterns in both captive and wild birds. More specifically, I found that plasma baseline corticosterone levels did not appear to be a strong lipogenic signal for fattening in male snow buntings, while in female eiders, baseline corticosterone showed stage-related elevations, reinforcing its proposed anabolic role on pre-breeding fattening (Hennin et al. 2015, 2016; Chapter 3). Temporal and stage-related changes in testosterone levels appeared to match the changes in muscle score in buntings, where eiders, on the other hand, exhibited daily fluctuations in circulating testosterone that may be more important

for regulating breeding behaviours than muscle hypertrophy, though there were no significant increases over the breeding stages (Chapter 3). Finally, I suggest that elevated levels of IGF-1 during the migration (buntings) and breeding (eiders) stages may be an important proliferative signal for testes growth and organ remodeling during and after spring migration, respectively. Similarly, fine-scale changes in IGF-1 levels may stimulate post-flight digestive tract recrudescence and growth of female reproductive tissue in eiders, despite a lack of large scale changes in IGF-1 levels across breeding stages (Chapter 3).

DISCUSSION OF FINDINGS

Using concepts from evolutionary genetics, the fact that multiple hormones can act simultaneously on different tissues is not surprising given that hormones display (1) *hormonal pleiotropy,* with multiple effects from hormone-specific receptors distributed in various tissues (Finch & Rose 1995), and (2) *phenotypic integration,* where co-expression of hormones and hormonal pathways may be tightly linked to regulate complex phenotypes (Cox et al. 2016). Here, I employ these concepts to guide our interpretation of hormone-mediated effects in this study. First, hormonal pleiotropic effects can occur *via* tissue-specific detection thresholds (i.e. sensitivity) from the elevation in hormone secretions (i.e. strength of signal; Ketterson & Nolan 1999). Seasonal variation of energy-regulating hormones may therefore mediate concentration-dependent effects on different target tissues. For example, natural and experimental elevations in testosterone levels of both sexes in the northern-temperate dark-eyed junco (*Junco hyemalis*) generated changes in multiple phenotypic traits (i.e. immune function, aggression, stress response, ornamentation), providing evidence of dose-response effects across a diversity of internal systems (Ketterson et al. 2009).

Applying these concepts to our study, slight elevations in testosterone levels may be enough to reach a minimum threshold for hypertrophy of skeletal muscle tissue in male buntings, and then further elevation during the migration and breeding stages may elicit a physiological “switch” for other testosterone-mediated effects such as the growth of reproductive tissue and the central

stimulation of breeding behaviours (Ball & Balthazart 2008). Along the path from hormone secretion to receptor binding, many intermediate molecules could regulate seasonal differences in tissue sensitivity, and thus, mediate changes in genomic effects, such as receptor density (Wacker et al. 2010), hormone binding proteins (i.e. free vs. total hormone; Malisch & Breuner 2010), enzymatic activity (i.e. aromatase in the brain; Rosvall et al. 2012), and transcriptional coactivators (i.e. cAMP response element binding protein-binding protein; Auger et al. 2002). Likewise, seasonal effects could be related to differences in upstream intermediate hormones in the hormonal signaling cascade or axis (i.e. LH receptors on testes effect on steroid production; Bartke 1987).

We found similar temporal patterns between testosterone and IGF-1 during energetic preparation and energetically demanding stages in both species (Chapter 3). Given the interaction of somatotropic (GH/IGF-1) signaling on the hypothalamic-pituitary-gonadal (HPG) axis in mammals (reviewed in Chandrashekar et al. 2004), it is perhaps not surprising to detect synchronized, concomitant increases in testosterone and IGF-1 that potentially co-evolved in preparation for reproductive investment, given that IGF-1 has only recently been shown to regulate performance-related traits in birds (i.e. clutch size and egg weight; Lodjak et al. 2017). Nonetheless, there is still a lack of empirical evidence supporting these hormonal effects on reproductive phenotypes in free-living, adult birds.

Importantly, while a qualitative assessment of temporal patterns suggested fine-scale, daily fluctuations in testosterone and IGF-1 levels in common eiders, quantitative analyses indicated no significant differences across breeding stages, unlike in snow buntings (Chapter 3). However, the current experimental approach of categorizing data into broad stages may reduce the potential of detecting finer scale changes in hormone concentrations over time. For instance, in eiders, both testosterone and IGF-1 displayed high levels followed by a distinct drop in circulating levels *within* the pre-recruiting stage (Figure 3.4E &F), likely generating a substantial amount of variation around the mean and reducing our ability to detect differences from other life-history stages

(Table 3.3). On the other hand, while breaking data into two-day groups does increase the temporal detail during the pre-breeding period, each category had small sample sizes reducing statistical power (although small standard errors of the means suggest accurate results given the high degree of individual variation in hormone levels during the breeding season; Williams 2012b). Therefore, I would argue that, the qualitative assessment of the temporal patterns provide valuable information on potential endocrine regulation of seasonal phenotypes both *within* and *across* life-history stages. Nevertheless, more robust statistical techniques, such as using approaches that can harness the power of temporal autocorrelation of multiple variables on repeated-measures data (i.e. Generalized Additive Mixed Models (GAMMs); Woods 2006) may provide a stronger and more relevant means of examining changes of hormone levels across stages.

DIFFICULTIES ASSOCIATED WITH MEASURING T_3 AND ACYLATED GHRELIN

My thesis originally proposed to measure the hormonal profiles of both the thyroid hormone, triiodothyronine (T_3), and acylated ghrelin; however, despite a great deal of effort, there were several logistical difficulties associated with the quantification of these candidate lipogenic hormones. In assessing T_3 status in circulation, most avian studies evaluate both the total and free (unbound) plasma portion of T_3 using radioimmunoassays (RIAs) either developed in-house (i.e. Chastel et al. 2003; Duriez et al. 2004; Vézina et al. 2009), or commercially available kits coated with goat-mouse IgG antibodies designed for humans or small rodent models (i.e. MP Biomedicals kit, Elliott et al. 2013; Welcker et al. 2013). Alternatively, T_3-specific enzyme immunoassays (EIAs) have been designed, however, these detection methods have not been as extensively validated in birds (McNabb 2007; except see Elarabany et al. 2012).

To test EIA detection of T_3 in birds, I attempted to measure circulating levels of total T_3 with both methanol-extracted (i.e. Reyns et al. 2002) and non-extracted pooled chicken plasma (Sigma Aldrich, Oakville, ON, CAN, #P3266; same steps for buntings and eider plasma) at multiple volumes (10 μL, 20 μL, 50

μL, and 100 μL) using three different commercial EIA kits (MP Biomedicals, Solon, OH, USA, #07BC-1005; Labor Diagnostika Nord, Nordhorn, Germany, #TFE-2300; Diagnostics Biochem Canada Inc., London, ON, CAN, #T3-4220). Our dose-response curve showed a non-parallel displacement with the plate's standard curve, indicating an inconsistent immunoactivity in detecting avian T_3. Furthermore, the lack of hormone detection could be attributed to (i) an incompatibility to bind to mammalian-derived antibodies (Brown et al. 2004), (ii) interference from binding proteins (i.e. albumin and transthyretin) or other plasma proteins (Selby 1999), or (iii) potentially low levels of T_3 in commercially purchased chicken plasma (Leclercq et al. 1988). Although RIAs are typically more sensitive (i.e. lower minimum detection limits) than EIAs, they nonetheless use potentially dangerous radioisotope labels (I^{125}, in the case of T_3 assays), which we currently do not have the facilities to safely handle. A potential solution may be to use high-performance liquid chromatography (HPLC) for high recoveries of plasma T_3 to increase the probability of antibody detection (Gika et al. 2005). However, we presently do not own the proper and expensive laboratory equipment (i.e. sample pumps and signal detector) to isolate thyroid hormones using this technique (Gika et al. 2005). Nonetheless, we found that three readily available T_3-based EIA kits were unable to appropriately measure plasma levels in avian plasma, and we caution the use of enzyme-immunoassays without proper validation from tests of both parallelism and relative binding affinity.

Acylated ghrelin has been a very difficult hormone to measure in this thesis for a number of reasons. First, plasma acylated ghrelin is highly susceptible to degradation from circulating esterases that hydrolyze the acyl-ester linkage and rapidly (within a few minutes) converts it into des-acyl ghrelin (De Vriese et al. 2004). After collection of a blood sample, protective reagents such as hydrochloric acid and broad-spectrum esterase inhibitors must be immediately added to the sample to, at a minimum, conserve the acyl attachment on ghrelin (Delhanty et al. 2012; see RAPID method as a more conservative approach, Stengel et al. 2009). Unfortunately, our samples were collected prior to

beginning this particular research project, and we therefore did not protect samples *a priori* at sample collection. I therefore assumed that most, if not all, acylated ghrelin was likely converted to des-acyl ghrelin in our plasma samples from both species (Hosoda et al. 2004). Presently, there are two different RIAs designed to detect acylated ghrelin at the N-terminus, considered the most conserved portion of the mature peptide across vertebrates, and des-acyl ghrelin at the C-terminus (Kaiya et al. 2007a, 2008). However, there are no current detection methods for des-acyl ghrelin in birds (mentioned in Goymann et al. 2017), as the C-terminus amino acid sequences of human and poultry (i.e. chicken, quail, turkey) are divergent, meaning that mammalian antibodies do not bind to avian des-acyl ghrelin (Kaiya et al. 2007a). These challenges prevented the quantifiction of both acylated (degraded) and des-acyl ghrelin (lack of biological importance to my study and indectectable methods). Despite all these potential issues, I still tried to quantify total ghrelin using a commercially available kit designed for rat/mouse species (EMD Millipore, Etobicoke, ON, CAN; #EZRGRT-91K), but due to the reasons above, it unsurprisingly failed to detect hormone concentrations.

Another issue with measuring acylated ghrelin is the extremely low plasma concentrations due to its enormous orexigenic potency (~<100 pg/mL), which by default as stated above, requires an RIA for high sensitivity (Kaiya et al. 2007b). Additionally, previous studies on acylated ghrelin in domestic chicks have used very large plasma volumes from sacrificed individuals to increase hormone analyte in samples (Kaiya et al. 2008, and references therein), which is simply not possible nor practical when using non-lethal blood collection techniques in smaller-bodied birds (i.e. songbirds). In this thesis, with amount of plasma being a constraint from assessment of multiple hormones from the same samples in small birds (i.e snow buntings) or used in other research (i.e. common eiders), we would not have been able to allocate similar amounts of plasma (>50 μL) as has been accomplished in domestic avian species. Altogether, we recommend that appropriate detection of acylated ghrelin in birds needs protection reagents, storage at -80°C, highly sensitive assays, and large plasma (sample) volumes.

FUTURE DIRECTIONS

A. From Observational Studies to Manipulative Experiments

With advances in ecological research on energetic physiology, both observational and manipulative studies can provide valuable mechanistic information about the regulation of energy-regulating hormones on energetic phenotypes in birds. Ramenofsky (2011) highlights how the difficulty in tracking migratory populations throughout the life cycle has led to most studies on migratory birds having been explored in a single species at only a single life-history stage. Long-term observational data in wild-caught birds can address this problem by providing an accurate depiction of natural hormone titers across the year, and then comparative analyses can then be used to examine similar energetic traits in closely related species (i.e., phylogenetic signal; Zheng et al. 2009), or in species sharing similar environmental conditions (i.e., convergent evolution; Losos 2011). In this case, my thesis implicitly assesses whether patterns of endocrine responses in two Arctic-breeding species are driven by similar environmental pressures (Chapter 3).

There is value to collecting endocrine profiles from observational (i.e. correlative) studies since they can reveal the biological relevance of natural endogenous hormone levels, which can then form the basis for manipulative hormone experiments aimed at confirming causal relationships of physiological phenomena within a life-history context (Eikenaar et al. 2011; Eikenaar et al. 2013; Ketterson et al. 1996). While I had originally aimed to conduct hormone (corticosterone) manipulations in the snow bunting population outlined in Chapter 3, unfortunately many birds were lethally infected with an avipoxvirus (*Poxviridae* family) in winter 2016, reducing sample sizes to the point that the planned experiments were impossible to conduct. Future manipulative experiments in snow buntings, particularly those using exogenous administration of IGF-1, may be particularly interesting and novel, as they would determine for the first time in wild adult birds whether this hormone causally induces growth in skeletal muscle and other somatic tissues in preparation for migration. Regardless, my thesis

suggests that any manipulations of avian energy-regulating hormones in free-living or captive populations should consider the natural life-history stage variation and ecological context (see considerations in Chapter 2), along with previously identified experimental designs such as dose concentration and the mode of hormone delivery (Crossin et al. 2016).

B. Integrative Tools to Identify Underlying Mechanisms

Endocrine regulation of peripheral energy stores is tightly integrated with other systemic signaling pathways, such as neural circuitry and metabolic processes (Figures 2.2 & 2.3; Chapter 2). Given the complexity of these interconnected relationships, it is critical that ecological researchers employ a variety of tools to identify the endogenous mechanisms of avian energy balance. First, with the rapid expansion in the number of avian species with sequenced genomes, transcriptomic profiling is rapidly allowing the field of evolutionary endocrinology to detect multiple, tissue-specific endocrine and metabolic markers involved in lipid and protein metabolism (Mello & Lovell 2017; Chapter 2). Besides the benefit of using genome-wide screening for differential expression of previously unknown molecules, these techniques can also measure mRNA expression of known proteins during life-history stages involving fat deposition and skeletal muscle growth, such as mRNA transcripts for hormone receptors, hormone and metabolite (i.e. fatty acids) binding proteins, hormone-converting enzymes, and cell membrane transporter proteins (Cox et al. 2016). From an ecological perspective, one issue with this approach is the animal sacrifice required to dissect target tissues, which may be arguably too invasive to assess seasonal patterns across the annual life cycle, or when studying a focal species within declining wild populations. Despite this, researchers can focus on comparisons between two stages with high and nil degrees of energetic storage to reveal hormonal effects on metabolic intermediates that generate fat and muscle phenotypes. Overall, the application and integration of plasma hormone levels and tissue-specific gene expression can connect endocrine function with

complex gene networks with the goal of completing these evolutionary mechanisms (Cox et al. 2016; Mello & Lovell 2017; Zera et al. 2007).

In addition to the use of these investigative tools on endogenous hardware, ecologists can examine how seasonal changes in hormone levels generate behavioural responses that proximately influence variation in motivation (i.e. food intake, nutrient preference) and performance (i.e. foraging trips, migratory restlessness) to ultimately affect energy stores (Bairlein 2002; Garland et al. 2016). For example, given that many behaviours are consistent within- and between-individuals in a population (Sih et al. 2012), future studies could examine the regulation of naturally circulating acylated ghrelin on feeding rates, as it would be expected that individuals with higher acylated ghrelin suppress appetite (i.e. decrease food intake) to a greater degree relative to individuals with lower levels (Kaiya et al. 2013). Testing these correlative relationships has the potential to bridge the gap between seasonal variation in energy-regulating hormones and the behaviours they control, providing a predictive utility for the hormone-behaviour regulation of nutritional intake and energetic storage.

C. Thyroidal Control of Seasonal Cyclicity in the Brain

The longstanding belief in vertebrates is that changes in thyroid hormones are essential for signaling the appropriate timing of circannual rhythms, with pioneering discoveries that showed thyroidectomy inhibits the photoperiod-induced responses in birds and mammals (Hazlerigg & Loudon 2008). Recently, Ebling (2014, 2015) reviewed the central control mechanisms of seasonal weight gain in mammals, suggesting that transition to long days stimulates the pars tuberalis of the pituitary gland to secrete thyroid stimulating hormone β-subunit (TSHβ) that binds to TSHβ receptors on tanycytes (i.e. glial cells) of the arcuate nucleus in the hypothalamus and up-regulates the production of deiodinase enzymes II (DIO2; converts T_4 to T_3) and III (DIO3; converts T_4 to inactive forms, reverse T_3 and T_2). Importantly, the high intracellular ratio of T_3:T_4 is thought to promote increases in food intake and body mass (Dardente et al. 2014);

however, the exact intermediate steps between increases in T_3 and these peripheral (anabolic) effects are currently unknown.

Nonetheless, it has been proposed that T_3 may be the master regulator of seasonal neuroplasticity in the mediobasal region of the adult hypothalamus that controls key metabolic functions (Ebling 2014; Migaud et al. 2015). Furthermore, hypothalamic neurogenesis in mammals gaining fat stores seasonally leads to concurrent expression of neuropeptides (i.e. POMC, Cart, NPY, AgRP) and responsiveness to hormones involved in energy balance, which indicates the importance of neural reorganization to modulate effects from peripheral signals (reviewed in Langlet 2014). Additionally, despite the anatomical differences of daylight transmission between mammals and birds (i.e. deep brain photoreception in birds rather than photoreceptors in the eyes of mammals; Nakane et al. 2010), both similarly activate T_3 conversion in the hypothalamus and in birds this conversion has been linked to priming of reproduction through stimulation of gonadotropin releasing-hormone neurons in Japanese quail (*Coturnix japonica*; Nakane & Yoshimura 2010; Nakao et al. 2008). Since the central mechanism of thyroid hormones on seasonal changes in physiology appears highly conserved in photosensitive vertebrates (Ebling 2015), we propose that future research in birds living and breeding in seasonal environments should focus on: (1) the intermediate steps of T_3 regulation on increases in food intake and weight gain; (2) whether these neural networks stimulate release of plasma energy-regulating hormones from peripheral tissues; and (3) the effect of photoperiod-induced neuroplasticity on appetite-stimulating (i.e. NPY and AgRP) neuropeptide expression.

D. Implications of IGF-1 for Life-History Variation

Using our comparative approach across species, we found that plasma IGF-1 levels were much higher in short-lived snow buntings (~up to 5.5-fold magnitude) than long-lived common eiders. Interestingly, Kenyon (2005, 2011) and collaborators' ground-breaking research found that mutations in the dauer formation (*daf*-2) gene, encoding IGF-1 receptors in *C. elegans*, extends lifespan,

with supporting results for receptor mutations in mammalian models (Bartke 2008). This inverse relationship between IGF-1 signaling and longevity may justify the differences in lifespan between snow buntings and common eiders. Although hormonal analysis on ageing was not a targeted objective of this study, our study design comparatively supports this relationship between circulating IGF-1 levels and lifespan, and this provides insight into promising avenues on life-history trade-offs between growth and longevity in a understudied ecological context in free-living vertebrates, including birds (Dantzer & Swanson 2012).

E. Comparing Energetic Gain Across Stages and Taxa: Importance of Context

To unravel the underlying mechanisms driving energy gain, an important contextual question arises: do all stages of energetic storage in vertebrates utilize the same hormones? Ultimately, although I would argue that the endogenous 'hardware' (i.e. common homeostatic mechanisms) should be theoretically conserved across stages, different ecological contexts should certainly be considered, including: (i) environment (Cornelius et al. 2013), (ii) life-history (Crossin et al. 2016), (iii) annual cycle (Wingfield 2008a), (iv) sex and age (Crespi et al. 2013), and (v) sociality (Hofmann et al. 2014). In my thesis, I provide context-dependent reasons for changes in hormone levels across life history stages (Chapter 3), while simultaneously controlling for many of these contexts (i.e. stage and sex); however, these other ecological contexts should be accounted for in future studies. First, although both species in this thesis naturally breed in the Arctic, unpredictable local (i.e. weather) conditions may differentially influence the physiology of free-living eiders in East Bay (Arctic) that are at a much higher latitude (~1,700 km) than captive buntings residing in Rimouski (sub-Arctic; Wingfield 2008b). Second, eiders are a longer-lived species with more total breeding opportunities compared to shorter-lived buntings, and therefore their life-history stages within the annual life cycle differ (Legagneux et al. 2016; Montgomerie & Lyon 2011), possibly affecting the seasonal differences in hormone titers. Sex is clearly important in eider

physiology, where only hens engage in pre-breeding energetic storage from female-biased parental investment in reproduction, while both male and female buntings require energy stores for spring migration (Chapter 1). Additionally, age may affect important stage-related strategies, such as foraging behaviour and rates of energetic gain (Descamps et al. 2011). Finally, hormone-behaviour interactions may affect the temporal and spatial differences in social groups of colonial-breeding eiders and migratory flocks of buntings (Hofmann et al. 2014). Taken together, these ecological contexts should be carefully considered when making predictions and interpretations about endocrine responses, despite using a common hormonal framework from other birds or even other vertebrates.

CONCLUSIONS

My thesis provides observational (correlative) information on the temporal patterns of hormone responses during stages of energetic gain in fat and skeletal muscle, showing evidence for changes in baseline corticosterone, testosterone, and IGF-1 across life-history stages in two Arctic species models. Here, I identified several issues with quantifying T_3 and acylated ghrelin, and provided some potential solutions for future work on these hormones. Future experimentation using hormone manipulations will identify cause-and-effect relationships with hormone-mediated traits. Transcriptomics, detection of tissue-wide mRNA expression levels, may be a useful genetic technique to seek new and confirm existing proteins in the hormone-metabolic interface. Behavioural assessment of food intake may provide another hormone-mediated response to complete the picture of these effects on seasonal energetic phenotypes. Further consideration should be given to the central regulation of thyroid hormones on seasonal neuroplasticity in key appetite-regulating centres in the brain. Although I aimed to control for different ecological contexts in both our study systems, I outline areas of consideration for future correlative and manipulative studies. Ultimately, my research contributes to the growing, yet incomplete literature on endocrine mechanisms that regulate fat accumulation and skeletal muscle

growth in birds breeding in highly seasonal environments, with implications on organismal performance and potentially indirect effects on fitness traits.

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