EXAMINATION OF THE PERIOD OF PREPARATION FOR BREEDING IN MALE AND FEMALE SONGBIRDS

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ABSTRACT

In virtually all species, reproduction must be precisely timed to coordinate breeding and rearing of offspring with favorable conditions. It is imperative for individuals to time the highest energetic cost to themselves or highest needs by offspring with greatest food availability in either abundance or nutrient make-up. To accomplish this, individuals must integrate signals conveying both internal and external status and accordingly alter the activity of the reproductive axis. To date most efforts to identify variation in control mechanisms for reproduction in seasonally breeding animals have focused on the brain as the initiator for reproduction. However, recent studies have re-directed attention to two other potential tissues, the pituitary and ovary, where variation in brake sensitivity may be important. To this end, a series of experiments were performed in two songbird species to elucidate the hormonal role in timing of breeding and the interaction of an energetic trade-off on the decision to breed in both males and females. I used two species, the wild dark-eyed junco (*Junco hyemalis*) and the wild-caught captive house sparrow (*Passer domesticus*) as model systems to address the following questions: 1) What physiological mechanisms explain individual variation in onset of gametogenesis? And 2) How are mechanisms of energy integrated with the reproductive axis' control of reproduction and timing of breeding to regulate energetic trade-offs? Specifically, we focused the role of testosterone in males (Chapter 2), and mechanisms downstream of the hypothalamus in females (Chapter 3). Next, in Chapter 4, the question of whether an energetic demand would reduce sperm quality was addressed. Lastly, in Chapter 5, we asked if an energetically costly immune challenge would result in the delay of clutch initiation. The results of these studies demonstrate the significant differences between sexes in the signals conveying an individual's internal and external status in order to alter activity of the reproductive axis, and therefore timing of breeding.

Collectively, these findings provide further evidence that females are ‘in the driver’s seat’ for onset of breeding and should be the focus of future research.

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HPG	Hypothalamic Pituitary Gonadal Axis
GnRH	Gonadotropin Releasing Hormone
LH	Luteinizing Hormone
FSH	Follicle Stimulating Hormone
T	Testosterone
E_2	Estradiol
VLDL	Very-low-density Lipoprotein
HPA	Hypothalamic Pituitary Adrenal Axis
CORT	Corticosteroid
LPS	Lipopolysaccharide
KLH	Keyhole Limpet Hemocyanin
GR	Glucocorticoid Receptor
MR	Mineralocorticoid Receptor
FSHR	Follicle-Stimulating Hormone Receptor
LHR	Luteinizing Hormone Receptor
ERα	Estrogen Receptor α

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CHAPTER 1: GENERAL INTRODUCTION

Introduction

In virtually all species, reproduction must be precisely timed to coordinate breeding and rearing of offspring with favorable environmental conditions (Lack, 1968; Naef-Daenzer and Keller, 1999; Newton and Marquiss, 1982; Sauther, 1998; Van Noordwijk et al., 1995). In small animals, investment into reproductive organs and rearing of offspring is often reduced during the less productive or even harsh environments of winter, and are restricted to the renewed resource abundance of spring and summer (Baker, 1938; Bronson, 1989). Therefore, animals in seasonal environments often restrict breeding to a limited time of year to align seasonal variation in food availability with reproduction and/or rearing of offspring (Naef-Daenzer and Keller, 1999; Perrins, 1970; Wayne et al., 1989), a period of high energetic demands.

Most temperate-zone animals breed seasonally to ensure that offspring are born at an optimal time for rearing and survival (Baker, 1938; Murton and Westwood, 1977). Before young arrive, parents must undergo extensive physiological and behavioral preparation for breeding. It is imperative for individuals to time the highest energetic cost to themselves or highest needs by offspring with greatest food availability in either abundance or nutrient make-up (Bronson, 1989; Holberton et al., 2005). To accomplish this, individuals must integrate signals conveying both internal (e.g., energy availability) and external (e.g., photoperiod) status and accordingly alter the activity of the reproductive axis, via activation of the reproductive endocrine axis.

Often, preparatory events must be initiated well in advance of seasonal increases in food availability (Gibb, 1950). Consequently, animals must be able to predict the onset of the breeding season by using local predictive environmental cues. While reproductive readiness is ultimately regulated by initial photoperiodic cues (Dawson et al., 2001), the exact onset of exponential gonadal growth is thought to be somewhat plastic and fine-tuned by supplementary cues (e.g., temperature and resource availability) (Marshall, 1959; Murton and Westwood, 1977; Wingfield et al., 1992; Wingfield and Kenagy, 1991). Plasticity in internal thresholds of ‘switches’ in response to interactions between the environment and physiological systems activated during critical windows can initiate or delay key life-history transitions that aid in adaptation to novel conditions (Nijhout, 2003; Suzuki and Nijhout, 2006; West-Eberhard, 1989; Wilczek et al., 2009).

The timing of the first clutch initiation in the spring has potential consequences for future nest attempts, life-history stages, and annual reproductive output (Stutchbury and Robertson, 1988; Winkler and Allen, 1996). Early breeding individuals tend to have offspring with higher survival and recruitment rates (Dobson and Michener, 1995; Galen and Stanton, 1991; Hochachka, 1990; Landa, 1992; Rieger, 1996; Verhulst et al., 1995). Offspring survival likelihood is positively related with offspring mass and condition and this relationship becomes increasingly important as the breeding season progresses (Naef-Daenzer et al., 2001; Rieger, 1996). Thus, delayed onset of reproduction has the potential to have profound and detrimental effects on both the male and female’s reproductive success (e.g., reduced recruitment) (Reed et al., 2009; Williams, 2012).

Seasonal breeding has been extensively studied, and the endocrine mechanisms controlling seasonal reproduction are becoming increasingly well understood (reviewed in: (Dawson, 2008; Dawson et al., 2001; Williams, 2012). However, the overwhelming male-bias in these studies has left researchers short on information on how environmental cues are integrated in the hypothalamic-pituitary-gonadal (HPG) axis to regulate female reproductive development and timing of egg-laying (Caro, 2012). Females and their physiological changes that occur in the final few days just prior to egg-laying are the key to understanding timing of breeding (Caro et al., 2009). The current model, described above, of day length as an initial predictive cue and supplemental environmental cues (e.g., temperature and food availability) fine-tuning actual timing of egg-laying is poorly supported by experimental data for female songbirds. Further, this model does not currently provide a well-founded mechanistic explanation for individual variation in timing of breeding in females (Williams, 2012).

Hormones like testosterone often demonstrate highly variable patterns of secretion, making it difficult to obtain 'physiologically meaningful' measures that can be related with traits of interest (Aschoff, 1979; Hau, 2007; Ketterson et al., 2009; Laucht et al., 2011). This dynamic nature of hormone secretion has made it inherently difficult for investigations aimed at understanding the impact of consistent individual differences in hormone levels on key fitness-related traits. In males, testosterone-mediated traits have often been related with morphological, physiological and behavioral traits such as dominance rank, plumage characteristics, immune function and genetic quality (Griffith et al., 1999; Jensen et al., 2004; Veiga, 1993). For example, morphological 'ornaments' have been observed to be related with testosterone. However, hormone levels in circulation are highly variable and actively respond to the environment and individual experiences (reviewed in: Ketterson et al., 2005; Wingfield et al., 1990) and also vary as a result of underlying endogenous rhythms (Bell-Pedersen et al., 2005; Daan et al., 1975; Gwinner, 1975, 1974; Morin et al., 1977; Takahashi and Menaker, 1980). Thus, relating an individual's circulating hormone levels sampled at a single time point poses challenges for interpretation and investigation of relationships with other traits of interest (Fusani, 2008) (e.g., sperm quality and ornamentation).

The overarching objective of the proposed research is to better understand the differences in the timing and duration of physiological preparation for breeding, and how energetic trade-offs in this critical period can lead to mis-timed breeding attempts in male and female songbirds. First, we aimed to characterize how the HPG axis is regulated in the period just prior to onset of breeding in a seasonally breeding bird. Second, now with a better understanding of the physiological mechanisms during the period leading up to breeding, we aimed to evaluate how resource-allocation-based trade-offs influenced an individual's decision to breed. Our research used both field and laboratory experiments to investigate these specific aims:

Specific Aim 1: What physiological mechanisms explain individual variation in onset of breeding?

Hypothesis 1: Will elevated nighttime levels of endogenous testosterone secreted by individuals relate to maximum testosterone secretion following stimulation with exogenous GnRH?

Hypothesis 2: What are the female-specific regulatory mechanisms for the onset of egg development downstream of hypothalamic control that is critical for regulation in timing of breeding?

Specific Aim 2: How are mechanisms of energy allocation integrated with HPG axis control of reproduction to regulate energetic trade-offs?

Hypothesis 3: Can mounting an immune response impose an energetic demand that will result in a short-term or long-term decrease in sperm viability, and will repeated exposure further reduce sperm viability?

Hypothesis 4: Can an energetically costly humoral immune challenge induce an energetic trade-off, thus delaying onset of clutch initiation?

Background for Aim 1

Role of Environmental Cues in Timing of Seasonal Breeding

Annual variation in photoperiod is experienced throughout the year in the temperate-zone, with the longest days occurring in the summer and shortest days in the winter. This variation in day-length throughout the year results in predictable seasons in the environment and seasonal breeding strategies by many animals (Caro et al., 2013a; Dawson et al., 2001). Most seasonal breeders undergo marked periods of gonadal regression, as well as marked gonadal recrudescence, undergoing physiological and morphological changes similar to those observed during puberty, in response to lengthening photoperiodic cues (Bronson and Heideman, 1994).

Animals use photoperiod as a reliable initial predictive cue, but must also rely on additional environmental cues such as rainfall, humidity, food availability (Wingfield and Farner, 1993), social cues, and internal cues (e.g., endocrine rhythmicity and energy reserves) to fine-tune individual timing decisions (Dawson et al., 2001; Holberton et al., 2005). Supplemental environmental cues are believed to be used to optimally time reproduction (Wingfield et al., 1997; Wingfield and Kenagy, 1991). However, the hormonal and neural mechanisms used to activate reproductive responses to supplementary cues are largely unknown, and it appears that males and females may respond differently (Ball and Ketterson, 2008). Ultimately, integration of such initial predictive and supplemental cues alter the activity of neuroendocrine cascades, further modifying the phenotypic expression of morphology, physiology, and behavior (Bronson, 1989; Jacobs and Wingfield, 2000; Wingfield, 2012).

In captivity, males only require photoperiod to acquire full gonadal size; however, females also require additional supplementary cues for follicular development (Ball and Ketterson, 2008). Even with the addition of ample food, moderate temperatures, and removal of predators or competition, females often fail to induce ovarian development in captivity. Females, requiring more external and internal stimuli (hormones), are therefore the limiting sex that drives onset of breeding season and reproductive fitness of both sexes (Ball and Ketterson, 2008).

The Hypothalamic-Pituitary-Gonadal (HPG) Axis

Environmental cues are perceived and transduced at the level of the hypothalamus, where gonadotropin-releasing hormone (GnRH) neurons trigger the endocrine cascade resulting in gonadal activation (Dawson, 2008; Jacobs and Wingfield, 2000). More specifically, neural signals relay sensory information to the HPG axis, where the release of GnRH from the hypothalamus triggers secretion of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary. LH stimulates the synthesis and release of steroid hormones, testosterone (T) and estradiol (E_2) from the gonads (Adkins-Regan, 2008; Wingfield, 2012). Both of these hormones are then transported to target tissues where they bind to receptors, thus shaping physiological, morphological, and behavioral traits.

Interaction of the HPG and the Hypothalamic-Pituitary-Adrenal (HPA) Axis

In conjunction with the HPG axis' role in preparation for breeding, the hypothalamic-pituitary-adrenal (HPA) axis, more specifically secretion of corticosterone (CORT), plays an essential role in metabolism and energy regulation (Landys et al., 2006; Sapolsky et al., 2000). Circulating CORT ultimately affects energy mobilization via binding to two intracellular receptors, mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) (De Kloet et al., 1998; Funder, 1997). Both GR and MR receptors influence the response to elevated CORT levels during an acute stressor (Hu et al., 2008; Landys et al., 2006). However, MR has a 6 to 10-fold higher affinity for CORT than GR, suggesting a two-tier system for CORT binding under baseline and stress-induced concentrations of hormone (De Kloet et al., 1998, 1990; Reul and

Kloet, 1985). Specifically, MR has a higher affinity for CORT and is therefore thought to mediate responses to lower levels of circulating CORT. Due to GR's lower affinity for binding CORT, it is likely more important for reacting to the stress-induced activation of the HPA axis.

It is now well known that secretions from the HPA axis are capable of reducing the activity of the HPG axis (reviewed in Wingfield and Sapolsky, 2003). By acting directly at the level of the gonad, CORT can influence gonadal steroidogenesis by suppressing transcription and translation of steroidogenic enzymes (Hu et al., 2008) without affecting LH secretion from the pituitary (Deviche et al., 2014, 2012; McGuire et al., 2013; Wingfield et al., 1982). However, the role the HPA axis plays in regulating the timing of seasonal growth of the gonads is less well understood (Schoech et al., 2009).

Role of Sex Steroids in Timing of Breeding

Hormones regulate a wide variety of traits and behaviors (Adkins-Regan, 2005; Nijhout, 1998), such as mate-guarding and territoriality (Adkins-Regan, 1981; Balthazart, 1983; Harding, 1983; Hutchison, 1978), and their ability to regulate these multiple traits (pleiotropic) simultaneously influence the evolution of complex phenotypes (Ketterson et al., 2009). To date most efforts to identify variation in control mechanisms for reproduction in seasonally breeding animals have focused on the brain as an initiator of breeding. However, recent studies have re-directed attention to two other potential tissues where variation in brake sensitivity may be important. One is variation in pituitary responsiveness to repeated exposure to pulses of stimulatory GnRH, likely as a result of sex-steroid negative feedback (Greives et al., 2016). The other is the gonad itself, which may play a greater role in controlling reproductive activity and timing than previously expected (Ball, 2007). Many hormones, including sex steroids, are known to vary temporally (i.e., annually and daily), as well as in response to the environment an individual experiences (Hegner and Wingfield, 1986; Ketterson et al., 2009).

Physiological parameters, such as circulating sex steroids, are frequently measured to attempt assessment of the stage of gonadal maturation in preparation for reproduction and hormonal variation between individuals. The steroid hormone T, through its pleiotropic effects, plays a crucial role in regulating and coordinating morphology, physiology and behavior (Adkins-Regan, 2005; Dixson and Anderson, 2004; Hasselquist et al., 1999; Hau, 2007; Wingfield et al., 1990; Wingfield and Hahn, 1994) and has been the focus of investigations aimed at understanding the evolution of hormone systems in males and females (Hau, 2007; Ketterson et al., 2009). Individual variation in T hormone profiles is thought to "orchestrate" variation in reproductive-related traits and behaviors, including mating strategies, territory, and nest defense behaviors by both social parents (Balthazart, 1983; Harding, 1983; Hutchison, 1978; Ketterson et al., 2009; Wingfield et al., 2001). Further, elevated T during the breeding season is thought to enhance short-term reproductive success by boosting behaviors such as courtship, copulation, song and territory defense, as well as enhance secondary sexual characteristics (e.g., sexual organ size), and sperm production (Goymann et al., 2007; Hau, 2007; Hegner and Wingfield, 1986; Ketterson and Nolan Jr, 1999; Moore, 1991; Nelson, 2005).

Most temperate-zone birds increase their neural steroid receptor densities, as well as aromatase activity/mRNA expression, concurrently with elevated circulating T levels during the breeding season (Canoine and Gwinner, 2002; Foidart et al., 1998; Riters et al., 2000; Silverin et al., 2000; Soma et al., 2003). Testosterone typically acts via nuclear receptors located at many targets, either by binding directly to androgen receptors (ARs), or being converted by the enzyme aromatase to E_2, which binds to estrogen receptors (ERs). Once bound, these receptors function as transcription factors to regulate gene expression, leading to various physiological, morphological, or behavior outcomes. Changes in hormone receptor expression are another important mechanism facilitating the release of some traits from their original hormone control (Canoine et al., 2007; Voigt and Goymann, 2007). Elevated mRNA expression functions to increase a tissue's sensitivity to lower levels of circulating hormones (Canoine et al., 2007).

The Use of GnRH Challenges

Research aimed at understanding the evolutionary role of hormones has often attempted to relate discreet phenotypic traits (Solís and Penna, 1997) and fitness (Comendant et al., 2003; Kempenaers et al., 2008) with circulating T levels (Balthazart, 1983; Dixson and Anderson, 2004; Harding, 1983; Hutchison, 1978; Moore, 1984). However, investigations of natural co-variation between circulating sex steroid levels sampled at one time point during the day, and traits of interest often fail to confirm the relationships established by manipulative studies (Apfelbeck et al., 2013; Book et al., 2001; Hau and Goymann, 2015; Husak et al., 2006; Johnson et al., 2011; Laucht et al., 2011, 2010; McGlothlin et al., 2008; Villavicencio et al., 2014; Weatherhead et al., 1993). Alternatively, manipulative experiments that alter hormones for prolonged periods at relatively static levels (e.g., implants) have demonstrated clear links between sex steroids, T and E_2, secreted during the breeding period and morphological and behavioral traits relevant for reproduction (Hegner and Wingfield, 1987; Jawor et al., 2007, 2006). However, these manipulation studies generally flood the system with higher levels of hormones than would naturally occur.

Another approach that researchers have begun to utilize more readily to investigate relationships between the sex steroid hormone T and fitness-related traits has been administration of exogenous GnRH. This approach attempts to generate a standardized physiological maximum sex steroid value that can then be related with individual variation in other traits of interest (e.g., ornamentation and behavior) (Goymann and Wingfield, 2004; Jawor et al., 2006; McGlothlin et al., 2008; McGlothlin and Ketterson, 2008; Wingfield et al., 1979). The production of such short-term increases in sex steroids may underlie individual variation in the mating effort/parental effort trade-off identified by implant studies.

Preparation for Breeding in Males

Field experiments have demonstrated that elevations in T concentrations increase the extra-pair mating success of males (Ball and Wingfield, 1987; Enstrom et al., 1997; Raouf et al., 1997; Reed et al., 2006; Roulin et al., 2004; Sheldon, 1994), due to greater mating effort (McGlothlin et al., 2007). Male fertility and spermatogenesis are androgen-dependent (McLachlan et al., 2002; Sharpe, 1994; Fig. 1.1). Specifically, T in the testis is responsible for promoting spermatogenesis, which does not proceed if relatively high levels of intratesticular T levels are not present (Chang et al., 2004; De Gendt et al., 2004; Haywood et al., 2003; Sharpe, 1994; Zirkin et al., 1989). However, low plasma levels of T appear to be sufficient for gonadal recrudescence and spermatogenesis (Wingfield et al., 1990; Wingfield and Moore, 1987). In a previous study of the captive male house sparrow, it was found during the breeding season that intratesticular T levels increase in parallel with plasma LH levels, but not circulating T levels (Donham et al., 1982). They also report that spermatogenesis is promoted by T sequestered from the testis directly, and not from circulating T (Donham et al., 1982).

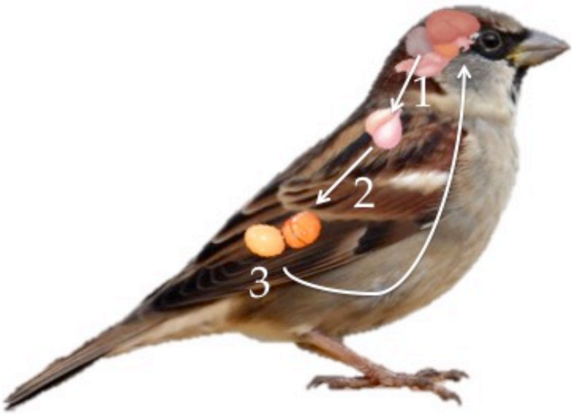

Figure 1.1. Reproduction in males is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. 1. Gonadotropin-Releasing Hormone (GnRH) stimulates 2. Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) released from the pituitary, which acts to 3. mature gonads and secrete sex steroids (testosterone, T; estrogen, E_2) to promote spermatogenesis.

Preparation for Breeding in Females

In female passerines, organization of the HPG axis in the period prior to breeding remains understudied (Fig. 1.2). To date we know the HPG axis is important for yolk formation, but we don't know the exact mechanisms dictating onset of egg production as females make this transition. Previous research has shown that the pituitary is capable of secreting high circulating levels of LH well before egg development begins (Greives et al., 2016), but ovaries do not increase circulating sex steroids levels until rapid yolk development (Jawor et al., 2007; Williams et al., 2004). This suggests that while the HPG axis is stimulated during the pre-breeding period, initiation of egg development itself is likely regulated somewhere downstream of the hypothalamus.

In preparation for breeding, female oviparous vertebrates undergo a period of rapid follicle growth in which follicles increase in size quickly in preparation for ovulation (Bêty et al., 2003; Drent and Daan, 1980; Rowe et al., 1994; Williams, 2012). Production of ovarian steroid hormones are essential for initiation of vitellogenesis (production of vitellogenin and VLDLy yolk precursors) by the liver (Johnson and Woods, 2007). The conversion of generic VLDL to VLDLy in the liver (Mitchell and Carlisle, 1991; Walzem et al., 1999) is mediated through the ERα receptor. Therefore, sex steroid production by the gonads of breeding female passerines are essential to stimulate the liver and in turn these yolk precursors lead to oocyte growth (Williams, 2012).

A previous study in European starlings (*Sturnus vulgaris*) found that circulating E_2 remains at low levels until the stage of rapid yolk development, and reaches maximal levels when birds have complete follicle hierarchies (Williams et al., 2004). Maintaining low levels of circulating E_2 until initiation of rapid follicle growth could be a beneficial mechanism to minimize negative pleitropic effects from elevated sex steroid levels over long periods of time. An understanding of the variability in liver yolk-precursor secretion and its relationship with the ability of the ovary to secrete E_2 (e.g., in response to GnRH stimulation) and lay date remains incomplete (Caro et al., 2013a, 2013b).

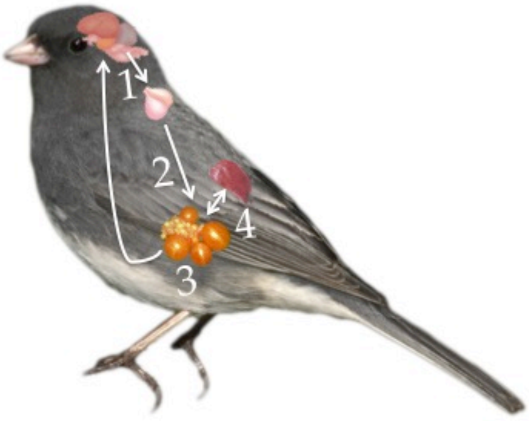

Figure 1.2. Reproduction in females is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. 1. Gonadotropin-Releasing Hormone (GnRH) stimulates 2. Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) released from the pituitary, which acts to 3. Stimulate sex steroid secretion (estrogen, E_2; testosterone, T) to mature gonads and further stimulates 4. Very-Low-Density Lipoprotein (VLDL) release from the liver to promote follicular growth and egg development.

Background for Aim 2

Costs of Reproduction

Environmental resources are believed to drive life history trait expression; therefore, allocation of limited resources would result in a tradeoff (Barnes & Partridge, 2003). Life history traits were binned into three categories, growth, maintenance, and reproduction, which all "compete" for limited resources (Barnes & Partridge, 2003). An individual is hypothesized to maximize its fitness by adjusting resource allocation between the competition of self maintenance versus offspring production (Calow, 1979; Levins, 1968). The classic view of reproduction confiscating resources (energy) from the somatic function of the individual has been challenged by a newer hypothesis that the pleitropic effects of the reproductive system have negative impacts on survival, rather than a solely resource-allocation based trade-off model (Barnes & Partridge, 2003; Harshman & Zera, 2006; Leroi, 2001; Reznick et al., 2000).

A resource-allocation based trade-off model, frequently diagramed as a "Y model" of allocation, each branch representing the degree of resource investment into either survival or reproduction, is highly dependent on nutrient input (Chippindale et al., 1993). If an individual has a fixed amount of internal resources to divert to either branch, then a higher investment into reproduction would result in a cost of reproduction and trade-off (Chippindale et al., 1993; Harshman & Zera, 2006; Reznick et al., 2000). That individual's ability to acquire, convert, and allocate nutrients to usable resources for growth, maintenance, or reproduction is a theoretical perspective of life history genetic variation (Reznick et al., 2000). Following this logic, some individuals are essentially "wealthier" and can allocate more resources to all branches of their life history (Reznick et al., 2000). Assuming evolution maximizes present reproduction, a life history trade-off of reduced survival and fecundity would be the resulting cost (Barnes &

Partridge, 2003; Harshman & Zera, 2006; Reznick et al., 2000). The physiological and ecological costs of reproduction are genetic, environmental, and physiological combined (Rose & Bradley, 1998).

Hormone regulation, immune function, intermediary metabolism and allocation, reproductive proteins, and defenses against stress and toxicity are the five fundamental mechanisms underlying the cost of reproduction (Harshman & Zera, 2006). Reproduction and up-regulation of reproductive hormones (e.g., androgens) compromise an individual's immune system, decreasing its ability to protect against stress, infection, or parasites (Greives et al., 2006; Gustafsson et al., 1994; Nordling et al., 1998). Female birds must adjust behaviors to compensate for energy demanding activities (Vezina & Salvante, 2010). Specifically, egg laying in female birds is energetically costly (Ricklefs, 1974; Williams, 2005), in part due to the increased mass and metabolic activity of organs such as the liver, which produces yolk precursors, or reproductive organs, such as the ovary and oviduct (Williams, 2005). In order for oviparous females (songbirds) to prepare for breeding, they must initiate yolking and final follicular maturation in the spring at a time when resources in the environment are still limited, as chick-rearing usually takes place during peak food abundance (Perrins, 1996). Once rapid final maturation of follicles begins, females are committed to a major energetic investment to lay eggs. A deficit in energy or specific nutrients during this time could restrain these energetically expensive and nutrient intensive reproductive processes (Aho et al., 1999; Brodmann et al., 1997; Svensson and Nilsson, 1995). Females are therefore incapable of escaping the costs of reproduction and must alter their allocation of energy by minimizing behaviors with energy requirements during reproduction (Gittleman & Thompson, 1988; Koivula et al., 2003). However, there are opposing theories on costs of reproduction in males. The first is that a male's energetic investment of testicular mass on metabolism is thought to be equivalent to a female's maximal energy investment during egg production (Nilsson & Raberg, 2001; Vezina & Salvante, 2010). While others have followed the theory that sperm is 'cheap' (Nakatsuru and Kramer, 1982; Olsson et al., 1997).

Immune-Reproductive Energetic Trade-Offs

The negative relationship shown between reproductive success and immune response is a key pathway for cost of reproduction in birds (Deerenberg et al., 1997; Gustafsson et al., 1994). Mounting an immune response, and the metabolic requirements of immune up-regulation, is energetically costly (Demas, 2004; Lochmiller and Deerenberg, 2000; Nelson and Demas, 2004). As preparation for breeding is an energetically demanding period (Kwan, 1994; Sandberg and Moore, 1996; Williams, 2005), unexpected energetic challenges such as mounting an immune response, are likely to induce an energetic trade-off (Bonneaud et al., 2003; Ilmonen et al., 2003; Raberg et al., 2000) during this critical time. Previous research has found that investment in reproductive processes can result in a self-maintenance trade-off by suppressing immune function and increasing risk of infection (Cartledge et al., 2005; Klein, 2000; Olsson et al., 2009; Skarstein et al., 2001).

Cost of Immune-Reproductive Trade-Offs in Males

Testosterone mediates many male reproductive tradeoffs, simultaneously promoting traits such as sperm production, aggression, sexual behaviors and ornaments at the expense of immunity and parental care (Folstad and Karter, 1992; Ketterson and Nolan, 1992; Wingfield et al., 2001). Endogenous levels of T have been shown to inversely correlate with innate immunity in free-living juncos (Greives et al., 2006). For males, if an infection occurs when individuals are actively investing in other costly processes (e.g., reproduction), immune activation may act to decrease or impede investment in current reproductive efforts, such as sperm production.

Males living in close proximity to conspecifics are at an increased risk of infection because of higher rates of exposure to parasites and pathogens (Altizer et al., 2003; Møller et al., 1993; Sadd and Schmid-Hempel, 2006). In a lekking species, the houbara bustard (Chlamydotis undulata undulata), it was shown that mounting an acute immune response imposed a cost on lower hatching rates due to reduced fertilization power (Chargé et al., 2010). Additionally, previous research has found immune activation suppressed luteinizing hormone levels (Owen-Ashley et al., 2006) and serum T production (Bosmann et al., 1996). However, in adult male rats, while a reduction in serum LH levels was noted, intratesticular T concentrations remained at a sufficient level to promote normal spermatogenesis (O'Bryan et al., 2000). For males, reduction in sperm quality can have detrimental effects on a male's fitness by impeding fertilization potential and likelihood of paternity (Birkhead and Møller, 1998; Parker, 1998; Pizzari et al., 2008; Pizzari and Birkhead, 2000; Pizzari and Parker, 2009; Snook, 2005).

Cost of Immune-Reproductive Trade-Offs in Females

Females faced with an energetic immune challenge during reproductive preparation can either 1) continue with clutch initiation and risk pathogen-induced death, or 2) they can mount an antibody response, which may delay reproduction, thus negatively impacting reproductive success. There are nutritional costs associated with immune activation in response to a pathogen, including the redistribution of nutritional resources from processes such as reproduction to the needs of the immune system (Lee, 2006; Ricklefs and Wikelski, 2002). The nutritional status of females prior to breeding has a positive interaction with reproductive success and negative interaction with parasitic susceptibility (Gustafsson et al., 1994). Access to key proteins and amino acids necessary for initiating breeding can be seasonally variable and limitations in key nutrients may further drive trade-offs between reproduction (egg production) and production of immune cells (Iseri and Klasing, 2014). Therefore, redirected nutritional and energetic demands can have adverse effects on a female's ability to initiate egg production.

Studies that experimentally manipulated investment in immune function (Adelman et al., 2010; Ilmonen et al., 2000; Merino et al., 2000; Raberg et al., 2000; Uller et al., 2006) or reproductive effort (Cox and John-Alder, 2007; French et al., 2007; Knowles et al., 2009) have found trade-offs between the two systems. However, other studies have not found the same well-defined trade-offs during experimental manipulations (Cox et al., 2010; Williams et al., 1999). Such discrepancies may be due to the fact that these studies were conducted after the energetic investment into clutch initiation was already made. Furthermore, these studies were carried out under controlled laboratory conditions, thus suggesting the need for research in free-living females, especially during the pre-breeding period.

Study Species

Dark-eyed junco (*Junco hyemalis*)

The dark-eyed junco (*Junco hyemalis*) is a seasonally breeding songbird distributed throughout the United States and breeds at high altitudes and latitudes (Nolan, 2002; Rowan, 1938). Dark-eyed juncos are an excellent model organism because they have been extensively studied (Ketterson and Atwell, 2016), are abundant, relatively easy to capture (Ketterson et al., 2009, 1998; Ketterson and Nolan, 1982), and were one of the first species found to show a photoperiod response linked to reproductive timing (Rowan, 1938; Wolfson, 1942). Juncos are a socially monogamous, ground-nesting species that lay between 3 – 5 (mean and median = 4) eggs per nest attempt from May to June (Nolan, 2002). Although only females incubate eggs, both sexes are known to contribute to care of nestlings and fledglings.

The Carolina junco (*Junco hyemalis carolinensis*), an Appalachian subspecies of Slate-colored junco, has received more than 25 years of study at the Mountain Lake Biological Station in Virginia. In contrast, the white-winged junco (*Junco hyemalis aikeni*), which breeds in the Black Hills of South Dakota, has received little direct observation or research.

House sparrows (*Passer domesticus*)

House sparrows (*Passer domesticus*) are a highly social species that engage in extra-pair copulations with intra- and inter- sexual selection pressures for high-quality sperm (Birkhead et al., 1994; Summers-Smith, 1963; Wetton and Parkin, 1991). House sparrows are gregarious, largely sedentary, thrive in populated areas, are a socially monogamous species, and are commonly found in close proximity to animal agricultural settings (Summers-Smith, 2010). The species is sexually dimorphic and males display a black bib (Anderson, 2006). Clutch sizes vary from 1 – 8 eggs (average of 4) and successful birds generally have an average of four clutches per breeding season (Anderson, 2006). Males spend time on the nest during incubation, likely to avoid heat loss and predation while the female is off the nest; however, males do not develop a brood patch (Anderson, 2006). During the nestling stage males generally contribute about half of the feedings (Anderson, 2006).

References

Adelman, J.S., Córdoba-Córdoba, S., Spoelstra, K., Wikelski, M., Hau, M., 2010. Radiotelemetry reveals variation in fever and sickness behaviours with latitude in a free-living passerine. Funct. Ecol. 24, 813–823.

Adkins-Regan, E., 2008. Do hormonal control systems produce evolutionary inertia? Philos. Trans. R. Soc. Lond. B Biol. Sci. 363, 1599–1609.

Adkins-Regan, E., 2005. Hormones and animal social behavior. Princeton University Press.

Adkins-Regan, E., 1981. Hormone Specificity, Androgen Metabolism, and Social Behavior. Am. Zool. 21, 257–271.

Aho, T., Kuitunen, M., Suhonen, J., Jäntti, A., Hakkari, T., 1999. Reproductive success of Eurasian treecreepers, *Certhia familiaris*, lower in territories with wood ants. Ecology 80, 998–1007.

Altizer, S., Nunn, C.L., Thrall, P.H., Gittleman, J.L., Antonovics, J., Cunningham, A.A., Dobson, A.P., Ezenwa, V., Jones, K.E., Pedersen, A.B., others, 2003. Social organization and parasite risk in mammals: integrating theory and empirical studies. Annu. Rev. Ecol. Evol. Syst. 34, 517–547.

Anderson, T.R., 2006. Biology of the ubiquitous house sparrow: from genes to populations. Oxford University Press.

Apfelbeck, B., Mortega, K., Kiefer, S., Kipper, S., Vellema, M., Villavicencio, C.P., Gahr, M., Goymann, W., 2013. Associated and disassociated patterns in hormones, song, behavior and brain receptor expression between life-cycle stages in male black redstarts, *Phoenicurus ochruros*. Gen. Comp. Endocrinol. 184, 93–102.

Aschoff, J., 1979. Circadian rhythms: general features and endocrinological aspects. Endocr. Rhythms 1–61.

Baker, J.R., 1938. The evolution of breeding seasons. Evolution 161, 177.

Ball, G.F., 2007. The ovary knows more than you think! New views on clock genes and the positive feedback control of luteinizing hormone. Endocrinology 148, 3029–3030.

Ball, G.F., Ketterson, E.D., 2008. Sex differences in the response to environmental cues regulating seasonal reproduction in birds. Philos. Trans. R. Soc. B Biol. Sci. 363, 231–246.

Ball, G.F., Wingfield, J.C., 1987. Changes in plasma levels of luteinizing hormone and sex steroid hormones in relation to multiple-broodedness and nest-site density in male starlings. Physiol. Zool. 191–199.

Balthazart, J., 1983. Hormonal correlates of behavior. Avian Biol. 7, 221–365.

Barnes, A.I., Partridge, L., 2003. Costing reproduction. Anim. Behav. 66, 199–204. doi:10.1006/anbe.2003.2122

Bell-Pedersen, D., Cassone, V.M., Earnest, D.J., Golden, S.S., Hardin, P.E., Thomas, T.L., Zoran, M.J., 2005. Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat. Rev. Genet. 6, 544–556.

Bêty, J., Gauthier, G., Giroux, J., 2003. Body condition, migration, and timing of reproduction in snow geese: a test of the condition-dependent model of optimal clutch size. Am. Nat. 162, 110–121.

Birkhead, T.R., Møller, A.P., 1998. Sperm competition and sexual selection. Academic Press.

Birkhead, T.R., Veiga, J.P., Moller, A.P., 1994. Male sperm reserves and copulation behaviour in the house sparrow, *Passer domesticus*. Proc. R. Soc. Lond. B Biol. Sci. 256, 247–251.

Bonneaud, C., Mazuc, J., Gonzalez, G., Haussy, C., Chastel, O., Faivre, B., Sorci, G., 2003. Assessing the cost of mounting an immune response. Am. Nat. 161, 367–379.

Book, A.S., Starzyk, K.B., Quinsey, V.L., 2001. The relationship between testosterone and aggression: A meta-analysis. Aggress. Violent Behav. 6, 579–599.

Bosmann, H.B., Hales, K.H., Li, X., Liu, Z., Stocco, D.M., Hales, D.B., 1996. Acute in vivo inhibition of testosterone by endotoxin parallels loss of steroidogenic acute regulatory (StAR) protein in Leydig cells. Endocrinology 137, 4522–4525. doi:10.1210/endo.137.10.8828518

Brodmann, P.A., Reyer, H.-U., Bollmann, K., Schläpfer, A.R., Rauter, C., 1997. The importance of food quantity and quality for reproductive performance in alpine water pipits (*Anthus spinoletta*). Oecologia 109, 200–208.

Bronson, F.H., 1989. Mammalian reproductive biology. University of Chicago Press.

Bronson, F.H., Heideman, P.D., 1994. Seasonal regulation of reproduction in mammals. Physiol. Reprod. 2, 541–584.

Calow, P., 1979. The cost of reproduction–a physiological approach. Biol. Rev. 54, 23–40.

Canoine, V., Fusani, L., Schlinger, B., Hau, M., 2007. Low sex steroids, high steroid receptors: increasing the sensitivity of the nonreproductive brain. Dev. Neurobiol. 67, 57–67.

Canoine, V., Gwinner, E., 2002. Seasonal differences in the hormonal control of territorial aggression in free-living European stonechats. Horm. Behav. 41, 1–8.

Caro, S.P., 2012. Avian ecologists and physiologists have different sexual preferences. Gen. Comp. Endocrinol. 176, 1–8.

Caro, S.P., Charmantier, A., Lambrechts, M.M., Blondel, J., Balthazart, J., Williams, T.D., 2009. Local adaptation of timing of reproduction: females are in the driver's seat. Funct. Ecol. 23, 172–179.

Caro, S.P., Schaper, S.V., Dawson, A., Sharp, P.J., Gienapp, P., Visser, M.E., 2013a. Is microevolution the only emergency exit in a warming world? Temperature influences egg laying but not its underlying mechanisms in great tits. Gen. Comp. Endocrinol. 190, 164–169.

Caro, S.P., Schaper, S.V., Hut, R.A., Ball, G.F., Visser, M.E., 2013b. The case of the missing mechanism: how does temperature influence seasonal timing in endotherms? PLoS Biol. 11, e1001517.

Cartledge, V.A., Gartrell, B., Jones, S.M., 2005. Adrenal and white cell count responses to chronic stress in gestating and postpartum females of the viviparous skink *Egernia whitii* (Scincidae). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 141, 100–107.

Chang, C., Chen, Y., Yeh, S., Xu, Q., Wang, R., Guillou, F., Lardy, H., Yeh, S., 2004. Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. Proc. Natl. Acad. Sci. U. S. A. 101, 6876–6881.

Chargé, R., Saint Jalme, M., Lacroix, F., Cadet, A., Sorci, G., 2010. Male health status, signalled by courtship display, reveals ejaculate quality and hatching success in a lekking species. J. Anim. Ecol. 79, 843–850.

Chippindale, A.K., Leroi, A.M., Kim, S.B., Rose, M.R., 1993. Phenotypic plasticity and selection in Drosophila life-history evolution. I. Nutrition and the cost of reproduction. J. Evol. Biol. 6, 171–193.

Comendant, T., Sinervo, B., Svensson, E.I., Wingfield, J., 2003. Social competition, corticosterone and survival in female lizard morphs. J. Evol. Biol. 16, 948–955.

Cox, R.M., John-Alder, H.B., 2007. Increased mite parasitism as a cost of testosterone in male striped plateau lizards *Sceloporus virgatus*. Funct. Ecol. 21, 327–334.

Cox, R.M., Parker, E.U., Cheney, D.M., Liebl, A.L., Martin, L.B., Calsbeek, R., 2010. Experimental evidence for physiological costs underlying the trade-off between reproduction and survival. Funct. Ecol. 24, 1262–1269.

Daan, S., Damassa, D., Pittendrigh, C.S., Smith, E.R., 1975. An effect of castration and testosterone replacement on a circadian pacemaker in mice (Mus musculus). Proc. Natl. Acad. Sci. 72, 3744–3747.

Dawson, A., 2008. Control of the annual cycle in birds: endocrine constraints and plasticity in response to ecological variability. Philos. Trans. R. Soc. Lond. B Biol. Sci. 363, 1621–1633.

Dawson, A., King, V.M., Bentley, G.E., Ball, G.F., 2001. Photoperiodic control of seasonality in birds. J. Biol. Rhythms 16, 365–380.

De Gendt, K., Swinnen, J.V., Saunders, P.T., Schoonjans, L., Dewerchin, M., Devos, A., Tan, K., Atanassova, N., Claessens, F., Lécureuil, C., others, 2004. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc. Natl. Acad. Sci. U. S. A. 101, 1327–1332.

De Kloet, E.R., Reul, J., Sutanto, W., 1990. Corticosteroids and the brain. J. Steroid Biochem. Mol. Biol. 37, 387–394.

De Kloet, E.R., Vreugdenhil, E., Oitzl, M.S., Joëls, M., 1998. Brain corticosteroid receptor balance in health and disease. Endocr. Rev. 19, 269–301.

Deerenberg, C., Arpanius, V., Daan, S., Bos, N., 1997. Reproductive effort decreases antibody responsiveness. Proc. R. Soc. Lond. B Biol. Sci. 264, 1021–1029.

Demas, G.E., 2004. The energetics of immunity: a neuroendocrine link between energy balance and immune function. Horm. Behav. 45, 173–180.

Deviche, P., Beouche-Helias, B., Davies, S., Gao, S., Lane, S., Valle, S., 2014. Regulation of plasma testosterone, corticosterone, and metabolites in response to stress, reproductive stage, and social challenges in a desert male songbird. Gen. Comp. Endocrinol. 203, 120–131.

Deviche, P., Gao, S., Davies, S., Sharp, P.J., Dawson, A., 2012. Rapid stress-induced inhibition of plasma testosterone in free-ranging male rufous-winged sparrows, *Peucaea carpalis*: characterization, time course, and recovery. Gen. Comp. Endocrinol. 177, 1–8.

Dixson, A.F., Anderson, M.J., 2004. Sexual behavior, reproductive physiology and sperm competition in male mammals. Physiol. Behav. 83, 361–371.

Dobson, F.S., Michener, G.R., 1995. Maternal Traits and Reproduction in Richardson's Ground Squirrels. Ecology 76, 851–862. doi:10.2307/1939350

Donham, R.S., Wingfield, J.C., Mattocks, P.W., Farner, D.S., 1982. Changes in testicular and plasma androgens with photoperiodically induced increase in plasma LH in the house sparrow. Gen. Comp. Endocrinol. 48, 342–347.

Drent, R.H., Daan, S., 1980. The Prudent Parent: Energetic Adjustments in Avian Breeding 1. Ardea 68, 225–252.

Enstrom, D.A., D Ketterson, E., Val Nolan, J., 1997. Testosterone and mate choice in the dark-eyed junco. Anim. Behav. 54, 1135–1146.

Foidart, A., Silverin, B., Baillien, M., Harada, N., Balthazart, J., 1998. Neuroanatomical distribution and variations across the reproductive cycle of aromatase activity and aromatase-immunoreactive cells in the pied flycatcher (*Ficedula hypoleuca*). Horm. Behav. 33, 180–196.

Folstad, I., Karter, A.J., 1992. Parasites, Bright Males, and the Immunocompetence Handicap. Am. Nat. 139, 603–622.

French, S.S., DeNardo, D.F., Moore, M.C., 2007. Trade-Offs between the Reproductive and Immune Systems: Facultative Responses to Resources or Obligate Responses to Reproduction? Am. Nat. 170, 79–89.

Funder, J.W., 1997. Glucocorticoid and mineralocorticoid receptors: biology and clinical relevance. Annu. Rev. Med. 48, 231–240.

Fusani, L., 2008. Endocrinology in field studies: problems and solutions for the experimental design. Gen. Comp. Endocrinol. 157, 249–253.

Galen, C., Stanton, M.L., 1991. Consequences of Emergence Phenology for Reproductive Success in *Ranunculus adoneus* (*Ranunculaceae*). Am. J. Bot. 78, 978–988. doi:10.2307/2445177

Gibb, J., 1950. The breeding biology of the great and blue titmice. Ibis 507–539.

Gittleman, J.L., Thompson, S.D., 1988. Energy allocation in mammalian reproduction. Am. Zool. 28, 863–875.

Goymann, W., Landys, M.M., Wingfield, J.C., 2007. Distinguishing seasonal androgen responses from male–male androgen responsiveness—revisiting the challenge hypothesis. Horm. Behav. 51, 463–476.

Goymann, W., Wingfield, J.C., 2004. Competing females and caring males. Sex steroids in African black coucals, *Centropus grillii*. Anim. Behav. 68, 733–740.

Greives, T.J., Fudickar, A.M., Atwell, J.W., Meddle, S.L., Ketterson, E.D., 2016. Early spring sex differences in luteinizing hormone response to gonadotropin releasing hormone in co-occurring resident and migrant dark-eyed juncos (*Junco hyemalis*). Gen. Comp. Endocrinol. 236, 17–23.

Greives, T.J., Mcglothlin, J.W., Jawor, J.M., Demas, G.E., Ketterson, E.D., 2006. Testosterone and innate immune function inversely covary in a wild population of breeding Dark-Eyed Juncos (*Junco hyemalis*). Funct. Ecol. 20, 812–818. doi:10.1111/j.1365-2435.2006.01167.x

Griffith, S.C., Owens, I.P.F., Burke, T., 1999. Female choice and annual reproductive success favour less-ornamented male house sparrows. Proc. R. Soc. B Biol. Sci. 266, 765. doi:10.1098/rspb.1999.0703

Gustafsson, L., Nordling, D., Andersson, M.S., Sheldon, B.C., Qvarnstrom, A., 1994. Infectious diseases, reproductive effort and the cost of reproduction in birds. Philos. Trans. R. Soc. B Biol. Sci. 346, 323–331.

Gwinner, E., 1975. Effects of season and external testosterone on the freerunning circadian activity rhythm of European starlings (Sturnus vulgaris). J. Comp. Physiol. 103, 315–328.

Harding, C.F., 1983. Hormonal influences on avian aggressive behavior, in: Hormones and Aggressive Behavior. Springer, pp. 435–467.

Harshman, L.G., Zera, A.J., 2007. The cost of reproduction: the devil in the details. Trends Ecol. Evol. 22, 80–86.

Hasselquist, D., Marsh, J.A., Sherman, P.W., Wingfield, J.C., 1999. Is avian humoral immunocompetence suppressed by testosterone? Behav. Ecol. Sociobiol. 45, 167–175.

Hau, M., 2007. Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. BioEssays 29, 133–144.

Hau, M., Goymann, W., 2015. Endocrine mechanisms, behavioral phenotypes and plasticity: known relationships and open questions. Front. Zool. 12, 1–15.

Haywood, M., Spaliviero, J., Jimemez, M., King, N.J., Handelsman, D.J., Allan, C.M., 2003. Sertoli and germ cell development in hypogonadal (hpg) mice expressing transgenic follicle-stimulating hormone alone or in combination with testosterone. Endocrinology 144, 509–517.

Hegner, R.E., Wingfield, J.C., 1987. Effects of experimental manipulation of testosterone levels on parental investment and breeding success in male house sparrows. The Auk 462–469.

Hegner, R.E., Wingfield, J.C., 1986. Behavioral and endocrine correlates of multiple brooding in the semicolonial house sparrow *Passer domesticus* I. Males. Horm. Behav. 20, 294–312.

Hochachka, W., 1990. Seasonal Decline in Reproductive Performance of Song Sparrows. Ecology 71, 1279–1288. doi:10.2307/1938265

Holberton, R.L., Dufty Jr, A.M., Greenberg, R., Marra, P.P., 2005. Hormones and variation in life history strategies of migratory and non-migratory birds. Birds Two Worlds Ecol. Evol. Migr. 290–302.

Hu, G., Lian, Q., Lin, H., Latif, S.A., Morris, D.J., Hardy, M.P., Ge, R., 2008. Rapid mechanisms of glucocorticoid signaling in the Leydig cell. Steroids 73, 1018–1024.

Husak, J.F., Fox, S.F., Lovern, M.B., Bussche, R.A., 2006. Faster lizards sire more offspring: sexual selection on whole-animal performance. Evolution 60, 2122–2130.

Hutchison, J.B., 1978. Hypothalamic regulation of male sexual responsiveness to androgen. Biol. Determinants Sex. Behav. 277–317.

Ilmonen, P., Hasselquist, D., Langefors, A., Wiehn, J., 2003. Stress, immunocompetence and leukocyte profiles of pied flycatchers in relation to brood size manipulation. Oecologia 136, 148–154.

Ilmonen, P., Taarna, T., Hasselquist, D., 2000. Experimentally activated immune defence in female pied flycatchers results in reduced breeding success. Proc. R. Soc. Lond. B Biol. Sci. 267, 665–670.

Iseri, V.J., Klasing, K.C., 2014. Changes in the amount of lysine in protective proteins and immune cells after a systemic response to dead *Escherichia coli*: implications for the nutritional costs of immunity. Integr. Comp. Biol. 54, 922–930.

Jacobs, J.D., Wingfield, J.C., 2000. Endocrine control of life-cycle stages: a constraint on response to the environment? The Condor 102, 35–51.

Jawor, J.M., Mcglothlin, J.W., Casto, J.M., Greives, T.J., Snajdr, E.A., Bentley, G.E., Ketterson, E.D., 2007. Testosterone response to GnRH in a female songbird varies with stage of reproduction: implications for adult behaviour and maternal effects. Funct. Ecol. 21, 767–775.

Jawor, J.M., McGlothlin, J.W., Casto, J.M., Greives, T.J., Snajdr, E.A., Bentley, G.E., Ketterson, E.D., 2006. Seasonal and individual variation in response to GnRH challenge in male dark-eyed juncos (*Junco hyemalis*). Gen. Comp. Endocrinol. 149, 182–189.

Jensen, H., SÆther, B.-E., Ringsby, T.H., Tufto, J., Griffith, S.C., Ellegren, H., 2004. Lifetime reproductive success in relation to morphology in the house sparrow Passer domesticus. J. Anim. Ecol. 73, 599–611.

Johnson, A.L., Woods, D.C., 2007. Ovarian dynamics and follicle development. Reprod. Biol. Phylogeny Aves 243–277.

Johnson, M.A., Cohen, R.E., Vandecar, J.R., Wade, J., 2011. Relationships among reproductive morphology, behavior, and testosterone in a natural population of green anole lizards. Physiol. Behav. 104, 437–445.

Kempenaers, B., Peters, A., Foerster, K., 2008. Sources of individual variation in plasma testosterone levels. Philos. Trans. R. Soc. B Biol. Sci. 363, 1711–1723.

Ketterson, E.D., Atwell, J.W., 2016. Snowbird: Integrative Biology and Evolutionary Diversity in the Junco. University of Chicago Press.

Ketterson, E.D., Atwell, J.W., McGlothlin, J.W., 2009. Phenotypic integration and independence: hormones, performance, and response to environmental change. Integr. Comp. Biol. 49, 365–379.

Ketterson, E.D., Nolan Jr, V., 1999. Adaptation, exaptation, and constraint: a hormonal perspective. Am. Nat. 154, S4–S25.

Ketterson, E.D., Nolan Jr, V., Sandell, M., 2005. Testosterone in females: mediator of adaptive traits, constraint on sexual dimorphism, or both? Am. Nat. 166, S85–S98.

Ketterson, E.D., Nolan, V., 1992. Hormones and Life Histories: An Integrative Approach. Am. Nat. 140, S33–S62.

Ketterson, E.D., Nolan, V., 1982. The Role of Migration and Winter Mortality in the Life History of a Temperate-Zone Migrant, the Dark-Eyed Junco, as Determined from Demographic Analyses of Winter Populations. The Auk 99, 243–259.

Ketterson, E.D., Parker, P.G., Raouf, S.A., Nolan, V., Ziegenfus, C., Chandler, C.R., 1998. The Relative Impact of Extra-Pair Fertilizations on Variation in Male and Female Reproductive Success in Dark-Eyed Juncos (*Junco hyemalis*). Ornithol. Monogr. 81–101.

Klein, S.L., 2000. Hormones and mating system affect sex and species differences in immune function among vertebrates. Behav. Processes 51, 149–166.

Knowles, S.C., Nakagawa, S., Sheldon, B.C., 2009. Elevated reproductive effort increases blood parasitaemia and decreases immune function in birds: a meta-regression approach. Funct. Ecol. 23, 405–415.

Koivula, M., Koskela, E., Mappes, T., Oksanen, T.A., 2003. Cost of reproduction in the wild: manipulation of reproductive effort in the bank vole. Ecology 84, 398–405.

Kwan, D., 1994. Fat reserves and reproduction in the green turtle, *Chelonia mydas*. Wildl. Res. 21, 257–265.

Lack, D.L., 1968. Ecological adaptations for breeding in birds.

Landa, K., 1992. Seasonal Declines in Offspring Fitness and Selection for Early Reproduction in Nymph-Overwintering Grasshoppers. Evolution 46, 121–135. doi:10.2307/2409809

Landys, M.M., Ramenofsky, M., Wingfield, J.C., 2006. Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes. Gen. Comp. Endocrinol. 148, 132–149.

Laucht, S., Dale, J., Mutzel, A., Kempenaers, B., 2011. Individual variation in plasma testosterone levels and its relation to badge size in House Sparrows *Passer domesticus*: It'sa night-and-day difference. Gen. Comp. Endocrinol. 170, 501–508.

Laucht, S., Kempenaers, B., Dale, J., 2010. Bill color, not badge size, indicates testosterone-related information in house sparrows. Behav. Ecol. Sociobiol. 64, 1461–1471.

Lee, K.A., 2006. Linking immune defenses and life history at the levels of the individual and the species. Integr. Comp. Biol. 46, 1000–1015.

Leroi, A.M., 2001. Molecular signals versus the Loi de Balancement. Trends Ecol. Evol. 16, 24–29.

Levins, R., 1968. Evolution in changing environments: some theoretical explorations. Princeton University Press.

Lochmiller, R.L., Deerenberg, C., 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88, 87–98.

Marshall, A.J., 1959. Internal and environmental control of breeding. Ibis 101, 456–478.

McGlothlin, J.W., Jawor, J.M., Greives, T.J., Casto, J.M., Phillips, J.L., Ketterson, E.D., 2008. Hormones and honest signals: males with larger ornaments elevate testosterone more when challenged. J. Evol. Biol. 21, 39–48.

McGlothlin, J.W., Jawor, J.M., Ketterson, E.D., 2007. Natural variation in a testosterone-mediated trade-off between mating effort and parental effort. Am. Nat. 170, 864–875.

McGlothlin, J.W., Ketterson, E.D., 2008. Hormone-mediated suites as adaptations and evolutionary constraints. Philos. Trans. R. Soc. B Biol. Sci. 363, 1611–1620.

McGuire, N.L., Koh, A., Bentley, G.E., 2013. The direct response of the gonads to cues of stress in a temperate songbird species is season-dependent. PeerJ 1, e139.

McLachlan, R.I., O'Donnell, L., Meachem, S.J., Stanton, P.G., De Kretser, D.M., Pratis, K., Robertson, D.M., 2002. Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. Recent Prog. Horm. Res. 57, 149–179.

Merino, S., Moreno, J., Sanz, J.J., Arriero, E., 2000. Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). Proc. R. Soc. Lond. B Biol. Sci. 267, 2507–2510.

Mitchell, M.A., Carlisle, A.J., 1991. Plasma zinc as an index of vitellogenin production and reproductive status in the domestic fowl. Comp. Biochem. Physiol. A Physiol. 100, 719–724.

Møller, A.P., Dufva, R., Allander, K., 1993. Parasites and the evolution of host social behavior. Adv. Study Behav. 22, 102.

Moore, M.C., 1991. Application of organization-activation theory to alternative male reproductive strategies: a review. Horm. Behav. 25, 154–179.

Moore, M.C., 1984. Changes in Territorial Defense Produced by Changes in Circulating Levels of Testosterone: A Possible Hormonal Basis for Mate-Guarding Behavior in White-Crowned Sparrows. Behaviour 88, 215–226.

Morin, L.P., Fitzgerald, K.M., Zucker, I., 1977. Estradiol shortens the period of hamster circadian rhythms. Science 196, 305–307.

Murton, R.K., Westwood, N.J., 1977. Avian breeding cycles. Clarendon Press Oxford.

Naef-Daenzer, B., Keller, L.F., 1999. The foraging performance of great and blue tits (*Parus major* and *P. caeruleus*) in relation to caterpillar development, and its consequences for nestling growth and fledging weight. J. Anim. Ecol. 68, 708–718.

Naef-Daenzer, B., Widmer, F., Nuber, M., 2001. Differential post-fledging survival of great and coal tits in relation to their condition and fledging date. J. Anim. Ecol. 70, 730–738.

Nakatsuru, K., Kramer, D.L., 1982. Is sperm cheap? Limited male fertility and female choice in the lemon tetra (Pisces, Characidae). Science 216, 753–755.

Nelson, R.J., 2005. An introduction to behavioral endocrinology . Sinauer Associates.

Nelson, R.J., Demas, G.E., 2004. Seasonal patterns of stress, disease, and sickness responses. Curr. Dir. Psychol. Sci. 13, 198–201.

Newton, I., Marquiss, M., 1982. Food, Predation and Breeding-season in sparrowhawks (*Accipiter nisus*). J. Zool. 197, 221–240.

Nijhout, H.F., 2003. Development and evolution of adaptive polyphenisms. Evol. Dev. 5, 9–18.

Nijhout, H.F., 1998. Insect hormones. Princeton University Press.

Nilsson, J.A., Raberg, L., 2001. The resting metabolic cost of egg laying and nestling feeding in great tits. Oecologia 128, 187–192.

Nolan, V., 2002. Dark-eyed junco: *Junco hyemalis*. Birds of North America, Incorporated.

Nordling, D., Andersson, M. ans, Zohari, S., Lars, G., 1998. Reproductive effort reduces specific immune response and parasite resistance. Proc. R. Soc. Lond. B Biol. Sci. 265, 1291–1298.

O'Bryan, M.K., Schlatt, S., Phillips, D.J., de Kretser, D.M., Hedger, M.P., 2000. Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels in vivo 1. Endocrinology 141, 238–246.

Olsson, M., Wilson, M., Uller, T., Mott, B., Isaksson, C., 2009. Variation in levels of reactive oxygen species is explained by maternal identity, sex and body-size-corrected clutch size in a lizard. Naturwissenschaften 96, 25–29.

Olsson, M., Madsen, T., Shine, R., 1997. Is sperm really so cheap? Costs of reproduction in male adders, Vipera berus. Proc. R. Soc. Lond. B Biol. Sci. 264, 455–459.

Owen-Ashley, N.T., Turner, M., Hahn, T.P., Wingfield, J.C., 2006. Hormonal, behavioral, and thermoregulatory responses to bacterial lipopolysaccharide in captive and free-living white-crowned sparrows (*Zonotrichia leucophrys gambelii*). Horm. Behav. 49, 15–29.

Parker, G.A., 1998. Sperm competition and the evolution of ejaculates: towards a theory base. Sperm Compet. Sex. Sel. 3, 54.

Perrins, C.M., 1996. Eggs, egg formation and the timing of breeding. Ibis 138, 2–15.

Perrins, C.M., 1970. The timing of birds 'breeding seasons. Ibis 112, 242–255.

Pizzari, T., Birkhead, T.R., 2000. Female feral fowl eject sperm of subdominant males. Nature 405, 787–789.

Pizzari, T., Parker, G.A., 2009. Sperm competition and sperm phenotype. Sperm Biol. Evol. Perspect. 207–245.

Pizzari, T., Worley, K., Burke, T., Froman, D.P., 2008. Sperm competition dynamics: ejaculate fertilising efficiency changes differentially with time. BMC Evol. Biol. 8, 332.

Raberg, L., Nilsson, J., Ilmonen, P., Stjernman, M., Hasselquist, D., 2000. The cost of an immune response: vaccination reduces parental effort. Ecol. Lett. 3, 382–386.

Raouf, S.A., Parker, P.G., Ketterson, E.D., Nolan, V., Ziegenfus, C., 1997. Testosterone affects reproductive success by influencing extra–pair fertilizations in male dark–eyed juncos (Aves: *Junco hyemalis*). Proc. R. Soc. Lond. B Biol. Sci. 264, 1599–1603.

Reed, T.E., Warzybok, P., Wilson, A.J., Bradley, R.W., Wanless, S., Sydeman, W.J., 2009. Timing is everything: flexible phenology and shifting selection in a colonial seabird. J. Anim. Ecol. 78, 376–387.

Reed, W.L., Clark, M.E., Parker, P.G., Raouf, S.A., Arguedas, N., Monk, D.S., Snajdr, E., Nolan Jr, V., Ketterson, E.D., 2006. Physiological effects on demography: a long-term experimental study of testosterone's effects on fitness. Am. Nat. 167, 667–683.

Reul, J., Kloet, E. de, 1985. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117, 2505–2511.

Reznick, D., Nunney, L., Tessier, A., 2000. Big houses, big cars, superfleas and the costs of reproduction. Trends Ecol. Evol. 15, 421–425.

Ricklefs, R.E., 1974. Energetics of reproduction in birds. Avian Energ. 15, 152–292.

Ricklefs, R.E., Wikelski, M., 2002. The physiology/life-history nexus. Trends Ecol. Evol. 17, 462–468.

Rieger, J.F., 1996. Body size, litter size, timing of reproduction, and juvenile survival in the Unita ground squirrel, *Spermophilus armatus*. Oecologia 107, 463–468. doi:10.1007/BF00333936

Riters, L.V., Eens, M., Pinxten, R., Duffy, D.L., Balthazart, J., Ball, G.F., 2000. Seasonal changes in courtship song and the medial preoptic area in male European starlings (*Sturnus vulgaris*). Horm. Behav. 38, 250–261.

Rose, M.R., Bradley, T.J., 1998. Evolutionary physiology of the cost of reproduction. Oikos 443–451.

Roulin, A., Müller, W., Sasvári, L., Dijkstra, C., Ducrest, A.-L., Riols, C., Wink, M., Lubjuhn, T., 2004. Extra-pair paternity, testes size and testosterone level in relation to colour polymorphism in the barn owl Tyto alba. J. Avian Biol. 35, 492–500.

Rowan, W., 1938. Light and seasonal reproduction in animals. Biol. Rev. 13, 374–401.

Rowe, L., Ludwig, D., Schluter, D., 1994. Time, condition, and the seasonal decline of avian clutch size. Am. Nat. 143, 698–722.

Sadd, B.M., Schmid-Hempel, P., 2006. Insect immunity shows specificity in protection upon secondary pathogen exposure. Curr. Biol. 16, 1206–1210.

Sandberg, R., Moore, F.R., 1996. Fat stores and arrival on the breeding grounds: reproductive consequences for passerine migrants. Oikos 577–581.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions 1. Endocr. Rev. 21, 55–89.

Sauther, M., 1998. Interplay of phenology and reproduction in ring-tailed lemurs: implications for ring-tailed lemur conservation. Folia Primatol. (Basel) 69, 309–320.

Schoech, S.J., Rensel, M.A., Bridge, E.S., Boughton, R.K., Wilcoxen, T.E., 2009. Environment, glucocorticoids, and the timing of reproduction. Gen. Comp. Endocrinol. 163, 201–207.

Sharpe, R.M., 1994. Regulation of spermatogenesis. Physiol. Reprod. 1, 1363–1434.

Sheldon, B.C., 1994. Male phenotype, fertility, and the pursuit of extra-pair copulations by female birds. Proc. R. Soc. Lond. B Biol. Sci. 257, 25–30.

Silverin, B., Baillien, M., Foidart, A., Balthazart, J., 2000. Distribution of aromatase activity in the brain and peripheral tissues of passerine and nonpasserine avian species. Gen. Comp. Endocrinol. 117, 34–53.

Skarstein, F., Folstad, I., Liljedal, S., 2001. Whether to reproduce or not: immune suppression and costs of parasites during reproduction in the Arctic charr. Can. J. Zool. 79, 271–278.

Snook, R.R., 2005. Sperm in competition: not playing by the numbers. Trends Ecol. Evol. 20, 46–53.

Solís, R., Penna, M., 1997. Testosterone levels and evoked vocal responses in a natural population of the frog *Batrachyla taeniata*. Horm. Behav. 31, 101–109.

Soma, K.K., Schlinger, B.A., Wingfield, J.C., Saldanha, C.J., 2003. Brain aromatase, 5α-reductase, and 5β-reductase change seasonally in wild male song sparrows: Relationship to aggressive and sexual behavior. J. Neurobiol. 56, 209–221.

Stutchbury, B.J., Robertson, R.J., 1988. Within-season and age-related patterns of reproductive performance in female tree swallows (*Tachycineta bicolor*). Can. J. Zool. 66, 827–834.

Summers-Smith, D., 2010. The sparrows. A&C Black.

Summers-Smith, J.D., 1963. The house sparrow. Collins London.

Suzuki, Y., Nijhout, H.F., 2006. Evolution of a polyphenism by genetic accommodation. Science 311, 650–652.

Svensson, E., Nilsson, J.-A., 1995. Food supply, territory quality, and reproductive timing in the blue tit (*Parus caeruleus*). Ecology 76, 1804–1812.

Takahashi, J.S., Menaker, M., 1980. Interaction of estradiol and progesterone: effects on circadian locomotor rhythm of female golden hamsters. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 239, R497–R504.

Uller, T., Isaksson, C., Olsson, M., 2006. Immune challenge reduces reproductive output and growth in a lizard. Funct. Ecol. 20, 873–879.

Van Noordwijk, A.J., McCleery, R.H., Perrins, C.M., 1995. Selection for the timing of great tit breeding in relation to caterpillar growth and temperature. J. Anim. Ecol. 451–458.

Veiga, J.P., 1993. Badge size, phenotypic quality, and reproductive success in the house sparrow: a study on honest advertisement. Evolution 1161–1170.

Verhulst, S., Van Balen, J.H., Tinbergen, J.M., 1995. Seasonal decline in reproductive success of the great tit: variation in time or quality? Ecology 76, 2392–2403.

Vézina, F., Salvante, K.G., 2010. Behavioral and physiological flexibility are used by birds to manage energy and support investment in the early stages of reproduction. Curr. Zool. 56.

Villavicencio, C.P., Apfelbeck, B., Goymann, W., 2014. Parental care, loss of paternity and circulating levels of testosterone and corticosterone in a socially monogamous song bird. Front. Zool. 11, 1.

Voigt, C., Goymann, W., 2007. Sex-role reversal is reflected in the brain of African black coucals (*Centropus grillii*). Dev. Neurobiol. 67, 1560–1573.

Walzem, R.L., Hansen, R.J., Williams, D.L., Hamilton, R.L., 1999. Estrogen Induction of VLDLy Assembly in Egg-Laying Hens. J. Nutr. 129, 467S–472S.

Wayne, N.L., Malpaux, B., Karsch, F.J., 1989. Social cues can play a role in timing onset of the breeding season of the ewe. J. Reprod. Fertil. 87, 707–713.

Weatherhead, P.J., Metz, K.J., Bennett, G.F., Irwin, R.E., 1993. Parasite faunas, testosterone and secondary sexual traits in male red-winged blackbirds. Behav. Ecol. Sociobiol. 33, 13–23.

West-Eberhard, M.J., 1989. Phenotypic plasticity and the origins of diversity. Annu. Rev. Ecol. Syst. 20, 249–278.

Wetton, J.H., Parkin, D.T., 1991. An association between fertility and cuckoldry in the house sparrow, *Passer domesticus*. Proc. R. Soc. Lond. B Biol. Sci. 245, 227–233.

Wilczek, A.M., Roe, J.L., Knapp, M.C., Cooper, M.D., Lopez-Gallego, C., Martin, L.J., Muir, C.D., Sim, S., Walker, A., Anderson, J., others, 2009. Effects of genetic perturbation on seasonal life history plasticity. Science 323, 930–934.

Williams, T.D., 2012. Physiological adaptations for breeding in birds. Princeton University Press.

Williams, T.D., 2005. Mechanisms underlying the costs of egg production. Bioscience 55, 39–48.

Williams, T.D., Christians, J.K., Aiken, J.J., Evanson, M., 1999. Enhanced immune function does not depress reproductive output. Proc. R. Soc. Lond. B Biol. Sci. 266, 753–757.

Williams, T.D., Kitaysky, A.S., Vézina, F., 2004. Individual variation in plasma estradiol-17β and androgen levels during egg formation in the European starling *Sturnus vulgaris*: implications for regulation of yolk steroids. Gen. Comp. Endocrinol. 136, 346–352.

Wingfield, J.C., 2012. Regulatory mechanisms that underlie phenology, behavior, and coping with environmental perturbations: an alternative look at biodiversity. The Auk 129, 1–7.

Wingfield, J.C., Breuner, C., Jacobs, J., 1997. Corticosterone and behavioral responses to unpredictable events. Perspect. Avian Endocrinol. 267–278.

Wingfield, J.C., Crim, J.W., Matfocks, P.W., Farner, D.S., 1979. Responses of photosensitive and photorefractory male white-crowned sparrows (*Zonotrichia leucophrys gambelii*) to synthetic mammalian luteinizing hormone releasing hormone (Syn-LHRH). Biol. Reprod. 21, 801–806.

Wingfield, J.C., Farner, D.S., 1993. Endocrinology of reproduction in wild species. Avian Biol. 9, 327.

Wingfield, J.C., Hahn, T.P., 1994. Testosterone and territorial behaviour in sedentary and migratory sparrows. Anim. Behav. 47, 77–89.

Wingfield, J.C., Hahn, T.P., Levin, R., Honey, P., 1992. Environmental predictability and control of gonadal cycles in birds. J. Exp. Zool. 261, 214–231.

Wingfield, J.C., Hegner, R.E., Dufty Jr, A.M., Ball, G.F., 1990. The " challenge hypothesis": theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. Am. Nat. 829–846.

Wingfield, J.C., Kenagy, G.J., 1991. Natural regulation of reproductive cycles. Vertebr. Endocrinol. Fundam. Biomed. Implic. 4, 181–241.

Wingfield, J.C., Lynn, S.E., Soma, K.K., 2001. Avoiding the "costs" of testosterone: ecological bases of hormone-behavior interactions. Brain. Behav. Evol. 57, 239–251.

Wingfield, J.C., Moore, M.C., 1987. Hormonal, social, and environmental factors in the reproductive biology of free-living male birds. Psychobiol. Reprod. Behav. Evol. Perspect. 149–175.

Wingfield, J.C., Sapolsky, R.M., 2003. Reproduction and resistance to stress: when and how. J. Neuroendocrinol. 15, 711–724.

Wingfield, J.C., Smith, J.P., Farner, D.S., 1982. Endocrine responses of white-crowned sparrows to environmental stress. Condor 399–409.

Winkler, D.W., Allen, P.E., 1996. The seasonal decline in avian clutch size: strategy or physiological constraints. Ecology 77, 922–932.

Wolfson, A., 1942. Regulation of spring migration in juncos. The Condor 44, 237–263.

Zirkin, B.R., Santulli, R., Awoniyi, C.A., Ewing, L.L., 1989. Maintenance of advanced spermatogenic cells in the adult rat testis: quantitative relationship to testosterone concentration within the testis. Endocrinology 124, 3043–3049.

CHAPTER 2: CONSISTENT INDIVIDUAL VARIATION IN DAY, NIGHT, AND GNRH-INDUCED TESTOSTERONE CONCENTRATIONS IN HOUSE SPARROWS (*PASSER DOMESTICUS*)[1]

Abstract

The hypothalamic-pituitary-gonadal (HPG) axis, with gonadotropin-releasing hormone (GnRH) initiating the endocrine cascade, regulates testosterone secretion. Testosterone, through its pleiotropic effects, plays a crucial role in coordinating morphology, physiology and behavior in a reproductive context. The concentration of circulating testosterone, however, varies over the course of the day and in response to other internal or external stimuli, potentially making it difficult to relate testosterone sampled at one time point with traits of interest. Many researchers now utilize the administration of exogenous GnRH to elicit a standardized stimulation of testosterone secretion. However, it has remained unclear if and how this exogenously stimulated activation of the HPG axis is related with endogenously regulated testosterone that is capable of influencing testosterone related traits. Repeated measures of a hormone can uncover consistent individual variation in hormonal differences at the HPG axis level, variation that potentially stems from underlying genetic variation in a population experiencing identical environmental cues. Thus, we asked, using the house sparrow (*Passer domesticus*), how daily endogenous variation in testosterone profiles relates to GnRH-induced testosterone secretion. Further, we

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explore the relationship between endogenous daily testosterone peaks and GnRH-induced testosterone with badge size, a morphological trait related with status within a social group. We found that GnRH-induced testosterone levels reflect a highly repeatable hormonal phenotype that is strongly correlated with nighttime testosterone levels. The results demonstrate the usefulness of GnRH-induced testosterone in studies aimed at understanding individual variation and selection on endogenously regulated testosterone levels and the potential importance of nighttime testosterone levels to physiology and behavior.

Introduction

Researchers aiming to understand the role hormones may play in evolutionary processes have often attempted to relate circulating hormone levels with discreet phenotypic traits and direct fitness-related traits (Balthazart, 1983; Comendant et al., 2003; Dixson and Anderson, 2004; Harding, 1983; Hau et al., 2010; Hutchison, 1978; Kempenaers et al., 2008; Moore, 1984; Ouyang et al., 2011b; Solís and Penna, 1997). Testosterone-mediated traits have often been related with morphological, physiological and behavioral traits such as dominance rank, plumage characteristics, immune function and genetic quality (Griffith et al., 1999; Jensen et al., 2004; Veiga, 1993). For example, morphological ‘ornaments’ have been observed to be related with testosterone. However, hormone levels in circulation are highly variable and actively respond to the environment and individual experiences (reviewed in: Ketterson et al., 2005; Wingfield et al., 1990) and also vary as a result of underlying endogenous rhythms (Bell-Pedersen et al., 2005; Daan et al., 1975; Gwinner, 1975, 1974; Morin et al., 1977; Takahashi and Menaker, 1980). Thus, relating an individual’s circulating hormone levels sampled at a single time point poses challenges for interpretation and investigation of relationships with other traits of interest (Fusani, 2008).

Classic manipulation experiments have enabled investigators to research the broad effects of hormones such as testosterone at the population level. However, these types of manipulations do not easily allow for natural temporal changes in these levels or individual variation in endogenously produced levels (Balthazart et al., 1984; Desjardins and Turek, 1977; Lofts et al., 1973; Stetson, 1972).

An additional approach being utilized by evolutionary endocrinologists is a hormone 'challenge' to probe the capability of endocrine systems to respond to upstream stimulation or feedback. This approach attempts to generate a standardized physiological response produced by an individual, while allowing for individual variation in maximal capacity of the system to remain. These values can then be related with individual variation in other traits of interest that may influence individual variation in fitness. For example, injections of the glucocorticoid receptor agonist dexamethasone or with adrenocorticotropic hormone (ACTH) allow researchers to assess negative feedback and maximal stimulation components, respectively, of the endocrine stress response (Bauer et al., 2015; Brown et al., 2001; Romero et al., 2008, 1998; Romero and Wikelski, 2010, 2006). Similarly, 'gonadotropin-releasing hormone (GnRH) challenges' (GnRH is endogenously released from the hypothalamus and activates the reproductive endocrine axis) have been employed to relate maximum sex steroid secretion with reproductive success (McGlothlin et al., 2010), parental care behavior (Goymann and Wingfield, 2004) and sexually selected traits (McGlothlin et al., 2008; McGlothlin and Ketterson, 2008).

While these 'challenges' are providing important insight into relationships between to daytime baseline levels and magnitude of responsiveness (Apfelbeck and Goymann, 2011; Goymann et al., 2015, 2007; Greives et al., 2006; McGlothlin et al., 2008; Moore et al., 2002), further research is needed to better understand how these exogenously stimulated short-term increases in testosterone relate to endogenous levels experienced by an individual at other key points in time. Said another way, given the temporal fluctuations in hormone titers exhibited within an individual throughout the day, does the ‘challenge’ approach provide insight into the levels that an individual endogenously produces at a given point throughout the day? Specifically, in this investigation we attempt to better understand the relationship between endogenous daily variations in testosterone and levels observed following a ‘GnRH challenge.’

Here, we ask if elevated nighttime levels of endogenous testosterone secreted by individuals are related to maximum testosterone secretion following stimulation with exogenous GnRH. To address this, we measured daytime baseline and GnRH-induced testosterone titers as well as circulating baseline testosterone levels at night from captive house sparrows (Passer domesticus). Badge size in house sparrows has been related with nighttime testosterone levels (Laucht et al., 2011) and amount of white in tail feathers in dark-eyed juncos (Junco hyemalis) is related with GnRH-induced testosterone (McGlothlin et al., 2008). Therefore, we related testosterone levels with badge size, a well-studied phenotypic trait in the house sparrow (Cordero et al., 1999; Laucht et al., 2011, 2010; Liker and Barta, 2001; Veiga, 1993; Whitekiller et al., 2000).

Further, we explored the repeatability of these endogenous hormones levels as well as GnRH-induced levels by sampling once per week for four weeks. Previous research evaluating diurnal changes in testosterone have observed a significant testosterone peak during the nighttime in house sparrows (Laucht et al., 2011). Such studies have begun to explore if and how these night levels were related to phenotypic traits and behaviors (Balthazart, 1976; Foerster et al., 2002; Laucht et al., 2011). However, in many species, obtaining circulating testosterone levels during the night would be very difficult in the wild. Thus, if peak night testosterone is correlated with GnRH-induced levels and both are repeatable, the use of a GnRH ‘challenge’ approach may provide additional opportunities to address research questions aimed at understanding links between the endogenous activity of the reproductive endocrine system and phenotypic traits of interest (e.g., ornamentation, behavior).

Methods

Study Individuals

Seventeen male house sparrows were captured in baited potter traps in Fargo, ND during October 2014. All individuals were held in captivity for a total of six months. Each house sparrow was individually housed at the North Dakota State University animal housing facility in 59.7 x 39.4 x 30.5 cm wire cages. All cages were visually, but not acoustically isolated. The birds had ad libitum access to food (canary seed), drinking water, bath water, and grit. Vitamin water was provided one week out of every month (eCOTRITION Pro Ultra-Care Vita-Sol for caged birds). After capture, individuals were maintained on a light-dark cycle of 8L: 16D for eight weeks to ensure a photosensitive state (Farner et al., 1966; King and Farner, 1963). The population was then photostimulated with a long day light-dark cycle of 16L: 8D (light on: 8:00 am, light off: 12:00am) to trigger gonadal recrudescence and a reproductive state (King and Farner, 1963; Small et al., 2007). The longest day length experienced by house sparrows in Fargo, ND is 15 hours and 52 minutes of light (US Naval Observatory, http://aa.usno.navy.mil/). Temperature was held between 22.2 – 23.9 °C. Animal care guidelines were followed and approved by our institutional IACUC (#A14044).

Individual Sampling and Measuring

Four weeks following the light transition to 16L: 8D, all 17 individuals were blood sampled. Birds were determined to be in reproductive state based on presence of an enlarged cloacal protuberance and an ability to produce an ejaculate. All birds were subjected to four weeks of sampling, with blood taken each week at three time points: daytime, post-GnRH, and nighttime. Blood samples to measure baseline day and night hormone levels were always collected within thirty minutes of entering the animal room. For the daytime blood sampling, we captured all males two hours after lights on, a time chosen to mimic a common morning sampling time obtained in field studies. Males then received an intramuscular injection of chicken GnRH-I (1.25 µg dissolved in 50 µL PBS for a final concentration of 25 ng/µL; American Peptide product #54-8-23, Sunnyvale, CA, USA). A dose of 2 mg/kg GnRH-I was administered (Jawor et al., 2007, 2006). Individuals were then held in cloth bags and bled a second time 30 minutes after the GnRH injection. A GnRH challenge induces a standardized response of the pituitary and gonads to secrete testosterone (Jawor et al., 2007, 2006). For nighttime sampling, all males were captured 2.5 hours after lights off (2:30 am), approximately 40 hours after the daytime sampling. Testosterone levels in juncos return to baseline levels within 2 hours following GnRH injection (Jawor et al., 2006), thus the daytime sampling 40 hours prior to nighttime sampling likely had minimal effect on circulating testosterone levels. All three bleeding sample time points (daytime, GnRH-induced, and nighttime) were repeated for 4 weeks to assess individual repeatability.

Approximately 50 µL of blood collected in a heparinized micro-hematocrit capillary tube was taken from the wing vein during each sampling event. Blood was kept on ice until centrifugation, and plasma was aspirated and stored at –80°C until assayed for testosterone. The order in which birds were sampled was random across all sampling events. There was no effect of time passed from entering the room to end of blood sampling on baseline daytime testosterone levels ($p = 0.719$, $df = 58$, $t = 0.361$) or nighttime testosterone levels ($p = 0.063$, $df = 55$, $t =$

1.901; package lme4: testosterone as dependent variable, time since entering room as fixed effect and ID as random variable).

To relate testosterone levels with a morphological trait known to be linked with reproductive behaviors and fitness in house sparrows (Buchanan et al., 2001; Laucht et al., 2011), we calculated badge length: each bird was held ventrally with his throat and bib stretched out and a ruler was used to measure length of the badge to the nearest millimeter (Griffith et al., 1999).

Determination of Plasma Testosterone Levels

Plasma testosterone was measured using an enzyme immunoassay EIA kit (Enzo Life Sciences, ADI-900-065) (Gerlach and Ketterson, 2013; Greives et al., 2006; Iserbyt et al., 2015; Wilcoxen et al., 2015). This assay has low cross reactivity with 19-hydroxytestosterone (14.6 %), androstendione (7.2 %), dehydroepiandrosterone (0.72 %), estradiol (0.4 %) and <0.001 %: dihydrotestosterone, estriol, aldosterone, corticosterone, cortisol, cortisone, estrone, progesterone, pregenolone. Hormones were extracted using diethyl ether (2x extractions) and dried under nitrogen gas on a hot block set to 25 °C. When available, 30 μL of plasma was used during extraction. A total of 33 samples out of 204 total had less than 30 μL of plasma available for extraction (daytime: 14, post-GnRH: 12, and nighttime: 7). Of these only four samples were below 20 μL of plasma, the kit's recommended amount. For these samples where 30 μL of plasma was unavailable, plasma volume used was recorded and the concentration was adjusted accordingly in the final calculation. It is likely that our extraction efficiencies were very high (~90 %) based on our experience and a previous study that used the same extraction protocol (Ouyang et al., 2011a). A standard from the kit was run three times on each plate in duplicate as an unknown, following extraction. These unknowns were used to calculate within plate variation, and the average of unknowns for each plate was used to calculate between plate variation.

Each sample was plated in duplicate, 100 μL per well following the manufacturer's guidelines. All samples were reconstituted with 300 μL of assay buffer, vortexed, and allowed to reconstitute overnight in the refrigerator. Samples were assayed on six plates in total with all samples from an individual kept on the same plate, but an individual's samples were randomized with respect to week and bleeding time across the plate. Concentrations of testosterone were calculated using a five-parameter logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories, Inc.). The coefficients of variation (CV) for intraplate testosterone ranged from 0.8 – 8.6% (Six plates: 1: 4.9 %; 2: 4.2 %; 3: 3.0 %; 4: 0.8 %; 5: 1.1 %; 6: 8.6 %) and interplate CV was 17.4 %. Samples that were below detection limit of our assay were assigned a value of lowest kit sensitivity, corrected for plasma volume (5.67 pg/ml). Of the 19 samples below detection limit, 15 were daytime samples, zero were post-GnRH samples and four were nighttime samples (two of which were the same individual over two consecutive weeks).

Statistical Analyses

We performed all statistical analyses using R 3.2.3 (R Core Team, 2014). Plasma testosterone levels were natural-log (ln)-transformed, after which they satisfied normality assumptions (based on qq plots of studentized residuals). We simultaneously estimated the repeatability of each sampling time point within each of the three bleeding times (daytime, post-GnRH, nighttime) and the among-individual correlations of the sampling methods. The among-individual correlation corresponds to the repeatable part of a correlation (Dingemanse et al., 2012) and is the portion of a phenotypic correlation that provides an upper estimate of underlying genetic correlation (Dingemanse and Dochtermann, 2014). To estimate the repeatabilities and among-individual correlations, we used a Bayesian Markov chain Monte Carlo multi-response linear mixed effects model using the (MCMCglmm) package in R (Dingemanse and Dochtermann, 2013; Hadfield, 2010). We included sample week (weeks 1 – 4) as a fixed effect and individual as a random variable. The prior used was uniform from –1 to 1 for the correlation and thus uninformative, with a MCMC chain of 1.3 x 106 iterations, a 300000 burn-in period, and a thinning interval of 1000 (all analyses obtained effective sample sizes that were around 1000). All 17 individuals had complete testosterone profiles across the four weeks and three bleeding times.

Adjusted repeatabilities (Nakagawa and Schielzeth, 2010) were estimated based on the random factor intercept variance and residual variance and is reported as τ along with credibility intervals (Dingemanse and Dochtermann, 2013). The 95 % lower and upper credible intervals (CI) are the levels of uncertainty around repeatability estimates (Edwards et al., 1963). Among-individual correlations between bleeding times are reported as r along with 95 % CI. Among-individual correlations that have CI levels that do not overlap zero can be considered "significant." Whether sample week affected testosterone level was determined based on whether a week's estimated effect had a 95 % CI that overlapped zero. Repeatabilities and among-individual correlations are reported based on MCMC posterior modes and 95 % CI.

We also used posterior modal estimates of best linear unbiased predictors (BLUPs) for each individual in subsequent analyses to determine the relationship between an individual's testosterone level and badge size. BLUPs provide an estimate of an individual average after controlling for variance of fixed effects. This relationship between an individual's testosterone level (BLUP) and badge size was estimated using Pearson's product moment correlation.

Results

Testosterone levels were highly repeatable across all four weeks: daytime (τ = 0.53; CI: 0.33 – 0.74; Fig. 2.1A), GnRH-induced (τ = 0.80; CI: 0.67 – 0.91; Fig. 2.1C), and nighttime (τ = 0.46, CI: 0.22 – 0.64; Fig. 2.1E; Table 2.1). Daytime and GnRH-induced testosterone levels did not have slopes different from zero across the four sampled weeks (daytime: p = 0.52, CI: –0.159 – 0.301; GnRH: p= 0.48, CI: –0.288 – 0.169), but nighttime levels did significantly differ across weeks, with levels generally declining in later sampling weeks (CI: –0.724 – –0.085; Fig. 2.1B, D, and F respectively). Correlations across bleeding times were biologically meaningful between daytime and nighttime testosterone, as well as the correlation between post-GnRH and nighttime testosterone (Table 2.2). Individual estimates from the population mean of all bleeding times (BLUPs) were not correlated with badge length.

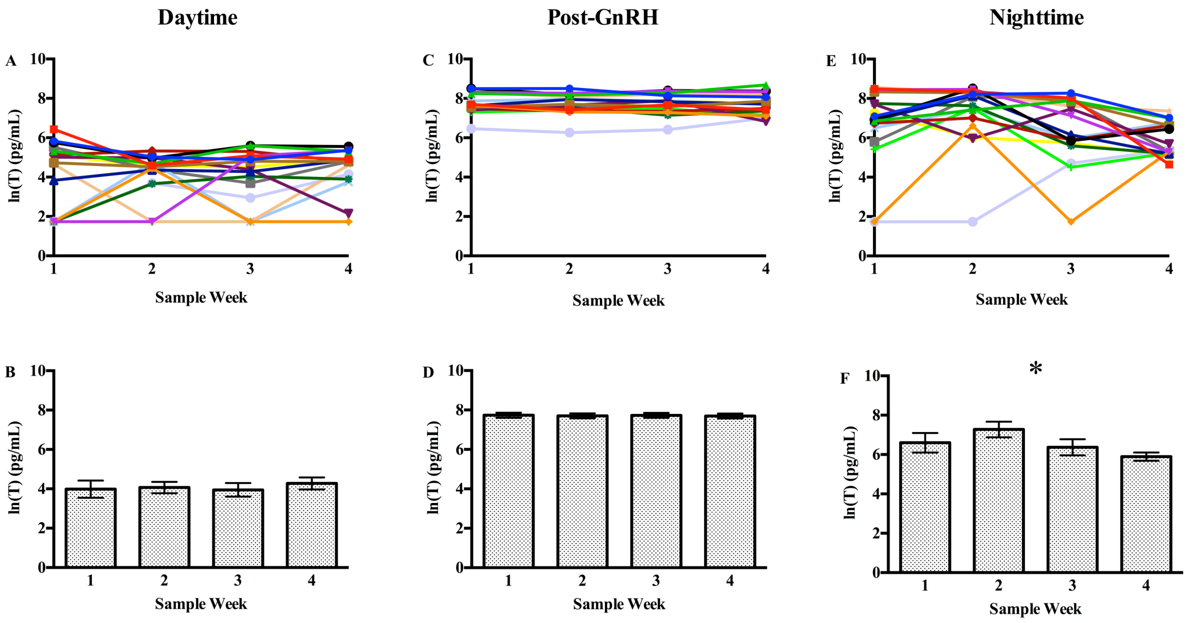

Figure 2.1. Individual male house sparrows' testosterone levels (A, C, E) and mean SE levels (B, D, F) across the four sampled weeks. Asterisk denotes a biologically meaningful difference between sample weeks.

Table 2.1. Repeatability of ln-transformed testosterone (pg/mL) with among and within-individual variances for each time point in 17 captive, wild-caught, male house sparrows (*Passer domesticus*) over four sampled weeks. Numbers in brackets are 95% credible intervals.

Bleeding Time	**Repeatability**	**Among-individual Variance**	**Within-individual Variance**
Daytime	τ=0.53 [0.33:0.74]	var=1.19 [0.49:2.42]	var=1.02 [0.70:1.50]
Post-GnRH	τ=0.80 [0.67:0.91]	var=0.35 [0.21:0.80]	var=0.10 [0.08:0.17]
Nighttime	τ=0.46 [0.22:0.64]	var=1.22 [0.52:2.73]	var=1.33 [1.02:2.17]

Table 2.2. Among-individual correlations between bleeding times of ln-transformed testosterone in 17 captive, wild-caught, male house sparrows (*Passer domesticus*) over four sampled weeks. Asterisks indicate a biologically meaningful correlation between bleeding times (credible intervals did not cross zero).

Bleeding Times	**Correlation of Testosterone (pg/mL)**	
Daytime : Post-GnRH	r = 0.19	CI: -0.192 – 0.700
Daytime : Nighttime	r = 0.41*	CI: 0.015 – 0.835
Post-GnRH : Nighttime	r = 0.44*	CI: 0.021 – 0.797

Discussion

Hormones like testosterone often demonstrate highly variable patterns of secretion, making it difficult to obtain ‘physiologically meaningful’ measures that can be related with traits of interest (Aschoff, 1979; Hau, 2007; Ketterson et al., 2009; Laucht et al., 2011). This dynamic nature of hormone secretion has made it inherently difficult for investigations aimed at understanding the impact of consistent individual differences in hormone levels on key fitness-related traits. Here, we demonstrated that both nighttime testosterone and GnRH-induced testosterone levels were highly repeatable and that these measures were correlated. GnRH-induced testosterone levels are predictive of nighttime levels; however, this relationship underestimates the among- and within-individual variation for nighttime testosterone because among individual variance is lower for post-GnRH testosterone than nighttime levels (Table 1).

This observation provides a framework to continue research aimed at understanding the role variation in hormonal systems plays in evolutionary and ecological processes of interest (e.g. (Crews, 1980; Real, 1994; Sheldon and Verhulst, 1996; Sih et al., 2004; Voesenek and Blom, 1996; Wingfield, 2013; Wingfield et al., 1997)).

Many past studies seeking to relate circulating levels of hormones with phenotypic traits or fitness often have, primarily for logistical reasons, utilized one baseline blood sample for measurement of hormones and often have observed weak to null relationships with fitness related traits (reviewed in: Bonier et al., 2009; Hau, 2007). Repeated measures of a trait over time under similar conditions are beneficial when addressing questions focused on relationships between hormonal variation and fitness among individuals (Hau and Goymann, 2015). One of the primary roles of hormones is to coordinate organismal level responses to actual or predicted changes in their environment (Ketterson et al., 2009). Thus, hormone levels are dynamic as opposed to unfluctuating. Therefore, repeated measures provide an approach to quantify relationships between hormones and behavior, as well as characterizing responses to the environment (Hau and Goymann, 2015). In addition to uncovering the underlying repeatability of hormone responses, given the response of hormones and response to GnRH in relation to variation in the environment, a reaction norm approach evaluating levels across different seasonal, environmental and social conditions will likely provide additional valuable insight (Hau and Goymann, 2015).

Here we demonstrate that under controlled captive conditions, the testosterone response to GnRH is highly repeatable. Repeatability of GnRH-induced testosterone secretion has been previously reported from field studies, but with lower repeatability estimates than those observed here (Jawor et al., 2007, 2006; McGlothlin et al., 2008). The variation between these field and lab repeatability estimates likely indicate a degree of plasticity in the system that may enable appropriate responses at different stages of the reproductive season or under varying environmental conditions experienced in the wild (Calisi and Bentley, 2009; Huey and Rosenzweig, 2009). Regardless, in both this study and previous studies, the repeatability of this trait suggests underlying genetic variation that selection could act upon (Boake, 1989; Jawor et al., 2006; Meyer et al., 1990).

Many environmental conditions vary predictably on a daily basis (e.g., light and temperature vary between day and night). Most animals display robust rhythms, with changes in physiology and behavior varying across the 24-hour cycle (Dunlap et al., 2004). Previous work, primarily from lab experiments and observations, has demonstrated that vertebrate sex steroid levels display daily (i.e., circadian) rhythmicity (Aschoff, 1979; Laucht et al., 2011; Plant, 1981). Yet many field and lab studies often are unable to fully track these changes to account for the possible influence daily variation in hormone levels has on traits of interest. Thus, the inability to establish a clear link between endogenous hormone levels and traits under investigation may arise from sampling of sex steroid levels at only one point in time. If influential levels are experienced at points in a daily cycle not sampled, key information in hormonal profiles may be missed.

Interestingly, where it has been measured, sex steroid concentrations in birds peak during the night (Balthazart, 1976; Foerster et al., 2002; Laucht et al., 2011). We also observed circulating levels of testosterone several times higher than levels observed during the daytime. While the reasons for the nightly elevation in testosterone is as of yet not fully known, one possible hypothesis may be promotion of sperm production, which occurs overnight in songbirds (Birkhead et al., 1994). Additionally, male songbirds engage in a wide-array of behaviors during the pre-dawn and dawn periods that may influence reproductive success (Poesel et al., 2006), thus, elevated levels of the sex steroid testosterone may influence the occurrence and intensity of these behaviors. For practical and logistical reasons, primarily because birds tend to be most active and easiest to catch during the morning (Hester, 1963; Poesel et al., 2006), the majority of field and laboratory studies sampling for hormones tend to collect blood during the daylight hours (e.g. (Apfelbeck et al., 2013; Apfelbeck and Goymann, 2011; Cain et al., 2012; Greives et al., 2006; McGlothlin et al., 2010, 2008; Villavicencio et al., 2014; Wingfield et al., 1992)).

One goal of the 'GnRH Challenge' has been to provide a measure of individual variation in GnRH-induced sex steroid production capability (Jawor et al., 2007, 2006; McGlothlin et al., 2007). The among-individual variation and correlation between GnRH-induced testosterone and nighttime testosterone observed here suggests that GnRH-induced testosterone is strongly related with an endogenously expressed testosterone levels experienced during the night. Thus, while it is often impractical to sample blood from birds at night, GnRH injections may be capable of approximating peak testosterone levels an individual may endogenously produce during the night. Further work in additional species will however be needed to confirm the generality of this relationship and further studies are needed to demonstrate the relevance of these high night-time levels. Taken together, the repeatability of GnRH-induced and nighttime testosterone coupled with strong correlations between these two sampling times is indicative of an HPG axis 'phenotype' that may be selected upon. Future studies should investigate repeatability of hormones across different social and seasonal time scales, but within the context of reproductive condition.

Daytime testosterone levels were very low (often undetectable) from week to week. Captivity has been shown to induce low levels of circulating testosterone (Wingfield et al., 1990, 1987), thus the low levels reported here may be a results of stress associated with captivity (Wingfield et al., 1990). Further, a lack of social interactions with females or other males likely also contributed to the observed low circulating daytime baseline levels. Thus, while these levels were found to be repeatable, we suggest caution in interpretation of what this repeatability estimate may mean, as the low variance introduced in estimating levels at or below detection likely inflated this estimate. Given these limitations, care should be taken when interpreting relationships between daytime, nighttime and post-GnRH testosterone levels.

Nighttime testosterone levels varied across the four sampled weeks, with circulating nighttime levels declining in the later sampling periods, and exhibited a moderately high repeatability. The variation in nighttime testosterone levels across the four sampled weeks may reflect natural seasonal variation across the breeding season. Effects of handling and repeated sampling across the study period could also have induced stress in these individuals. Stress is capable of suppressing reproductive function (reviewed in: Wingfield and Sapolsky, 2003). Another possibility for these observed reductions in endogenous nighttime testosterone levels could be due to lack of social stimulation (Dey et al., 2014; Gonzalez et al., 2002; Liker and Barta, 2001; McGraw et al., 2003; McGraw and Hill, 2000; Riters et al., 2004); males had no access to females and no male-male interactions were possible. Our findings of substantially elevated levels of testosterone at night as compared to daytime circulating levels are consistent with previous research evaluating diurnal changes in testosterone (Laucht et al., 2011).

Badge size is often measured as a sexually selected ornament in house sparrows (Griffith et al., 1999; Jensen et al., 2004; Laucht et al., 2011, 2010; Veiga, 1993). Hormonal control of traits, such as badge size, are often primed by social feedback systems, such as dominance rank within a population or flock (Duckworth et al., 2004; McGraw and Hill, 2000; Parker et al., 2002; Rubenstein and Hauber, 2008). Generally, a less dominant individual will have lower levels of circulating testosterone and suppressed sexual signals, as seen in some house sparrow and barn swallow populations (Buchanan et al., 2001; Laucht et al., 2011; Safran et al., 2008). A recent study conducted on a large flock of mixed-sex captive house sparrows observed a lack of relationship between daytime levels of baseline testosterone and badge size, but interestingly nighttime testosterone levels were found to be correlated with badge length (Laucht et al., 2011). Our captive set-up did not allow for social interactions and sample size for this study was considerably smaller than the previously mentioned study. Social feedback may be important for the bidirectional feedback between sexual signals and physiological condition, potentially mediated by testosterone (Safran et al., 2008). Therefore, badge size could not be used to gather information about an individual's social status within a social hierarchy, preventing a bidirectional feedback from occurring.

Future work allowing for greater natural social interactions and a larger sample size will be needed to further clarify the relationship between nighttime testosterone levels, GnRH-induced testosterone levels and morphological traits (e.g. badge size) in the house sparrow and other passerines.

Conclusions

Here we report that nighttime levels of sex steroids in photo-stimulated house sparrows are significantly higher than circulating daytime/morning levels. Our study mimicked 'morning' sampling by taking a blood sample two hours after lights on in the animal room. Mornings are the common time for trapping and sampling of hormones in birds. However, the current study indicates a single sample obtained during morning hours may not be indicative of peak endogenously produced levels. Further, we demonstrated that the maximum testosterone level produced following exogenous stimulation with GnRH is highly correlated with circulating nighttime levels. Both GnRH-induced and nighttime testosterone levels were highly repeatable across multiple sampling periods. These data suggest a GnRH-induced testosterone measure may be indicative of a HPG 'phenotype,' which could serve as an alternate means to investigate this endocrine trait in animals where obtaining a night sample is impractical. Combined, our data suggest the need for a better understanding of the role that variation endocrine systems exhibit within a day (e.g., day and night) and peak levels experienced has on traits of interest. Such an understanding may provide important insights into the role endocrine systems play in regulating and coordinating responses to selection.

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References

Apfelbeck, B., Goymann, W., 2011. Ignoring the challenge? Male black redstarts (*Phoenicurus ochruros*) do not increase testosterone levels during territorial conflicts but they do so in response to gonadotropin-releasing hormone. Proc. R. Soc. Lond. B Biol. Sci. 278, 3233–3242.

Apfelbeck, B., Mortega, K., Kiefer, S., Kipper, S., Vellema, M., Villavicencio, C.P., Gahr, M., Goymann, W., 2013. Associated and disassociated patterns in hormones, song, behavior and brain receptor expression between life-cycle stages in male black redstarts, *Phoenicurus ochruros*. Gen. Comp. Endocrinol. 184, 93–102.

Aschoff, J., 1979. Circadian rhythms: general features and endocrinological aspects. Endocr. Rhythms 1–61.

Balthazart, J., 1983. Hormonal correlates of behavior. Avian Biol. 7, 221–365.

Balthazart, J., 1976. Daily variations of behavioural activities and of plasma testosterone levels in the domestic duck *Anas platyrhynchos*. J. Zool. 180, 155–173.

Balthazart, J., Schumacher, M., Malacarne, G., 1984. Relative potencies of testosterone and 5α-dihydrotestosterone on crowing and cloacal gland growth in the Japanese quail (*Coturnix coturnix japonica*). J. Endocrinol. 100, 19–23.

Bauer, C.M., Needham, K.B., Le, C.N., Stewart, E.C., Graham, J.L., Ketterson, E.D., Greives, T.J., 2015. Hypothalamic-pituitary-adrenal axis activity is not elevated in a songbird (*Junco hyemalis*) preparing for migration. Gen. Comp. Endocrinol.

Bell-Pedersen, D., Cassone, V.M., Earnest, D.J., Golden, S.S., Hardin, P.E., Thomas, T.L., Zoran, M.J., 2005. Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat. Rev. Genet. 6, 544–556.

Birkhead, T.R., Veiga, J.P., Moller, A.P., 1994. Male sperm reserves and copulation behaviour in the house sparrow, Passer domesticus. Proc. R. Soc. Lond. B Biol. Sci. 256, 247–251.

Boake, C.R., 1989. Repeatability: its role in evolutionary studies of mating behavior. Evol. Ecol. 3, 173–182.

Bonier, F., Martin, P.R., Moore, I.T., Wingfield, J.C., 2009. Do baseline glucocorticoids predict fitness? Trends Ecol. Evol. 24, 634–642.

Brown, J.L., Bellem, A.C., Fouraker, M., Wildt, D.E., Roth, T.L., 2001. Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring. Zoo Biol. 20, 463–486.

Buchanan, K.L., Evans, M.R., Goldsmith, A.R., Bryant, D.M., Rowe, L.V., 2001. Testosterone influences basal metabolic rate in male house sparrows: a new cost of dominance signalling? Proc. R. Soc. Lond. B Biol. Sci. 268, 1337–1344.

Cain, K.E., Burns, C.M.B., Ketterson, E.D., 2012. Testosterone production, sexually dimorphic morphology, and digit ratio in the dark-eyed junco. Behav. Ecol. ars186. doi:10.1093/beheco/ars186

Calisi, R.M., Bentley, G.E., 2009. Lab and field experiments: are they the same animal? Horm. Behav. 56, 1–10.

Comendant, T., Sinervo, B., Svensson, E.I., Wingfield, J., 2003. Social competition, corticosterone and survival in female lizard morphs. J. Evol. Biol. 16, 948–955.

Cordero, P.J., Wetton, J.H., Parkin, D.T., 1999. Extra-pair paternity and male badge size in the house sparrow. J. Avian Biol. 97–102.

Crews, D., 1980. Interrelationships among ecological, behavioral, and neuroendocrine processes in the reproductive cycle of *Anolis carolinensis* and other reptiles. Adv. Study Behav. 11, 1–74.

Daan, S., Damassa, D., Pittendrigh, C.S., Smith, E.R., 1975. An effect of castration and testosterone replacement on a circadian pacemaker in mice (*Mus musculus*). Proc. Natl. Acad. Sci. 72, 3744–3747.

Desjardins, C., Turek, F.W., 1977. Effects of testosterone on spermatogenesis and luteinizing hormone release in Japanese quail. Gen. Comp. Endocrinol. 33, 293–303.

Dey, C.J., Dale, J., Quinn, J.S., 2014. Manipulating the appearance of a badge of status causes changes in true badge expression. Proc. R. Soc. Lond. B Biol. Sci. 281, 20132680.

Dingemanse, N.J., Dochtermann, N.A., 2014. Individual behaviour: behavioural ecology meets quantitative genetics. Oxford University Press.

Dingemanse, N.J., Dochtermann, N.A., 2013. Quantifying individual variation in behaviour: mixed-effect modelling approaches. J. Anim. Ecol. 82, 39–54.

Dingemanse, N.J., Dochtermann, N.A., Nakagawa, S., 2012. Defining behavioural syndromes and the role of “syndrome deviation”in understanding their evolution. Behav. Ecol. Sociobiol. 66, 1543–1548.

Dixson, A.F., Anderson, M.J., 2004. Sexual behavior, reproductive physiology and sperm competition in male mammals. Physiol. Behav. 83, 361–371.

Duckworth, R.A., Mendonça, M.T., Hill, G.E., 2004. Condition-dependent sexual traits and social dominance in the house finch. Behav. Ecol. 15, 779–784.

Dunlap, J.C., Loros, J.J., DeCoursey, P.J., 2004. Chronobiology: biological timekeeping. Sinauer Associates.

Edwards, W., Lindman, H., Savage, L.J., 1963. Bayesian statistical inference for psychological research. Psychol. Rev. 70, 193.

Farner, D.S., Follett, B.K., King, J.R., Morton, M.L., 1966. A quantitative examination of ovarian growth in the white-crowned sparrow. Biol. Bull. 130, 67–75.

Foerster, K., Poesel, A., Kunc, H., Kempenaers, B., 2002. The natural plasma testosterone profile of male blue tits during the breeding season and its relation to song output. J. Avian Biol. 33, 269–275.

Fusani, L., 2008. Endocrinology in field studies: problems and solutions for the experimental design. Gen. Comp. Endocrinol. 157, 249–253.

Gerlach, N.M., Ketterson, E.D., 2013. Experimental elevation of testosterone lowers fitness in female dark-eyed juncos. Horm. Behav. 63, 782–790.

Gonzalez, G., Sorci, G., Smith, L.C., De Lope, F., 2002. Social control and physiological cost of cheating in status signalling male house sparrows (*Passer domesticus*). Ethology 108, 289–302.

Goymann, W., Landys, M.M., Wingfield, J.C., 2007. Distinguishing seasonal androgen responses from male–male androgen responsiveness—revisiting the challenge hypothesis. Horm. Behav. 51, 463–476.

Goymann, W., Villavicencio, C.P., Apfelbeck, B., 2015. Does a short-term increase in testosterone affect the intensity or persistence of territorial aggression?—An approach using an individual's hormonal reactive scope to study hormonal effects on behavior. Physiol. Behav. 149, 310–316.

Goymann, W., Wingfield, J.C., 2004. Competing females and caring males. Sex steroids in African black coucals, Centropus grillii. Anim. Behav. 68, 733–740.

Greives, T.J., Mcglothlin, J.W., Jawor, J.M., Demas, G.E., Ketterson, E.D., 2006. Testosterone and innate immune function inversely covary in a wild population of breeding Dark-Eyed Juncos (*Junco hyemalis*). Funct. Ecol. 20, 812–818. doi:10.1111/j.1365-2435.2006.01167.x

Griffith, S.C., Owens, I.P.F., Burke, T., 1999. Female choice and annual reproductive success favour less-ornamented male house sparrows. Proc. R. Soc. B Biol. Sci. 266, 765. doi:10.1098/rspb.1999.0703

Gwinner, E., 1975. Effects of season and external testosterone on the freerunning circadian activity rhythm of European starlings (*Sturnus vulgaris*). J. Comp. Physiol. 103, 315–328.

Gwinner, F., 1974. Testosterone induces“ splitting” of circadian locomotor activity rhythms in birds. Science 185, 72–74.

Hadfield, J.D., 2010. MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package. J. Stat. Softw. 33, 1–22.

Harding, C.F., 1983. Hormonal influences on avian aggressive behavior, in: Hormones and Aggressive Behavior. Springer, pp. 435–467.

Hau, M., 2007. Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. BioEssays 29, 133–144.

Hau, M., Goymann, W., 2015. Endocrine mechanisms, behavioral phenotypes and plasticity: known relationships and open questions. Front. Zool. 12, 1–15.

Hau, M., Ricklefs, R.E., Wikelski, M., Lee, K.A., Brawn, J.D., 2010. Corticosterone, testosterone and life-history strategies of birds. Proc. R. Soc. Lond. B Biol. Sci. 277, 3203–3212.

Hester, A.E., 1963. A plastic wing tag for individual identification of passerine birds. Bird-Band. 213–217.

Huey, R.B., Rosenzweig, F., 2009. Laboratory evolution meets catch-22: balancing simplicity and realism. Exp. Evol. Concepts Methods Appl. Sel. Exp. 671–702.

Hutchison, J.B., 1978. Hypothalamic regulation of male sexual responsiveness to androgen. Biol. Determinants Sex. Behav. 277–317.

Iserbyt, A., Eens, M., Müller, W., 2015. Sexually antagonistic selection during parental care is not generated by a testosterone-related intralocus sexual conflict–insights from full-sib comparisons. Sci. Rep. 5.

Jawor, J.M., Mcglothlin, J.W., Casto, J.M., Greives, T.J., Snajdr, E.A., Bentley, G.E., Ketterson, E.D., 2007. Testosterone response to GnRH in a female songbird varies with stage of reproduction: implications for adult behaviour and maternal effects. Funct. Ecol. 21, 767–775.

Jawor, J.M., McGlothlin, J.W., Casto, J.M., Greives, T.J., Snajdr, E.A., Bentley, G.E., Ketterson, E.D., 2006. Seasonal and individual variation in response to GnRH challenge in male dark-eyed juncos (*Junco hyemalis*). Gen. Comp. Endocrinol. 149, 182–189.

Jensen, H., SÆther, B.-E., Ringsby, T.H., Tufto, J., Griffith, S.C., Ellegren, H., 2004. Lifetime reproductive success in relation to morphology in the house sparrow Passer domesticus. J. Anim. Ecol. 73, 599–611.

Kempenaers, B., Peters, A., Foerster, K., 2008. Sources of individual variation in plasma testosterone levels. Philos. Trans. R. Soc. B Biol. Sci. 363, 1711–1723.

Ketterson, E.D., Atwell, J.W., McGlothlin, J.W., 2009. Phenotypic integration and independence: hormones, performance, and response to environmental change. Integr. Comp. Biol. 49, 365–379.

Ketterson, E.D., Nolan Jr, V., Sandell, M., 2005. Testosterone in females: mediator of adaptive traits, constraint on sexual dimorphism, or both? Am. Nat. 166, S85–S98.

King, J.R., Farner, D.S., 1963. The relationship of fat deposition to Zugunruhe and migration. Condor 200–223.

Laucht, S., Dale, J., Mutzel, A., Kempenaers, B., 2011. Individual variation in plasma testosterone levels and its relation to badge size in House Sparrows *Passer domesticus*: It'sa night-and-day difference. Gen. Comp. Endocrinol. 170, 501–508.

Laucht, S., Kempenaers, B., Dale, J., 2010. Bill color, not badge size, indicates testosterone-related information in house sparrows. Behav. Ecol. Sociobiol. 64, 1461–1471.

Liker, A., Barta, Z., 2001. Male badge size predicts dominance against females in house sparrows. The Condor 103, 151–157.

Lofts, B., Murton, R.K., Thearle, R.J.P., 1973. The effects of testosterone propionate and gonadotropins on the bill pigmentation and testes of the house sparrow (*Passer domesticus*). Gen. Comp. Endocrinol. 21, 202–209.

McGlothlin, J.W., Jawor, J.M., Greives, T.J., Casto, J.M., Phillips, J.L., Ketterson, E.D., 2008. Hormones and honest signals: males with larger ornaments elevate testosterone more when challenged. J. Evol. Biol. 21, 39–48.

McGlothlin, J.W., Jawor, J.M., Ketterson, E.D., 2007. Natural variation in a testosterone-mediated trade-off between mating effort and parental effort. Am. Nat. 170, 864–875.

McGlothlin, J.W., Ketterson, E.D., 2008. Hormone-mediated suites as adaptations and evolutionary constraints. Philos. Trans. R. Soc. B Biol. Sci. 363, 1611–1620.

McGlothlin, J.W., Whittaker, D.J., Schrock, S.E., Gerlach, N.M., Jawor, J.M., Snajdr, E.A., Ketterson, E.D., 2010. Natural selection on testosterone production in a wild songbird population. Am. Nat. 175, 687–701.

McGraw, K.J., Dale, J., Mackillop, E.A., 2003. Social environment during molt and the expression of melanin-based plumage pigmentation in male house sparrows (*Passer domesticus*). Behav. Ecol. Sociobiol. 53, 116–122.

McGraw, K.J., Hill, G.E., 2000. Carotenoid-based ornamentation and status signaling in the house finch. Behav. Ecol. 11, 520–527.

Meyer, K., Hammond, K., Parnell, P.F., Mackinnon, M.J., Sivarajasingam, S., 1990. Estimates of heritability and repeatability for reproductive traits in Australian beef cattle. Livest. Prod. Sci. 25, 15–30.

Moore, I.T., Perfito, N., Wada, H., Sperry, T.S., Wingfield, J.C., 2002. Latitudinal variation in plasma testosterone levels in birds of the genus Zonotrichia. Gen. Comp. Endocrinol. 129, 13–19.

Moore, M.C., 1984. Changes in Territorial Defense Produced by Changes in Circulating Levels of Testosterone: A Possible Hormonal Basis for Mate-Guarding Behavior in White-Crowned Sparrows. Behaviour 88, 215–226.

Morin, L.P., Fitzgerald, K.M., Zucker, I., 1977. Estradiol shortens the period of hamster circadian rhythms. Science 196, 305–307.

Nakagawa, S., Schielzeth, H., 2010. Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. Biol. Rev. 85, 935–956.

Ouyang, J.Q., Hau, M., Bonier, F., 2011a. Within seasons and among years: when are corticosterone levels repeatable? Horm. Behav. 60, 559–564.

Ouyang, J.Q., Sharp, P.J., Dawson, A., Quetting, M., Hau, M., 2011b. Hormone levels predict individual differences in reproductive success in a passerine bird. Proc. R. Soc. Lond. B Biol. Sci. 278, 2537–2545.

Parker, T.H., Knapp, R., Rosenfield, J.A., 2002. Social mediation of sexually selected ornamentation and steroid hormone levels in male junglefowl. Anim. Behav. 64, 291–298.

Plant, T.M., 1981. Time courses of concentrations of circulating gonadotropin, prolactin, testosterone, and cortisol in adult male rhesus monkeys (*Macaca mulatta*) throughout the 24 h light-dark cycle. Biol. Reprod. 25, 244–252.

Poesel, A., Kunc, H.P., Foerster, K., Johnsen, A., Kempenaers, B., 2006. Early birds are sexy: male age, dawn song and extrapair paternity in blue tits, *Cyanistes* (formerly *Parus*) *caeruleus*. Anim. Behav. 72, 531–538.

R_Core_Team, 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2012. ISBN 3-900051-07-0.

Real, L., 1994. Behavioral mechanisms in evolutionary ecology. University of Chicago Press.

Riters, L.V., Teague, D.P., Schroeder, M.B., 2004. Social status interacts with badge size and neuroendocrine physiology to influence sexual behavior in male house sparrows (*Passer domesticus*). Brain. Behav. Evol. 63, 141–150.

Romero, L.M., Meister, C.J., Cyr, N.E., Kenagy, G.J., Wingfield, J.C., 2008. Seasonal glucocorticoid responses to capture in wild free-living mammals. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 294, R614–R622.

Romero, L.M., Soma, K.K., Wingfield, J.C., 1998. The Hypothalamus and Adrenal Regulate Modulation of Corticosterone Release in Redpolls (*Carduelis flammea*—An Arctic-Breeding Song Bird). Gen. Comp. Endocrinol. 109, 347–355. doi:10.1006/gcen.1997.7048

Romero, L.M., Wikelski, M., 2010. Stress physiology as a predictor of survival in Galapagos marine iguanas. Proc. R. Soc. Lond. B Biol. Sci. 277, 3157–3162. doi:10.1098/rspb.2010.0678

Romero, L.M., Wikelski, M., 2006. Diurnal and nocturnal differences in hypothalamic–pituitary–adrenal axis function in Galapagos marine iguanas. Gen. Comp. Endocrinol. 145, 177–181.

Rubenstein, D.R., Hauber, M.E., 2008. Dynamic feedback between phenotype and physiology in sexually selected traits. Trends Ecol. Evol. 23, 655–658.

Safran, R.J., Adelman, J.S., McGraw, K.J., Hau, M., 2008. Sexual signal exaggeration affects physiological state in male barn swallows. Curr. Biol. 18, R461–R462.

Sheldon, B.C., Verhulst, S., 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol. Evol. 11, 317–321. doi:10.1016/0169-5347(96)10039-2

Sih, A., Bell, A., Johnson, J.C., 2004. Behavioral syndromes: an ecological and evolutionary overview. Trends Ecol. Evol. 19, 372–378.

Small, T.W., Sharp, P.J., Deviche, P., 2007. Environmental regulation of the reproductive system in a flexibly breeding Sonoran Desert bird, the rufous-winged sparrow, *Aimophila carpalis*. Horm. Behav. 51, 483–495.

Solís, R., Penna, M., 1997. Testosterone levels and evoked vocal responses in a natural population of the frog *Batrachyla taeniata*. Horm. Behav. 31, 101–109.

Stetson, M.H., 1972. Feedback regulation of testicular function in Japanese quail: Testosterone implants in the hypothalamus and adenohypophysis. Gen. Comp. Endocrinol. 19, 37–47.

Takahashi, J.S., Menaker, M., 1980. Interaction of estradiol and progesterone: effects on circadian locomotor rhythm of female golden hamsters. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 239, R497–R504.

Veiga, J.P., 1993. Badge size, phenotypic quality, and reproductive success in the house sparrow: a study on honest advertisement. Evolution 1161–1170.

Villavicencio, C.P., Apfelbeck, B., Goymann, W., 2014. Parental care, loss of paternity and circulating levels of testosterone and corticosterone in a socially monogamous song bird. Front. Zool. 11, 1.

Voesenek, L., Blom, C., 1996. Plants and hormones: an ecophysiological view on timing and plasticity. J. Ecol. 111–119.

Whitekiller, R.R., Westneat, D.F., Schwagmeyer, P.L., Mock, D.W., 2000. Badge size and extra-pair fertilizations in the house sparrow. The condor 102, 342–348.

Wilcoxen, T.E., Horn, D.J., Hogan, B.M., Hubble, C.N., Huber, S.J., Flamm, J., Knott, M., Lundstrom, L., Salik, F., Wassenhove, S.J., Wrobel, E.R., 2015. Effects of bird-feeding activities on the health of wild birds. Conserv. Physiol. 3, cov058.

Wingfield, J.C., 2013. Ecological processes and the ecology of stress: the impacts of abiotic environmental factors. Funct. Ecol. 27, 37–44.

Wingfield, J.C., Ball, G.F., Dufty, A.M., Hegner, R.E., Ramenofsky, M., 1987. Testosterone and aggression in birds. Am. Sci. 75, 602–608.

Wingfield, J.C., Hegner, R.E., Dufty Jr, A.M., Ball, G.F., 1990. The“ challenge hypothesis”: theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. Am. Nat. 829–846.

Wingfield, J.C., Jacobs, J., Hillgarth, N., 1997. Ecological Constraints and the Evolution of Hormone-Behavior Interrelationshipsa. Ann. N. Y. Acad. Sci. 807, 22–41.

Wingfield, J.C., Sapolsky, R.M., 2003. Reproduction and resistance to stress: when and how. J. Neuroendocrinol. 15, 711–724.

Wingfield, J.C., Vleck, C.M., Moore, M.C., 1992. Seasonal changes of the adrenocortical response to stress in birds of the Sonoran Desert. J. Exp. Zool. 264, 419–428.

CHAPTER 3: REGULATORY MECHANISMS DOWNSTREAM OF HYPOTHALAMIC CONTROL FOR THE ONSET OF RAPID FOLLICLE GROWTH

Abstract

To date, mechanisms related to seasonal reproductive timing decisions in vertebrates have received far more study in males than in females, but ultimately it is female timing decisions that dictate when rearing of offspring will occur. Reproduction is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. Production and release of gonadotropin-releasing hormone (GnRH) stimulates the pituitary to secrete luteinizing hormone and follicle stimulating hormone, initiating the beginning stages of gonadal recrudescence and production of sex steroids, such as testosterone and estradiol. Gonadal sex steroid production is essential to prime the liver for secretion of yolk precursors in breeding female passerines. While the hypothalamus is capable of being stimulated during the pre-breeding period, egg development itself is likely regulated somewhere downstream of the hypothalamus. In this study we aimed to uncover mechanisms related with the onset of follicular maturation. It is possible that the ovary may become more sensitive to gonadotropins, and release higher levels of estradiol, or the liver may become more sensitive to estrogens. Alternatively, both the ovary and liver may become less sensitive to glucocorticoids, which could be inhibiting reproduction. We used GnRH challenges to examine variation in breeding-stage specific (pre-recruiting versus egg development stages) patterns of pituitary and ovarian responsiveness in free-living female dark-eyed juncos (Junco hyemalis). We also examined mRNA expression of several candidate genes in the gonad and liver to assess relative sensitivity to gonadotropins, estradiol, and stress hormones. Combined, our data suggest there is seasonal variation in female sensitivity downstream of the

hypothalamus, which may provide further insight into the physiological mechanisms regulating onset of clutch initiation.

Introduction

Most temperate-zone animals breed seasonally to ensure that offspring are born at an optimal time for rearing and survival (Baker, 1938; Murton and Westwood, 1977). Before young arrive, parents must undergo extensive physiological and behavioral preparation for breeding. Often, preparatory events must be initiated well in advance of seasonal increases in food availability (Gibb, 1950). Therefore, animals must be able to predict the onset of the breeding season by using local predictive environmental cues. While reproductive readiness is ultimately regulated by initial photoperiodic cues (Dawson et al., 2001), the exact onset of exponential gonadal growth is thought to be somewhat plastic and fine-tuned by supplementary cues (e.g., temperature and resource availability) (Marshall, 1959; Murton and Westwood, 1977; Wingfield et al., 1992; Wingfield and Kenagy, 1991).

Seasonal breeding has been extensively studied, and the neuroendocrine mechanisms controlling reproductive processes are becoming increasingly well understood (avian species reviewed in: (Dawson, 2008; Dawson et al., 2001; Williams, 2012)). Environmental cues are perceived and transduced at the level of the hypothalamus, where gonadotropin-releasing hormone (GnRH) neurons trigger the endocrine cascade resulting in gonadal activation (Dawson, 2008; Jacobs and Wingfield, 2000). More specifically, neural signals relay sensory information to the hypothalamo-pituitary-gonadal (HPG) axis, where the release of GnRH from the hypothalamus triggers secretion of gonadotropins, such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary. LH stimulates the synthesis and release of steroids such as testosterone (T) and estradiol (E_2) from the gonads (Adkins-Regan, 2008;

Wingfield, 2012). These hormones are then transported to target tissues where they bind to receptors, thus shaping physiological, morphological, and behavioral traits. However, studies on avian reproductive physiology have typically focused on males (Caro, 2012), despite the recognition that females often play a critical role in many important reproductive decisions, such as timing of egg laying (Caro et al., 2009). Therefore, it is likely selection acts differentially on males and females, and that sexes may differ substantially in the mechanisms underlying breeding phenology (Ball and Ketterson, 2008; Caro, 2012). Additionally, it is unknown whether seasonal, sex, or individual variation in sex steroid response to GnRH is attributable to the ability of the pituitary to respond to GnRH, or of the gonad to respond to LH.

In conjunction with the HPG axis' role in preparation for breeding, the hypothalamic-pituitary-adrenal (HPA) axis, more specifically secretion of corticosterone (CORT), plays an essential role in metabolism and energy regulation (Landys et al., 2006; Sapolsky et al., 2000). Egg laying in female birds is energetically costly (Ricklefs, 1974; Williams, 2005), in part due to the increased mass and metabolic activity of organs, for example, production of yolk precursors in the liver or recrudescence of reproductive organs, such as the ovary and oviduct (Williams, 2005). Metabolic fuels are required for gamete and sex steroid production, and periods of low energy intake (e.g., food restriction) can inhibit reproduction and sexual behavior (Schneider, 2004). Glucocorticoid production, essential for metabolic processes, varies in response to stress in a season-specific manner (Romero, 2002) and vertebrate gonads are physiologically capable of detecting stress cues through glucocorticoids receptors (Abraham et al., 2013; Bambino and Hsueh, 1981; Denari and Ceballos, 2006; Hsueh and Erickson, 1978; Kwok et al., 2007; Lattin et al., 2011). CORT ultimately affects energy mobilization via binding to two intracellular receptors, mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) (De Kloet et al., 1998; Funder, 1997). By quantifying relative expression of GR and MR in tissues essential for egg-laying (i.e., ovary and liver) at different stages of follicular maturation, we can assess an animal's capacity to respond to CORT-mediated metabolic demands (Lattin and Romero, 2015) and energy mobilization for reproductive processes.

In this study, we focused on a very specific time point in which wild dark-eyed junco (*Junco hyemalis*) females are transitioning into producing their first follicle hierarchy of the breeding season. To begin to search for meaningful variation in sensitivity at different levels of the reproductive axis downstream of the hypothalamus, we aimed to address two specific questions: 1) How do pituitary and gonadal sensitivity to GnRH and LH change as a follicle hierarchy is initiated? And 2) How do ovarian and liver sensitivity to glucocorticoids and gonadotropins change as birds are transitioning into a follicle hierarchy?

Methods

Study Species

The dark-eyed junco (*Junco hyemalis*) is a seasonally breeding songbird located throughout North America that breeds at high altitudes and latitudes (Nolan, 2002). Dark-eyed juncos are an excellent model organism because they have been studied extensively, are abundant, and are relatively easy to capture. Juncos are a ground-nesting species that lay eggs from May to June, and clutches range between 3–5 eggs (mean and median = 4) per nest attempt (Nolan, 2002). Our field site was located in the Black Hills National Forest, South Dakota, USA. This study was conducted in two phases, with one field season taking place in 2009 and the other in 2016 (see below for details). Fieldwork took place in April and early May, prior to the onset of the breeding season.

Trapping and Handling

All birds were captured passively in continuously monitored seed-baited mist-nets or walk-in traps for ~ 4 wks (4/20/2009-5/15/2009 and 4/17/2016-5/14/2016). Morphometric measures such as body mass (to nearest 0.1 g), condition, tarsus length (to nearest 0.1 mm), wing length, tail length, wing white, tail white, and presence/absence of a brood patch were recorded for all individuals. Both furcular and abdominal fat stores were visually scored on a 0 – 5 scale with a score of zero meaning no visible fat and five meaning visible bulging fat (Ketterson and Nolan, 1983; O'Neal et al., 2011). All individuals received a unique numbered metal band and color band combination to enable later identification. Females were differentiated from males by plumage differences during the very early breeding season and by the presence of a brood patch (female) or an enlarged cloacal protuberance (male) thereafter. Females were also examined for the presence of pronounced abdominal swelling indicative of egg development (Cain and Ketterson, 2012).

Blood Sampling

Baseline blood samples (~100 µL in 2009 for LH and T, <50 µL in 2016 for VLDL) were collected into heparinized microcapillary tubes from the alar vein. Immediately following an initial blood sample all individuals in both years (excluding pilot data females- see supplementary figure 1) received an intramuscular injection of chicken GnRH-I (American Peptide product #54-8-23, Sunnyvale, CA, USA) into the pectoralis muscle. Females received a dose of 1.25 µg dissolved in 50 µL PBS for a final concentration of 25 ng/µL (Bergeon Burns et al., 2014; Jawor et al., 2007, 2006). This dose is capable of fully activating the HPG axis in the dark-eyed junco (Jawor et al., 2006). Individuals were then held in cloth bags until blood was sampled at either 5, 15, or 30 min.

Post challenge blood samples varied as follows. LH levels were predicted to be elevated in circulation much sooner than sex steroid levels (e.g., 2 min following intrajugular injection on rufous-winged sparrows (Deviche et al., 2010)). In order to characterize the time course for LH response to an intramuscular injection of GnRH in juncos, females were randomly assigned to 5, 15, or 30 min delays between GnRH injection and post-challenge blood sampling during the 2009 field season. However, as the results of this time course were not yet known, once a sample size of 6 was reached for each time point, blood was sampled at 15 min post-GnRH injection for the remainder of the 2009 season (n = 40), and assayed for LH and T. T levels peak at 30 min following GnRH injection and decline to baseline levels within 2 hrs following injection (Jawor et al., 2006). T was assayed only when sufficient plasma (>30 μL) remained following apportionment for LH, so the sample sizes for LH analysis are larger than they are for T.

In 2016, females received a GnRH challenge and only a post-GnRH challenge blood sample was taken to measure E_2 levels during the pre-recruiting (n = 41) and rapid follicle growth (n = 18) stages. Therefore, each E_2 measurement represents a unique individual. All post-GnRH challenge blood samples were taken 30 min post-injection.

Pilot Data: To assess the ability of GnRH to increase E_2 levels, a subset of females was sampled for baseline circulating E_2 or post-GnRH challenge E_2 levels during the pre-recruiting (Baseline: n = 6; Post-GnRH: n = 6) and rapid follicle growth (Baseline: n = 5; Post-GnRH: n = 3) stages as pilot data (see supplementary Figure 1). Because E_2 measurement requires a large amount of plasma (>100 μL), individuals were only sampled for baseline circulating E_2 or post-GnRH E_2, never both. Therefore, each E_2 measurement represents a unique individual. Baseline E_2 levels were measured immediately following capture. As stated above, all post-GnRH challenge blood samples were taken 30 min post-injection. The rapid follicle growth post-GnRH group had lower capture rates (n = 3) and two females were removed from this group due to the finding that they had finished laying and had likely initiated incubation.

All blood samples were stored on ice until centrifugation to separate red blood cells from plasma. Plasma samples were stored at –20°C until further analysis.

Luteinizing Hormone (LH) Assay

Plasma LH was measured using the heterologous radioimmunoassay (RIA) that has been utilized extensively in songbirds (Follett et al., 1975, 1972; Sharp et al., 1987; Wingfield et al., 1991), including juncos (Bergeon Burns et al., 2014; Greives et al., 2016; Jawor et al., 2007, 2006; Rosvall et al., 2013). This RIA employed a double-antibody, post-precipitation process with antisera raised against purified chicken LH. Each sample was run in duplicate (20 µL each) on a single assay. The minimum detectable concentration was 0.078 ng/mL and intra-assay variation was 10.2 ± 1.3 %.

Testosterone (T) Assay

Plasma testosterone was assayed using an enzyme immunoassay (EIA) kit (Testosterone; ADI-901-065; Enzo Life Sciences, Farmingdale, NY, USA) as described previously (Clotfelter et al., 2004). Hormones were doubly extracted using diethyl ether, dried under nitrogen gas, and approximately 2000 cpm of 3H-T was added to each sample (40 µL) for determination of sample recovery. Extracts were then re-suspended in 50 µL ethanol and diluted with assay buffer (to a volume of 350 µL). Each sample was plated in duplicate (100 µL per well) following manufacturer's guidelines. Concentrations of testosterone levels were determined using a four-parameter logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories, Inc.). Average recovery of 3H-T after extraction was 90%, and T concentrations were corrected to reflect incomplete recovery. Intra-plate variations ranged from 5.0% – 13.2% (two plates: 1: 5.0 % and 2: 13.2 %) and inter-plate variation was 13.2 %.

Estradiol (E_2) Assay

Plasma E_2 was assayed using an EIA kit (17β–estradiol high sensitivity; ADI-900-174; Enzo Life Sciences, Farmingdale, NY, USA). This kit was previously validated in a songbird species (Gall et al., 2013; Wilcoxen et al., 2015). When available, 100 µL of plasma was used during extraction. A total of 19 samples out of 94 had less than 100 µL of plasma available for extraction. For samples where 100 µL of plasma was unavailable, plasma volume was recorded and the concentration was adjusted accordingly. Hormones were triply extracted using diethyl ether, dried under nitrogen gas, and reconstituted in assay buffer (260 µL). Each sample was plated in duplicate (100 µL per well) following the manufacturer's guidelines. Concentrations of E_2 levels were calculated using a four-parameter logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories, Inc.). Intra-plate variation ranged from 3.0 % – 8.8 % (three plates: 1: 4.2 %, 2: 3.0 %, 3: 8.8 %) and inter-plate variation was 5.4 %. Samples that were below detection limit of the assay were assigned a value of lowest kit sensitivity (14.0 pg/mL).

Very-Low-Density Lipoprotein (VLDL) Assay

VLDL levels were measured following previously described methods (Challenger et al., 2001; Mitchell and Carlisle, 1991; Needham et al., 2017; Salvante and Williams, 2003; Williams, 1999; Williams et al., 2003; Williams and Martyniuk, 2000). Total VLDL, using triglyceride methods, were originally developed in the domestic hen (Mitchell and Carlisle, 1991) and later validated in passerines (Challenger et al., 2001; Williams and Christians, 1997; Williams and Martyniuk, 2000). A non-breeder plasma pool (Sigma-Aldrich, P3266-1mL) was used for calculation of inter- and intra-assay variation. The intra-plate variation ranged from 0.6 % – 4.8 % (3 plates: 1: 4.8 %, 2: 0.6 %, 3: 3.5 %) and inter-plate variation was 3.0 %.

Breeding Stage Assignment

Females sampled in 2009 were assigned to either an early breeding or egg development stage by visual assessment (Cain and Ketterson, 2012), or backdating if the date of first egg was known, assuming a 12-day incubation period (Nolan, 2002). Females captured in early spring were categorized as "early breeding" if they showed no signs of a brood patch and no indication of egg development (early breeding; LH: n = 45, T: n = 23 samples). Females that demonstrated a pronounced and distinctive swelling of the abdomen characteristic of egg formation or presence of a brood patch at the time of capture were classified as "egg development" (egg development; LH: n = 14, T: n = 15).

In 2016, based on circulating VLDL levels, we divided females into two groups: 1) pre-recruiting stage, which is the period preceding rapid follicle growth (E_2: n = 44 samples), and 2) the rapid follicle growth stage (E_2: n = 16 samples) (Lamarre et al., 2017). The pre-recruiting stage of ovarian development is a slow process in which a large number of pre-vitellogenic "white" follicles are present in the ovary (Williams, 2012). The second stage, rapid follicle growth, can last from days to weeks. During this stage of ovarian development, the majority of proteins and lipids are added to yolk via uptake of yolk precursors from the blood and then produce large yolky yellow follicles (Williams, 2012). The secretion dynamics of these physiological parameters shifts, so that there is a marked increase in circulating VLDL at the rapid follicle growth stage to promote yolking in birds (Challenger et al., 2001; Hennin et al., 2014). This noted increase in VLDL aided in categorizing females into breeding stages in the current study while minimizing invasiveness (i.e., laparotomy).

Plasma levels of VLDL were evaluated in five individual male birds sampled in the pre-breeding period to assign an upper limit for classification of non-egg-producing individuals (Vanderkist et al., 2000), since males have basal levels of plasma VLDL (Mitchell and Carlisle, 1991; Williams and Christians, 1997). The highest value of VLDL observed in the sampled males was 0.96 mg/mL. In order to be conservative, we doubled this maximum value (Vanderkist et al., 2000), so that a female with a VLDL level below 1.92 mg/mL was considered to be in the pre-breeding stage. Thus, birds not in the rapid follicle growth stage (<1.92 mg/mL) are similar to pre-breeding females from the 2009 collection and females considered to be in the rapid follicle growth stage (>1.92 mg/mL) are intended to be equivalent to the egg development females in 2009. No plasma was available from 2009 for VLDL assays.

Tissue Processing and Molecular Methods

To assess mRNA expression of candidate genes in the liver and ovary, a subset of juncos were harvested off of the main study site for tissue. Birds were collected at two time points in 2016 that were anticipated to have females at pre-recruiting follicles (early breeding: April 25–27, 2016, n = 14; VLDL = 1.4 ± 0.09 mg/mL) or onset of rapid follicle growth (late breeding: May 12–14, which was several days after the first known egg of the population was laid, n = 8; VLDL = 3.4 ± 0.83 mg/mL) stages. Following morphometric measures, individuals received an overdose of isoflurane followed by rapid decapitation. Ovaries and livers were collected, rapidly frozen on powdered dry ice, and stored at –80 °C until RNA extraction. During late breeding collection, any ovaries with follicles that had entered a hierarchy were collected, stored, and analyzed separately from the ovary head (n = 3 ovaries).

Total RNA was extracted from tissue (entire ovary head; theca and granulosa layers of follicles; partial section of liver) using a RNAzol®RT isolation reagent (Sigma Aldrich, St.

Louis, MO) according to the manufacturer's instructions. Following spectrophotometry to quantify total RNA concentration and optical density, 1 μg was treated with TURBO DNAse and removal reagents as per manufacturer's guidelines (Life Technologies, Carlsbad, CA). After again determining RNA concentration, we then reverse transcribed 500 ng of RNA (ovary, follicles, or liver) using qScript reverse transcriptase and oligo(DT) primers (Quanta Biosciences, Beverly, MA) in a total reaction volume of 5 μL. To verify that samples were not contaminated with DNA, we ran a portion of our samples without reverse transcriptase.

The resulting cDNA was used as a template for quantitative real-time PCR (qPCR) to measure relative abundance of mRNA expression of estrogen receptor α (ERα), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) in the liver, as well as follicle-simulating hormone receptor (FSHR), luteinizing hormone receptor (LHR), GR, and MR in the ovary. We also quantified expression of PPIA and RPL4 reference genes (Zinzow-Kramer et al., 2014) for normalization of the expression of each gene of interest. We performed qPCR using a Stratagene Mx3000P machine equipped with MxPro software (v.4.10, Agilent, Santa Clara, CA, USA). Reactions (10 μL) were run in triplicate using 5 μL Perfecta SYBR green low ROX (Quanta Biosciences, no. 95056-100), 3 μL cDNA (diluted 1:40), and primers at a concentration of 0.3 μM, including no template controls (see supplementary material for primers). Thermocycling conditions for LHR, ERα, PPIA, and RPL4 reactions were as follows: 10 min at 95°C, 40 cycles of 95°C for 30s, 60°C for 1 min, and 72°C for 1 min. Thermocycling conditions for GR, MR, and FSHR reactions were as follows: 10 min at 95°C, 40 cycles of 95°C for 30s, 65°C for 1 min, and 72°C for 1 min. A final melting phase of 95°C for 1 min, 55°C for 30s, and 95°C for 30s was run for each primer to confirm single-product specificity of each sample.

A pooled sample of cDNA made for each tissue group (10 µL per liver and 15 µL per ovary sample from the first 10 females collected) was run on every qPCR plate, serving as a calibrator to which each individual sample was compared. An average of the two reference genes (PPIA and RPL4) was calculated for each individual by tissue type. We used the 2-ΔΔCt method of quantification (Livak and Schmittgen, 2001), in which the abundance of each gene of interest is expressed as the fold change expression relative to a pooled standard, normalized by the expression of the reference genes (PPIA and RPL4). We assessed amplification efficiencies for each gene using a 5-point standard dilution curve in MxPro (efficiencies ranged from 89.0% – 98.7%). See supplemental Table 1 for primer sequence information.

Statistical Analyses

All statistical analyses were performed using R v3.2.3 (R Core Team, 2014).

Time Course of LH Response to GnRH Challenge

To examine the time course of LH response to a GnRH challenge, we conducted a linear mixed-effects model, with treatment group (5, 15, or 30 min latency) and GnRH time point (pre- or post-GnRH injection) as fixed factors. All linear mixed effects models used package lme4 (Bates et al., 2015). Individual identity was included as a random repeated factor. A treatment group*GnRH time point interaction was included to ask whether the tendency to elevate LH in response to GnRH varied by time-course classification. To further probe this interaction, a Tukey's post-hoc analysis comparing a family of four estimates was performed to identify differences between the pilot study of initial and post-GnRH challenge LH within each time-course classification (5, 15, or 30 min.; Table 3.1).

Response of LH and T to GnRH Challenge

Baseline and 15 min post-GnRH LH and T levels (in separate models) were analyzed using a linear mixed-effects model, with breeding stage and blood sample time point (pre- or post-challenge) as fixed effects and the individual ID as a random effect to control for repeated measures from the same individual. A breeding stage*time point interaction was included to ask whether tendency to elevate LH or T in response to GnRH varied by stage of breeding. Tukey's post-hoc analyses explored the breeding stage*time point interactions, identifying pairwise differences between initial and post-GnRH challenge LH or T within each breeding stage (Table 3.2 and 3.3).

Breeding Stage Variation in E_2

To assess the ability of GnRH to maximize E_2 levels, we used a Welch's two sample t-test and tested for differences between GnRH-induced E_2 levels across breeding stages (pre-recruiting or rapid follicle growth). A Welch's two sample t-test was also used to test for a difference in VLDL levels between breeding stages.

Tissue Gene Expression

A linear model was performed with collection period as a fixed effect to test for differences in candidate gene expression between breeding stages on each tissue. Values are provided as means ± SEM.

Results

Time Course of LH Response to GnRH Challenge

There were significant main effects of both the treatment groups (5, 15, or 30 min latency; $F_{2,96} = 10.8$, $p < 0.0001$) and GnRH time point (pre- or post-GnRH injection; $F_{1,50} = 30.4$, $p < 0.0001$) on LH, as well as a significant interaction between treatment group and GnRH time point ($F_{2,50} = 11.6$, $p < 0.0001$). Post-hoc tests revealed that LH was significantly elevated from pre-injection levels at 5 ($p < 0.001$; Pre-injection: 1.7 ± 0.53 ng/mL, n = 6; Post-injection: 4.8 ± 0.98 ng/mL, n = 6) and 15 ($p = 0.001$; Pre-injection: 1.4 ± 0.19 ng/mL, n = 40; Post-injection: 2.2 ± 0.21 ng/mL, n = 40) min post-challenge, but did not differ from pre-injection levels by 30 minutes post-challenge ($p = 1.000$; Pre-injection: 0.9 ± 0.24 ng/mL, n = 6; Post-injection: 0.9 ± 0.20 ng/mL, n = 6) (Fig. 3.1).

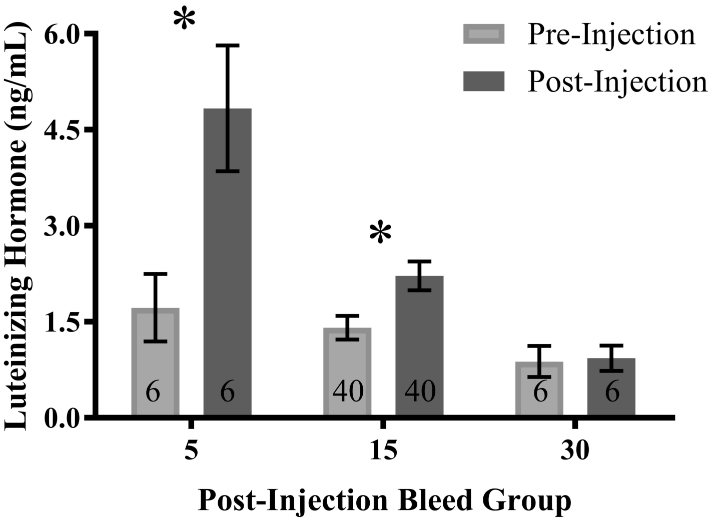

Figure 3.1. Time course of female luteinizing hormone response to a GnRH challenge. Females were sampled for initial LH levels, injected with GnRH, and bled again at 5, 15, or 30 min post-injection. Figure shows means ± SEM.

Breeding Stage Variation of LH and T in Response to a GnRH Challenge

There was a significant main effect of time point (pre-injection or 15 min post injection) ($F_{1,51} = 13.6$, $p < 0.001$) and of breeding stage (early breeding or egg development) ($F_{1,40} = 11.1$, $p < 0.010$) on LH levels. There was also a significant interaction between time point and breeding stage ($F_{1,51} = 4.3$, $p = 0.044$) on LH levels. Baseline LH levels significantly increased from early breeding to egg development ($t = –3.855$, df = 53, $p = 0.002$). Post-hoc pairwise comparisons within each breeding stage revealed that following a GnRH challenge, females significantly elevated LH during early breeding ($t = –5.53$, $df = 52$, $p < 0.0001$; pre-injection: 1.0 ± 0.15 ng/mL; post-injection: 2.0 ± 0.21 ng/mL), but did not increase LH from initial levels during egg development ($t = –0.95$, $df = 52$, $p = 0.778$; pre-injection: 2.8 ± 0.54 ng/mL; post-injection: 3.1 ± 0.54 ng/mL). Post-GnRH challenge LH levels did not significantly differ across breeding stages ($t = –2.35$, $df = 53$, $p = 0.100$) (Fig. 3.2).

There was a significant main effect of time point (pre-injection or 15 min post injection) ($F_{1,32} = 28.7$, $p < 0.0001$), but not of breeding stage (early breeding or egg development) ($F_{1,32} = 0.03$, $p = 0.875$) on T levels. There was not a significant interaction between time point and breeding stage ($F_{1,32} = 0.12$, $p = 0.731$) on T levels. Post-hoc pairwise comparisons within each breeding stage revealed that following a GnRH challenge, females significantly elevated T from initial levels during both early breeding ($t = –4.58$, $df = 26$, $p = 0.001$; pre-injection: 0.5 ± 0.02 ng/mL; post-injection: 0.7 ± 0.05 ng/mL) and egg development ($t = –3.08$, $df = 19$, $p = 0.029$; pre-injection: 0.5 ± 0.05 ng/mL; post-injection: 0.7 ± 0.06 ng/mL). Baseline T levels did not significantly differ between breeding stages ($t = –0.40$, $df = 32$, $p = 0.978$). Post-GnRH T levels also did not significantly increase across breeding stages ($t = 0.12$, $df = 31$, $p = 0.999$) (Fig. 3.2).

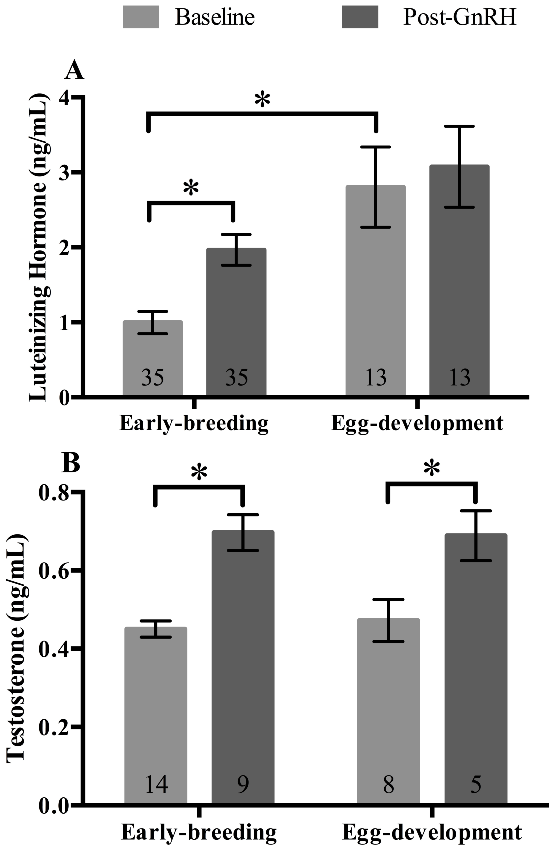

Figure 3.2. Luteinizing hormone and testosterone readily respond to a GnRH challenge in the early breeding season. Breeding stage variation in (A) luteinizing hormone and (B) testosterone responses to GnRH challenges in the female dark-eyed junco (*Junco hyemalis*). Figures show means ± SEM. Significant differences between breeding stages and time points are denoted with asterisks (*).

Breeding Stage Variation in E_2 and VLDL

GnRH-induced E_2 levels did not significantly differ across the two breeding stages (Pre-recruiting: 43.6 ± 4.33 pg/mL; Rapid follicle growth: 64.2 ± 11.67 pg/mL; $t = -1.65$, $df = 18$, $p = 0.116$; Fig. 3.3A). VLDL levels were significantly different between breeding stages because we assigned a cut-off value to categorize females as either pre-recruiting or rapid follicle growth ($t = -11.19$, $df = 39$, $p < 0.0001$; pre-recruiting: 1.3 ± 0.05 mg/mL; rapid follicle growth: 2.4 ± 0.08 mg/mL; Fig. 3.3B).

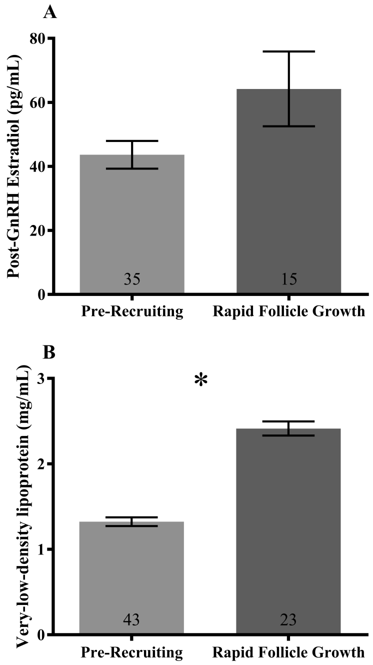

Figure 3.3. Females did not significantly elevate GnRH-induced levels of estradiol (A), but did significantly elevate very-low-density lipoprotein levels (VLDL; B) in the rapid follicle growth period of preparation for breeding. Each measurement represents a unique individual female dark-eyed junco (Junco hyemalis). Figure shows mean ± SEM. Significant difference between breeding stage is denoted with asterisk (*).

Ovary and Liver Gene Expression

In the liver, GR, MR, and ERα mRNA expression levels were significantly higher during the pre-recruiting stage, as compared to rapid follicle growth stage (GR: $t = -3.85$, $F_{1,20} = 14.72$, $p = 0.001$; early-season: $\bar{X} = 1.7 \pm 0.17$; late-season: $\bar{X} = 0.8 \pm 0.14$; MR: $t = -6.56$, $F_{1,20} = 43.03$, $p < 0.0001$; early-season: $\bar{X} = 1.4 \pm 0.10$; late-season: $\bar{X} = 0.5 \pm 0.07$; ERα: $t = -4.07$, $F_{1,20} = 16.53$, $p < 0.001$; early-season: $\bar{X} = 1.6 \pm 0.21$; late-season: $\bar{X} = 0.5 \pm 0.09$; Fig. 3.4).

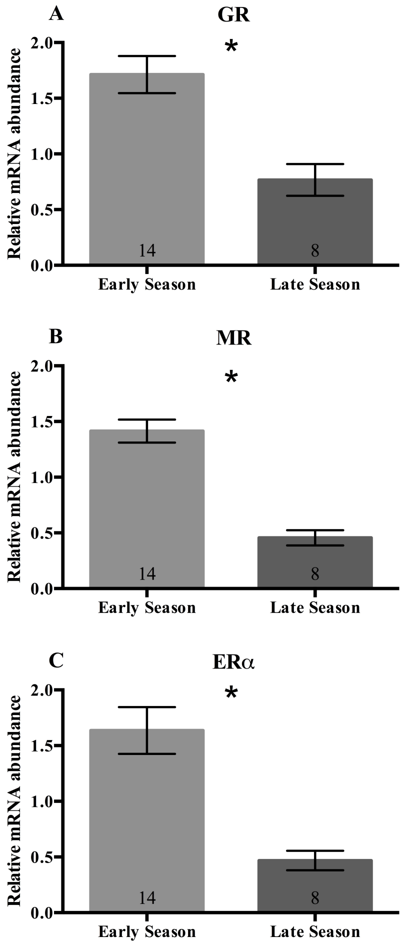

Figure 3.4. mRNA expression of candidate genes in the liver are higher in the early season than in late season when female dark-eyed juncos (*Junco hyemalis*) enter rapid follicle growth. Liver gene expression is shown across breeding stages for (A) glucocorticoid receptor, (B) mineralocorticoid receptor, and (C) estrogen receptor α. Gene expression is unit-less and quantified on a log2-fold change relative to an arbitrary calibrator. Figures show means ± SEM. Significant differences between breeding stages are denoted with asterisks (*).

In the ovary, both FSHR and GR mRNA expression levels were higher during the pre-recruiting stage, compared to females initiating rapid follicle growth (FSHR: $t = –3.36$, $F_{2,22} = 9.14$, $p = 0.003$; early-season ovary: $\bar{X} = 2.0 \pm 0.27$; late-season ovary: $\bar{X} = 0.8 \pm 0.17$; GR: $t = –2.73$, $F_{2,22} = 5.67$, $p = 0.012$; early-season ovary: $\bar{X} = 0.9 \pm 0.14$; late-season ovary: $\bar{X} = 0.4 \pm 0.05$; Fig. 3.5). Similarly, FSHR and GR mRNA expression levels were higher during the pre-recruiting stage, compared to hierarchy follicles from the late collection (FSHR: $t = –3.42$, $F_{2,22} =$

9.14, p = 0.003, $\bar{X}$ = 0.2 ± 0.10; GR: t = –2.60, $F_{2,22}$ = 5.67, p = 0.016, $\bar{X}$ = 0.2 ± 0.09; Fig. 3.5). The mRNA expression levels in the ovary did not significantly change between pre-recruiting and rapid follicle growth stages for LHR (t = 0.093, $F_{2,22}$ = 16.13, p = 0.926; early-season ovary: $\bar{X}$ = 0.1 ± 0.03; late-season ovary: $\bar{X}$ = 0.2 ± 0.07; Fig. 3.5) and MR (t = –1.48, $F_{2,22}$ = 1.18, p = 0.154; early-season ovary: $\bar{X}$ = 2.0 ± 0.35; late-season ovary: $\bar{X}$ = 1.2 ± 0.35; Fig. 3.5). However, LHR mRNA expression levels were lower during the pre-recruiting stage compared to hierarchy follicles from the late collection (t = 5.52, $F_{2,22}$ = 16.13, p < 0.0001, $\bar{X}$ = 3.4 ± 1.75; Fig. 3.5). There was no change in MR mRNA expression between pre-recruiting and hierarchy follicles (t = –0.79, $F_{2,22}$ = 1.18, p = 0.440, $\bar{X}$ = 1.4 ± 0.97; Fig. 3.5).

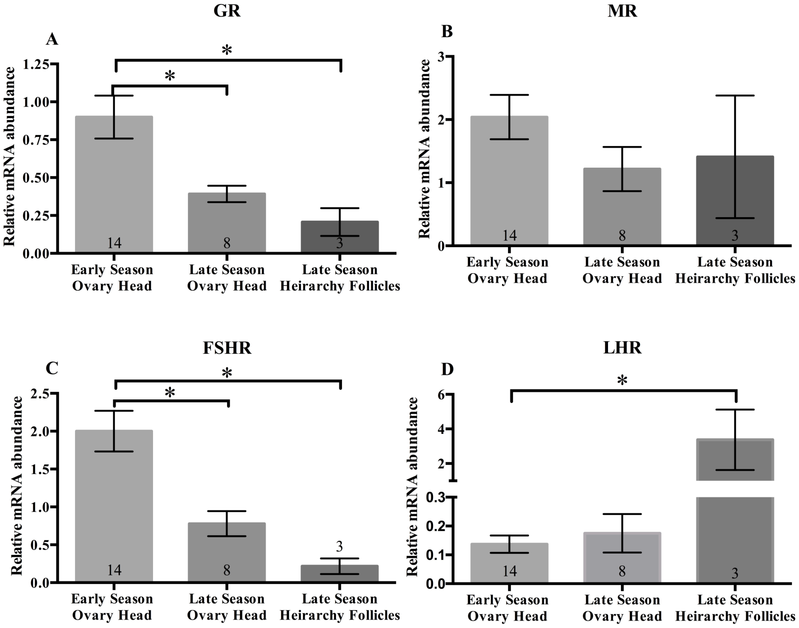

Figure 3.5. mRNA expression in the ovary is higher for glucocorticoid receptor and follicle-stimulating hormone receptor in the early season compared to the late season, when female dark-eyed juncos (*Junco hyemalis*) enter rapid follicle growth. Ovary gene expression is shown across breeding stages for (A) glucocorticoid receptor, (B) mineralocorticoid receptor, (C) follicle stimulating hormone receptor, and (D) luteinizing hormone receptor. During the late season, all hierarchy follicles were separated from the ovary head and follicle layers were combined into one tissue sample. Gene expression is unit-less and quantified on a log2-fold change relative to an arbitrary calibrator. Figures show means ± SEM. Significant differences between breeding stages are denoted with asterisk (*).

Discussion

Mechanisms regulating seasonal reproduction in songbirds have received far more attention in males than in females (Caro, 2012; Dawson, 2015, 2003), despite the recognition that females play a critical role in timing of breeding (Ball and Ketterson, 2008; Caro et al., 2009). In particular, organization of the female HPG axis in the period prior to breeding remains understudied. To date we know the HPG axis is important for yolk formation, but we don't know the exact mechanisms dictating onset of egg production as females make this transition. Here, we assessed whether there are changes in the HPG axis, downstream of the hypothalamus, at a very specific and crucial time point when female birds transition from partial (i.e., recrudesced gonads) to full reproductive status (i.e., yolking and egg development).

We found that LH and T are responsive to exogenous GnRH in the early-breeding stage. Only T is capable of elevating levels in response to exogenous GnRH during egg development. Baseline LH levels are high in the egg-development stage of the pre-breeding season, and females are likely not capable of further responding to exogenous GnRH. In the liver, relative mRNA expression levels were higher in the early season compared to late season levels. Ovarian mRNA expression showed a similar pattern for GR and FSHR, but levels did not significantly change in the ovary head for MR and LHR. Combined, our data suggest that downstream of the hypothalamus, there are multiple changes in HPG-axis as female songbirds make this critical transition. Our data also support the previous finding that captive dark-eyed junco females are capable of pituitary and gonadal hormonal responses in the pre-breeding stage, even in the absence of yolky follicles (Rosvall et al., 2013).

To begin to evaluate the HPG axis' sensitivity to upstream regulation by the hypothalamus at the key time point when females are shifting into egg development, we probed the pituitary's response to exogenous GnRH. Baseline LH levels were significantly lower compared to GnRH-induced LH levels during pre-breeding, but not during egg development. In two previous studies in dark-eyed junco females it was found that LH levels during the pre-breeding stage showed an elevation in response to GnRH stimulation (Greives et al., 2016; Jawor et al., 2007) similar to LH responsiveness in the current study. The lack of difference between initial and post-challenge LH levels during egg development in our study is likely attributable to a ceiling effect, where LH was already at maximal secretion, thus leaving little scope for further increase. Alternatively, post-GnRH LH levels at 5 min showed the greatest response to exogenous GnRH; therefore, it is possible that some individual variation may have been lost at the 15 min measure, which is when LH measurements were taken for seasonal comparisons. Future work should utilize the 5 min post-GnRH measure of LH to fully evaluate the maximum capacity of the pituitary to respond to exogenous GnRH. These data reflect a stage-specific variation in LH response to GnRH.

It appears that baseline LH levels remain low until hypothalamic GnRH release is increased in females that are closer to developing a follicle hierarchy, likely to aid in initiation of yolking. Furthermore, our LH data suggests that the pituitary is 'online' and responsive to GnRH across pre-breeding stages. However, variation in baseline levels may indicate a degree of plasticity in the system that enables a more appropriate response to varying environmental conditions (e.g., temperature (Schaper et al., 2011)) at different stages of the reproductive season due to endogenous GnRH release.

Downstream of the pituitary, we tested whether the gonads are responsive to stimulation prior to and during initiating a follicle hierarchy. We found a significant increase from baseline T levels following a GnRH challenge during both the early breeding and egg development stages.

However, neither baseline nor post-GnRH T significantly differed across the two breeding stages. Similar to our findings, female Northern cardinals (*Cardinalis cardinalis*) have been shown to elevate T in response to GnRH prior to egg development, during the non-breeding season (i.e., " reproductive quiescence") (DeVries et al., 2011), presumably also stimulated via increased LH release. In contrast to our findings, a study on dark-eyed juncos breeding in Virginia, where T was measured at 30 min after a GnRH challenge, T significantly elevated only during the egg development stage, but not during the earlier pre-breeding stage (Jawor et al., 2007).

The breeding environment in South Dakota is thought to be more extreme than in Virginia (Bergeon Burns et al., 2014), which could result in a delay in laying despite physiological readiness to breed in South Dakota. In white-crowned sparrows (*Zonotrichia leucophrys*), subspecies experiencing less predictable environments appear to be more sensitive to temperature cues, perhaps to fine-tune timing of breeding. This sensitivity to temperature is reflected in ovarian development, however, and not in seasonal circulating LH levels (Wingfield et al., 2003, 1997, 1996). An alternative, but not mutually exclusive explanation for this apparent difference in a female junco's capacity to elevate T in response to GnRH during pre-breeding, is a potential difference between subspecies in sensitivity to the same supplementary cues, such as temperature, food availability, and social cues. In two separate studies comparing male junco subspecies, it was found that circulating hormone levels of T did not differ and therefore may be less prone to evolutionary change than the responsiveness of individual hormone targets (Bergeon Burns et al., 2014; Burns et al., 2013).

During the breeding season, the ability to produce short-term increases in T may provide fitness benefits to females. For example, previous work in female dark-eyed juncos found a positive relationship between T response to GnRH during egg development and aggression toward a same-sex intruder, and more aggressive females had a greater likelihood of producing a nest that was successful (Cain and Ketterson, 2012). During the early breeding season, elevated T in females may also facilitate mating behavior (Leboucher et al., 1998). E_2 plays a central role in female reproduction (Williams, 2012) and is converted from T by one enzymatically-catalyzed reaction step (aromatase). In a previous dark-eyed junco study, post-GnRH challenge T and E_2 levels were positively correlated when pooled E_2 samples were used (Rosvall et al., 2013). Thus, T and E_2 could be co-regulated due to shared dependence on upstream components of the endocrine cascade (i.e., hypothalamus and pituitary).

In the current study, GnRH-induced E_2 levels did not significantly differ across breeding stages (pre-recruiting and rapid follicle growth). This coincides with GnRH-induced testosterone levels in this study, which also did not significantly elevate across breeding stages. In the dark-eyed junco, post-injection T and E_2 levels were positively correlated when pooled E_2 samples were used (Rosvall et al., 2013). Therefore, T and E_2 levels could be co-regulated and therefore co-evolve due to shared dependence on upstream stimulation of the HPG axis (Rosvall et al., 2013). It has been suggested that approximately 87.5% of estradiol output is attributed to ovarian tissue containing follicles less than 5 mm in diameter (Senior and Furr, 1975). It is likely that further capacity by the ovaries to respond to exogenous GnRH challenges in the rapid follicle growth stage is unnecessary for gonadal maturation. All E_2 levels reported in this study were from unique individuals, which did not allow us to look at an individual's response to GnRH stimulation. Further work should be done in larger birds to look at an individual female's ability to respond to exogenous GnRH across the breeding season.

While we were unable to measure baseline levels of E_2 due to a limitation in plasma volume, we were able to collect pilot data from unique individuals to assess the ability of the ovary to respond to GnRH across the two breeding stages and compare that with circulating (baseline) levels of E_2 at the same time points. This pilot data (supplementary material figure 1) shows that circulating E_2 levels were lower during the pre-recruiting stage than in the rapid follicle growth stage. Similarly, a previous study in European starlings (*Sturnus vulgaris*) found that circulating E_2 remains at low levels until the stage of rapid yolk development, and reaches maximal levels when birds have complete follicle hierarchies (Williams et al., 2004). Maintaining low levels of circulating E_2 until initiation of rapid follicle growth could be a beneficial mechanism to minimize negative effects from elevated sex steroid levels over long periods of time. In the pre-recruiting stage the ovaries appear to respond robustly to a GnRH challenge, but in the rapid follicle growth GnRH-induced levels of E_2 were not significantly higher than circulating levels. Sample sizes for each grouping is low and especially for the GnRH challenged females during rapid follicle growth, which may account for the large variation and lack of significant difference noted here.

Rapid follicle growth occurs quickly in preparation for ovulation in birds (Bêty et al., 2003; Drent and Daan, 1980; Rowe et al., 1994; Williams, 2012). We aimed to explore variability in liver yolk-precursor secretion, its relationship with the ability of the ovary to secrete E_2, and lay date (a poorly understood relationship (Caro et al., 2013a, 2013b)). To accomplish this aim, we looked at how mRNA expression of gonadotropins and glucocorticoids change in these two tissues as birds initiate rapid follicle growth for egg development. In the liver, all genes (GR, MR, and ERα) were expressed at higher levels in the pre-recruiting period of ovarian development compared to the rapid follicle growth stage. Measurement of MR and

GR expression gives an indication of gonadal and liver sensitivity to glucocorticoids (CORT). Specifically, MR has a higher affinity for CORT and is therefore thought to mediate responses to lower levels of circulating CORT. Both GR and MR receptors influence the response to elevated CORT levels during an acute stressor (Hu et al., 2008; Landys et al., 2006). Higher GR and MR allows the liver to be sensitive to stressors, so that yolk precursor synthesis cannot be activated until the female is experiencing a relatively non-stressful environment, or until sensitivity has decreased. However, if high GR and MR expression is indicative of future GR and MR abundance (which would be likely during rapid follicle growth), then perhaps the liver is getting primed for this energetically expensive task.

By acting directly at the level of the gonad, CORT can influence gonadal steroidogenesis by suppressing transcription and translation of steroidogenic enzymes (Hu et al., 2008) without affecting LH secretion from the pituitary (Deviche et al., 2014, 2012; McGuire et al., 2013; Wingfield et al., 1982). In the European starling, the gonads of both sexes were able to directly detect cues to stress and respond to these cues in a season-specific manner by modulating sex steroid secretion (McGuire et al., 2013). Production of ovarian steroid hormones are essential for initiation of vitellogenesis by the liver (Johnson and Woods, 2007). Sex steroid hormones are also important for protein synthesis to transition from generic VLDL to VLDLy yolk precursor synthesis is estrogen-dependent (Mitchell and Carlisle, 1991; Wallace, 1985; Walzem, 1996; Williams and Martyniuk, 2000) via the ERα receptor. A complex relay of gene expression and cell signaling within the follicles occurs during the transition to rapid follicle growth and results in increased sex steroid production and secretion. High expression levels of ERα in the early collection period is indicative of the liver preparing for the ovaries' signal that it is time to start increasing yolk precursors production.

Ovarian GR levels were highly expressed in the pre-recruiting stage compared to rapid follicle growth stage and MR expression did not change across the two breeding stages. MR has a 6 to 10-fold higher affinity for CORT than GR, suggesting a two-tier system for CORT binding under baseline and stress-induced concentrations of hormone (De Kloet et al., 1998, 1990; Reul and Kloet, 1985). Due to GR's lower affinity for binding CORT, it is likely more important for reacting to the HPA-axis, which may explain why it is differentially expressed across the two breeding stages.

Vertebrates display seasonal changes in circulating (unstressed and stressed) glucocorticoid release and in many species, including birds, and glucocorticoid concentrations are commonly elevated during the breeding season (Romero, 2002). One explanation for changes in GR across the season is the ovary's requirement for energy mobilization as it gears up for an energetically expensive process. In the early stages of gonadal recrudescence individuals face high metabolic costs (Vézina and Salvante, 2010) and supplementary cues are important in making the decision to delay or advance the onset of breeding (Ball, 1993; Dawson, 2008; Hinde and Steel, 1976; Wingfield and Sapolsky, 2003). Once an individual is committed to breeding supplementary cues become less influential (Ball, 1993; Dawson, 2008; Hinde and Steel, 1976; Wingfield and Sapolsky, 2003) and the gonads becoming capable of dampening responsiveness to stress hormone and metabolic stress (McGuire et al., 2013). Modulation of the response to stress is likely more important in the period prior to onset of full gonadal recrudescence because the gonads are not yet maximally stimulated by components of the HPG axis, such as gonadotropins (LH and FSH). Therefore, the ovary should be sensitive to stressors during the pre-breeding period, so that an individual avoids initiating egg development too early and risks a mis-timed breeding attempt.

FSHR was highly expressed in the pre-recruiting stage of follicle growth, but showed lower levels during rapid follicle growth. Follicle-stimulating hormone receptor mRNA in the granulosa layer is the earliest marker of selection of follicle recruitment (Woods and Johnson, 2005). Increasing FSH responsiveness is thought to lead to expression of luteinizing hormone receptor mRNA. Similar to our findings, previous research in the European starling showed elevated FSHR expression when follicles were developing, followed by a decline as follicles continued to grow (Perfito et al., 2014), which is consistent with results in chickens (Zhang et al., 1997). Previous work has also demonstrated a decline in FSH binding as follicles develop, suggesting that the mature protein for FSHR is similarly declining (Bahr and Johnson, 1984).

Expression of LHR did not change across the two breeding stages in our population. In contrast to our findings, expression of LHR increased drastically across the pre-breeding season and remained elevated until follicles were nearing ovulation in the European starling (Perfito et al., 2014). However, this study did not separate out follicles, and instead measured the entire ovary intact. In our study, the ovary head, made up of all small white follicles, did not show an increase in LHR mRNA expression across the pre-breeding season, but the hierarchy follicles show a large elevation in LHR mRNA expression. However, there was a small sample size for hierarchy follicle samples. Similar to the study in European starlings, a study in chickens found that LHR mRNA and LHR binding continue to increase as follicles mature (Zhang et al., 1997). However, LHR transcript (mRNA) abundance may not impact actual sensitivity to LH (Bergeon Burns et al., 2014) because it is indicative of future LHR abundance and not of current receptor abundance. It is possible that the ovary and liver maintain high mRNA expression levels of key genes in the period prior to rapid follicle growth to halt further ovarian development until environmental cues signal appropriate timing. It is also important to note that we cannot assume transcript abundance always reflects actual protein abundance, and sensitivity can be affected by variables other than receptor density (Bergeon Burns et al., 2014).

In summary, we found that (1) both the pituitary and gonads are responsive to GnRH, (2) circulating LH levels are higher during rapid follicle growth, (3) GnRH-induced levels of E_2 do not change over the pre-breeding season, and (4) mRNA expression suggests that both the liver and ovary change their responsiveness to stress hormones and gonadotropins during this critical time period. Prior to developing a follicle hierarchy, both the pituitary and gonads show evidence of sensitivity to upstream stimulation by the HPG-axis. Therefore, it is possible that the communication between the ovary and liver is a potential mechanism regulating onset of egg development. Gonadotropin-inhibitory hormone (GnIH) is another potential mechanism, as it has been shown to act not just at the level of the hypothalamus, but also directly on the pituitary and gonad (Ubuka and Bentley, 2009). Future research should focus on identifying if the stage of initial cell proliferation is determining subsequent steroidogenic and/or yolk uptake capacity during rapid follicle growth (Williams, 2012).

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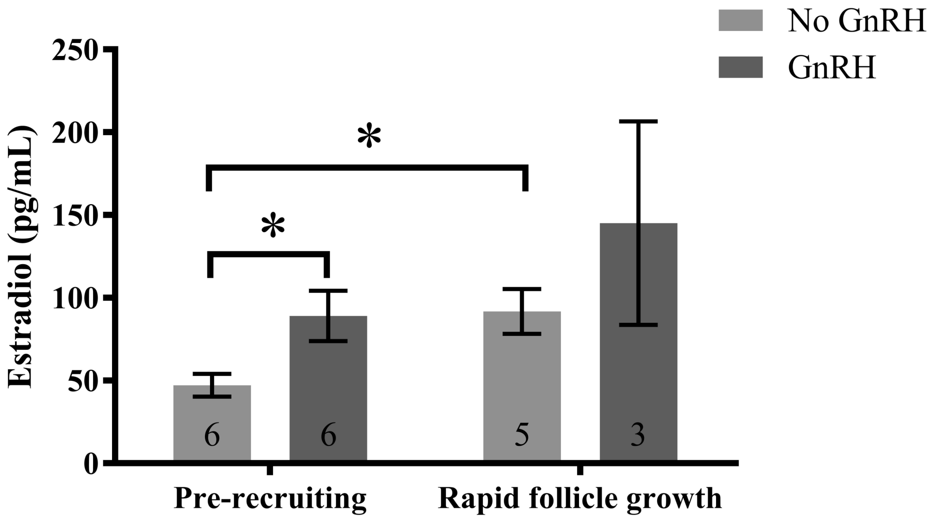

Figure 3.6. Pilot data of circulating E_2 levels compared to GnRH-induced E_2 levels across breeding stages. Each measurement represents a unique individual female dark-eyed junco (*Junco hyemalis*). During the pre-recruiting stage, circulating E_2 (also referred to as "no GnRH") levels were significantly lower compared to GnRH-induced E_2 levels (No GnRH: 47.3 ± 6.88 pg/mL; GnRH: 89.0 ± 15.19 pg/mL; t = 2.74, df = 7.4, p = 0.027). Circulating E_2 levels did not significantly differ from GnRH-induced E_2 levels during the rapid follicle growth stage (No GnRH: 91.8 ± 13.47 pg/mL; GnRH: 145.1 ± 61.42 pg/mL; t = 1.29, df = 3.6, p = 0.274; Fig. 4). During the pre-recruiting stage, circulating E_2 levels were significantly lower compared to the rapid follicle growth stage (t = –3.19, df = 6.5, p = 0.017). However, GnRH-induced E_2 levels did not significantly differ across breeding stages (t = –1.34, df = 3.7, p = 0.257). Figure shows mean ± SEM. Significant difference between breeding stage is denoted with asterisk (*).

References

Abraham, S.B., Rubino, D., Sinaii, N., Ramsey, S., Nieman, L.K., 2013. Cortisol, obesity, and the metabolic syndrome: A cross-sectional study of obese subjects and review of the literature. Obesity 21, E105–E117.

Adkins-Regan, E., 2008. Do hormonal control systems produce evolutionary inertia? Philos. Trans. R. Soc. Lond. B Biol. Sci. 363, 1599–1609.

Bahr, J.M., Johnson, A.L., 1984. Regulation of the follicular hierarchy and ovulation. J. Exp. Zool. Part Ecol. Genet. Physiol. 232, 495–500.

Baker, J.R., 1938. The evolution of breeding seasons. Evolution 161, 177.

Ball, G.F., 1993. The neural integration of environmental information by seasonally breeding birds. Am. Zool. 33, 185–199.

Ball, G.F., Ketterson, E.D., 2008. Sex differences in the response to environmental cues regulating seasonal reproduction in birds. Philos. Trans. R. Soc. B Biol. Sci. 363, 231–246.

Bambino, T.H., Hsueh, A.J., 1981. Direct inhibitory effect of glucocorticoids upon testicular luteinizing hormone receptor and steroidogenesis in vivo and in vitro. Endocrinology 108, 2142–2148.

Bates, D., Maechler, M., Bolker, B., Walker, S., Christensen, R.H.B., Singmann, H., Dai, B., Grothendieck, G., Eigen, C., Rcpp, L., 2015. Package "lme4." convergence 12, 1.

Bergeon Burns, C.M., Rosvall, K.A., Hahn, T.P., Demas, G.E., Ketterson, E.D., 2014. Examining sources of variation in HPG axis function among individuals and populations of the dark-eyed junco. Horm. Behav. 65, 179–187. doi:10.1016/j.yhbeh.2013.10.006

Bêty, J., Gauthier, G., Giroux, J., 2003. Body condition, migration, and timing of reproduction in snow geese: a test of the condition-dependent model of optimal clutch size. Am. Nat. 162, 110–121.

Burns, B., Rosvall, K.A., Ketterson, E.D., 2013. Neural steroid sensitivity and aggression: comparing individuals of two songbird subspecies. J. Evol. Biol. 26, 820–831.

Cain, K.E., Ketterson, E.D., 2012. Competitive females are successful females; phenotype, mechanism, and selection in a common songbird. Behav. Ecol. Sociobiol. 66, 241–252.

Caro, S.P., 2012. Avian ecologists and physiologists have different sexual preferences. Gen. Comp. Endocrinol. 176, 1–8.

Caro, S.P., Charmantier, A., Lambrechts, M.M., Blondel, J., Balthazart, J., Williams, T.D., 2009. Local adaptation of timing of reproduction: females are in the driver's seat. Funct. Ecol. 23, 172–179.

Caro, S.P., Schaper, S.V., Dawson, A., Sharp, P.J., Gienapp, P., Visser, M.E., 2013a. Is microevolution the only emergency exit in a warming world? Temperature influences egg laying but not its underlying mechanisms in great tits. Gen. Comp. Endocrinol. 190, 164–169.

Caro, S.P., Schaper, S.V., Hut, R.A., Ball, G.F., Visser, M.E., 2013b. The case of the missing mechanism: how does temperature influence seasonal timing in endotherms? PLoS Biol. 11, e1001517.

Challenger, W.O., Williams, T.D., Christians, J.K., Vézina, F., 2001. Follicular development and plasma yolk precursor dynamics through the laying cycle in the European starling (*Sturnus vulgaris*). Physiol. Biochem. Zool. 74, 356–365.

Clotfelter, E.D., O'neal, D.M., Gaudioso, J.M., Casto, J.M., Parker-Renga, I.M., Snajdr, E.A., Duffy, D.L., Nolan, V., Ketterson, E.D., 2004. Consequences of elevating plasma testosterone in females of a socially monogamous songbird: evidence of constraints on male evolution? Horm. Behav. 46, 171–178.

Dawson, A., 2015. Annual gonadal cycles in birds: modeling the effects of photoperiod on seasonal changes in GnRH-1 secretion. Front. Neuroendocrinol. 37, 52–64.

Dawson, A., 2008. Control of the annual cycle in birds: endocrine constraints and plasticity in response to ecological variability. Philos. Trans. R. Soc. Lond. B Biol. Sci. 363, 1621–1633.

Dawson, A., 2003. Photoperiodic control of the annual cycle in birds and comparison with mammals. Ardea 90, 355–367.

Dawson, A., King, V.M., Bentley, G.E., Ball, G.F., 2001. Photoperiodic control of seasonality in birds. J. Biol. Rhythms 16, 365–380.

De Kloet, E.R., Reul, J., Sutanto, W., 1990. Corticosteroids and the brain. J. Steroid Biochem. Mol. Biol. 37, 387–394.

De Kloet, E.R., Vreugdenhil, E., Oitzl, M.S., Joëls, M., 1998. Brain corticosteroid receptor balance in health and disease. Endocr. Rev. 19, 269–301.

Denari, D., Ceballos, N.R., 2006. Cytosolic glucocorticoid receptor in the testis of *Bufo arenarum*: seasonal changes in its binding parameters. Gen. Comp. Endocrinol. 147, 247–254.

Deviche, P., Beouche-Helias, B., Davies, S., Gao, S., Lane, S., Valle, S., 2014. Regulation of plasma testosterone, corticosterone, and metabolites in response to stress, reproductive stage, and social challenges in a desert male songbird. Gen. Comp. Endocrinol. 203, 120–131.

Deviche, P., Gao, S., Davies, S., Sharp, P.J., Dawson, A., 2012. Rapid stress-induced inhibition of plasma testosterone in free-ranging male rufous-winged sparrows, Peucaea carpalis: characterization, time course, and recovery. Gen. Comp. Endocrinol. 177, 1–8.

Deviche, P.J., Hurley, L.L., Fokidis, H.B., Lerbour, B., Silverin, B., Silverin, B., Sabo, J., Sharp, P.J., 2010. Acute stress rapidly decreases plasma testosterone in a free-ranging male songbird: potential site of action and mechanism. Gen. Comp. Endocrinol. 169, 82–90.

DeVries, M.S., Holbrook, A.L., Winters, C.P., Jawor, J.M., 2011. Non-breeding gonadal testosterone production of male and female Northern Cardinals (*Cardinalis cardinalis*) following GnRH challenge. Gen. Comp. Endocrinol. 174, 370–378.

Drent, R.H., Daan, S., 1980. The Prudent Parent: Energetic Adjustments in Avian Breeding 1. Ardea 68, 225–252.

Follett, B.K., Farner, D.S., Mattocks, P.W., 1975. Luteinizing hormone in the plasma of white-crowned sparrows (*Zonotrichia leucophrys gambelii*) during artificial photostimulation. Gen. Comp. Endocrinol. 26, 126–134.

Follett, B.K., Scanes, C.G., Cunningham, F.J., 1972. A radioimmunoassay for avian luteinizing hormone. J. Endocrinol. 52, 359–378.

Funder, J.W., 1997. Glucocorticoid and mineralocorticoid receptors: biology and clinical relevance. Annu. Rev. Med. 48, 231–240.

Gall, M.D., Salameh, T.S., Lucas, J.R., 2013. Songbird frequency selectivity and temporal resolution vary with sex and season. Proc. R. Soc. Lond. B Biol. Sci. 280, 20122296.

Gibb, J., 1950. The breeding biology of the great and blue titmice. Ibis 507–539.

Greives, T.J., Fudickar, A.M., Atwell, J.W., Meddle, S.L., Ketterson, E.D., 2016. Early spring sex differences in luteinizing hormone response to gonadotropin releasing hormone in co-occurring resident and migrant dark-eyed juncos (*Junco hyemalis*). Gen. Comp. Endocrinol. 236, 17–23.

Hennin, H.L., Legagneux, P., Bêty, J., Williams, T.D., Gilchrist, H.G., Baker, T.M., Love, O.P., 2014. Pre-breeding energetic management in a mixed-strategy breeder. Oecologia 177, 235–243. doi:10.1007/s00442-014-3145-x

Hinde, R.A., Steel, E., 1976. The effect of male song on an estrogen-dependent behavior pattern in the female canary (*Serinus canarius*). Horm. Behav. 7, 293–304.

Hsueh, A.J.W., Erickson, G.F., 1978. Glucocorticoid inhibition of FSH-induced estrogen production in cultured rat granulosa cells. Steroids 32, 639–648.

Hu, G., Lian, Q., Lin, H., Latif, S.A., Morris, D.J., Hardy, M.P., Ge, R., 2008. Rapid mechanisms of glucocorticoid signaling in the Leydig cell. Steroids 73, 1018–1024.

Jacobs, J.D., Wingfield, J.C., 2000. Endocrine control of life-cycle stages: a constraint on response to the environment? The Condor 102, 35–51.

Jawor, J.M., Mcglothlin, J.W., Casto, J.M., Greives, T.J., Snajdr, E.A., Bentley, G.E., Ketterson, E.D., 2007. Testosterone response to GnRH in a female songbird varies with stage of reproduction: implications for adult behaviour and maternal effects. Funct. Ecol. 21, 767–775.

Jawor, J.M., McGlothlin, J.W., Casto, J.M., Greives, T.J., Snajdr, E.A., Bentley, G.E., Ketterson, E.D., 2006. Seasonal and individual variation in response to GnRH challenge in male dark-eyed juncos (*Junco hyemalis*). Gen. Comp. Endocrinol. 149, 182–189.

Johnson, A.L., Woods, D.C., 2007. Ovarian dynamics and follicle development. Reprod. Biol. Phylogeny Aves 243–277.

Ketterson, E.D., Nolan, V., 1983. Autumnal Zugunruhe and migratory fattening of dark-eyed juncos apparently suppressed by detention at the wintering site. Wilson Bull. 628–635.

Kwok, A.H.Y., Wang, Y., Wang, C.Y., Leung, F.C., 2007. Cloning of chicken glucocorticoid receptor (GR) and characterization of its expression in pituitary and extrapituitary tissues. Poult. Sci. 86, 423–430.

Lamarre, V., Franke, A., Love, O.P., Legagneux, P., Bêty, J., 2017. Linking pre-laying energy allocation and timing of breeding in a migratory arctic raptor. Oecologia 1–14.

Landys, M.M., Ramenofsky, M., Wingfield, J.C., 2006. Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes. Gen. Comp. Endocrinol. 148, 132–149.

Lattin, C.R., Romero, L.M., 2015. Seasonal variation in glucocorticoid and mineralocorticoid receptors in metabolic tissues of the house sparrow (*Passer domesticus*). Gen. Comp. Endocrinol. 214, 95–102.

Lattin, C.R., Waldron-Francis, K., Richardson, J.W., Breuner, C.W., Romero, L.M., 2011. Quantification of glucocorticoid and mineralocorticoid receptors in House Sparrow brain and peripheral tissues, in: Integrative and comparative biology. Oxford Univ Press Inc Journals Dept, 2001 Evans Rd, Cary, NC 27513 USA, pp. E217–E217.

Leboucher, G., Béguin, N., Mauget, R., Kreutzer, M., 1998. Effects of fadrozole on sexual displays and reproductive activity in the female canary. Physiol. Behav. 65, 233–240.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2- $^{\Delta\Delta}$CT method. methods 25, 402–408.

Marshall, A.J., 1959. Internal and environmental control of breeding. Ibis 101, 456–478.

McGuire, N.L., Koh, A., Bentley, G.E., 2013. The direct response of the gonads to cues of stress in a temperate songbird species is season-dependent. PeerJ 1, e139.

Mitchell, M.A., Carlisle, A.J., 1991. Plasma zinc as an index of vitellogenin production and reproductive status in the domestic fowl. Comp. Biochem. Physiol. A Physiol. 100, 719–724.

Murton, R.K., Westwood, N.J., 1977. Avian breeding cycles. Clarendon Press Oxford.

Needham, K.B., Cook, N.J., Rutherford, A.R., Greives, T.J., 2017. A pre-breeding immune challenge delays reproduction in the female dark-eyed junco (*Junco hyemalis*). J. Avian Biol.

Nolan, V., 2002. Dark-eyed junco: *Junco hyemalis*. Birds of North America, Incorporated.

O'Neal, D.M., Kiley, R.P., Ketterson, E.D., 2011. The effect of winter sex ratio on immune function and condition in a differential migrant. Physiol. Behav. 102, 406–413.

Perfito, N., Guardado, D., Williams, T.D., Bentley, G.E., 2014. Social cues regulate reciprocal switching of hypothalamic Dio2/Dio3 and the transition into final follicle maturation in European starlings (*Sturnus vulgaris*). Endocrinology 156, 694–706.

R Core Team, 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2012. ISBN 3-900051-07-0.

Reul, J., Kloet, E. de, 1985. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117, 2505–2511.

Ricklefs, R.E., 1974. Energetics of reproduction in birds. Avian Energ. 15, 152–292.

Romero, L.M., 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. Gen. Comp. Endocrinol. 128, 1–24.

Rosvall, K.A., Bergeon Burns, C.M., Hahn, T.P., Ketterson, E.D., 2013. Sources of variation in HPG axis reactivity and individually consistent elevation of sex steroids in a female songbird. Gen. Comp. Endocrinol. 194, 230–239.

Rowe, L., Ludwig, D., Schluter, D., 1994. Time, condition, and the seasonal decline of avian clutch size. Am. Nat. 143, 698–722.

Salvante, K.G., Williams, T.D., 2003. Effects of corticosterone on the proportion of breeding females, reproductive output and yolk precursor levels. Gen. Comp. Endocrinol. 130, 205–214.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions 1. Endocr. Rev. 21, 55–89.

Schaper, S.V., Dawson, A., Sharp, P.J., Gienapp, P., Caro, S.P., Visser, M.E., 2011. Increasing temperature, not mean temperature, is a cue for avian timing of reproduction. Am. Nat. 179, E55–E69.

Schneider, J.E., 2004. Energy balance and reproduction. Physiol. Behav. 81, 289–317.

Senior, B.E., Furr, B.J.A., 1975. A preliminary assessment of the source of oestrogen within the ovary of the domestic fowl, *Gallus domesticus*. J. Reprod. Fertil. 43, 241–247.

Sharp, P.J., Dunn, I.C., Talbot, R.T., 1987. Sex differences in the LH responses to chicken LHRH-I and-II in the domestic fowl. J. Endocrinol. 115, 323–331.

Ubuka, T., Bentley, G.E., 2009. Identification, localization, and regulation of passerine GnRH-I messenger RNA. J. Endocrinol. 201, 81–87.

Vanderkist, B.A., Williams, T.D., Bertram, D.F., Lougheed, L.W., Ryder, J.L., 2000. Indirect, Physiological Assessment of Reproductive State and Breeding Chronology in Free-Living Birds: An Example in the Marbled Murrelet (*Brachyramphus marmoratus*). Funct. Ecol. 14, 758–765.

Vézina, F., Salvante, K.G., 2010. Behavioral and physiological flexibility are used by birds to manage energy and support investment in the early stages of reproduction. Curr. Zool. 56.

Wallace, R.A., 1985. Vitellogenesis and oocyte growth in nonmammalian vertebrates, in: In Developmental Biology, Oogenesis. Springer, pp. 127–177.

Walzem, R.L., 1996. Lipoproteins and the laying hen: form follows function. Poult. Avian Biol. Rev. U. K.

Wilcoxen, T.E., Horn, D.J., Hogan, B.M., Hubble, C.N., Huber, S.J., Flamm, J., Knott, M., Lundstrom, L., Salik, F., Wassenhove, S.J., Wrobel, E.R., 2015. Effects of bird-feeding activities on the health of wild birds. Conserv. Physiol. 3, cov058.

Williams, T.D., 2012. Physiological adaptations for breeding in birds. Princeton University Press.

Williams, T.D., 2005. Mechanisms underlying the costs of egg production. Bioscience 55, 39–48.

Williams, T.D., 1999. Avian reproduction, overview. Encycl. Reprod. 1, 325–336.

Williams, T.D., Christians, J.K., 1997. Female reproductive effort and individual variation: neglected topics in environmental endocrinology, in: Proceedings of the 13th International Congress of Comparative Endocrinology. Monduzzi Editore Rome, pp. 1669–1675.

Williams, T.D., Kitaysky, A.S., Vézina, F., 2004. Individual variation in plasma estradiol-17β and androgen levels during egg formation in the European starling *Sturnus vulgaris*: implications for regulation of yolk steroids. Gen. Comp. Endocrinol. 136, 346–352.

Williams, T.D., Martyniuk, C.J., 2000. Tissue Mass Dynamics during Egg-Production in Female Zebra Finches *Taeniopygia guttata*: Dietary and Hormonal Manipulations. J. Avian Biol. 31, 87–95.

Williams, T.D., Miller, M., Murphy, M., 2003. Individual and resource-dependent variation in ability to lay supranormal clutches in response to egg removal. The Auk 120, 481–489.

Wingfield, J.C., 2012. Regulatory mechanisms that underlie phenology, behavior, and coping with environmental perturbations: an alternative look at biodiversity. The Auk 129, 1–7.

Wingfield, J.C., Hahn, T.P., Levin, R., Honey, P., 1992. Environmental predictability and control of gonadal cycles in birds. J. Exp. Zool. 261, 214–231.

Wingfield, J.C., Hahn, T.P., Maney, D.L., Schoech, S.J., Wada, M., Morton, M.L., 2003. Effects of temperature on photoperiodically induced reproductive development, circulating plasma luteinizing hormone and thyroid hormones, body mass, fat deposition and molt in mountain white-crowned sparrows, *Zonotrichia leucophrys oriantha*. Gen. Comp. Endocrinol. 131, 143–158.

Wingfield, J.C., Hahn, T.P., Wada, M., Astheimer, L.B., Schoech, S., 1996. Interrelationship of day length and temperature on the control of gonadal development, body mass, and fat score in white-crowned sparrows, *Zonotrichia leucophrys gambelii*. Gen. Comp. Endocrinol. 101, 242–255.

Wingfield, J.C., Hahn, T.P., Wada, M., Schoech, S.J., 1997. Effects of day length and temperature on gonadal development, body mass, and fat depots in white-crowned sparrows, *Zonotrichia leucophrys pugetensis*. Gen. Comp. Endocrinol. 107, 44–62.

Wingfield, J.C., Hegner, R.E., Lewis, D.M., 1991. Circulating levels of luteinizing hormone and steroid hormones in relation to social status in the cooperatively breeding white-browed sparrow weaver, *Plocepasser mahali*. J. Zool. 225, 43–58.

Wingfield, J.C., Kenagy, G.J., 1991. Natural regulation of reproductive cycles. Vertebr. Endocrinol. Fundam. Biomed. Implic. 4, 181–241.

Wingfield, J.C., Sapolsky, R.M., 2003. Reproduction and resistance to stress: when and how. J. Neuroendocrinol. 15, 711–724.

Wingfield, J.C., Smith, J.P., Farner, D.S., 1982. Endocrine responses of white-crowned sparrows to environmental stress. Condor 399–409.

Woods, D.C., Johnson, A.L., 2005. Regulation of follicle-stimulating hormone-receptor messenger RNA in hen granulosa cells relative to follicle selection. Biol. Reprod. 72, 643–650.

Zhang, C., Shimada, K., Saito, N., Kansaku, N., 1997. Expression of messenger ribonucleic acids of luteinizing hormone and follicle-stimulating hormone receptors in granulosa and theca layers of chicken preovulatory follicles. Gen. Comp. Endocrinol. 105, 402–409.

Zinzow-Kramer, W.M., Horton, B.M., Maney, D.L., 2014. Evaluation of reference genes for quantitative real-time PCR in the brain, pituitary, and gonads of songbirds. Horm. Behav. 66, 267–275.

CHAPTER 4: REPEATED IMMUNE CHALLENGES AFFECT TESTOSTERONE BUT NOT SPERM QUALITY[2]

Abstract

Mounting an immunological response is energetically demanding and necessarily redirects allocation of resources towards immune system activation and away from other energetically expensive processes, such as reproduction. Lipopolysaccharide (LPS), a major component of the outer membrane of the cell wall of gram-negative bacteria *Escherichia coli*, mimics a bacterial infection without producing the cost of replicating the pathogen and is one of the most commonly used agents to induce an acute phase immune response. Here, we ask if a trade-off can be induced between activation of the acute phase immune response and sperm function, a key indicator of sperm competitive ability. Further, we ask whether repeated exposure to this endotoxin in a social species such as the house sparrow (*Passer domesticus*), where repeated pathogen exposure may be common, may have a more pronounced effect. To address our questions we exposed individuals to two rounds of LPS treatment or control, to mimic a repeated pathogen exposure in the wild. We predicted that repeated pathogen exposure would have detrimental effects on sperm quality, and therefore, reproductive success. We compared a measure of sperm quality (straight-line velocity) in captive male house sparrows between LPS treated and control individuals. We found that although LPS treatment impaired circulating

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testosterone and induced a hypothermic state when compared with controls, it did not affect sperm quality within days or weeks following a single or repeated LPS exposure.

Introduction

Mounting an immune response and the metabolic requirements of immune up-regulation, are energetically costly (Demas, 2004; Lochmiller and Deerenberg, 2000; Nelson and Demas, 2004). These costs are likely to induce energetic trade-offs (Bonneaud et al., 2003; Ilmonen et al., 2003; Raberg et al., 2000). If an infection occurs when individuals are actively investing in other costly processes (e.g. reproduction), immune activation may act to decrease or impede investment in current reproductive efforts, such as sperm production.

Reduction in sperm quality can have detrimental effects on a male's fitness by impeding fertilization potential and likelihood of paternity (Birkhead and Møller, 1998; Parker, 1998; Pizzari et al., 2008; Pizzari and Birkhead, 2000; Pizzari and Parker, 2009; Snook, 2005). In birds, sperm fertilizing ability and the number of sperm ejaculated are influential for sperm competition in the wild (Birkhead et al., 1994, 1995; Birkhead and Møller, 1995). Sperm quality can be measured as sperm swimming velocity, which is related to the fertilizing efficiency of an ejaculate (Birkhead et al., 1999; Pizzari et al., 2007; Pizzari and Parker, 2009) and likely determines sperm competitive ability (Pizzari et al., 2008).

Male fertility and spermatogenesis are androgen-dependent (McLachlan et al., 2002; Sharpe, 1994). Specifically, testosterone in the testis is responsible for promoting spermatogenesis, which does not proceed if relatively high levels of testosterone are not present (Chang et al., 2004; De Gendt et al., 2004; Haywood et al., 2003; Sharpe, 1994; Zirkin et al., 1989). Acute inflammation may impair testicular steroidogenesis (O'Bryan et al., 2000) and further disrupt the entire spermatogenic process (cross-section of seminiferous tubule; Reddy et al., 2006) and male reproductive function (Henkel and Schill, 1998; Sanocka-Maciejewska et al., 2005; Urata et al., 2001). An acute inflammatory response can also increase oxidative stress (Sorci and Faivre, 2009). Production of highly reactive oxygen species by phagocytes during the acute phase response (Swindle and Metcalfe, 2007) are harmful to the membrane of germ cells and have been associated with complete arrest of Sertoli cell maturation (Koksal et al., 2003).

For social animals, living in close proximity to conspecifics comes with a cost of increased risk of infection because of higher rates of exposure to parasites and pathogens (Altizer et al., 2003; Møller et al., 1993; Sadd and Schmid-Hempel, 2006). The house sparrow (*Passer domesticus*) is a highly gregarious species, adapted to urban environments, and commonly found in close proximity to animal agricultural settings (Anderson, 2006). A study evaluating prevalence of common avian diseases in free-living passerines found that 17.2% of 373 sampled house sparrows had presence of *Escherichia coli* in the cloaca and this bacteria was the most commonly found in all sampled birds (Morishita et al., 1999). *Escherichia coli* is a gram-negative bacteria with a wide host range that is associated with fecal contamination (Morishita et al., 1999). Social life makes individuals more prone to repeated exposure to the same pathogens established in a colony (Sadd and Schmid-Hempel, 2006) and may be a cost of sociality due to rate of contact between individuals (Alexander 1974, Hoogland 1979, 1981, Duffy 1983, Pulliam and Caraco 1984, Brown and Brown 1986). Repeated exposure to pathogens, such as *Escherichia coli*, could result in a more pronounced diversion of resources away from reproduction and towards immune activation, potentially leading to reproductive consequences.

To investigate potential trade-offs between activation of an acute phase immune response and sperm quality (straight-line velocity) we injected house sparrows with lipopolysaccharide (LPS) to induce immune function activation and assessed sperm competitive ability (sperm quality) (Birkhead et al., 1999; Pizzari et al., 2007; Pizzari and Parker, 2009). LPS, a major component of the outer membrane of the cell wall of gram-negative bacteria *Escherichia coli,* is recognized by the host's immune system as a bacterial infection, leading to release of cytokines and activation of the acute phase response (Bonneaud et al., 2003; Demas and Nelson, 2011; Dunn et al., 1999; Dunn and Wang, 1996; Turnbull et al., 1998; Turnbull and Rivier, 1996). LPS is known to induce hyperthermia, anorexia, as well as neuroendocrine alterations (Carlson, 1997; Turnbull and Rivier, 1995). Here, using the house sparrow, we will assess whether these LPS-induced changes in physiology and energy balance alter sperm performance.

In this study we addressed three hypotheses. Mounting an immune response will result in a: i) *short-term decrease* in sperm quality. This observation would likely be a result of hyperthermia, heat stress to the testes, and/or non-specific damage from inflammatory responses. ii) *Long-term reduction* in sperm quality. A depression of testosterone production and disruption of spermatogenesis would be the likely mechanism for this outcome. iii) *Repeated LPS exposure* will further reduce sperm quality. The first hypothesis was assessed as sperm quality at 24-and 48-hour sperm sampling post-LPS treatment, as well as the thermoregulatory response to LPS treatment. The second hypothesis was then assessed by measuring the testosterone profile at 24-hours post-LPS treatment, in conjunction with sperm quality measured at 14-days post-LPS treatment. Finally, the third hypothesis was assessed as sperm quality following a second round of LPS-treatment.

Methods

Study Subjects

We used baited mistnets and potter traps to capture 16 adult male house sparrows from Fargo, ND in October, 2014. Birds were individually housed at the North Dakota State

University animal housing facility in 23.5 x 15.5 inch wire cages and held in captivity for a total of 6 months. Individuals were visually, but not acoustically isolated. Access to food (canary seed), drinking water, bath water, and blue grit was provided ad libitum. Vitamin water was provided one week out of every month (eCOTRITION Pro Ultra-Care Vita-Sol for caged birds). Following capture, individuals were maintained on a light-dark cycle of 8L : 16D for eight weeks to ensure a photosensitive state (King and Farner, 1963; Farner et al., 1966). To trigger gonadal recrudescence and a reproductive state, the population was photostimulated with a long day light-dark cycle of 16L : 8D (lights on: 8:00am, lights off: 12:00am) (King and Farner, 1963; Small et al., 2007), approximating the longest day length experienced by house sparrows in Fargo, ND (15 hours and 52 minutes of light)(US Naval Observatory, http://aa.usno.navy.mil/). Temperature was maintained between 22.2 – 23.9 °C. Immediately prior to the current study, we collected blood samples from all individuals and analyzed their day, night, and gonadotropin-releasing hormone (GnRH)-induced testosterone levels (Needham et al., 2016). Animal care guidelines were followed and approved by our institutional IACUC (#A14044).

Immune Challenge

Males were randomly assigned to either an experimental or control treatment group.

Experimental males (n = 8) received a 2.0 mg/kg intramuscular injection of LPS (from *E. coli* 055:B5 #L2880) diluted in PBS (Adelman et al., 2010; Sköld-Chiriac et al., 2014). A second LPS injection of the same dose was administered 3 weeks after the first injection. Control birds (n = 8) were given an equal volume injection of 1X phosphate-buffered saline (PBS). Injections occurred at ~10:00am, immediately following sperm collection (see below).

Temperature and Mass Measurements

To assess the effectiveness of LPS treatment, we collected body temperature and body mass. Cloacal body temperature was measured with a probe thermometer (Testo 925, Testo, Lenzkirch, Germany) paired with a Type T rectal probe for mice thermocouple sensor (RET-4, Physitemp Instruments Inc., Clifton, NJ, USA) at 6 hours and 24 hours after the first injection. Percent change in body temperature at 6 hours was calculated as: ((temperature at 6 hours post injection – baseline temperature)/baseline temperature)*100. Percent change in body temperature at 24 hours was calculated as: ((temperature at 24 hours post injection – baseline temperature)/baseline temperature)*100. Using a pesola scale, birds were weighed to the nearest 0.25 grams prior to injection and then again 24 hours post-injection. Percent change in mass was measured as ((mass at 24 hours post injection - baseline mass)/baseline mass)*100.

Blood Sampling

Blood samples were taken from all individuals 24 hours post-injection to assess the effect of LPS on testosterone levels as compared to control birds. During each sampling event, we collected ~100 µL of blood from the wing vein into 50 µL heparinized micro-hematocrit capillaries. Blood was stored on ice until centrifuged and plasma was separated. Plasma was stored at –80°C until later assayed for hormone analysis.

Determination of Plasma Testosterone Levels

Plasma testosterone was measured using a commercially available enzyme immunoassay (Testosterone ELISA Kit, Enzo Life Sciences, Farmingdale, NY, USA, #ADI-900-065) following previously described methods (Needham et al., 2016). Briefly, hormones were extracted using diethyl ether (2x extractions) and dried under nitrogen gas on a heat block set to 25°C, using 30 µL of plasma. All samples were reconstituted with 300 µL of assay buffer, vortexed, and allowed to reconstitute overnight in the refrigerator. Each sample was plated in duplicate following manufacturer's guidelines. To calculate intra- and inter- assay variation, a standard of known concentration was run three times on each plate in duplicate. The coefficients of variation (CV) for intra-plate testosterone were as follows: plate 1= 5.7% and plate 2= 3.9%, and the inter-plate CV was 4.8%. Samples that were below detection level by our assay (n = 18 out of 64 total) were assigned a value of lowest kit sensitivity, corrected for plasma volume (5.67 pg/ml).

Sperm Sampling and Analyses

To assess the effect of an immune challenge on sperm quality, sperm samples were collected on the day of injection, prior to treatment, and 24 hr, 48 hr, and 14 days post-injection. Sampling sperm quality at 14 days post-injection was chosen because spermatogenesis is thought to last between 11 – 14 days in birds (Amir et al., 1973). This allows us to assess the impact of LPS on the testosterone-dependent beginning stages of spermatogenesis (Sharpe, 1994). Sperm samples were collected within 2 minutes of capture by gently massaging the male's cloaca (Gee and Temple, 1978; Tuttle et al., 1996) and ejaculates were collected in 5 μL capillary tubes (Wheaton, Millville, NJ, USA, Product #851321) (Kast et al., 1998). Sperm samples were immediately mixed in 50 μL of room temperature PBS. A 9 μL sperm/buffer solution was then transferred onto a glass slide and placed under a compound laboratory microscope at 100X magnification (VWR 89404-470). Sperm motion video was recorded for two minutes using a microscope eyepiece camera (AmScope MU1400) and analyzed with the CASA plug-in to ImageJ (Losdat et al., 2011; Wilson-Leedy and Ingermann, 2007). The CASA program quantified straight line velocity (VSL) of all sperm samples (King et al., 2000; Wilson-Leedy and Ingermann, 2011). The CASA output also includes calculations of the percent motility, velocity curvilinear (VCL), velocity average path (VAP), linearity (LIN), wobble (WOB), progression (PROG), beat cross frequency (BCF), and number of sperm tracked of each sperm sample (Wilson-Leedy and Ingermann, 2011). Passerine spermatozoa travel in a straight line and in general all other sperm measures were correlated with VSL except the number of sperm. Therefore, we chose to focus on VSL as a measure of sperm quality (Losdat et al., 2011). For more information on the other sperm measures included in our CASA output see supplementary material Table 1.

Statistical Analyses

To examine the influence of treatment on testosterone levels, temperature change, and change in mass we used a Welch's two-sample t-test. Sperm quality (VSL) was analyzed using a linear mixed-effects model (lmer function in package lme4), with treatment and sperm sampling time (24-hour, 48-hour or 14-days) as fixed effects and individual ID as a random effect to control for repeated measures from the same individual. Separate models were run for each injection (one or two). All statistical analyses were performed using R v3.2.3 (R Core Team, 2014) and effects were considered significant at $\alpha = 0.05$ level. Values are provided as mean ± SEM.

Results

Temperature and Mass Responses

LPS injected birds had significantly greater changes in body temperature (drop in temperature) than control birds at 6 hours post-treatment ($p = 0.036$, df = 12.3, $t = 2.35$, CI: 0.003 – 0.080; PBS: 1.47 ± 1.00%, LPS: –2.71 ± 1.47%; Fig. 4.1A). After 24 hours, changes in body temperature no longer differed between treatment groups ($p = 0.409$, df = 13.9, $t = 0.85$, CI: –0.01 – 0.03; PBS: 1.74 ± 0.79%, LPS: 0.82 ± 0.74%; Fig. 4.1B). LPS-treated birds did not lose more body mass than control birds at 24 hours post-treatment (p = 0.967, df = 12.0, t = –0.04, CI: –0.02 – 0.02; PBS: –0.68 ± 0.41%, LPS: –0.65 ± 0.64%; Fig. 4.1C). For more information on the descriptive statistics of body mass and temperature see supplementary material Table 4.2.

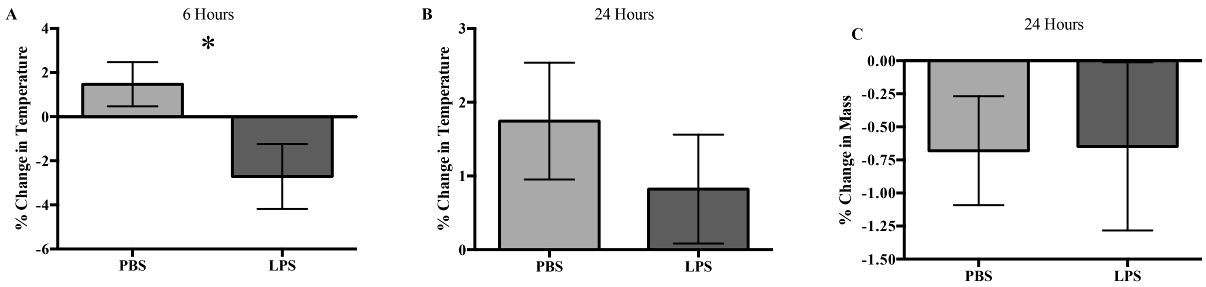

Figure 4.1. LPS-treated house sparrow (*Passer domesticus*) males had lower temperatures than control males at 6 hours post-treatment. The change in temperature between baseline temperature and 6 hours (Fig. 1A) or 24 hours (Fig. 1B). Mass of individuals was compared between baseline and 24 hours post-injection, within treatment groups (Fig. 1C). All graphs compare control (PBS) or treatment (LPS) injected individuals. * denotes p <0.05.

Testosterone Levels

Circulating testosterone levels of LPS treated birds were significantly lower than control birds when compared at 24 hours following LPS injection round 1 (p = 0.017, df = 7, t = 3.11, CI: 9.39 – 68.66; PBS: 44.70 ± 12.53 pg/mL, LPS: 5.67 ± 0.00 pg/mL (all samples below detection limit)) and LPS injection round 2 (p = 0.047, df = 13.5, 2.19, CI: 0.53 – 63.17; PBS: 56.79 ± 11.26 pg/mL, LPS: 24.94 ± 9.21 pg/mL; Fig. 4.2).

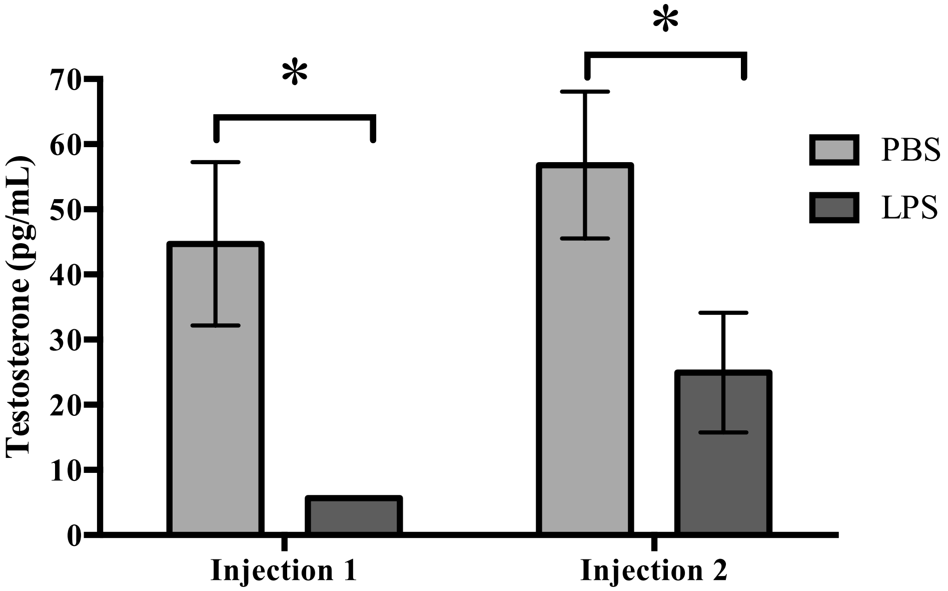

Figure 4.2. LPS-treated house sparrow (*Passer domesticus*) males had lower circulating testosterone levels compared to control males after both LPS injections. The comparison of circulating testosterone levels between treatment (LPS) and control (PBS) house sparrows 24 hours after each injection (1 and 2). The two rounds of injections were administered 21-days apart. * denotes $p < 0.05$ between LPS and PBS individuals.

Sperm Quality

During the first round of treatment there was no main effect of treatment (LPS or PBS; $F_{1,14} < 0.001$, $p = 0.994$) or time (24-hour, 48-hour, or 14-days; $F_{3,42} = 1.94$, $p = 0.138$) on VSL. VSL levels did not change over time when compared to individual's baseline VSL or due to an effect of treatment (Fig. 4.3A). There was no interaction between treatment and time on VSL ($F_{3,42} = 0.44$, $p = 0.723$).

Following the second round of treatment VSL was not impacted by the LPS treatment (LPS or PBS; $F_{1,14} = 0.035$, $p = 0.855$; Fig. 4.3B). VSL speed declined over time when compared to an individual's baseline VSL (24-hour, 48-hour, or 14-days; $F_{3,42} = 16.87$, $p < 0.001$; Fig. 4.3B). There was no interaction between treatment and time on VSL ($F_{3,42} = 0.74$, $p = 0.533$).

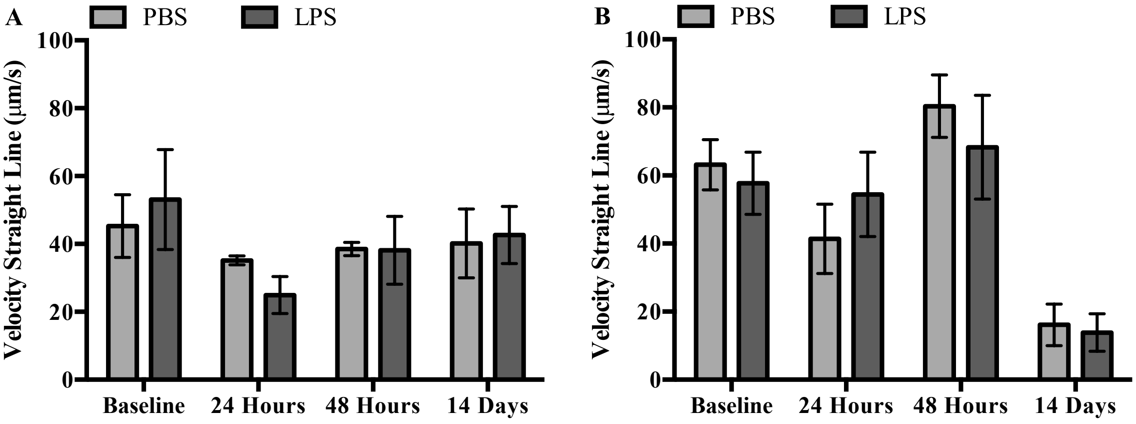

Figure 4.3. LPS-treated house sparrow (*Passer domesticus*) males did not suppress sperm quality compared to control males at any time point. Straight-line velocity of sperm over time: prior to injection (baseline), 24 hours, 48 hours and 14 days post-injection. Each time point compares control (PBS) to treatment (LPS) individuals. The two graphs represent straight-line velocity of sperm for injection 1 (A) and injection 2 (B).

Discussion

In this study, we asked if mounting an immune response, mediated by exposure to lipopolysaccharide (LPS), would induce a trade-off between the immune system and sperm quality. Our measure of sperm quality, VSL, is strongly correlated with other sperm measures (e.g., motility; see supplementary material) and has been associated with sperm competition in passerines (Kleven et al., 2009). We found that although LPS treatment is associated with a reduction in circulating testosterone and a hypothermic state, it did not affect sperm quality over short (24h or 48h) or longer time scales (14 days).

While LPS influenced testosterone and body temperature, treated individuals did not show other typical responses to pathogen exposure, such as significant mass loss, or hyperthermia (fever) (Dantzer and Kelley, 2007; Faggioni et al., 1997; Hart, 1988; Johnson et al., 1993; Klasing and Leshchinsky, 1999; Matson et al., 2005). In general other studies have shown that LPS exposure results in body mass loss and suppressed feeding behavior in birds and mammals (Cook et al., 1993; Parmentier et al., 1998; Van Heugten et al., 1996). However, similar to our findings, other studies also using LPS to elicit an inflammatory response, did not observe a change in body mass (Zebra finch (*Taeniopygia guttata*): Alonso-Alvarez et al., 2004; House sparrow: Lee et al., 2005). Lee et al. (2005) hypothesized that house sparrows may have a more plastic immune defense strategy and have adopted low investment into inflammation (Lee et al., 2005). Systemic inflammatory responses are costly and have the potential to be overly vigorous and misdirected when elicited by novel pathogens (Lee and Klasing, 2004). Thus, a dampened systemic inflammatory response may allow a host to avoid a trade-off between immune function, and other important processes (e.g., growth and reproduction) (Lee and Klasing, 2004).

Our first hypothesis was that LPS would induce hyperthermia responses, with fever leading to a short-term decrease in sperm quality. LPS-treated individuals in our study did not display the predicted hyperthermic response that is observed in some other bird species (D'alecy and Kluger, 1975; Gray et al., 2005; Johnson et al., 1993; Maloney and Gray, 1998; Nomoto, 2003; Pittman et al., 1976), but rather a hypothermic response. Similar to our findings, other studies have also shown a LPS-treated short-term hypothermic state is small passerines (captive white-crowned sparrow (*Zonotrichia leucophyrys gambelii*) (Owen-Ashley et al., 2006); free-living northwestern song sparrows (*Melospiza melodia morphna*) (Owen-Ashley and Wingfield, 2006)). Small passerines are endothermic homeotherms that maintain high body temperatures (38-42°C (Prinzinger et al., 1991)) (reviewed in: McKechnie and Lovegrove, 2002; Owen-Ashley and Wingfield, 2007). If body temperatures rise above a safe upper limit, due to a fever response, thermal death can occur (Boulant, 2000; Mackowiak, 1994; Mackowiak and Boulant, 1996). Additionally, a fever response is accompanied by antimicrobial and immunostimulating benefits, but these can be easily offset by the high energy costs (Romanovsky and Szekely, 1998). While a fever ensures the active attack against pathogens, hypothermia secures the defense of the host's vital systems (Romanovsky and Szekely, 1998) and conserves energy. However, to date it still remains unclear if a hypothermic response is an adaptive component of the acute phase response in small passerines (reviewed in: Owen-Ashley and Wingfield, 2007). This observation that LPS-treated males displayed a hypothermic response induced by LPS treatment likely contributed to a lack of treatment effect on short-term sperm quality.

We also hypothesized a short-term influence on sperm as a result of the LPS-induced inflammatory response that lasts 24 to 72 hours; however, no short-term treatment effect was observed here. In contrast to our findings, in a lekking species, the houbara bustard (*Chlamydotis undulata undulata*), it was shown that mounting an acute immune response imposed a cost on lower hatching rates due to reduced fertilization power (Chargé et al., 2010). While the pattern of a reduction in sperm VSL (quality) in LPS treated birds was observed at 24hr following injection 1 and 48hr following injection 2, no statistical effect of LPS on sperm quality was found. This lack of statistical effect may indeed imply no relationship between an acute phase response to LPS and sperm quality, or it could be that we were unable to detect a relationship, potentially because of limitations in housing space (and thus sample size), combined with an observed high variance between individuals. Alternatively, the captive environment in our study (e.g. ad libitum food and vitamin water) may have provided adequate resources to minimize damage by the inflammatory response on reproductive effort.

A longer-term (14 days) reduction in sperm quality in response to a depression in circulating testosterone production, and disruption of spermatogenesis was also hypothesized. LPS-treated birds had suppressed circulating testosterone levels 24 hours after each treatment compared to control birds, but treatments did not differ in sperm quality at 14-days post-treatment. Similar to our findings, others have found LPS-treated suppression of luteinizing hormone levels (Owen-Ashley et al., 2006) and serum testosterone production (Bosmann et al., 1996). However, in adult male rats, while LPS reduced serum LH levels, intratesticular testosterone concentrations remained at a sufficient level to promote normal spermatogenesis (O'Bryan et al., 2000). In a previous study of the captive male house sparrow, it was found during the breeding season that intratesticular testosterone levels increase in parallel with plasma LH levels, but not circulating testosterone levels (Donham et al., 1982). They also report that spermatogenesis is promoted by testosterone sequestered from the testis directly, and not from circulating testosterone (Donham et al., 1982). While we are unable to remark on the effects of LPS on intratesticular testosterone, since LPS did not alter sperm quality it is probable that only circulating testosterone was altered by LPS treatment. While investigation of effects on intratesticular testosterone would be needed to confirm this speculation, measuring intratesticular testosterone levels requires sacrificing the individual and does not allow for repeated sampling, as was conducted in the current study.

Observed testosterone levels in this study were low, as all sample concentrations were less than 60 pg/mL (0.06 ng/mL). Circulating testosterone levels of wild house sparrow males vary widely across the breeding season, with peaks during the breeding period and drastic declines within the incubation and parental care stages (Hegner and Wingfield, 1986; Wingfield et al., 1987). During the pre-breeding period (February and March) testosterone levels can range from 1000 – 3000 pg/mL (1.00 – 3.00 ng/mL) (Hegner and Wingfield, 1986; Wingfield et al., 1987). Testosterone levels can reach as high as 5000 pg/mL (5.00 ng/mL) in the early breeding stages (Hegner and Wingfield, 1986; Wingfield et al., 1987), when testosterone levels are at their highest in birds (Nelson, 1996). Thereafter, circulating levels drop to pre-breeding levels, around 1000 pg/mL (1.00 ng/mL), until the next breeding attempt (Hegner and Wingfield, 1986; Wingfield et al., 1987). Captive house sparrows, tend to have decreased circulating testosterone levels during the breeding season, typically ranging from ~100 – 700 pg/mL (0.1 – 0.7 ng/ml) (Buchanan et al., 2003; Laucht et al., 2011; Greenman et al., 2005). While we are not certain of why our males had levels lower than those observed in other wild and captive male house sparrow studies, differences in hormone extraction and assay methods (EIA versus RIA) may be one cause. Interestingly, these males were responsive to GnRH, with average post-stimulated testosterone levels of 2981 pg/mL (2.98 ng/mL) (Needham et al., 2016). This suggests that the testes were capable of producing breeding-season like circulating levels and males in this study reliably provided sperm samples, indicating that testes were indeed in breeding condition. Finally, the males in this study were able to hear other males and see a female house sparrow; however, they were unable to interact and receive the normal social cues that would be experienced in a wild setting. House sparrows are a social species (Anderson, 2006), and our housing conditions (individually and visually isolated from other males) may limit that amount of social interaction with conspecifics (Wingfield et al., 1990), which could have led to the low circulating levels observed here (Donham et al., 1982).

Finally, our study addressed the prediction that sperm quality, would display stronger negative effects due to a repeated exposure to LPS. Sperm quality did not change over time in birds re-exposed to LPS. In contrast, in humans and domestic animals acute or chronic infections can result in inflammation of the genitourinary tract, resulting in male infertility (Kauffold et al., 2007). This suggests that despite the fact that repeated exposure to LPS continued to suppress circulating testosterone production in our birds, LPS-treated males were able to maintain sperm viability. Individuals from both treatment groups began to cease sperm production by the 14-day collection following the second injection, likely due to males entering a photorefractory phase, which is why a difference in VSL was observed over time following the second injection..

A social species with high risk of cuckoldry, such as the house sparrow (Wetton et al., 1995; Wetton and Parkin, 1991), may have mechanisms in place to avoid consequences to repeated pathogen exposure (Lee et al., 2005) during the breeding season when loss of genetic offspring is a risk. The specific mechanisms that may maintain sperm viability in the face of repeated endotoxin exposure as well as the long-term consequences (e.g. overwinter survival) of exposure were not assessed here, but repeated exposure to pathogens may pose an increased risk in wild birds. Future work will be needed to further explore these possibilities.

Conclusion

Repeated LPS challenges did not readily induce an energetic trade-off in resource allocation towards immune activation and away from one aspect of sperm quality, VSL, in the captive house sparrow. Shutting-down reproductive efforts, such as courtship behavior and sperm production, would put a male at risk of losing a mate or being cuckolded. Future work should measure reactive oxygen species (ROS) damage and its effect on reproductive efforts in wild populations that would likely experience greater energy demands than captive animals. In addition, future research should examine how and at what point in time during the fertilization process an immune response is most likely to impact male fertility.

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Table 4.1. The correlations between ImageJ CASA output sperm measurements and straight-line velocity (VSL) for sixteen male house sparrows (*Passer domesticus*) from injection one (top panel) and injection two (bottom panel).

	Control (Saline)	**Treatment (Lipopolysaccharide)**
Percent Motility	p < 0.0001 , df = 30, t = 4.79, r = 0.66	p <0.001, df = 30, t = 3.97, r = 0.59
Velocity Curvilinear (VCL)	p = 0.423, df = 30, t = 0.81, r = 0.15	p = 0.27, df = 30, t = 1.12, r = 0.20
Velocity Average Path (VAP)	p < 0.0001, df = 30, t = 5.49, r = 0.71	p = 0.89, df = 30, t = –0.14, r = –0.03
Linearity (LIN)	p < 0.0001, df = 30, t = 24.84, r = 0.98	p = < 0.0001, df = 30, t = 14.36, r = 0.93
Wobble (WOB)	p < 0.0001, df = 30, t = 14.52, r = 0.94	p < 0.0001, df = 30, t = 17.99, r = 0.96
Progression (PROG)	p < 0.0001, df = 30, t = 18.00, r = 0.96	p < 0.0001, df = 30, t = 30.19, r = 0.98
Beat Cross Frequency (BCF)	p < 0.0001, df = 30, t = 5.69, r = 0.72	p < 0.0001, df = 30, t = 4.75, r = 0.66
Number of Sperm	p = 0.22, df = 30, t = –1.26, r = –0.22	p = 0.33, df = 30, t = –0.99, r = –0.18
Percent Motility	p <0.001, df = 30, t = 4.19, r = 0.61	p < 0.0001, df = 30, t = 5.10, r = 0.68
Velocity Curvilinear (VCL)	p = 0.43, df = 30, t = 0.79, r = 0.14	p = 0.24, df = 30, t = 1.21, r = 0.22
Velocity Average Path (VAP)	p < 0.0001, df = 30, t = 9.38, r = 0.86	p < 0.0001, df = 30, t = 10.62, r = 0.89
Linearity (LIN)	p < 0.0001, df = 30, t = 10.14, r = 0.88	p < 0.0001, df = 30, t = 8.81, r = 0.85
Wobble (WOB)	p < 0.0001, df = 30, t = 16.24, r = 0.95	p < 0.0001, df = 30, t = 19.06, r = 0.96
Progression (PROG)	p < 0.0001, df = 30, t = 23.11, r = 0.97	p < 0.0001, df = 30, t = 19.68, r = 0.96
Beat Cross Frequency (BCF)	p <0.001, df = 30, t = 4.44, r = 0.63	p <0.01, df = 30, t = 3.53, r = 0.54
Number of Sperm	p = 0.33, df = 30, t = –0.98, r = –0.18	p = 0.35, df = 30, t = –0.94, r = –0.17

Table 4.2. The average temperature and mass measurements for sixteen male house sparrows (*Passer domesticus*). Values are reported as mean ± SEM.

	Control (Saline)	**Treatment (Lipopolysaccharide)**
Baseline Temperature (°C)	43.2 ± 0.30	43.1 ± 0.20
Temperature at 6 hours (°C)	43.8 ± 0.38	41.9 ± 0.59
Temperature at 24 hours (°C)	43.9 ± 0.10	43.5 ± 0.29
Baseline Mass (mm)	27.7 ± 0.35	28.1 ± 0.79
Mass at 24 hours (mm)	27.5 ± 0.37	27.9 ± 0.78

References

Adelman JS, Bentley GE, Wingfield JC, Martin LB, Hau M. 2010. Population differences in fever and sickness behaviors in a wild passerine: a role for cytokines. J Exp Biol 213:4099–4109.

Alonso-Alvarez C, Bertrand S, Devevey G, Gaillard M, Prost J, Faivre B, Sorci G. 2004. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. Am Nat 164:651–659.

Altizer S, Nunn CL, Thrall PH, Gittleman JL, Antonovics J, Cunningham AA, Dobson AP, Ezenwa V, Jones KE, Pedersen AB, others. 2003. Social organization and parasite risk in mammals: integrating theory and empirical studies. Annu Rev Ecol Evol Syst 34:517–547.

Amir D, Braun-Eilon B, Schindler H. 1973. Passage and disappearance of labelled spermatozoa in the genital tract of the male Japanese quail in segregation or cohabitation. In: Annales de Biologie Animale Biochimie Biophysique. Vol. 13. EDP Sciences. p 321–328.

Anderson TR. 2006. Biology of the ubiquitous house sparrow: from genes to populations. Oxford University Press.

Birkhead TR, Martinez JG, Burke T, Froman DP. 1999. Sperm mobility determines the outcome of sperm competition in the domestic fowl. Proc R Soc Lond B Biol Sci 266:1759–1764.

Birkhead TR, Møller AP. 1995. Extra-pair copulation and extra-pair paternity in birds. Anim Behav 49:843–848.

Birkhead TR, Møller AP. 1998. Sperm competition and sexual selection. Academic Press.

Birkhead TR, Veiga JP, Fletcher F. 1995. Sperm competition and unhatched eggs in the house sparrow. J Avian Biol:343–345.

Birkhead TR, Veiga JP, Moller AP. 1994. Male sperm reserves and copulation behaviour in the house sparrow, *Passer domesticus*. Proc R Soc Lond B Biol Sci 256:247–251.

Bonneaud C, Mazuc J, Gonzalez G, Haussy C, Chastel O, Faivre B, Sorci G. 2003. Assessing the cost of mounting an immune response. Am Nat 161:367–379.

Bosmann HB, Hales KH, Li X, Liu Z, Stocco DM, Hales DB. 1996. Acute in vivo inhibition of testosterone by endotoxin parallels loss of steroidogenic acute regulatory (StAR) protein in Leydig cells. Endocrinology 137:4522–4525.

Boulant JA. 2000. Role of the preoptic-anterior hypothalamus in thermoregulation and fever. Clin Infect Dis 31:S157–S161.

Buchanan KL, Evans MR, Goldsmith AR. 2003. Testosterone, dominance signalling and immunosuppression in the house sparrow, *Passer domesticus*. Behav Ecol Sociobiol 55:50–59.

Carlson DE. 1997. Adrenocorticotropin correlates strongly with endotoxemia after intravenous but not after intraperitoneal inoculations of E. coli. Shock 7:65–69.

Chang C, Chen Y, Yeh S, Xu Q, Wang R, Guillou F, Lardy H, Yeh S. 2004. Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. Proc Natl Acad Sci U S A 101:6876–6881.

Chargé R, Saint Jalme M, Lacroix F, Cadet A, Sorci G. 2010. Male health status, signalled by courtship display, reveals ejaculate quality and hatching success in a lekking species. J Anim Ecol 79:843–850.

Cook ME, Miller CC, Park Y, Pariza M. 1993. Immune modulation by altered nutrient metabolism: nutritional control of immune-induced growth depression. Poult Sci 72:1301–1305.

D'alecy LG, Kluger MJ. 1975. Avian febrile response. J Physiol 253:223–232.

Dantzer R, Kelley KW. 2007. Twenty years of research on cytokine-induced sickness behavior. Brain Behav Immun 21:153–160.

De Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M, Devos A, Tan K, Atanassova N, Claessens F, Lécureuil C, others. 2004. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc Natl Acad Sci U S A 101:1327–1332.

Demas G, Nelson R. 2011. Ecoimmunology. Oxford University Press.

Demas GE. 2004. The energetics of immunity: a neuroendocrine link between energy balance and immune function. Horm Behav 45:173–180.

Donham RS, Wingfield JC, Mattocks PW, Farner DS. 1982. Changes in testicular and plasma androgens with photoperiodically induced increase in plasma LH in the house sparrow. Gen Comp Endocrinol 48:342–347.

Dunn AJ, Wang J. 1996. Cytokine effects on CNS biogenic amines. Neuroimmunomodulation 2:319–328.

Dunn AJ, Wang J, Ando T. 1999. Effects of cytokines on cerebral neurotransmission. In: Cytokines, stress, and depression. Springer. p 117–127.

Faggioni R, Fuller J, Moser A, Feingold KR, Grunfeld C. 1997. LPS-induced anorexia in leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice. Am J Physiol-Regul Integr Comp Physiol 273:R181–R186.

Farner DS, Follett BK, King JR, Morton ML. 1966. A quantitative examination of ovarian growth in the white-crowned sparrow. Biol Bull 130:67–75.

Gee GF, Temple SA. 1978. Artificial insemination for breeding non-domestic birds.

Gray DA, Maloney SK, Kamerman PR. 2005. Lipopolysaccharide-induced fever in Pekin ducks is mediated by prostaglandins and nitric oxide and modulated by adrenocortical hormones. Am J Physiol-Regul Integr Comp Physiol 289:R1258–R1264.

Greenman CG, Martin II LB, Hau M. 2005. Reproductive state, but not testosterone, reduces immune function in male house sparrows (Passer domesticus). Physiol Biochem Zool 78:60–68.

Hart BL. 1988. Biological basis of the behavior of sick animals. Neurosci Biobehav Rev 12:123–137.

Haywood M, Spaliviero J, Jimemez M, King NJ, Handelsman DJ, Allan CM. 2003. Sertoli and germ cell development in hypogonadal (hpg) mice expressing transgenic follicle-stimulating hormone alone or in combination with testosterone. Endocrinology 144:509–517.

Hegner RE, Wingfield JC. 1986. Behavioral and endocrine correlates of multiple brooding in the semicolonial house sparrow *Passer domesticus* I. Males. Horm Behav 20:294–312.

Henkel R, Schill W-B. 1998. Sperm separation in patients with urogenital infections. Andrologia 30:91–97.

Ilmonen P, Hasselquist D, Langefors A, Wiehn J. 2003. Stress, immunocompetence and leukocyte profiles of pied flycatchers in relation to brood size manipulation. Oecologia 136:148–154.

Johnson RW, Curtis SE, Dantzer R, Bahr JM, Kelley KW. 1993. Sickness behavior in birds caused by peripheral or central injection of endotoxin. Physiol Behav 53:343–348.

Kast TL, Ketterson ED, Nolan Jr V. 1998. Variation in ejaculate quality in dark-eyed juncos according to season, stage of reproduction, and testosterone treatment. The Auk:684–693.

Kauffold J, Henning K, Bachmann R, Hotzel H, Melzer F. 2007. The prevalence of chlamydiae of bulls from six bull studs in Germany. Anim Reprod Sci 102:111–121.

King JR, Farner DS. 1963. The relationship of fat deposition to Zugunruhe and migration. Condor:200–223.

King LM, Holsberger DR, Donoghue AM. 2000. Correlation of CASA velocity and linearity parameters with sperm mobility phenotype in turkeys. J Androl 21:65–71.

Klasing KC, Leshchinsky TV. 1999. Functions, costs, and benefits of the immune system during development and growth. Ostrich 69:2817–2832.

Kleven O, Fossøy F, Laskemoen T, Robertson RJ, Rudolfsen G, Lifjeld JT. 2009. Comparative evidence for the evolution of sperm swimming speed by sperm competition and female sperm storage duration in passerine birds. Evolution 63:2466–2473.

Koksal IT, Usta M, Orhan I, Abbasoglu S, Kadioglu A. 2003. Potential role of reactive oxygen species on testicular pathology associated with infertility. Asian J Androl 5:95–100.

Laucht S, Dale J, Mutzel A, Kempenaers B. 2011. Individual variation in plasma testosterone levels and its relation to badge size in House Sparrows *Passer domesticus*: It'sa night-and-day difference. Gen Comp Endocrinol 170:501–508.

Lee KA, Klasing KC. 2004. A role for immunology in invasion biology. Trends Ecol Evol 19:523–529.

Lee KA, Martin II LB, Wikelski MC. 2005. Responding to inflammatory challenges is less costly for a successful avian invader, the house sparrow (*Passer domesticus*), than its less-invasive congener. Oecologia 145:243–250.

Lochmiller RL, Deerenberg C. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88:87–98.

Losdat S, Richner H, Blount JD, Helfenstein F. 2011. Immune activation reduces sperm quality in the great tit. Plos One 6:e22221.

Mackowiak PA. 1994. Fever: blessing or curse? A unifying hypothesis. Ann Intern Med 120:1037–1040.

Mackowiak PA, Boulant JA. 1996. Fever's glass ceiling. Clin Infect Dis 22:525–536.

Maloney SK, Gray DA. 1998. Characteristics of the febrile response in Pekin ducks. J Comp Physiol [B] 168:177–182.

Matson KD, Ricklefs RE, Klasing KC. 2005. A hemolysis–hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. Dev Comp Immunol 29:275–286.

McKechnie AE, Lovegrove BG. 2002. Avian facultative hypothermic responses: a review. The Condor 104:705–724.

McLachlan RI, O'Donnell L, Meachem SJ, Stanton PG, De Kretser DM, Pratis K, Robertson DM. 2002. Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. Recent Prog Horm Res 57:149–179.

Møller AP, Dufva R, Allander K. 1993. Parasites and the evolution of host social behavior. Adv Study Behav 22:102.

Morishita TY, Aye, Ley EC, Harr BS. 1999. Survey of Pathogens and Blood Parasites in Free-Living Passerines. Avian Dis 43:549–552.

Needham KB, Dochtermann NA, Greives TJ. 2016. Consistent individual variation in day, night, and GnRH-induced testosterone concentrations in house sparrows (*Passer domesticus*). Gen Comp Endocrinol.

Nelson RJ. 1996. An introduction to behavioral endocrinology. Anim Behav 51:1193–1194.

Nelson RJ, Demas GE. 2004. Seasonal patterns of stress, disease, and sickness responses. Curr Dir Psychol Sci 13:198–201.

Nomoto S. 2003. Role of prostaglandin E2 and indomethacin in the febrile response of pigeons. Jpn J Physiol 53:253–258.

O'Bryan MK, Schlatt S, Phillips DJ, de Kretser DM, Hedger MP. 2000. Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels in vivo 1. Endocrinology 141:238–246.

Owen-Ashley NT, Turner M, Hahn TP, Wingfield JC. 2006. Hormonal, behavioral, and thermoregulatory responses to bacterial lipopolysaccharide in captive and free-living white-crowned sparrows (*Zonotrichia leucophrys gambelii*). Horm Behav 49:15–29.

Owen-Ashley NT, Wingfield JC. 2006. Seasonal modulation of sickness behavior in free-living northwestern song sparrows (Melospiza melodia morphna). J Exp Biol 209:3062–3070.

Owen-Ashley NT, Wingfield JC. 2007. Acute phase responses of passerine birds: characterization and seasonal variation. J Ornithol 148:583–591.

Parker GA. 1998. Sperm competition and the evolution of ejaculates: towards a theory base. Sperm Compet Sex Sel 3:54.

Parmentier HK, Walraven M, Nieuwland MG. 1998. Antibody responses and body weights of chicken lines selected for high and low humoral responsiveness to sheep red blood cells. 1. Effect of *Escherichia coli* lipopolysaccharide. Poult Sci 77:248–255.

Pittman QJ, Veale WL, Cockeram AW, Cooper KE. 1976. Changes in body temperature produced by prostaglandins and pyrogens in the chicken. Am J Physiol Content 230:1284–1287.

Pizzari T, Birkhead TR. 2000. Female feral fowl eject sperm of subdominant males. Nature 405:787–789.

Pizzari T, Cornwallis CK, Froman DP. 2007. Social competitiveness associated with rapid fluctuations in sperm quality in male fowl. Proc R Soc Lond B Biol Sci 274:853–860.

Pizzari T, Parker GA. 2009. Sperm competition and sperm phenotype. Sperm Biol Evol Perspect:207–245.

Pizzari T, Worley K, Burke T, Froman DP. 2008. Sperm competition dynamics: ejaculate fertilising efficiency changes differentially with time. BMC Evol Biol 8:332.

Prinzinger R, Pressmar A, Schleucher E. 1991. Body temperature in birds. Comp Biochem Physiol A Physiol 99:499–506.

R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2012. ISBN 3-900051-07-0.

Raberg L, Nilsson J, Ilmonen P, Stjernman M, Hasselquist D. 2000. The cost of an immune response: vaccination reduces parental effort. Ecol Lett 3:382–386.

Reddy MM, Mahipal SV, Subhashini J, Reddy MC, Roy KR, Reddy GV, Reddy PR, Reddanna P. 2006. Bacterial lipopolysaccharide-induced oxidative stress in the impairment of steroidogenesis and spermatogenesis in rats. Reprod Toxicol 22:493–500.

Romanovsky AA, Szekely M. 1998. Fever and hypothermia: two adaptive thermoregulatory responses to systemic inflammation. Med Hypotheses 50:219–226.

Sadd BM, Schmid-Hempel P. 2006. Insect immunity shows specificity in protection upon secondary pathogen exposure. Curr Biol 16:1206–1210.

Sanocka-Maciejewska D, Ciupińska M, Kurpisz M. 2005. Bacterial infection and semen quality. J Reprod Immunol 67:51–56.

Sharpe RM. 1994. Regulation of spermatogenesis. Physiol Reprod 1:1363–1434.

Sköld-Chiriac S, Nord A, Nilsson J, Hasselquist D. 2014. Physiological and behavioral responses to an acute-phase response in zebra finches: immediate and short-term effects. Physiol Biochem Zool 87:288–298.

Small TW, Sharp PJ, Deviche P. 2007. Environmental regulation of the reproductive system in a flexibly breeding Sonoran Desert bird, the rufous-winged sparrow, *Aimophila carpalis*. Horm Behav 51:483–495.

Snook RR. 2005. Sperm in competition: not playing by the numbers. Trends Ecol Evol 20:46–53.

Sorci G, Faivre B. 2009. Inflammation and oxidative stress in vertebrate host–parasite systems. Philos Trans R Soc Lond B Biol Sci 364:71–83.

Swindle EJ, Metcalfe DD. 2007. The role of reactive oxygen species and nitric oxide in mast cell-dependent inflammatory processes. Immunol Rev 217:186–205.

Turnbull AV, Lee S, Rivier C. 1998. Mechanisms of Hypothalamic-Pituitary-Adrenal Axis Stimulation by Immune Signals in the Adult Rata. Ann N Y Acad Sci 840:434–443.

Turnbull AV, Rivier C. 1995. Regulation of the HPA axis by cytokines. Brain Behav Immun 9:253–275.

Turnbull AV, Rivier C. 1996. Cytokines within the neuroendocrine system. Curr Opin Endocrinol Diabetes Obes 3:149–156.

Tuttle EM, Pruett-Jones S, Webster MS. 1996. Cloacal protuberances and extreme sperm production in Australian fairy-wrens. Proc R Soc Lond B Biol Sci 263:1359–1364.

Urata K, Narahara H, Tanaka Y, Egashira T, Takayama F, Miyakawa I. 2001. Effect of endotoxin-induced reactive oxygen species on sperm motility. Fertil Steril 76:163–166.

Van Heugten E, Coffey MT, Spears JW. 1996. Effects of immune challenge, dietary energy density, and source of energy on performance and immunity in weanling pigs. J Anim Sci 74:2431–2440.

Wetton JH, Burke T, Parkin DT, Cairns E. 1995. Single-locus DNA fingerprinting reveals that male reproductive success increases with age through extra-pair paternity in the house sparrow (*Passer domesticus*). Proc R Soc Lond B Biol Sci 260:91–98.

Wetton JH, Parkin DT. 1991. An association between fertility and cuckoldry in the house sparrow, *Passer domesticus*. Proc R Soc Lond B Biol Sci 245:227–233.

Wilson-Leedy J, Ingermann R. 2011. Computer assisted sperm analysis using ImageJ; description of necessary components and use of free software. Idaho.

Wilson-Leedy JG, Ingermann RL. 2007. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 67:661–672.

Wingfield JC, Ball GF, Dufty AM, Hegner RE, Ramenofsky M. 1987. Testosterone and aggression in birds. Am Sci 75:602–608.

Wingfield JC, Hegner RE, Dufty Jr AM, Ball GF. 1990. The“ challenge hypothesis”: theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. Am Nat:829–846.

Zirkin BR, Santulli R, Awoniyi CA, Ewing LL. 1989. Maintenance of advanced spermatogenic cells in the adult rat testis: quantitative relationship to testosterone concentration within the testis. Endocrinology 124:3043–3049.

CHAPTER 5: A PRE-BREEDING IMMUNE CHALLENGE DELAYS REPRODUCTION IN THE FEMALE DARK-EYED JUNCO (*JUNCO HYEMALIS*)[3]

Abstract

Precise timing of life-history transitions in predictably changing environments is hypothesized to aid in individual survival and reproductive success, by appropriately matching an animal's physiology and behavior with prevailing environmental conditions. Therefore, it is imperative for individuals to time energetically costly life-history stages (i.e., reproduction) so they overlap with seasonal peaks in food abundance and quality. Female lifetime reproductive fitness is affected by several factors that influence energy balance, including arrival date, timing of egg production, and energetic condition. Therefore, any extra energetic costs during reproduction may negatively affect timing of egg production, and ultimately a female's fitness. For example, mounting an immunological response elicits a high energetic cost, and this transfer of resources towards cell and immune system maintenance could have direct negative effects on reproductive timing. In order to determine whether an immune challenge delays onset of breeding (i.e., egg production), we administered either a humoral immune challenge (keyhole limpet hemocyanin (KLH)) (treatment) or physiological saline (control) to free-living female dark-eyed juncos (*Junco hyemalis*) in the period immediately prior to egg-laying (~4 weeks). We

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found that KLH-injected females artificially delayed clutch initiation when compared to control females. These data help to refine our understanding of how free-living birds allocate resources between reproduction and self-maintenance processes during the critical pre-laying period of the annual cycle.

Introduction

In virtually all species, reproduction must be precisely timed to coordinate breeding and rearing of offspring with favorable environmental conditions (Lack 1968, Newton and Marquiss 1982, Van Noordwijk et al. 1995, Sauther 1998, Naef-Daenzer and Keller 1999). Animals in seasonal environments therefore often restrict breeding to a limited time of year to align seasonal variation in food availability with reproduction and/or rearing of offspring (Perrins 1970, Wayne et al. 1989, Naef-Daenzer and Keller 1999).

The timing of the first clutch initiation in the spring has potential consequences for future nest attempts, life-history stages and annual reproductive output (Stutchbury and Robertson 1988, Winkler and Allen 1996). Early breeding individuals tend to have offspring with higher survival and recruitment rates (Hochachka 1990, Galen and Stanton 1991, Landa 1992, Dobson and Michener 1995, Verhulst et al. 1995, Rieger 1996). Offspring survival likelihood is positively related with offspring mass and condition and this relationship becomes increasingly important as the breeding season progresses (Rieger 1996, Naef-Daenzer et al. 2001), therefore suggesting a greater energetic investment by parents is needed to successfully raise late hatched/born offspring. Thus, delayed onset of reproduction has the potential to have profound and detrimental effects on a female’s reproductive success (e.g., reduced recruitment) (Reed et al. 2009, Williams 2012).

In order for oviparous females to breed early, they must initiate yolking and final follicular maturation in the spring at a time when resources in the environment are still limited, as chick-rearing usually takes place during peak food abundance (Perrins 1996). A deficit in energy or specific nutrients during this time could restrain these energetically expensive and nutrient intensive reproductive processes (Svensson and Nilsson 1995, Brodmann et al. 1997, Aho et al. 1999). In addition to energetic resources directed towards reproduction, an individual's finite energy budget must allocate energy towards several other important mechanisms, including immune function (Calow 1979, Harshman and Zera 2007). Activation of the humoral immune system induces production of antibodies in response to an exposure to antigens (Demas et al. 2011). Mounting an immune response increases basal metabolic rate in both mammals and birds following an immune challenge (Demas et al. 1997, Ots et al. 2001, Martin et al. 2003). As preparation for breeding is an energetically demanding period (Kwan 1994, Sandberg and Moore 1996, Williams 2005), unexpected energetic challenges such as mounting an immune response, are likely to induce an energetic trade-off during this critical time.

Females faced with an immune challenge during reproductive preparation can either continue with clutch initiation and risk pathogen-induced death, or they can mount an antibody response, which may delay reproduction, thus negatively impacting reproductive success. Previous research has found that investment in reproductive processes can result in a self-maintenance trade-off by suppressing immune function and increasing risk of infection (Klein 2000, Skarstein et al. 2001, Cartledge et al. 2005, Olsson et al. 2009). Studies that experimentally manipulated investment in immune function (Ilmonen et al. 2000, Merino et al. 2000, Raberg et al. 2000, Uller et al. 2006, Adelman et al. 2010) or reproductive effort (Cox and John-Alder

2007, French et al. 2007, Knowles et al. 2009) have found trade-offs between the two systems. However, other studies have not found the same well-defined trade-offs during experimental manipulations (Williams et al. 1999a, Cox et al. 2010). Such discrepancies may be due to the fact that these studies were conducted after the energetic investment into clutch initiation was already made. Furthermore, these studies were carried out under controlled laboratory conditions, thus suggesting the need for research in free-living females, especially during the pre-breeding period.

Here, using a wild free-living songbird model, the dark-eyed junco (*Junco hyemalis*), we directly test the hypothesis that an energetically costly humoral immune challenge will induce an energetic trade-off, thus delaying onset of clutch initiation. An induced trade-off could decrease reproductive success in these females, as earlier breeding individuals tend to have higher annual reproductive success (Daan et al. 1990, Hochachka 1990, Verhulst et al. 1995). To experimentally test this hypothesis we captured female dark-eyed juncos just prior to nest initiation and injected individuals with either an antigen known to generate a humoral immune response, or a control vehicle. Egg lay date and reproductive output measures (e.g., number of eggs hatched and nestling mass) were quantified.

Methods

Study Species and Capture

The dark-eyed junco (*Junco hyemalis*) is a seasonally breeding songbird found throughout the United States and breeds at high altitude and latitudes (Nolan 2002). Juncos are an excellent model organism because they have been extensively studied (Ketterson and Atwell 2016), are abundant, and are relatively easy to capture (Ketterson and Nolan 1982, Ketterson et al. 1998, 2009). Female dark-eyed juncos were captured in baited potter traps during the pre-breeding period (April-May). Individuals were given unique color band combinations for later identification. Morphometric measures including body mass and condition, fat score, tarsus length, wing length, and tail length were taken for all individuals. We were unable to age individuals in this study. Our field site is located on the Western edge of South Dakota, southeast of Lead, SD within the Black Hills National Forest and is characterized as an isolated mountain range with high-density forest cover. All procedures used in this study were approved by the NDSU Institutional Animal Care and Use Committee (Protocol # A13063). Capture of birds was conducted under license #22 from the Sate of South Dakota Department of Game, Fish, and Parks.

Humoral Immune Challenge

To induce a humoral immune response, experimental juncos received an injection of keyhole limpet hemocyanin (KLH). KLH is an innocuous respiratory protein derived from the giant keyhole limpet (*Megathura crenulata*), and serves as a novel non-pathogenic protein antigen that is highly immunogenic, but does not induce traits of an acute phase immune response (e.g., inflammation, fever, or sickness behavior) (Dixon et al. 1966, Demas et al. 1997, Hasselquist et al. 2001). While KLH does not cause a fever response or sickness behaviors, it does induce antibody production (Hasselquist et al. 1999, O'Neal et al. 2011) and increases metabolic rate (Demas et al. 1997). We prepared the KLH solution by emulsifying 1 mg KLH in 1 mL distilled water (Enzo Life Sciences, Lot # 01091429, Farmingdale, NY, USA) and further mixed in 1 mL of Freund's incomplete adjuvant (Thermo-Scientific 77145, Lot # OJ190038, Rockford, IL, USA) (Hasselquist et al. 1999, O'Neal et al. 2011, Graham et al. 2017), using the previously described rapid vortex method (Flies and Chen 2003).

Immune challenged birds received a single intramuscular injection of 100 μL of Freund's containing 50 μg of KLH (Hasselquist et al. 1999, O'Neal et al. 2011). Control individuals received a 100 µL intramuscular injection of sterile physiological saline- we used saline as a vehicle as Freund's incomplete adjuvant can magnify antibody production in response to an antigen (Dixon et al. 1966, French et al. 1970). To randomize treatment, the first individual captured was placed in a treatment group based on a coin toss and every other bird was thenceforth assigned to an experimental (n = 22 females; n = 1 male) or control group (n= 20 females; n = 2 males). The three individuals later identified as males, by observation of singing during nesting, were removed from the study. Dates of injections ranged from April 28, 2015 to May 22, 2015. In previous studies, a significant increase in KLH-specific antibodies in response to KLH in Freund's incomplete adjuvant in songbirds (Hasselquist et al. 1999), including dark-eyed juncos (O'Neal et al. 2011) has been observed. Because we could not reliably recapture females during the peak of antibody production we were unable to assess the post-KLH immune response.

Very-Low-Density Lipoprotein (VLDL)

Plasma very-low-density lipoprotein (VLDL) is one primary source of yolk protein for egg production (Williams 1999, 2012). Circulating levels of plasma lipid concentrations increase as females enter the laying period, leading to oocyte growth (Williams 1999, 2012). Assessment of total VLDL using triglyceride (TRIG) methods were originally developed in the domestic hen (Mitchell and Carlisle 1991) and later validated in passerines (Williams and Christians 1997, Williams and Martyniuk 2000, Challenger et al. 2001). We measured plasma TRIG concentrations (VLDL) from plasma samples taken prior to KLH or control administration to confirm a similar state in preparation for breeding between the two treatment groups.

We measured plasma TRIG concentrations in 5 µL plasma samples, using a previously validated colorimetric endpoint assay and by subtracting free glycerol (Sigma-Aldrich, F6428) from total glycerol (Sigma-Aldrich T2249) (Williams et al. 1999b, Guglielmo et al. 2002, Anteau and Afton 2008). Briefly, samples were read in duplicate at 570 nm in 96-well polystyrene microtitre plates on a microplate spectrophotometer (Microplate Manager; Bio-Rad Laboratories, Inc.). Using a chicken plasma pool (Sigma-Aldrich, P3266-1mL), we calculated inter- and intra-assay variation (interassay CV: 0.43%; intra-assay CV, plate 1: 1.26%, plate 2: 1.89%).

Determination of Egg One Date

Extensive nest searching was conducted May 18th to July 31st 2015 to determine egg one lay date in treatment and control females. Juncos are a ground-nesting species that lay 3 – 5 (mean and median = 4) eggs per nest attempt from May to June (Nolan 2002). If nests were found during nest construction, the nest was checked every other day for initiation of egg laying. If a nest was found during the incubation or nestling phase, we backdated to determine the first egg date based on known incubation and nestling mass timescales (Nolan 2002). As we only wanted to include first nest attempts in this study, we used a cut-off date (Julian day 142) to differentiate likely first versus possible second nest attempts of the season. The cut-off date was calculated as 2 standard errors from the mean egg lay date (to construct a 95 % confidence interval around the mean) of un-manipulated females in the population. For each group, nests were found for 10 KLH and 11 saline females.

Reproductive Output Measures

For nests found during egg laying or incubation, the number of eggs laid and how many eggs hatched or remained unhatched was noted. For nests that survived to hatching or were found during the nestling stage, individual nestling mass was recorded on hatch day (Day 0), day 3 and day 6. Nests were checked daily for activity from a distance with binoculars from day 7 until day 10 of the nestling stage. On day 11 nests were checked for number of nestlings preparing to fledge. A nest was also considered fledged if active on day 10, but nestlings not present and the nest was without evidence of nest predation when checked on the morning of day 11. Due to variation in number of nests found prior to hatch and duration each nest remained active, we report the varying sample sizes for the different measures in each treatment in Table 5.2.

Statistical Analyses

We performed all statistical analyses in R 3.2.2 (R Core Team, 2014) package lme4 and significance was considered at $\alpha = 0.05$ level.

To confirm no differences prior to assignment to experimental or control groups, measures of female condition (mass, tarsus, wing tail, fat score) and VLDL levels of all injected individuals were compared. Further, we assessed whether or not differences in these variables were observed between treatment and control individuals from a reduced dataset for which egg one date could be assigned. To confirm there was no seasonal bias in when individuals from each treatment were injected, we tested for a difference in Julian day of injection between the two treatment groups. Analyses were run using a Welch's two-sample t-test. Values are provided as mean ± SEM.

Next, we tested for the effects of KLH treatment on clutch initiation using a generalized linear model (GLM; family = poisson, link = log), which included treatment (KLH or control), VLDL levels, and Julian day of injection as fixed effects. The interaction between treatment and VLDL levels were not significant, and were therefore removed from the final model. The number of days between injection administration and first egg lay date of a clutch were calculated and will henceforth be referred to as "days until egg 1." Given that females were captured over a 4-week period, we estimated immune challenge cost on clutch initiation as the number of days between injection and first egg laid (mean injection date did not differ between treatments, see below).

We tested whether treatment had an effect at different nesting stages. First, we compared the following nesting measures between treatments: hatch day mass, day 3 mass, day 6 mass, number of eggs laid, number of eggs that hatched or remained unhatched, and number of nestlings that fledged. A Welch's two-sample t-test was performed between the two treatment groups for each aforementioned measure. Values are provided as mean ± SEM. Second, we aimed to address if treatment or egg 1 lay date (i.e., seasonal effect) influenced the probability of at least one nestling successfully fledging. Using a generalized linear model (GLM; family = binomial, link = logit), treatment (KLH or control) and Julian day of egg 1 were included as fixed effects. Fledge success was included as the response variable. Third, we asked if treatment or Julian day of egg 1 (i.e., seasonal trend) had a significant effect on nestling mass. Using a nested ANOVA with the lmer function, treatment and Julian day of egg 1 were included as fixed effects and nestling mass as the response variable. Further, an analysis of variance of type III with Satterthwaite approximation for degrees of freedom was performed. A separate model was run for each day that nestling mass was recorded (hatch day, day 3 or day 6). Nest ID was converted to a factor and included as the random variable. A post-hoc comparison of means using the glht function was used to further evaluate a difference in nestling mass between treatments.

Results

Prior to treatment, females assigned to the KLH (n = 22) or vehicle (n = 20) groups did not differ in body mass, tarsus, wing length, tail length, fat score, or VLDL levels (Table 5.1).

Individuals with known egg 1 dates (KLH: n = 10, Saline: n = 11) also did not differ in any of the previously mentioned measures prior to treatment (Table 5.1). Average Julian day of injection did not significantly differ between treatments (KLH: 131.3 ± 2.43; Control: 134.3 ± 2.19) ($p = 0.88$, $t = -0.16$, $df = 18$).

Treatment significantly affected the number of days between injection and egg lay date (days until egg 1; $Z_{1,17} = -3.76$, $p = 0.0002$; Fig. 5.1). Compared to saline females, KLH-injected females significantly delayed onset of clutch initiation after injection. On average, females injected with saline laid their first egg 12 ± 1.29 days after vehicle injection, while KLH injected females laid on average 20 ± 2.15 days after injection. VLDL levels also significantly predicted the days until egg 1 ($Z_{1,17} = -2.10$, $p = 0.04$), but Julian day of injection did not have a significant impact ($Z_{1,17} = -0.46$, $p = 0.65$). The number of nests with date of first eggs laid, categorized by treatment, are provided (Fig. 5.2).

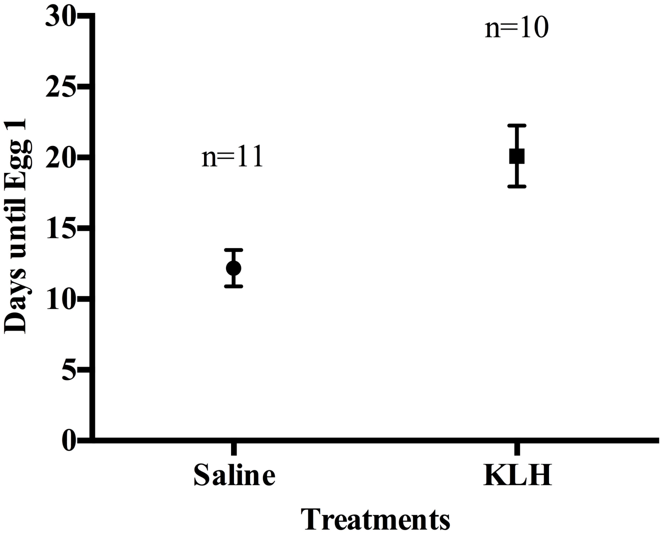

Figure 5.1. The number of days between injection and egg lay date ($p = 0.007$, $t = -3.05$, $df = 18$) in female dark-eyed juncos (*Junco hyemalis*). Females were either injected with physiological saline (control) or keyhole limpet hemocyanin (KLH) (treatment).

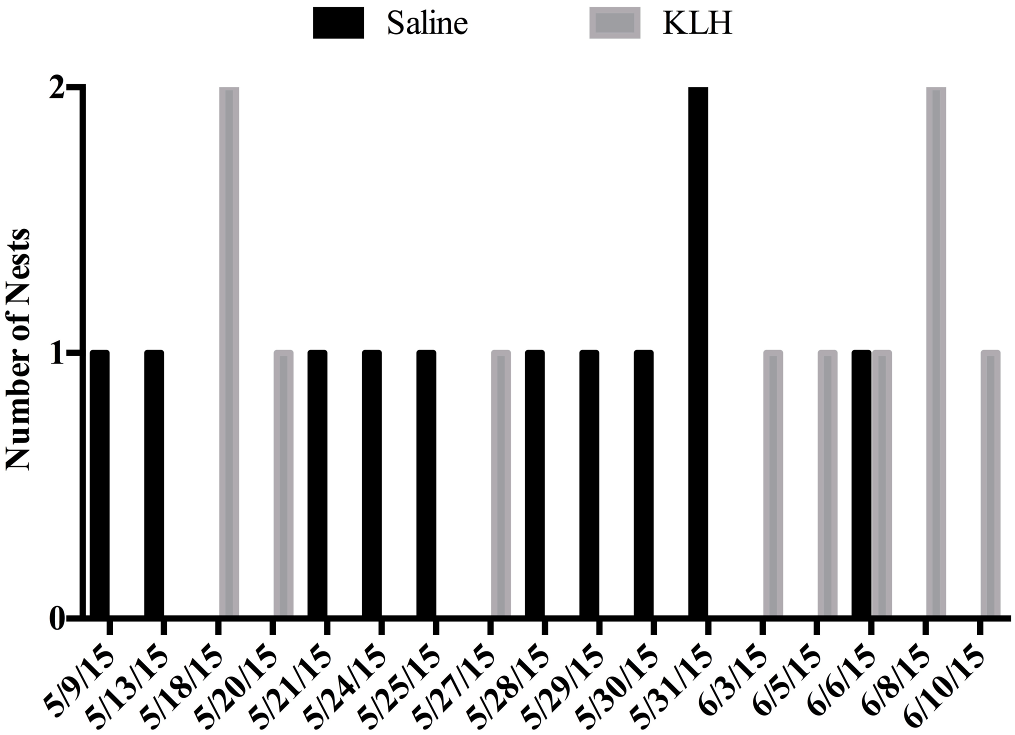

Figure 5.2. The number of nests with date of first eggs laid and by treatment (keyhole limpet hemocyanin (KLH) or physiological saline) in the free-living dark-eyed junco (*Junco hyemalis*).

Treatment ($Z_{1,18} = -0.13$, $p = 0.90$) and Julian day of egg 1 ($Z_{1,18} = 0.54$, $p = 0.59$) did not have a significant impact on the probability that at least one nestling would survive to fledging. There was no significant difference in number of eggs laid, number of eggs hatched or unhatched, nestling mass (hatch day, day 3, or day 6), or number of nestlings fledged between treatments (Table 5.2). Treatment and Julian day of egg 1 did not significantly affect nestling mass at hatch day, day 3 or day 6 (Treatment: Hatch day $p = 0.65$, $F_{1,21} = 0.21$, Day 3 $p = 0.56$, $F_{1,28} = 0.36$, and Day 6 $p = 0.86$, $F_{1,28} = 0.03$; Julian day of egg 1: Hatch day $p = 0.10$, $F_{1,21} = 3.02$, Day 3 $p = 0.35$, $F_{1,28} = 0.89$, Day 6 $p = 0.35$, $F_{1,28} = 0.92$).

Table 5.1. Measurements, prior to treatment, of all keyhole limpet hemocyanin (KLH; n = 22) and physiological saline (control; n = 20) injected female dark-eyed juncos (*Junco hyemalis*). In bolded italics are the subset of KLH (n = 10) and saline (n = 11) females with known egg 1 dates. Values are means ± SEM. VLDL = very-low-density lipoprotein.

Measures	**KLH**	**Saline**	**t (df)**	**p**
Mass (g)	23.8 ± 0.37	23.9 ± 0.33	–0.41 (39.9)	0.92
	23.7 ± 0.62	***24.0 ± 0.39***	***–0.41 (15.4)***	***0.89***
Tarsus (mm)	21.3 ± 0.13	21.1 ± 0.10	1.49 (37.9)	0.14
	21.4 ± 0.25	***21.0 ± 0.15***	***1.23 (15.6)***	***0.24***
Wing (mm)	81.4 ± 0.60	82.1 ± 0.55	–0.79 (39.9)	0.44
	81.3 ± 1.00	***81.4 ± 0.86***	***–0.05 (18.0)***	***0.96***
Tail (mm)	72.6 ± 0.42	73.1 ± 0.44	–0.91 (39.6)	0.37
	73.1 ± 0.50	***72.6 ± 0.53***	***0.76 (19.0)***	***0.46***
Fat Score	0.6 ± 0.21	1.1 ± 0.21	–1.38 (39.9)	0.18
	0.7 ± 0.36	***1.2 ± 0.26***	***–1.13 (17.5)***	***0.28***
VLDL (mg/mL)	4.6 ± 0.88	4.5 ± 0.78	0.12 (39.8)	0.90
	3.6 ± 1.20	***5.3 ± 1.21***	***–1.01 (18.9)***	***0.33***

Table 5.2. Differences in reproductive output measures between keyhole limpet hemocyanin (KLH) and physiological saline (control) injected female dark-eyed juncos (*Junco hyemalis*), with a known egg 1 date, prior to treatment.

Reproductive Measure	**KLH Mean ± SEM**	**n**	**Saline Mean ± SEM**	**n**	**t (df)**	**p**
Hatch Day Mass	3.7 ± 0.26	3	3.3 ± 0.38	5	–0.46 (21)	0.65
Day 3 Mass	7.6 ± 0.40	4	8.1 ± 0.29	6	0.60 (28)	0.56
Day 6 Mass	14.4 ± 0.41	4	14.8 ± 0.68	6	–0.18 (28)	0.86
No. of Eggs	3.8 ± 0.41	8	4.2 ± 0.40	6	–0.72 (11.8)	0.48
Hatched Eggs	2.0 ± 0.63	8	2.5 ± 1.0	6	–0.42 (8.6)	0.69
Unhatched Eggs	1.9 ± 0.64	8	1.7 ± 0.80	6	0.20 (10.4)	0.84
No. Fledged	1.6 ± 0.45	10	2.3 ± 0.62	11	–0.88 (17.9)	0.39

*n = the number of nests in each treatment

Discussion

Here we show that a humoral immune challenge significantly delayed clutch initiation in free-living female dark-eyed juncos. The immune response stimulated via KLH injection likely induced an energetic trade-off, resulting in our observation that KLH-injected females had significantly later egg 1 lay dates compared to saline-injected females. These findings suggest that when faced with a choice between delaying reproduction and risking mortality due to an infection, individuals preferentially invest in self-maintenance at the cost of reduced reproductive output resulting from delayed clutch initiation. However, our study was unable to detect any crude costs of delay in terms of fledging success or nestling weights.

No differences were observed between treatment groups at the beginning of the study for mass, fat score or skeletal size, suggesting that delay in clutch initiation was not due to initial differences in size or energy stores between the two treatment groups. Further, average VLDL levels did not differ between the two treatment groups before treatment. Very-low density lipoprotein (VLDL) is a yolk precursor protein used as a measure of egg production or reproductive state (Vanderkist et al. 2000) and also as a measure of an individual’s condition (Griffin and Whitehead 1982). The lack of difference in VLDL levels suggests that control birds were not physiologically closer to egg laying than experimental KLH-injected birds. However, VLDL was a significant predictor of the number of days between when a female was treated until a clutch was initiated. Together, these data suggest that, despite females in both groups being similar in current condition prior to the breeding season, a humoral immune challenge altered an individuals ‘decision’ to initiate egg production.

One explanation for why we see a trade-off in our study is due to the fact that KLH induces an energetic challenge. In mice it was shown that KLH increased oxygen consumption by ~25% (Demas et al. 1997), supporting a metabolic cost of response to KLH. While KLH is often referred to as a mild antigen (Dixon et al. 1966, Demas et al. 1997, Krug et al. 2004, Dube and Bertozzi 2005), our findings combined with the observation that KLH-injected females have high nest failure rates when nestlings are present (Graham et al. 2017), suggests that the humoral immune response induced by KLH has severe implications for reproductive effort in wild animals.

The delay in clutch initiation observed in this study was likely the result of energy resources being redirected to humoral immune activation and antibody production (Demas et al. 1997, Hasselquist et al. 1999). Egg production is an energetically demanding stage for females and available energy can have crucial effects on reproductive timing decisions (Visser and Lessells 2001, Williams 2012); therefore, redirected nutritional and energetic demands can have adverse effects on a female’s ability to initiate egg production. Further, there are nutritional costs associated with immune activation in response to a pathogen, including the redistribution of nutritional resources from processes such as reproduction to the needs of the immune system

(Ricklefs and Wikelski 2002, Lee 2006). Access to key proteins and amino acids necessary for initiating breeding can be seasonally variable. Limitation in key nutrients may further drive trade-offs between reproduction (egg production) and production of immune cells (Iseri and Klasing 2014).

Our low sample size of nests at the nestling stage was likely due to high predation rates in our population, and may be the reason we did not detect a difference in nestling mass or number of nestlings between KLH and saline treatment. Because ground-nesting species face high predation rates (Smith and Andersen 1982, Clotfelter et al. 2007), it is often difficult to acquire high sample sizes of reproductive output measures within each stage (eggs, nestlings). In this study we were unable to determine the number of offspring recruited to the breeding population in the following breeding season (reproductive success), as very few individuals were re-sighted in the following season, either due to high mortality and/or dispersal rates (Ketterson and Nolan 1982). Dark-eyed junco natal dispersal of juveniles can be high and lead to inflated estimates of mortality at the juvenile stage (Nolan 2002, McGlothlin et al. 2005). Despite the fact that we did not find a direct effect of delayed breeding on various nesting stages, there was a potential indirect cost on the social parents from the KLH manipulation in this study. Since likelihood of offspring survival is positively related to mass and condition (Rieger 1996, Naef-Daenzer et al. 2001) at fledging, late season breeders likely have an increased workload through nestling care. Future research is needed to assess the total reproductive cost directly associated with delayed breeding (i.e., smaller clutches or fewer fledglings recruited to the breeding population) in wild populations.

Conditions in the wild where resources are more limiting can uncover energetic trade-offs that might have been obscured by lab-based conditions where temperatures are often mild and food abundant. Here, our data indicate that even a mild antigen can incur significant costs to females during physiological preparation for breeding, thus leading to delayed onset of seasonal reproduction.

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References

Adelman, J. S., Córdoba-Córdoba, S., Spoelstra, K., Wikelski, M. and Hau, M. 2010. Radiotelemetry reveals variation in fever and sickness behaviours with latitude in a free-living passerine. - Funct. Ecol. 24: 813–823.

Aho, T., Kuitunen, M., Suhonen, J., Jäntti, A. and Hakkari, T. 1999. Reproductive success of Eurasian treecreepers, *Certhia familiaris*, lower in territories with wood ants. - Ecology 80: 998–1007.

Anteau, M. J. and Afton, A. D. 2008. Using plasma-lipid metabolites to index changes in lipid reserves of free-living lesser scaup (*Aythya affinis*). - The Auk 125: 354–357.

Brodmann, P. A., Reyer, H.-U., Bollmann, K., Schläpfer, A. R. and Rauter, C. 1997. The importance of food quantity and quality for reproductive performance in alpine water pipits (*Anthus spinoletta*). - Oecologia 109: 200–208.

Calow, P. 1979. The cost of reproduction–a physiological approach. - Biol. Rev. 54: 23–40.

Cartledge, V. A., Gartrell, B. and Jones, S. M. 2005. Adrenal and white cell count responses to chronic stress in gestating and postpartum females of the viviparous skink *Egernia whitii* (*Scincidae*). - Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 141: 100–107.

Challenger, W. O., Williams, T. D., Christians, J. K. and Vézina, F. 2001. Follicular development and plasma yolk precursor dynamics through the laying cycle in the European starling (*Sturnus vulgaris*). - Physiol. Biochem. Zool. 74: 356–365.

Clotfelter, E. D., Pedersen, A. B., Cranford, J. A., Ram, N., Snajdr, E. A., Nolan, V. and Ketterson, E. D. 2007. Acorn mast drives long-term dynamics of rodent and songbird populations. - Oecologia 154: 493–503.

Cox, R. M. and John-Alder, H. B. 2007. Increased mite parasitism as a cost of testosterone in male striped plateau lizards *Sceloporus virgatus*. - Funct. Ecol. 21: 327–334.

Cox, R. M., Parker, E. U., Cheney, D. M., Liebl, A. L., Martin, L. B. and Calsbeek, R. 2010. Experimental evidence for physiological costs underlying the trade-off between reproduction and survival. - Funct. Ecol. 24: 1262–1269.

Daan, S., Dijkstra, C. and Tinbergen, J. M. 1990. Family planning in the kestrel (*Falco tinnunculus*): the ultimate control of covariation of laying date and clutch size. - Behaviour 114: 83–116.

Demas, G. E., Chefer, V., Talan, M. I. and Nelson, R. J. 1997. Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. - Am. J. Physiol.-Regul. Integr. Comp. Physiol. 273: R1631–R1637.

Demas, G. E., Zysling, D. A., Beechler, B. R., Muehlenbein, M. P. and French, S. S. 2011. Beyond phytohaemagglutinin: assessing vertebrate immune function across ecological contexts. - J. Anim. Ecol. 80: 710–730.

Dixon, F. J., Jacot-Guillarmod, H. and McConahey, P. J. 1966. The antibody responses of rabbits and rats to hemocyanin. - J. Immunol. 97: 350–355.

Dobson, F. S. and Michener, G. R. 1995. Maternal Traits and Reproduction in Richardson's Ground Squirrels. - Ecology 76: 851–862.

Dube, D. H. and Bertozzi, C. R. 2005. Glycans in cancer and inflammation— potential for therapeutics and diagnostics. - Nat. Rev. Drug Discov. 4: 477–488.

Flies, D. B. and Chen, L. 2003. A simple and rapid vortex method for preparing antigen/adjuvant emulsions for immunization. - J. Immunol. Methods 276: 239–242.

French, V. I., Stark, J. M. and White, R. G. 1970. The influence of adjuvants on the immunological response of the chicken: II. Effects of Freund's complete adjuvant on later antibody production after a single injection of immunogen. - Immunology 18: 645.

French, S. S., DeNardo, D. F. and Moore, M. C. 2007. Trade-Offs between the Reproductive and Immune Systems: Facultative Responses to Resources or Obligate Responses to Reproduction? - Am. Nat. 170: 79–89.

Galen, C. and Stanton, M. L. 1991. Consequences of Emergence Phenology for Reproductive Success in *Ranunculus adoneus* (*Ranunculaceae*). - Am. J. Bot. 78: 978–988.

Graham, J. L., Mady, R. P. and Greives, T. 2017. Experimental immune activation using a mild antigen decreases reproductive success in free-living female Dark-eyed Juncos (*Junco hyemalis*). - Can. J. Zool. in press.

Griffin, H. D. and Whitehead, C. C. 1982. Plasma lipoprotein concentration as an indicator of fatness in broilers: Development and use of a simple assay for plasma very low density lipoproteins. - Br. Poult. Sci. 23: 307–313.

Guglielmo, C. G., O'Hara, P. D., Williams, T. D. and Blem, C. 2002. Extrinsic and intrinsic sources of variation in plasma lipid metabolites of free-living western sandpipers (*Calidris mauri*). - The Auk 119: 437–445.

Harshman, L. G. and Zera, A. J. 2007. The cost of reproduction: the devil in the details. - Trends Ecol. Evol. 22: 80–86.

Hasselquist, D., Marsh, J. A., Sherman, P. W. and Wingfield, J. C. 1999. Is avian humoral immunocompetence suppressed by testosterone? - Behav. Ecol. Sociobiol. 45: 167–175.

Hasselquist, D., Wasson, M. F. and Winkler, D. W. 2001. Humoral immunocompetence correlates with date of egg-laying and reflects work load in female tree swallows. - Behav. Ecol. 12: 93–97.

Hochachka, W. 1990. Seasonal Decline in Reproductive Performance of Song Sparrows. - Ecology 71: 1279–1288.

Ilmonen, P., Taarna, T. and Hasselquist, D. 2000. Experimentally activated immune defence in female pied flycatchers results in reduced breeding success. - Proc. R. Soc. Lond. B Biol. Sci. 267: 665–670.

Iseri, V. J. and Klasing, K. C. 2014. Changes in the amount of lysine in protective proteins and immune cells after a systemic response to dead *Escherichia coli*: implications for the nutritional costs of immunity. - Integr. Comp. Biol. 54: 922–930.

Ketterson, E. D. and Nolan, V. 1982. The Role of Migration and Winter Mortality in the Life History of a Temperate-Zone Migrant, the Dark-Eyed Junco, as Determined from Demographic Analyses of Winter Populations. - The Auk 99: 243–259.

Ketterson, E. D. and Atwell, J. W. 2016. Snowbird: Integrative Biology and Evolutionary Diversity in the Junco. - University of Chicago Press.

Ketterson, E. D., Parker, P. G., Raouf, S. A., Nolan, V., Ziegenfus, C. and Chandler, C. R. 1998. The Relative Impact of Extra-Pair Fertilizations on Variation in Male and Female Reproductive Success in Dark-Eyed Juncos (*Junco hyemalis*). - Ornithol. Monogr.: 81–101.

Ketterson, E. D., Atwell, J. W. and McGlothlin, J. W. 2009. Phenotypic integration and independence: hormones, performance, and response to environmental change. - Integr. Comp. Biol. 49: 365–379.

Klein, S. L. 2000. Hormones and mating system affect sex and species differences in immune function among vertebrates. - Behav. Processes 51: 149–166.

Knowles, S. C., Nakagawa, S. and Sheldon, B. C. 2009. Elevated reproductive effort increases blood parasitaemia and decreases immune function in birds: a meta-regression approach. - Funct. Ecol. 23: 405–415.

Krug, L. M., Ragupathi, G., Ng, K. K., Hood, C., Jennings, H. J., Guo, Z., Kris, M. G., Miller, V., Pizzo, B., Tyson, L. and others 2004. Vaccination of small cell lung cancer patients with polysialic acid or N-propionylated polysialic acid conjugated to keyhole limpet hemocyanin. - Clin. Cancer Res. 10: 916–923.

Kwan, D. 1994. Fat reserves and reproduction in the green turtle, *Chelonia mydas*. - Wildl. Res. 21: 257–265.

Lack, D. L. 1968. Ecological adaptations for breeding in birds. in press.

Landa, K. 1992. Seasonal Declines in Offspring Fitness and Selection for Early Reproduction in Nymph-Overwintering Grasshoppers. - Evolution 46: 121–135.

Lee, K. A. 2006. Linking immune defenses and life history at the levels of the individual and the species. - Integr. Comp. Biol. 46: 1000–1015.

Martin, L. B., Scheuerlein, A. and Wikelski, M. 2003. Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? - Proc. R. Soc. Lond. B Biol. Sci. 270: 153–158.

McGlothlin, J. W., Parker, P. G., Nolan and Ketterson, E. D. 2005. Correlational Selection Leads to Genetic Integration of Body Size and an Attractive Plumage Trait in Dark-Eyed Juncos. - Evolution 59: 658–671.

Merino, S., Moreno, J., Sanz, J. J. and Arriero, E. 2000. Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). - Proc. R. Soc. Lond. B Biol. Sci. 267: 2507–2510.

Mitchell, M. A. and Carlisle, A. J. 1991. Plasma zinc as an index of vitellogenin production and reproductive status in the domestic fowl. - Comp. Biochem. Physiol. A Physiol. 100: 719–724.

Naef-Daenzer, B. and Keller, L. F. 1999. The foraging performance of great and blue tits (*Parus major* and *P. caeruleus*) in relation to caterpillar development, and its consequences for nestling growth and fledging weight. - J. Anim. Ecol. 68: 708–718.

Naef-Daenzer, B., Widmer, F. and Nuber, M. 2001. Differential post-fledging survival of great and coal tits in relation to their condition and fledging date. - J. Anim. Ecol. 70: 730–738.

Newton, I. and Marquiss, M. 1982. Food, predation and breeding-season in Sparrowhawks (*Accipiter-nisus*), - J. Zool. 197: 221–240.

Nolan, V. 2002. Dark-eyed junco: *Junco hyemalis*. - Birds of North America, Incorporated.

Olsson, M., Wilson, M., Uller, T., Mott, B. and Isaksson, C. 2009. Variation in levels of reactive oxygen species is explained by maternal identity, sex and body-size-corrected clutch size in a lizard. - Naturwissenschaften 96: 25–29.

O'Neal, D. M., Kiley, R. P. and Ketterson, E. D. 2011. The effect of winter sex ratio on immune function and condition in a differential migrant. - Physiol. Behav. 102: 406–413.

Ots, I., Kerimov, A. B., Ivankina, E. V., Ilyina, T. A. and Hõrak, P. 2001. Immune challenge affects basal metabolic activity in wintering great tits. - Proc. R. Soc. B Biol. Sci. 268: 1175–1181.

Perrins, C. M. 1970. The timing of birds 'breeding seasons. - Ibis 112: 242–255.

Perrins, C. M. 1996. Eggs, egg formation and the timing of breeding. - Ibis 138: 2–15.

R Core Team 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2012. - ISBN 3-900051-07-0.

Raberg, L., Nilsson, J., Ilmonen, P., Stjernman, M. and Hasselquist, D. 2000. The cost of an immune response: vaccination reduces parental effort. - Ecol. Lett. 3: 382–386.

Reed, T. E., Warzybok, P., Wilson, A. J., Bradley, R. W., Wanless, S. and Sydeman, W. J. 2009. Timing is everything: flexible phenology and shifting selection in a colonial seabird. - J. Anim. Ecol. 78: 376–387.

Ricklefs, R. E. and Wikelski, M. 2002. The physiology/life-history nexus. - Trends Ecol. Evol. 17: 462–468.

Rieger, J. F. 1996. Body size, litter size, timing of reproduction, and juvenile survival in the Unita ground squirrel, *Spermophilus armatus*. - Oecologia 107: 463–468.

Sandberg, R. and Moore, F. R. 1996. Fat stores and arrival on the breeding grounds: reproductive consequences for passerine migrants. - Oikos: 577–581.

Sauther, M. 1998. Interplay of phenology and reproduction in ring-tailed lemurs: implications for ring-tailed lemur conservation. - *Folia Primatol*. (Basel) 69: 309–320.

Skarstein, F., Folstad, I. and Liljedal, S. 2001. Whether to reproduce or not: immune suppression and costs of parasites during reproduction in the Arctic charr. - Can. J. Zool. 79: 271–278.

Smith, K. G. and Andersen, D. C. 1982. Food, Predation, and Reproductive Ecology of the Dark-Eyed Junco in Northern Utah. - The Auk 99: 650–661.

Stutchbury, B. J. and Robertson, R. J. 1988. Within-season and age-related patterns of reproductive performance in female tree swallows (*Tachycineta bicolor*). - Can. J. Zool. 66: 827–834.

Svensson, E. and Nilsson, J.-A. 1995. Food supply, territory quality, and reproductive timing in the blue tit (*Parus caeruleus*). - Ecology 76: 1804–1812.

Uller, T., Isaksson, C. and Olsson, M. 2006. Immune challenge reduces reproductive output and growth in a lizard. - Funct. Ecol. 20: 873–879.

Van Noordwijk, A. J., McCleery, R. H. and Perrins, C. M. 1995. Selection for the timing of great tit breeding in relation to caterpillar growth and temperature. - J. Anim. Ecol.: 451–458.

Vanderkist, B. A., Williams, T. D., Bertram, D. F., Lougheed, L. W. and Ryder, J. L. 2000. Indirect, Physiological Assessment of Reproductive State and Breeding Chronology in Free-Living Birds: An Example in the Marbled Murrelet (*Brachyramphus marmoratus*). - Funct. Ecol. 14: 758–765.

Verhulst, S., Van Balen, J. H. and Tinbergen, J. M. 1995. Seasonal decline in reproductive success of the great tit: variation in time or quality? - Ecology 76: 2392–2403.

Visser, M. E. and Lessells, C. M. 2001. The costs of egg production and incubation in great tits (*Parus major*). - Proc. R. Soc. Lond. B Biol. Sci. in press.

Wayne, N. L., Malpaux, B. and Karsch, F. J. 1989. Social cues can play a role in timing onset of the breeding season of the ewe. - J. Reprod. Fertil. 87: 707–713.

Williams, T. D. 1999. Avian reproduction, overview. - Encycl. Reprod. 1: 325–336.

Williams, T. D. 2005. Mechanisms underlying the costs of egg production. - Bioscience 55: 39–48.

Williams, T. D. 2012. Physiological adaptations for breeding in birds. - Princeton University Press.

Williams, T. D. and Christians, J. K. 1997. Female reproductive effort and individual variation: neglected topics in environmental endocrinology. - Proc. 13th Int. Congr. Comp. Endocrinol.: 1669–1675.

Williams, T. D. and Martyniuk, C. J. 2000. Tissue Mass Dynamics during Egg-Production in Female Zebra Finches *Taeniopygia guttata*: Dietary and Hormonal Manipulations. - J. Avian Biol. 31: 87–95.

Williams, T. D., Christians, J. K., Aiken, J. J. and Evanson, M. 1999a. Enhanced immune function does not depress reproductive output. - Proc. R. Soc. Lond. B Biol. Sci. 266: 753–757.

Williams, T. D., Guglielmo, C. G., Egeler, O. and Martyniuk, C. J. 1999b. Plasma lipid metabolites provide information on mass change over several days in captive Western Sandpipers. - The Auk: 994–1000.

Winkler, D. W. and Allen, P. E. 1996. The seasonal decline in avian clutch size: strategy or physiological constraints. - Ecology 77: 922–932.

CHAPTER 6: CONCLUDING DISCUSSION

Most non-tropical animals exhibit seasonal changes in reproduction, and photoperiod acts as the main environmental cue regulating the timing of breeding. The integration of photoperiodic cues ensures offspring are born during favorable environmental conditions for seasonal breeders. However, mechanisms regulating seasonal reproduction in songbirds have received far more attention in males than in females, despite the recognition that females play a critical role in timing of breeding. In particular, organization of the female HPG axis in the period prior to breeding remains understudied. To this end, a series of experiments were performed in photoperiodic songbirds to examine 1) how male and females initiate the onset of breeding, and 2) how an applied energetic demand may alter the decision to breed in both sexes.

In Chapter 2, I described a laboratory experiment in which I assessed the hormonal variation of testosterone in the period prior to onset of spermatogenesis in the wild-caught captive house sparrow (*Passer domesticus*) (Hypothesis 1). Additionally, I also tested the relationship between testosterone profiles and breeding readiness in the field using wild-caught male dark-eyed juncos (*Junco hyemalis*) (Appendix Figures A1, A2, A3, A4, A5). In Chapter 3, using wild female dark-eyed juncos we probed and tested multiple levels of the reproductive axis to assess the mechanisms directly related to onset of follicle development (Hypothesis 2).

After evaluating the reproductive axis in the period prior to breeding, we aimed to assess how energetic demands in this critical period of preparation can alter both male and females decisions to breed. In Chapter 4, we delivered a repeated energetic challenge onto the wild-caught captive male house sparrows to determine at what point spermatogenesis would halt and therefore become detrimental to a male’s reproductive fitness (Hypothesis 3). Similarly, wild female dark-eyed juncos received an immune challenge to stimulate an energetic demand in the period prior to egg development to assess whether a trade-off between the immune and reproductive systems delayed the decision to breed (Hypothesis 4). This experiment was described in Chapter 5.

The results of the first two studies demonstrates that testosterone is repeatable in the period leading up to sperm production, suggesting that once the HPG axis is activated early in the season it becomes established much sooner than females are preparing for breeding. This allows for males to be ready for testosterone-based behaviors, such as courtship and mating, while females are fine-tuning their reproductive axis to environmental and social cues. Likewise, delay onset of egg development could be beneficial to the female by minimizing negative effects, such as elevated sex steroid levels over long periods of time, having to maintain high energetic costs of follicular development, and the potential for mis-timed attempts, which could have detrimental effects on the fitness of both sexes in a social pair.

The findings of the second set of studies establish that males appear capable of maintaining sperm production in the face of an energetic demand, while females are forced to delay the decision to breed. If we follow the theory that sperm are “cheap” and eggs are “expensive” to produce, then it is logical that males are capable of enduring an energetic demand, while females are forced to invest energy and resources away from reproduction. It is possible that males face the highest energetic demands of reproduction and sperm production in the period of testicular recrudescence. Therefore, an energetic demand during the period prior to onset of sperm production could be more telling. In this case, future research would need to look at onset of sperm production rather than if males shut-down their ability to produce sperm.

Collectively, these findings provide further evidence that females should be the focus of future research when attempting to identify regulation of onset of breeding. A potential mechanism that should be explored further, is the role of Gonadotropin-Inhibitory Hormone (GnIH) in timing of breeding of both males and females, as it has been shown to act not just at the level of the hypothalamus, but also directly on the pituitary and gonad.

APPENDIX

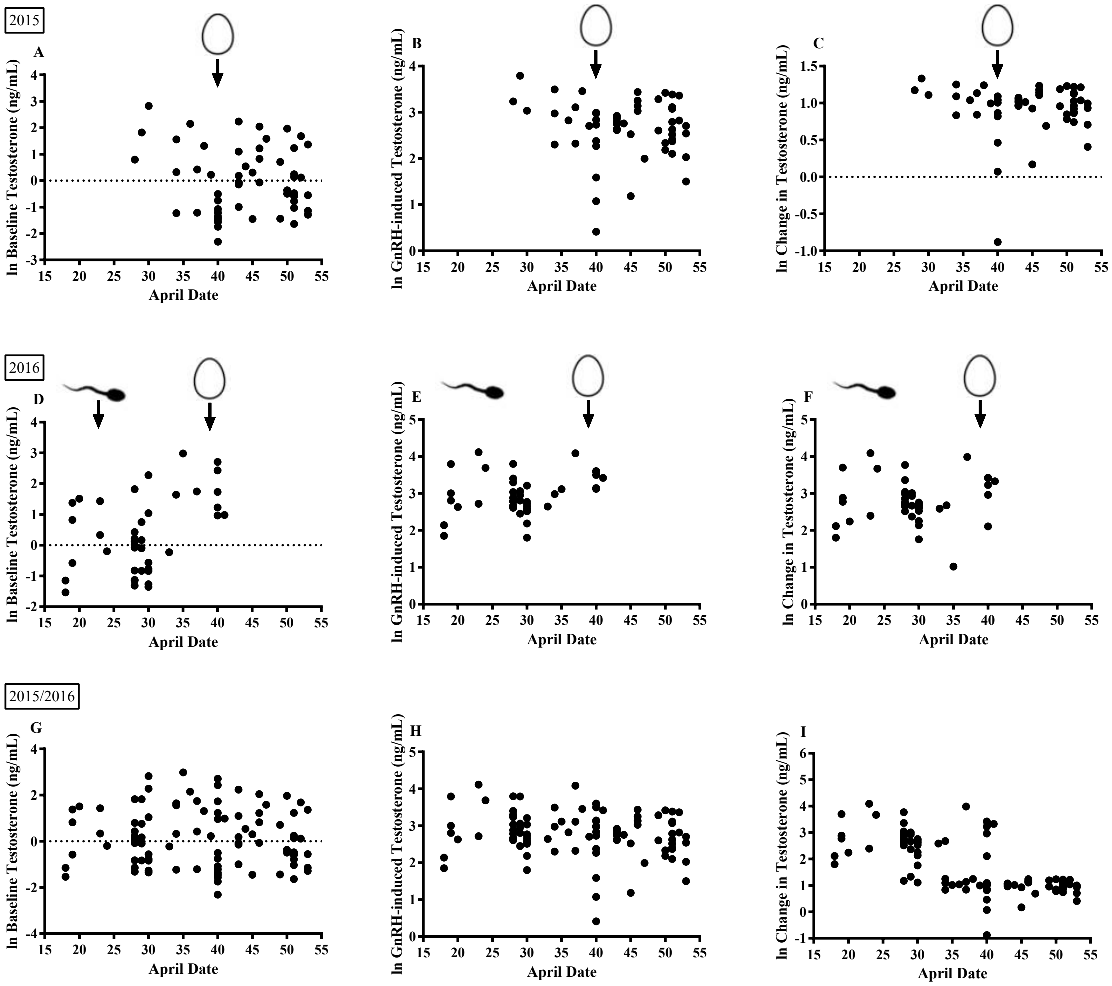

Figure A1. The testosterone profiles of wild dark-eyed junco (*Junco hyemalis*) males across the breeding season. The top panel (A, B, and C) represent data from 2015 only. The middle panel (D, E, and F) represent data from 2016 males only and also display the first date of sperm production and the first egg laid in the population for that year. The bottom panel (G, H, and I) represent the combination of data from 2015 and 2016. All plasma testosterone levels were natural-log (ln)-transformed. Change in testosterone was calculated as the amount testosterone levels increased from baseline to GnRH-induced levels. A: p = 0.248, df = 53, t = –1.17, r = –0.16; B: p = 0.319, df = 53, t = –1.01, r = –0.14; C: p = 0.700, df = 53, t = –0.39, r = –0.05; D: p = 0.005, df = 40, t = 2.97, r = 0.43; E: p = 0.048, df = 40, t = 2.04, r = 0.31; F: p = 0.510, df = 40, t = 0.67, r = 0.10; G: p = 0.734, df = 95, t = –0.34, r = –0.04; H: p = 0.105, df = 95, t = –1.63, r = –0.17; I: p <0.0001, df = 95, t = –8.50, r = –0.66.

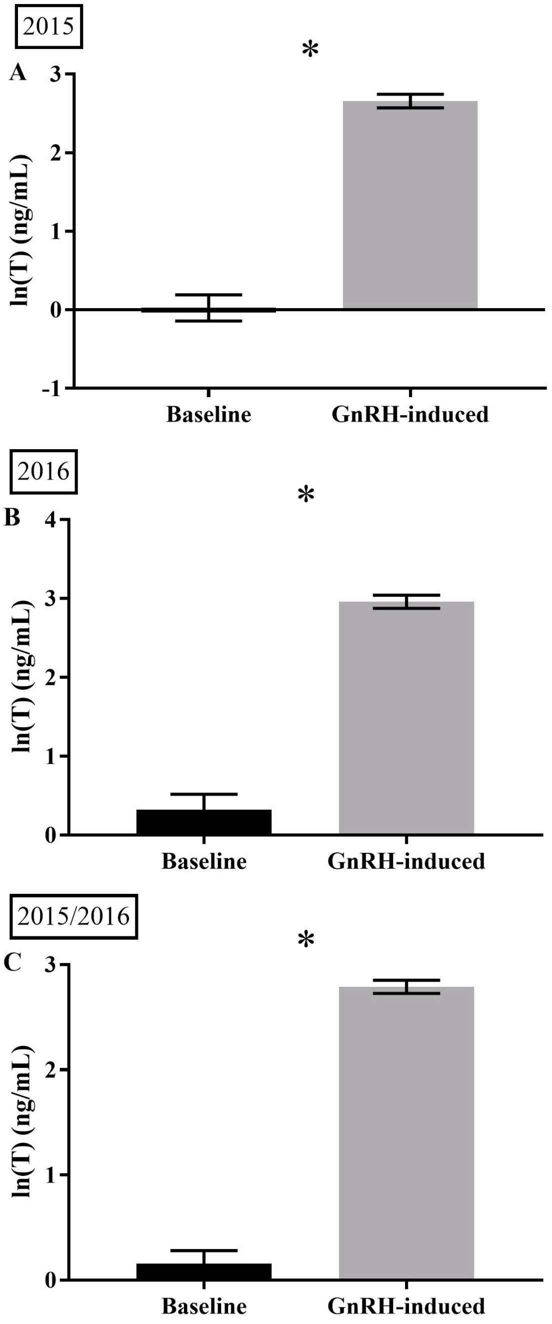

Figure A2. The elevation of testosterone from baseline to GnRH-induced levels of wild dark-eyed junco (*Junco hyemalis*) males from the combined 2015 and 2016 breeding seasons. All plasma testosterone levels were natural-log (ln)-transformed. A: $p < 0.0001$, df = 108, t = 14.1; B: $p < 0.0001$, df = 82, t = 12.6; C: $p < 0.0001$, df = 192, t = 18.7.

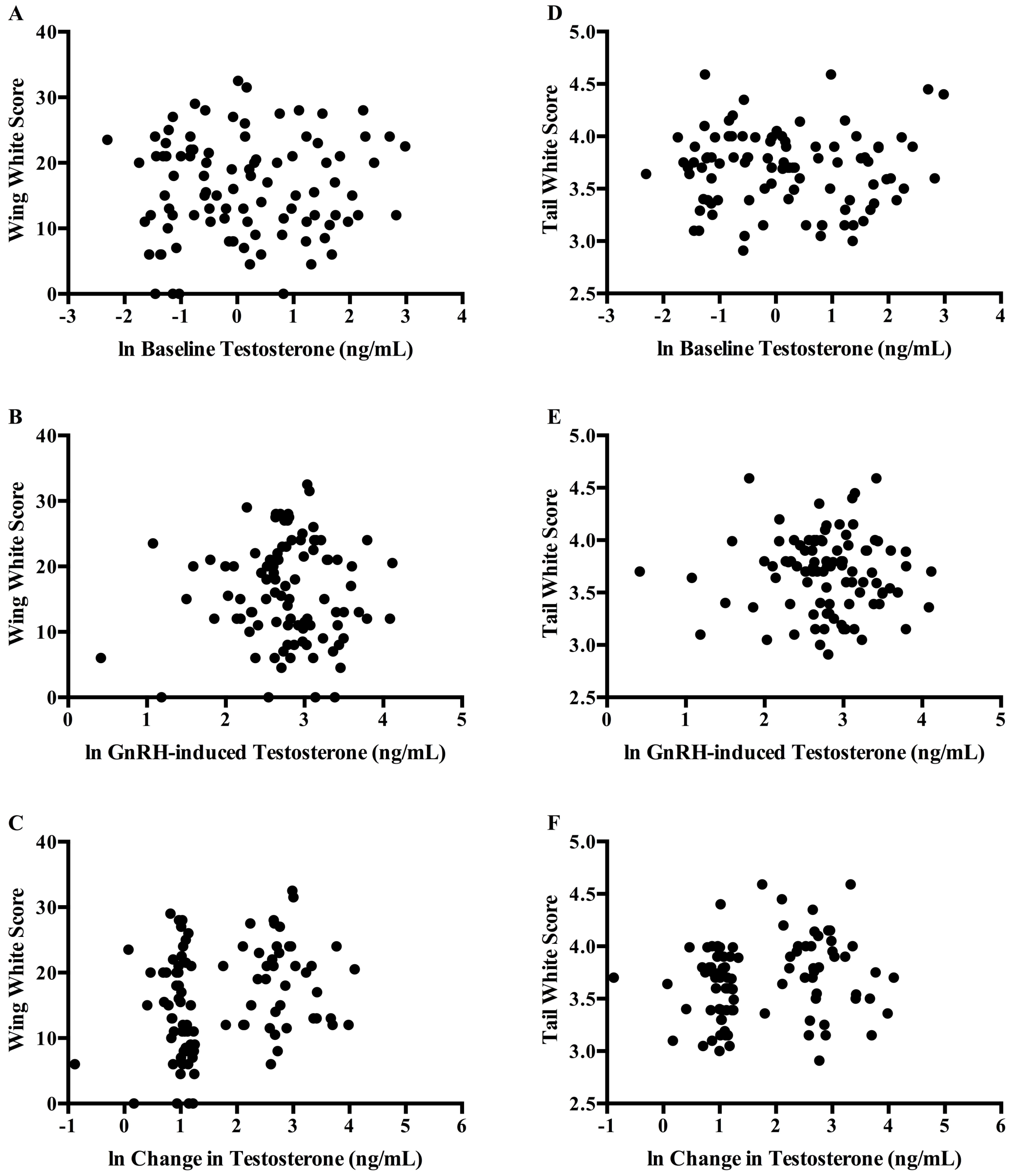

Figure A3. The relationship of white ornamentation with testosterone profiles of wild dark-eyed junco (*Junco hyemalis*) males from the combined 2015 and 2016 breeding seasons. All plasma testosterone levels were natural-log (ln)-transformed. Change in testosterone was calculated as the amount testosterone levels increased from baseline to GnRH-induced levels. Wing white was measured as the length of the white patch along the rachis with calipers (mm) of both the top and bottom wing bars (if present). An individual's score was the sum of the wing white values on the right wing. We measured the tail white value of a rectrix as the percentage of its area that was white. An individual's score was the sum of the tail white values on the right side of the tail. A: p = 0.592, df = 94, t = 0.54, r = 0.06. B: p = 0.839, df = 94, t = 0.20, r = 0.02. C: p = 0.009, df = 94, t = 2.68, r = 0.27. D: p = 0.928, df = 95, t = –0.09, r = –0.01. E: p = 0.844, df = 95, t = –0.20, r = –0.02. F: p = 0.170, df = 95, t = 1.38, r = 0.14.

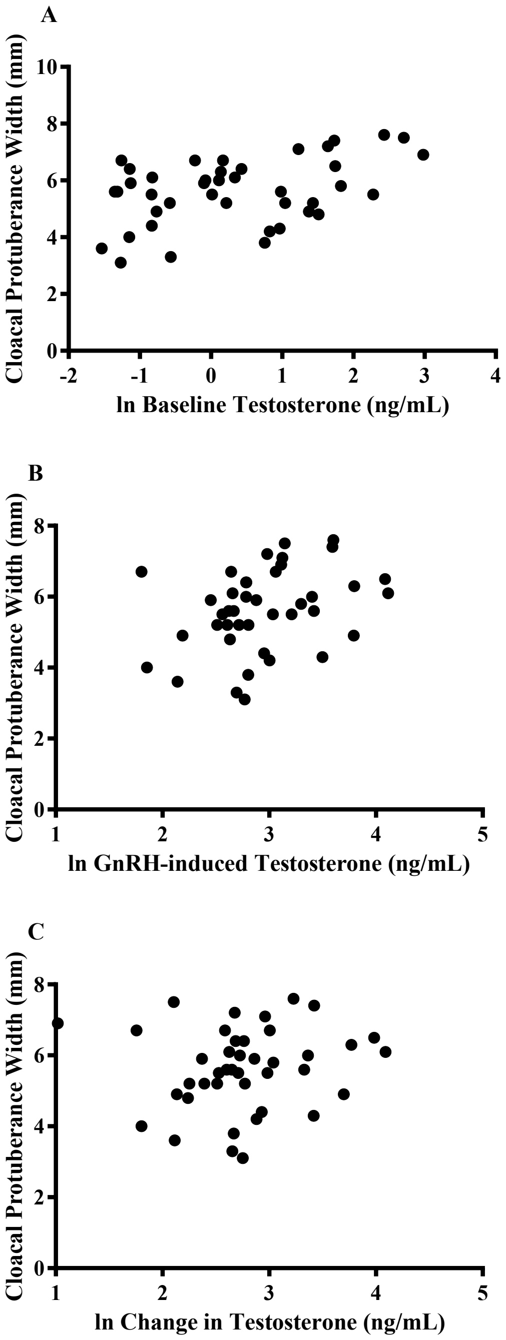

Figure A4. The relationship of the cloacal protuberance width with testosterone profiles of wild dark-eyed junco (*Junco hyemalis*) males from the 2016 breeding season. All plasma testosterone levels were natural-log (ln)-transformed. Change in testosterone was calculated as the amount testosterone levels increased from baseline to GnRH-induced levels. An individual's cloacal protuberance was measured with calipers (mm). A: p = 0.006, df = 39, t = 2.91, r = 0.42. B: p = 0.031, df = 39, t = 2.24, r = 0.34. C: p = 0.503, df = 39, t = 0.68, r = 0.11.

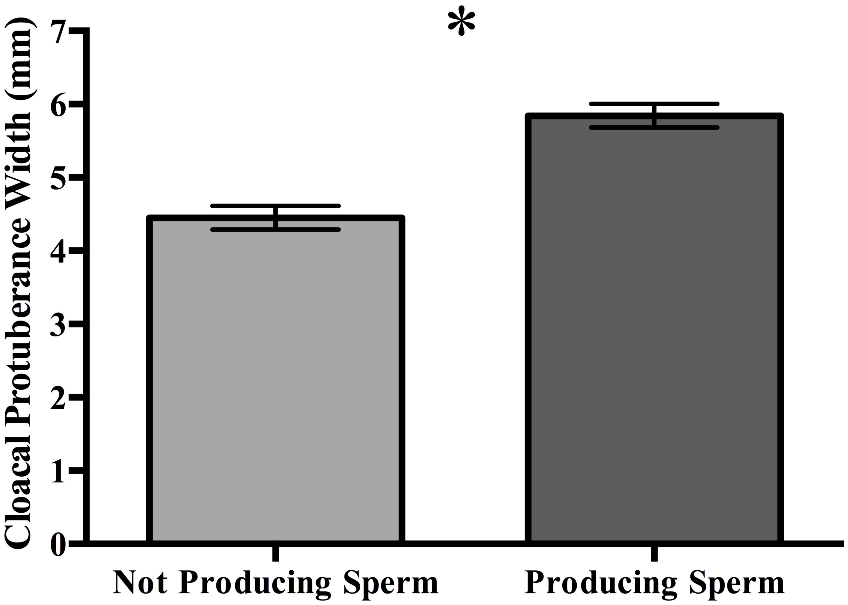

Figure A5. The difference in size of the cloacal protuberance width between wild dark-eyed junco (*Junco hyemalis*) males producing or not producing sperm from the 2016 breeding season. An individual's cloacal protuberance was measured with calipers (mm). Sperm samples were collected within two minutes of capture by gently massaging the male's cloaca and ejaculates were collected in 5 µL capillary tubes. Values are reported as mean ± SEM; p = 2.915 e –08, df = 85, t = –6.11.

Discussion of Appendix Figures

In the wild dark-eyed junco (*Junco hyemalis*) we found that males initiate testosterone (T) production (baseline T; Fig. A1 - A, D, and G), are responsive to exogenous GnRH stimulation (Fig. A1 - B, E, and H), and begin sperm production (Fig. A1 - D, E, and F) before the first egg is laid in the population. In Figure A2 we show that exogenous GnRH induces the testes to maximize output of testosterone (Fig. A2 – A, B, and C). White ornamentation is a sexually selected trait that refers to the amount of white on the tail feathers or wing bars. In our population we found that, when we combine collection years 2015 and 2016, wing white scores are only correlated with the change in T from baseline to GnRH-induced levels (Fig. A3 - C) and not with baseline or GnRH-induced T (Fig. A - A and B). Tail white scores were not correlated with any measure of T (Fig. A3 - D, E, and F). Clocacal protuberance (CP) width was only measured in the 2016 field season when sperm samples were also collected. CP width was correlated with baseline and GnRH-induced T levels (Fig. A4 - A and B), but not the change in T (Fig. A4 - C). Further, males that were producing sperm, as supported by collected ejaculates, had significantly larger CPs than those males not producing sperm (Fig. A5).