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From Fur to Feathers: Mechanisms Regulating Reproductive Flexibility and Life History Trade-off Dynamics

By

Mattina M. Alonge

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Integrative Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor George E. Bentley, Chair
Professor Caroline M. Williams
Professor Lance J. Kriegsfeld
Professor Eileen Lacey

Spring 2023

Abstract

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Life History Trade-off Dynamics

By

Mattina M. Alonge

Doctor of Philosophy in Integrative Biology

University of California, Berkeley

Professor George E. Bentley, Chair

Reproduction is a physiologically costly life history stage that varies widely in resource and behavioral demands depending on the specific stage of courting, mating, and period of parental care. Seasonal shifts in environmental conditions influence animal energy budgets in ways which shape species' life histories. Animals allocate energy and nutrients among a suite of competing physiological demands across short-, and long-term, timescales and the expression of specific traits or allocation strategy is tightly linked to the environment. Constraints on behavior and/or physiology have important implications for individual fitness and life history evolution, often leading to tradeoffs between processes supporting survival versus those supporting reproduction.

In **Chapter 1**, I provide the first neuroanatomical description of RFRP (precursor peptide of gonadotropin-inhibitory hormone, GnIH) expression and localization in the brain of any bat using a widespread temperate species (*Eptesicus fuscus*, big brown bat) as a model. We show that RFRP transcripts occur in the hypothalamus, testes, and ovaries of big brown bats. Cellular RFRP immunoreactivity was observed across the hypothalamus, arcuate nucleus, and median eminence (ME), most similar to what is known in naked mole rats. RFRP fiber immunoreactivity was widespread as in other vertebrates. Putative interactions between RFRP-ir fibers and gonadotropin-releasing hormone cell bodies were observed in 16% of GnRH cells, suggesting potential for direct regulation of GnRH via RFRP signaling. This strengthens our fundamental knowledge of how the HPG axis may be regulated in bats and may now guide functional approaches to understanding how reproductive neurophysiology responds, or be well-adapted, to seasonal environmental cues and stressors.

Animals face unpredictable challenges that require rapid, facultative physiological reactions to support survival but may compromise reproduction. Bats have a longstanding reputation for being highly sensitive to stressors, with sensitivity and resilience varying both within and among species. yet little is known about how stress affects the signaling that regulates reproductive physiology. **Chapter 2** provides the first description of the molecular response of the hypothalamic-pituitary gonadal (HPG) axis of male big brown bats (*E. fuscus*) in response to short-term stress using a

standardized restraint manipulation. This acute stressor was sufficient to upregulate plasma corticosterone and resulted in a rapid decrease in circulating testosterone. While we did not find differences in the mRNA expression of key steroidogenic enzymes (StAR, aromatase, 5-alpha reductase), seminiferous tubule diameter was reduced in stressed bats coupled with a 5-fold increase in glucocorticoid receptor (GR) mRNA expression in the testes. These changes, in part, may be mediated by RFamide-related peptide (RFRP) because fewer immunoreactive cell bodies were detected in the brains of stressed bats compared to controls—suggesting a possible increase in secretion—and increased RFRP expression locally in the gonads. The rapid sensitivity of the bat testes to stress may be connected to deleterious impacts on tissue health and function as supported by significant transcriptional upregulation of key pro-apoptotic signaling molecules (Bax, cytochromec). Experiments like this broadly contribute to our understanding of the stronger ecological predictions regarding physiological responses of bats within the context of stress which may impact decisions surrounding animal handling and conservation approaches.

Chapter 3 shifts from examining molecular mechanisms and neuroendocrinology regulating reproduction to a whole organism approach. Using a well-established captive colony of zebra finches, I characterize the behavioral and physiological conflicts between self-maintenance and reproductive investment and the factors that influence responses in line with energy limitation and/or trade-offs. Animals are faced with unpredictable challenges – such as pathogen exposure or infection – requiring quick physiological responses that support survival and may compromise resource allocation to reproduction. Stereotypical "sickness behaviors" may represent an energetic strategy that reduces investment in unnecessary activities to allocate resources toward – and support – the immune system. I use a framework of avian breeding stages to demonstrate the context-dependent behavioral conflict that may/may not be present across a species' breeding period, and the importance of examining these conflicts through a holistic lens that takes species' sociality and mating system into account as these variables directly affect routine individual energetic demands.

Dedication

For my first scientific mentor, Dr. Jason Bystriansky, who exposed me to the world of strong scientific inquiry and collaborative research and believed in me from the very beginning.

&

For my dogs, Olive and Jules, who have taught me valuable lessons in patience and trust and remind me that you can savor small moments while chasing what you love at full speed.

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First, my earliest mentors in science at DePaul University, because without their faith in me I would have never had the confidence to explore scientific research or fallen in love with the process:

Jason Bystriansky – Thank you for giving a bright-eyed, bushy-tailed master's student a first chance at research. I learned the value of meaningful and genuine collaboration in science, the type of relationships that blossom into friendships. My best habits in the lab came from the time you invested training your students hand-on in the lab, and have since influenced my own approach to teaching and training. Moreover, my time as a master's student entirely shaped my first interests in comparative animal physiology.

I am grateful for inspiring conversations surrounding science shared with **Jalene LaMontagne**, **Carolyn Martineau**, and **Elizabeth LeClair**; each who have opened their doors to me and continued to support me as I took time to decide what my academic path would be. All have modeled for me, in their ways, how to be a strong woman in science.

I have experienced immense growth during my time at UC Berkeley – personally, academically, and intellectually – and largely due to the transformational interactions and honest conversations with my mentors, colleagues, and friends:

George Bentley – I am forever grateful for the faith and freedom he granted me at the early stages of my PhD career and I do not know that I would have survived graduate school, and the challenges and hurdles that life brought, without his unwavering support. My scientific inquiry is stronger, my self-confidence raised, and my independence powerful; all a result of his mentorship. This lab has, in many ways, felt like home. And I will walk away from graduate school with strong values of work-life balance, compassion for mental health, and validation that dogs are the best animals on the planet.

Caroline Williams – Has improved my ability to ask novel questions about the natural world and undoubtedly has improved my work through her own expertise and integrative approach to physiology. I look up to her strength as a woman, her intellectual success within academia, and her warmth in welcoming colleagues and mentees into her time and spaces. Her commitment to fostering community is unmatched and has been an integral part of my sense of belonging during graduate school.

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Valentina Squicciarini – Her energy was just what I needed to get my through the final years of my PhD.

Kevin Roberts – For long walks through campus and North Berkeley neighborhoods that got me out of my head, accepting me for all that I am and helping me to do the same.

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My parents, **Susan** and **Jack Alonge**, for always encouraging me to chase my passions in life and have supported me as I have taken my time to identify them.

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Dissertation Introduction

1.1 Life History Theory and Environmental Influence

"To know the organism, it is necessary to know its environment." – George Bartholemew

Successful organisms grow, maintain their bodies and overall homeostasis, and (once mature) reproduce. While summarized simply in theory, no organism exists in isolation within the natural world and optimal physiology and behavior is shaped by interactions within the ecosystem as well as small-scale molecular processes within tissues and cells. Energy is the critical currency which supports, but may also constrain, physiological and behavioral processes for an animal and this has led to evolutionarily selected strategies for energy allocation across processes supporting growth, somatic maintenance, and reproduction; all which act together to support individual fitness through selection pressures that existed over evolutionary timescales.

Seasonal shifts in environmental conditions influence animal energy budgets in ways which shape species' life histories (Stearns 1992; Ricklefs and Wikelski 2002). Life history encompasses the entire lifespan of an organism, from its birth to its death, including age or stage-specific patterns of maturation, survival, mating, and reproduction (Flatt and Heyland 2011). A life history trait, therefore, is any phenotype or characteristic that is directly related to survival and reproduction with some examples being lifespan, number of offspring, age at maturity, or growth rate. At its beginnings, life history theory developed with the broad aim to understand how evolution shapes organisms to achieve reproductive success given selection pressures imposed by ecological challenges. Secondly, life history theory aimed to identify the ways in which (or whether) internal constraints (i.e. trade-offs) can optimize a set of life history traits to maximize reproductive success and fitness.

Classical life history theory posits that organisms are continually challenged with allocating resources between competing processes. Animals must often allocate energy and nutrients among a suite of competing physiological demands across short-, and long-term, timescales and the expression of specific traits or allocation strategy is tightly linked to the environment. Constraints on behavior and/or physiology have important implications for individual fitness and life history evolution, often leading to tradeoffs where processes supporting somatic maintenance are upregulated at the expense of those supporting reproduction (i.e. the "Y" model) (Stearns 1989; Zera and Harshman 2001).

A classic and well-studied example of reproductive versus somatic investment is seen in wing-polymorphic crickets where flight-capable (long-winged) female individuals develop musculature permitting dispersal, but possess smaller ovaries compared to flightless (short-winged) females, referred to as "flight oogenesis syndrome" (Harrison 1980; Roff 1986; Zera 2005; Tanaka and Suzuki 1998). Birds exhibit temporal separation of reproduction from other costly life history stages, as seen in separate periods of molt, migration, and breeding birds. While these examples are well known and strong examples of the conflict between somatic maintenance and reproductive investment, detecting trade-offs between life history traits is often difficult in nature. The

occurrence of trade-offs is impacted, in part, by an individual's broad energetic "strategy", composed of two mutually inclusive components: 1) how an animal interacts with its environment (e.g. behavior), and 2) the physiological mechanisms regulating an animal's metabolism and homeostasis (9). Understanding the fundamental constraints and flexibility of energetic strategy is essential to identifying selection pressures that shape temporal aspects of species' life history, as well as to developing strong predictions regarding species' resilience within increasingly variable environments.

1.2 Regulation of Reproductive Physiology and Behavior via the Hypothalamic-Pituitary-Gonadal (HPG) Axis

The coordination of physiological processes – such as metabolism, movement, sensory perception, and fertility – relies on chemical cues. In 1849, Arnold Berthold became the first to propose that male testes secreted something into the blood which was a critical signal for male-specific characteristics. Later, Bayliss and Starling provided the first evidence for what we now know as a hormone, coining the term in 1905 from the Greek word "*hormon*" which means *to excite*. We now define something as a hormone if it is a chemical messenger that is released into the circulation or tissue that then effects function of target cells (Köllner 2018). Extrinsic and intrinsic factors impact an animal's physiology, and sensory cells in the brain and nervous system work alongside the endocrine system to modulate overall whole-organism homeostasis. The endocrine system provides critical chemical signals that integrate information about an organism's environment and stimulate adaptive responses from the molecular to behavioral level. This complex coordination is essential to the survival and reproductive success.

Successful reproduction is a key component of evolutionary fitness. The timing of reproductive investment is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. This is one of several endocrine axes that is highly conserved among vertebrate evolutionary history with variations in neuropeptide signaling molecules, sensitivity to photoperiodic signals, and/or overall seasonality. The hypothalamus serves as a direct link between the nervous and endocrine systems, integrating cues of individual condition or environment into regulatory signals via a set of highly conserved reproductive neuropeptides. The dynamics among these neuropeptides can either stimulate or inhibit reproductive physiology and behavior.

The HPG axis is regulated to best ensure that reproductive investment will not compromise parent or offspring survival within a certain set of extrinsic and intrinsic conditions. Gonadotropin-releasing hormone (GnRH) is a central mediator within the HPG axis upon which many other signaling molecules converge to act. GnRH is synthesized and secreted from a relatively specific region of the hypothalamus, traveling through the hypophyseal portal system to act on cells in the anterior pituitary. These pituitary cells, when stimulated by GnRH, will then release luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the circulation – lending these cells to be named gonadotrophs. Working in tandem, LH and FSH then act on the gonads to modulate steroidogenesis and gamete development (i.e. fertility). Within this signaling pathway, there are important negative feedback loops involving sex steroid and pituitary gonadotropin communication to the brain.

After the initial characterization of GnRH, researchers later identified additional reproductive neuropeptides that function as upstream regulators of the synthesis and secretion of GnRH. With its discovery in 2000 (Tsutsui et al. 2000), gonadotropin-inhibiting-hormone (GnIH) came to the forefront within the field of reproductive neuroendocrinology as a key inhibitory signaling molecule with the potential to downregulate the HPG axis and suppress reproductive physiology/fertility. Later, along with the mammalian orthology RFRP-3, GnIH was found to interact with melatonin secreting pathways in response to seasonal photoperiod changes. Others have found a relationship between GnIH expression and stress in cases where reproductive behavior and physiology are suppressed. Later still, in mammals, kisspeptin, was identified along with neurokynin B and dynorphin, constitute another key signaling pathway within the brain that interact closely with GnRH-secreting cells (Skorupskaite, George, and Anderson 2014) and regulating pulsatile LH signaling that controls ovulation as well as negat feedback.

1.3 Reproductive Flexibility in the Face of Unpredictable Challenges

While organismal physiology can influence trajectories of life history evolution, so too can resultant life history patterns impose energetic conflict between critical fitness-defining processes such as those supporting reproduction. Upon reaching maturity, an animal's life is subdivided into a series of life stages that vary in energetic demands and – in some cases – phenotypic characteristics. Some animals reproduce only once in their lifetime (semelparous), however most species have the capacity to reproduce multiple times throughout their lifespan (iteroparous) with a greater degree of flexibility inherent to this facet of their life history. Challenges to an individual's homeostasis – which I will define here as *stress* – likely impacts the allocation strategy for an organism and dictates whether sufficient energy is available to invest successfully in reproduction at a given time. Species' coping mechanisms within a dynamic world are a cumulative function of 1) extrinsic environmental conditions (e.g. temperature, food availability), and 2) investment in intrinsic physiological processes (e.g. plumage, reproduction, immune function). Often, the evolutionary adaptations and phenotypes that a species may present in response to challenges are referred to as *resilience*. John Wingfield and others went have gone on to further develop this definition, where a mechanistic perspective shifts this terminology to "*resilience potential*" of species and/or individuals (Wingfield, Kelley, and Angelier 2011).

1.4 Areas of Opportunity and Selected Focal Species

Understanding the fundamental constraints and flexibility of energetic strategy is essential to shaping strong predictions regarding species' resilience in increasingly variable environments. Energetic constraints on reproductive investment have led to the evolution of different reproductive strategies and variation in the flexibility in behavior and molecular responses within the reproductive (HPG) axis.

Bats are intriguing models for exploring physiological constraints, especially those pertaining to reproduction. Like all small mammals, they maintain extremely high metabolic rates, however – being uniquely volant – they require additional energy to support concurrent demands of flight, gestation, and lactation. Further, many bat species exhibit a decoupling of copulation and gestation separated by the hibernation period; mating occurs in the fall, and female physiology permits storage of sperm throughout winter with delayed ovulation in the spring (Gustafson 1979; Oxberry

1979). Bats are also physiologically amenable to a spectrum of ecosystems and environments and naturally exhibit intriguing interindividual and interspecific variation in foraging behavior, roost selection, and microhabitat use. While we now understand more about when various life history stages occur in bats, little is known about which environmental cues stimulate changes in biology, what mechanisms regulate this, or the individual physiology that permits energetic investment in reproduction to occur. Understanding the ways in which reproductive physiology is regulated at the individual level will provide knowledge essential to making predictions about both fitness and strategies for survival within bat populations. Here, I suggest that we may exploit the uniqueness in temperate bat reproductive life-history to deepen our understanding of ecologically-relevant mechanisms regulating reproduction.

Environmental and social stressors can have a negative effect on fertility and/or reproductive outcomes across a variety of vertebrate taxa, including humans (Moberg, 1985; Sapolsky, 1987; Phillips, 1989; Knol, 1991; Fenster et al., 1997; Sheiner et al., 2002; Wingfield and Sapolsky, 2003). While the negative relationship between stress and reproduction has been recognized since the earliest studies of stress physiology in a wide variety of classic model species (e.g. Selye, 1939), there is a significant gap in knowledge pertaining to how stress influences the hypothalamic-pituitary-gonadal (HPG) reproductive axis in bats and may impact reproductive behavior and physiology. The neural interaction of GnIH (mammalian ortholog RFRP-3) and GnRH is widely conserved across taxa, suggesting a broadly-conserved role in reproduction and thus lifetime fitness. There are, however, substantial species differences in the localization of cell bodies producing RFRP-3 as well as in patterns of fiber immunoreactivity in the brain. These differences raise the question of functional differences of GnIH in different species and variation in the molecular signals that may be critical for organismal responses to unpredictable physiological stressors.

Big brown bats (*Eptesicus fuscus*) are a resilient and widely distributed species across North America. This species hibernates over winter, with females storing sperm in the reproductive tract during this overwintering period and delaying ovulation until arousal in the spring. This species is not currently threatened in the wild and can be easily captured in the wild. Additionally, these animals are able to maintained in captivity and are known to progress through natural annual life history stages (mating, hibernation, reproduction, weaning) within semi-natural captive settings. This presents a perfect opportunity to examine molecular mechanisms underlying reproductive physiology in a system with many variables controlled compared to a purely wild setting.

We often turn to highly seasonal species as models for exploring patterns of reproductive life history trade-offs, however I posit that this approach limits our understanding of life history and energetic strategy to broad scale changes within a particular season or time of year when predictable change (whether environmental or physiological) is known to occur. In reality, the natural world and the organisms thriving within are inherently dynamic. A breeding season, for example, can be broken down into much smaller periods of time which vary in behavioral and energetic demands, and short term shifts in behavior will alter individual expenses and capacity for resource allocation. Furthermore, the ecology and social system of a given species will also influence energetic strategy and evolution of life history trade-offs.

I propose that the flexibility of opportunistic breeders provides a unique opportunity and significant utility for exploring intricacies of acute trade-off dynamics particularly in the face of unpredictable environmental or social challenges. No study to date has examined ways in which reproductive and self-maintenance priorities (including facets of parental care behavior) may vary in response to a physiological challenge depending on specific reproductive stage (direct comparison between incubation versus nestling provisioning). In order to best predict when, or whether, compromises in reproductive investment will occur in the face of a homeostatic stressor we must integrate the complexities of individual physiological and social status into our framework of life history trade-offs. Zebra finches (*Taeniopygia guttata*) are opportunistic breeders – meaning they will reproduce any time that sufficient basic requirements are met, such as food, water, and climate. As birds, they have very discrete and identifiable periods of reproduction. Additionally, *T. guttata* exhibit mating strategies which include social monogamy and biparental care. These characteristics make zebra finches an ideal model to explore the dynamic nature of behavioral trade-offs across distinct reproductive stages (i.e. incubation and nestling provisioning) as well as the interaction with social/energetic support or lack thereof. Like *E. fuscus*, this avian species also is known to easily be maintained as a colony in captive settings and will reproduce readily.

1.5 Dissertation Objectives and Structure

This dissertation explores how physiological challenges influence reproduction at both a mechanistic and whole organism (behavioral) level, with a particular interest in trade-offs between the two tenets of fitness: survival and reproduction. I aim to take an integrative approach through *three overarching objectives*:

(1) Characterize the localization and expression of gonadotropin-inhibitory hormone homolog, RFRP, in a bat species for the first time,
(2) Use this information as the foundation to experimentally test the response of the bat HPG axis (from brain to gonads) to acute stress, and
(3) Examine the dynamic nature of reproductive and somatic trade-offs using a model that integrates both physiological challenge and social support into the energetic framework.

Going forward, this dissertation is structured into three chapters that will summarize three distinct, but connected, projects. I begin by providing a the first characterization of key inhibitory reproductive neuropeptide, RFRP, in the brain and gonads of any bat species using the big brown bat (*Eptesicus fuscus*) as a model. To follow this descriptive work, I then designed an experiment aimed at identifying molecular responses of the brain and gonads to acute stress in this temperate bat species (*E. fuscus*). Lastly, I characterize the behavioral and physiological conflicts between self-maintenance and reproductive investment and the factors that influence responses in line with energy limitation and/or trade-offs using an opportunistic avian breeder, the zebra finch.

Chapter 1

Immunoreactive distribution of gonadotropin-inhibitory hormone precursor, RFRP, in a temperate bat species (*Eptesicus fuscus*)

2.1 Abstract

Gonadotropin-inhibitory hormone (GnIH, also known RFRP-3 in mammals) is an important regulator of the hypothalamic-pituitary-gonadal (HPG) axis and downstream reproductive physiology. Substantial species differences exist in the localization of cell bodies producing RFRP-3 and patterns of fiber immunoreactivity in the brain, raising the question of functional differences. Many temperate bat species exhibit unusual annual reproductive patterns. Male bats upregulate spermatogenesis in late spring which is asynchronous with periods of mating in the fall, while females have the physiological capacity to delay their reproductive investment over winter via sperm storage or delayed ovulation/fertilization. Neuroendocrine mechanisms regulating reproductive timing in male and female bats are not well-studied. We provide the first description of RFRP – precursor peptide of gonadotropin-inhibitory hormone – expression and localization in the brain of any bat using a widespread temperate species (*Eptesicus fuscus,* big brown bat) as a model. RFRP mRNA expression was detected in the hypothalamus, testes, and ovaries of big brown bats. Cellular RFRP-immunoreactivity was observed within the PVN, DMH, arcuate nucleus (Arc) and median eminence (ME). As in other vertebrates, RFRP fiber immunoreactivity was widespread, with greatest density observed in the hypothalamus, POA, ARC, ME, midbrain, and thalamic nuclei. Putative interactions between RFRP-ir fibers and gonadotropin-releasing hormone cell bodies were observed in 16% of GnRH-ir cells, suggesting direct regulation of GnRH via RFRP signaling. This characterization of RFRP distribution contributes to deeper understanding of bat neuroendocrinology which serves as foundation for manipulative approaches examining changes in reproductive neuropeptide signaling in response to environmental and physiological challenges within, and among, bat species.

2.2 Introduction

Many bat species breed seasonally when reproductive timing is correlated with optimal resource supply and environmental conditions (Racey 1982). Temperate bat species, however, often exhibit annual reproductive life history patterns that – while not uncommon in reptiles, amphibians, and fish – are atypical for mammals. Hibernation is a common overwintering strategy among small mammals, but the timing of reproductive investment surrounding this period is unique in bats. Unlike many hibernating rodents, in which both males and females are reproductively quiescent in the fall and then upregulate reproduction in the spring, temperate-zone bats mate in the autumn prior to overwintering. This results in differing temporal periods of active reproductive investment between males and females. Often, male bats exhibit peaks of spermatogenesis in early summer and then engage in mating behavior in the fall. Once fall mating has occurred, female bats delay major reproductive investment (gestation/lactation) over winter, and they have the capacity to store sperm, delay ovulation, or postpone fertilization depending on the species (Richardson 1977, Gustafson 1979, Hosken 1996, Oxberry 1979, Crichton 2000, Willis 2017). While there is a body of literature highlighting the phenology of these events and some important factors (i.e., temperature and food availability) that may serve as cues for reproduction (Racey 1973, Racey

and Swift 1981), study of the neuroendocrine mechanisms involved in regulating reproduction in bats has not progressed significantly since the 1980s and early 1990s. Intraspecific variation in reproductive phenology and physiological capabilities (e.g. delayed ovulation, delayed embryonic development), as well as diversity of the environments inhabited, invites questions surrounding the neuroendocrine control of bat reproduction.

Like most other vertebrates, bats have a classic hypothalamo-pituitary-gonad (HPG) axis that is regulated by gonadotropin-releasing hormone (GnRH). GnRH induces release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland, and these gonadotropins regulate downstream gonadal activity with, presumably, associated steroid feedback. Seasonal variation in pituitary gonadotropes was described in greater mouse-eared bats (*Myotis myotis*) as early as 1956 (Herlant 1956) and later expanded to species including the California leaf-nosed bat (*Macrotus californicus*) (Richardson 1980, 1981) and little brown bat (*Myotis lucifugus*) (Anthony and Gustafson 1984). Female bats show greater change in pituitary LH-immunoreactivity across the annual cycle, with significant decreases during pregnancy and lactation as well as significant gonadotrope hypertrophy during hibernation. King et al. (1984) first characterized the distribution of GnRH-producing cell bodies in the Chiropteran brain demonstrating a similar distribution in *M. lucifugus* as in primates but differing from known cellular immunoreactivity in rodents (Douglas 1976). Localization of GnRH cells was later characterized in big brown bats (*Eptesicus fuscus*) with immunoreactivity in the periventricular nuclei (PVN), arcuate (Arc), and dorsomedial nucleus of the hypothalamus (DMH) as well as in the preoptic area (POA), median eminence (ME), and olfactory region (Oelschlager and Northcutt 1992). GnRH-immunoreactive (GnRH-ir) fibers were predominantly located in the hypothalamus, infundibular stalk, POA, suprachiasmatic nuclei (SCN), and bed nucleus of the stria terminalis (BNST). Cells immunoreactive for GnRH were also found within the habenular nuclei of the epithalamus, suggesting a role for this brain area in processing environmental stimuli within the context of bat reproduction (Oelschlager and Northcutt 1992). Studies exploring the temporal patterns of GnRH expression in bats reveal that GnRH-ir cell bodies and projection of GnRH-ir fibers decrease in quantity in post-ovulatory animals, suggesting that this neuropeptide can play a key role in regulating ovulation, as observed in other taxa (Anthony et al. 1989, Anthony 2000). GnRH immunoreactivity also shows seasonal fluctuations, where fewer GnRH-ir cells are present in the summer than during winter hibernation period, with greater seasonal variation within the hypothalamus than the POA; a pattern consistent across both sexes (Kawamoto et al. 1998). In 1998, the coexistence of FMRFamide-related protein (a neuropeptide from a larger family all possessing an -Arg-Phe-NH_2 at their C-terminus) and GnRH-immunoreactivity in the ARC and ME of the big brown bat was described, with colocalization between these two neuropeptides also occurring in the nervus terminalis. The FMRFamide fibers projected into the brainstem (Oelschlager et al. 1998). This FMRFamide peptide has not been definitively identified.

While the specific identity of the immunoreactive FMRF-amide peptide from Oelschlager et al (1998) is unclear, its close association with GnRH suggests the potential for functional interaction of the two peptides. Gonadotropin-inhibitory hormone (GnIH, mammalian ortholog RFRP-3), a member of the RFamide family of peptides, was discovered in 2000 (Tsutsui et al. 2000) and has a neuroanatomical and/or functional interaction with GnRH in many vertebrate species (Bentley et al. 2003, 2006). In addition to interacting directly with GnRH neurons via cell membrane receptors, GnIH/RFRP-3 also acts on gonadotrophs within the anterior pituitary (Ubuka et al.

2006). Further, GnIH/RFRP-3 and its cognate receptor are synthesized in the gonads of all vertebrates studied and appears to regulate gonadal physiology (Bentley et al. 2008, Zhao et al. 2010, McGuire and Bentley 2010; Dickens and Bentley 2014).

In the present study, we aimed to map the neural distribution of the precursor peptide for gonadotropin-inhibitory hormone, RFRP (interchangeably referred to as neuropeptide-VF, NPVF), immunoreactivity in the big brown bat (*Eptesicus fuscus*), one of the most common and widespread temperate insectivorous bats in North America (Kurta and Baker 1990). In temperate zones, *E. fuscus* mate in the fall and females arrest ovulation during winter hibernation (Wimsatt 1960, 1969). Male reproductive physiology (i.e., spermatogenesis and androgen production) occurs in late spring and early summer, and corresponds to periods of peak food availability and optimal climatic conditions (McWilliam 1987). During winter, circulating estrogen in female pallid bats (*Antrozous pallidus)* is low but rises rapidly in the spring upon arousal from hibernation (Oxberry 1979). Our goal was to examine the distribution of RFRP and determine a potential role for this peptide in regulating reproductive activity. The prior description of FMRFamide immunoreactivity in close proximity to GnRH in the big brown bat brain (Oelschlager et al 1998) guided us to also explore putative neuroanatomical interactions between RFRP-ir fibers and GnRH-ir cell bodies. A basic characterization of RFRP distribution is required before we begin exploring the complex subtleties involved in reproductive neuroendocrine signaling in response to environmental and physiological challenges within, and among, bat species. Fundamental knowledge of how the HPG axis is regulated in a temperate bat species provides the groundwork for how reproductive physiology might respond, and be well-adapted to the unique challenges that bat populations face in terms of seasonal environmental change and increased urbanization. This will ultimately move us toward greater understanding not only of species-level differences, but also the subtle variation that exists at the individual level including degree of plasticity within rapidly changing environments.

2.3 Materials and Methods

Animals and Tissue Sampling

Big brown bats (*E. fuscus*) were captured from the wild in southern Ontario, Canada and housed in a free flight colony at McMaster University where the temperature and lighting varied with ambient conditions (Skrinyer et al. 2017). Animals freely progressed through annual cycles of breeding and hibernation. Bats were given *ad libitum* access to food (mealworms) and water. Four male bats (fall 2017) and four nonreproductive female bats (spring 2018) from the colony were used in this study. Four male bats and four (non-reproductive) female bats were captured and heavily anesthetized with isoflurane and perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Remaining bats, from which fresh gonadal tissue was collected, were euthanized by barbiturate overdose with 0.4 or 0.5 ml sodium pentobarbital (Pentobarbital Sodique, 54.7 mg/ml; Ceva Santé Animale, Libourne, France) via intraperitoneal injection. Gonadal tissues were subsequently collected, immediately frozen on dry ice and stored at -80°C. Whole brains of all individuals were removed and fixed in 4% paraformaldehyde in PBS for 24-48 hours before they were transferred to a 30% sucrose in PBS cryoprotectant solution for 5 days at 4°C. Brains were then frozen on dry ice and stored at -80°C until further processing.

Presence of RFRP mRNA expression in Brain and Gonads

One hypothalamus, ovary, and testis were used for the identification of cDNA encoding big brown bat RFRP. Total RNA was isolated from tissues using TriZOL (Invitrogen, Carlsbad, CA) and 1 ug of RNA was reverse transcribed into cDNA (iScript cDNA synthesis kit, Bio-Rad, Hercules, California). Gene-specific primers (Table 1) were designed to amplify target regions corresponding to RFRP, as well as beta-actin as a positive control in each of the tissues. PCR products were visualized using ethidium bromide on a 1.5% agarose gel (Figure 1A) where known target amplicon size was confirmed.

Identification of RFRP (NPVF) Sequence

Amplification products from endpoint PCR for RFRP were submitted to the UC Berkeley Functional Genomic Laboratory (FGL) for Sanger sequencing. The returned sequence was used as a query for nucleotide BLAST (/blast.ncbi.nlm.nih.gov) whereby the resulting search reported a 91% identical match (Fig. 2A) for the predicted *Eptesicus fuscus* RFRP mRNA sequence (Accession number XM_008150846.1). A multiple sequence alignment was generated using predicted big brown bat RFRP mRNA sequence and known sequences in quail, mouse, rhesus macaque, and human (Fig. 2B).

Immunohistochemistry – RFRP & GnRH

Fixed and frozen male (n=4) and female brains (n=4) were cryostat-sectioned (40 µm) into antifreeze prior to labeling via floating immunohistochemistry. Each brain was organized into 4 parallel series during sectioning with one brain of each sex cut on the sagittal plane and the remaining three cut on the coronal plane. Brain sections were stored at -20 degrees C until further immunohistochemical processing.

Sections were washed in 1X PBS (0.1M) before incubating in 0.05% hydrogen peroxide diluted in 0.1M PBS (pH for 15 min at room temperature. After washing again in 0.1M PBS, a blocking solution of 0.2% PBS-T/2% normal goat serum (NGS) was added, and sections were gently agitated for 2 h at room temperature. For RFRP single-labeling, tissue was incubated in anti-RFRP primary antibody (PAC123,124 1:5000, generated by George Bentley, in 0.2% PBS-T/1% NGS) for 48 hours at 4°C. Following incubation in primary antibody, sections were washed in 0.2% PBS-T and incubated in goat anti-rabbit biotinylated secondary antibody (1:200 in PBS-T, Vector Laboratories, #BA-1000) for 1 hr, followed by Vectastain Elite ABC as described by the manufacturer (Vector Laboratories, #PK-6100) for 1 hr. Sections were washed in 0.2% PBS-T and color development for RFRP immunoreactivity was performed using VIP substrate according to manufacturer's instructions (Vector Laboratories, #SK-4100).

For double-labeling RFRP alongside GnRH, sections were first incubated in anti-HU60 primary antibody (HU60, gift from H. Urbanski, at 1:10,000 in 0.2% PBS-T/1% NGS) for 48 hours at 4°C. Sections were incubated in goat anti-rabbit biotinylated secondary antibody as above before Vectastain Elite ABC and color development of GnRH-immunoreactive material was performed according to manufacturer's instructions using Vector DAB substrate (Vector Laboratories, #SK-4600). Following incubation with the DAB solution, sections were washed 5x in 1X PBS before applying antibody for RFRP after which tissue was washed thoroughly 5x with 0.2% PBS-T and then incubated in anti-RFRP primary antibody (PAC123,124 1:5000 in 0.2% PBS-T/1% NGS) for 48 hrs at 4 °C replicating the protocol as described above.

GnRH/RFRP double-labeled sections were used to determine putative interactions between RFRP-immunoreactive fibers and GnRH-immunoreactive cell bodies. Oil immersion microscopy (Zeiss Imager M.1, Zeiss Plan-NEOFLUAR oil 1018-595 100x objective) was used to visualize GnRH cells at high magnification throughout each the brain of each animal. For each bat, the total number of GnRH-ir cells were counted as well as the number of these cells that possessed RFRP-ir fibers touching or crossing over the soma in the same plane of focus, from which we calculated a percentage.

RFRP Cell Location and Fiber Density Analysis

Cell locations were documented by brain region using an unpublished *E. fuscus* brain atlas (atlas researched and produced by Dr. Matt Carter with the guidance of Dr. John Casseday and Dr. Ellen Covey, Department of Psychology, University of Washington) in conjunction with a cytoarchitectural brain atlas of the common vampire bat (Bhatnagar, 2008). Two brains from non-reproductive female *E. fuscus* (collected in spring) were used to generate fiber distribution data. Color images were converted in Adobe Photoshop to 8-bit black and white with a resolution of 360 pixels/inch. Fiber density was analyzed using ImageJ. The immunoreactivity threshold in these images was standardized across all representative tissue sections to ensure that any background coloration was not included in fiber density calculations. The density of RFRP immunoreactive fibers was quantified by determining the percent area of immunoreactivity within 200 μm x 200 μm regions of interest (ROI) placed as a grid over each brain section (ImageJ). Based on minimum (3%) and maximum (79%) immunoreactivity output for all tissue sections analyzed, ranges of fiber density were set where LOW = 5-19%, MED = 20-30%, and HIGH = >30%.

Antibody Characterization

One series of *E. fuscus* brain sections (coronal) was used to perform an anti-RFRP antibody (PAC123,124) preadsorption using chicken-derived GnIH peptide (as performed in Bentley et al. 2003) to confirm specificity of this antibody in this particular species. No immunoreactivity was observed in these sections via microscopy. This antibody has been shown to be highly specific for RFRP across several mammalian and avian species.

2.4 Results

Bats exhibit RFRP-3 precursor (RFRP) mRNA expression in the hypothalamus and gonads.

We first aimed to determine whether big brown bats express RFRP within the hypothalamic-pituitary-gonadal (HPG) axis. RFRP mRNA was detected in hypothalamic and gonadal tissue isolated from both male and female *E. fuscus* (Fig. 1). A comparison of the amplified partial mRNA sequence for RFRP in the big brown bat to known RFamide-related peptide mRNA sequences for rat, human, and macaque (Fig. 2B) indicated that bats show the greatest degree of homology with non-human primates with respective similarities of 58.33%, 60.83%, and 63.33% (via ClustalOmega2.1multiple alignment).

RFRP cellular immunoreactivity is localized in the hypothalamus, arcuate nucleus, and median eminence while RFRP fiber-ir is widespread in the bat brain.

Histological localization of neuron fibers and cell bodies containing mature RFRP peptide was determined using immunohistochemistry. In non-reproductive male and female big brown bat

brains, RFRP immunoreactive cell bodies were concentrated in the arcuate nucleus (Arc). Nearly all cells observed were bipolar, and the broad distribution did not differ between the sexes (Table 2). As shown in Fig. 3, cells were commonly seen closely surrounding the third ventricle (3V) including the arcuate nucleus (Arc), paraventricular nucleus (PVN), dorsomedial nucleus (DMH), ventromedial nucleus (VMH), and median eminence (ME). A single small population of RFRP-immunoreactive cells was observed caudally at the top of the spinal column (Fig. 5B), and was surrounded by network of dense RFRP-ir fibers. RFRP fiber immunoreactivity, as in other vertebrates, was observed in multiple areas of the bat brain, ranging from anterior regions such as the nucleus accumbens (nAc) to posterior projections extending down the spinal tract (Figs. 4 and 5). Regions such as the preoptic area (POA), arcuate, median eminence, paraventricular and ventromedial thalamic nuclei, and cuneiform nuclei showed greatest density of RFRP immunoreactive fibers (Fig. 6). Fibers were seen to project beyond these dense regions, extending bilaterally from the third ventricle toward the lateral hypothalamus (LH) and throughout the midbrain. These patterns of fiber density were consistent across individuals examined, with no noted differences between sexes.

RFRP immunoreactive fibers project to subset of GnRH cells.
RFRP fibers were in high density within the preoptic area (POA), arcuate, and median eminence, so we explored potential interactions between RFRP-ir cell projections and GnRH-ir neuron cell bodies. Putative neuroanatomical connections between RFRP-ir fibers and GnRH-ir cells were observed in approximately 16% of GnRH-ir cells, with the majority of GnRH-ir cells lacking any detectable contact with RFRP-ir fibers and no difference between sexes (Figure 7).

2.5 Discussion

Over evolutionary time, species become well-adapted to the local and annual pattern of the environmental conditions they experience. Energetic constraints on reproductive investment have led to the evolution of different reproductive strategies and variation in the flexibility within the reproductive (HPG) axis. The neural interaction of GnIH (mammalian ortholog RFRP-3) and GnRH is widely conserved across taxa (e.g., Bentley et al. 2003), suggesting a broadly-conserved role in reproduction and thus lifetime fitness. There are, however, quite substantial species differences in the localization of cell bodies producing RFRP-3 as well as in patterns of fiber immunoreactivity in the brain. These differences raise the question of functional differences of GnIH in different species. In this study, we provide the first description of RFRP distribution in any bat species, characterizing sequence information as well as highlighting cellular localization in the brain. The latter varies in some ways from what we know in other, traditionally studied species in laboratory environments. This may not be surprising as the reproductive life history strategies of many bat species is quite different from other mammals and may be a result of energetic constraints imposed by flight and, for females of temperate species, the somewhat unique need to pause reproductive investment during overwintering. Having said that, we still do not know the function of gonadotropin-inhibitory hormone in bats.

Big brown bats express RFRP transcripts in the brain, ovaries, and testes. This agrees with what is seen in other mammals examined (Kriegsfeld et al. 2006, McGuire and Bentley 2010, Zhao et al. 2010, Singh et al. 2011, reviewed in Bentley et al. 2017), including humans (Oishi et al. 2012), as first described in birds (Bentley et al. 2008). Like other hibernating mammals, temperate bats are

seasonal breeders, upregulating their reproductive physiology at a specific time within the annual cycle. Bats offer a unique opportunity for future studies examining the pattern of reproductive neuropeptide expression within the context of male versus female reproductive physiology, including mechanisms involved in regulating extended reproductive suppression in overwintering female animals and HPG axis reactivation in the spring. While extended torpor and hibernation is not uncommon in small mammals, temperate-zone bats present an opportunity to examine the potential role of an inhibitory reproductive neuropeptide, RFRP-3, in the lengthy period of reproductive quiescence exhibited by hibernating females.

We found that big brown bats show cellular RFRP immunoreactivity in the dorsomedial hypothalamus (DMH), paraventricular nuclei of the hypothalamus (PVN), ventromedial nuclei of the hypothalamus (VMH), as well as lateral regions of the hypothalamus and the arcuate (Arc). This is a wider distribution than has been observed in rodent model systems (mouse, rat, hamster), where RFRP is confined primarily to cells of the DMH with few in the region of the VMH (Kriegsfeld et al. 2018, Tsutsui and Ubuka 2018, Hinuma et al. 2000, Legagneux et al. 2009). Primates (macaque) and naked mole rats possess RFRP-ir cells in the PVN, but naked mole rats are the only species reported to exhibit RFRP-ir cells in the arcuate, as seen in the present study on big brown bats (Peragine et al. 2017). The arcuate is known to have abundant beta-endorphin neurons across taxa. Within the macaque brain GnIH-immunoreactive fibers were seen to have putative interactions with beta-endorphin expressing cells, suggesting that there may be an interactive inhibitory effect on reproduction (Ubuka et al. 2009). While we did not explore it in the present study, RFRP presence in the Arc suggests that this is a possibility for big brown bats as well.

Big brown bats used in this study possessed abundant RFRP fiber immunoreactivity in multiple brain areas. Regions with the greatest density of RFRP-ir fibers were hypothalamic regions surrounding the third ventricle, the lateral hypothalamus, POA, median eminence, midbrain, and ventral posteromedial nucleus of the thalamus (VPM). However we consistently observed sparse RFRP-3 fibers in the olfactory bulb as well as apparent connectivity between the POA and hypothalamus, and lengthwise down the spinal tract across all individuals examined. GnIH-ir fiber distribution has been well-characterized in other taxa. Japanese quail exhibit GnIH fibers mainly in the hypothalamus, POA, median eminence, telencephalon, optic tectum, dorsal motor nucleus in the medulla oblongata (Ukena et al. 2003). In rats, the lateral septal nucleus in the telencephalon, PVN and other nearby hypothalamic nuclei, periaqueductal region of the midbrain, and pons were the areas with the greatest RFRP immunoreactive fiber labeling (Yano et al. 2003). Primates – with which we show that bats share the greatest RFPR genetic similarity – have been found to have RFRP-ir fibers in the nucleus of the stria terminalis, habenula, PVN, POA, arcuate, ME, dorsal hypothalamus (diencephalon area), medial region of the superior colliculus, midbrain, and pons (Ubuka et al. 2009). In seasonally-reproducing mammals, reproductive physiology is activated or inhibited in synchrony with environmental factors, most notably photoperiod. Directional change in day length is integrated into the mammalian neuroendocrine system via the retinohypothalamic tract which generates a signal to the suprachiasmatic nucleus (SCN) and regulates melatonin synthesis and secretion from the pineal gland (Elliot and Tamarkin 1994, Moore 1995). The duration of melatonin secretion is transduced in the hypothalamus to regulate downstream reproductive physiology (Carter and Goldman 1983, Bittman and Karsch 1984, Nakane and Yoshimura 2018). No study to date has examined how RFRP expression, synthesis, or secretion is

impacted by environmental cues in a bat species. Our map of RFRP fiber distribution suggests that it may be acting on cell populations across the brain via an extensive network, including the potential for signaling within the SCN. Given the annual reproductive patterns of temperate bat species, future controlled experiments should be designed to explore potential interactions between photoperiodic machinery and regulation of the HPG axis in bats.

We found that approximately 16% of GnRH-immunoreactive cell bodies receive putative contacts from RFRP-ir fibers, with GnRH neurons located primarily in the median eminence (ME), arcuate, PVN, with some scattered within the DMH/VMH and preoptic area (POA). The localization of GnRH cell bodies we see in the big brown bat differs from what is well-characterized in rodents, where GnRH cell bodies are localized primarily in the anterior hypothalamus and POA (Silverman et al. 1979). Our findings are consistent with early immunohistochemical characterization of GnRH in the little brown bat (King et al. 1984), big brown bat (Oelschläger and Northcutt 1992), Japanese long-fingered bat (Mikami et al.1988) and that which is seen in primates/humans (Parker et al. 1980, Silverman et al. 1982). Our findings also support the overlap in distribution described in 1998 between GnRH (previously named LHRH) and FMRFamide-like immunoreactivity in the brains of *E. fuscus* (Oelschläger and Northcutt 1998), the same study species used here. The putative neuroanatomical interaction we describe between RFRP-ir fibers and GnRH cells provides some evidence that RFRP may have the potential to regulate GnRH secretion directly at some level, however RFRP-ir fibers also projected to regions beyond those containing GnRH cells suggesting functions that may extend beyond upstream HPG regulation. Only one study has described a reproductive neuropeptide during hibernation in bats, finding a greater number of GnRH-immunoreactive neurons within the arcuate and noting that these cells were also larger in size than that seen in non-hibernating bats (Mikami et al. 1998). It has been suggested that low ambient temperature and declines in food availability may provide cues to downregulate reproductive biology in female bats entering hibernation (Racey 1982). It is possible that RFRP is acting upstream of GnRH to provide an inhibitory signal that maintains the HPG axis in a quiescent state throughout the challenges of a harsh winter. If this is the case, it would then be important to understand what cues – stress, environmental temperature, resource availability, social stimuli, etc. – that may cause upregulation of RFRP in the brain of temperate bats.

2.6 Tables and Figures

Table 1. List of polymerase chain reaction (PCR) primer sequences.

Gene	Forward Primer	Reverse Primer
beta Actin (ref)	TCCCTGGAGAAGAGCTACGA	ACAGGTCCTTACGGATGTCG
GAPDH (ref)	GGAGCGAGATCCCGCCAACAT	GGGAGTTGTCATACTTGTCATGG
RFRP / NPVF	ATGAGCACACCTGCAGTCAA	GCTGTTGTTGTCCCAAACCT

Figure 1. Neuropeptide VF (NPVF) mRNA expression was observed in isolated hypothalamus, ovary, and testis of big brown bats. Amplification products matched the predicted size for both NPVF (155 bp) and actin (178 bp) controls.

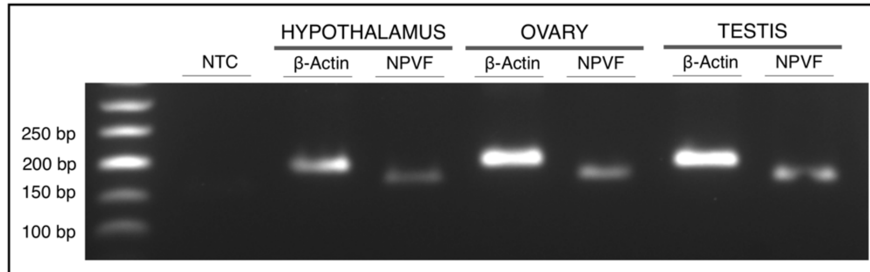

Figure 2. (A) Neuropeptide VF (NPVF) amplification product showed 91% match to the big brown bat (*Eptesicus fuscus*) predicted sequence (NCBI Ref: XM_008150846.1). (B) Multiple mRNA sequence alignment including partial big brown bat transcript, where * indicates regions most conserved.

(a)

Score	Expect	Identities	Gaps	Strand
141 bits(76)	5e-30	96/106(91%)	6/106(5%)	Plus/Plus

```
Amplification Product  1    AAGAAAGCACNNNGGGCCATNACCACCCTCGCNCTCTCGAGAAGGGAAGACATATAAAGG
                            ||||||||||   |||| || |||||||| || |||| |||||||||||||||||||||||
RFRP Predicted         278  AAGAAAGCAC-TGGGGCAATGACCACCCT-GC-CTCT-GAGAAGGGAAGACATATAAAGG

Amplification Product  61   AAAGCATCTAATCTCGCCACAAAGGTTTGGGACAACAACAGC-AAA  105
                            |||||||||||||| |||||||||||||||||||||||||||| |||
RFRP Predicted         334  AAAGCATCTAATCT-GCCACAAAGGTTTGGGACAACAACAGCCAAA  378
```

(b)

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Eptesicus(amplified)    --------------------------NNTNNNAG--CCANNNGANAAGAAAGCAC--NNN    30
rat                     ACTCAGCAGCCAACCTTCCCCTGAGGTTTGGGAGGAACATAGAAGACAGAAGAAGCCCCA    313
macaque                 ACTCAGTCACCAACTTGCCATTGAGATTTGGGAGGACCACTGAAGAAGAAAGAAGCGCTG    354
human                   ACTCCTTCGCCAACTTGCCATTGAGATTTGGGAGGAACGTTCAAGAAGAAAGAAGTGCTG    354
                                                    *   **   *     * *   *** *

Eptesicus(amplified)    GGGCCATNACCACCCTCGCNCTCTCGAGAAGGGAAGACATATAAAGGAAAGCATC-----    85
rat                     GGGCACGGGCCAAC--------------------------ATGGAGGCAGGGACCATGAG    347
macaque                 GAGCAACAGCCAACCTGCCT--CTGAGATCTGGAAGAAATATGGAGGTGAGCCTCGTGAG    412
human                   GAGCAACAGCCAACCTGCCT--CTGAGATCTGGAAGAAATATGGAGGTGAGCCTCGTGAG    412
                        * **     *** *                          **  ***   *   *

Eptesicus(amplified)    --------TAATCTCGCCACAAAGGTTTGGGACAAC---AACAGCAAA------------    122
rat                     CCATTTTCCCAGCCTGCCCCAAAGGTTTGGGAGAAC---AACAGCCAGACGCATCACCAA    404
macaque                 ACGGGTTCTTAACCTGCCCCAAAGGTTTGGGAGAACGACAACAGCCAAAAGTGTCTGCAG    472
human                   ACGTGTTCCTAACCTGCCCCAAAGGTTTGGGAGAACAACAACAGCCAAAAGTGTCTGCAG    472
                                  * *  *** ************* ***   ****** *
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Figure 3. RFRP immunoreactive cell bodies were observed in the brain with predominant localization within the hypothalamus. (A,C) Distribution was most dense in the arcuate nucelus (Arc) and median eminence, however (B) cells were also seen surrounding the third ventricle (3V) within the DMH and PVN.

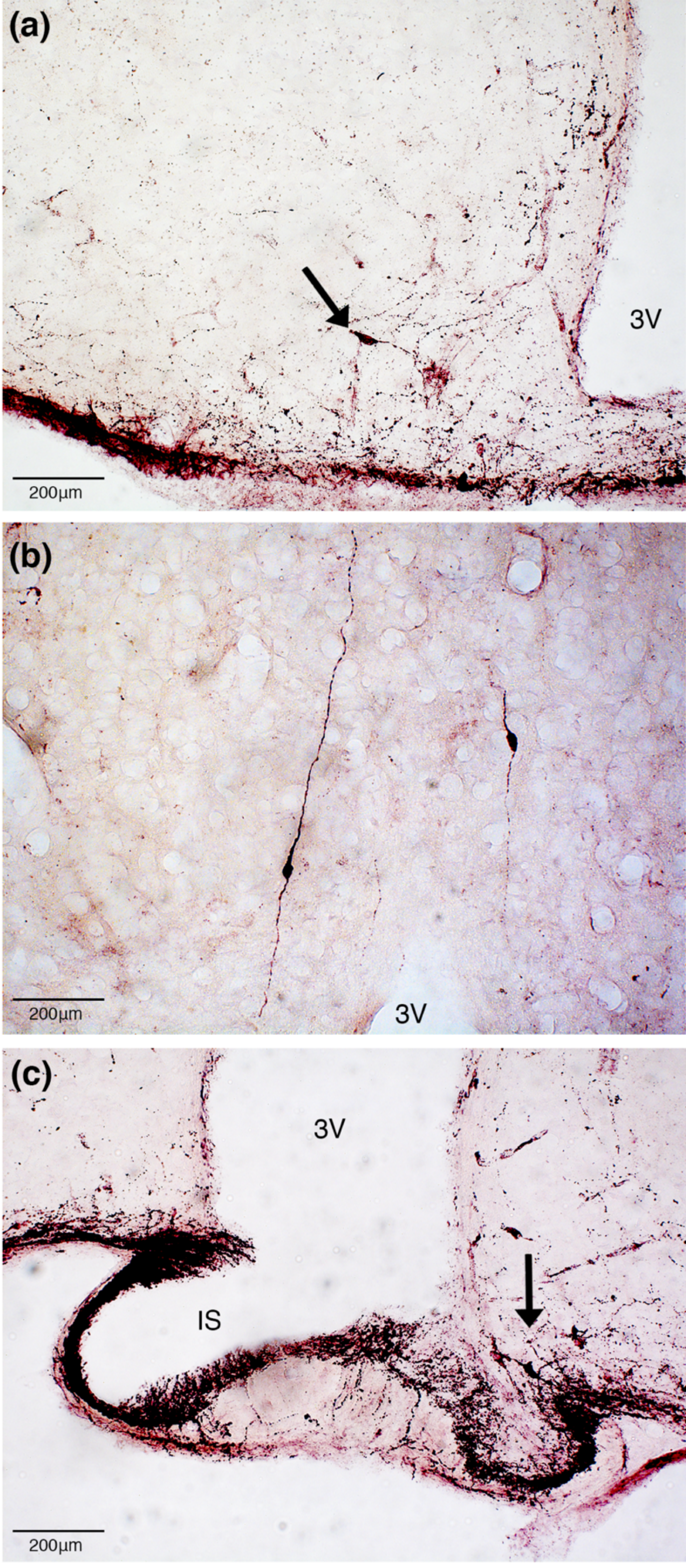


Figure 4. Coronal sections showing RFRP immunoreactive fiber labeling in the brain. Fiber-ir was greatest in the (A) preoptic area and (B) lateral hypothalamus, regions surrounding the third ventricle (3V) including (C) PVN, DMH, Arc, and median eminence, as well as (D) posteriorly toward the spinal tract and dorsal third ventricle.

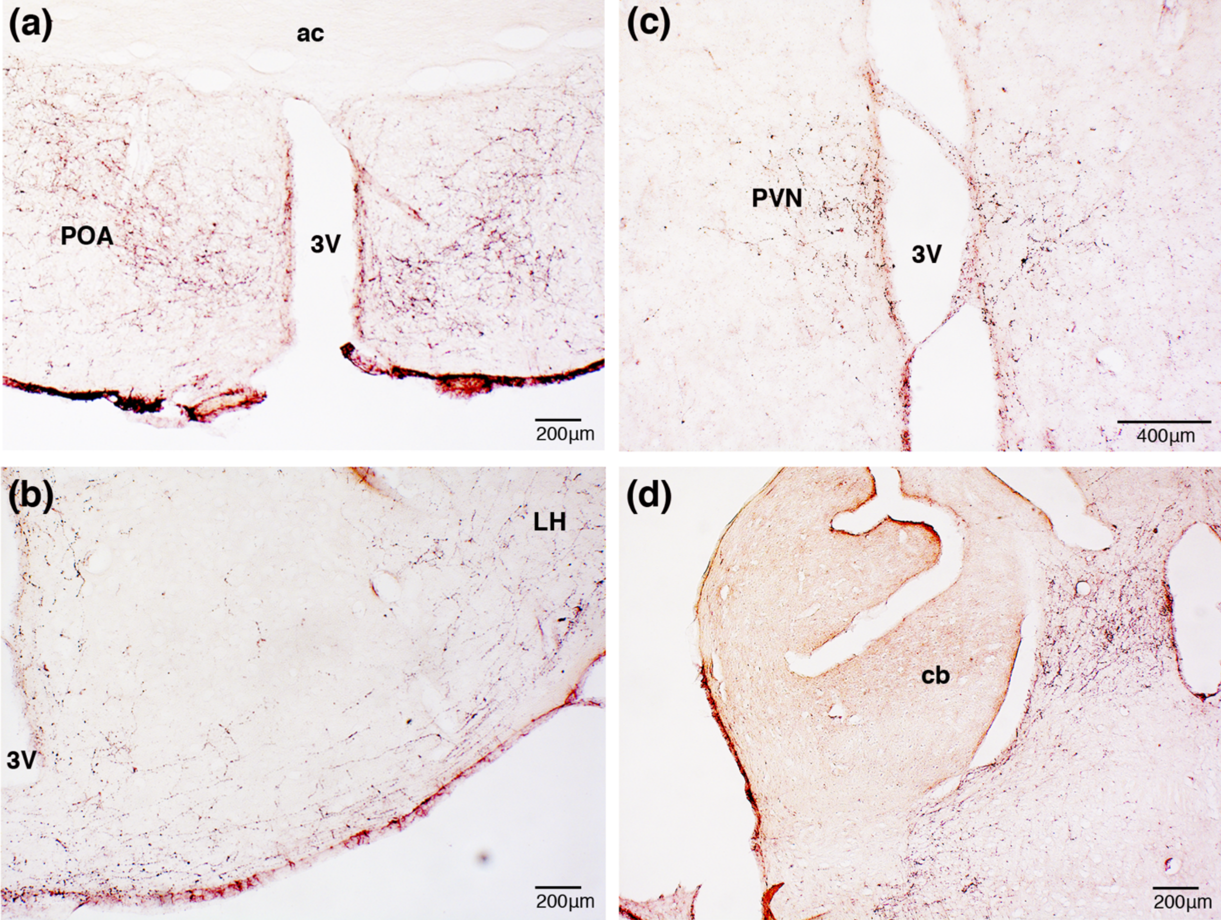


Figure 5. Sagittal sections showing RFRP immunoreactive fiber labeling in the brain. Fiber-ir could be seen extending across the midbrain toward the cerebellum, and connecting the POA and hypothalamus, two dense regions of RFRP immunoreactivity.

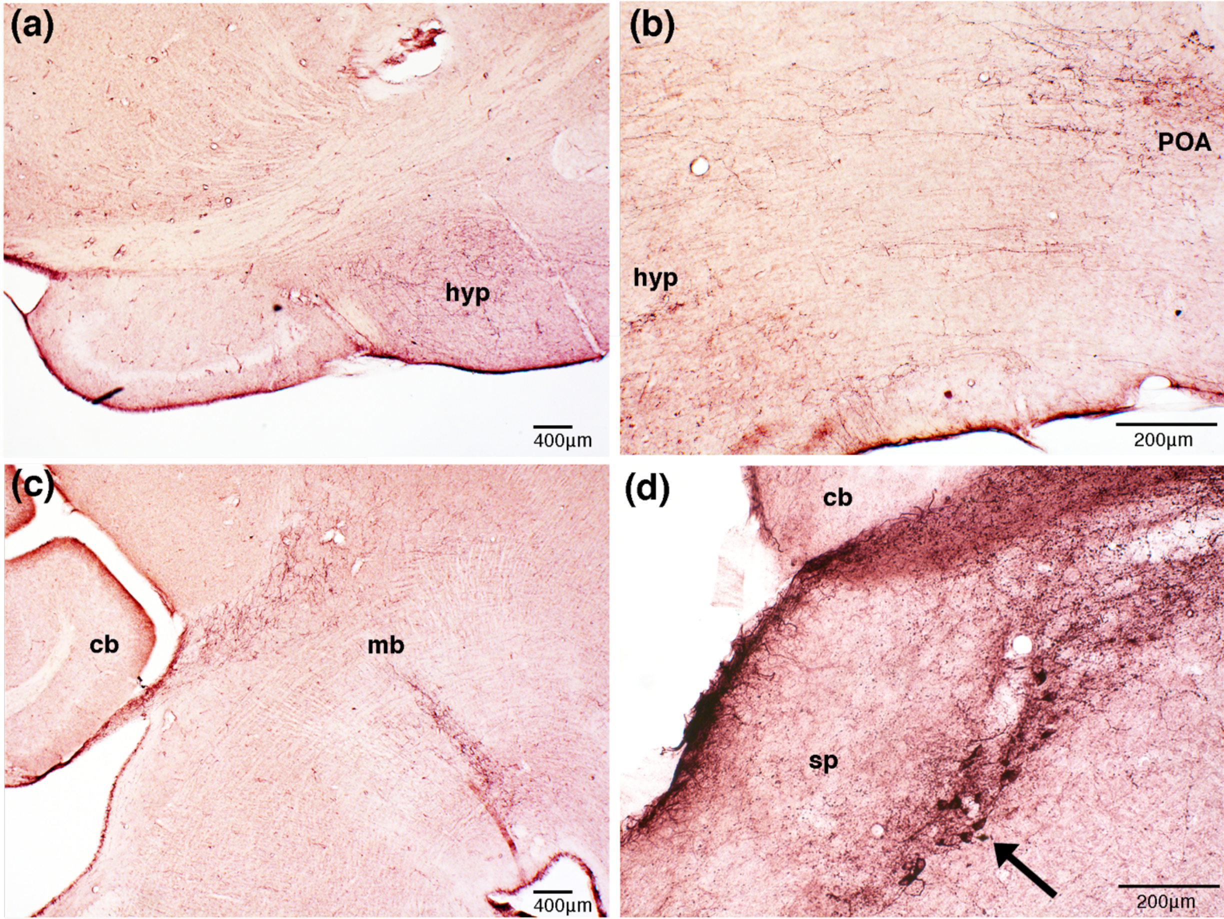


Figure 6. Immunoreactive RFRP fibers were quantified to map regions of low (blue), medium (pink), and high (red) density across the entire brain. Fibers were observed consistently from anterior to posterior (A-H) with pre-optic area (POA), median eminence (ME), VPM, and cn being areas of highest density. Representative microscopy images highlighting (I) low, (J) medium, and (K) high fiber density in the brain.

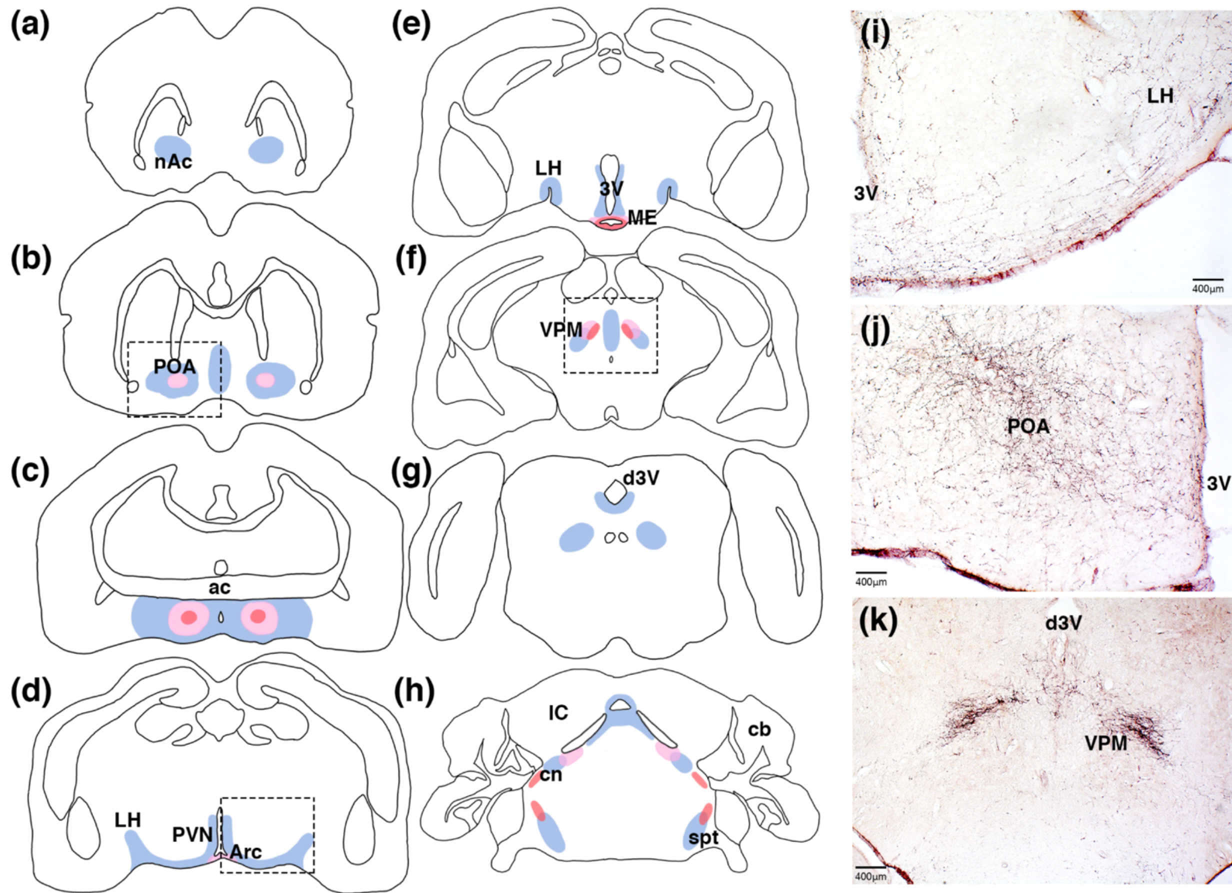
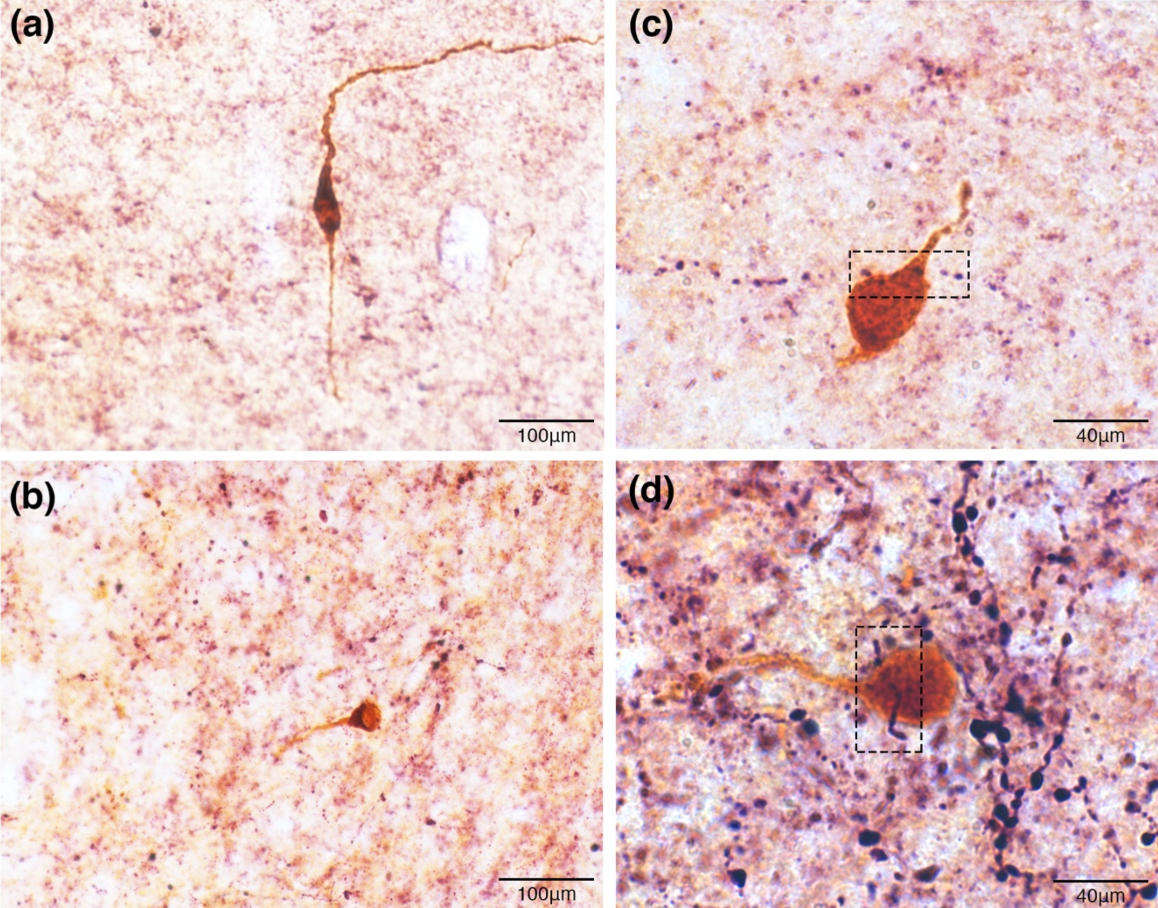


Figure 7. The majority of GnRH-ir cell bodies observed in the brain of big brown bats did not appear to interact with RFRP-ir cells (A-B), however putative contacts (C-D) were observed between RFRP immunoreactive fibers and GnRH-immunoreactive cell bodies in approximately 16% of cells.



Chapter 2

Acute restraint stress rapidly impacts reproductive neuroendocrinology and downstream gonad function in big brown bats (*Eptesicus fuscus*)

3.1 Abstract

Animals face unpredictable challenges that require rapid, facultative physiological reactions to support survival but may compromise reproduction. Bats have a long-standing reputation for being highly sensitive to stressors, with sensitivity and resilience varying both within and among species. yet little is known about how stress affects the signaling that regulates reproductive physiology. Here we provide the first description of the molecular response of the hypothalamic-pituitary gonadal (HPG) axis of male big brown bats (*Eptesicus fuscus*) in response to short-term stress using a standardized restraint manipulation. This acute stressor was sufficient to upregulate plasma corticosterone and resulted in a rapid decrease in circulating testosterone. While we did not find differences in the mRNA expression of key steroidogenic enzymes (StAR, aromatase, 5-alpha reductase), seminiferous tubule diameter was reduced in stressed bats coupled with a 5-fold increase in glucocorticoid receptor (GR) mRNA expression in the testes. These changes, in part, may be mediated by RFamide-related peptide (RFRP) because fewer immunoreactive cell bodies were detected in the brains of stressed bats compared to controls—suggesting a possible increase in secretion—and increased RFRP expression locally in the gonads. The rapid sensitivity of the bat testes to stress may be connected to deleterious impacts on tissue health and function as supported by significant transcriptional upregulation of key pro-apoptotic signaling molecules (Bax, cytochrome-c). Experiments like this broadly contribute to our understanding of the stronger ecological predictions regarding physiological responses of bats within the context of stress which may impact decisions surrounding animal handling and conservation approaches.

3.2 Introduction

Bats are critical for the maintenance and stability of many terrestrial ecosystems. Acting as pollinators, seed dispersers, voracious insectivores, and prey for a variety of birds and mammals, the maintenance of bat populations is critical to the success of plants and animals globally. Of the approximate 6,500 mammalian species currently recognized, roughly 1,400 (21%) are bats. Despite their importance to ecosystem health, relatively little is known about the endocrine and molecular mechanisms regulating the reproductive physiology of bats, including how the reproductive system responds to stress. As anthropogenic change continues and urban sprawl expands, it is important to understand how various acute and chronic stressors impact reproductive signaling and physiology in bats.

Reproduction is an energetically costly process. In nature, animals are often faced with unpredictable challenges that require rapid, facultative physiological responses that support survival (Wingfield, 2015) and that often come at the expense of reproduction (Wingfield and Sapolsky, 2003). Environmental and social stressors can have a negative effect on fertility and/or reproductive outcomes across a variety of vertebrate taxa, including humans (Moberg, 1985; Sapolsky, 1987; Phillips, 1989; Knol, 1991; Fenster et al., 1997; Sheiner et al., 2002; Wingfield and Sapolsky, 2003). While the negative relationship between stress and reproduction has been

recognized since the earliest studies of stress physiology in a wide variety of classic model species (e.g. Selye, 1939), very little is known about how stress influences the hypothalamic-pituitary-gonadal (HPG) reproductive axis in bats.

Sensitivity and resilience to stress appear to vary both within and among species, even though the basic molecular components of the hypothalamic-pituitary-adrenal (HPA) axis are highly conserved. The endocrine system is essential for regulating circadian rhythms and seasonal energy balance, and it impacts metabolism, cognition, cardiovascular function, reproduction, and general behavior collectively as part of preserving the internal milieu (i.e. homeostasis) of the organism (Selye, 1950; reviewed in Sapolsky et al., 2000). The HPA axis is also critical for proper physiological response following physical or psychological stress. A variety of stressors result in the synthesis and secretion of corticotropin-releasing hormone (CRH; Vale et al., 1981) as well as arginine vasopressin from the paraventricular nucleus and supraoptic nucleus of the hypothalamus, which further stimulates the production and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. Subsequently, ACTH initiates release of glucocorticoids (GCs; corticosterone and/or cortisol, depending on the species) from the adrenal cortex (Whitnall, 1993; Smith and Vale, 2006; Watts, 2007). These GCs impact cellular activity in a variety of tissues and are subsequently regulated via negative feedback through binding with glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) at the level of the brain and anterior pituitary gland (Reul and DeKloet, 1985; Dallman 2007; Herman, 2010).

In many species, CRH and adrenal GCs are known to interact directly with the HPG axis. For example, stress-induced increases in CRH and GCs can suppress secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) through actions on gonadotropin releasing hormone (GnRH)-producing neurons and the anterior pituitary gland (Rivier, 1986; McClusky et al., 1988; Jasoni et al., 2005; Acevedo-Rodriguez et al., 2018; Dubey, 1985; Petraglia et al., 1987; Ciechanowska et al., 2011; Phumsatitpong and Moenter, 2018; Rivier and Rivest, 1991). Changes within the HPG axis are also associated with alterations in sexual behavior (Retana-Marquez et al., 2003; Bentley et al., 2006) and fertility (Berga and Loucks, 2005; Joseph and Whirledge, 2017). In mammals, peripheral glucocorticoid injection inhibits the secretion of gonadotropins (LH and FSH) (Dubey, 1985; Petraglia et al., 1987) and, in males, results in the regression of accessory sex organs associated with decreased reproductive success with female mates (Lerman et al., 1997).

The interaction of GCs with the neuropeptide gonadotropin-inhibitory hormone (GnIH) (Kirby et al., 2009) and its mammalian ortholog RFamide-related peptide (RFRP) (Kriegsfeld et al., 2006; Johnson et al., 2007; Murakami et al., 2008) raises questions regarding whether RFRP exerts actions that downregulate the reproductive axis in response to stress, particularly given that GnRH neurons in mammals and birds contact and express receptors for GnIH/RFRP (Bentley et al, 2003; Ubuka et al., 2008; Kirby et al., 2009; reviewed in Son et al., 2022). Acute and chronic stress in male rats causes increased RFRP transcription in the dorsomedial hypothalamus and adrenalectomy prevents this upregulation (Kirby et al., 2009), further supporting the hypothesis that GC signaling is part of the neural RFRP response to stressors. Furthermore, chronic stress in female rats reduces reproductive success, although this can be rescued by knockdown of RFRP (Geraghty et al., 2015). Because GnIH/RFRP and its receptors are expressed in the steroidogenic cells of the testes and ovaries in birds and mammals, suggesting a role in local regulation of fertility (Bentley et al., 2008; McGuire and Bentley 2010; Zhao et al., 2010; Squicciarini et al., 2018).

While prior work suggests that acute stress reliably leads to declines in plasma testosterone in some bat species (e.g. Reeder et al., 2004a, 2006b), the mechanisms underlying this effect have not been explored. Because reproduction is a key component supporting individual fitness and, more broadly, the success of wild animal populations, our study was designed to evaluate the response of the HPG axis to acute restraint stress in male big brown bats (*Eptesicus fuscus*) examining changes in neurobiology, circulating hormone concentrations, and downstream gonadal function. We hypothesized that acute stress leads to a decrease in reproductive neuropeptide signaling associated with declines in testosterone production (i.e. fertility). Specifically, we predicted that acute stress will activate the HPA axis, elevate plasma glucocorticoid concentrations, and decrease plasma testosterone concentrations through changes in steroidogenic enzymes within the gonads. Compared to non-stressed control animals, we predicted stressed bats will exhibit decreased GnRH, increased RFRP immunoreactivity in the brain, and an increased number of RFRP to GnRH neuron contacts, which together would support active inhibition of the HPG axis. The findings from this work provide a necessary foundation for building a more informed framework for ecological predictions regarding how the physiology of bats changes within the context of stressors.

3.3 Materials & Methods

Animals, Stress Protocol, and Tissue Sampling

Our study used 16 adult male big brown bats (*E. fuscus*) from a captive, mixed sex, breeding colony housed at McMaster University. Animal housing included free-flight indoor and outdoor areas where bats experienced natural, seasonal fluctuations in lighting and temperature, and had ad libitum access to food (yellow mealworms, Tenebrio molitor) and water (as per Skrinyer et al., 2017). Bats selected for the experiment were housed indoor in groups of four in stainless steel wire mesh holding cages (28 x 22 x 18 cm; l x w x h; ¼-inch mesh) for at least 72 hours to acclimate to the holding room. On days of tissue collection, a male bat was removed from each cage and randomly assigned to either the stress or control condition. Bats in the stress condition (n = 8) were secured ventrum-up for 1 h in a custom restrainer (Ceballos-Vasquez et al., 2014) with their wings outstretched and held in place with Velcro® straps. Body restraint with outspread wings is known to be highly effective in activating a physiological stress response in another small insectivorous bat from North America (Reeder et al., 2004b). Following restraint, bats were deeply anesthetized via isoflurane inhalation and euthanized by decapitation, after which trunk blood was immediately collected. Blood samples were temporarily stored on ice until they were centrifuged (2000 g for 10 min), after which isolated blood plasma was stored at -80°C for later hormone analysis.

Whole brains were extracted and immediately placed in 4% paraformaldehyde for fixation (48-72 h) after which they were cryoprotected (30% sucrose / PBS) before freezing at -80°C. Testes were also removed immediately following euthanasia and were flash frozen on dry ice prior to -80°C storage. Tissue collection for bats in the control condition (n = 8) followed identical protocols as the stress condition, with blood collection occurring within 3 min of removal from their home cage (mean: 82 seconds, range: 71-93 seconds). All procedures were approved by the Animal Research Ethics Board of McMaster University and conformed to the Guide to the Care and Use of Experimental Animals published the Canadian Council on Animal Care.

Hormone Analysis
Circulating plasma corticosterone concentrations were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical #501320, Ann Arbor, Michigan, USA). Control plasma was diluted 1:160 (sample:kit buffer) and stress plasma was diluted 1:600 prior to plating. Percent binding for all samples was between 32-53%. Circulating plasma testosterone concentrations were also determined with a commercially available ELISA kit (Enzo Life Sciences #ADI-901-065, Farmingdale, New York, USA), with control plasma diluted 1:640 and stress plasma diluted 1:100. Percent binding for individual testosterone samples ranged from 32-86%. Prior to sample processing, both kits were validated for use in E. fuscus by testing for parallelism of serially diluted plasma pools for bats from stress and control treatments (n = 8 control samples, n = 8 stress samples) alongside the kit standard and recommended standard curve dilution series. For the corticosterone kit, pooled plasma was diluted 1:137-1:880 for stress samples and 1:20-1:160 for control samples . For the testosterone kit, pooled plasma was diluted 1:20-1:640 for both control and stress samples. For both hormones, results of the parallelism test confirmed that the target analyte was recognized by kit reagents in a predictable, dose-dependent manner.

Double Label Immunohistochemistry - RFRP and GnRH Immunoreactivity
Brains were sectioned (40 µm) with a cryostat and tissue slices were organized into three parallel series. Sections were immediately transferred to antifreeze solution (5.47 g sodium phosphate dibasic, 1.59 g sodium phosphate monobasic, 9 g NaCl, 10 g PVP-40, 300 g sucrose, 300 mL ethylene glycol, 10 mL 0.1 M sodium azide, in 1 L dH2O) for storage at -20 C prior to immunohistochemistry. Six brains from each treatment group were sectioned coronally, while sagittal sections were collected from the remaining two brains per treatment group. Sections were washed 5 times with 1X PBS (pH 7.4) prior to incubating in 0.5% hydrogen peroxide in PBS for 15 min at room temperature. After washing again in PBS, a blocking solution of 2% normal goat serum (NGS) in PBS / 0.2% Triton X100 (PBST) was added, and sections were gently agitated for 2 h at room temperature. To quantify the number of cells containing GnRH, tissue sections were incubated in a solution containing anti-GnRH primary antibody (HU60, gift from H. Urbanski, 1:10,000 in 0.2%PBST/1%NGS) for 48 h at 4°C. This was followed by incubation in goat anti-rabbit biotinylated secondary antibody (1:200 in PBST) before Vectastain Elite ABC (Vector Laboratories Inc. #PK-6100, Newark, California, USA) and color development of GnRH immunoreactive (GnRH-ir) material was performed using Vector DAB substrate (Vector Laboratories Inc. #SK-4100, Newark, California, USA) as per manufacturer's instructions. Sections were washed 3 times in 0.2% PBST, then incubated in a solution of anti-GnIH primary antibody (PAC123,124 1:5000 in 0.2%PBST/1%NGS) for 48 h at 4°C to simultaneously label cells containing mature RFRP peptide. Following primary antibody incubation, sections were transferred to goat anti-rabbit biotinylated secondary antibody solution (1:200 in PBST) before Vectastain Elite ABC and color development of RFRP immunoreactive material was performed using Vector VIP substrate (Vector Laboratories #SK-4600, Newark, California, USA). The specificity of the anti-GnIH (white-crowned sparrow) antibody in the big brown bat was previously validated in our lab using a standard preadsorption method with GnIH peptide which resulted in a complete loss of immunoreactivity, as expected (see Alonge et al., 2022). The total numbers of GnRH and RFRP-immunoreactive cell bodies were counted separately across one entire series of sections for each bat brain by individuals blind to the experimental treatment. One individual from

the stress group was excluded from analysis of the RFRP cell number data due to a problem during the immunolabeling protocol which made the tissue unable to process.

RNA Isolation and Quantitative PCR

One half of one testis from each male bat was used for RNA isolation, conducted using 500 µL PureZOL (Bio-Rad #7326890, Hercules, California, USA) in an RNase-free snap cap tube. Tissue was homogenized over ice before adding PureZOL to 1.0 ml total volume and following reagent manufacturer's instructions. Samples were eluted in 30 µl nuclease-free water. RNA integrity was determined for all samples (Agilent Bioanalyzer, Functional Genomics Laboratory, UC Berkeley, Berkeley, California, USA) with resulting RIN numbers >7. For each sample, 1 µg RNA was converted to cDNA (Bio-Rad, iScript cDNA synthesis kit #1708890, Hercules, California, USA) which was then diluted 1:5 with nuclease-free water as a working stock solution that was stored at -20°C until use. All primers for reference genes and genes of interest (Table 1) were validated for amplification of a single product in big brown bat testes using endpoint PCR (Invitrogen, Platinum Taq High Fidelity #11304-011, Waltham, Massachusetts, USA) and visualized on an agarose gel.

Primer efficiencies were determined using 4-fold serially diluted five-point standard curve for all gene targets made from pooled cDNA from bat testes; calculated efficiencies fell within a range of 90 to 110% for all primer pairs used. The cDNA samples from each individual bat testis were used at 1:20 dilution for quantitative PCR (qPCR) for all genes, except for Beta-actin which was plated at 1:80 dilution. Reactions were prepared following the protocols provided by the kit (Bio-Rad SsoAdvanced SYBR Green Supermix #1725271, Hercules, California, USA) using primers at a final concentration of 0.5 µM per reaction. Absence of contamination was confirmed by lack of amplification of no-template (no cDNA) and no-reverse transcriptase controls during qPCR. Amplification of a single product was verified by the final dissociation/melting curve. Relative mRNA expression was calculated using the delta-delta Ct (Livak) method (Livak and Schmittgen 2001) using two reference genes (GAPDH and Beta-actin) confirmed to be unaffected by stress treatment. Genes of interest (GOI) were selected based on critical roles in glucocorticoid sensitivity (e.g. GR, MR), sex steroidogenesis pathway (e.g. aromatase, 5-alpha reductase, StAR), broad inhibitory role within the HPG axis (e.g. RFRP), and pro-apoptotic signaling (e.g. Bax, Apaf-1, cytochrome-c, Figure 6D). These data reflect potentially rapid transcriptional changes within the gonads in response to our stress treatment.

Testes Histology, Morphology, and TUNEL labeling

A subset of control (n = 5) and stress (n = 4) bats provided sufficient testis tissue to use for cryosectioning and hematoxylin and eosin (H&E) Y staining. Slides were prepared with a series of three 25 µm thaw-mounted testis sections from individual bats and fixed for 10 min in 4% paraformaldehyde before proceeding to standard protocol for H&E staining. Images were captured at 200X total magnification and imported into ImageJ where the diameter of each circular seminiferous tubule in cross section was measured by drawing a straight line from basement membrane to basement membrane across the center of the tubule; all circular tubules were measured within each image. Mean seminiferous tubule diameter (µm) for each individual bat was determined by converting the unitless ImageJ output measures into µm using calibrated scale bar (1.32 raw ImageJ units = 200 µm) and calculating the average for 16 total tubules per individual. Seminiferous tubular diameters of bats from the stress and control groups were compared with Welch's t-test.

A series of sections (16 µm) was collected from one testis of each bat and thaw-mounted directly on to slides for quantifying the amount and localization of TUNEL labeled cells. Due to limited tissue samples remaining from the animals in the study, a subset of control (n = 6) and stress (n = 5) bats were processed for TUNEL labeling. Slides were stored at -20°C until processing. Following the protocol supplied with a commercially available kit (R&D Systems Inc., TACS 2Tdt-DAB In Situ Apoptosis Detection Kit #4810-30-K, Minneapolis, MN, USA), sections were removed from -20°C and allowed to dry completely overnight and equilibrate to room temperature before proceeding to labeling. Sections were then gently rehydrated through a graded series of ethanol incubations (100%, 95%, 70%) and fixed on slides in 4% paraformaldehyde/PBS for 10 min at room temperature. Slides were then washed in 1X PBS for 10 min and the sections were permeabilized in cytonin solution (R&D Systems Inc. #4876-05-01, Minneapolis, MN, USA) for 45 min at room temperature inside a humidity box. Tissue was washed in distilled water two times (5 min each) before quenching endogenous peroxidase activity in a 1:10 solution of 30% hydrogen peroxide in methanol for 5 min. A positive control slide was generated by treating tissue with TACS nuclease for 10 min at room temperature and then processed alongside the experimental slides after this point. All slides were washed in 1X PBS and incubated in 1X TdT labeling buffer (provided by kit) for 5 min before adding TdT enzyme reaction solution. Tissue was incubated with this reaction mix for 1 h in a humidity box at 37°C. After incubation with enzyme, tissue was incubated in provided Stop Solution for 5 min and washed subsequently in 1X PBS two times (5 min each). Following kit instructions, a strep-horseradish peroxidase (HRP) solution was prepared and added to tissue for 10 min at 37°C, after which slides were thoroughly washed in 1X PBS to remove excess antibody and minimize background labeling. Color development of TUNEL-positive immunoreactive material was performed using Vector DAB substrate (Vector Laboratories, #SK-4100, Newark, California, USA). Tissue was counterstained using 1% methyl green and dehydrated in a graded ethanol series (70%, 95%, 100%) before clearing in Histoclear (5 min) and cover slipping using Permount medium (Thermo Fisher #SP15-500, Waltham, MA, USA).

Microscopy images (400X total magnification, Zeiss Imager A1 and AxioCam MRC5) were collected and used for TUNEL labeling analyses. Eight images were collected per individual bat and the total number of cells (via methyl green staining) was quantified using Fiji. Within the software, each 300ppi microscope image was converted to 16-bit greyscale, background subtracted (value = 40 standardized across all images), and threshold adjusted to highlight cells. Particle analysis was modified such that only particles greater than 180-pixel units were counted with standardized degree of circularity (0.3-1.0, where 1.0 is a perfect circle), and the total number of particles (i.e. cells) within each image was automatically counted. Following this, the number of TUNEL-positive cells within each image was manually counted by volunteers blind to treatment (ImageJ, multipoint tool) and the percentage of TUNEL-labeled cells was calculated per image. From this, a mean percentage of TUNEL-positive cells was calculated for each individual based on the cumulative data across 8 images per bat.

Statistical Analysis

All data are reported as the mean ± standard error (s.e.m.). No individuals were excluded from statistical comparisons of treatment (stress) and control groups unless otherwise noted below. Prior to all analyses of treatment-level differences, a Shapiro-Wilk test was used to evaluate the

normality of data for bats in the stress and the control groups. Homogeneity of variances among the groups was evaluated using Fligner-Killeen test, which is appropriate when data are non-parametric. When data were normally distributed and homoscedastic, a two-sample Welch's t-test was used to compare the means of treatment and control groups; for each comparison, the test statistic, degrees of freedom, and associated p-value are reported. In cases where the data were not normally distributed and/or variances were heteroscedastic, a Wilcoxon rank sum test was used to compare the treatment and control groups; for each comparison, the associated W and Z values are reported along with calculated p-values. In all cases, statistically significant differences between treatment and control groups ($p < 0.05$) are indicated graphically using an asterisk (*). We used Pearson's correlation coefficient (R) to evaluate the linear relationship between two variables, with the direction/strength, and significance reported through R- and p-values, respectively. In the case of analyzing the relationship between RFRP and GR expression (**Figure 5B**), one stress-treated individual was excluded from the analysis as an outlier (RFRP mRNA relative expression value = 14.41; > 1.5 X IQR) however inclusion of this individual does not change the significant relationship detected ($R = 0.6$, $p = 0.016$).

3.4 Results

Acute restraint rapidly activates the HPA axis, decreases plasma testosterone, and alters glucocorticoid sensitivity of bat testes.

Continuous restraint for 60 minutes resulted in a significant increase in circulating plasma corticosterone concentrations compared to baseline controls ($W = 0$, $Z = -3.31$, $p = 0.00093$, **Figure 1A**), thereby validating the efficacy of the restraint protocol in activating the hypothalamic-pituitary-adrenal (HPA axis. Bats experiencing acute restraint stress also had significantly reduced plasma testosterone compared to control animals ($W = 57$, $Z = -2.57$, $p = 0.01$; **Figure 1B**). The endocrine response to restraint was accompanied by an approximately 5-fold increase in glucocorticoid receptor (GR) mRNA transcription in the bat testes ($W = 9$, $Z = -2.43$, $p = 0.014$; Figure 2A), although no change in mineralocorticoid receptor (MR) expression was observed ($W = 27$, $Z = -0.46$, $p = 0.645$; **Figure 2B**).

Steroidogenic enzyme mRNA expression unchanged in the bat testes following acute restraint, but stressed bats exhibit decreased seminiferous tubule diameter

Despite a significant reduction on circulating testosterone with stress, we did not detect transcriptional changes in enzymes associated with testosterone regulation. Specifically, stress did not alter the relative expression of steroidogenic acute regulatory (StAR) protein ($W = 40$, $Z = -0.788$, $p = 0.430$; **Figure 3A**), aromatase ($df = 9.25$, $p = 0.122$; Figure 3B), or 5-alpha reductase ($W = 42$, $Z = -0.977$, $p = 328$; **Figure 3C**) in the testes of E. fuscus, demonstrating that rapid declines in testosterone were not a result of changes in steroidogenic enzyme expression. Seminiferous tubule diameter tended to be smaller in stress-treated versus control bats (group means were found to be 109.14 μm and 148.08 μm, respectively), although the difference was not quite significant ($p = 0.059$, $df = 5.0273$, $t = 2.4223$; **Figures 3D** and **E**). Individuals with higher concentrations of plasma testosterone exhibited larger seminiferous tubule diameters ($R = 0.68$, $p = 0.05$; **Figure 3F**). There was a moderate negative correlation between glucocorticoid receptor (GR) expression in the testes and seminiferous tubule diameter that was not significant ($R = -0.65$, $p = 0.067$; **Figure 3G**).

Acute restraint stress impacts RFRP immunoreactivity in the bat brain and upregulates RFRP expression in the testes

Histological localization of hypothalamic cell bodies containing immunoreactive GnRH and RFRP peptides measured with floating immunohistochemistry revealed that the number of GnRH-immunoreactive (GnRH-ir) cells did not differ between stress-treated and control bats (W = 16, Z = -1.62, p = 0.104; **Figure 4A**). In contrast, there was a significant decrease in the number of RFRP-immunoreactive (RFRP-ir) cell bodies in bats subject to restraint stress (df = 9.21, p = 0.039; **Figure 4C**). Relative RFRP mRNA expression was significantly upregulated within the testes of stress -treated compared to control bats (W = 12, Z = -2.06, p = 0.037; Figure 5A). Although there was no correlation between within-individual RFRP expression and plasma testosterone concentration in either the stress-treated (R = -0.56, p = 0.19) or control groups (R = -0.67, p = 0.083), within the control group bats with greater RFRP cell numbers tend to exhibit lower concentrations of plasma testosterone (Figure 4E). There was a positive correlation between within-individual GR expression and RFRP expression (R = 0.55, p = 0.038; **Figure 5B**).

Rapid upregulation of apoptotic gene expression in the gonads but no significant difference in TUNEL-positive cell number

There was a significant upregulation of Bax (W = 13, Z = -1.96, p = 0.049; **Figure 6A**) and cytochrome-c (p = 0.0069, W = 7, Z = -2.69; **Figure 6B**) mRNA expression in the testes following 60 min of restraint stress; however, no change in Apaf-1 expression was observed (p = 0.441, W = 40, Z = -0.76; Figure 6C). Despite rapid changes in mRNA expression, there was no difference in the number of TUNEL-positive cells between the stress-treated and control groups (p = 0.2469, t = 1.2396, df = 8.87; **Figure 7A**) although there was a tendency for control bats to exhibit an overall higher percentage of TUNEL-labeled cells in the testes (**Figure 7B**). Finally, there was no correlation between within-individual degree of mRNA expression and the number of TUNEL positive cells for any genes of interest (statistics for each gene not shown).

3.5 Discussion

We used a holistic approach to explore the impacts of acute stress on the reproductive physiology of male bats by examining changes in key reproductive neuropeptides (GnRH and RFRP), circulating plasma hormones (corticosterone and testosterone), and downstream changes in testes gene expression associated with gonad health and fertility. Acute restraint stress resulted in changes in RFRP expression at both the hypothalamic and gonadal levels of the hypothalamic-pituitary-gonadal (HPG) axis. This was associated with a predictable decline in circulating testosterone, but no change in mRNA expression of key steroidogenic enzymes (StAR, aromatase, 5-alpha reductase) in the testes. Morphological differences in the gonads were observed between stress-treated and control animals, with the seminiferous tubule diameters of stressed bats smaller than those of control bats. In contrast, there was no apparent effect of restraint stress on cellular GnRH immunoreactivity in the bat brain. Stress likely increased gonadal sensitivity to glucocorticoids through a rapid 5-fold increase in GR expression. Whether mediated by neurobiological signaling or direct glucocorticoid effects on the gonads, we also found evidence for rapid pro-apoptotic gene expression changes in the testes in response to acute (60 minute) restraint stress. Together, our findings suggest that while RFRP may be involved in a broad downregulation of the HPG axis in male bats exposed to acute stress and support the possibility

that testes may receive direct endocrine signals that influence fertility at the sex steroid and cellular-health levels.

Previous descriptions of glucocorticoid responses in bats have focused largely on stress associated with handling and manipulating individuals. For example, in captive variable flying foxes (*Pteropus hypomelanus*), restraint during handling resulted in a 4-fold rise in adrenocorticotropic hormone (ACTH) concentration after 1 h, with cortisol becoming significantly elevated after only 20 min and peaking at levels almost 800% greater than baseline measurements (Widmaier and Kunz, 1993; Widmaier et al., 1994). Similar patterns of GC dynamics have been reported for *P. vampyrus* and *P. pumulis* following restraint-stress (Reeder et al., 2004b, 2006a; Widmaier et al., 1994). Our experiment was designed specifically to examine impacts of restraint stress on the HPG and HPA axes, so we used these previous studies to determine the appropriate time for sampling tissues to quantify physiological changes associated with HPA activation. Our design produced a clear and reliable timeline for detecting neuroendocrine and gonadal changes in response to a highly standardized acute stressor.

Acute restraint is sufficient to induce changes in reproductive neuroendocrinology

Stress-induced decreases in androgen concentrations have been observed in other bats (Reeder et al., 2004a, 2006a), although neither the mechanism(s) underlying the declines nor the implications for reproductive success have been examined. Our data indicate that 60 min of restraint stress is sufficient to induce neurobiological as well as gonadal changes within the HPG axis. Although restraint stress did not influence the number of GnRH-ir cells in the brain, there was a decrease in the number of RFRP-ir cells. While this may seem counterintuitive given the inhibitory role of RFRP within the reproductive axis, it may reflect active secretion of this neuropeptide in response to the restraint treatment. This is an interesting contradictory finding as acute immobilization stress increases the number of hypothalamic RFRP-ir cells in rats (Kirby et al., 2009) and the number of GnIH-ir cells in birds (Calisi et al., 2008), with the latter varying by season. In sheep, acute restraint stress did not affect concentrations of RFRP mRNA or mature peptide in the brain (Papargiris et al., 2011), but the same group later found that the number of close contacts (via immunohistochemical methods) between RFRP-immunoreactive fibers and GnRH cell bodies increased following acute stress (~10% of cells in control group vs. ~40% after stress) and increased the number of RFRP cells co-labeled with Fos (Clarke et al., 2016). Without being able to measure RFRP release into the bat hypothalamo-pituitary-portal system, it is difficult to determine the dynamics of RFRP release in response to our manipulation. It is possible that in E. fuscus, GnRH may be more important for regulation of seasonal reproduction and less involved than RFRP in facultative responses to an unpredictable, acute stressor.

In our study, GnRH-ir cell bodies were more abundant than RFRP-ir cells, regardless of treatment. In agreement with our data, previous studies in other taxa have revealed a lack of change in GnRH, even in response to a chronic stress paradigm (Du Ruisseau et al., 1979; Kirby et al., 2009). Our results may reflect the inhibitory actions of RFRP secretion on GnRH cells; such effects would prevent subsequent GnRH release without changing GnRH-ir cell number and would ultimately inhibit LH and FSH secretion, thereby reducing testosterone production (as seen in Ducret et al., 2009; Gojska et al., 2014). Repeated cortisol injection (used to mimic chronic stress pharmacologically) increases the number of neuron-fiber contacts between RFRP and GnRH cells

in the sheep brain (Clarke et al., 2016), but the functional consequences of this change have yet to be identified.

In mammals (Kirby et al., 2009; Gojska and Belsham, 2014) and birds (Calisi et al., 2010; Son et al., 2014) RFRP-producing cells are known to express GR and there is putative molecular evidence for transcriptional upregulation of GnIH/RFRP under stress through GC-stimulated recruitment of GR to the GnIH promoter region (Son et al., 2014). As a result, glucocorticoid signaling to RFRP-producing cells in the hypothalamus may trigger the production and/or secretion of RFRP, leading to subsequent suppression of GnRH release. This may inhibit the synthesis or secretion of gonadotropins into the circulation which could influence downstream function of Leydig and Sertoli cells of the male gonads, including spermatogenesis and sex steroid production (Shalet, 2009) as reflected in our data.

Glucocorticoid sensitivity increases rapidly at the level of the bat gonad
In the present study, we demonstrate that stress-induced declines in the concentration of circulating testosterone may be linked to negative impacts on male bat fertility at the level of the testes. Our data show that acute stress can impact male gonads directly, resulting in rapidly increased transcription of GR and RFRP. The approximate 5-fold increase in GR expression we observed indicates the onset of a heightened sensitivity to GCs which, depending on the cell type, may impact downstream testosterone secretion. Furthermore, it has been shown in mice that in vitro application of RFRP to isolated testes results in the simultaneous reduction in spermatogenesis and germ cell proliferation (Anjum et al., 2014) which suggests that the upregulation of RFRP expression we observed in the bat testes may directly affect male bat fertility in a similar fashion. Furthermore, Orr and Mann (1990) found that restraint stress-induced decreases in testosterone in rats was LH independent. It is possible that the decrease in plasma testosterone we observed in bats is occurring through direct action of glucocorticoids on Leydig cells via GR, a mechanism known to suppress testes sensitivity to gonadotropins and damper testosterone production in rats (Orr and Mann, 1992). The upregulation of GR mRNA expression we observed in the bat gonads provides some support for this, although we cannot say specifically what cell types within the testes may be altering their transcriptional activity.

Seasonal or life history stage-dependent GC responses to stress have been observed in other taxa (McGuire et al., 2013; Vera et al., 2011; Wingfield et al., 1992), including bats (Gustafson and Belt, 1981; Klose et al., 2006; Reeder et al., 2004b). Sex differences in HPA activation have been observed in *P. hypomelanus*; while males displayed no detectable impact of handling on glucocorticoid levels, reproductive females were characterized by differential responses to stress throughout gestation (Reeder et al., 2004a). Glucocorticoid concentrations in little brown myotis (*Myotis lucifugus*) are generally higher in females, with both sexes showing elevations during autumn swarming (i.e., the primary reproductive season), and reproductive females showing elevated baselines during mid-to-late pregnancy in late-spring (Reeder et al., 2004b). Although we were unable to characterize the sensitivity of the HPG axis to stress across annual seasons or between sexes in our study, it is possible that highly seasonal species such as E. fuscus (that only reproduce once per year) may exhibit different responses to stress during their annual reproductive cycle or even between early versus late periods of reproductive investment (i.e. May through September). Because stress and RFRP have broad links to suppression of sexual behavior (Retana-Marquez et al., 2003; Dubey and Plant, 1985; Sato et al., 1996), it is possible the stress-induced

changes in reproductive neuroendocrinology we observed may lead to further organismal-level changes in mating behavior or individual reproductive success which could have negative fitness impacts.

Acute restraint significantly impacts testes morphology and upregulates pro-apoptotic gene expression.

We found no changes in mRNA expression of steroidogenic enzymes in the testes (StAR, aromatase, 5-alpha reductase); however, there was a decrease in seminiferous tubule diameter in stressed compared to non-stressed bats., which suggests a rapid regressive tissue response. It is possible that other enzymes in the testosterone synthesis pathway (e.g. cytochrome p450) may respond to acute restraint in bats, or that functional enzyme activity may be altered, thus providing a causal connection between stress, GC activity, and negative impacts on gonad tissue morphology and function.

Given the apparent sensitivity of the bat testes to acute restraint and a concomitant absence of change in steroidogenic enzyme expression, the overall impacts of stress on gonadal cell health are not immediately clear. We detected transcriptional upregulation of two key signaling molecules — Bax and cytochrome-c — involved in the receptor-mediated apoptotic pathway in the testes of E. fuscus. Bax, a member of the bcl-2 protein family, is one of the core apoptotic pathway regulators that interacts with mitochondria and mediates outer membrane permeability (Westphal et al., 2014). An increase in membrane permeability leads to the subsequent release of cytochrome-c from the mitochondrial matrix, enabling a series of downstream caspase activation steps and cell death (Santucci et al., 2019). While we did detect increases in Bax and cytochrome-c mRNA expression, we did not find significant differences in the percent of TUNEL-labeled cells in the testes of stress-treated bats compared to controls. Because TUNEL-labeling is a method that detects DNA fragmentation, a molecular process that would occur only at late-stages of cell death, it is possible that we were not able to capture the expected response within the 60 minutes of our protocol. Interestingly, we found a tendency for control bats to exhibit greater total percent of TUNEL-positive cells in their testes. These data – while contradictory to our predictions – may reflect a higher degree of cell turnover, and thereby higher degree of gonadal activity, within the testes of control bats compared to those that experienced stress given that stressed animals decreased seminiferous tubule diameter and apparent testosterone production.

The upregulation of pro-apoptotic gene expression for Bax and cytochrome-c was a surprising finding given the short duration (60 minutes) of our stress manipulation; however, there is a precedent for pro-apoptotic effects in the gonads following longer periods of stress. For example, female mice restrained for 24 h displayed increased corticotropin-releasing hormone (CRH) in the ovaries, accompanied by decreased IGF-1 and upregulation of Bax mRNA expression (Liang et al., 2013). Additionally, chronic stress can cause increases in plasma glucocorticoids, decreases in testosterone, and an increase in the frequency of apoptotic Leydig cells in rat testes (Chen et al., 2012), a finding that is supported by Gao et al., (2003) where in vivo Leydig cell treatment with GCs resulted in apoptosis. Additionally, there is a link between intra-testicular RFRP and upregulated gonadal apoptosis. In mice, in vitro treatment of testes with RFRP was seen to cause dose-dependent increases in caspase-3 and PARP peptide cleavage indicative of receptor-mediated apoptosis signaling (Anjum et al., 2014). The fact that testes health and function may be suppressed without the direct influence of circulating gonadotropin signaling suggests that GCs are likely

acting directly on the gonads to downregulate cellular maintenance and sex steroid synthesis with potentially deleterious impacts on overall tissue health. Thus, while studies in other model systems support the pro-apoptotic effects we observed, the rapidity of this transcriptional response in bats is a novel finding and an exciting avenue for research. Future work should continue to explore gonad function and fertility in female and male bats, identifying details of the underlying cellular biology mechanisms and timeframes for recovery before we can make broad claims regarding the sensitivity of bat reproductive physiology and fertility to stressors.

3.6 Tables and Figures

Table 1. Validated forward and reverse primer sequences used for quantitative real-time PCR

Gene	Forward Primer	Reverse Primer
beta Actin (reference)	TCCCTGGAGAAGAGCTACGA	ACAGGTCCTTACGGATGTCG
GAPDH (reference)	GGAGCGAGATCCCGCCAACAT	GGGAGTTGTCATACTTGTCATGG
GR	AGACCAAGCGATTCCTCTCA	AGCCTAAGTCACAGGGAGCA
MR	AGCGTGAGTCCAGCAAAGAT	CACGCTGAAGATGTCACGTT
RFRP	ATGAGCACACCTGCAGTCAA	GCTGTTGTTGTCCCAAACCT
cytochrome-c	GCATTTCTTTCGAGGCAGTC	GCACTCACCACCTCTGTGAA
BAX	ATGGAGCTGCAGAGGATGAT	ATCAGCTCAGGCACTTTGGT
APAF1	ACTGCTTTCCTGGTCATTTGAT	AGAGGGGAAAGGAGTTCAAAAC
StAR	CTCTGGCCCTTACATGTCTACC	GCCCCTCTTGTAAGACCCTAAT
aromatase	CAAGTGCCTCAAATGCTGAA	GACAAGATCAGGCGACCAAT
5-alpha reductase	TGGTGAGATCATCGAATGGA	ATGAGGGCTTTCCGAAACTT

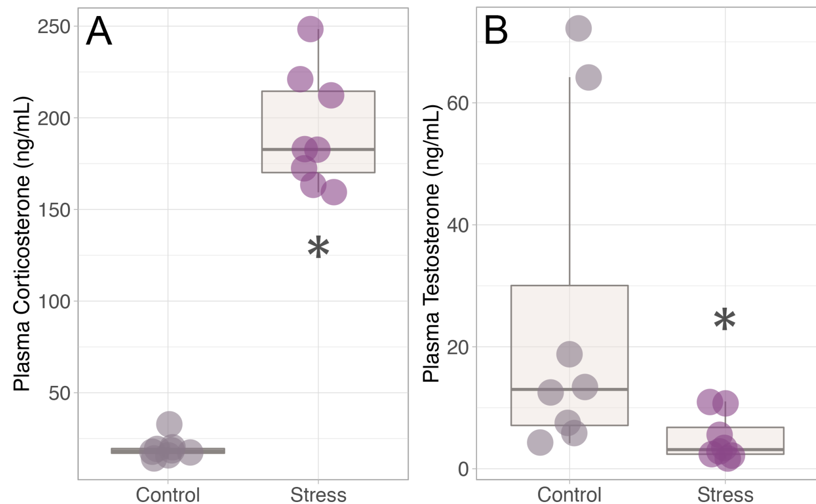


Figure 1. Impact of 60 minutes of restraint stress on (A) circulating corticosterone concentrations (ng/mL) and (B) testosterone (ng/mL). Stress significantly upregulated plasma corticosterone and resulted in significantly reduced testosterone in male bats compared to control animals, asterisk indicates $p < 0.05$. Boxplots display the group median (horizontal line), with shaded region representing the interquartile range (25%-75%) and vertical lines extending to group minimum and maximum values. Outliers are displayed as stand-alone points beyond these vertical lines.

Figure 2. Impact of 60 min of restraint stress on testes receptor gene expression. (A) Glucocorticoid receptor (GR) mRNA expression was significantly higher in male bats. (B) There was no effect on mineralocorticoid receptor (MR) expression. Bar plots represent mean ± SE (vertical bars) relative mRNA expression normalized to the control group. Asterisk indicates a significant difference ($p < 0.05$).

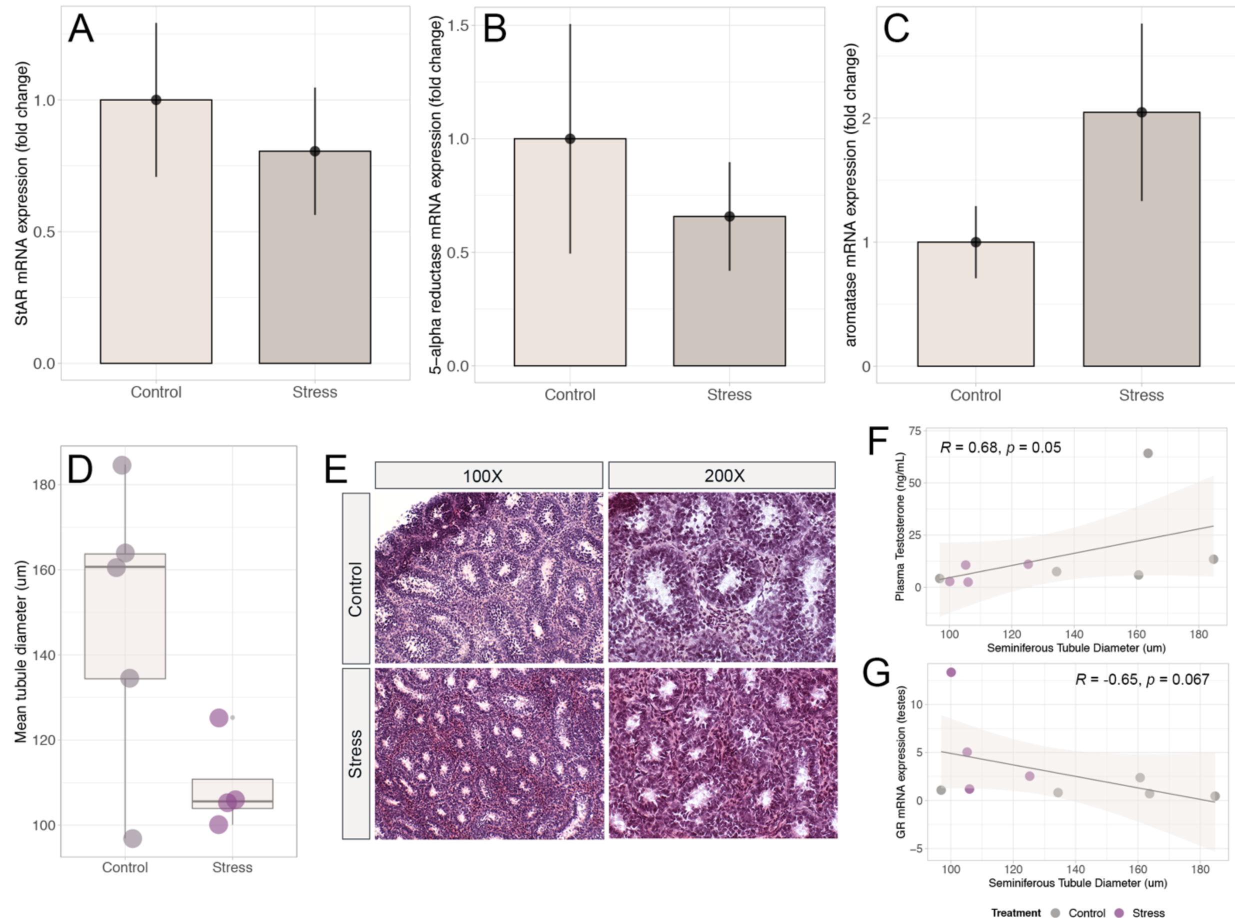
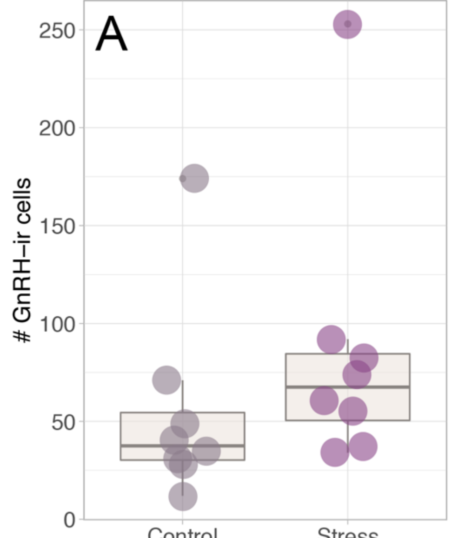
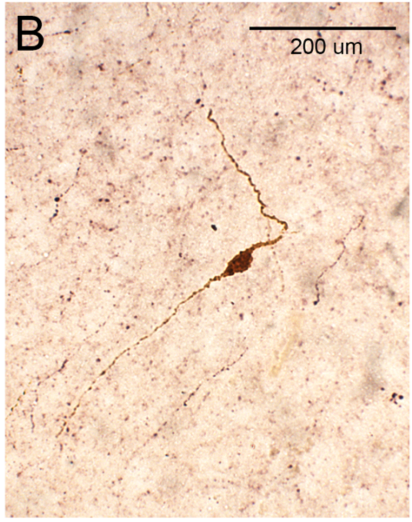
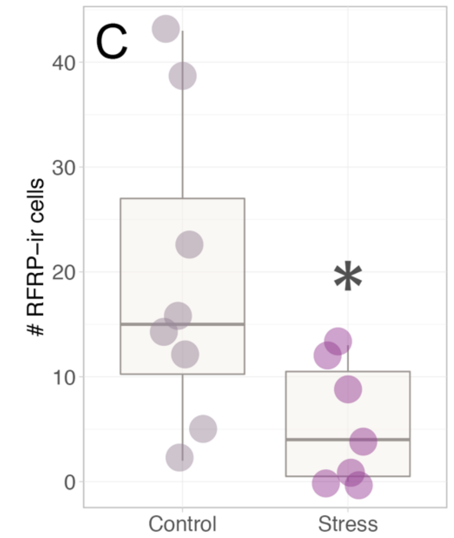
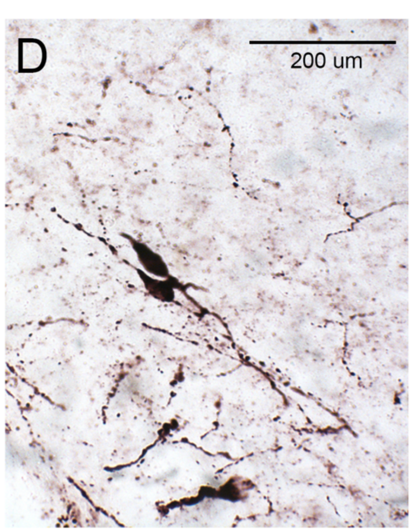


Figure 3. Expression of key steroidogenic enzymes in the bat testes and impact of stress on seminiferous tubule diameter. No significant transcriptional effect of acute restraint stress was observed for (A) steroidogenic acute regulatory (StAR) protein, (B) 5-alpha reductase, or (C) aromatase. Bar plots shown represent mean relative expression (normalized to control group) with standard error represented as vertical lines within each bar. (D) Individual mean seminiferous tubule diameter (µm) of control and stress-treated groups. Boxplots display the group median (*horizontal line*), with shaded region representing the interquartile range (25%-75%) and vertical lines extending to group minimum and maximum values. Outliers are displayed as stand-alone points beyond these vertical lines. (E) Representative testes microscope images following hematoxylin and eosin staining. (F) A significant positive relationship was detected between individual plasma testosterone concentration and seminiferous tubule diameter using spearman correlation coefficient, while (G) individuals with greater mRNA expression for gonadal GR tended to have smaller seminiferous tubule diameter. Shaded region represents 95% confidence interval.









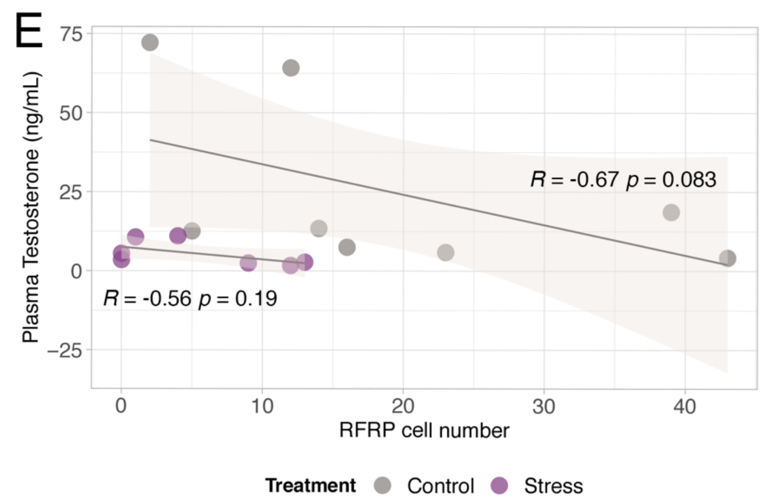


Figure 4. Impacts of restraint stress on key stimulatory and inhibitory reproductive neuropeptides. (A) The total number of immunoreactive cell bodies labeled for gonadotropin-releasing hormone (GnRH) in the brains of male bats was unchanged by acute restraint; (B) representative image of detected immunolabeling via DAB substrate (400X). However (C) significantly fewer RFRP-immunoreactive cell bodies were observed within stressed animals compared to controls, asterisk indicates $p < 0.05$; where (D) RFRP cells were labeled using Vector VIP substrate. Boxplots display the group median (horizontal line), with shaded region representing the interquartile range and vertical lines extending to group minimum and maximum values. Outliers are displayed as stand-alone points beyond these vertical lines. We found (E) no significant within-individual relationship between quantified RFRP-ir cell number and respective concentration of plasma testosterone, although there is a tendency ($p = 0.083$) within control animals for those with greater RFRP cell number to exhibit lower circulating testosterone. Shaded region represents 95% confidence interval.

Figure 5. Impact of 60 min of restraint stress on testes RFRP regulation and relationship with GR expression. (A) Acute restraint stress resulted in transcriptional upregulation of RFRP in the testes. Bar plots represent mean ± SE (*vertical bars*) relative expression normalized to the control group. (B) Increase in RFRP mRNA expression was positively correlated with within-individual testes GR expression, with no apparent effect within treatment groups ($p > 0.05$). Asterisk indicates a significant difference ($p < 0.05$). Shaded region in panel B represents 95% confidence interval.

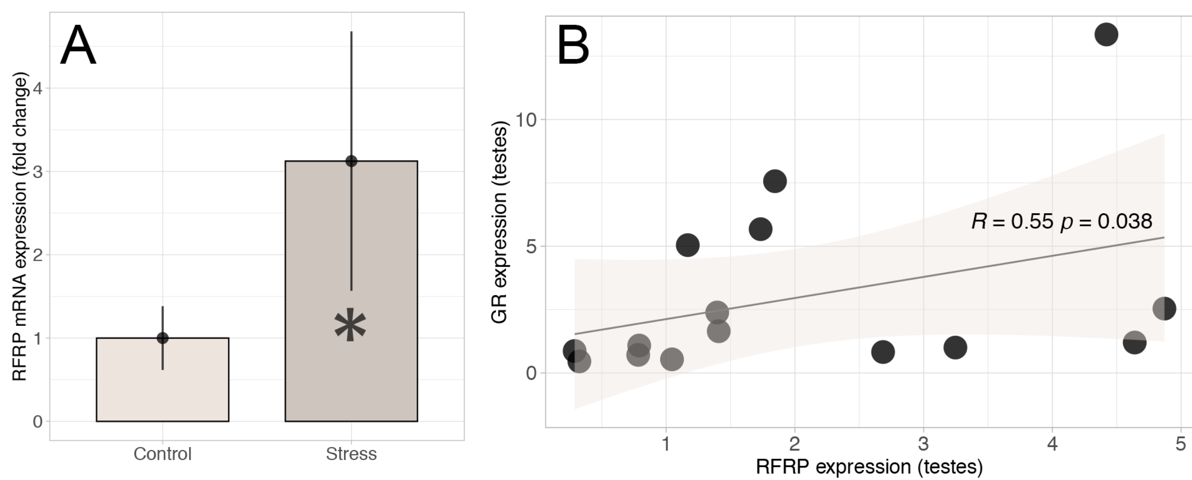
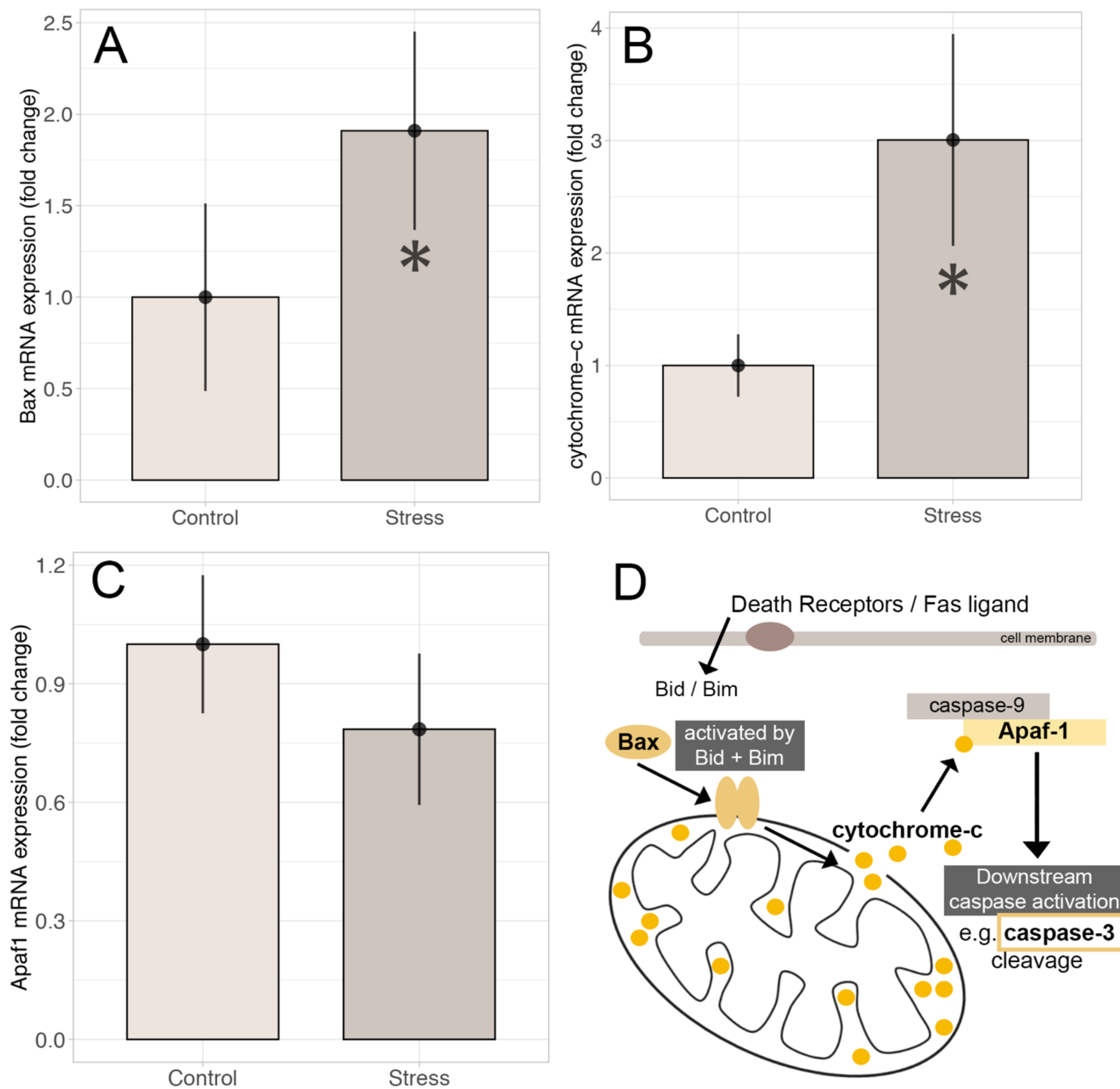


Figure 6. Effects of 60 min of restraint stress on pro-apoptotic mRNA expression in the testes. In bats exposed to restraint there was a significant transcriptional upregulation of (A) *Bax* and (B) *cytochrome-c*, but not in (C) *Apaf-1* mRNA expression. (D) These target molecules are involved in a mitochondrial mediated pro-apoptotic pathway that begins with activation of death receptors in the cell membrane. This signal of cellular stress stimulates Bid and Bim to activate Bax, which then dimerizes and induces mitochondrial membrane permeabilization and subsequent release of cytochrome-c from the outer mitochondrial matrix. Cytochrome-c binds to Apaf-1, permitting the interaction of Apaf-1 with caspase-9. Activation of caspase-9 is the first in a series of classical downstream caspase activation steps that ultimately leads to cellular apoptosis. Asterisk indicates a significant difference ($p < 0.05$). Bar plots represent mean ± SE (*vertical bars*) relative expression normalized to the control group.



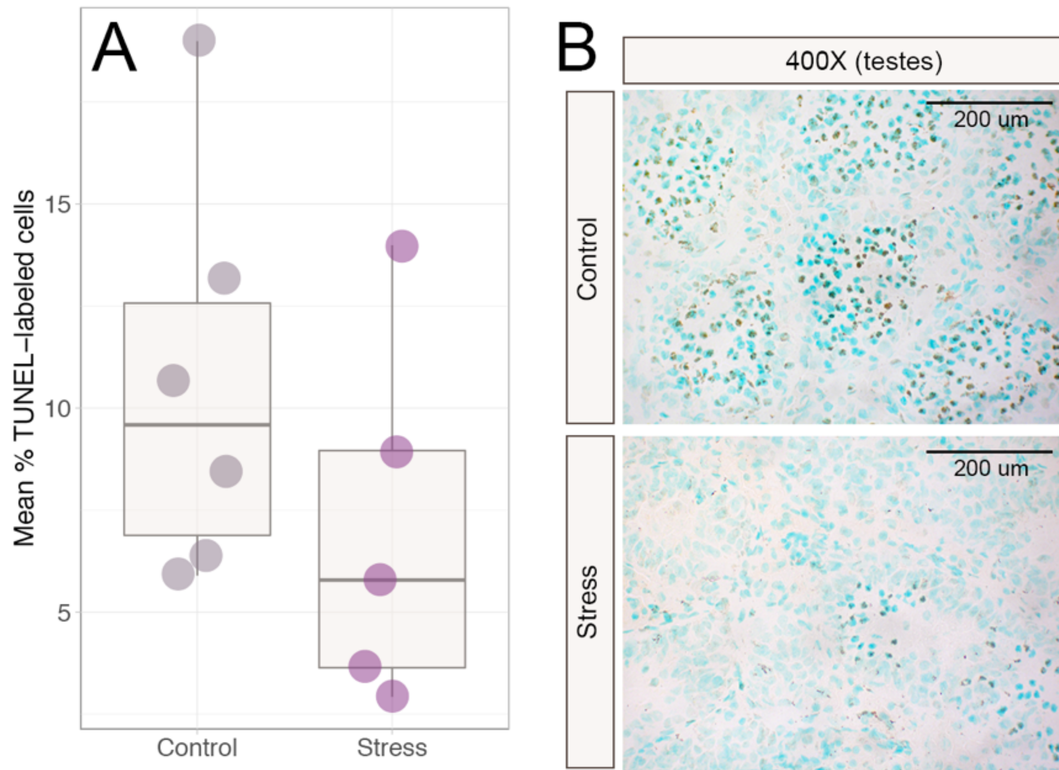


Figure 7. No effect of stress on late-stage apoptosis in the bat testes indicated by TUNEL-reactivity. (A) There was no difference in the percent of total TUNEL-positive cells in the testes of stressed bats compared to the un-stressed control group. Boxplots display the group median (horizontal line), with shaded region representing the interquartile range and vertical lines extending to group minimum and maximum values. Outliers are displayed as stand-alone points beyond these vertical lines. (B) There was a tendency for control bats to exhibit greater percent of TUNEL-positive cells. Individual cell nuclei visualized by methyl green and TUNEL-positive cells were labeled using DAB substrate (brown colorization).

Chapter 3

Sick and flying solo: Impact of single parenthood on trade-off dynamics between survival and reproduction in a socially monogamous songbird (*Taeniopygia guttata*)

4.1 Abstract

This project examines the importance of timing on shaping our predictions surrounding life history trade-offs through a direct comparison of organismal response across discrete stages within the reproductive period. I aim to provide behavioral and physiological measures indicative of investment in self-maintenance (i.e. processes that support survival) versus reproduction. Parental female zebra finches were treated with LPS either at midpoint of incubation or during nestling provisioning. We found that regardless of reproductive stage, total activity was reduced in LPS-treated females, but only a detectable increase in % time spent resting was seen during provisioning. There was a near significant negative body mass change in LPS-treated females only during the incubation period ($p = 0.09$). Parental care (# nest visits and % time in nest over 6 hours) was only compromised with LPS during nestling provisioning and sick females tended to also have longer intervals between nest visits during this reproductive stage. Within sick provisioning females, those with larger brood size tended to spend less time resting. LPS treatment did not affect baseline plasma corticosterone in females, or their healthy male mates. Because zebra finches are socially monogamous and provide biparental care, we repeated this experimental design adding removal of the male mate at time of LPS/saline treatment to explore how this may shift the trade-off thresholds within the reproductive stages or change the female response. Single parenthood increases energetic demand for each female, and thus we found a conflict between self-maintenance and reproductive investment (either behavioral or physiological) during incubation and, in some cases, more extreme compromises in parental care during provisioning with potentially negative repercussions on offspring.

4.2 Introduction

Seasonal environmental variation influences animal energy budgets in ways which shape species' life histories. Classical life history theory posits that organisms are continually challenged with allocating resources between competing processes. Constraints on resources, or behavioral and/or physiological capacity, have important implications for individual fitness and life history evolution – often leading to trade-offs. Most frequently, life history trade-offs are examined through a physiological lens, focusing on the allocation of macromolecules and nutrients or upregulation of signaling that supports one aspect of an organism's physiology at the expense of another. Time, however, is arguably an equally important resource within the context of managing and maximizing individual fitness, and selection pressures may have led to the evolution of behavioral trade-offs which arise when investing in behaviors supporting one facet of life history (e.g., survival) may conflict with investment in other behaviors (e.g., reproduction). Understanding the flexibility and context-dependence of trade-offs is essential to identifying selection pressures that

shape temporal aspects of species' life history, as well as to developing strong predictions regarding individual resilience within increasingly variable environments.

There are fluctuations in environment that are predictable (e.g. day/night, seasonal temperature), but organismal responses to unpredictable change might require rapid, facultative shifts in behavior and physiology during – or immediately following – a stressor (Wingfield 2015). Pathogen exposure is one such unpredictability that may challenge organismal physiology and alter behavior as well as apply strong selection pressures on species' life history evolution. Exposure to an immunogenic compound results in a well-defined acute phase response (APR) which may include expression of stereotypical "sickness behaviors" that accompany the upregulation of physiological mechanisms that support individual survival such as cytokine release, APR protein production, and fever response (Hart 1988; Aubert et al. 1995; Dantzer et al. 2008). The ability to mount this rapid response through the innate immune system a – a first line of defense common among all vertebrates – often correlates negatively with reproductive investment or overall reproductive success. Social context can influence behavioral responses, including predicted sickness behaviors, during an immune challenge (Lopes et al. 2012, Lopes et al. 2013, Lopes et al. 2021; Hennessy et al. 2020). For example, male zebra finches treated with lipopolysaccharide to induce the acute phase response do not exhibit the expected decline in locomotor activity or body mass when housed in a group (colony) setting but do exhibit sickness behavior and significant body mass loss in isolation (Lopes et al. 2012).

Periods of parental care - any trait, behavioral or physiological, that enhance the fitness of offspring (Clutton-Brock 1991; Royle, Smiseth, and Kölliker 2012) – are a demanding life history stage for reproducing animals both in terms of energy, and time allocation. It is broadly postulated within life history theory that parental resources (time, energy) allocated to offspring cannot therefore be used to support self-maintenance (Stearns 1992; others). Seminal work describing demands of parental care emphasized high levels of activity required and postulated that physiological costs are incurred which sacrifice self-maintenance and survival (Kendeigh 1952; Royama 1966; Drent and Daan 1980). Birds are excellent models for exploring trade-offs within the context of reproduction as they have very identifiable and discrete phases of breeding and – across species – express a wide array of mating systems and optimal parental care strategies. In altricial avian species, average metabolic energy expenditure in chick-rearing adults is approximately 3-times basal metabolic rate (BMR) with reports of maximum daily expenditures of up to 6-times BMR (Piersma and van Gils 2011). These high energetic demands required suggest that physiological costs are likely to compromise investment in self-maintenance and survival. Furthermore, while the vast majority of species operate under female-only parental care systems, or no parental care at all, birds are an interesting evolutionary exception where approximately 85% of avian species exhibit biparental care (Cockburn 2006). While there are clear synergistic fitness benefits in biparental care systems, the inherent sexual conflict between male and females can lead to variation in the type and degree of parental care investment between them and suggests that individual fitness is maximized by a certain level of 'self-interest' (Wesolowski 1994; Takahashi et al. 2017). Thus, we hypothesize that the presence or absence of social support will impact energetic demands experienced by one sex, with potential impacts on energetic thresholds associated with trade-offs between reproduction and self-maintenance (i.e. survival).

We often turn to highly seasonal species as models for exploring life history trade-offs, however this approach limits our understanding of life history and energetic strategy to broad scale changes within a particular season or time of year when predictable change (whether environmental or physiological) is known to occur. In reality, the natural world and the organisms thriving within are inherently dynamic. A breeding season, for example, can be broken down into much smaller periods of time which vary in behavioral and energetic demands, and short-term shifts in behavior will alter individual expenses and capacity for resource allocation. Furthermore, the ecology and social system of a given species will also influence energetic strategy and evolution of life history trade-offs. We therefore propose that the flexibility of opportunistic breeders provides a unique opportunity and significant utility for exploring intricacies of acute trade-off dynamics particularly in the face of unpredictable environmental or social challenges.

Many studies have provided detailed characterization of the APR in a variety of bird species including *T. guttata* (Owen-Ashley and Wingfield 2007; Burness et al. 2010; Sköld-Chiriac et al. 2014; Scalf et al. 2019) and others examining variation in activity and parental energetic demands during avian reproduction. No study to date has examined ways in which reproductive and self-maintenance priorities (including facets of parental care behavior) may vary in response to a physiological challenge depending on specific reproductive stage (direct comparison between incubation versus nestling provisioning). In order to best predict when, or whether, compromises in reproductive investment will occur in the face of a homeostatic stressor we must integrate the complexities of individual physiological and social status into our framework of life history trade-offs. Here we harnessed the opportunistic nature of zebra finch reproduction, along with their mating strategies which include social monogamy and biparental care, to explore the dynamic nature of behavioral trade-offs across distinct reproductive stages of incubation and nestling provisioning.

Using an acute immune challenge via lipopolysaccharide (LPS) injection – a nonlethal immunogenic compound isolated from bacteria cell walls – we accumulated a suite of behavioral and physiological data divided into specific output measures associated with parental care investment and behaviors supporting self-maintenance, and coupled this with non-terminal physiological data indicative of immune response and metabolic activity (e.g. plasma corticosterone concentrations). To test trade-off thresholds, we took a novel approach of combined LPS treatment and (male) mate removal, increasing parental demand and workload for the female. We aimed to determine how specific timing within the reproductive period influences the likelihood for trade-offs to occur, and when behaviors supporting reproductive investment versus self-maintenance are in conflict, with the broad hypothesis that social/parental support may impact trade-off thresholds. We predicted that there will only be a conflict between behaviors supporting self-maintenance and those supporting reproduction during nestling provisioning, and therefore female finch behavior and physiology will be more stable following an immune challenge during incubation. Further, biparental care may alleviate some energetic demand for immune-challenged females, therefore we predicted that loss of this social support of the male would result in shifts in physiology and behavior that strongly favor self-maintenance of the female in support of individual survival.

4.3 Materials and Methods

Animal Husbandry

Adult (greater than 180 days of age) captive zebra finches were colony-housed in a free-flight aviary at the Field Station for the Study of Behavior, Ecology and Reproduction at the University of California, Berkeley. All birds were supplied with ad libitum food (millet and canary seed), water, nest boxes, nesting material, and allowed to pair freely of their choosing. Aviary spaces are designed to expose animals to natural changes in daylength and contain natural branch perches for enrichment. Each bird was uniquely banded prior to the onset of the experiment for identification and the behavior of each pair was monitored regularly prior to treatment for association with a specific numbered nest box and to record progression through reproductive stages (i.e. day of incubation, nestling hatching). All animal husbandry and experimental manipulations were carried out following approval by the University of California, Berkeley Office of Laboratory Animal Care (OLAC) and in accordance to animal welfare laws and accepted practices.

Injection Protocol, and Mate Removal

Incubation experiments were standardized to occur between day 7-9 of their 14-day incubation period across all breeding pairs. Prior to incubation experiments, egg number was standardized to 4 eggs per nest to equilibrate perceived parental workload across breeding pairs. During nestling provisioning, all experiments occurred 7 days after hatching of the clutch was complete. Females and males of each pair were briefly captured no less than 2 days prior to treatment and were uniquely marked with colored sharpie on their chest for quick identification within the colony. On the day of injection, both female and male parental birds were rapidly caught (< 3-5 min) by using a soft butterfly net within the aviary (8:30AM) and approximately 40uL blood was collected by brachial venipuncture into one heparinized capillary tube and specific bleed times were recorded with the aim of collecting all blood samples within 3 minutes of entering the aviary. Body mass of female and males was recorded, as well as body mass of individual nestlings in the case of experiments occurring during the provisioning period. The female of each pair was injected either with 50uL lipopolysaccharide (LPS, Sigma Aldrich #L4005, 0.5mg/mL) or vehicle (0.9% saline, sterile). The specific dose chosen (approximately 2mg/kg body mass) is known to induce sickness behaviors within zebra finch colonies previously used for study in our aviary system (Lopes et al. 2012, Lopes et al. 2013, Lopes et al. 2014).

Birds were then returned to the colony and parental nest activity was recorded for 6 consecutive hours (9:00AM-3:00PM) by video camera. Parental birds were observed 5 hours post-injection to record stereotypical sickness behavior (outlined below). Following observation, parental birds were captured, blood was collected a second time from the opposite wing (< 3 minutes after entering the aviary), and body mass recorded before release back into the colony. A final body mass was recorded for both parental birds, as well as all nestlings when present, the following day 24 hours after injection.

To assess the impact of social and parental support on trade-off dynamics, mate removal experiments were performed using a parallel injection paradigm as described above. At the time of female injection, the male mate of each pair was simultaneously removed and relocated to a separate aviary room where no visible interaction or clear communication could occur between

parental birds. Following the 24-hour experiment period for each pair, the male mate was returned to the aviary room with its respective female partner to support ongoing reproductive investment.

Quantifying Activity and Parental Care Behavior

Live behavioral observations of zebra finch activity within the colony were collected between 13:30 and 14:30 (5 hours post-injection time) by trained volunteers blind to treatment. Expression of stereotypical avian sickness behavior was assessed by quantifying the number of flights, number of hops, and time spent at rest (excluding feeding time) for each parental bird (females and males) in 5 minutes time bins. Each focal bird was observed over 6 separate time bins for a total of 30 minutes.

Video cameras were used to record nest box activity continuously for focal breeding pairs between 9:00 and 15:00 each day of an experiment upon release of parental birds following injection (female) and body mass measures of both parents. Trained observers blind to treatment recorded nest box entrance and exit times for both male and female of each focal pair used in the study; colored chest markings and unique leg bands were used to confirm the identity of these individuals in video recordings. Using acquired entrance/exit times, total percent of time each bird spent in the nest (of 6 hours total continuous recording time), mean nest visit duration (minutes), and mean nest visit interval (i.e. time between individual visits) were calculated for each parent.

Hormone Analysis

On the day of experiments, fresh whole blood was centrifuged for 10 minutes at 1200g to isolate and store plasma at -80°C until processing. Circulating (baseline) plasma corticosterone concentrations – the major circulating glucocorticoid in birds – were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Arbor Assays #K014-H1, Ann Arbor, Michigan). Plasma was diluted 1:15 with assay buffer provided, and 1:24 in cases where plasma volume collected was low for a given individual. Dilution factor was corrected for prior to data analysis. Prior to assaying experimental samples, this kit was validated for use in T. guttata by testing for parallelism of serially diluted plasma pools, generated by treatment (n = 8 saline injected individuals, n = 8 LPS injected individuals) alongside the provided kit standard and recommended standard curve dilution series. Results of the parallelism test confirmed that the target analyte was recognized by kit reagents in a predictable, dose-dependent manner. The dilution factor for plasma was selected from the results of this parallelism, with 1:15 falling approximately in the mid-range of the provided standard curve. Percent binding for individual corticosterone samples ranged from 26.34%-79.68%. Degree of inter-assay variation was calculated by determining the percent variation (%IAV) in corticosterone concentration of the same plasma sample across each assay plate.

Inflammatory Gene Expression (qPCR)

Quantitative PCR was used to examine the differential expression of candidate genes known to be upregulated in response to LPS treatment in birds (Scalf et al. 2019). On injection days, 25uL of the whole blood collected in the afternoon (5 hours post-injection of either 0.9% saline or LPS) was transferred to 500uL PureZOL (Bio-Rad RNA isolation reagent, #7326880), vortexed well to lyse cellular components of the blood, and frozen at -80°C until RNA extraction. RNA was isolated using a combined TriZOL and column purification (Zymo Research #R2070) method (Scalf et al. 2019; Wilsterman et al. 2020), using a total volume of 1.0mL PureZOL for 25uL whole blood.

Samples were eluted in 25 µl nuclease-free water. RNA integrity was determined for all samples (Agilent Bioanalyzer, Functional Genomics Laboratory, UC Berkeley) with resulting RIN numbers > 9. For each sample, 0.5 µg RNA was converted to cDNA (Bio-Rad, iScript cDNA synthesis kit #1708890) which was then diluted 1:5 with nuclease-free water as a working stock solution that was stored at -20°C until use. All primers for reference genes and genes of interest (Table 1) were validated for amplification of a single product in *T. guttata* blood using endpoint PCR (Invitrogen, Platinum Taq High Fidelity #11304-011) and visualized on an agarose gel. Primer efficiencies were determined using serially diluted five-point standard curve (see Table 1 for details) for all gene targets made from pooled zebra finch blood cDNA; calculated efficiencies fell within a range of 100 - 116% for all primer pairs used. When plating quantitative PCR (qPCR) assays, the cDNA samples from each individual zebra finch were used at either 1:20 dilution (18S, PGK) or 1:10 dilution (TLR3, IL1ß). Reactions (12ul total volume) were prepared following the protocols provided by the kit (Bio-Rad SsoAdvanced SYBR Green Supermix #1725271) using primers at a final concentration of 0.4 or 0.5 µM per reaction (Table 1). Absence of contamination was confirmed by lack of amplification of no-template (no cDNA) and no-reverse transcriptase controls during qPCR. Amplification of a single product was verified by the final dissociation/melting curve. Relative fold-change in mRNA expression was calculated using Pfaffl method (Pfaffl 2001) where specific amplification efficiencies are accounted for during analysis using one reference gene (18S) which was confirmed to be unaffected by stress treatment. We selected genes of interest (GOI) based on their critical roles in the inflammatory/acute phase response (TLR3, IL1ß). These data reflect potentially rapid transcriptional changes within the blood in response to LPS treatment and influence of mate removal.

Plasma Bactericidal Killing Ability (BKA)

Response to *S. aureus* – a gram positive bacteria – requires complement-independent immune signaling pathways and includes phagocytosis. Plasma microbiocidal activity against *Staphylococcus aureus* (*S. aureus*) for individual zebra finches used in this study was determined following ex vivo the 96-well plate-based method validated by French and Neuman-Lee (2012) originally adapted from spectrophotometer method established by Liebl and Martin (2009). All steps were carried out within an ethanol-sterilized laminar flow cell culture hood using autoclaved equipment and sterile consumable items. One day prior to assay, microbe stock solution was prepared by reconstituting one lyophilized S. aureus pellet (ATCC 6538, Epower #0485E7, Microbiologics Inc, Saint Cloud, MN) in 10mL pre-warmed (37°C) sterile 1X PBS to 6.2 X 107 CFU/10mL, incubating at 37°C for 30 minutes before storing at 4°C until use. A working solution of S. aureus (1 X 105 CFU/mL) diluted in sterile 1X PBS was prepared fresh on the day of assay. Plasma samples were thawed (each having been thawed once prior for hormone assays) and diluted 1:12 with sterile 1X PBS and kept on ice during plating. Prior to assay, validation of this method and identification of the appropriate plasma dilution was performed using serial dilution series of pooled plasma (1:2-1:64) and spectrophotometer readings collected at 2, 4, 6, 8, 10, 12, and 24 hours, testing the resultant absorbance values at all available wavelengths at each timepoint to identify appropriate time within log phase bacterial growth for to standardize calculation of %BKA per sample.

Samples were plated (96-well sterile tissue culture plate, round bottom, Falcon #353077, Corning, NY) in triplicate for every individual to enable greater accuracy of results, using 20uL plasma dilution per well. Bacteria working solution (1 X 105 CFU/mL) was plated by adding 4uL to

plasma and positive control wells. Positive controls (PBS, S. aureus, tryptic soy broth) were used to validate viability of bacteria stock used for assays and negative control wells (PBS and tryptic soy broth only) were used to ensure sterility of all solutions used. After addition of bacteria, plates were placed on a shaker for 30 seconds and then incubated (lidded) at 37°C for 30 minutes after which 130uL pre-warmed (37°C) sterile tryptic soy broth (TSB) was added to all wells. Baseline absorbance readings were collected at 340nm before incubating at 37°C. Plates were temporarily removed from incubator and absorbance measured (microplate reader, Bio-Rad 680XR) at 2, 4, 6, 8, and 11 hours after plating. 22 Plasma samples (35% of the total number of individuals) had to be excluded from this assay due to low plasma volume. Raw data used were from the 11 hour timepoint (340nm) and intra-assay variation between the two plates required to run all samples in hand was 2.85%. Negative control wells were used to verify that no contamination was present in assay materials and solutions but were not a required part of the final data calculations. Percent BKA (% BKA) data for female zebra finches used in the study was calculated using the following equation as performed in French and Neuman-Lee (2012):

% BKA = [(1 – mean abs. across sample in triplicate) / mean abs. positive control] X 100

Summary of Statistical Analyses

All data were analyzed within each reproductive stage independently and all groups consist of unique individual birds, and unique breeding pairs, with no individual used more than once in the overall study design. All data analyses were run in R Studio v.1.2.1335.

Prior to all analyses of treatment-level differences, Shapiro-Wilk tests were used to evaluate the normality of data at the group level and homogeneity of variance verified using Levene's test. Finches used in this study represent random individuals from the colony population with each individual only used once within the study design (and each pair unique) therefore resultant data are presumed to be independent. Two-way analysis of variance (ANOVA) was used to assess potential effects of treatment (LPS or 0.9% saline), mate removal, and any interaction between these variables using a fixed effects two-factor design. For each output measure of interest, group means were calculated, and standard error (SE) reported (see **Tables 1** and **2**). In cases were a significant interaction between treatment and mate removal was detected, type III ANOVA was used; otherwise in all other cases type II ANOVA was the default method for statistical analyses. All data were included in ANOVA calculations (i.e. no suspected outliers removed) to maintain the highest amount of statistical power given limited sample sizes in our design. When a significant effect was detected via two-way ANOVA, a Tukey test for Honest Significant Differences (Tukey HSD) was used to perform multiple pairwise comparisons. In all cases, statistically significant differences among our four groups ($p < 0.05$) are indicated within the figures by the presence of a different letter notation (a, b, c) above the plotted data.

Parental care index (PCI) data were non-parametric for both the incubation and nestling provisioning stage, with heteroskedastic variance (via F test) across groups. Therefore, PCI data were analyzed using Kruskal-Wallis test followed by Wilcoxon rank sum multiple pairwise comparisons to detect individual differences between our experimental groups. There were issues with cameras used during some of the experiments which led to loss of parental care data points within some groups. Due to these relatively small sample sizes for parental care measures, all data were retained for the statistical analyses of PCI and no outliers were removed. We used Pearson's

correlation coefficient (R) to evaluate the linear relationship between two variables, with the direction/strength, and significance reported through R- and p-values, respectively. In the case of significant main effect of mate removal on change in nestling body mass during provisioning, two sample t-test was used to identify significant difference between nestlings of paired vs. single parent females (**Figure 5B**) after testing for normality and homogeneity of variance within and between groups respectively.

4.4 Results

Female zebra finches exhibit sickness behavior during incubation and nestling provisioning, with suggested impact of mate removal on its expression.

An analysis of variance (two-way ANOVA) revealed that there was significant effect of treatment on the amount of total activity (quantified as number of hops + number of flights) of female parental birds during both the incubation (df = 1. F = 35.8307, $p < 0.0001$). and nestling provisioning (df = 1, F = 6.397, $p < 0.05$). stages (see **Tables 1** and **2**). LPS-treated incubating females exhibited significantly lower activity levels compared to saline-treated controls ($p = 6.10 \times 10^{-6}$, TukeyHSD) (**Figure 1A**). This overall pattern of decreased activity with LPS-treatment is consistent when comparing LPS- and saline-treated single parent females ($p = 0.06$, TukeyHSD). During incubation, we also detected a significant effect of mate status in our model (df = 1, F = 4.4908, $p < 0.05$) where mate removal itself resulted in lower total activity even in healthy females compared to healthy paired controls ($p = 0.00016$). During the nestling provisioning stage there was no significant individual effect of treatment or mate status on total activity, however we did detect a barely statistically significant interaction (treatment x mate status, df = 1, F = 4.051, $p = 0.05$) which indicates that any change in this behavioral metric in response to LPS is dependent on the presence or absence of the male mate. During the provisioning period, LPS-treated females tended to exhibit lower activity than saline controls ($p = 0.151$, TukeyHSD), however this pattern was not statistically different via pairwise comparison. Further, this pattern was abolished with mate removal during nestling provisioning as saline-single parent females were generally less active than paired saline females ($p = 0.110$, TukeyHSD) with a group mean/median more similar to LPS single-parent birds (**Figure 1A**). No significant differences in percent of time at rest (of 30 minutes total binned observation time) during the incubation period however there was a significant main effect of treatment during nestling provisioning (df = 1, F = 8.5383, $p = 0.0067$) with no effect of mate removal (**Figure 1B**).

Visual interpretation of data highlights that – as expected – incubating female finches tend to spend a greater percentage of time at rest than provisioning females and this may mask some traditional behavior metrics of sickness behavior. There were no significant effects of the experimental manipulations on female body mass (change over 24 hrs post-injection) during either reproductive stage (**Figure 1C**). There were also no significant associated group-level differences in overall activity or body mass change of male finches in response to immune challenge experienced by female mates (**Table 4**). Like females, male zebra finches tend to exhibit greater overall activity (total number of hops + flights) during period of nestling provisioning ($\bar{x}$ = 313.57 +/- 47.16) relative to incubation ($\bar{x}$ = 133.29 +/- 45.92).

Immune challenged females tend to modify time in the nest and number of nest visits in a stage-specific manner, with varied impact of mate removal.

Individual metrics of parental care (number of nest visits, % of total time in next box, mean visit interval, and mean visit duration) were quantified from 6-hours of continuous video footage to tease apart the type of behaviors that are critical to incubation and provisioning periods, and how this behavioral investment may shift in response to a physiological challenge and/or shift in social support. Results of the two-way ANOVA indicated a significant main effect ($p < 0.05$) of mate removal on the total percent of time a female parent spent in the nest box during both the incubation and nestling provisioning periods (**Figure 2A**). Single-parent females tended to spend a greater percent of time in their nest box when compared to paired females, regardless of LPS or saline treatment. This pattern of increased nest presence of single parent females was not as strong during nestling provisioning, but there was a notable increase in interindividual variation within mate removal groups regardless of LPS or saline treatment (**Figure 2A**). When the total number of individual nest visits was quantified for each female parent (between 9:00-15:00) we found a significant stage-specific effect of LPS treatment ($p < 0.05$) where LPS-treated females exhibited relatively fewer visits to their nests during nestling provisioning, with no change in nest visits during incubation (**Figure 2B**). During incubation, there was a significant interaction effect detected between treatment and mate status ($p = 0.025$). Interestingly, saline-treated single parent females exhibited significantly more nest visits than healthy paired females ($p = 0.011$) but this compensatory increase was not observed when single parent females simultaneously experienced LPS treatment. Instead, LPS single parent females displayed a lower total nest visit numbers comparable to those having support of their male mates.

There was no effect of mate status or treatment on the mean duration of nest visits during incubation. During provisioning, ANOVA identified mate removal as a significant main effect ($p < 0.05$) with no effect of immune challenge (**Figure 2C**). LPS treatment alone did not significantly affect the mean duration of nest visits during either reproductive stage however these data may suggest that females experiencing an immune challenge under single-parent conditions tend to display longer nest visit duration (low statistical power within incubating mate removal groups, $n = 4$ each, makes differences difficult to analyze). This stage-specific effect during incubation is coupled with a significant main effect of mate status on the mean time between nest visits (i.e. mean nest visit interval, $p < 0.05$), with an overall decrease in mean interval time seen in saline-treated single parent females compared to healthy controls ($p = 0.018$, Tukey HSD). In contrast, there was no effect of mate status on nest visit interval detected during nestling provisioning, but instead a significant effect of immune challenge ($p < 0.05$) where LPS treated females tended to spend a greater amount of time away from their nests between visits than saline-treated controls (**Figure 2D**).

Immune-challenged female finches compromise parental care during only nestling provisioning, however lack of mate support abolishes this LPS effect and increases parental care during incubation.

To consolidate individual parental care measures into one integrative metric of behavioral parental investment, principal component analyses (PCA) were used to generate variable correlation plots for each reproductive period separately, including all independent behavioral variables quantified during the study (**Figure 3**). By assessing the relationship among variables by stage, it was possible to determine which individual metrics common between incubation and provisioning provide

unique contributions along behavioral dimensions. Based on the resultant relationships among variables and common units, we selected three: 1) total time in the nest (min), 2) mean duration of nest visits (min), and 3) mean interval between visits (min) which were used to calculate individual Parental Care Index (PCI) for each bird; where PCI = [total time in nest (min) + mean visit duration (min)] / [mean visit interval (min)].

Kruskal-Wallis test detected significant differences across our experimental groups during both the incubation (df = 3, X2 = 10.27, p = 0.0164) and nestling provisioning (df = 3, X2 = 8.747, p = 0.0328) periods. Multiple pairwise comparisons detected an overall increase in parental care index (PCI) during incubation under single parent conditions, regardless of LPS or saline treatment, with significant separation (p = 0.036) between single parent saline-treated females and saline-treated females with their mates (**Figure 4A**). During provisioning, there was a significant decrease in PCI with LPS treatment (Saline vs. LPS, p = 0.033) however this response was not detected under mate removal conditions. Thus, females provisioning to nestlings exhibited a decrease in calculated PCI with LPS-treatment when their male mate was present, with no apparent compromise to parental investment during incubation. Further, mate removal resulted in an increase in calculated parental investment during incubation and abolished any behavioral response to LPS during nestling provisioning. However, the calculated PCI for male mates did not significantly differ via Welch's two-sample t-test between saline-treated and LPS-treated females during incubation (df = 5.90, t = 1.4674, p = 0.193) or provisioning periods (df = 10.75 , t = 0.781, p = 0.451). No significant within-pair relationship was detected between female PCI and male PCI. Females did not differ significantly in pre-treatment body mass at the start of the experiment however subtle differences among individuals allowed us to detect an overall significant relationship between pre-treatment female body mass (i.e. crude reflection of starting body condition) and PCI, where females with higher body mass possess a higher calculated PCI (**Figure 4B**). When data were separated by reproductive stage, this relationship was stronger within the incubation period (p = 0.024, R = 0.49) compared to nestling provisioning (p = 0.13, R = 0.3 (**Figure 4C**).

Lack of biparental care has significant impact on nestling body mass, with no effect of acute immune challenge.

We analyzed potential effects of immune challenge and mate removal on nestling mass during the nestling provisioning period. Two-way analysis of variance (type II) detected a significant main effect of mate removal (df = 1, F = 6.68, p = 0.011) on the change in nestling mass (24 hours after female parent treatment) with no significant effect of LPS treatment of the female parent (**Figure 5A**). When data were pooled to examine this effect of mate removal further, two-sample t-test detected a significant difference between the mean change in nestling mass between offspring of paired females and those that had their male mate removed. Nestlings of single-parent females gained significantly less body mass after 24 hours than nestlings with both parents, regardless of the immune status of their female parent (**Figure 5B**). Furthermore, a greater proportion of nestlings of single-parent females also lost body mass after 24 hours compared to those with biparental care (**Figure 5C**) highlighting potentially severe impacts on growth and development.

No significant impact of immune challenge and/or mate removal on baseline plasma corticosterone concentrations.

Among saline-treated (paired) females, paired t-tests indicated there was no statistical difference between mean baseline plasma corticosterone concentrations during the incubation period vs. nestling provisioning when analyzed in the morning (df = 41, t = 0.9682, p = 0.3386) or afternoon (df = 42, t = -1.7269, p = 0.0915) (**Figure 6**). Mean circulating corticosterone tended to increase more from morning to afternoon during nestling provisioning (AM mean = 11.48 ng/ml, PM mean = 17.80 ng/ml) compared to incubation (AM mean = 13.25 ng/ml, PM mean = 13.49 ng/ml). Analysis of variance did not detect a statistical difference in baseline corticosterone among female birds pre-treatment (time zero) across treatment groups. Furthermore, there was no effect of LPS treatment and/or mate removal on the change in baseline CORT concentration of female parental finches in our experiment during either reproductive stage examined (**Figure 7A**). Given this lack of effect, we pooled all female CORT data to assess the relationship between within-individual change in plasma CORT and calculated parental care index (PCI). Correlation analysis revealed a significant negative relationship (R = -0.4, p = 0.026) in which females with a greater increase in plasma CORT (regardless of treatment) also exhibited lower PCI (**Figure 7B**), highlighting a connection between physiology and degree of parental investment. While there were significant shifts in female behavior in response to our experimental manipulations, treatment seemingly had no significant impact on the change in plasma corticosterone concentrations of their male mates (**Figure 7C**).

Inflammatory gene expression and functional immunity are impacted by treatment or mate removal in a reproductive stage-dependent manner.

We first analyzed potential changes in IL-6 and Il1-β expression within each reproductive stage. Two-way ANOVA did not reveal a significant effect of mate removal for any of these three genes of interest during incubation or nestling provisioning, therefore data were pooled to examine specific effect of LPS treatment on red blood cell (RBC) inflammatory gene expression and potential differences between reproductive stages. Wilcoxon rank-sum tests (non-parametric distributions and heteroskedastic variance) revealed that inflammatory mRNA expression was extremely stable during incubation, regardless of saline or LPS treatment, however there was a tendency for LPS-treated females to exhibit elevated Il1-β (W = 18 , Z = -1.403, p = 0.160) RBC expression with no apparent effect on IL-6 (**Figure 8A** and **B**). Thus, LPS-treated females tend to elevate red blood cell TLR-3 and Il1-β expression in a stage-specific way, independently of mate removal.

Analysis of variance detected a stage-specific effect of mate removal on female percent bactericidal killing ability (%BKA), a functional measure of immunity. There was a statistically significant main effect of mate removal (df = 1, F = 20.46, p = 0.0008) during the incubation period only (**Figure 9A**) where single parent females exhibited higher %BKA than those of paired females, regardless of LPS/saline treatment. In contrast, there was no effect of mate removal during nestling provisioning, and instead a nearly significant (df = 1, F = 3.69, p = 0.070) effect of LPS treatment (**Figure 9B**). Data were pooled by LPS or saline treatment within each reproductive stage to examine these stage-specific effects and Welch's two-sample t-test indicated that bactericidal killing ability was significantly higher in single-parent females during the incubation period (df = 5.04, t = -4.0819, p = 0.0093) compared to those with male mate present (**Figure 9C**).

Furthermore, at the time point of our %BKA calculation, provisioning females exhibited more elevated bactericidal killing ability relative to incubating females overall.

Immune challenge results in reproductive stage-specific behavioral trade-offs and, in some cases, acute positive impacts on parental investment.

To determine the ways in which specific behavioral output measures contribute to characterizing a specific period of reproductive investment (i.e. incubation vs. nestling provisioning) principle component analyses were performed including saline-treated (healthy) female birds only (**Figure 10A**). Based on this output, we sought to assess the circumstances under which trade-offs between behaviors supporting self-maintenance versus those that support reproductive investment may be observed and how we may predict differences between reproductive stages.

When exploring potential trade-offs between self-maintenance and reproduction in immune-challenged (i.e. LPS treated) female zebra finches, stage-specific significant relationships were found. From PCA data (**Figure 10A**), we focused our correlational analyses by selecting specific behavioral metrics strongly characterizing each reproductive period with the prediction that trade-offs will be observed in those behaviors only in the associated reproductive stage. Contrary to our prediction, we identified a significant positive relationship ($R = 0.61$, $p < 0.05$) during incubation between percent of time a female spent at rest and the total percent of time she was within the nest box (**Figure 10B**) with no relationship between these behaviors during the nestling provisioning period. However, a negative correlation between percent of time at rest and the total number of individual nest visits was seen among LPS-treated females only during the nestling provisioning period (**Figure 10C**), suggesting a compromise in this facet of parental investment in favor of a behavior supporting individual self-maintenance. Furthermore, females provisioning to offspring also experienced greater declines in body mass (24 hours post-injection) when exhibiting longer mean nest visit duration ($R = -0.71$, $p < 0.05$; **Figure 10D**). There was no relationship between change in female body mass and nest visit duration during incubation.

We found a strong stage-specific relationship between within-individual IL-6 mRNA expression and calculated parental care index (PCI, $R = 0.95$, $p < 0.05$). During the incubation period females that exhibited greater relative mRNA expression of IL-6 in isolated red blood cells also had higher PCI values (**Figure 10E**). This pattern was not echoed during nestling provisioning and instead there was almost no change entirely in female PCI as IL-6 expression varied. There was no significant relationship between PCI and the other genes of interest in this study (IL1-b, TLR-3).

4.5 Discussion

Reproduction is an energetically costly life history stage that varies widely in resource and behavioral demands depending on the specific stage of courting, mating, and period of parental care. Animals are faced with unpredictable challenges – such as pathogen exposure or infection – requiring quick physiological responses that support survival (Wingfield, 2015), often at the expense of reproduction (Wingfield and Sapolsky, 2003). Hart (1988) was the first to suggest that the expression of “sickness behaviors” is an energetic strategy that reduces investment in unnecessary activities in order to allocate resources toward – and support – the immune system. Many examine the occurrence of trade-offs during reproduction without accounting for the variation in demand (behavioral and energetic) across life history sub-stages of the breeding period

nor the degree of social support for a given species. Through our approach and focal species, we were able to explore the inherent interaction between the trade-off and energy limitation hypotheses which shape rapid facultative responses in behavior and physiology in response to ecologically-relevant challenges.

Expression of sickness behaviors are not reproductive stage-specific.

The use of LPS stimulates the acute phase response in birds and the APR has been posited to be the most demanding collection of immune defenses (Lochmiller and Deerenberg 2000; Klasing 2004). In our study, LPS injection resulted in predictable declines in activity regardless of reproductive stage that reflect stereotypical expression of sickness behavior (Hart 1988) and our data match with other studies (Bonneaud et al. 2003, Adelman 2010; Lopes et al. 2013). The reduction in (hopping and flying) activity may allow for a stronger APR in LPS-treated females, as zebra finches tend to mount a relatively weaker immune response when maintaining a high degree of activity (Lopes et al. 2014). The interaction between immune challenge and loss of social/parental support of the male mate on overall female activity was particularly evident during nestling provisioning as even saline-treated single parent females tended to exhibit reduced numbers of hops and flights akin to that seen in LPS-treated groups however there was a high degree of interindividual variation within these single parent groups. This may indicate that the demands of zebra finch single parenthood require a conservative energy use strategy regardless of the presence of a physiological (e.g. immune) challenge, and the degree of energy conservation could be linked to individual facets of condition or status (e.g. starting body mass, baseline corticosterone concentrations, etc.). We quantified hopping and flying activity separately from calculating the percent of time an individual female spent at rest and the separation of these metrics allows us to consider differences in motivation behind these behaviors. Zebra finches are a highly social and curious species, often interacting with other members of their colony. They also, in the wild and within our captive setting, forage for food resources primarily among tree branches and on the surface of the ground. Therefore, changes in total activity quantified in our study may cumulatively reflect significant broad shifts in social behaviors and foraging activity, while the calculated percent of time at rest may – in part – more accurately represent time dedicated strictly toward overall energy conservation, or prioritization of self-maintenance when immune-challenged.

Injection of LPS/endotoxin is known to reduce feeding behavior/anorexia in many taxa, including birds (Johnson et al. 1993; Cheng, Freire, and Pajor 2004; Owen-Ashley et al. 2006, Owen-Ashley and Wingfield 2007). Despite potential changes in foraging behavior or energy use/conservation, we did not detect a significant effect of immune challenge or mate removal on body mass change 24 hours post-injection. This was a surprising finding as other studies often find that LPS treatment results in significant decrease in body mass within this time frame (Klasing et al. 1987; Kozak, Conn, and Kluger 1994). Changes in behaviors that may be associated with decreased foraging activity were observed. However, it is possible that decreases in hopping and flying activity in our study may not reflect changes in energy intake as we were not able to quantify individual food consumption within the colony-housed setting. Only during incubation did we find a tendency for paired females to lose more body mass (between 0.5-1.0 gram) after LPS injection although these data were not statistically significant. Given that incubating birds may not be able to forage as freely, subtle decreases in body mass may be expected and we might therefore expect mass loss to be exacerbated when faced with an immune challenge.

There are stage-specific negative and positive relationships between behaviors supporting parental care and those supporting self-maintenance.
There are obvious energetic benefits to expressing sickness behavior, but there may be significant opportunity costs or physiological constraints. We may shape our predictions regarding how resources – energy and time – will be allocated in the context of two broad hypotheses (reviewed in Owen-Ashely and Wingfield 2007). First, the trade-off hypothesis predicts that investment in one life history stage (e.g., reproductive period) will come at the expense of maximal immune response via APR, or vice-versa. At the same time, we should predict that the APR will be suppressed if there is a threat of depleting energy stores; the energy limitation hypothesis.

Potential trade-offs between survival and reproductive investment are of great interest across species. Female lizards have been found to lay smaller eggs when treated with LPS compared to controls (Uller, Isaksson, and Olsson 2006) with no effect on total clutch size. Similarly, female dung beetles laid smaller brood balls with no impact on total ball number (Reaney and Knell 2010). LPS-injection of maternal mice did not significantly effect litter weight, amount of pup carrying time, or time to retrieve (Aubert et al. 1997). In birds, female house sparrows (Passer domesticus) have been found to exhibit a reduced feeding rate (quantified as visits/nestling/hour) when challenged with LPS compared to PBS-treated controls and, for a given brood size, the likelihood of brood abandonment increased with LPS treatment (Bonneaud et al. 2003). Common Eiders (Somateria mollissima), a long-lived capital breeding bird species, increased provisioning care to offspring despite greater energetic costs and mass loss during incubation when injected with sheep red blood cells (SRBCs) to challenge the immune system (Hanssen 2006). Effects of LPS on prenatal investment into avian eggs have also been observed in house wrens where LPS-treated females produced eggs with larger yolk and higher concentration of corticosterone suggestive of increased investment in reproduction with immune challenge (Bowers et al. 2015). Conflicting data regarding shifts in parental investment make it difficult to generate broad-sweeping predictions regarding the occurrence of life history trade-offs based on classic frameworks. Instead, this highlights the importance of considering the subtle differences in behavioral patterns, energetic demands, and social interactions or support within a given system and across taxa.

Zebra finches are opportunistic breeders, meaning that they can upregulate fertility and reproduce at any time during the year if health is good and resources abundant (Zann et al. 1995). Opportunistic breeders therefore should have a high potential for future reproductive events (relatively higher residual reproductive value), diminishing the individual value for a current attempt if faced with an unpredictable environmental or physiological challenge. We found the predicted effect of LPS challenge between reproductive stages, where parental care was not compromised during incubation but individuals did appear to alter parental effort during the provisioning period. During incubation, zebra finches – and many other bird species – spend most of their time incubating their eggs to regulate developmental temperature and simultaneously deter predators and defend their reproductive effort. Given that incubating birds may not be able to forage as freely, subtle decreases in body mass may be expected and we might therefore expect mass loss to be exacerbated. But while there may be energetic costs during this reproductive stage, we did not find that parental care was compromised and, in some cases, LPS-treated females actually spent a greater amount of time in their nest which may actually suggest a developmental advantage for offspring and potential fitness benefit as a result of a negatively perceived parental

challenge. In contrast, there is inherent conflict between behaviors associated with the nestling provisioning and stereotypical sickness behaviors and thus we predicted behavioral trade-offs to occur in a stage-specific manner. Indeed, we found that LPS-treated females not only decreased the number of visits to their nest of offspring, but also tended to spend a greater amount of time away from their offspring between nest visits. By quantifying individual metrics of parental care effort (e.g. time in the nest box, nest visits, visit duration, etc.) we were able to highlight not only the fine-scale and subtle differences in daily patterns between reproductive stages, but also the specific facets of behavior that are more/less sensitive to trade-off (LPS challenge) and how energy limitation (mate removal) may shift the degree of behavioral flexibility within our system.

At the individual level, some incubating paired female finches treated with LPS provided more parental care to their nest than saline control birds despite the physiological immune challenge. This manifested as a greater total percent of time in the nest box, more nest visits, and a longer mean duration across nest visits. This is an interesting positive relationship between self-maintenance and reproductive investment because of a perceived negative physiological stressor.

Corticosterone may not be a critical endocrine mediator of trade-off expression.

The stress response has often been proposed as an important mediator of life history trade-offs, including a facet of chemical and endocrine signals that may impact reproductive physiology and behavior. For many years now, interactions and reciprocal effects have been proposed between the hypothalamic-pituitary-adrenal (HPA) axis and the immune system (Bateman et al. 1989) and we now know that secretion of glucocorticoids is associated with an initial stimulation of the immune response (Dhabhar 2002). As early as 1957 Wexler et al. discovered that peripheral injection of LPS resulted in elevated plasma glucocorticoids, and since then supported by many other studies (Johnson et al., 1993; Nakamura et al. 1998; reviewed in Beishuizen and Thijs 2003). While we expected to see an increase in baseline concentrations of corticosterone with LPS injection in our study, we did not detect any effect of LPS on corticosterone concentrations among female birds. This is supported by previous studies out of our research group that also found no significant effect of LPS treatment on plasma CORT in male zebra finches (Lopes, Wingfield, and Bentley 2012). We collected blood plasma from the birds in our study 6 hours after the time of injection and mate removal. It may be that there was a rapid increase in circulating corticosterone earlier in the timeline of our experiment that we missed by our 6-hour blood collection however thorough studies performed in white crowned sparrows show that plasma CORT remained elevated following LPS injection at 6 hours (Owen-Ashley et al. 2006). Surprisingly, removal of parental support from their male mate also did not have a significant impact on plasma corticosterone of females suggesting that females did not experience exacerbated stress with this change in social support. Additionally, parental male zebra finches did not experience apparent stress (if change in baseline plasma corticosterone concentration is an indicator) in response to the immune status of their female mate. Taken together, it may be that the behavioral changes observed here may be independent of corticosterone concentration but may instead be regulated by other endocrine and chemical signals such as prolactin (Angelier and Chastel 2009; Angelier et al. 2016; Smiley and Adkins-Regan 2018), leptin (O'Connor et al. 2005; Elmquist et al. 1998; Ahima and Flier 2000), and pro-inflammatory cytokines (Rivest and Rivier 1995). It is well-accepted that behavior can change facultatively without concurrent change in circulating stress hormones (Romero, Reed, and Wingfield 2000).

Suppression of the HPA axis in response to an acute immune challenge may suggest an energy allocation strategy that attempts to rapidly balance reproductive demands with the costs of self-maintenance. Interestingly, there is some evidence that – across bird species – the stress response may tend to vary in strength with perceived value of the current reproductive attempt; those with high value on the current attempt exhibit relatively dampened stress responses (Bokony et al., 2009). The same comparative analysis revealed a tendency for relatively weaker changes in circulating corticosterone responses in systems with female-biased parental care. Our study revealed that females with relatively lower changes in baseline corticosterone also tended to have higher parental care index (PCI), regardless of treatment. It is important to acknowledge that it is difficult to associate changes in baseline corticosterone within one day with one of our experimental manipulations or activity of the HPA axis (i.e. individual stress) as there is a number of external variables that influence synthesis and secretion of this hormone. The broad relationship between within-individual change in CORT and calculated PCI may suggest that an increased shift in energy mobilization through greater change in plasma CORT tends to be connected to a relatively lower degree of parental investment. This increase in metabolic activity could be the result of increased metabolic rates to accommodate glucose use, activation of immune responses, changes in overall colony activity, or upregulation of the stress axis; all of which may reasonably be associated with our system and experimental design but which we cannot pinpoint concretely here.

Lastly, there is some suggestion for a role of glucocorticoids in the maintenance of sociality under stressful conditions which is particularly relevant to our study design. In an adrenalectomized mouse model, exogenous treatment with GCs fully recovered social exploration that had been reduced by pro-inflammatory cytokine signals (Goujon et al. 1997). In zebra finches – a highly social species that naturally lives within a large colony setting – it is possible that there is a tight balance between inflammatory signals during the immune challenge we used here and glucocorticoid concentrations in order to preserve ecologically relevant social behaviors that may benefit individual survival in the wild.

Molecular facets of immunity are sensitive to reproductive stage and the presence (or absence) of social support.

The APR evoked by LPS injection is an essential defense mechanism against pathogen infection, initiating rapid repair processes and protections that prevent severe tissue damage along with regulating homeostatic functions. Our understanding of cytokines began solely within the context of understanding molecular biology associated with the immune response, but we now know that these signaling molecules are also involved in regulating endocrinology and cues to, and within, the brain. Interleukin 1-beta (IL1-) and IL-6 are released upon activation of the innate immune signaling cascade and are a characteristic part of the APR (Vilcek 2003; Dantzer 2004). In 1989, Dantzer and Kelley posited that cytokines directly induce stereotypical sickness behaviors through integration of signals and bidirectional communication between the brain and the immune system (Dantzer and Kelley 1989). Circulating IL-6 plays a partial role in regulating LPS-induced sickness behaviors, including a decrease in voluntary activity (locomotion, eating, drinking, social interaction) (Harden et al. 2006, Harden et al. 2008). Use of IL-6 antibodies to block their biological actions caused a rapid decrease in fatigue in humans with disease associated with IL-6 overproduction (Spathe-Schwalbe et al. 1998; Nishimoto 2005; Nishimoto and Kishimoto 2006). Additionally, IL1-beta is known to provide a direct line of communication to the central nervous

system and influence locomotor activity and food consumption (Anforth et al. 1998; Nadjar et al. 2005; Chaskiel et al. 2019). These signaling molecules may be important holistic mediators that act in a synergistic way (Harden et al. 2008) to influence behavior and physiology beyond the immune system alone, influencing physiological and behavioral trade-off thresholds.

In the present study, we found a clear stage-specific difference in female transcriptional response to LPS treatment. During incubation, RBC mRNA expression was extremely stable for the two pro-inflammatory cytokines we measured (IL1-beta, IL-6). During the provisioning stage, females appeared to upregulate transcription of these cytokines in response to LPS treatment. Interpretation of this finding is interesting, because on the one hand this may suggest that incubating females do not need to upregulate innate immune defenses when faced with an immune challenge, perhaps because they are not reaching a threshold of energetic limitation and are able to rely on constitutively expressed immune defenses. However, if energetic limitation drives changes in cytokine mRNA expression it is surprising that provisioning females increase gene expression while that may also be more rapidly using energy stores to support relatively greater activity and foraging/feeding demands. These findings make more sense when we examine them alongside the patterns of parental care investment we observed, where we see a significant impact of LPS-treatment on parental care investment during nestling provisioning and not incubation. Provisioning females may have the capacity to upregulate pro-inflammatory responses because they simultaneously compromise parental behavior, or perhaps an increase in cytokine signaling to the brain occurs first which then may impact changes in prioritization and reproductive/parental investment. Additionally, sex steroids have been shown to impact cytokine production in vitro (Wang et al. 1993; Wang et al. 1999) which suggests that fluctuations in hormones across life history sub-stages such as estrogen may also modulate the APR and associated sickness behaviors.

Classic life history trade-off theory predicts that immune function will be compromised with investment in reproduction. There is evidence for negative correlations between reproductive effort and individual %BKA in birds. Bactericidal killing ability (BKA), a crude measure of functional constitutive/innate immunity, in common eiders (Somateria mollissima) decreased with larger clutch size as well as total incubation time (Neggazi et al. 2016). More recently, Chang van Oordt et al. (2022) showed that tree swallows with a higher BKA fed their nestlings less, and that the specific time that an individual breeds during the year also effects whether this reproductive/BKA trade-off occurs. Despite stage-specific significant changes in parental care behavior with LPS treatment, we were surprised to see no effect of LPS treatment on calculated killing ability in our study during incubation or provisioning periods. This may be due to having only a single timepoint at which plasma was assayed (6 hours after the start of the experiment for each pair) which could have missed the optimal time for capturing complement-mediated immunity, despite being an appropriate time to detect behavioral response and other physiological responses to LPS. Interestingly, male mate removal resulted in significantly higher %BKA of female parents in our study but only during the incubation period. This stage-specific result may be due to higher overall %BKA seen during nestling provisioning compared to the incubation period regardless of treatment. It may be that loss of parental support caused a low but sustained level of stress (whether social or energetic), so subtle we were not able to detect significant changes in plasma corticosterone. This “eustress” may actually have short-term positive effects on facets of physiology and organismal function, including enhanced innate immunity (Tort 2011; Fleshner et al. 2022; Dhabhar 2014; Liu et al. 2023).

4.6 Tables and Figures

Table 1. Quantitative real-time PCR primers used to quantify red blood cell inflammatory mRNA expression.

Gene	Forward Primer	Reverse Primer	Conc. (μM)
18S (reference)	CATCCAATCGGTAGTAGCG	TAACCCGTTGAACCCCAT	0.4
TLR-3	TTTGGCCCACCTTTCAG	GCCAATCAGCAAACCAA	0.5
IL1-beta	TTCCGGTGCATCAGAGGCAGTTAT	GCACGAAGCACTTGTGGTCAATGT	0.5

Table 2. Two-way ANOVA parameters and significance (female zebra finches) based on incubation period data. Statistically significant effects are bolded ($p \leq 0.05$) and shaded for visualization.

INCUBATION				
Output Measure	**Predictors**	**d.f.**	***F***	***p***
Total activity (hops + flights)	**treatment**	1	35.8307	**6.10×10^{-6}**
	mate status	1	4.4908	**0.0461**
	treatment X mate	1	2.9920	0.0983
% Time at Rest	treatment	1	2.9432	0.1010
	mate status	1	0.4549	0.5074
	treatment X mate	1	0.4186	0.5247
Change in Body Mass (g)	treatment	1	1.9271	0.1796
	mate status	1	3.2493	0.0852
	treatment X mate	1	0.2308	0.6359
% Time in Nest Box	treatment	1	0.2112	0.6513
	mate status	1	9.3868	**0.0067**
	treatment X mate	1	0.0733	0.7896
Nest Visit Duration (mean, min)	treatment	1	3.9832	0.0622
	mate status	1	0.7135	0.4100
	treatment X mate	1	0.1768	0.6794
Nest Visit Interval (mean, min)	treatment	1	0.039	0.8451
	mate status	1	13.791	**0.0017**
	treatment X mate	1	0.928	0.3487
# Nest Visits	treatment	1	0.0020	0.9647
	mate status	1	12.4155	**0.0024**
	treatment X mate	1	5.9086	**0.0257**
Parental Care Index	treatment	1	0.9368	0.3459
	mate status	1	5.9609	**0.0252**
	treatment X mate	1	2.0353	0.1708

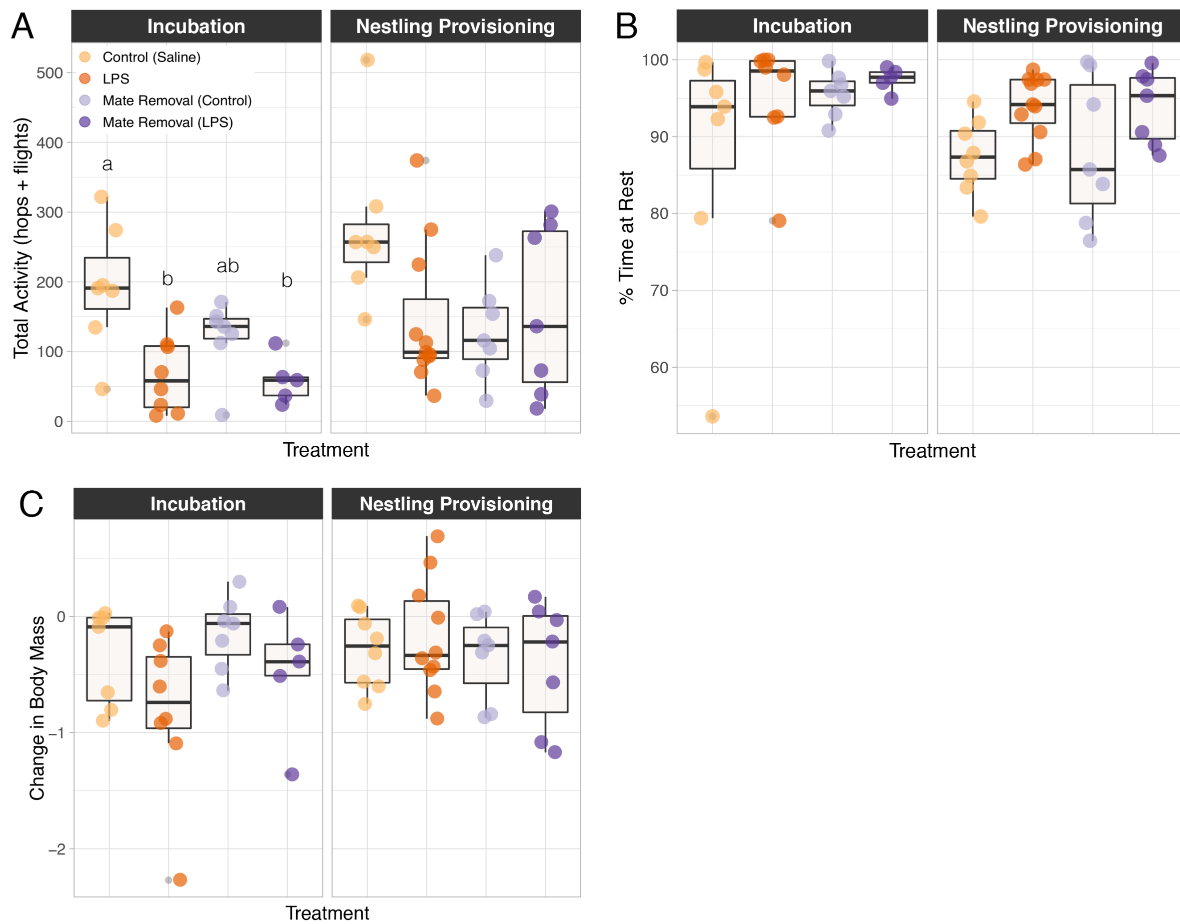
Table 3. Two-way ANOVA parameters and significance (female zebra finches) based on nestling provisioning period data. Statistically significant effects are bolded ($p \leq 0.05$) and shaded for visualization.

NESTLING PROVISIONING				
Output Measure	**Predictors**	**d.f.**	***F***	***p***
Total activity (hops + flights)	treatment	1	0.742	0.3968
	mate status	1	1.603	0.2166
	treatment X mate	1	4.051	0.0546
% Time at Rest	**treatment**	1	8.5383	**0.0067**
	mate status	1	0.0341	0.8548
	treatment X mate	1	0.0437	0.8360
Change in Body Mass (g)	treatment	1	0.0486	0.8272
	mate status	1	0.8734	0.3580
	treatment X mate	1	0.3010	0.5876
% Time in Nest Box	treatment	1	0.7463	0.3969
	mate status	1	5.2443	**0.0320**
	treatment X mate	1	1.2167	0.2819
Nest Visit Duration (mean, min)	treatment	1	0.6901	0.4154
	mate status	1	4.3548	**0.0493**
	treatment X mate	1	1.7541	0.1996
Nest Visit Interval (mean, min)	**treatment**	1	6.8963	**0.0158**
	mate status	1	0.5202	0.4787
	treatment X mate	1	0.4815	0.4954
# Nest Visits	**treatment**	1	6.6401	**0.0185**
	mate status	1	0.8931	0.3565
	treatment X mate	1	0.3108	0.5839
Parental Care Index	treatment	1	0.9611	0.3377
	mate status	1	4.1960	**0.0526**
	treatment X mate	1	0.0434	0.8369

Table 4. Impact of female treatment on male behavior and body mass. No significant effects were detected within either reproductive stage.

	Stage	Treatment	Mean ± SE
Total Activity (hops + flights)	Incubation	*Saline*	133.29 ± 45.92
		LPS	147 ± 39.47
	Provisioning	*Saline*	313.57 ± 47.16
		LPS	338.9 ± 44.6
% Time at Rest	Incubation	*Saline*	95.65 ± 1.7
		LPS	95.83 ± 1.3
	Provisioning	*Saline*	87.5 ± 2.4
		LPS	86.93 ± 2.18
Change in Body Mass (grams)	Incubation	*Saline*	-0.25 ± 0.14
		LPS	-0.49 ± 0.14
	Provisioning	*Saline*	-0.3 ± 0.18
		LPS	-0.28 ± 0.08

Figure 1. Impact of immune challenge and/or mate removal on parental female zebra finch activity measures and body mass change over 24 hours. Activity was quantified by (A) total sum of flights and hops and (B) percent of time spent resting during live behavior observations (5-minute bins, 30 minutes total per animal). (C) Neither treatment or mate removal had a significant effect on change in female body mass after 24 hours. Boxplots display the group median (horizontal line) with shaded region corresponding the interquartile range (25% - 75%) and vertical lines extending to group minimum and maximum values. Significant differences ($p \leq 0.05$) between groups (via Tukey HSD) indicated by different letters.



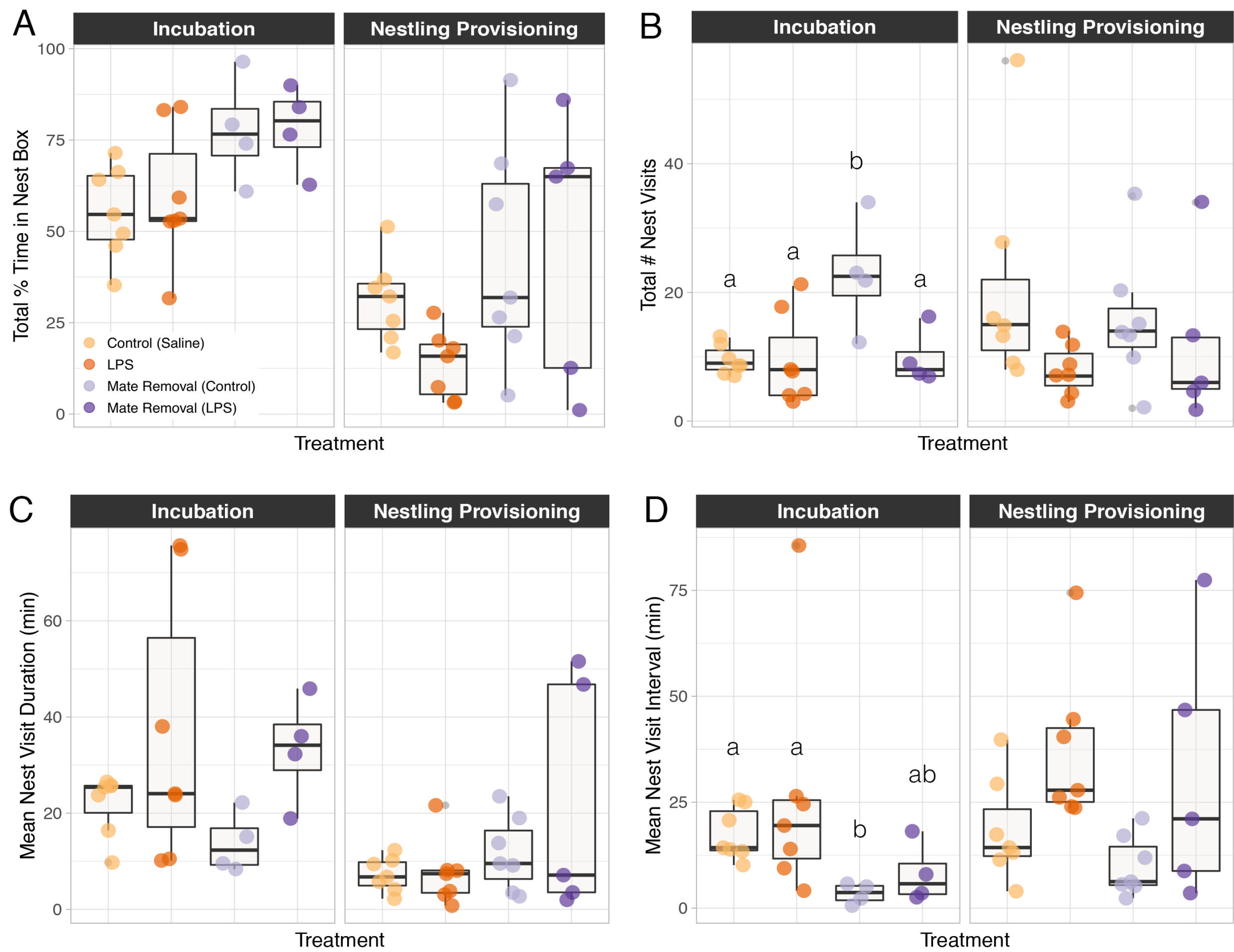


Figure 2. Impact of immune challenge and/or mate removal on female parental care, quantified by (A) total percent of time in the next box, (B) total number of individual nest visits, (C) the mean duration of each nest visit, and (D) the mean interval between nest visits. Boxplots display the group median (horizontal line) with shaded region corresponding the interquartile range (25%-75%) and vertical lines extending to group minimum and maximum values. Statistically significant differences ($p \leq 0.05$) between groups (via Tukey HSD) are indicated by different letters. Outliers are depicted as stand-alone points beyond the vertical range lines.

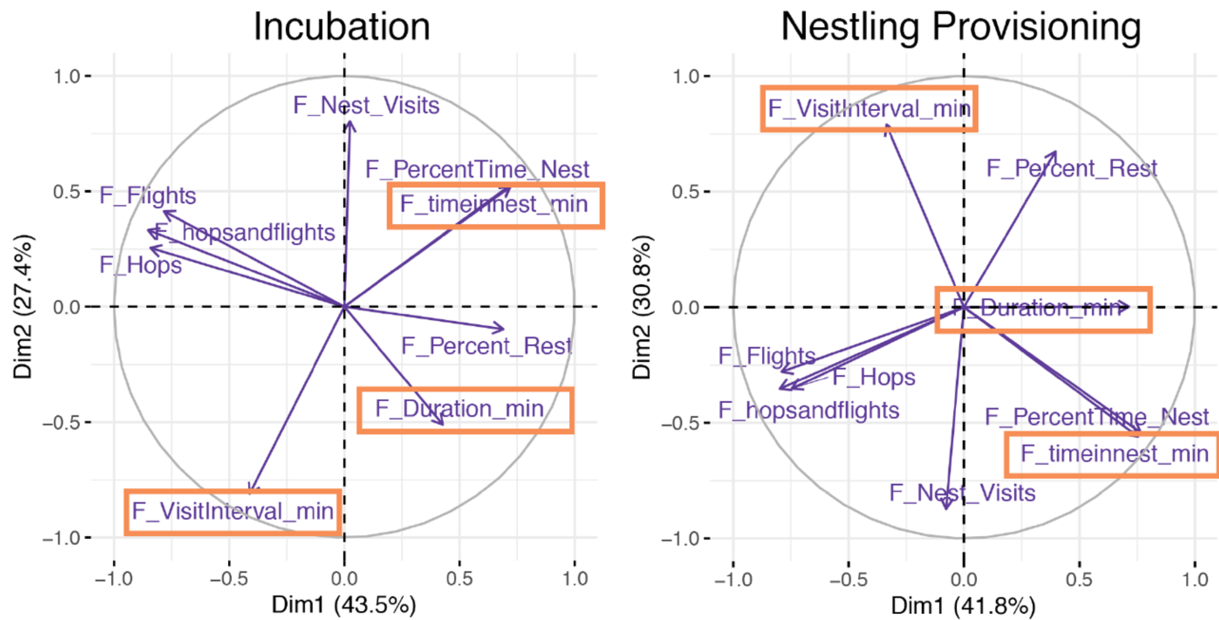


Figure 3. Variable correlation plot for incubation and nestling provisioning periods. Positively correlated behavioral metrics are grouped together in the same direction along the axes, negatively correlated variables are oriented oppositely on the plot, and greater distance from the origin reflects greater representation along the dimensions. The total time in the nest box, mean duration of nest visits, and mean interval between nest visits were then used to calculate an integrative index of parental care within each reproductive stage and experimental treatment group, where Parental Care Index (PCI) = [time in nest (min) + mean visit duration (min)] / mean visit interval (min).

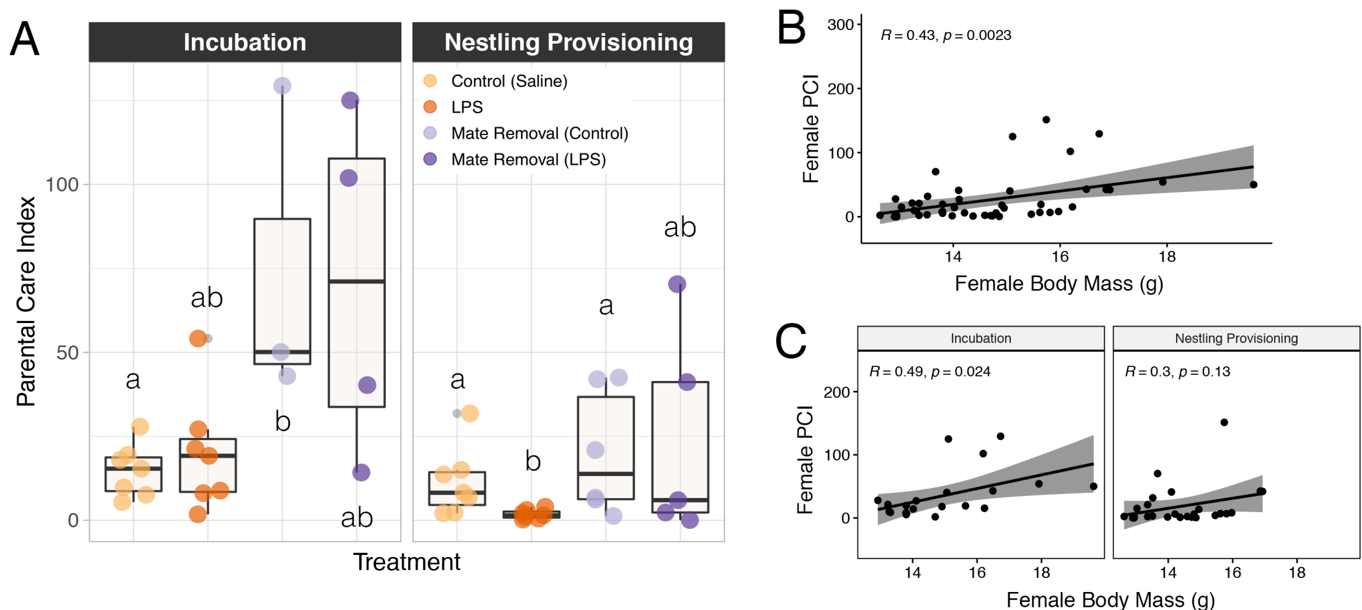


Figure 4. (A) Calculated parental care index (PCI) for female zebra finches across treatments during incubation and nestling provisioning periods of investment. Boxplots display the group median (horizontal line) with shaded region corresponding the interquartile range (25%-75%) and vertical lines extending to group minimum and maximum values. Statistically significant differences ($p \leq 0.05$) between groups (via Wilcoxon-rank sum) are indicated by different letters. Outliers are depicted as stand-alone points beyond the vertical range lines. Females did not differ significantly in pre-treatment body mass at the start of the experiment, however subtle differences among individuals allowed us to determine an overall significant relationship between (B) pre-treatment female body mass (i.e. crude reflection of starting body condition) and PCI, where females with higher body mass possess a higher calculated PCI. (C) This relationship was stronger within the incubation period ($p = 0.024$, $R = 0.49$) compared to nestling provisioning ($p = 0.13$,

Figure 5. Change in individual nestling mass across experimental treatment groups. (A) A significant effect of mate removal was detected where (B) nestlings of single parent females gained significantly less mass (grams) than paired females. Additionally, (C) a greater proportion of single parent nestlings experienced body mass loss after 24 hours. Asterisk indicates $p < 0.05$.

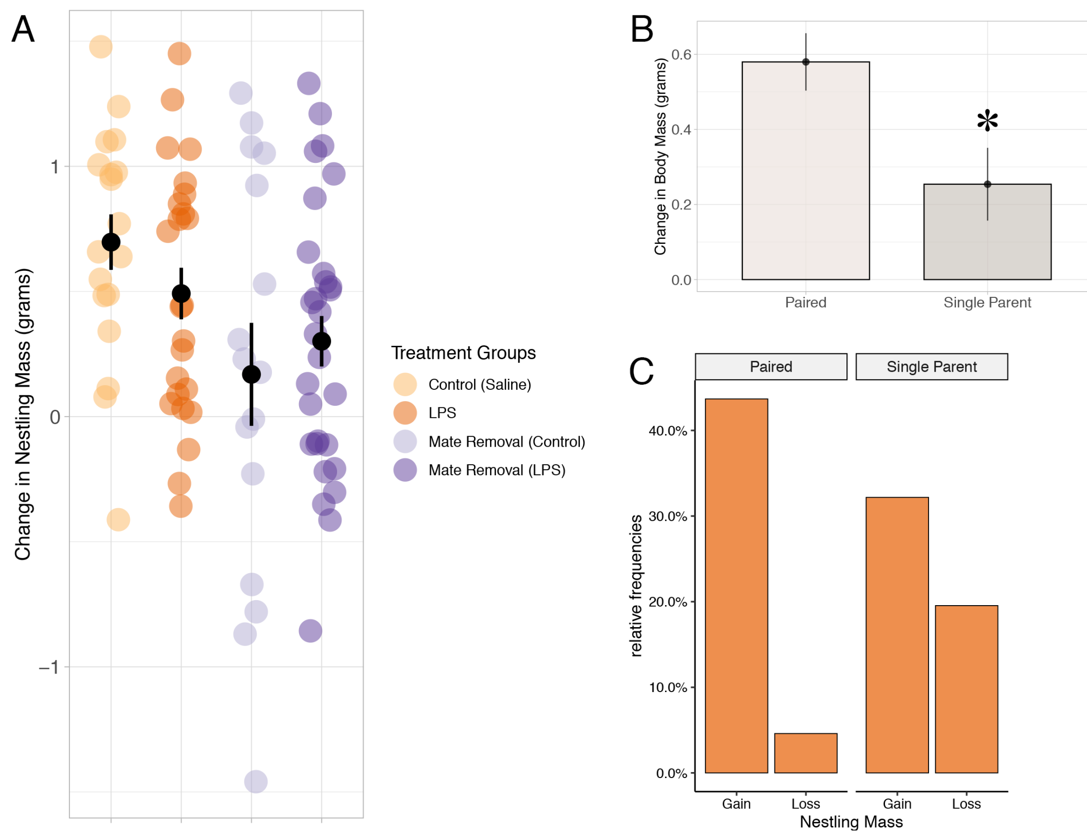


Figure 6. Concentrations (ng/ml) of baseline plasma corticosterone in saline-treated (healthy) paired female parental zebra finches by reproductive stage. There was no difference in (A) pre-treatment corticosterone or (B) concentrations measured in the afternoon. Bar plots indicate group means with standard error represented by vertical lines.

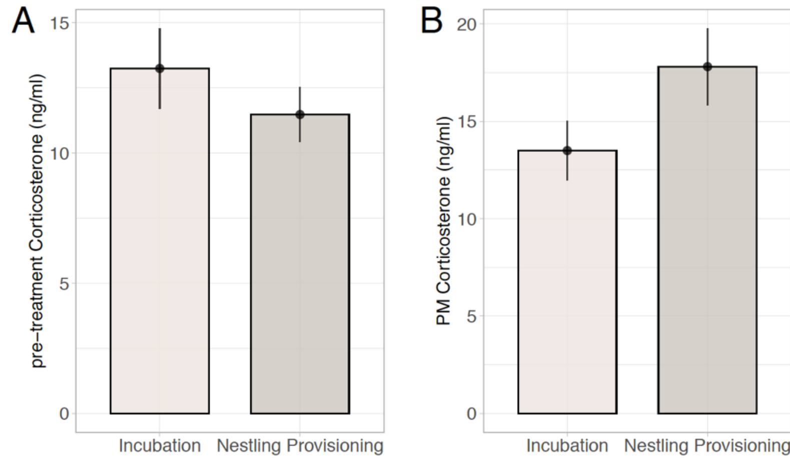
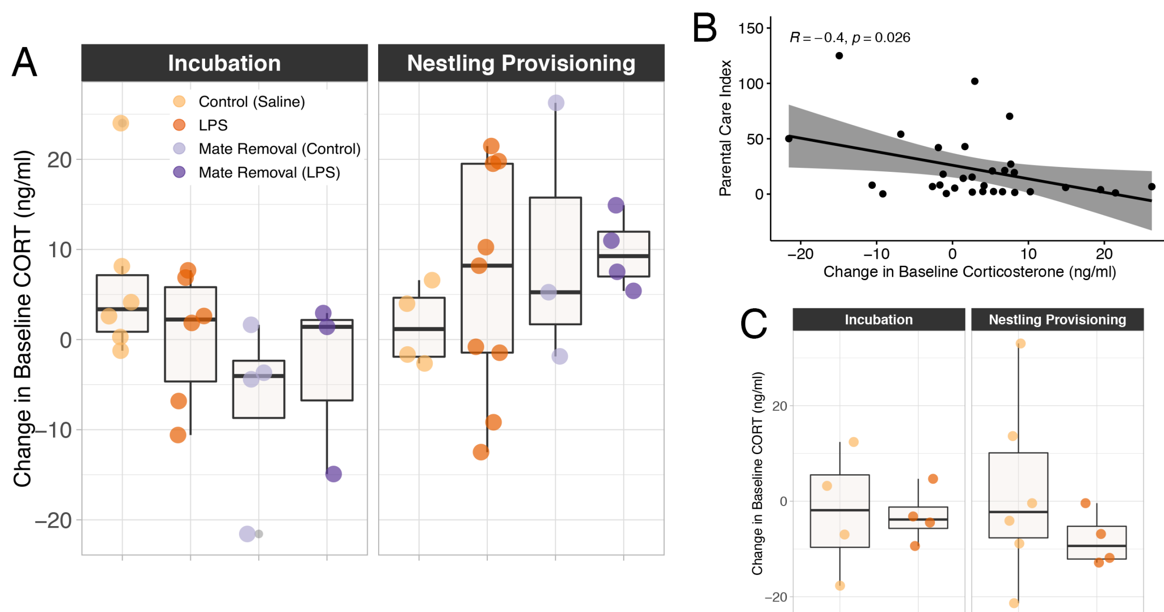


Figure 7. Impact of LPS and/or mate removal on change in plasma corticosterone. (A) No effect of immune challenge or mate removal on change in female baseline plasma corticosterone was detected. (B) A significant relationship between within-individual change in baseline corticosterone and calculated PCI was found (R = -0.4, $p < 0.05$), where females with greater increase in circulating corticosterone also tended to exhibit lower parental investment as reflected by individual parental care index value. (C) Immune status of females did not significantly impact change in plasma corticosterone of male mates, however variance in CORT change among males was significantly decreased (F test, $p < 0.05$) in both reproductive stages when females were treated with LPS.



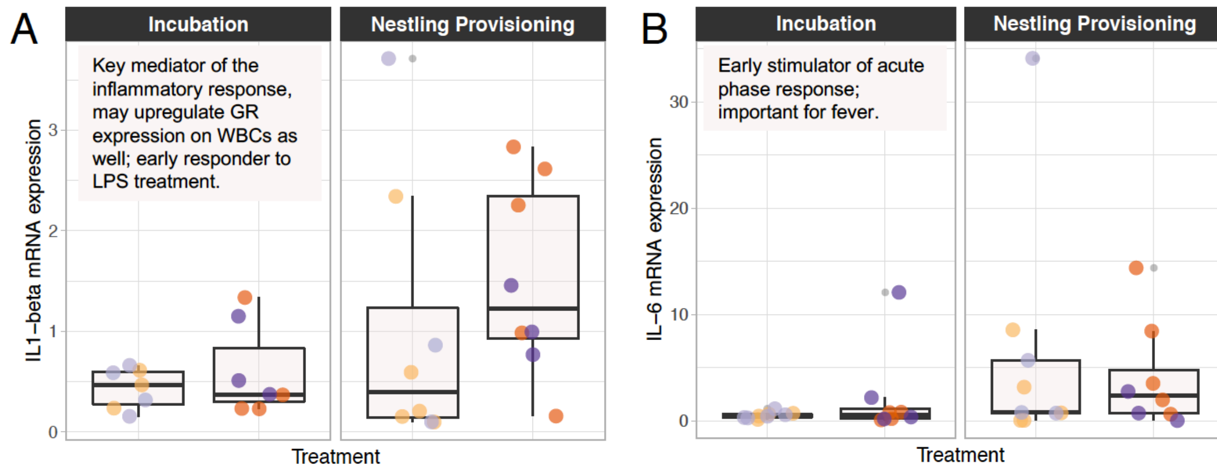


Figure 8. Female red blood cell mRNA expression of immune-associated cytokines (A) IL1-β , and (B) IL-6. Boxplots display the group median (horizontal line) with shaded region corresponding the interquartile range (25%-75%) and vertical lines extending to group minimum and maximum values.

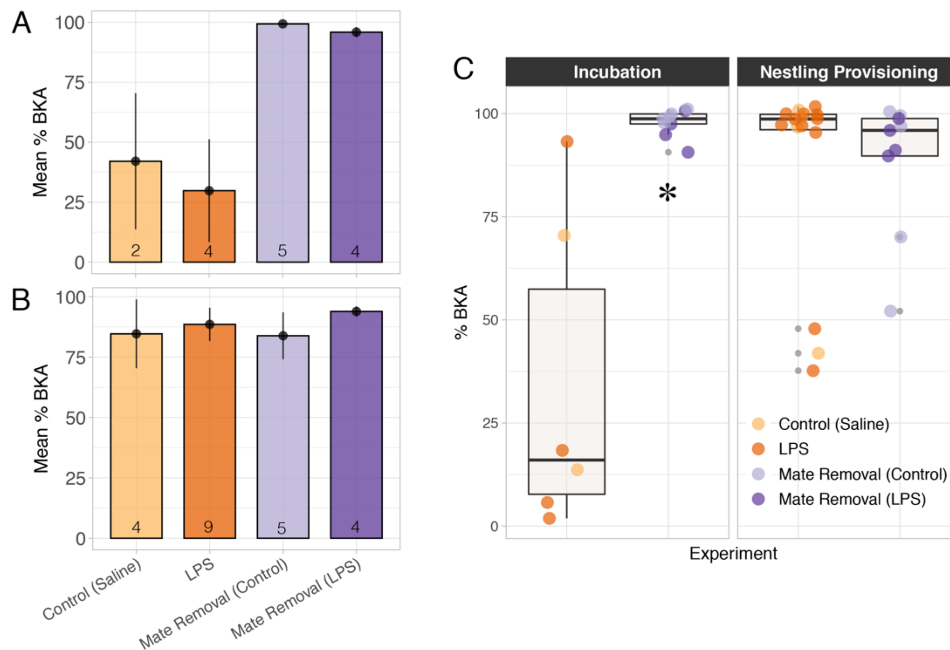


Figure 9. Mean percent bactericidal killing ability (%BKA) by experimental treatment group, separated by (A) incubation period and (B) nestling provisioning period. Sample sizes (n) indicated within bars. (C) Bactericidal killing ability is relatively higher during provisioning compared to incubation period, however females lacking their mate show significantly elevated %BKA during incubation regardless of LPS or saline treatment. Boxplots display the group median (horizontal line) with shaded region corresponding the interquartile range (25%-75%) and vertical lines extending to group minimum and maximum values. Statistically significant differences ($p \leq 0.05$) between groups are indicated by asterisk.

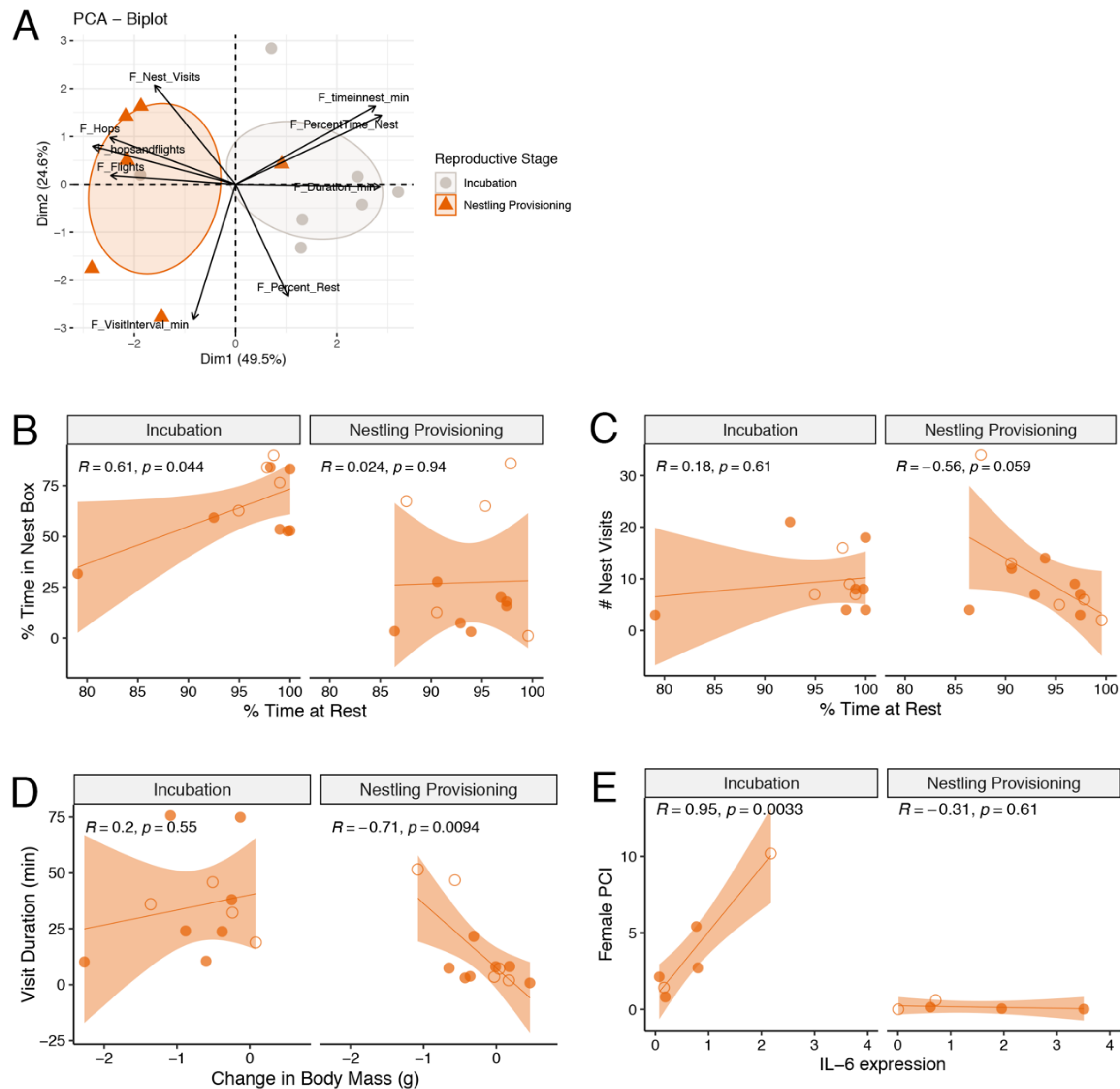


Figure 10. Among healthy females, (A) principal component analysis validated that there are distinct behavioral metrics that are important for and may be used to characterize incubation and nestling provisioning. Ellipses reflect 95% confidence intervals surrounding the group means. Stage specific significant relationships were found among female finches challenged with LPS treatment. (B) A positive relationship between % time at rest and total % of time inside the nest box was detected during the incubation period only, while (C) a significant negative relationship between % time at rest and total number of nest visits was found only during nestling provisioning. (D) Individual females with higher mean duration of nest visits lost significantly greater mass during provisioning with no relationship during incubation. Changes to molecular biology in response to LPS treatment resulted in a positive relationship between individual IL-6 mRNA expression and calculated parental care index (PCI) only during incubation. Filled circles = LPS, Empty circles = Mate Removal (LPS). Correlation analyses of these predicted trade-offs were conducted using generalized linear models with Pearson correlation coefficient. Shaded area represents 95% confidence interval surrounding the resultant model.

Broad Conclusions and Contributions to the Field

The brain is a critical regulator of seasonal reproduction, translating environmental cues into molecular signals that dictate proper timing for physiological investment in reproduction. Natural selection has led to the use of photoperiod as the most invariant environmental cue (for any particular date of the year) to determine timing of reproduction (Prendergast et al 2001, reviewed in Nakane and Yoshimura 2019), with a strong spring bias across mammals (Bronson 1989). The anomalous temporal delay in reproductive events that hibernating bat species exhibit makes bats an interesting model for examining neuroendocrine patterns associated with fertility and reproductive behavior across the annual cycle. Next steps should aim to characterize the expression pattern and dynamics of key reproductive neuropeptides in response to seasonal and environmental variation and physiological stressors, as well as mechanistic studies that can assess the direct downstream effects of these neuropeptides. Acquisition of this knowledge will allow us not only to understand the mechanisms regulating reproductive physiology/fertility in bats, but also help us make stronger predictions regarding population dynamics and inform decisions about their management in the wild. Furthermore, using bats as models within comparative studies advances the fields of reproductive neuroendocrinology and physiology through understanding the components of HPG axis regulation not only in a classic comparison between seasonal versus "opportunistic" breeders, but also emphasizes the value in exploring the variation in reproductive phenology and physiology that exists within those broad classifications.

Stress can lead to inhibition of reproductive physiology and behavior across a wide array of taxa and it is likely that the specific eco-physiological context a stressor is experienced within distinctly influences the neurobiological and gonadal responses of the organism (Bentley et al., 2017). Collectively, our findings indicate that the brain and the testes in E. fuscus respond to acute restraint stress with significant declines in circulating plasma testosterone and potential rapid apoptotic consequences in the gonads coupled with an increase in local RFRP and GR expression. The short timeframe of the gonadal response in *E. fuscus* is unprecedented in mammals, suggesting that bats are highly sensitive to acute stressors. As such, our data have implications for conservation management of bat roosts and hibernacula, especially in terms of disturbance. Finally, because stress responses are known to vary temporally, including seasonally, it is important that future studies examine the effects of acute stressors on the HPG axis across the seasons. Temperate, seasonally breeding bats like *E. fuscus* are a particularly appropriate model system for addressing such questions.

As emphasized throughout this dissertation, life history theory of trade-offs posits a negative relationship between investment in processes supporting reproduction and those supporting survival, or somatic maintenance, with predictable effects on behavioral and physiology. However, individual responses to an ecological stressor(s) are not static and the relationship – or degree of relationship – between a given perceived energetic challenge and individual fitness should also be dynamic, with both energy and time as valuable resources underlying these potential trade-offs. The present work emphasizes the ways that a social and physiological stressor may interact to shape an individual's energetic strategy and expression of trade-offs between reproduction and self-maintenance. We have used this framework of avian breeding stages to demonstrate the context-dependent behavioral conflict that may/may not be present across a species' breeding

period, and the importance of examining these conflicts through a holistic lens that takes species' sociality and mating system into account as these variables directly affect routine individual energetic demands.

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