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Environmental Factors Affecting Avian Reproduction and Behavior

by

Darcy Fay Kato Ernst

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
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Abstract

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Doctor of Philosophy in Integrative Biology

University of California, Berkeley

Professor George E. Bentley, Chair

Appropriate timing of reproduction is crucial to animals' reproductive success and fitness. Animals living in unpredictable environments do not constrain their breeding to one season, but rather breed any time of the year in which good conditions occur. These animals, termed "opportunistic" breeders, cannot predict when good conditions will occur based on the seasons, and must instead respond quickly to proximate environmental factors to successfully breed and raise young. Animals may respond to a number of different cues in the environment to decide when is the best time to breed, and thus the brain must integrate these varying sources of information to produce an appropriate decision.

Cue integration in the brain takes place in the hypothalamus located at the base of the brain and, if conditions are favorable, will result in a physiological reproductive cascade. In stimulatory conditions gonadotropin-releasing hormone (GnRH) is released from the hypothalamus, which stimulates the production and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland. These two hormones act on the gonads to increase production of testosterone, estradiol and gametes, leading to an increase in fertility. These changes in physiology are associated with an increase in reproductive behavior, including courtship and song behavior. Release of GnRH from the hypothalamus is considered the gateway for reproduction, so research on the mechanisms of reproductive timing often focus on how information is transmitted to the GnRH system and hormones that influence GnRH release.

The goal of this dissertation is to increase our understanding of the mechanisms underlying reproductive timing in response to social cues, stress, and food availability in an avian model of opportunistic breeding, the zebra finch (*Taeniopygia guttata*). Social cues and food availability stimulate and stress inhibits reproduction in zebra finches, but the endocrine underpinnings of these phenomena are undefined. Chapter 1 provides an overview of the current scientific knowledge regarding the effects of social cues, stress, and food availability on reproductive physiology. The remaining chapters detail experiments I conducted to further elucidate the mechanisms responsible for the effects of these cues on reproductive physiology and behavior, and the dissertation concludes with a discussion chapter

Chapter 2, "Social stimulation, reproductive behavior and the thyroid hormone deiodinase system in an opportunistically-breeding songbird", examines the role the presence of an available mate plays in activating reproduction. Isolated males were compared to those receiving a female stimulus or a male stimulus animal. I measured key genes in various tissues of the reproductive axis, activation and protein content of the hypothalamus, and reproductive behaviors. My findings suggest that the hypothalamus is indeed active in response to an available mate and appropriate reproductive behaviors increase, but more work needs to be done to determine how GnRH release might be governed by social cues.

Chapter 3, "Differential response of GnIH in the brain and gonads following acute stress in a songbird", looks at stress as a potential mechanism by which to time reproduction. Stress is a known inhibitor of reproduction across vertebrate taxa, and thus can be used to time breeding by increasing the stress hormone corticosterone (resulting in decreased reproductive axis function) during times when environmental conditions are poor. This chapter focuses on gonadotropin-inhibitory hormone (GnIH), a hormone known to inhibit reproduction at all levels of the reproductive axis, in the hypothalamus and gonads. While previous research has suggested a role for GnIH in stressed-induced reproductive inhibition, this is the first experiment to show a differential response of the GnIH system in the hypothalamus and the gonads in response to stress. This study provides more information about the role of GnIH, particularly that the GnIH response to stress may not be as conserved throughout vertebrate evolution as previously thought.

Chapter 4, "Corticosterone measurements differ in plasma sampled from a wing vein and trunk blood", is concerned with the methodology used to measure hormones in laboratory and field settings and the potential sources of intra-individual hormone variability. When comparing corticosterone levels between wing blood, a source commonly used in field studies, and trunk blood, a source commonly used in terminal studies, we found differences in corticosterone levels within an individual. This finding illustrates that the source of blood collection for hormone assay is an important source of variability and must be considered in experimental design and when comparing results across studies.

Chapter 5, "Perception of food availability determines reproductive response to food restriction in the zebra finch *Taeniopygia guttata*", investigates the role of perception in food-induced reproductive suppression and activation. Animals that were food restricted decreased reproductive parameters (GnRH, general activity), while animals that were food restricted but could see food during restriction maintained higher levels of GnRH and activity similar to controls. This suggests that the visual cue of food is enough to maintain reproductive activity even in conditions of starvation, and that perceptual information as well as metabolic information from food is important in these reproductive activity considerations. Additionally, measurement of expression of the orexigenic peptide neuropeptide Y (NPY) and its correlation with GnRH across groups indicates a potential novel role for this peptide in governing reproductive activation in response to the perception of food. Taken together, these studies indicate the importance of brain integration of social cues, stress, and food availability to initiate breeding during favorable conditions in opportunistic breeders.

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Chapter 1

Non-photic environmental factors affecting the timing of avian reproduction and sexual behavior

Introduction

Appropriate timing of reproduction is crucial to animals' reproductive success and fitness (Lack 1954, Perrins 1970). Production of young must occur when environmental conditions are most favorable to ensure the survival of young (Perrins 1996), thus there is strong evolutionary pressure on physiological mechanisms underlying reproductive timing. Much work has been done on the mechanisms of reproductive timing, with a particular focus on how photoperiod can initiate an increase in gonadal development and reproductive behavior. While changes in photoperiod drive reproduction in some species, food availability, social cues, and stress can act as supplementary cues to fine-tune the timing of breeding (Wingfield 1980, Wingfield 1983). Much of our current knowledge of the effects of these cues on the reproductive axis come from the study of photoperiodic breeders, though opportunistic or flexible breeders that do not rely on photoperiod may use these cues exclusively to time reproduction. Here I examine how these supplementary cues affect reproductive development, behavior, and reproductive timing in non-photoperiodic breeders, while contrasting how these cues may affect the timing of breeding in photoperiodic breeders.

Reproductive Physiology

Successful timing of reproduction is achieved by an appropriate physiological response to stimulatory cues. To initiate reproduction various cues are integrated by the hypothalamus at the base of the brain (Ball 1993, Ubuka et al. 2013) and, when conditions are deemed appropriate for breeding, there is upregulation of the hypothalamo-pituitary-gonadal (HPG) axis and an increase in associated behavior. In stimulatory conditions gonadotropin-releasing hormone (GnRH) is released from the hypothalamus, which stimulates the production and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland. These two hormones act on the gonads to increase production of testosterone, estradiol and gametes, leading to an increase in fertility. These changes in physiology are associated with an increase in reproductive behavior, including courtship and song behavior. Therefore, GnRH is often considered the "gateway" to reproduction, and study of the mechanisms of reproductive timing often focus on how information is transmitted to the GnRH system and hormones that influence GnRH release.

One hormone poised to "gate" reproduction is gonadotropin-inhibitory hormone (GnIH), which can inhibit reproduction at all levels of the HPG axis. GnIH neurons in the hypothalamus interact directly with GnRH neurons (Bentley et al. 2006, Ubuka et al. 2008, Ubuka et al. 2009), GnIH receptor is found on GnRH-producing neurons (Ubuka et al. 2008, Ubuka et al. 2013), and GnIH reduces firing of GnRH-producing neurons *in vivo* (Ducret et al. 2009). GnIH is also secreted into the hypothalamo-pituitary portal system to directly inhibit pituitary release of LH and FSH (Clarke et al. 2008, Johnson et al. 2007, Ubuka et al. 2006), and is synthesized in the gonads and can act to decrease steroidogenesis in the gonads (McGuire et al. 2011, Singh et al. 2011) via action on GnIH receptor (reviewed in Bentley et al. 2010, Ubuka et al. 2013). Hypothalamic GnIH responds to food cues (Chowdhury et al. 2012, Fraley et al. 2013) and stress (Calisi et al. 2008, Kirby et al. 2009, Geraghty et al. 2015), providing a potential mechanism by which these cues could be conveyed to the reproductive axis (Clarke et al. 2012). GnIH is beginning to be considered in the context of social cues (Calisi et al. 2011, Maruska and Fernald, 2011, Perfito et al. 2015)

Social Cues

Social interactions have profound effects on vertebrate reproductive physiology and behavior (Crews and Silver 1985, Maruska and Fernald 2011, Wingfield et al. 1994) and can determine the proximate timing of reproduction (Coulson 1966). A mate is required for reproduction in birds, and thus mate presence and availability can stimulate reproductive activity. Pair bond and courtship behaviors can induce activation of the reproductive axis, and many species require complex interaction with their mate to activate the reproductive axis. For example, ovarian growth and development in the ring dove is induced by a number of social interactions with her mate. The female hearing her own coo during courtship behavior induces ovarian development (Cheng 1986), and response to male (Cheng 1974) and nest-building activity (Cheng and Balthazart 1982) also influence ovarian development and egg production.

Social structure and the status of the animal in the social environment can greatly affect breeding opportunities and breeding success. In environments with limited resources necessary for breeding, such as cavities in which to nest, many animals have developed complex social structures to decide who gets access to these limited resources (Newton 1994). Birds cannot produce or build new cavities themselves, and instead must rely on already-existing structures. With the cavities as a limited resource, not all sexually mature adults will be able to breed in any given year. While the social structure and ramifications of these systems are well-studied, the effects on the reproductive axis are less well-known. Potentially, social structure or mate availability could stimulate GnRH release or interact with hormones that control GnRH release to govern reproductive activity.

Food Availability

Sufficient energetic resources are required for reproduction, and thus food availability is a potent stimulator of reproductive activity. Food availability is a proximate cue that seasonal breeders use to fine-tune breeding to coincide with favorable environmental conditions (Hahn 2005, Hau et al. 2000, Davies et al. 2015). Indeed, it is the predictable seasonal increase in food availability that is the ultimate cue that drives selection for animals to use changes in daylength to stimulate reproduction. In photoperiodic breeders, daylength is the primary driver of reproductive development, with food availability supplementing that information. Increased food availability has been shown to advance lay date (Kallander 1974, Kallander and Karlsson 1993), while food restriction can delay lay date, resulting in fewer nestlings of low quality (Barba et al. 1995). Lack of energetic resources can also halt reproductive activity entirely, causing animals to miss a full breeding season or not breed for multiple years (Martin 1987).

Food provides metabolic and energetic information to the reproductive axis, and these aspects of food have been shown to increase reproduction activity across vertebrate taxa (Batzli 1986, Rubenstein and Wikelski 2003, Sedinger and Raveling 1986). High quality food is particularly effective at increasing reproductive output and reproductive behavior (Hau et al. 2000, Schoech et al. 2004). Young often require different foodstuffs than adults, and thus certain types of food may stimulate reproduction as animals perceive an increase in the necessary food to provision young. For example, the hatching of the first clutch in zebra finches (*Taeniopygia guttata*) coincides with availability of grass seed necessary to provision young (Zann et al. 1995), and the timing of great tit reproduction is such that young hatch during peak caterpillar availability (Van Noordwijk et al 1995). Food also provides perceptual information to animals through visual, gustatory, and tactile cues. These cues could operate to stimulate reproduction independently of the metabolic cues associated with food, though very little research on the

effects of perceptual cues on the reproductive axis exists in birds (Furlonger et al. 2012, Hau et al. 2000).

The timing of reproduction on the basis of availability of certain foods is problematic in the face of climate change, as the yearly emergence of these plant and insect food sources is heavily dependent on temperature (Dewar and Watt 1992, Nebeker 1971, Parmesan 2006). Recent of examples of mistiming of reproduction of birds due to climate change show a reduction in populations of a number of avian species (Both et al. 2006, Visser et al. 2006). Animals that respond directly to food cues might then fare better than those that are predicting food availability based on photoperiodic or other cues. These flexible breeders could activate the reproductive axis when food is available, potentially adjusting when they reproduce more easily.

Stress

The cessation of reproductive function due to lack of food availability is inextricably linked with stress. Food restriction activates the endocrine stress response in vertebrates, which is characterized by an increase in circulating glucocorticoids. When a stressor, in this case lack of food availability, is perceived by the brain the hypothalamus releases corticotropin-releasing hormone (CRH) which stimulates release of adreno-corticotropic hormone (ACTH) from the pituitary gland. ACTH stimulates production and release of glucocorticoids from the adrenal glands. In birds and many mammals the primary glucocorticoid, and thus what is measured when considering stress, is corticosterone.

The primary purpose of this increase in corticosterone with food restriction is to utilize the catabolic action of corticosterone. Corticosterone breaks down food reserves (glycogen, adipose tissue) to increase blood glucose levels to fuel the animal in a stressed state. The increase in corticosterone with food restriction or other stress organizes a variety of other physiological functions and behaviors, to orchestrate an "emergency life-history stage" (Wingfield et al. 1995). The effects of corticosterone and the stress response include a shift of energetic resources away from growth and reproduction and towards survival, including finding food.

Lack of food availability is only one cause of corticosterone release and subsequent inhibition of breeding due to stress. Predation attempts are common initiators of the stress response, and presence of predators can affect when birds initiate reproduction (Lima 2009, Martin 1995, Scheuerlein et al. 2001). Birds will abandon nests and stop reproductive activity in severe weather, which has been linked to HPA axis activation during storms (Astheimer et al. 1995, Wingfield et al. 1998).

Stress is a known inhibitor of reproductive physiology and behavior across vertebrate taxa (Moore & Jessop 2003, Siegel 1980, Wingfield et al. 1998). Due to this inhibitory action stress can therefore time breeding. There are many mechanisms by which stress can inhibit reproductive function. Corticosterone can directly affect the reproductive axis via action on the glucocorticoid receptor, which is present in the hypothalamus, pituitary, and gonads (Gore et al. 2006, Rivier and Rivest, 1991). A growing body of literature addresses the role of GnIH in stress-induced reproductive inhibition (Calisi et al. 2008, Kirby et al. 2009, Geraghty et al. 2015), though the response of the GnIH system to stress does not appear to be universal. Numerous studies find increased corticosterone levels with stress and associated reproductive malfunction, but no measurable change in GnIH (Lopes et al. 2012, Papargiris et al. 2011). Due to their repressive effects on the reproductive axis, stress and corticosterone must be considered

as potential governors of reproductive timing and/or supplementary information to the reproductive axis.

Conclusions

Social cues, food availability and stress have profound effects on reproductive physiology and behavior. Birds may use any or all of these cues to effectively time reproduction such that young are born at a time when they are most likely to survive. This dissertation concerns the mechanisms underlying reproductive timing in the opportunistically-breeding zebra finch, addressing how these environmental factors interact with the reproductive axis.

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Chapter 2

Social stimulation, reproductive behavior and the thyroid hormone deiodinase system in an opportunistically-breeding songbird

Abstract

Recent studies of the onset of breeding in long-day photoperiodic breeders have focused on the roles of type 2 and 3 iodothyronine deiodinases (DIO2 and DIO3) in the conversion of thyroxine (T4) to triiodothyronine (T3) and subsequent activation of the reproductive axis. It has been hypothesized that an increase in DIO2 and reciprocal decrease in DIO3 causes the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, setting off a reproductive cascade, and that this DIO mechanism for GnRH release is conserved across vertebrate taxa. We sought to test whether social cues that are known to stimulate reproductive behaviors can activate the DIO system to initiate reproduction in a non-photoperiodic bird, the zebra finch (*Taeniopygia guttata*). Isolation of males and subsequent presentation of females did not increase DIO2 or GnRH expression in the hypothalamus, nor did it decrease gonadotropin-inhibitory hormone (GnIH) or DIO3. Males receiving a female stimulus showed significantly higher mRNA expression and immunoreactive cell count of the immediate early gene EGR-1 than isolated males, indicating hypothalamic activation in response to a female. Cells immunoreactive for EGR-1 were not co-localized with those immunoreactive for GnRH. Reproductive behaviors (singing, copulation attempts, and overall activity) were significantly higher in males receiving a female stimulus. This study presents a social effect on behavior and EGR-1 expression in the hypothalamus of males in response to females, but more research is needed to determine if the DIO2 system is responsive to social stimulation in this species.

Introduction

The physiological mechanisms allowing animals to time breeding appropriately in response to environmental cues have been the subject of research for almost a century. Recent studies of the onset of breeding in long day photoperiodic breeders have focused on the role of type 2 iodothyronine deiodinase (DIO2) in the conversion of thyroxine (T4) to triiodothyronine (T3) and subsequent activation of the reproductive axis. In seasonally-breeding Japanese quail, *Coturnix japonica*, long day lengths induce an increase in the expression of the thyroid stimulating hormone beta subunit (*TSHβ*) followed by an increase in local expression of *DIO2* and a decrease in type 3 iodothyronine deiodinase (*DIO3)* in the hypothalamus (Yoshimura et al. 2003, Nakao et al. 2008). In this DIO model the local photo-induced increase in expression of *DIO2* and decrease in *DIO3* is viewed as a “reciprocal switch”, causing an increase in locally-available T3 in the hypothalamus. While it has not been demonstrated directly, this model suggests the increase in hypothalamic T3 causes a release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, thus increasing expression and release of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary and activating the gonads.

The reciprocal switching of *DIO2* and *DIO3* is hypothesized to be a conserved mechanism of reproductive activation in all vertebrates (Nakane and Yoshimura, 2014), though this has only been tested in photoperiodic breeders (Yoshimura et al. 2003, Revel et al. 2006, Hanon et al. 2008). If the DIO system is an evolutionarily-conserved mechanism that regulates GnRH release then it is likely that it responds to cues other than day length, given that multiple cues can influence GnRH release. Species that do not respond reproductively to changes in day length and those that live in areas where day lengths do not differ throughout the year could instead activate the DIO system to cause a release in GnRH in response to stimulatory cues such as availability of mates, food, or nesting sites.

Non-photoperiodic breeders that breed whenever conditions are favorable are termed opportunistic breeders (Dawson et al. 2001). To time breeding appropriately, these species - such as zebra finch, *Taeniopygia guttata* - respond to and integrate proximate cues of food and water availability, mating opportunities, and other resources to time reproduction (Perfito et al. 2007, 2008, Zann et al. 1995, Hahn et al. 2005). The mechanism(s) underlying reproductive activation in response to food, water, or mate availability remain(s) unknown, thus we sought to investigate a potential role for the DIO system in reproductive activation in this species

The hypothalamic neuropeptides GnRH and gonadotropin-inhibitory hormone (GnIH) are two likely key integrators of environmental stimuli for reproduction. As such, they are also likely to interact with the DIO system to control breeding. While breeding zebra finches do not differ from non-breeders in hypothalamic immunoreactivity of GnRH and GnIH (Perfito et al. 2011), the DIO system could be acting to regulate GnRH release when breeding commences. This, together with the proposed role of DIO in activation of breeding in photoperiodic species, led us to test if the DIO, GnRH and GnIH systems respond to a social stimulus in zebra finches. Specifically, we sought to test whether there is a reciprocal switch between *DIO2* and *DIO3* expression following social stimulation of an opportunistic breeder, similar to that seen in response to long days in a photoperiodic species. When males were housed in isolation and deprived of social cues, we expected to see a decrease in *DIO2* expression, an increase in *DIO3* expression and decreases in GnRH, LH, and testosterone (T). Following presentation of a potential mate to these isolated males, we expected to observe an increase in *DIO2* expression, a decrease in *DIO3* and *GnIH* expression and increases in GnRH, LH and T. We expected to see these changes in association with an increase in reproductive behaviors in response to a new female and sought to characterize activity of the DIO system in a social paradigm.

Materials and Methods

Study Animals

Male zebra finches were bred and housed in mixed-sex free-flight aviaries at the Field Station for the Study of Behavior, Ecology, and Reproduction (FSSBER) at the University of California, Berkeley. Birds were exposed to natural changes in day length that were supplemented with full-spectrum artificial light to create a minimum 12L:12D photoperiod. Birds were supplied with water and German millet mixed with canary seed *ad libitum*. Food was supplemented with cuttlebone, grit, and lettuce weekly. All animal care and procedures were approved by the University of California Office of Laboratory Animal Care and conducted in accordance with local animal welfare laws and policies.

Study Design

Adult male zebra finches were caught from the colony between 10 and 11 am and transferred to an isolation cage (18”x8”x12”) inside a custom-made temperature and light-controlled insulated box. Within each box, males did not receive any visual or auditory input from the colony or adjacent isolation boxes. For the two days of isolation males were maintained on the 12L:12D photoperiod they had experienced in a colony setting and had *ad libitum* access to water and German millet mixed with canary seed.

After two days of isolation birds (n=8 per group) were randomly assigned to one of 3 treatments: 1) maintained in isolation (isolated), 2) presented with a novel female for 30 minutes (female stimulus), or 3) presented with a novel male for 30 minutes (male stimulus). Behavior was recorded for 30 minutes before and 30 minutes after stimulus presentation for all treatments.

The novel stimulus birds came from separate colony rooms, ensuring that the experimental males had had no previous experience with these stimulus animals.

After treatment, experimental males were deeply anesthetized with isoflurane (Phoenix Pharmaceuticals Inc., Burlingame, CA, U.S.A.) and rapidly decapitated, at which time trunk blood was collected. Brain and testis tissue were frozen immediately on dry ice (Experiment 1). Heads with pituitary tissue intact were frozen on dry ice and the pituitaries were subsequently extracted under a dissection microscope.

This experiment was repeated with six birds in each group. In this second experiment the brains were fixed in a 4% paraformaldehyde solution (PFA) for three days before being cryoprotected in 30% sucrose in 0.1 M phosphate-buffered saline (PBS) and frozen (Experiment 2).

Tissue Processing

Trunk blood was spun in a centrifuge at 4 ºC at 1500 g for 10 minutes to separate blood plasma. Plasma was stored at -80 ºC before assay for LH, testosterone, and corticosterone. Pituitaries were extracted from the skull and placed into 1 ml of TRIzol reagent (Invitrogen by Life Technologies, Grand Island, NY, U.S.A.) and homogenized. One testis from each bird was placed into 1 ml of TRIzol and homogenized.

Brains were cut into 40-μm thick slices on a cryostat (cm3050s, Leica Microsystems, Buffalo Grove, IL, U.S.A.) and mounted directly on to slides. Brain tissue was mounted onto slides beginning with the appearance of the tractus septomesencephalicus (TrSM), a neuroanatomical landmark anterior to the hypothalamus, and all slices were mounted until the appearance of the cerebellum. 3mm punches were taken through the hypothalamus from alternating sections and placed in TRIzol. All sections were mounted on slides for subsequent immunocytochemistry (ICC). The brains put in PFA immediately after collection were also cut into 40-μm thick slices, but were stored in antifreeze prior to ICC. Prior to freezing, length and width of testes were measured to assess fresh testis volume. Volume was calculated as $V=4/3\pi a^2 b$, where a is half the width and b is half the length (long axis) of the testis.

RNA was extracted using chloroform (as described in Perfito et al. 2012). RNA extracts were treated with a DNAse (Invitrogen by Life Technologies, Grand Island, NY, U.S.A.) to digest any single- and double-stranded DNA and subsequently reverse transcribed using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, U.S.A.) to create cDNA from each tissue.

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) on cDNA obtained from hypothalamic punches was run for a number of genes: *DIO2, DIO3, GnRH, GnIH,* and early growth response protein 1 (*EGR-1)*, an immediate-early gene (IEG). This IEG was taken as an indicator of general activation of neurons (Hoffman et al. 1993, Morgan and Curran 1989, 1995). The reference gene *18S* was used as a control gene in hypothalamic tissue as its expression did not change with treatment. cDNA was diluted 1:25 with water treated with diethylpyrocarbonate (DEPC). Primers were designed for each gene from previously published sequences in the zebra finch genome (see Table 1 for accession numbers) using Primer3 software (simgene.com). qPCR was performed using manufacturer's instructions for SYBR green reagent (Applied Biosystems by Life Technologies, Grand Island, NY, U.S.A.). Post-qPCR products were cloned, sequenced, and compared to GenBank to confirm identification of target genes. Raw fluorescent data were

analyzed with the RT-PCR Miner program (Zhao and Fernald, 2005) and cycle thresholds were obtained from this program. Expression values were calculated as $1/(1+E)\wedge Ct$, where E is the average PCR efficiency for the gene of interest as calculated by a standard curve and Ct is the cycle threshold. Data are shown as fold-change, which was calculated by dividing the expression of the gene of interest (corrected for the reference gene *18S*) by the average expression for the isolate group.

Immunocytochemistry

Immunocytochemistry (ICC) was performed on series of brain tissue to label GnRH and EGR-1 proteins. Alternating sections of brains from experiment 1 (mounted on slides) were single-labeled for GnRH and double-labeled for GnRH and EGR-1. EGR-1 single-label ICC was conducted on one set of brain tissue from experiment 2 (free-floating brain sections) that had been fixed at the time of collection. For GnRH single-label and GnRH/EGR-1 double-label slides were fixed in 4% paraformaldehyde (PFA) for 20 minutes and washed in 0.1M phosphate-buffered saline (PBS, pH 7.4) three times. Slides were then incubated in 0.03% hydrogen peroxide in methanol for 30 minutes followed by three PBS washes and one hour of incubation in 2% normal goat serum (Vector Laboratories, Burlingame, CA, U.S.A.) in 0.2% phosphate-buffered saline with Triton x-100 (PBS-T). Slides were incubated in GnRH primary antibody (HU60, a gift from Dr. Henryk Urbanski, Portland, OR, U.S.A.) 1:5000 in PBS-T for one hour at room temperature and subsequently for 48 hours at 4 ºC. After incubation in the primary antibody slides were washed with PBS, incubated in the secondary antibody biotinylated goat anti-rabbit (1:250 in 0.2% PBS-T, Vector Laboratories, Burlingame, CA, U.S.A.) for one hour, washed in PBS-T, and incubated in avidin/biotinylated enzyme complex (PK-6100, Vectastain Elite ABC Kit (Standard), Vector Laboratories, Burlingame, CA, U.S.A.) as per manufacturer's instructions. Slides were washed in PBS and incubated with DAB peroxidase substrate kit (SK-4100, Vector Laboratories, Burlingame, CA, U.S.A. For double-label slides were then incubated in the primary antibody for EGR-1 (c-19, sc-189, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) 1:1000 in 0.2% PBS-T for 24 hours. Slides were then washed and incubated as described above with biotinylated goat anti-rabbit secondary antibody, then ABC, then DAB with nickel sulfate. For an EGR-1 single-label all steps were the same as described above on free-floating tissue, then brain tissue was mounted onto slides. All slides were then dehydrated with graded ethanol, cleared in xylenes and coverslipped with Permount (SP 15-100, Fisher Scientific Inc., Waltham, MA, USA).

Quantification of Immunocytochemistry

Cell counts for all immunocytochemistry were obtained by a researcher unaware as to experimental treatment. All cells immunoreactive (-ir) for GnRH were counted for each animal and assessed for co-localization with EGR-1. Single-label EGR-1 was counted at 20x magnification centered around the midline with the base of the brain just visible in the microscope field from the pre-optic area (POA) through the appearance of the cerebellum. All EGR-1-ir cells in this space were counted for each individual and averaged over the total number of sections counted for analysis.

Hormone assays

Concentrations of testosterone and corticosterone in plasma were measured using enzyme immunoassay kits (EIA, Enzo Life Sciences, Farmingdale, NY, U.S.A.). These kits have been

validated and optimized for zebra finches (Lynn et al. 2007, 2010). Protocols for both hormones were followed as specified by Lynn et al. 2010. Plasma was diluted 1:40 for testosterone and corticosterone EIA. All samples were run in duplicate on one plate per hormone, with a standard curve also in duplicate for comparison.

Radioimmunoassay for Luteinizing Hormone

Plasma was assayed in 15 μl duplicate samples in a single assay using a micro-modification of the radioimmunoassay originally devised by Follett and colleagues (1975). Intra-assay coefficient of variation was 3.4% and the lower detection limit was 0.07 ng/ml.

Analysis of Behavior

Video recordings were taken before and after presentation of the stimulus animal with security cameras (SWADS-120CAM-US, Swann Communications, Santa Fe Springs, CA, U.S.A.) and recorded with a 4-channel DVR (Q-See, Anaheim, CA, U.S.A.). Animals were recorded for 30 minutes prior to stimulus presentation and 30 minutes during stimulus presentation. Individuals receiving no stimulus animal had their cages opened and handled in a similar fashion to those receiving a stimulus animal. Behavior was recorded for each individual animal by counting copulation attempts and number of songs and by timing the duration of total time resting. Activity levels were calculated by subtracting the time resting from total time of recording. Number of songs was determined by counting song bouts with five or more seconds between each song. Due to the low number of songs, directed (towards the stimulus animal) and undirected (with no obvious intended recipient) songs were combined and taken as a measure of total song activity. The first five minutes of each video were not included in the analysis to allow animals time to adjust to the experimenter opening the cage. Analysis of video was conducted by researchers blind to the treatment of the animals except when a stimulus animal was present.

Statistical Analysis

One-way analysis of variance (ANOVA) tests were performed for immunoreactive cell counts, gene expression as measured by qPCR, and hormone concentrations from RIA and ELISA tests. One-way ANOVA tests were also performed for behavioral measures of total time active, copulation attempts, and number of songs. Significance was set at $p<.05$. In cases of statistical significance, Tukey's test of multiple comparisons was used to assess which groups were significantly different. All statistical analyses were performed using Prism 6 (GraphPad, La Jolla, CA, U.S.A.).

Experiment 1 had 8 animals per group, though there are some data sets where all animals could not be used for analysis. For circulating plasma hormones, radioimmunoassay for luteinizing hormone was performed first, followed by ELISAs for testosterone and corticosterone. Thus, there was not enough plasma collected from some birds to run all three assays. For analysis of real-time quantitative PCR (qPCR), samples with expression levels 3 or more standard deviations above the mean were considered outliers and were excluded from analysis. This occurred for two samples for expression of *DIO3*, one from the male stimulus group and one from the female stimulus group giving a sample size of 8 for the isolate group and 7 each for the male stimulus and female stimulus groups.

Results

Real-time quantitative PCR

Hypothalamic expression of type-2 deiodinase (*DIO2*) and type-3 deiodinase (*DIO3*) did not differ across groups (Fig 1 A, B) (ANOVA, DIO2: F (2,21)=0.264 p=0.599, DIO3: F (2, 19)=0.624, p=0.547). Expression of GnRH and GnIH was also similar across groups (Fig 1 C, D) (ANOVA, GnRH: F (2,21)=1.907, p=0.173, GnIH: F (2,21)=2.321, p=0.123). Expression of the housekeeping gene *18S* did not differ across groups (F (2,21)=0.117, p=0.256). *EGR-1* expression was greater in the group receiving females as compared to the isolated group, while the group exposed to males had an intermediate level of *EGR-1* expression (ANOVA, F (2,21)=3.507, p<0.049). Tukey's multiple comparisons test produced a mean difference of -1.035 between the isolated group and the group receiving females, indicating a significant difference between these two groups (Fig 2 A).

Immunocytochemistry

Analysis of immunocytochemistry revealed no significant differences in GnRH immunoreactive cell number (GnRH-ir) between groups (F (2,21) = 0.606, p=0.555) (Fig 4). In sets of tissue double-labeled for EGR-1 and GnRH, EGR-1 was not found to be co-localized with GnRH-ir cells. Analysis of single-label EGR-1 ICC showed that number of EGR-1 labeled cells was significantly greater in the hypothalamus in the female stimulus group as compared to the isolate group and the male stimulus group (Figure 2B, F (2,21) = 6.686, P<0.04, ANOVA, Tukey's multiple comparisons test where p<0.05 as indicated by letters a, b).

Circulating Plasma Hormones and Testicular Volume

ELISA revealed circulating testosterone was significantly higher in males receiving a male stimulus and those receiving a female stimulus as compared to isolated males (Fig 5A, Multiple comparison t-tests with Sidak-Bonferroni correction, p<0.012 for isolated vs. male stimulus, p<0.021 for isolated vs. female stimulus). The male stimulus group did not differ in circulating testosterone level from the female stimulus group (Multiple comparison t-tests with Sidak-Bonferroni correction, p=0.848)

Luteinizing hormone (LH) did not differ across groups (Fig 5B, F (2,21)=1.958, p=0.166), nor did circulating corticosterone (Fig 5C, F (2, 14)=0.555, p=0.586). The RIA was run first followed by the ELISAs for testosterone and corticosterone, thus plasma was not available from all birds for measurement of testosterone (total of 8 samples in the isolated and female stimulus groups and 7 in the male stimulus group) and corticosterone (total of 6 samples for isolated and female stimulus groups and 5 for male stimulus group). Testis volume was not significantly different across groups (F (2,21)= 0.985, p=0.211).

Behavior

Prior to presentation of a stimulus, animals in all three groups had similar levels of activity (defined as time resting subtracted from the total time) (F(2,21)=0.212, p=0.81). The isolate and male groups maintained levels of activity similar to that of their baseline recording, while males presented with a female were more active (F(2,21)=14.42, p<0.0002) (Fig. 6A) Males in the presence of a female also showed significantly more copulation attempts (F (2,21)=14.11, p<0.0001) (Fig. 6B) and greater number of songs than isolated males and those in the presence of a male (F (2,21)=4.74, p<0.02, ANOVA) (Fig. 6C).

Discussion

In this experiment we sought to test whether social cues activate the DIO system in male zebra finches. If the mechanism of DIO-induced GnRH release is evolutionarily conserved across vertebrate taxa, and if there is no separate or additional mechanism for regulating GnRH release, we would expect *DIO2*/*DIO3* expression in non-photoperiodic species to respond to stimulatory cues other than photoperiod. We predicted that the HPG axis would be activated in male zebra finches that were presented with a female after isolation. Thus, we also predicted that there would be reciprocal switching of *DIO2* and *DIO3* expression in the hypothalamus, precipitating other indicators of reproductive activation (increased GnRH expression, increased circulating LH or T, increased reproductive behaviors). In our non-photoperiodic model and within this experimental paradigm, no changes in the DIO system were observed with a social stimulus.

Males receiving a female stimulus showed increased levels of activity, song behavior, and copulation attempts compared to isolates or those receiving a male stimulus, suggesting males were responding to females with appropriate reproductive effort. Males with a female stimulus also showed a physiological response to these females in the form of an increase in *EGR-1* expression and EGR-1 protein immunoreactivity in the hypothalamus. It is unknown, however, what cell type is being activated in response to the social stimulus. In double-label immunocytochemistry EGR-1 was not found to be co-localized with GnRH. While this indicates that GnRH-ir cells might not be directly activated with presentation of a female, EGR-1-ir cell count in the hypothalamus did increase, including in the pre-optic area where GnRH cells are located. A similar pattern in immediate early gene immunoreactive (IEG-ir) cells surrounding but not co-localized with GnRH cells was observed in white-crowned sparrows following treatment with NMDA (Meddle et al. 1999). In that study increased C-FOS immunoreactivity was associated with an increase in LH release, suggestive of GnRH release. While the activation of hypothalamic cells shown with an increase in EGR-1 indicates a hypothalamic response to female stimulus, theoretically this increase in expression could be associated with the change in behavior seen with the presence of a female as opposed to causing an increase in activity in the reproductive axis.

Males exhibited a behavioral response to the presence of a female, but other reproductive parameters usually associated with reproductive activation, including increased GnRH expression and increased circulating LH, did not change. Likewise, no changes in expression of *DIO2* or *DIO3* were found. While thirty minutes is considered sufficient time for changes in expression to be seen, it is possible that dynamic changes of *DIO2, DIO3,* and *GnRH* expression were missed due to the analysis of a single time point. Experiments inducing *DIO2* expression in photoperiodic breeders with a photic stimulus show great variation in timing of *DIO2* expression after stimulus presentation. Japanese quail show a change in expression a few hours into the first long day (Yoshimura et al. 2003), while Syrian hamsters showed increases in *DIO2* expression 8 days after transfer from short to long day lengths (Yasuo et al. 2009). *DIO2* expression can be stimulated by social cues in photoperiodic breeders several days after stimulus presentation (Perfito et al. 2015), and thus an experiment with many time points after social stimulation would be required to provide a definitive conclusion as to whether the *DIO* system responds to the presence of a potential mate in zebra finches. Alternately, a time-course study of zebra finches could show an increase in *GnRH* expression and release independently from changes in *DIO2* expression, as was found in European starlings (Bentley et al. 2013). Testosterone was significantly higher in males receiving a female stimulus than isolated males.

Testosterone was also higher in males receiving a male stimulus, indicating an increase in testosterone with all social stimuli, not with female stimulus only.

In summary, this study provides evidence that male zebra finches respond to potential mates with increased reproductive effort and increased activity of the hypothalamus. The lack of changes seen in *DIO2, DIO3, GnIH* and *GnRH* expression across groups indicate that either these components of the reproductive axis are not influenced by social cues in this species, or that our experimental time-course was not sufficient to reveal the influence of social cues reproductive activation. If the DIO system is truly an evolutionarily conserved mechanism in the initiation of vertebrate reproduction, then further experiments over different time-frames will elucidate changes in the DIO response to social cues.

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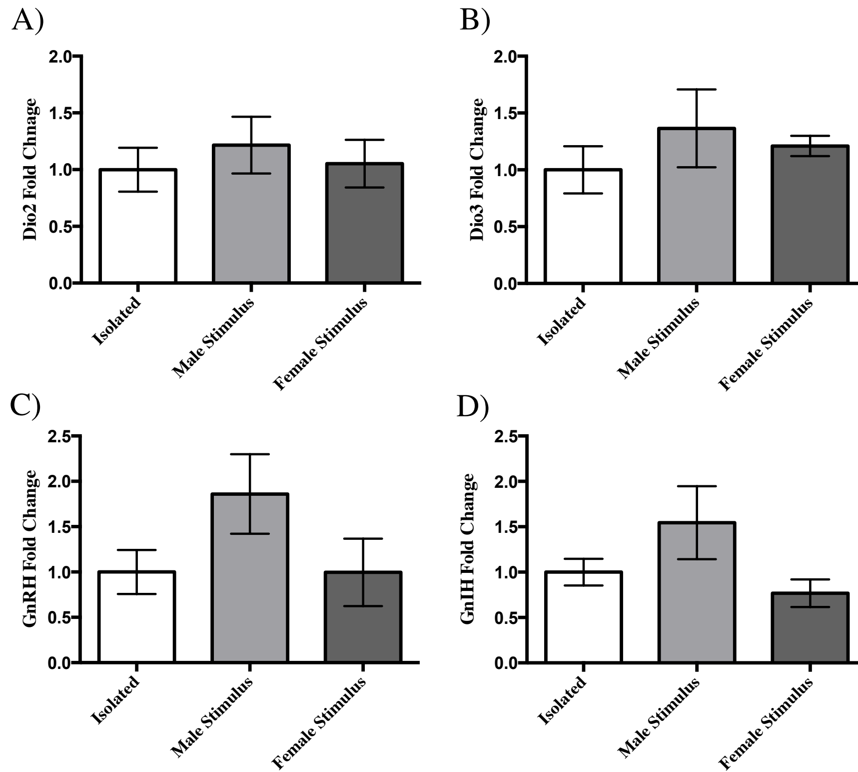

Figure 1. Fold change in mRNA expression of *DIO2* (A), *DIO3* (B), *GnRH* (C), and *GnIH* (D). The isolated group is shown in white, group given a male stimulus in light gray, and group given a female stimulus in dark gray. Fold change across groups after 30 minutes of treatment was not significantly different for any of these genes (ANOVA, *DIO2*: F (2,21)=0.264 p=0.599, *DIO3*: F (2, 19)=0.624, p=0.547, *GnRH*: F (2,21)=1.907, p=0.173, *GnIH*: F (2,21)=2.321, p=0.123).

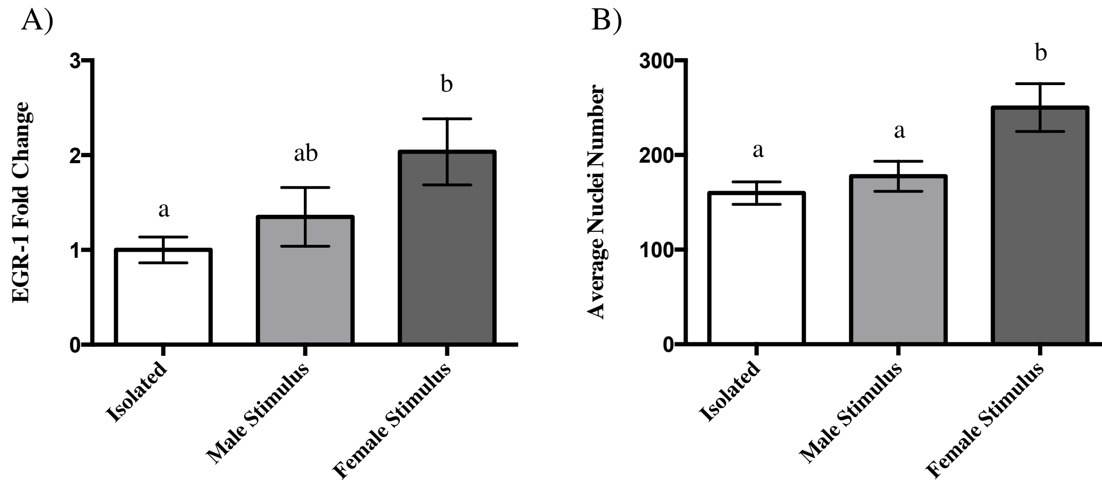

Figure 2. EGR-1 mRNA expression (A) and protein (B). A. Fold change in immediate-early gene *EGR-1* mRNA expression (mean +/- SE) in the hypothalamus was significantly higher (ANOVA, F (2,21)=3.507, p<0.049) in birds given a female stimulus as compared to isolated animals, shown here where *a* is significantly different from *b* Birds given a male stimulus show an intermediate amount of *EGR-1* expression, not significantly different from the level of expression in the isolates or those receiving a female, indicated by *ab*. B. There were significantly more EGR-1-ir cells in the hypothalamus of males receiving a female as compared to isolates and those receiving a male stimulus (ANOVA, F (2,21) = 6.686, P<0.04).

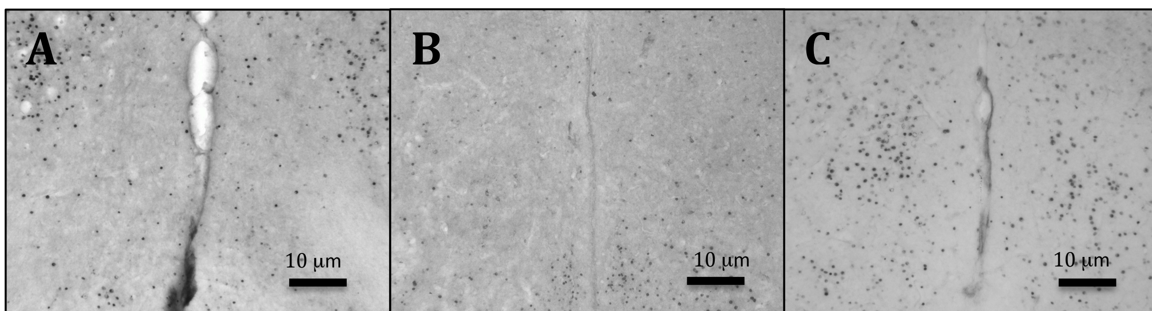

Figure 3. Single-label EGR-1 immunocytochemistry. Representative photographs of EGR-1 positive cells in the pre-optic area (POA) of the hypothalamus of isolated males (A), males receiving a male stimulus (B), and males receiving a female stimulus (C).

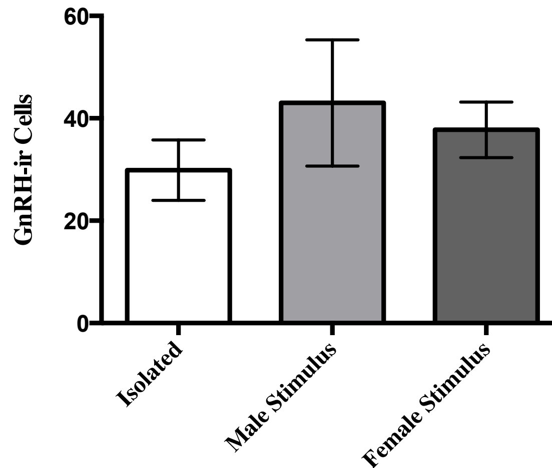

Figure 4. Single-label GnRH Immunocytochemistry. No significance difference in GnRH-ir cell count was found between groups (F (2,21) = 0.606, p=0.555).

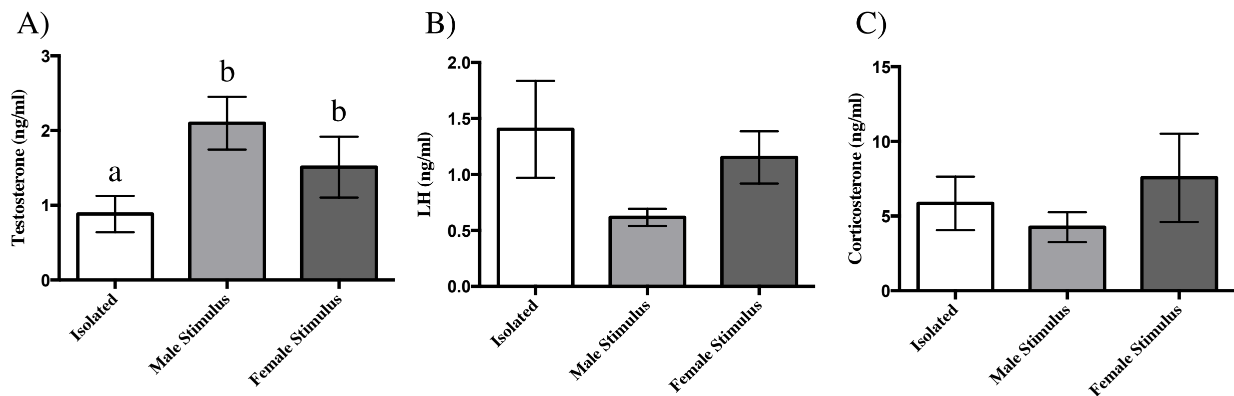

Figure 5. Circulating concentrations of testosterone (A), luteinizing hormone (B), and corticosterone (C). Testosterone (mean +/- SE) was significantly higher in males receiving a male stimulus and those receiving a female stimulus as compared to isolated males (Multiple comparison t-tests with Sidak-Bonferroni correction, p<0.012 for isolated vs. male stimulus, p<0.021 for isolated vs. female stimulus). The male stimulus group did not differ in circulating testosterone level from the female stimulus group (Multiple comparison t-tests with Sidak-Bonferroni correction, p=0.848). Luteinizing hormone (mean +/- SE) did not differ across groups (F (2,21)=1.958, p=0.166), nor did circulating corticosterone (mean +/- SE, F (2, 14)=0.555, p=0.586).

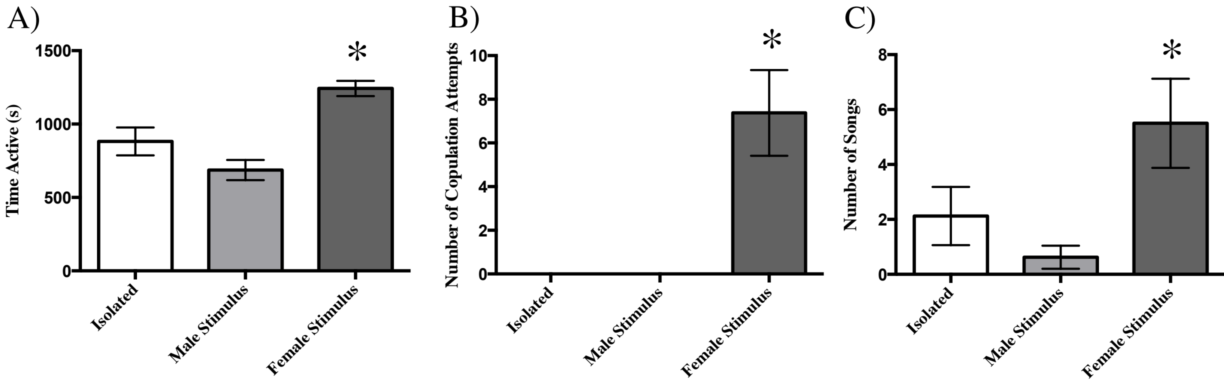

Figure 6. Behavioral Output. In the presence of a female, males were more active and exhibited increased sexual behavior. Males given a female stimulus for 30 minutes showed increased activity (A) ($F(2,21)=14.42$, $p<0.0002$), number of copulations (B) ($F(2,21)=14.11$, $p<0.0001$), and number of songs (C) ($F(2,21)=4.74$, $p<0.02$, ANOVA) as compared to isolated males and those given a male stimulus.

Gene	Forward Primer	Reverse Primer	bp	Genbank Accession #
Dio2	CAGGTCAAACTGGGAGGAGA	CACACTTGCCACCAACATTC	103	NM_001270969
Dio3	TACAACATCCCCAAGCACCA	TCTGCCTCCCTGGTACATCA	186	XM_004174551
GnRH	ACTCCACAACCTCTCTCAGG	CTCTGCTGCTCCTCCTCTAA	209	NM_001142320, XM_002197400
GnIH	CCCTGAGATTTGGAAGAGC	CAGATTGACAGGCAGTGAC	152	AB522971.1
EGR-1	AACGAGAAAACCCTGCCAGA	TCCACTGACGAGGCTGAAGA	147	NM_001080957.1
18S	CCATCCAATCGGTAGTAGCG	GTAACCCGTTGAACCCCATT	151	HQ873432.1

Table 1. Primer sequences used for real-time quantitative PCR in hypothalamic tissue and anticipated size of amplified products.

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Chapter 3

Differential response of GnIH in the brain and gonads following acute stress in a songbird

Abstract

Gonadotropin–inhibitory hormone (GnIH) acts to inhibit reproduction at all levels of the hypothalamo–pituitary–gonad axis. GnIH expression and/or immunoreactivity in the hypothalamus increase with acute stress in some birds and mammals, and thus may be involved in stress-induced reproductive inhibition. Much is known about GnIH and stress in seasonal and continuous breeders, but far less is known about these interactions in opportunistic breeders. For opportunistically breeding animals, reproductive readiness is closely associated with unpredictable environmental cues, and thus the GnIH system may be more sensitive to stress. To test this, we collected tissues from zebra finches immediately following capture or after 60 minutes of restraint. Restraint significantly increased plasma corticosterone in males and females but, contrary to studies on other species, restrained birds had significantly fewer GnIH immunoreactive (GnIH–ir) cell bodies than control birds. GnIH-ir cell number did not differ between the sexes. Stressed females had lower mRNA expression of the beta subunit of follicle stimulating hormone (FSHβ) in the pituitary, suggesting that the reduction in observed GnIH immunoreactivity in females may have been due to increased GnIH release in response to acute stress. GnIH expression increased in the testes, but not the ovaries, of restrained animals. Our data suggest that although GnIH responsiveness to stress appears to be conserved across species, specific tissue response and direction of GnIH regulation is not. Variation in the GnIH response to stress between species might be the result of ecological adaptations or other species differences in the response of the GnIH system to stress.

Introduction

Stress is a known inhibitor of reproductive physiology and behavior across vertebrate taxa (Moore & Jessop 2003, Siegel 1980, Wingfield et al. 1998). Although some components of the pathways involved in stress-induced reproductive impairment are well known (e.g., adrenal activation, opioid production) the exact mechanisms underlying this phenomenon have yet to be fully elucidated. Gonadotropin-inhibitory hormone (GnIH; Tsutsui et al. 2000) has emerged as a potentially important component of pathways involved in stress-induced reproductive dysfunction (Breen & Mellon 2014, Geraghty et al. 2015, Parhar et al. 2012). Hypothalamic GnIH can act to inhibit firing of GnRH neurons (Ducret et al. 2009); it also acts directly on the anterior pituitary to decrease synthesis and release of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Ciccone et al. 2004, Sari et al. 2009, Tsutsui et al. 2000). Hypothalamic GnIH neurons express glucocorticoid receptor (GR), and restraint stress stimulates GnIH activity in birds and mammals (Calisi et al. 2008, Calisi et al. 2010, Kirby et al. 2009, Son et al. 2014). Adrenalectomy of male rats blocks stress-induced increases in GnIH expression, and functional knock-down of GnIH in female rats prevents stress-induced decreases in fertility (Geraghty et al. 2015). Further, GnIH is synthesized by and acts in the gonads to inhibit their activity (Bentley et al. 2008; McGuire & Bentley 2010, Zhao et al. 2010). Testicular expression of GnIH increases in culture in response to corticosterone, while ovarian expression of GnIH does not change with treatment (McGuire et al. 2013). Thus, stress-induced elevation of glucocorticoids can act directly on GnIH-producing cells in the hypothalamus and gonads, thereby inhibiting reproductive function via inhibition of GnRH, the gonadotropins, and/or steroidogenesis.

A strong body of evidence indicates an effect of stress on the mammalian GnIH system, resulting in decreased reproductive activity. For example, recent use of a GR agonist *in vitro*

caused an increase in transcription of both GnIH and its receptor (GnIH-R) (Gojska & Belsham, 2014), and treatment of a GnIH-expressing neuronal cell line with corticosterone increased expression of GnIH mRNA (Son et al. 2014). Treatment of neonatal mice with synthetic glucocorticoid dexamethasone (DEX) yields delayed pubertal onset associated with increased GnIH-R expression in the pre-optic area (POA) of the hypothalamus and increased GnIH immunoreactive cell number in the dorsomedial hypothalamus (DMH) (Soga et al. 2012). Overall, the GnIH system is activated in response to stress, resulting in decreased reproductive activity, in a number of species.

Some stressors known to activate the hypothalamo-pituitary-adrenal (HPA) axis have less clear effects on the GnIH system. Transfer of European starlings, *Sturnus vulgaris,* from outdoor to indoor aviaries, a transfer that suppresses reproduction in this species, did not affect GnIH expression or immunoreactivity in the hypothalamus of either sex but did increase gonadal GnIH expression in females (Dickens & Bentley 2014). Likewise, elevated corticosterone associated with fasting in zebra finches was unrelated to hypothalamic GnIH expression or immunoreactivity, but was significantly associated with an increase in testicular GnIH expression (Lynn et al. 2015). Injection of lipopolysaccharide as an immune-challenge in zebra finches increased circulating corticosterone concentration and decreased GnRH-I mRNA expression and immunoreactivity, but did not change GnIH expression or immunoreactivity (Lopes et al. 2012). Restraint stress in ewes caused increased corticosterone, decreased LH, no change in GnIH expression or peptide content, and no change in GnIH cell activation as measured by the immediate early gene Fos (Papargiris et al. 2011). Thus, the response of the GnIH system to stress or increased corticosterone does not appear to be universal.

Given the wide variation in interactions of stress and the GnIH system, we sought to characterize the response of GnIH in the hypothalamus and gonads to acute restraint stress in a flexible or opportunistic breeder. We hypothesized that the relationship between GnIH and stress may differ from that seen in seasonal species, and thus used a common laboratory model of opportunistic breeding, the zebra finch (*Taeniopygia guttata*). Specifically, we sought to test whether these opportunistic breeders respond to stress via increased hypothalamic expression and immunoreactivity of GnIH as has been suggested by previous work in house sparrows (*Passer domesticus*, Calisi et al. 2008). Zebra finches do not show changes in hypothalamic GnIH immunoreactivity with changes in breeding status (Perfito et al. 2011). However, GnIH is sensitive to food restriction in birds and mammals (Fraley et al. 2013, Lynn et al. 2015), an environmental cue known to decrease reproduction in zebra finches. GnIH could act to transiently inhibit reproduction in times of stress or under poor breeding conditions (Perfito et al. 2011), supported by recent research in which food restriction was shown to affect the GnIH system (Lynn et al. 2015).

To assess the responsiveness of the zebra finch GnIH system to stress we used acute restraint stress, known to elevate corticosterone in birds. In addition to measuring GnIH in the hypothalamus and gonads we also examined GnRH expression, FSHβ expression, and circulating testosterone to characterize the effects of restraint stress on reproduction in this species. We predicted that, if zebra finches respond to stress in a manner that is similar to seasonally breeding birds, finches exposed to acute restraint would show increase in GnIH immunoreactivity and expression, a reduction in GnRH and FSHβ expression, and a reduction in circulating testosterone.

Materials and Methods

Animals

Adult male and female zebra finches were collected from mixed-sex free-flight aviaries at the UC Berkeley Field Station for the Study of Behavior, Ecology, and Reproduction (FSSBER). The birds were allowed to pair and breed freely in this colony setting and were fed German millet mixed with canary seed *ad libitum* supplemented with lettuce and egg-based food.

Males and females were collected as either control animals (within 4 minutes of entering the aviary) or stressed animals (held for 60 minutes in a ventilated cloth bag). This common capture-handling stress paradigm is used in birds as a way to elevate glucocorticoid levels reliably (Wingfield et al. 1992, Wingfield 1994). The experiment took place at 11 am on May 3rd, 2013, a day with approximately 14 hours of light. Sample sizes were as follows: ten control males, ten control females, seven stressed males and eight stressed females. To obtain tissue, animals were deeply anesthetized with isoflurane (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) and rapidly decapitated, at which time trunk blood was collected. Control animals were collected within four minutes of investigators entering each aviary, with the average time from entering the aviary to collection being one minute and fifty-four seconds. Birds were collected from two aviaries by multiple researchers to obtain the control samples.

Brain tissue was fresh frozen on dry ice and stored at -80 ºC prior to sectioning. The remaining head with pituitary intact was submerged in nucleic acid preservation buffer (Camacho-Sanchez et al. 2013) and frozen for seven days at -20 ºC. Once thawed, the pituitary gland was extracted from the skull under a dissecting microscope and placed in RLY buffer (Isolate II RNA mini kit, Bio-52073, Bioline USA Inc, Taunton, MA).

Trunk blood was spun in a centrifuge at 4 ºC at 1500 g for 10 minutes to separate blood plasma, which was then stored at -80 ºC prior to assay. Gonadal tissue was fresh frozen on dry ice and stored at -80 ºC prior to RNA extraction. Prior to freezing, testes were measured with calipers and volume was calculated as $V=4/3\pi a^2 b$, where a is half the width and b is half the length (long axis) of the testis. Ovarian stage was assessed prior to freezing (Bahr & Johnson 1984, Perfito et al. 2015).

Hormone assays

Concentrations of corticosterone in both sexes and testosterone in males were measured by enzyme immunoassay kits (EIA, Enzo Life Sciences, Farmingdale, NY, USA). These kits have been validated and optimized for zebra finches (Lynn et al. 2007, 2010). Protocols for both hormones were followed as specified by Lynn et al. 2010. Plasma was diluted 1:20 for testosterone and 1:40 for corticosterone EIA. All samples were run in triplicate with a standard curve run in duplicate on each plate. Testosterone samples were run on a single plate and corticosterone samples were run on a total of two plates. Average intraassay variation was 5.87% for testosterone and 9.24% for corticosterone. Average interassay variation was 7.06% for corticosterone. Sensitivity was 0.091 ng/ml for the testosterone assay and 0.072 ng/ml for the corticosterone assay.

Brain Processing

Brains were sectioned at 40 μm on a cryostat, mounted directly onto gelatin-coated slides, and frozen at -20 ºC. Alternate sections were punched with a 3mm circular punch (Harris Uni-core, catalog number 69036; Electron Microscopy

Sciences, Ft Washington, PA) centered at the bottom of the brain beginning approximately at plate A3.5 (Stokes et al. 1974), when the tractus septomesencephalicus (TrSM) emerges, until a total of 36 punches were obtained for each animal. Tissue punches were homogenized in RLY buffer (Isolate II RNA mini kit (Bio-52073, Bioline USA Inc, Taunton, MA) and stored at -80 ºC until all samples were ready for RNA extraction. Whole brain sections mounted directly onto slides were used for immunocytochemistry while punched hypothalamic tissue was processed and used for quantitative PCR. This technique provides an equal total volume of tissue per animal for assessment of gene expression.

Immunocytochemistry

For assessment of GnIH with immunocytochemistry (ICC) one series of slides (every fourth section) was used per animal. To label for GnIH peptide, tissue on slides was first fixed in 4% paraformaldehyde (PFA) for 20 minutes and washed in 0.1M phosphate-buffered saline (PBS- pH 7.4) three times. Slides were then incubated in 0.03% hydrogen peroxide in methanol for 30 minutes followed by three PBS washes and one hour of incubation in 2% normal goat serum in 0.2% phosphate-buffered saline with Triton x-100 (PBS-T) (S-1000, Vector Laboratories, Burlingame, CA, USA). Slides were incubated in GnIH primary antibody (PAC 123,124, antigen sequence: SIKPFSNLPLRF-NH, Bentley, Berkeley, CA, USA) 1:5000 in PBS-T for 24 hours at 4 ºC. After incubation in the primary antibody, slides were washed three times for five minutes with PBS, incubated in biotinylated goat anti-rabbit antiserum (BA-1000, Vector Laboratories, Burlingame, CA, USA) made 1:250 in 0.2% PBS-T for one hour, washed in PBS, and incubated in avidin/biotinylated enzyme complex (PK-4005, Vectastain ABC, Vector Laboratories, Burlingame, CA, USA) for amplification as per manufacturer's instructions. Slides were washed three times for five minutes each in PBS and incubated with DAB peroxidase substrate kit (SK-4100, Vector Laboratories, Burlingame, CA, USA). All slides were then dehydrated with graded ethanol, cleared in xylenes and coverslipped with Permount (SP 15-100, Fisher Scientific Inc., Waltham, MA, USA).

Cell counts for all immunocytochemically-labeled cells were obtained by a researcher who was unaware of the sex and experimental treatment of animals. Cells were counted using a Zeiss Axio Imager A1 microscope and AxioVision 4.5 software.

Real-time quantitative PCR

To measure levels of gene expression, real-time quantitative PCR (RT-qPCR) was conducted on hypothalamic, pituitary, and gonadal tissue. This technique was used to measure expression of GnIH and GnRH in the hypothalamus, FSHβ in the pituitary, and GnIH in the gonads. Tissues were extracted and reverse-transcribed using manufacturer's instructions for the Isolate II RNA mini kit (Bio-52073, Bioline USA Inc, Taunton, MA). Four hypothalamic samples (two control males, one control female, and one restrained female), three pituitary samples (one control male, two control females), and one ovarian sample (control female) were not analyzed for expression due to having exceptionally low yield (below 10 ng/μl as measured by a NanoDrop 2000 spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE)).

To assess gonadal GnIH expression one testis from each male was homogenized and RNA was extracted and reverse transcribed as described above. For females, each follicle type (small white follicles, small yellow follicles, dominant follicle, post-ovulatory ovary) was homogenized and run separately.

For all tissues, cDNA was diluted 1:25 with water treated with diethylpyrocarbonate (DEPC). Primers were designed for each gene from previously published or predicted sequences in the zebra finch genome (see Table 1 for accession numbers) using Primer3 software (simgene.com), and purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Primer sets were optimized for purity and specificity and melt curves were analyzed prior to use. Primers were not used if secondary structure or dimerization was found in no-template controls. The reference genes 18S and GAPDH were used in hypothalamic, pituitary, and testis tissue as their expression did not change with treatment. We calculated a geometric mean of these reference genes for each sample. The 18S-GAPDH geometric mean did not change with treatment for any tissue. The reference gene β-actin was used for ovarian tissue as it did not change with treatment. Quantitative PCR was performed using manufacturer's instructions for SYBR green reagent (Applied Biosystems by Life Technologies, Grand Island, NY, USA), with 5 μm of primer and 3.75 μl of cDNA for a final reaction volume of 15 μl. Raw fluorescent data were analyzed with the RT-PCR Miner program (Zhao and Fernald, 2005) and cycle thresholds were obtained from this program. Expression values were calculated as $1/(1+E)^{Ct}$, where E is the average PCR efficiency for the gene of interest as calculated by a standard curve and Ct is the cycle threshold. Data are shown as fold-change, which was calculated by dividing the expression of the gene of interest (corrected for the geometric mean of the control genes) by the average expression for the male control group

Statistical Analysis

All statistical analyses were performed using Prism (version 6.0d, 2013, Graphpad). Measurements of corticosterone, immunocytochemistry, and qPCR of hypothalamus and pituitary tissue were compared between control and restraint treatments and between sexes using Two-Way ANOVA with Tukey's post-hoc comparison. Sex and treatment were considered independent variables, and female and male data were not pooled so sex differences could be assessed. Unpaired t-tests were performed for gonadal gene expression within each sex, for testicular volume, and to assess differences in circulating testosterone between restrained and control males.

Results

Circulating Hormones and Testicular Volume

As expected, one hour of restraint stress raised corticosterone levels significantly in both males and females (Two-way ANOVA, $F(1,31)=42.17$, $p<0.0001$). Corticosterone levels did not differ significantly between the sexes in the control group or the stressed group (Fig 1). Circulating testosterone in males did not differ between the control group and the restrained group (unpaired t-test, $t=0.114$, $df=11$, $p=0.911$) (Fig 2). Testis volume did not differ between control and restrained males (Unpaired t-test, $t=0.423$, $df=15$, $p=0.679$).

GnIH Immunocytochemistry

The number of GnIH immunoreactive (GnIH-ir) cells was significantly lower in stressed males and females than in controls (Fig 3). Two-way ANOVA showed a significant effect of treatment ($F(1,18)$, $p=0.0002$)) but not sex ($F(1,18)$, $p=0.545$).

Gene Expression

Hypothalamic Gene Expression

There were no overall effects and no interaction of sex and treatment on GnIH expression in the hypothalamus ($F_{(1,25)}=0.199$, $p=0.66$) (Fig 4a). Likewise, GnRH expression did not differ by sex or treatment ($F_{(1, 26)}=0.002$, $p=0.961$) (Fig 4b).

Pituitary Gene Expression

Quantitative PCR (qPCR) for expression of the beta subunit of FSH in the pituitary revealed a significant effect of stress on expression, though this effect was only observed in females. There was a significant interaction of sex and treatment on FSHβ expression ($F(1,26)=4.678$, $p=0.04$). Post-hoc analysis revealed control females had significantly higher FSHβ expression in the pituitary than stressed females and both control and stressed males ($F(1,26)=5.817$, $p=0.023$).

Gonadal Gene Expression

Stress significantly increased GnIH expression in the testes of males (Unpaired t-test, (Unpaired t-test, $t=2.271$, $df=14$, $p<0.04$) (Fig 6). Though RNA was extracted for each follicle individually, not enough females were sampled in each follicular stage to assess GnIH expression for follicle types individually. To better understand total GnIH expression in the ovary for each female all expression values for all follicle types available were averaged. Females were further divided into those with an established follicular hierarchy at the time of experiment and those with small white follicles only, and t-tests were performed to compare GnIH expression between stressed and control females in each ovarian condition. Ovarian GnIH expression was not significantly different between control females and those subjected to 60 minutes of restraint stress in females with an established follicular hierarchy (Unpaired t-test, $t=1.099$, $df=4$, $p=0.334$) or those with small white follicles only ($t=1.602$, $df=9$, $p=0.144$) (Table 2).

Discussion

In this study we sought to characterize the response of the zebra finch GnIH system to stress in both males and females. We examined GnIH in the hypothalamus and gonads as both populations of GnIH-producing cells have been shown to be active in response to stress (Calisi et al. 2008, Kirby et al. 2009, McGuire et al. 2013). Glucocorticoid receptor is present in GnIH-producing cells in the hypothalamus (Calisi et al. 2010, Kirby et al. 2009, Son et al. 2014) and in gonadal tissue (Lattin et al. 2012, McGuire et al. 2013), while it is unknown if GnIH-producing cells in the gonads express GR. Previous studies have shown that hypothalamic GnIH immunoreactivity increased (Calisi et al. 2008, Kirby et al. 2009) or did not change (Papargiris et al. 2011, Dickens & Bentley 2014, Lynn et al. 2015) in response to stress. For the first time we show the number of GnIH immunoreactive cells in the hypothalamus decreasing with stress.

In females, the decrease in GnIH immunoreactivity is associated with a decrease in FSHβ expression in the pituitary. This association implies that GnIH is being released to the hypophyseal portal system in response to stress and inhibiting FSHβ expression. GnIH is a potent inhibitor of gonadtropes both *in vivo* and *in vitro* (reviewed by Clarke & Parkington, 2014, Tsutsui et al. 2010), raising the possibility that GnIH had a direct effect on FSHβ–producing cells in our study. Alternatively, GnIH could have inhibited GnRH synthesis or secretion, thus decreasing FSHβ expression through an indirect mechanism; no difference in GnRH expression was observed, but GnIH could be decreasing GnRH release, thereby decreasing FSHβ expression. An effect on FSHβ was not seen in males, possibly because

mechanisms of GnIH activity differ between males and female or because control males had significantly lower FSHβ expression than females. Understanding how acute restraint affected LHβ expression or circulating LH would provide more evidence for this hypothesis, but unfortunately we were unable to investigate these aspects of the reproductive axis as the tools have not yet been developed.

In contrast to other studies investigating GnIH responses to restraint stress, we saw no changes in GnIH expression in the hypothalamus. A possible explanation is that restraint stress does not affect GnIH expression in this species, or, alternatively, time course of the experiment (60 minutes) was such that GnIH expression changes were missed. Though an effect of stress on GnIH expression has been described in mammals (Kirby et al. 2009, Geraghty et al. 2015), no study to date has shown significant differences in GnIH expression with stress in the hypothalamus of birds (Dickens & Bentley 2014, Lynn et al. 2015), although changes in GnIH peptide have been observed (Calisi et al. 2008). A thorough time-course of restraint and tissue collection would be necessary to state definitively that GnIH expression does not change with restraint stress in these animals.

Consistent with other findings (McGuire et al. 2013), we found that GnIH expression in the gonads increased in response to stress in males but not females. We expected that this increase in GnIH would be associated with a decrease in circulating testosterone, though in our study testosterone did not differ between control and restrained males. Other studies have shown circulating testosterone decreasing (Deviche et al. 2010, 2012, Lynn et al. 2010) or increasing (Van Hout et al. 2010) with acute stress in songbirds, thus we suggest further research into the dynamics of gonadal GnIH expression and testosterone synthesis and secretion is necessary. There appears to be variation in the response of circulating testosterone to acute stress, and thus these results could be due to the specific timing of testosterone sampling. GnIH upregulation in the testes could be affecting expression of steroidogenic enzymes, with effects on circulating testosterone level seen before or after our sample was taken.

Our results supports *in vitro* findings that corticosterone administration to European starling gonads affected GnIH expression in testes but not ovaries, (McGuire et al. 2013), we believe more detailed study of the effects of acute stress on GnIH expression in relation to ovarian stage is needed. Females in our aviaries were randomly assigned to either the control or the stressed group, and thus we completed the experiment with females in various stages of ovarian development. Numbers of females at each ovarian stage were too limited to analyze expression in each type of follicle separately, and thus ovarian data was divided only into those with an established follicular hierarchy and those with only small white follicles. Ovarian GnIH expression has been shown to increase when females are moved from outdoor to indoor housing (Dickens & Bentley 2014), providing evidence that ovarian GnIH is responsive to stressful conditions, so more careful study of GnIH expression in different ovarian stages in zebra finches could reveal a similar effect of stress on ovarian GnIH.

We have shown decreased hypothalamic GnIH expression and increased GnIH expression in the testis of these opportunistic breeders, which raises questions about the interactions of GnIH and acute stressors in the context of flexible breeding strategies. As opportunistic breeders are able to capitalize on transient availability of resources, zebra finches must maintain reproductive and behavioral flexibility (Lynn et al. 2010). The decrease in GnIH immunoreactivity in the hypothalamus (proposed here to result from increased GnIH release) and increase in testicular GnIH expression in our restrained groups suggest that zebra finches could be modulating both hypothalamic and gonadal GnIH in response to stressful conditions to

temporarily inhibit breeding. How long these effects are maintained upon release from the stressor and what this could mean for timing of breeding in the wild remain to be tested. Knowledge of the dynamics of GnIH receptor would provide more information about the function of the GnIH system in response to acute stress. GnIH receptor expression in the hypothalamus increases with stress in rats (Geraghty et al. 2015), though whether this effect is present in zebra finches remains to be tested. Additional work on GnIH receptor expression in the gonads is warranted, as presence of this receptor in the gonads is known (Bentley et al. 2008, McGuire & Bentley, 2010), but expression has not been examined under stressful conditions.

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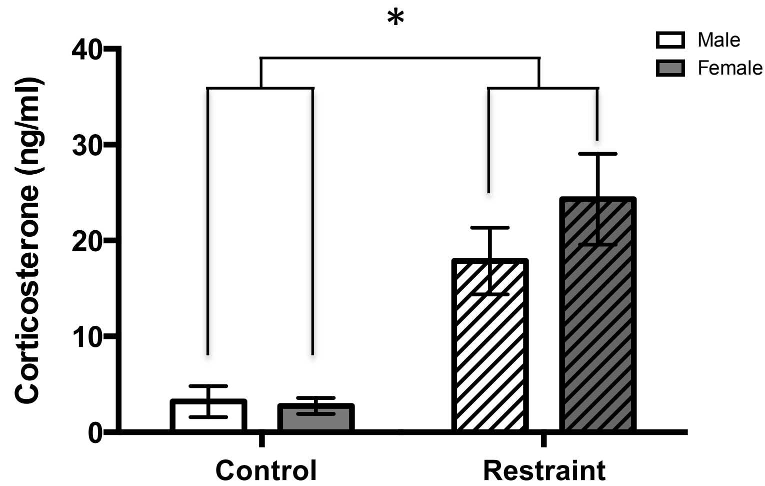

Figure 1. Circulating corticosterone in control and restraint groups. Corticosterone (mean +/- SE), was significantly higher in zebra finches subjected to 60 minutes of restraint than in control birds (F(1,31)=42.17, p<0.0001), regardless of sex.

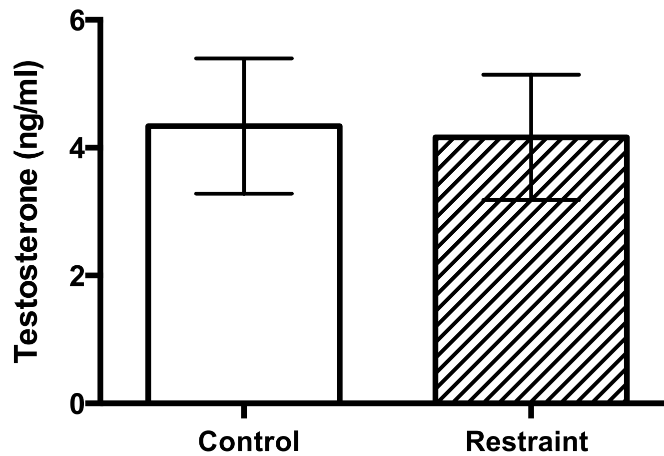

Figure 2. Circulating testosterone in control and restrained males. Testosterone (mean +/- SE), did not differ between zebra finch males collected within 4 minutes of entering the aviary (control) and males restrained for 60 minutes (unpaired t-test, t=0.114, df=11, p=0.911).

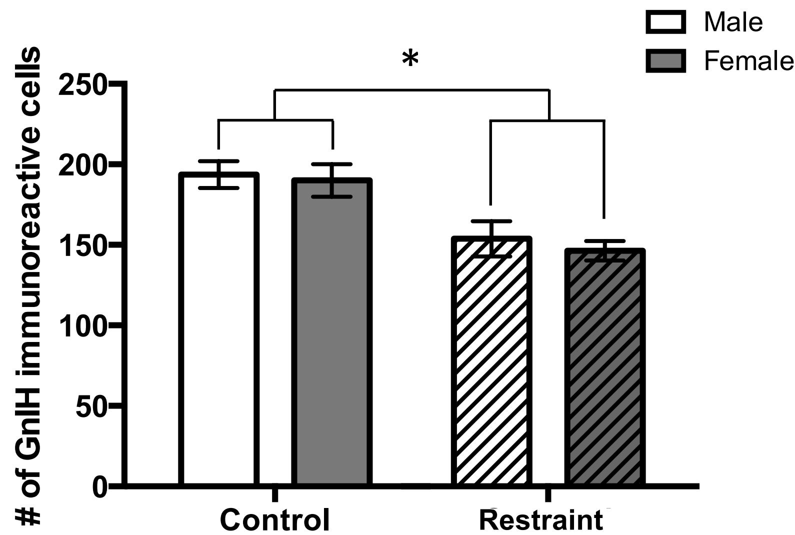

Figure 3. GnIH immunoreactivity in the hypothalamus of control and restrained birds. Sixty min of restraint significantly decreased the number of GnIH immunoreactive (GnIH-ir) cells in the hypothalamus (Two-way ANOVA, $F(1,30)=45.72$, $p<.001$). GnIH-ir cell number did not differ between males and females ($F(1,30)=0.017$, $p=0.896$).

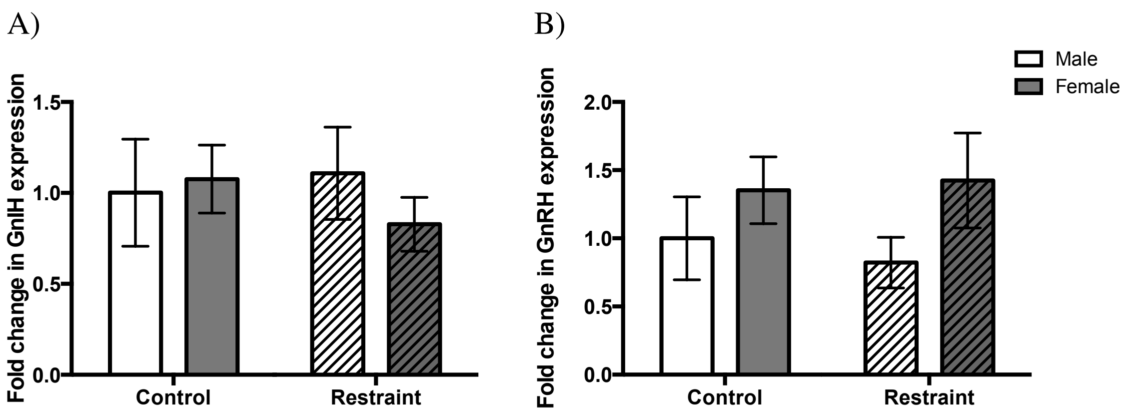

Figure 4. Hypothalamic gene expression. Mean +/- SE for (a) fold change in GnIH expression and (b) fold change in GnRH expression. Expression of GnIH did not differ significantly between control and restrained birds, nor did it differ by sex (Two-way ANOVA ($F(1,25)=0.199$ $p=0.66$)). Likewise, GnRH expression did not differ by sex or treatment ($F(1, 26)=0.002$, $p=.961$).

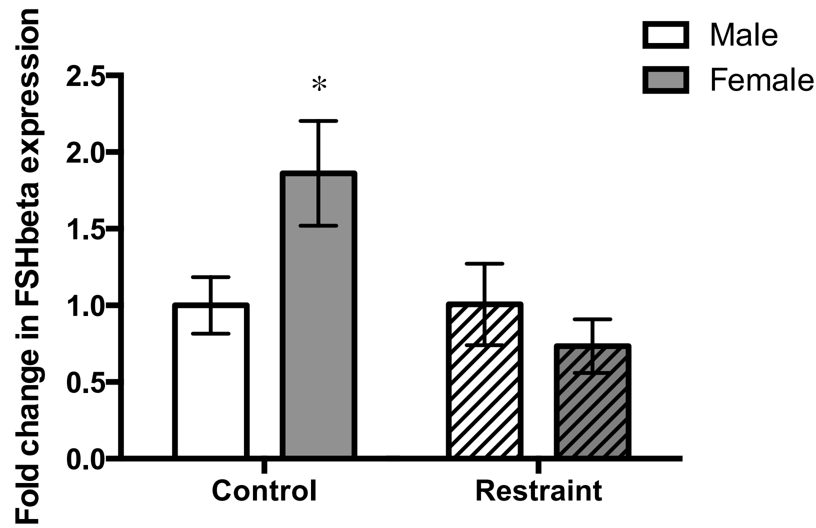

Figure 5. Expression of FSHβ in the pituitary. Restraint significantly decreased expression of the beta subunit of FSH in the pituitary in females but not in males ($F(1,26)=5.817$, $p=0.023$)

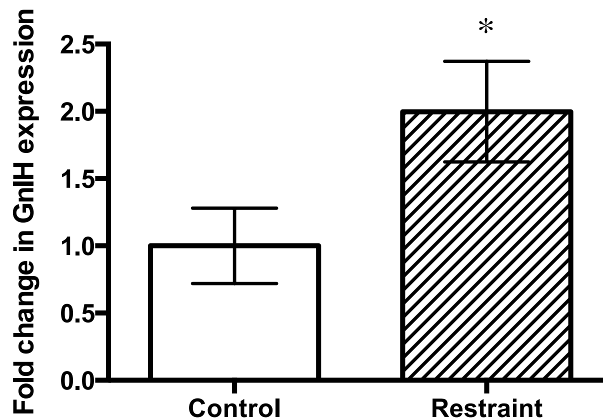

Figure 6. GnIH expression in the testes. GnIH expression (fold change, mean +/- SE), was significantly higher in the testes of zebra finch males subjected to 60 minutes of restraint than in control birds (Unpaired t-test, $t=2.271$, $df=14$, $p<0.04$).

Gene	Forward Primer	Reverse Primer	bp	Genbank Accession #
GnIH	CCCTGAGATTTGGAAGAGC	CAGATTGACAGGCAGTGAC	152	AB522971.1
GnRH	ACTCCACAACCTCTCTCAGG	CTCTGCTGCTCCTCCTCTAA	209	NM_001142320 XM_002197400
FSHβ	GCTTCACAAGGGATCCAGTA	AAAGATTCAGGGTGGTCTCC	124	XM_002194731.2
18S	CCATCCAATCGGTAGTAGCG	GTAACCCGTTGAACCCCATT	151	HQ873432.1
β-Actin	GTGCGTGACATCAAGGAGAA	AGGAGCTAGAGGCAGCTGTG	79	AY045726
GAPDH	AGCAATGCTTCCTGCACTAC	CTGTCTTCTGTGTGTGGCTGTG	121	AF_416452

Table 1. Primers used for Real-time quantitative PCR in hypothalamic, pituitary, and gonadal tissues.

	Control	Restraint
Follicular Hierarchy	2.767	0.045
	0.060	0.020
	0.173	0.020
	Mean: 1	Mean: 0.028
Small White Follicles	1.302	0.473
	0.500	0.278
	2.861	0.409
	0.131	0.082
	0.736	0.133
	0.470	
	Mean: 1	Mean: 0.275

Table 2. Individual GnIH expression in the ovaries of control and restrained females, separated by ovarian status of the female at the time of the experiment. Ovarian GnIH expression was not significantly different between control females and those subjected to 60 minutes of restraint stress in females with an established follicular hierarchy (Unpaired t-test, $t=1.099$, $df=4$, $p=0.334$) or those with small white follicles only ($t=1.602$, $df=9$, $p=0.144$).

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Chapter 4

Corticosterone measurements differ in plasma sampled from a wing vein and trunk blood

Abstract

Understanding sources of variation in endocrine measurements is important in assessing how endocrine mechanisms may influence the life history and evolutionary history of animals. Particularly, understanding how our methodologies might influence endocrine measurements is of critical importance in experimental design. Field and laboratory experiments often measure corticosterone in studies of stress and endocrinology, and there is much scientific interest in understanding within-individual variation in corticosterone and the stress response. We hypothesized that corticosterone measurements would differ between two common methods of blood collection: wing venipuncture and trunk blood collection. To test this, we subjected male and female zebra finches (*Taeniopygia guttata*) to sixty minutes of restraint in a cloth bag, a common restraint stress paradigm that reliably elevates corticosterone levels in birds. Blood was then collected from a wing vein and subsequently trunk blood when animals were euthanized. To control for the effects of the time difference between the wing bleed and the trunk bleed a separate group of animals were bled from a wing vein and subsequently bled from that same wing vein two minutes later. Plasma corticosterone was significantly different between wing blood and trunk blood within individuals (p=0.010), with corticosterone lower in trunk than wing blood. This was driven by a sex-specific effect, as when males and females are analyzed separately the effect was only significant in males (Males: p=0.035, Females: p=0.171). Plasma corticosterone levels did not differ between two successive wing bleeds taken at 60 minutes and 62 minutes of restraint (p=0.900), nor was there a linear relationship between plasma corticosterone value and the time between the 60 minute and the 62 minute bleed (p=0.783). Source of blood can contribute to individual variability in corticosterone measurements, and therefore should be considered when designing experiments or attempting to compare studies of different types.

Introduction

Understanding sources of variation in endocrine measurements is important in assessing how endocrine mechanisms may influence the life history and evolutionary history of animals. Increased interest in the degree and basis of variation in endocrine responses has led to a variety of studies of this type, particularly those on sources of variation for circulating hormones. The stress response and circulating levels of the stress hormone corticosterone have been of particular interest as they have been linked to behavior (MacDougall-Shackleton et al. 2009, Vitousek et al. 2014), reproductive success (Vitousek et al. 2014), and fitness (Blas et al. 2007). Understanding of the links between corticosterone and these traits will only increase with further knowledge of the basis of individual variation in corticosterone secretion (reviewed by Breuner et al. 2008, Cockrem et al. 2007).

Stress, as defined by an increase in hypothalamic-pituitary-adrenal (HPA) axis activation resulting in the release of glucocorticoids (corticosterone or cortisol), has widespread effects on physiology and behavior. As the primary output of the endocrine stress response, baseline and stress-induced corticosterone levels are often measured and correlated with changes in immune function and reproductive output, among other traits. Knowledge of the sources of variation in corticosterone measurements are needed to establish repeatability measures and make accurate comparisons across studies. Recent research has elucidated many potential sources of variation in corticosterone levels between animals of the same species and within a single animal. Between animals of the same species, sex (Wada et al. 2008, Small and Schoech, 2015), age

(Blas et al. 2006, Sims et al. 2000, Walker et al. 2015), HPA axis organization during development (Liu et al. 2000, Plotsky and Meaney 1993), and genotype (Cockrem et al. 2010, Evans et al. 2006) can all contribute to differences seen in corticosterone measurements. Within an individual, corticosterone released in response to stress varies with season and photoperiod (Romero et al. 1997, Romero and Reed, 2008), migratory status (Romero et al. 1997), physiological condition (stage of molt, for example, Romero and Reed, 2008), environmental conditions (such as enrichment, Belz et al. 2003), housing conditions (Brown and Grunberg, 1995, Craig et al. 1986), and amount of human interaction (Walker et al. 2005).

In this study we sought to determine whether plasma obtained from different sources of blood could be a source of variability in corticosterone measurement. Studies of birds vary in the source of blood for corticosterone measurement, with field or survival studies often taking a blood sample from the wing (Crino et al. 2013, Dickens and Bentley 2014, Krause et al. 2014), while terminal studies of birds in the field or the laboratory take blood from the trunk when an animal is decapitated (Ernst et al. 2015, Yadav and Haldar, 2014). To better compare studies that use different methods it is necessary to know whether the source of blood could contribute to variation in corticosterone. We hypothesized that circulating corticosterone measurements would differ in blood taken from the wing vein and that obtained from trunk blood.

Materials and Methods

Comparison of hormone level in wing and trunk blood

Adult male and female zebra finches were collected from mixed-sex free-flight aviaries at the UC Berkeley Field Station for the Study of Behavior, Ecology, and Reproduction (FSSBER) in Berkeley, CA. The birds were allowed to pair and breed freely in this colony setting and were fed German millet mixed with canary seed *ad libitum* supplemented with lettuce and egg-based food.

To compare corticosterone in wing blood and trunk blood, seven males and eight females were subjected to restraint stress by being placed in a ventilated cloth bag for 60 minutes. This common capture-handling stress paradigm reliably elevates glucocorticoid levels in birds (Wingfield et al. 1992, Wingfield 1994). After 60 minutes of restraint a small sample of blood (~100ml) was collected by puncturing a wing vein with a 26-gauge needle. Birds were subsequently anesthetized with isoflurane (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) and rapidly decapitated, at which time trunk blood was collected. Trunk blood samples were collected approximately 2 minutes after the wing blood sample was obtained for each bird (average time between bleeds 121.7 ± 10.04). The experiment took place on May 3rd, 2013, a day with approximately 14 hours of light.

Comparison of hormone level in wing blood over time

Animals were housed in single-sex aviaries at the College of Wooster in Wooster, OH, though males and females were housed in the same room and hence able to see and hear each other. Birds were fed finch and canary seed mix *ad libitum* supplemented with spinach and egg-based food, and the light cycle was held at LD 14:10.

To address whether corticosterone levels differed over time from a wing vein only, we subjected nine males and ten females in The College of Wooster colony to 60 min of restraint stress as described above. The period of restraint was initiated within 3 min of the researcher entering the room in which birds were housed. Following the 60-min restraint period, a blood sample (~50 μl) was collected from by puncturing a wing vein with a 26-gauge needle (this

sample is hereafter referred to as Wing-60). The bird was held in the hand, and a second blood sample was collected from the same wing vein approximately two minutes later (average time between bleeds was 112.7 ± 3.2 s). This sample is hereafter referred to as Wing-62. Samples were collected on 9 different days between February 11th, 2015, and February 25th, 2015. Following collection, all blood samples were centrifuged to separate plasma, which was then stored at -80 ºC prior to assay.

Hormone assays

Corticosterone concentrations in plasma samples were measured by enzyme immunoassay kits (EIA, Enzo Life Sciences, Farmingdale, NY, USA). These kits have been validated and optimized for zebra finches (Lynn et al. 2007, 2010, 2015). Plasma was incubated in steroid displacement buffer (Enzo Life Sciences, Farmingdale, NY, USA) and then diluted with assay buffer for a final dilution of 1:40. Manufacturer's instructions were followed for the remainder of the assay. All samples were run in triplicate with a standard curve run in duplicate on each plate. Corticosterone samples were run on a total of four plates. Due to the paired nature of the design of this study, samples from individual birds were always run on the same plate. Average intraassay and interassay variation was 8.6% and 12.7% respectively. Sensitivity was 0.04 ng/ml.

Statistical Analysis

All statistical analyses were performed using Prism (version 6.0d, 2013, Graphpad). Comparison of corticosterone values were made using paired t-tests, and a linear regression was conducted to determine if there was a relationship between the difference in time between bleeds and corticosterone measurement.

Results

Plasma corticosterone in wing blood and trunk blood

Plasma corticosterone was significantly different between wing blood and trunk blood within individuals (Fig. 1, $p=0.010$, $t=2.957$, $df=14$). This is driven by a sex-specific effect, as when males and females are analyzed separately the effect is only significant in males (Males: $p=0.035$, $t=2.721$, $df=6$, Females: $p=0.171$, $t=1.525$, $df=7$).

Plasma corticosterone in wing blood over time

Plasma corticosterone levels did not differ between two successive wing bleeds taken at 60 minutes and 62 minutes of restraint (Fig. 2, Paired t-test, $p=0.900$, $t=0.128$, $df=20$). Analyzing males and female separately, corticosterone levels still did not differ between two successive wing bleeds (Males: $p=0.219$, $t=1.334$, $df=8$, Females: $p=0.220$, $t=1.319$, $df=9$).

No linear relationship was found between plasma corticosterone value and the time between the 60 minute bleed and the 62 minute bleed (Fig. 3, $y=-0.007x+.1473$, r squared 0.005, $p=0.783$).

Discussion

Here we report another potential source of variation in circulating corticosterone levels, which is the source of the plasma for assay. Within an individual, corticosterone values were significantly different between trunk blood and wing blood. This effect was driven by a sex-specific effect, as males differed significantly in their trunk and wing values within an individual

while females did not. This effect is not likely an artifact of the additional time of restraint or necessary order of wing sample being taken before trunk sample, as corticosterone levels did not differ between two successive wing bleeds at 60 and 62 minutes of restraint. Additionally, there was no correlation between the time between the two wing bleeds and the change in corticosterone.

In this study, wing blood was collected exclusively from the brachial vein, while trunk blood was a sample of all veins and arteries feeding the head tissue including carotid arteries and the jugular vein (Evans and Heiser, 2004), and thus differences in supply or vascular carriage of corticosterone could be responsible for the differences seen between wing and trunk blood. One proposed mechanism for differences in corticosterone measurements between trunk blood and wing blood is the secretion of neurosteroids into jugular blood (Newman et al. 2008). However, contrary to past studies on varying levels of corticosterone in jugular and wing blood (Newman et al. 2008), we found that trunk blood had significantly lower levels of corticosterone than wing blood within an individual.

We found a sex-specific effect of this difference in wing and trunk blood measurements of corticosterone, with males having a significant difference in corticosterone between the two sources of plasma, while females did not differ. This suggests there are sex differences in physiology or carriage of hormones in the blood. It is unlikely this is an effect of sex differences in amount of corticosteroid binding globulin (CBG) because only the effect of trunk versus wing blood differed in males and females as opposed to the corticosterone measurements themselves. CBG could however be functioning differently in males and females. Further research is necessary to understand the mechanism of this difference in corticosterone measurements due to source of blood and the mechanisms of sex difference in this effect.

In conclusion, we report blood source to contribute to within-animal variation in corticosterone measurements. It is important to consider this source of variation, especially when designing experiments and comparing studies. In studies of endocrinology, corticosterone levels are commonly measured by taking a blood sample and conducting an ELISA or RIA on the plasma obtained from this sample. Field studies often only allow for a single blood sample of an individual, therefore making the understanding of the sources of variation in corticosterone important to allow for comparison.

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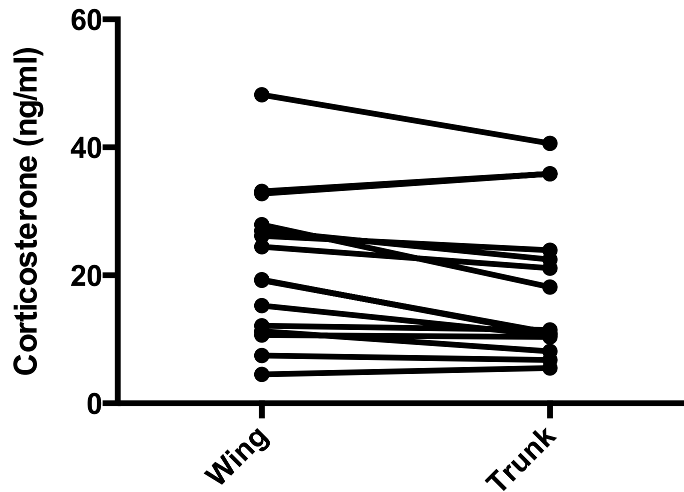

Figure 1. Corticosterone in wing and trunk blood. Corticosterone measurements in wing blood were significantly different from corticosterone measurements in trunk blood (Fig. 1, Paired t-test, p=0.010, t=2.957, df=14).

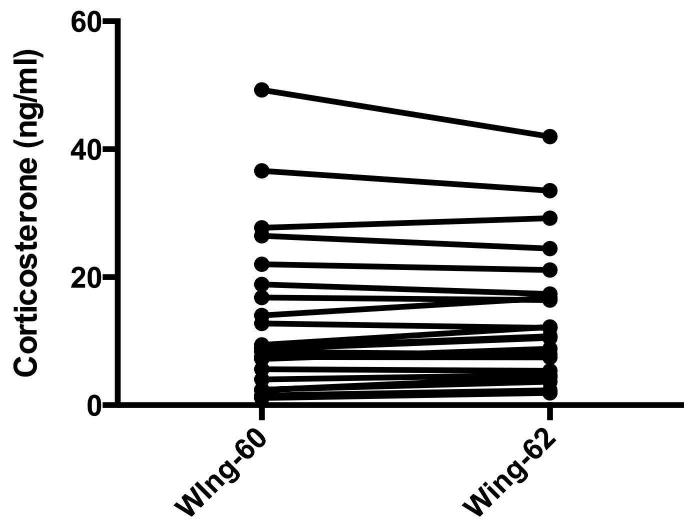

Figure 2. Corticosterone in wing blood over time. Corticosterone values did not differ in wing bleeds taken two minutes apart (at 60 and 62 minutes of restraint). Paired t-test, p=0.900, t=0.128, df=20.

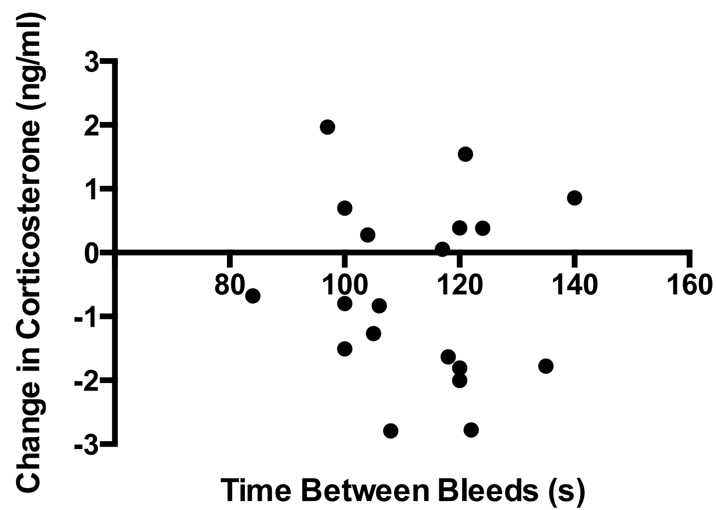

Figure 3. Correlation of time between bleeds and change in corticosterone. The difference in corticosterone level between the Wing-60 sample and the Wing-62 sample was not related to the time between bleeds ($y=-0.007x+.1473$, $R^2=0.005$, $p=0.783$)

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Chapter 5

Perception of food availability determines reproductive response to food restriction in the zebra finch *Taeniopygia guttata*

Abstract

An increase in food availability stimulates reproductive activity in zebra finches. It is not known if it is increased energy intake, the visual stimulus of food, or both that affects reproductive status. We hypothesize that physical interaction with food as well as visualization of food affects reproduction. To test this, singly-housed birds were videotaped for 1 hour and then randomly assigned to one of four groups: control (ad libitum food), 7 hours of complete food restriction, 7 hours of exposure to a food dish with seed hulls only (no nutritive value), or 7 hours of a food dish covered in clear plastic so birds were able to see food but not touch it (n=10 per group). At the end of treatment birds were videotaped for an hour followed by collection of tissue. Corticosterone was significantly higher in the food restricted group and the group receiving seed hulls than in controls ($p<05$). Birds exposed to seed hulls spent significantly more time at their food dish ($p<.02$) than did control birds or birds with plastic-covered food dishes, while food restricted birds spent an intermediate amount of time at their food dish. Total activity was higher than controls in all experimental groups ($p<.05$). Perception of food affected expression of neuropeptide Y and gonadotropin-releasing hormone in the hypothalamus ($p<.05$). Overall, our data suggest that, while metabolically similar, a visual food stimulus affects zebra finches differently from a food stimulus that they can interact with but receive no nutritional value. These data highlight the importance of visualization of food when considering how food availability stimulates reproductive activity.

Introduction

Animals must time breeding appropriately so that energetically-costly activities (i.e. provisioning of young) coincide with the times of the highest food availability. In environments in which this resource availability does not vary predictably with the seasons animals cannot rely on photoperiodic cues to predict when favorable conditions may occur and must instead be flexible or opportunistic breeders, reproducing quickly at the onset of good conditions. In many opportunistic breeders, reproductive physiology and behavior are stimulated by an increase in food availability, though how cues of food availability cause this increase in reproductive activity is still unknown.

An animal is considered reproductively active when there is an upregulation of the hypothalamo-pituitary-gonadal (HPG) axis and an increase in associated behavior. In stimulatory conditions gonadotropin-releasing hormone (GnRH) is released from the hypothalamus, which stimulates the production and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland. These two hormones act on the gonads to increase production of testosterone, estradiol and gametes, leading to an increase in fertility. These changes in physiology are associated with an increase in reproductive behavior, including courtship and song behavior. Therefore, GnRH is often considered the “gateway” to reproduction, and much scientific thought has been given to the control of GnRH.

One hormone poised to “gate” reproduction is gonadotropin-inhibitory hormone (GnIH), which can inhibit reproduction at all levels of the HPG axis. GnIH neurons in the hypothalamus interact directly with GnRH neurons (Bentley et al. 2006, Ubuka et al. 2008, Ubuka et al. 2009) and release GnIH into the hypothalamo-pituitary portal system to directly inhibit pituitary release of LH and FSH. GnIH is also synthesized in the gonads and can act to decrease steroidogenesis in the gonads via action on GnIH receptor. Hypothalamic GnIH has been shown to increase with food restriction in some species (Chowdhury et al. 2012, Fraley et al. 2013), providing a

potential mechanism by which food availability could be conveyed to the reproductive axis (Clarke et al. 2012).

GnIH and GnRH neurons in the hypothalamus interact with neurons that produce neuropeptide Y (NPY), a peptide that stimulates feeding behavior in response to low energy availability (Boswell 2001, Pralong 2010). NPY neurons contact GnRH (Kuenzel 2000, Rastogi et al. 2015) and GnIH neurons (Klingerman et al. 2011, Singh et al. 2013), providing a network by which information of food availability could be conveyed to the reproductive axis. Studies in mammals suggest that NPY can indeed modulate reproductive activity (Hill et al. 2008, Pralong 2010), and this has recently begun to be explored in birds (Davies and Deviche, 2015).

In addition to the mechanisms by which food availability affects the reproductive axis, the type of cue food provides to stimulate reproduction is in question. GnIH and GnRH may be responding to the metabolic component of food availability, though other research suggests perceptual cues may impact reproductive activation or suppression as well (reviewed in Hahn et al. 2005). For example, in the spotted antbird, a photoperiodic breeder, reproductive behavior was stimulated by the sight of preferred food (Hau et al. 2000). Using a common model of opportunistic breeding, the zebra finch (*Taeniopygia* guttata), we sought to test whether visual or tactile interaction with food could affect reproduction. We hypothesized that physical interaction with food as well as visualization of food could stimulate reproduction, even in the absence of metabolic food cues. We predicted that birds that could see or interact with food when food restricted would blunt their response to food restriction, maintaining higher levels of GnRH and lower levels of NPY and GnIH than food restricted birds in order to maintain reproduction.

Materials and Methods

Experimental Design

Adult male zebra finches were housed in mixed-sex free-flight aviaries at the Field Station for the Study of Behavior, Ecology, and Reproduction at UC Berkeley. Forty males were weighed and transferred to individual cages that were visually but not acoustically isolated from other birds for two weeks prior to the experiment. As in the mixed-sex colony setting, birds were exposed to natural changes in photoperiod will supplemental full-spectrum light for a minimum 12:L 12:D photoperiod. In the colony and prior to the experiment birds were fed German millet mixed with canary seed *ad libitum* supplemented weekly with lettuce and egg-based food.

On the experimental day males were randomly assigned to one of four groups:

1) Control – 7 hours of *ad libitum* access to food
2) Fasted – 7 hours with no access to food (empty food dish)
3) Tactile stimulus – 7 hours with access to seed hulls only (no nutritive value)
4) Visual stimulus - 7 hours with access only to a food dish covered in plastic, so animals could see the food but not interact with it.

At sunrise all birds were allowed to feed for one hour prior to administration of the experimental paradigm. During this hour video recordings were taken of behavior so levels of activity and feeding behavior could be assessed. One hour after sunrise a single investigator entered the room containing the individual cages and removed all food dishes and briefly swept out each cage and the floor of the room to remove any seeds. At this time control birds received a full food dish, fasted birds received an empty food dish, tactile stimulus birds received a food

dish with only seed hulls, and visual stimulus birds received a food dish covered in plastic. After 6 hours of treatment video recordings were again taken of behavior, and tissue was collected 7 hours after the beginning of treatment. An investigator was only present in the room containing cages when food dishes were replaced.

After seven hours of treatment birds were terminally anesthetized with isofluorane (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) and rapidly decapitated, at which time trunk blood was collected. All animals were collected within three minutes of entering the experiment room. Brain tissue was fresh frozen on dry ice and stored at -80 ºC prior to processing. The rest of the head with pituitary intact was placed in nucleic acid preservation buffer (Camacho-Sanchez et al. 2013) for one week, at which time pituitaries were extracted using a dissection microscope and frozen at -80 ºC. Testes were measured with calipers, fresh frozen on dry ice and stored at -80 ºC. Volume of testes was calculated as $V=4/3pa^2b$, where a is half the width and b is half the length (long axis) of the testis.

Hormone Assays

Concentrations of corticosterone and testosterone were determined by enzyme immunoassay kits (EIA, Enzo Life Sciences, Farmingdale, NY, USA). These kits have been validated and optimized for use in zebra finches (Lynn et al., 2007, 2010), and protocols were followed as described in Lynn et al. 2010. Plasma was diluted 1:20 for testosterone and 1:40 for corticosterone EIA. All samples were run in triplicate with a standard curve run in duplicate on each plate. Testosterone and corticosterone were each run on one plate to decrease intraassay variation. Average intraassay variation was 4.85% for testosterone and 6.85% for corticosterone. Sensitivity was 0.087 ng/ml for the testosterone assay and 0.071 ng/ml for the corticosterone assay.

Real-time quantitative PCR

To measure levels of gene expression, real-time quantitative PCR (RT-qPCR) was conducted on hypothalamic, pituitary, and gonadal tissue. To quantify expression of genes in the hypothalamus brains were sectioned at 40 µm on a cryostat, mounted directly onto gelatin-coated slides, and frozen at -20 ºC. Alternate sections were punched with a 3mm circular punch (Harris Uni-core, catalog number 69036; Electron Microscopy Sciences, Ft Washington, PA). The punch was centered at the bottom of the brain beginning approximately at plate A3.5 (Stokes et al. 1974), when the tractus septomesencephalicus (TrSM) emerges, until a total of 36 punches were obtained for each animal. Hypothalamic tissue punches were homogenized in RLY buffer (Isolate II RNA mini kit, Bio-52073, Bioline USA Inc, Taunton, MA) and stored at -80 ºC until all samples were ready for RNA extraction. Tissues were extracted and purified using manufacturer's instructions for the Isolate II RNA mini kit (Bio-52073, Bioline USA Inc, Taunton, MA), and reverse-transcribed with iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, U.S.A.).

Primers were designed for each gene from previously published or predicted sequences in the *T. guttata* genome (see Table 1 for accession numbers) using Primer3 software (simgene.com), and were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Primer pairs were optimized for purity and specificity with melt curves analysis, and primers were not used if secondary structure or dimerization was found in no-template controls. β-actin and 18S were run as reference genes, but 18S in the hypothalamus was significantly different across groups ($F(3,35)= 2.785$, $p=0.017$) so it was excluded as a reference gene and β-actin

alone was used for reference.

Behavior

Behavior was recorded with security cameras (SWADS-120CAM-US, Swann Communications, Santa Fe Springs, CA, U.S.A.) and a 4-channel DVR (Q-See, Anaheim, CA, U.S.A.). Analysis of time at food dish and total activity was completed by an experimenter blind to the experimental treatment of the animal. For measurements of total activity videos were analyzed using BioVision 3.0 software (Boncher et al. 2013).

Data Analysis

Morphometrics, circulating levels of hormones, expression values from qPCR, and quantification of behavior were compared between control and experimental groups using one-way ANOVA. When significance was found, Tukey's post-hoc comparison test was used to determine which groups were significantly different from each other. We used linear regression to explore relationships between gene expression and behavior. Data are presented as means ± standard error. All statistical analyses were performed using Prism software (version 6.0d, 2013, Graphpad).

Results

Mass and testicular volume

Comparisons of mass lost between groups revealed a significant effect of treatment (Fig. 1, One-way ANOVA, $F(3,36) = 0.747$, $p<0.0001$). Post hoc multiple comparisons tests revealed that birds in the fasted, tactile stimulus, and visual stimulus groups all lost significantly more mass (calculated as percent loss) than the control group. The fasted group and the visual stimulus group were also significantly different from each other. Testis volume was not different between groups ($F(3,36) = 1.797$, $p=0.693$; average testis volume 26.3 ± 2.912).

Circulating testosterone and corticosterone

Corticosterone was significantly different between groups ($F(3,34)= 2.529$, $p=0.017$), with the control group significantly different from the fasted and tactile stimulus groups. (Fig 2A). Testosterone was not significantly different between groups (Fig 2B, $F(3,33)= 1.675$, $p=0.188$). There was no correlation found between circulating corticosterone and testosterone ($Y = 0.01292*X + 1.691$, $R^2 = 0.037$, $p=0.267$).

Gene expression

GnRH expression in the hypothalamus was significantly affected by treatment (Fig 3A, $F(3,35) = 3.13$, $p=0.008$). Posthoc analysis revealed that birds receiving a visual stimulus had significantly higher GnRH expression than fasted and tactile stimulus birds. Control birds were not significantly different from any other group. Hypothalamic GnIH expression did not differ across groups (Fig 3B, $F(3,35) = 0.921$, $p = 0.135$), though GnIH expression was correlated with GnRH expression (Fig 3C, $Y = -0.388X + 1.495$, $R^2 = 0.107$, $p = 0.045$). FSHβ expression in the pituitary did not differ across groups ($F(3,32)= 0.164$, $p=0.367$), and FSHβ expression was not correlated with expression of GnRH ($Y = 0.093X+0.787$, $R^2 = 0.027$, $p = 0.336$). GnIH expression in the testes did not differ with treatment (Fig 6, $F(3,32)= 0.604$, $p = 0.443$).

Expression of NPY was affected by treatment (Fig 4A, $F(3,33) = 2.151$, $p = 0.0132$), with NPY expression higher in the tactile stimulus group than controls and the visual stimulus

group. The fasted group was not significantly different from any other group. NPY expression was found to be correlated to the time spent at the food dish in the last hour of the experiment (Fig 4C, Y = 696.5*X – 224.6, $R^2 = 0.355$, $p < 0.0001$).

Behavior

Food restriction increased activity over control levels (Fig 5, $F (3,16)= 2.384$, $p < 0.02$), with the tactile stimulus and visual stimulus group significantly higher than the control group and the fasted group not significantly different from any other group. Activity in the first hour of the experiment (prior to treatment) did not differ between groups ($F (3, 12) = 1.539$, $p = 0.227$).

In the last hour of the experiment the tactile stimulus group spent the most time at the food dish ($F (3,36) = 4.025$, $p = 0.011$). The tactile stimulus group was significantly different from the control and the visual stimulus group, and the fasted group was not significantly different from any other group. Time spent at the food dish in the first hour of the experiment did not differ between groups ($F (3,36) = 0.370$, $p = 0.878$).

Discussion

Though all three experimental groups (fasted, tactile stimulus, visual stimulus) received no metabolic input, we found significant differences in the animals' response to food restriction. All three groups lost mass as compared to controls, with the visual stimulus group losing less mass than the fasted group. Corticosterone levels rose predictably in the fasted and tactile stimulus group, with the visual stimulus group showing an intermediate level of corticosterone secretion not significantly different from any other group.

Expression of GnRH and NPY in the hypothalamus were affected by perception of food availability. Fasted animals had increased NPY and decreased GnRH due to food restriction, but a visual cue of food masked these effects in food restricted animals, keeping NPY low and GnRH as high as control levels. While it is possible that NPY and GnRH could be reacting to food restriction and visual cues independently, we suggest that in this case NPY is controlling GnRH, decreasing GnRH expression in fasted conditions and allowing it to stay high when the animal perceives that food is available, i.e. the visual stimulus condition. Further research is necessary to test this hypothesis and determine if it is a direct, mechanistic link.

Consistent with other studies of zebra finches (Lynn et al. 2015) we found no significant effect of treatment on GnIH expression in the hypothalamus or gonads. Taking these data together it appears that zebra finches do not respond to food restriction with activation of the GnIH system as has been seen in other species (Chowdhury et al. 2012, Fraley et al. 2013). However, in our study GnIH expression was inversely correlated with GnRH expression, suggesting some relationship is maintained between these two peptides even without significant effects on GnIH. Additionally, an effect of stress on GnIH in the testes has been described in zebra finches (Ernst et al. 2015), but the current data suggest that corticosterone increases from food restriction do not elevate gonadal GnIH in the same way.

Combined, these data suggest a perceptual component to the effects of food availability on the reproductive axis, corticosterone secretion, and behavior. Receiving a visual food cue prevents the NPY increase and reproductive axis decrease usually associated with food restriction. This is a novel effect of perception on expression of NPY, and suggests a new potential mechanism for GnRH control not previously explored in opportunistically-breeding birds.

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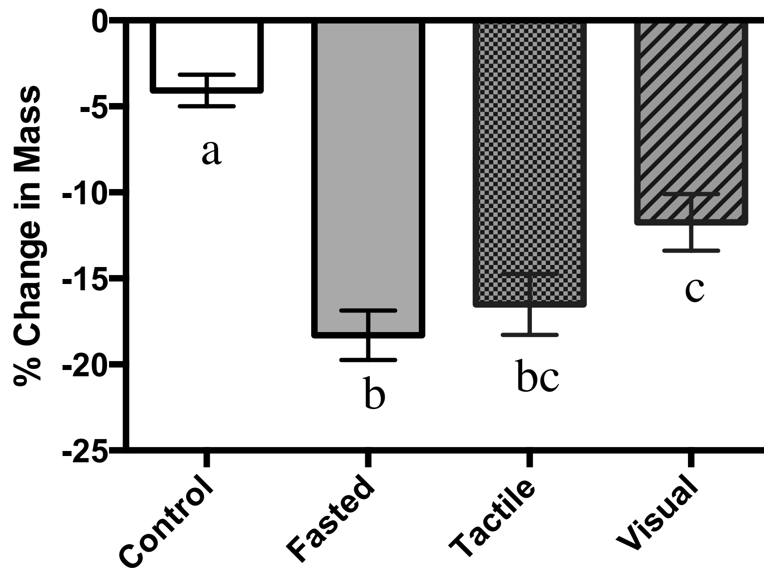

Figure 1. Mass lost with treatment. Fasted, tactile stimulus and visual stimulus groups all lost significantly more mass during the experiment than the control group (F (3,36) = 0.747, p<0.0001). The fasted and the visual stimulus group were also significantly different from each other,

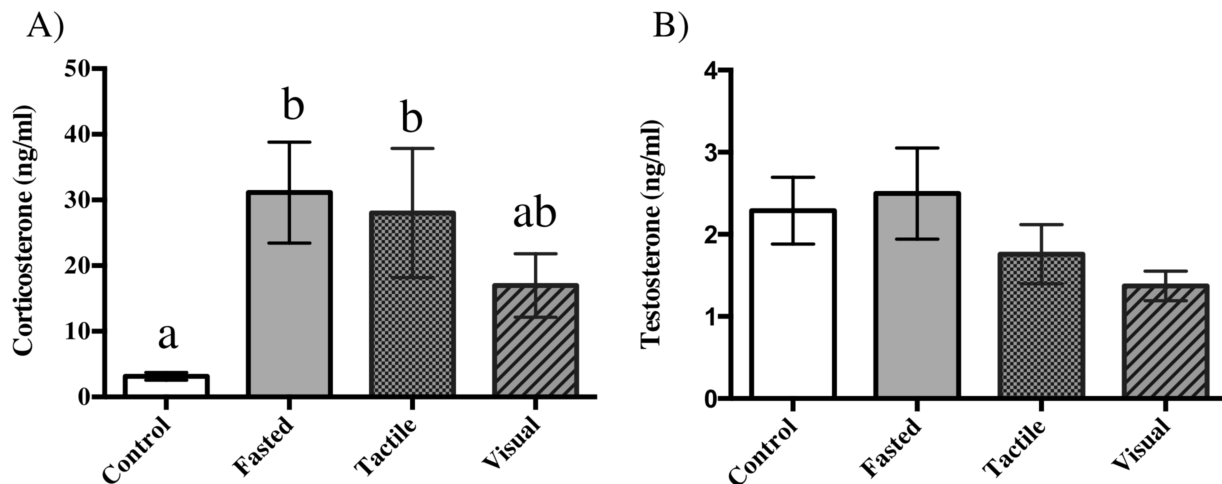

Figure 2. Circulating corticosterone and testosterone. Circulating corticosterone (A) was significantly different across groups (F (3,34)= 2.529, p=0.017), with the control group significantly different from the fasted and tactile stimulus groups as indicated by a vs. b. The visual stimulus group was not significantly different from any other group, thus is labeled ab. Circulating testosterone (B) was not significantly different across groups (F (3,33)= 1.675, p=0.188).

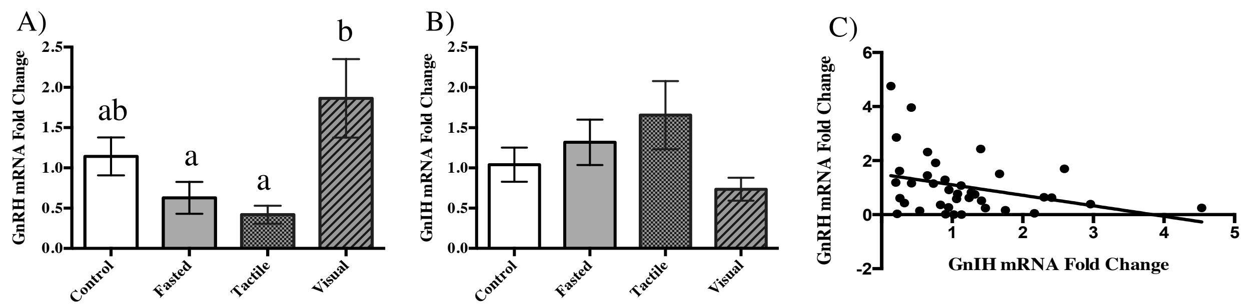

Figure 3. Hypothalamic gene expression. GnRH mRNA expression in the hypothalamus (A) was significantly higher in the visual stimulus group as compared to the fasted and tactile stimulus group (F (3,35) = 3.13, p=0.008). No other group was significantly different from the control group (Tukey's post hoc comparison at $p < 0.05$ indicated by different letters). GnIH mRNA expression in the hypothalamus (B) did not differ with treatment (F (3,32) = 0.604, p = 0.443), though GnIH expression was correlated with GnRH expression (C, Y = -0.388X + 1.495, $R^2 = 0.107$, p = 0.045).

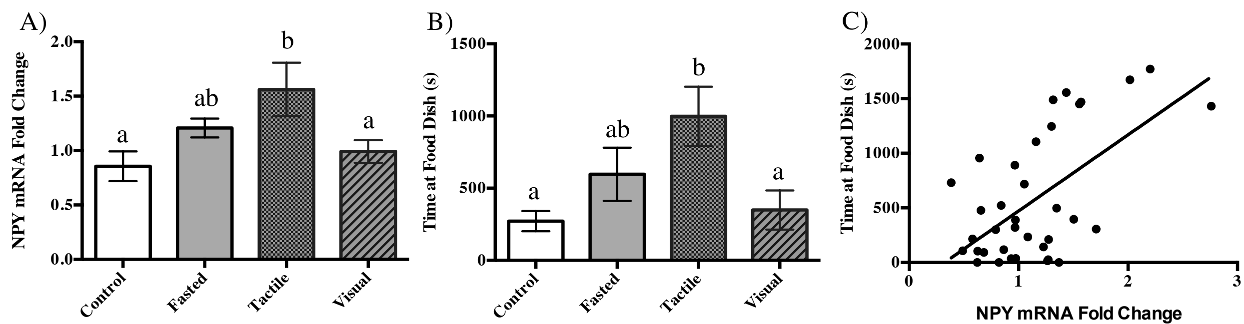

Figure 4. NPY expression and feeding behavior. Expression of NPY in the hypothalamus (A) was affected by treatment (F (3,33) = 2.151, p = 0.0132), with NPY expression higher in the tactile stimulus group than control and the visual stimulus group (Tukey's post hoc comparison at $p<0.05$ indicated by different letters). The fasted group was not significantly different from any other group (indicated by ab). Time spent at the food dish in the last hour of the experiment (B) also differed between groups, with the tactile group spending significantly more time at the food dish than the control and visual stimulus group. NPY expression was correlated with time spent at the food dish (C) in the last hour of the experiment ($Y = 696.5*X – 224.6$, $R^2 = 0.355$, $p < 0.0001$).

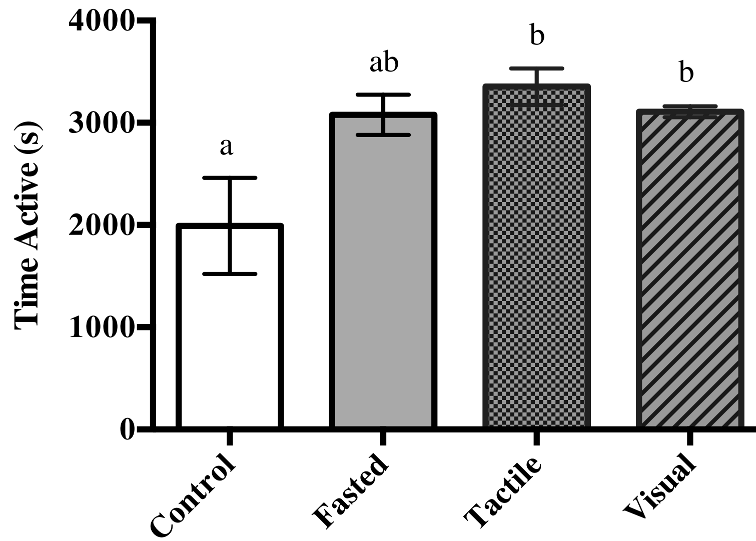

Figure 5. Total activity in the last hour of treatment. Food restriction increased activity over control levels (F (3,16)= 2.384, p < 0.02), with the tactile stimulus and visual stimulus group significantly higher than the control group and the fasted group not significantly different from any other group.

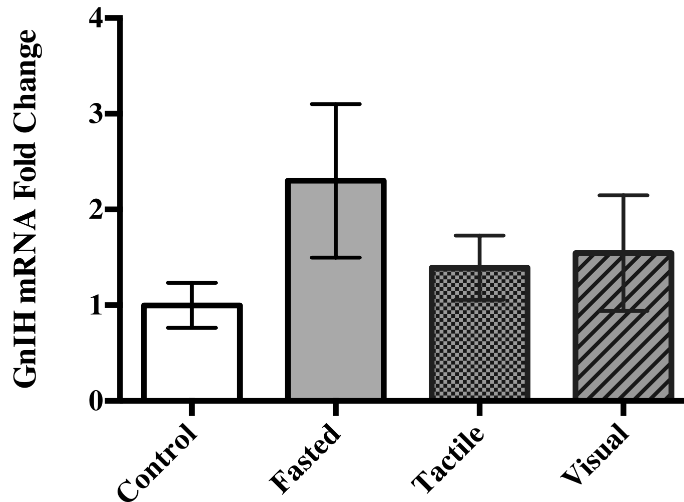

Figure 6. GnIH expression in the testes. GnIH expression in the testes did not differ with treatment (F (3,32)= 0.604, p = 0.443).

Gene	Forward Primer	Reverse Primer	bp	Genbank Accession #
GnIH	CCCTGAGATTTGGAAGAGC	CAGATTGACAGGCAGTGAC	152	AB522971.1
GnRH	ACTCCACAACCTCTCTCAGG	CTCTGCTGCTCCTCCTCTAA	209	NM_001142320 XM_002197400
FSHb	GCTTCACAAGGGATCCAGTA	AAAGATTCAGGGTGGTCTCC	124	XM_002194731.2
NPY	TGCTGACTTTCGCCCTGTC	AGCATCTGGCCATGTCCTCT	114	XM_012571586.1
18S	CCATCCAATCGGTAGTAGCG	GTAACCCGTTGAACCCCATT	151	HQ873432.1
b-Actin	GTGCGTGACATCAAGGAGAA	AGGAGCTAGAGGCAGCTGTG	79	AY045726
GAPDH	AGCAATGCTTCCTGCACTAC	CTGTCTTCTGTGTGTGGCTGTG	121	AF_416452

Table 1. Primers sequences and anticipated size of real-time quantitative PCR products.

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Chapter 6

Conclusions

Opportunistic breeding is an ideal strategy in unpredictable environments in which animals have only transient availability of good conditions in which to raise young. Animals that use this breeding strategy respond quickly to favorable environmental conditions by activating the reproductive axis and reproductive behavior in response to stimulatory cues. The mechanisms by which these cues are integrated in the brain and transmitted to the reproductive axis have remained elusive despite 100 years of endocrine research on this and related subjects. The experiments done for this dissertation provide novel evidence of the mechanisms underlying reproductive timing in response to social cues, stress, and food availability in the opportunistically-breeding zebra finch.

Chapter 2 describes an experiment that examined the role of social stimulation in reproductive activity and asked whether social cues could stimulate DIO2 and result in GnRH release as has been described in a photoperiodic model (Nakao et al. 2008, Yoshimura et al. 2003). While no evidence was found for changes in DIO2 with social stimulation, males given a female stimulus did show increases in activation in the hypothalamus and reproductive behavior. This hypothalamic activation indicates that males did perceive the females as stimulatory, and suggests the potential for downstream effects on the reproductive axis over a longer time period than was examined. Future research will further explore the role of social cues in activating the reproductive axis and sexual behavior in zebra finches.

In chapter 3 the role of gonadotropin-inhibitory hormone (GnIH) was examined as a mediator of stress to the reproductive axis. GnIH has been implicated in stress-induced reproductive inhibition in a number of species (Calisi et al. 2008, Kirby et al. 2009, Geraghty et al. 2015) and could thus be used to time reproduction in opportunistic breeders. In times of poor environmental conditions (low food and water availability, lack of appropriate social cues or nesting sites, inclement weather) animals could activate the stress response, therefore activating GnIH and suppressing reproductive function. Contrary to other studies (Calisi et al. 2008, Kirby et al. 2009), activating the stress response in zebra finches decreased GnIH in the hypothalamus, though we believe this to be due to a release of GnIH into the hypothalamo-pituitary portal system. This belief is supported by a decrease seen in expression of FSHβ in the pituitary of females, potentially due to GnIH action at the level of the pituitary. Additionally, GnIH was increased in the gonads of males but not females in response to stress, consistent with other findings (McGuire et al. 2013). This is the first description of a differential response of the GnIH system in the hypothalamus and gonads and the first study to detail these sex differences in the avian GnIH system.

Chapter 4 describes differences found in corticosterone measurement within one animal when comparing wing blood to trunk blood. This is an important look at the methodology used to measure hormones in laboratory and field settings and details a source of intra-individual hormone variability. Therefore, the source of blood collection for hormone assay is an important source of variability and must be considered in experimental design and when comparing results across studies. Understanding the nature of within-individual variation in hormonal measurements is critically important for interpretation of the studies described in this dissertation and elsewhere, and therefore this chapter has broad implications for the field of endocrinology.

In addition to cues of mate availability and stress, food availability is of critical importance in determining reproductive timing in zebra finches. Chapter 5 illustrates the importance of non-metabolic cues of food when assessing food availability and determining reproductive timing. The stress hormone corticosterone increases with food restriction (Kitaysky et al. 2001, Lynn et al. 2010), so this study considered both the stress response and the

reproductive response to food restriction and provided supplementary perceptual cues. Animals that were food restricted but could see food (visual stimulation group) maintained higher levels of GnRH expression and lower levels of NPY expression not significantly different than control levels. Corticosterone levels were not significantly different from either the control group nor the food restricted group, while the food restricted group was higher than the control group. This indicates that food restriction in the visual stimulation group activated the stress response to increase corticosterone, but that animals perceived food availability and thus did not down-regulate reproductive parameters. These novel findings highlight the role of visual cues in activating reproduction in zebra finches, and suggest that reproduction may be activated without metabolic cues from food.

These studies detail important differences between the sexes, and highlight the need to consider how and why males and females might differ in their endocrine responses. In chapter 3, restraint stress decreased hypothalamic GnIH in both males and females but a decrease in FSHβ was only seen in females. Gonadal GnIH was only shown to increase in males. Further experiments may show that males and females use hypothalamic and gonadal GnIH systems differently in their control of the reproductive axis. In chapter 4, corticosterone was different in wing and trunk blood within one animal, and this effect was driven by males. Further research is necessary to understand why males have more intra-individual variation due to source of blood than females.

Taken together, the chapters in this dissertation present new evidence for the mechanisms underlying reproductive timing in response to environmental cues in an opportunistic breeder. Social cues and food availability can stimulate reproduction, potentially via different mechanisms. Whether food availability or social cues are the primary driver of reproduction in these animals in the wild remains to be tested, though it is possible that both are required (Perfito 2010, Perfito et al. 2011). The activation of the stress response due to lack of food or mate availability must also be considered, as stress can decrease reproductive axis activity by increasing activity of the GnIH system. These data underscore the complexity the mechanisms necessary to activate reproduction and the importance of brain integration of various cues for reproductive success and fitness.

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