Consequences of Negative Energy Balance on Avian Reproductive Physiology:

Endocrine and Metabolic Mediators

by

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ABSTRACT

Reproduction is energetically costly and seasonal breeding has evolved to capitalize on predictable increases in food availability. The synchronization of breeding with periods of peak food availability is especially important for small birds, most of which do not store an extensive amount of energy. The annual change in photoperiod is the primary environmental cue regulating reproductive development, but must be integrated with supplementary cues relating to local energetic conditions. Photoperiodic regulation of the reproductive neuroendocrine system is well described in seasonally breeding birds, but the mechanisms that these animals use to integrate supplementary cues remain unclear. I hypothesized that (a) environmental cues that negatively affect energy balance inhibit reproductive development by acting at multiple levels along the reproductive endocrine axis including the hypothalamus (b) that the availability of metabolic fuels conveys alterations in energy balance to the reproductive system. I investigated these hypotheses in male house finches, *Haemorhous mexicanus*, caught in the wild and brought into captivity. I first experimentally reduced body condition through food restriction and found that gonadal development and function are inhibited and these changes are associated with changes in hypothalamic gonadotropin-releasing hormone (GnRH). I then investigated this neuroendocrine integration and found that finches maintain reproductive flexibility through modifying the release of accumulated GnRH stores in response to energetic conditions. Lastly, I investigated the role of metabolic fuels in coordinating reproductive responses under two different models of negative energy balance, decreased energy intake (food restriction) and increased energy expenditure (high temperatures). Exposure to high temperatures lowered body condition and reduced food intake. Reproductive development was inhibited under both energy challenges, and occurred with decreased gonadal gene expression of enzymes involved in steroid synthesis. Minor changes in fuel utilization occurred under food restriction but not high temperatures. My results support the hypothesis that negative energy balance inhibits reproductive development through multilevel effects on the hypothalamus and gonads. These studies are among the first to demonstrate a negative effect of high temperatures on reproductive development in a wild bird. Overall, the

above findings provide important foundations for investigations into adaptive responses of breeding in energetically variable environments.

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CHAPTER 1

INTRODUCTION

Obtaining adequate energy is essential for all organisms to survive and reproduce. Reproduction requires an increase in energy expenditure. Thus, in many organisms the timing of seasonal breeding has evolved to capitalize on changing food availability (Lack, 1968; Nager et al. 1997), such that an increase in energy expenditure can be matched with an increase in energy intake. Most small passerines are income breeders in which breeding costs must be covered by daily foraging rather than energy store utilizations. The breeding period must, therefore, be matched with the time(s) of year when ambient conditions, including temperature, are relatively mild and food is readily available (Charmentier et al., 2008; Hořák et al., 2015; Thomas et al., 2001; Wingfield, 1983). Synchronizing breeding with periods of peak food availability maximizes fitness across a variety of species (Both et al., 2006; Daan et al., 1990; Thomas et al., 2001; Williams, 2012a).

Birds that breed seasonally undergo extensive annual changes in their reproductive system, such that gonads grow several hundred fold (Dawson et al., 2001) in preparation for the breeding season and regress at the end of this season. Environmental cues that reliably precede changes in food availability are often used to activate the reproductive neuroendocrine system and ultimately gonadal development. The annually changing photoperiod (day length) is the primary cue that most temperate region-breeding birds utilize to stimulate reproductive onset (Dawson and Sharp, 2007; Farner, 1985). The regulation of the hypothalamic-pituitary-gonadal (HPG) axis by photoperiod is well described in seasonally breeding birds, and the general mechanism is highly conserved across vertebrates. In response to long days, gonadotropin-releasing hormone-I (GnRH) release from the hypothalamus stimulates the anterior pituitary gland to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Hattori et al., 1986; Sharp et al., 1990). LH and FSH then act on the gonads to increase steroid hormone production and secretion, gonadal growth, and gametogenesis (Deviche et al., 2005; Kirby and Froman, 2000).

Annual changes in photoperiod are consistent but local environmental conditions can vary from year to year. As a result, birds use supplementary factors (e.g., temperature, food availability, rainfall, and social interactions) to synchronize gonadal development and breeding with optimal local environmental conditions (Dawson and Sharp, 2007). The mechanisms by which the HPG axis integrates supplementary environmental information are presently unclear. Energy availability is responsible for the timing of breeding in both an evolutionary and physiological context. Understanding its role in modifying metabolic and reproductive physiology as well as the mechanisms through which it acts is, therefore, especially important in furthering our understanding of adaptive breeding responses under fluctuating energetic conditions. There is extensive evidence in wild birds relating changes in food availability to reproduction, with food supplementation advancing breeding onset and success (Ruffino et al., 2014), and food manipulations in captive birds affecting multiple aspects of reproductive physiology and behavior (Davies and Deviche, 2014). To date, investigations into the mechanisms regulating reproductive physiology in response to food availability have primarily used domestic birds (Bruggeman et al., 1998; Ciccone, 2007) or wild birds that breed opportunistically rather than seasonally (Hahn, 1995; O'Brien and Hau, 2005; Perfito et al., 2008). Information obtained from these species does not necessarily extend to wild, seasonally breeding species. Indeed, chickens, for example, have been artificially selected with regard to both reproduction and metabolism, and opportunistic breeders are thought to rely primarily on non-photic cues and/or endogenous circannual clocks to time changes in their reproductive physiology. Furthermore, experimental food manipulations sometimes influence body condition (Davies et al., 2015a; Fokidis et al., 2012; Meijer, 1991; Perez-Rodriguez et al., 2006), but the concurrent measurement of reproductive effects and specific markers of energy homeostasis in any wild birds is rare.

In addition to the influence of energy intake, situations that increase energy demand and expenditure related to non-reproductive activities can also affect reproductive timing and investment. Experimental increases in energy expenditure, for example in locomotor activity (Deerenberg et al., 1998; Martin et al., 2003), immune activity (Wilkelski et al., 1999), or thermoregulation (Smith et al., 2015), elevate metabolic rate and alter a bird's energy budget.

While partial compensation is possible (Deerenberg et al., 1998; Hawley et al., 2012; Wilkelski et al., 1999), the timing of reproduction may be delayed and/or the energy allocated to reproduction may be reduced (Lynn et al., 2003). Circannual rhythms have been hypothesized to track and respond to energy turnover, independent of external environmental factors (Wilkelski et al., 2008), but how the circannual activity of the HPG axis, specifically, responds to energy turnover, and the mechanisms by which the HPG axis integrates energy-related information remain unresolved.

Non-photoperiodic cues are generally thought to affect HPG axis activity through converging on GnRH cells in the hypothalamus to influence their activity (Ciccone, 2007; Dawson and Sharp, 2007; Mantei et al., 2008; Moore et al., 2006; Small et al., 2007; Stevenson and Ball, 2009). However, these cues can exert effects at multiple levels of the HPG axis, in some cases directly influencing gonadal function (McGuire et al., 2013). Functional redundancies and plasticity in these mechanisms are thought to exist, as integrating information regarding energy intake, storage, and expenditure is crucial to species survival (Bellefontaine et al., 2014). Investigating the central and peripheral mechanisms by which animals integrate energetic information will help in understanding the plasticity of breeding responses and, ultimately, how populations succeed or fail in adjusting to environmental changes.

Study Species

The house finch, *Haemorhous mexicanus,* is a seasonally breeding songbird of the subfamily Carduelinae (in the Fringillidae family) that is found across the western and eastern U.S. The desert southwest, including Mexico, is their native range, where they are especially abundant and inhabit rural, urban, and suburban environments (Howell and Webb, 1995). House finches are seasonal breeders and have been used to investigate photoperiodism both in early and more recent studies (Cho et al., 1998; Hamner, 1966, 1968; Salvante et al., 2013). They exhibit a photoperiodic cycle of gonadal growth (Hamner, 1966) and GnRH activity (Cho et al., 1998) typical of northern temperate zone birds. Therefore, the photoperiodic responses of the HPG axis of this species are well-characterized, providing a model in which to study the additional influences of non-photic factors on HPG axis activity.

Proper synchronization of reproductive development between male and female birds is necessary for breeding to occur (Caro et al., 2009), and so obtaining information from both sexes is ideal. However, as is commonly the case in wild birds, captive female house finches failed to undergo follicular development in response to photostimulation in my pilot studies. Therefore, I, conducted my studies only on male finches. Male house finches feed their mate during breeding and incubation, and they provide food to the hatchlings and fledglings (Thompson, 1960). Food availability in this species is, therefore, likely to affect both male and female reproductive efforts. Female songbirds of other species have been described as being more reliant than males on non-photic cues, including food availability (Ball and Ketterson, 2008; Caro et al., 2009; Caro, 2012). If this is true in house finches, effects of food manipulations on the male reproductive system likely occur also in females, perhaps to an even greater extent.

The ability of house finches to inhabit and breed in hot, desert environments with temperatures often above their thermoneutral zone (TNZ) provides a unique opportunity to examine energy homeostasis and reproductive physiology under high, albeit natural temperatures. Additionally, the Carduelinae subfamily is comprised of species with diverse breeding patterns, including opportunistic breeders, and these other members of this subfamily have been used to investigate the reproductive responses to photic and non-photic cues (Hahn, 1995; Hahn et al., 1995). These studies facilitate the potential for comparative analysis of relationships between breeding strategies and environmental regulation of the HPG axis in a phylogenetic context (Hahn and MacDougall-Shackleton, 2008).

Dissertation Overview

The aim of my dissertation is to investigate the endocrine and metabolic mechanisms that mediate the effects of negative energy balance on reproductive physiology. I tested the hypotheses that (1) negative energy balance inhibits gonadal maturation through multiple regulatory points along the HPG axis, including the hypothalamus and gonads and (2) changes in energy balance are relayed to the HPG axis directly though fuel availability and indirectly through metabolic hormones.

In Chapter 2, I experimentally reduced food availability to a level that decreased body condition. I then examined the effect of this reduction on all levels of the HPG axis through measuring hypothalamic GnRH expression, gonadal growth, morphology, testosterone release, as well as the responsiveness to stimulation at both the pituitary gland and gonadal levels. I also examined the potential role of the stress hormone, corticosterone (CORT), and hypothalamic circuits in mediating these effects.

In Chapter 3, I further investigated the central hypothalamic mechanisms that mediate inhibited HPG axis activity and gonadal development under negative energy balance. In food-restricted finches, I measured a correlate of GnRH release, plasma LH, and the capacity of the hypothalamus to release GnRH in response to pharmacological stimulation.

In Chapter 4, I used two models of negative energy balance, food restriction and high temperatures, to relate changes in key metabolites (glucose and fatty acids) to changes in gonadal development and function. I also examined the peripheral mechanisms that integrate energetic information through measuring gonadal expression of key genes involved in steroid synthesis, spermatogenesis, and the stress response.

CHAPTER 2

FOOD RESTRICTION NEGATIVELY AFFECTS MULTIPLE LEVELS OF THE REPRODUCTIVE AXIS IN MALE HOUSE FINCHES, *HAEMORHOUS MEXICANUS*

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Abstract

Nutrition influences reproductive functions across vertebrates, but the effects of food availability on the functioning of the hypothalamic– pituitary–gonadal (HPG) axis in wild birds and the mechanisms mediating these effects remain unclear. We investigated the influence of chronic food restriction on the HPG axis of photostimulated house finches, *Haemorhous mexicanus.* Food-restricted birds had underdeveloped testes with smaller seminiferous tubules than *ad libitum*-fed birds. Baseline plasma testosterone increased in response to photostimulation in *ad libitum*-fed but not in food-restricted birds. Food availability did not, however, affect the plasma testosterone increase resulting from a gonadotropin-releasing hormone-I (GnRH) or a luteinizing hormone (LH) challenge. The number of hypothalamic GnRH immunoreactive (ir) but not proGnRH-ir perikarya was higher in food-restricted than in *ad libitum*-fed finches, suggesting inhibited secretion of GnRH. Hypothalamic gonadotropin-inhibitory hormone (GnIH)-ir and neuropeptide Y (NPY)-ir were not affected by food availability. Plasma corticosterone (CORT) was also not affected by food availability, indicating that the observed HPG axis inhibition did not result from increased activity of the hypothalamic–pituitary– adrenal (HPA) axis. This study is among the first to examine multilevel functional changes in the HPG axis in response to food restriction in a wild bird. The results indicate that food availability affects both hypothalamic and gonadal function, but further investigations are needed to clarify the mechanisms by which nutritional signals mediate these effects.

Introduction

For many animals, the decision of when to breed is a critical one because timing reproduction to coincide with favorable environmental conditions can substantially impact reproductive success and fitness (Both et al., 2006; Davies and Deviche, 2014; Olsson and Shine, 1997; Thomas et al., 2001). Food availability has long been considered the ultimate

environmental factor influencing breeding seasons in seasonally breeding birds (Hořák et al., 2015; Lack, 1968; Murton and Westwood, 1977; Wingfield, 1983). As most passerines do not store large amounts of energy, timing energetically costly breeding with periods of peak food abundance is crucial. This is especially true for females, who experience an additional energetic cost associated with egg formation (Nager, 2006). During periods of energetic scarcity, birds may delay egg laying and have smaller clutches (Meijer et al., 1990; Rodenhouse and Holmes, 1992), whereas food supplementation can result in birds advancing laying and producing larger clutches (Ruffino et al., 2014). Food availability, therefore, acts both as the ultimate factor and as a proximate factor to control the timing of breeding.

In most birds, reproductive development is seasonally activated through the hypothalamic–pituitary–gonadal (HPG) axis, with long days stimulating gonadotropin-releasing hormone-I (GnRH) secretion from the hypothalamus (Follett et al., 1977; for review, see Dawson, 2015). GnRH stimulates the anterior pituitary gland to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Hattori et al., 1986; Sharp et al., 1990). In male birds, LH and FSH act on the gonads to increase testosterone production and secretion. The overall result is increased gonadal size (Farner and Follett, 1979; Farner and Gwinner, 1980) and an increase in testosterone-mediated secondary sex characteristics and behavior (Kirby and Froman, 2000). The effects of food availability on the HPG axis have been primarily investigated in opportunistically breeding birds, in which food rather than photoperiodc may serve as a primary proximate signal. In opportunistic species, food restriction can inhibit HPG axis activity. For example, in the zebra finch, *Taeniopygia guttata,* food restriction leads to underdeveloped testes (Perfito et al., 2008) and in the red crossbill, *Loxia curvirostra*, it attenuates long day-induced LH secretion (Hahn, 1995). Whether similar inhibition of the HPG axis occurs in more strictly photoperiodic avian species in response to decreased food availability is uncertain.

Non-photoperiodic signals are generally thought to affect the HPG axis by converging on GnRH cells to influence their activity (Dawson and Sharp, 2007), and there is some evidence that the effects of nutritional signals are mediated at the hypothalamic level. Gonadotropin-inhibitory hormone (GnIH) (Tsutsui et al., 2000), which inhibits both LH release from the pituitary gland and

GnRH cells directly (for reviews, see Clarke, 2011; Kriegsfeld et al., 2015; Tsutsui, 2009; Tsutsui et al., 2010, 2012), is one potential candidate in this mediation. Short-term energy deprivation stimulates GnIH activity in the Pekin duck, *Anas platyrhynchos domestica* (Fraley et al., 2013), and GnIH can stimulate food intake in birds and mammals (for reviews, see Clarke et al., 2012; Kriegsfeld et al., 2015; Tsutsui, 2009; Tsutsui et al., 2010, 2012). Neuropeptide Y (NPY) is another neuropeptide that may be involved in transducing metabolic information to GnRH cells. The orexigenic role of NPY is well known (Bungo et al., 2011; Bechtold and Loudon, 2013; Davies and Deviche, 2015; Kuenzel et al., 1987; Richardson et al., 1995) and there are potential links between NPY cells and GnRH release (Contijoch et al., 1993; McShane et al., 1992) as well as between NPY and GnIH activity (Klingerman et al., 2011). The GnIH–NPY axis is thus hypothesized to play an important role in relating energy homeostasis to reproduction (Davies and Deviche, 2014).

Alternatively or concurrently, food availability may influence the HPG axis activity at levels other than the hypothalamus. The sensitivity of the pituitary gland to GnRH, or of the testes to LH, may be regulated by environmental factors (Jawor et al., 2006; Perfito et al., 2011). This sensitivity can be probed using hormonal challenges, in which subjects receive GnRH or LH, and the downstream hormonal response, either LH or testosterone, is then measured (Bergeon Burns et al., 2014; Jawor et al., 2006; Perfito et al., 2011). In domestic chickens, *Gallus gallus domesticus*, food restriction alters the LH response of the pituitary gland to GnRH (Bruggeman et al., 1998; Tanabe et al., 1981). To our knowledge, the HPG axis responsiveness of male birds under different energetic states has only been investigated in Abert's towhees, *Melozone aberti* (Davies et al., 2015a). As the coordination of breeding with energetically favorable conditions is crucial, we hypothesized that there are multiple sites of regulation on the HPG axis, and that altering the sensitivity of the pituitary gland and gonads to upstream hormones may serve as energy-dependent regulatory mechanisms. The effects of food availability on the HPG axis may be mediated directly by metabolic information (e.g. glucose and fatty acids), but intermediate metabolic hormones may cause indirect effects. In particular, decreased food availability may in some circumstances be perceived as a stressor and increase glucocorticoid secretion (Fokidis et

al., 2012; Lynn et al., 2010). Glucocorticoids, including corticosterone (CORT), negatively affect reproduction by inhibiting HPG axis activity (Sapolsky et al., 2000; Schoech et al., 2009) and thus could potentially serve as mediators between energetic state and reproductive function. Few studies, however, have examined the effects of food restriction simultaneously on plasma CORT and HPG axis activity.

The objective of the present study was to evaluate the effects of energetic deficit on HPG axis functionality and on reproductive morphology in a seasonally breeding, photoperiodic male songbird, the house finch, *Haemorhous mexicanus* (Müller 1776).Male House finches exhibit increased HPG axis activity and gonadal growth in response to long days (Cho et al., 1998; Hamner, 1966, 1968). To determine whether adequate energetic balance is necessary during this time to stimulate the HPG axis, we manipulated food availability in captive birds exposed to long days. We hypothesized that food availability is an important factor affecting HPG axis activity and testicular development, and that it acts at multiple levels of the HPG axis to precisely modulate this activity.

We assessed reproductive morphology by measuring growth of the testes and the cloacal protuberance, an androgen-dependent secondary sexual characteristic, as well as the size of the seminiferous tubules. HPG axis functionality was assessed at multiple levels. Hypothalamic regulation was examined via neuropeptide (GnRH, its precursor proGnRH, GnIH and NPY) immunoreactivity (ir). Baseline plasma testosterone was examined throughout the experiment, and the plasma testosterone response to exogenous administration of GnRH and LH was measured. Lastly, to determine whether CORT mediates the effects of negative energetic balance on reproductive function, we measured plasma CORT throughout the experiment. We predicted that food- restricted birds would exhibit smaller gonads and lower baseline plasma testosterone, attenuated LH- and GnRH-induced plasma testosterone, and lower GnRH (-ir) in the hypothalamus. We further predicted the GnIH–NPY system and/or plasma CORT to change with energetic state, such that brain GnIH-ir and NPY-ir, and plasma CORT would increase in response to food restriction.

Methods

All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee. All necessary permits to capture animals were obtained through the US Fish and Wildlife Service and the Arizona Game and Fish Department.

Capture and initial conditions. Adult male House finches (N=16) were caught in Tempe, AZ, USA, between 4 and 21 January 2014, at which time they were naturally exposed to a non- stimulating photoperiod. Birds were caught using food-baited traps and were sexed based on plumage coloring, and aged based on molt patterns (Pyle, 1997). Only post-second year (i.e. hatched in 2012 or earlier) males were selected. Birds were transported to Arizona State University Animal Care Facilities and placed in visually isolated, individual cages at 25°C. Birds were exposed to a short, non-stimulatory photoperiod (10 h light: 14 h dark, lights on at 07:30 h) similar to natural conditions. House finches regain photosensitivity by the end of October (Hamner, 1966) and were thus photosensitive at the time of the experiment. Birds were initially given sunflower seeds *ad libitum* and transferred to Mazuri small bird breeding diet (PMI Nutrition International, Richmond, IN, USA) over the course of 1 week.

Food restriction and photostimulation. The individual daily food consumption of each bird was measured over the course of 2 weeks. Birds were then randomly divided into two groups (N=8): (1) *ad libitum* food availability (controls) and (2) food restricted. Food-restricted birds were given a daily ration equal to 70% of their *ad libitum* food intake. We selected this amount based on a pilot study, which showed that a 70% food restriction resulted in an approximate 10% decrease in body mass. All birds were weighed daily to the nearest 0.1 g. At the start of the food restriction period (time 0), all birds were transferred to a moderately stimulatory day length (13 h light: 11 h dark, lights on at 06:00 h) for the remainder of the study (7 weeks).

Morphology. In addition to daily monitoring of body mass, body fat and muscle stores were estimated 3 times throughout the study: prior to photostimulation and the food restriction treatment (time 0), after 4 weeks of treatment, and after 7 weeks of treatment. The amount of furcular fat was visually estimated using a scale of 0–5, with 0 for no fat and 5 for bulging fat (Helms and Drury, 1960). As the pectoral muscles are the largest store of protein in birds, their size was estimated using a scale of 0–3, with 0 for concave pectoral muscles and a prominent

keel and 3 for convex pectoral muscles that protrude above the keel (Salvante et al., 2007). At each of these three time points, cloacal protuberance width (±0.1 mm) was also measured using digital calipers.

Blood sampling and hormone challenges. The effect of food restriction on the plasma testosterone response to acute GnRH and ovine LH treatment was investigated after 4 weeks of photostimulation. An initial blood sample (100 µl) was taken from the jugular vein of each finch into a heparinized microsyringe and immediately placed on ice. This blood sample was obtained within 3 min of reaching into the bird's cage. Each bird then received an intramuscular injection of either synthetic GnRH (Sigma Chemical Co., MO, USA; 5 mg/kg) or freshly prepared ovine LH solution (Batch AFP5551B; The National Peptide and Hormone Program, Harbor-UCLA Medical Center, Torrance, CA, USA; 1mg/kg) dissolved in 100 µl of sterile saline solution. Birds were returned to their cage and bled again (100 µl) 30 min later. Each bird received the opposite hormone treatment 1 week later. Injected hormone concentrations and sampling time were based on previous studies successfully showing treatment effects on plasma testosterone (Deviche et al., 2012; Jawor et al., 2006). Blood was centrifuged within 3 h, and plasma was collected and stored at −80°C until assayed. All samples were collected between 10:00 h and 15:00 h.

Additional blood samples for baseline hormone measurements (plasma testosterone and CORT) were obtained prior to the start of the treatment and after 7 weeks of treatment. At each time, blood (100 µl) was taken and plasma was stored as described above for initial blood sampling. The initial blood samples obtained at weeks 4 and 5 (i.e. before hormone challenges) were also used to compare baseline plasma testosterone and CORT throughout the study.

Tissue processing. After 7 weeks of photostimulation and food treatment, and 2 weeks after the last hormone challenge, birds received an intramuscular injection of 400 µl anesthetic solution (0.9% NaCl containing 20 mg/ml xylacine and 100 mg/ml ketamine). Birds were perfused transcardially with 35 ml wash solution (0.9% NaCl and 0.1% $NaNO_2$ in 0.1 mol/l phosphate buffer, PB) followed by 35 ml of fixative (4% paraformaldehyde and 0.1% $NaNO_2$ in 0.1 mol/l PB). Birds were then decapitated. The testes were removed and the brains were dissected out.

Testes were rinsed in PB and weighed to the nearest 0.01 mg. Both testes from each bird were placed together in molds containing tissue freezing medium and flash-frozen in an alcohol/dry ice slurry. They were kept at −80°C until sectioned. Testes were cryostat-sectioned (25 µm thick sections) at −15°C and sections were collected onto glass slides. Slides were kept at 4°C until stained with hematoxylin and eosin for histological examination.

Brains were placed in fixative overnight at 4°C and washed three times in wash solution. They were cryoprotected in 30% sucrose in 0.1 mol/l PB at 4°C for 2 days until they had sunk. Brains were then placed in plastic molds containing tissue freezing medium and flash-frozen in an alcohol/dry ice slurry. They were kept at −80°C until sectioned. Brains were cryostat- sectioned coronally (25 µm thick sections) at −20°C using the canary brain stereotaxic atlas as a reference (Stokes et al., 1974). Sections were collected in five parallel series, one for each immunocytochemical procedure (GnRH, proGnRH, GnIH, NPY) plus one extra series. Sections were placed in wells containing cryoprotectant solution (Watson et al., 1986) and kept at −20°C until immunolabeling.

Testis histology. For measurement of the diameter of seminiferous tubules, one right and one left testis section from each bird, stained with hematoxylin and eosin, was used. These sections were taken from the largest part of the testis. One photograph of each testis section was taken using an Olympus DEI-750D digital camera mounted on an Olympus BX60 light microscope (Olympus Optical Co. Ltd, Tokyo, Japan) at 40× magnification. PhotograpHT were analyzed using Image-Pro Plus software (Media Cybernetics, LP, Silver Spring, MD, USA). First, a line from the upper left corner to the lower right corner was drawn on the image. The smallest diameter of the first five tubules that intersected with the line was measured using the automated measurement tool, with manual selection of the tubule outline. If fewer than five tubules intersected the line, the next tubules outward were measured until five tubules per testis (i.e. 10 tubules per bird) were measured. The smallest diameter of each tubule was averaged for each bird to obtain a single seminiferous tubule diameter value.

Plasma testosterone and CORT assays

Testosterone. A validated (Deviche and Cortez, 2005) commercial enzyme-linked immunoassay (Enzo Life Sciences, Farmingdale, NY, USA) was used to measure plasma testosterone. Instructions outlined by the manufacturer were followed. Plasma was diluted 15×in assay buffer containing 1 µl displacement reagent: 99 µl plasma. Samples were assayed in duplicate with all samples from each bird on a single plate, but with samples randomized on each plate, and with birds randomized across three plates. Each plate included a complete standard curve. Three additional house finch plasma samples were used as an internal control across the three plates. The assay sensitivity was 29.5 pg/ml and the inter- and intra-assay coefficients of variation were 3.2% (N=3samples assayed on each plate) and 2.2% (N=95 samples), respectively.

CORT. A commercial enzyme-linked immunoassay (Enzo Life Sciences), was used to measure plasma CORT. Instructions outlined by the manufacturer were followed. Plasma was diluted 20× in assay buffer containing 2.5 µl displacement reagent: 97.5 µl plasma. Samples were assayed in duplicate with all samples from each bird on a single plate, but with all samples randomized on each plate, and with birds randomized across two plates. Each plate included a complete standard curve. Two additional house finch plasma samples were used as an internal control across the two plates. The assay sensitivity was 48.75 pg/ml and the inter- and intra-assay coefficients of variation were 3.5% (N=2 samples assayed on each plate) and 2.1% (N=64 samples), respectively. A house finch plasma dilution curve was parallel to a standard curve run on the same plate ($F_{1,12}$=0.0044, P=0.95), validating the use of this assay in house finches.

GnRH, ProGnRH, GnIH and NPY immunocytochemistry. Brain sections were labeled for chicken GnRH-like-immunoreactivity (cGnRH-ir), proGnRH-ir, GnIH-ir and NPY-ir. The region containing each neuropeptide was located using anatomical landmarks (see below), and one parallel series collected was used for each assay, with sections on either side of the region of interest included in the assay. Between two and four assays were performed for each neuropeptide, with birds from each treatment group equally represented on each assay. Immunocytochemical labeling was done using a previously published procedure (Deviche et al., 2000; Small et al., 2008). Briefly, free-floating sections were washed three times for 20 min in 0.1

mol/l PB, incubated in 0.36% hydrogen peroxide, washed 3 times for 5 min in 0.1 mol/l PB, incubated for 1 h in normal blocking serum, and incubated overnight at 4°C in primary antibody. The next day, sections were washed 3 times for 10 min in 0.1 mol/l PB with 0.1% Triton X-100 (Sigma-Aldrich Co., St Louis, MO, USA; 0.1% PBT), incubated for 1 h in secondary antibody, washed 3 times in 0.1% PBT, incubated for 1 h in Vectastain ABC solution (Vector Laboratories, Inc., Burlingame, CA, USA), washed 3 times for 10 min in 0.1% PBT, incubated in Vector SG chromagen, and washed two times for 5 min in 0.1 mol/l PB. Sections were mounted on glass slides, dried overnight, and coverslipped using Cytoseal 60 (Stephens Scientific, Kalamazoo, MI, USA).

GnRH. The tractus septomesencephalicus (TrSM) was used as an anatomical landmark for identifying the preoptic area (POA), the region where GnRH cells are located (Stokes et al., 1974). The primary antibody (6DL31/4 provided by P. J. Sharp, University of Edinburgh, UK) was used at a 1:20,000 dilution in 0.3% PBT. The blocking serum used was normal rabbit serum (Vector Laboratories) at a 1:66 dilution in 0.3% PBT. The secondary antibody used was biotinylated rabbit anti-sheep IgG (Vector Laboratories) at a 1:200 dilution in normal rabbit blocking serum. Sections were incubated in chromagen for 3.5 min.

ProGnRH. Again, the TrSM was used as a landmark for identifying the POA. The primary antibody (1947; Roberts et al., 1989; Dutlowet al., 1992) was used at a 1:1000 dilution in 0.3% PBT. The blocking serum used was normal horse serum (Vector Laboratories) at a 1:33 dilution in 0.3% PBT. The secondary antibody used was biotinylated horse anti-mouse/rabbit IgG (Vector Laboratories) at a 1:100 dilution in 0.3% PBT. Sections were incubated in chromagen for 3 min.

GnIH. The anterior commissure was used as a landmark for identifying the paraventricular nucleus (PVN), the region containing GnIH cells (Tsutsui et al., 2000). The primary antibody (anti-quail GnIH antibody; Tsutsui et al., 2000) was used at a 1:10,000 dilution in 0.3% PBT. The blocking serum and secondary antibody solutionswere the same as in the proGnRH staining, and sections were incubated in chromagen for 3 min.

NPY. The median eminence (ME) was used as a landmark for identifying the infundibular nucleus/median eminence area, the region where NPY cells involved in HPG axis activation are

found (Walsh and Kuenzel, 1997). The primary antibody (Bachem Laboratories, Torrence, CA, USA) was used at a 1:20,000 dilution in 0.3% PBT. The blocking serum and secondary antibody solutions were the same as in the proGnRH staining, and sections were incubated in chromagen for 2.5 min.

Immunocytochemical data collection. The number of cGnRH-ir, proGnRH-ir, GnIH-ir and NPY-ir perikarya were counted throughout the hypothalamus using an Olympus BX60 light microscope. For NPY-ir, data could not be collected for one bird as the ME was damaged during sectioning.

For cGnRH-ir and GnIH-ir, the size and optical density of perikarya was quantified. The quality of immunostaining made it impossible to quantify these additional measurements for proGnRH-ir and NPY-ir. Digital photograpHT were taken using an Olympus DEI-750D digital camera mounted on the Olympus BX60 light microscope at 400× magnification. Camera settings were standardized across all photograpHT. Perikarya were randomly selected using a grid and photographed with one central cell in focus. Six cells on every brain section were photographed. For each section, an out-of-focus background photograph of the neostriatum was taken at the same magnification and at the same time to standardize for variation in background immunolabeling and illumination. The neostriatum was selected as it does not contain any GnRH or GnIH cells. Images were analyzed using Image-Pro Plus software. First, the background image was subtracted from the perikaryon-containing image. Each perikaryon was outlined, and the immunolabeling area and optical density (arbitrary units: 0=no staining, 1=complete saturation) were measured. An average value for both perikaryon area and optical density was calculated for each bird.

For cGnRH-ir and GnIH-ir, the density of fibers in the ME was quantified. Three photograpHT spanning the ME were taken for each bird at 100× magnification. The entire cross-sectional area of the ME was present on each photograph. A background image of the neostriatum was also taken at the same time. The background image was subtracted using Image-Pro Plus. The ME was then outlined and the mean optical density of this region was

measured. The three values were averaged to obtain a single value for each bird. Data could not be collected for one bird as the ME was damaged during sectioning.

For GnIH-ir, the density of fibers in the POA was quantified in sections adjacent to those containing GnRH cell bodies. For each of two sections, a photograph on each hemisphere of the brain was taken at 100× magnification. A background image was also taken and subtracted in the same manner as above. One field (650×450 μm^2) was selected on each brain hemisphere. Each field was taken with the bottom side just next to the medial ventricle and the top just below the TrSM. In this area, the mean optical density was measured. The average value for each of the four fields was averaged to obtain one value for each bird.

Statistical analyses. The effects of treatment on body mass, morphological characteristics and plasma hormones were analyzed using two-way repeated measures ANOVA, with time as the within-subject factor and food availability as the between-subjects factor. For ordinal scale data (fat and muscle scores), data were ranked before proceeding with the analysis. Data sets that were not normally distributed or homoscedastic, according to the Shapiro–Wilk test and Levene's test, respectively, were transformed prior to analysis, using either a square root or natural log transformation. For data sets that did not display sphericity, according to Mauchly's sphericity test, degrees of freedom were deflated using a ε-derived Greenhouse–Geisser correction. When a statistically significant treatment × time interaction was detected using ANOVA, SNK tests were used to perform pair-wise comparisons. Effects of treatment on testis mass, seminiferous tubule diameter and neuropeptide expression were analyzed using Student's t-test, except in the case of cell counts, in which non-parametric Mann–Whitney U-tests were used. Data sets that were not normally distributed or homoscedastic, according to the Shapiro–Wilk test and Levene's test, respectively, were transformed prior to analysis, using a natural log transformation. Data were analyzed using SPSS (version 22; IBM, Armonk, NY, USA) and SigmaPlot (version 12.0; Systat Software, Inc., San Jose, CA, USA). The significance level of all statistical tests was set at P=0.05.

Results

Body condition. Body mass was affected by food availability ($F_{1,13}$=16.37, P=0.001), time ($F_{7,91}$=5.70, P=0.003) and the interaction between these factors ($F_{7,91}$=24.08, P<0.001; Fig. 2.1A). *Ad libitum*-fed and food-restricted birds had a similar body mass at the start of the treatment [Student–Newman–Keuls (SNK) tests, P>0.05], and *ad libitum*-fed birds maintained roughly the same body mass throughout the study (SNK tests, P>0.05). By contrast, food-restricted birds lost mass within the first week of treatment and then maintained a lower body mass than *ad libitum*-fed birds for the duration of the study (SNK tests, P<0.05). Furcular fat was affected by food availability ($F_{1,13}$=4.92, P=0.045), time ($F_{2,26}$=5.16, P=0.013) and the interaction between these factors ($F_{1,13}$=4.92, P=0.006; Fig. 1B). *Ad libitum*- fed birds maintained the same amount of fat throughout the experiment (SNK tests, P>0.05), whereas food-restricted birds lost fat stores within the first 4 weeks of the treatment and had less fat than *ad libitum*-fed birds after 7 weeks of treatment (SNK tests, P<0.05). Muscle score was affected by food availability ($F_{1,13}$=5.18, P=0.040), time ($F_{2,26}$=6.66, P=0.005) and the interaction between these factors ($F_{2,26}$=5.72, P=0.009; Fig. 2.1C). *Ad libitum*-fed birds maintained the same amount of muscle throughout the experiment (SNK tests, P>0.05), whereas food-restricted birds lost muscle within the first 4 weeks of the treatment and had less muscle than *ad libitum*-fed birds after 7 weeks of treatment (SNK tests, P<0.05).

Cloacal protuberance. Cloacal protuberance width increased during exposure to long days in both *ad libitum*-fed and food-restricted birds ($F_{2,26}$=16.79, P<0.001; Fig. 2.1D), but was not affected by food availability ($F_{1,13}$=0.65, P=0.44), and there was no food availability × time interaction ($F_{2,26}$=2.42, P=0.11).

Testis morphology. Food-restricted birds had a lower gonadosomatic index (gonad mass as a percentage of body mass) than *ad libitum*-fed birds (t_{13}=5.43, P<0.001; Fig. 2.2). Seminiferous tubule diameter was also smaller in food-restricted birds than in *ad libitum*-fed birds (t_{13}=5.13, P<0.001; Fig. 2.3).

Plasma testosterone. Baseline plasma testosterone was affected by the interaction of food availability and time ($F_{3,39}$=5.82, P=0.002; Fig. 2.4A). Plasma testosterone did not change in response to photostimulation in food-restricted birds (SNK tests, P>0.05), but increased after 4

weeks of photostimulation in *ad libitum*-fed birds (SNK tests, $P<0.05$) before returning to initial levels after 7 weeks of photostimulation.

Plasma testosterone increased in response to GnRH challenge ($F_{1,14}=7.32$, $P=0.017$) and was influenced by food availability ($F_{1,14}=13.16$, $P=0.003$), but there was no interaction between the effect of the GnRH challenge and food availability ($F_{1,14}=0.91$, $P=0.36$; Fig. 2.5A). There was, therefore, no evidence that food availability influenced the plasma testosterone response to a GnRH injection. It is unlikely that this lack of effect resulted from low statistical power to detect differences between food-restricted and *ad libitum*-fed finches ($1-\beta=0.85$).

Similarly, plasma testosterone increased in response to LH challenge ($F_{1,14}=11.46$, $P=0.004$) and was influenced by food availability ($F_{1,14}=14.85$, $P=0.002$), but there was no interaction between LH injection and food availability ($F_{1,14}=0.73$, $P=0.41$; Fig. 2.5B). Thus, as was the case for GnRH, there was no evidence that the plasma testosterone response to LH challenge was modulated by food availability. Firm conclusions on this subject are, however, limited by the relatively low statistical power ($1-\beta=0.23$) of the ANOVA to detect group differences.

Plasma CORT. Baseline plasma CORT decreased during the study ($F_{3,39}=14.37$, $P<0.001$). Food-restricted birds had consistently lower plasma CORT than *ad libitum*-fed birds ($F_{1,13}=6.29$, $P=0.026$) but there was no interaction between time and food availability ($F_{3,39}=1.98$, $P=0.13$; Fig. 2.4B), indicating that the group difference was present throughout the study.

Brain neuropeptide immunoreactivity. We measured several parameters to estimate the hypothalamic content of proGnRH-ir, GnRH-ir, GnIH-ir and NPY-ir. Of these, only one (number of GnRH-ir perikarya) differed between the two experimental groups ($U=8$, $P=0.02$; Fig. 6, Table 2.1A), with food- restricted finches having more immunostained perikarya than *ad libitum*-fed finches.

Discussion

We used a seasonally breeding, photoperiodic songbird to test the hypotheses that (i) energy balance is an important factor affecting HPG axis activity and photoinduced reproductive development, and (ii) energy-mediated signals affect multiple levels of the HPG axis to modulate

this development. To test these hypotheses, we chronically food restricted finches under long-day conditions to induce negative energy balance. The functionality of the HPG axis was assessed based on the measurement of multiple parameters. The size of the testes, seminiferous tubules and cloacal protuberance provided measures of reproductive development. Baseline plasma testosterone and GnRH- and LH-induced plasma testosterone levels indicated HPG axis activity and responsiveness to acute stimulation. Central mechanisms were investigated by comparing proGnRH, GnRH, GnIH and NPY peptide expression in the hypothalamus of food-restricted and *ad libitum*-fed birds. Finally, plasma CORT was measured to assess whether food restriction-induced effects on the HPG axis are associated with enhanced hypothalamic–pituitary–adrenal (HPA) axis activity.

The results support our hypotheses. Food restriction inhibited reproductive development, with food-restricted birds having underdeveloped testes, narrower seminiferous tubules and lower baseline plasma testosterone levels than *ad libitum*-fed birds. Additional effects were seen at the hypothalamic level, with food- restricted birds having a greater number of GnRH perikarya than *ad libitum*-fed birds. However, we found no evidence that food restriction affects the responsiveness of the HPG axis to acute stimulation.

Testicular response to energetic deficit. Chronic food restriction was an effective method to induce negative energy balance. After just 1 week of food restriction, birds had lost body mass, due at least in part to decreased fat and muscle energy stores. The loss of energy stores was associated with profound changes in reproductive morphology. As predicted, food-restricted birds had smaller testes and narrower seminiferous tubules than *ad libitum*-fed birds. The effect of food availability on testicular growth is consistent with results obtained in opportunistic species (Hahn, 1995; O'Brien and Hau, 2005; Perfito et al., 2008). To our knowledge, however, our study is among the first to show an inhibition of testicular development in a predictably breeding, photoperiodic wild songbird under energetic deficit. European starlings, *Sturnus vulgaris,* with reduced body mass as a result of experimentally changing the daily duration of food availability also have underdeveloped testes (Dawson, 1986; Meijer, 1991), but testis growth is unaffected by food availability in Abert's towhees (Davies et al., 2015a). Seasonal

testicular growth is primarily due to proliferation of Sertoli cells, which make up the majority of the mass in developed testes (Deviche et al., 2011; Young et al., 2001). In this study, we found that the smaller testes under food restriction can be at least partially attributed to smaller seminiferous tubules. As seminiferous tubules are the sites of spermatogenesis, these data suggest that food restriction reduced sperm production.

Body condition in free-living house finches correlates positively with plasma testosterone (Duckworth et al., 2001). Consistent with this observation, food-restricted finches were in lower body condition and had lower plasma testosterone than *ad libitum*-fed finches. Chronic food restriction (Davies et al., 2015a; Pérez- Rodríguez et al., 2006) and fasting (Lynn et al., 2010) also decrease plasma testosterone in other avian species, indicating the generality of the plasma testosterone response to energetic challenges. Taken together, these morphological and hormonal data reveal inhibition of both endocrine and exocrine testicular functions during energetic deficit. Food availability is generally thought to be a more important proximate cue in species that rely less on photoperiod to time breeding, but the present results suggest that the inhibitory action of energetic deficit on gonadal development and function may be conserved across species with diverse breeding patterns.

In *ad libitum*-fed birds, plasma testosterone first increased in response to photostimulation, and then returned to initial levels after 7 weeks. There are two potential explanations for this decline in plasma testosterone. The first is that birds at the end of the study had become photorefractory and were undergoing gonadal regression. Captive house finches exposed to 12 h light: 12 h dark begin testicular regression only after 12 weeks, but those exposed to 18 h light: 6 h dark begin regression after 5 weeks (Hamner, 1966). An intermediate day length and duration of exposure (13 h light: 11 h dark, 7 weeks) as used here makes it difficult to determine whether photorefractoriness would have developed. Regardless, birds had large testes after 7 weeks of photostimulation, indicating that gonadal regression might have begun but was not completed. The second and potentially more likely explanation for the observed decline in plasma testosterone is that house finches experience fluctuations in plasma testosterone throughout the breeding season that are not closely associated with gonadal

development. This situation is commonly observed in other photoperiodic species, especially in those such as the house finch that are double-brooded (Dawson, 1983; Wingfield, 1984). This fluctuation has been hypothesized to serve changing behavioral needs during territorial defense, courtship and nesting activity (Wingfield et al., 1987), but the mechanism responsible for these fluctuations is unclear.

Cloacal protuberance growth did not mirror that of the testis. In contrast to testicular size, cloacal protuberance growth in finches was not affected by food availability. Cloacal protuberance size usually varies in parallel with testis size (Perfito et al., 2005; Small et al., 2008) and both are influenced by circulating testosterone levels (Deviche and Cortez, 2005). Although food-restricted finches had lower plasma testosterone than *ad libitum*-fed finches, it appears that the precise relationship between plasma testosterone and cloacal protuberance growth is somewhat dissociated, an observation that is not without precedent (Wingfield et al., 2012). This dissociation may result from the threshold level of plasma testosterone necessary to stimulate cloacal protuberance growth being lower than that necessary for testis growth.

The absence of a plasma testosterone increase in photostimulated, food-restricted finches in the present study may reflect lower plasma LH, resulting in attenuated stimulation of Leydig cells. Supporting this hypothesis, other avian studies found that food restriction can decrease plasma LH (Hahn, 1995; Kobayashi et al., 2002). Alternatively or additionally, food restriction may decrease the sensitivity of Leydig cells to LH. The present results do not favor this hypothesis because LH administration had a similar relative stimulatory effect on plasma testosterone in *ad libitum*-fed and food-restricted finches. However, relatively small sample sizes and high inter-individual variation in the plasma testosterone response to LH injection led to a low statistical power to reveal group differences. Furthermore, our companion study on Abert's towhees found that photostimulated, food-restricted males show a reduced plasma testosterone response to LH injection compared with *ad libitum*-fed males (Davies et al., 2015b). The difference between the results of the two studies may be due to differences in experimental design and statistical analysis. Alternatively, house finches and towhees may use different endocrine mechanisms to regulate the activity of their HPG axis during periods of energy scarcity.

Pituitary gland responsiveness under energetic deficit. GnRH administration increased plasma testosterone in *ad libitum*- fed and food-restricted finches, but we found no evidence for a modulation of this increase by food availability. These data suggest that the energetic state did not influence the sensitivity of the pituitary gland to GnRH. In other species, this sensitivity can fluctuate seasonally, increasing during the breeding season (Jawor et al., 2006; Hirschenhauser et al., 2000). Food restriction attenuated the LH response to GnRH in chickens (Bruggeman et al., 1998; Tanabe et al., 1981) and the testosterone response to GnRH in our study on Abert's towhees (Davies et al., 2015b). The absence of this effect in the current study again suggests either species differences or differences due to experimental design.

Hypothalamus-mediated effects of energetic deficit. As is commonly the case in photoperiodic species (Dawson and Goldsmith, 1997; Hahn and Ball, 1995; Saldanha et al., 1994), the hypothalamic expression of GnRH in the house finch changes seasonally, increasing in preparation for breeding (through increased synthesis) and decreasing at the end of the breeding season as a result of decreased release and synthesis (Cho et al., 1998). In the present study, food restriction resulted in an increased number of hypothalamic cGnRH-ir perikarya, but other measures of GnRH system activity (perikaryon immunostaining area and optical density; density of the median eminence cGnRH-ir fibers) were not affected by the treatment. In previous studies, increased hypothalamic cGnRH-ir perikaryon number (under short-day conditions) was interpreted to reflect cellular build-up of the peptide as a result of decreased transport and/or secretion (Foster et al., 1988), and decreased cGnRH-ir perikaryon number can occur with increased cellular activation and release of the peptide (Lee et al., 1990). Inhibited secretion of GnRH, rather than increased production of GnRH, is a probable explanation based on the observation that food restriction did not influence hypothalamic proGnRH-ir expression, which correlates with GnRH production (Parry et al., 1997). We therefore propose that prior to photostimulation, GnRH in the present study increased through renewed synthesis associated with the development of photosensitivity (Dawson and Goldsmith, 1997; Deviche et al., 2000; Parry et al., 1997; Stevenson et al., 2012a), and that during photostimulation, food restriction

decreased GnRH secretion, resulting in increased brain GnRH stores and a larger number of visible cGnRH-ir perikarya in food-restricted than in *ad libitum*-fed finches.

The present results do not preclude the possibility that energetic factors regulate GnRH function indirectly, i.e. by acting at the testicular rather than hypothalamic level. Steroid feedback by testosterone negatively affects hypothalamic cGnRH-ir (Deviche et al., 2006). As food-restricted birds had lower plasma testosterone than *ad libitum*-fed birds, a decrease in gonadal steroid feedback may have stimulated GnRH synthesis. Again, however, the observation that proGnRH-ir expression did not differ between treatment groups does not support the proposition that food restriction increased hypothalamic GnRH production.

In mammals, energy deficits influence the activity of the HPG axis by acting primarily on the GnRH system (Wade et al., 1996). This system is speculated to also be the primary site of regulation in birds, but there is little research to resolve this. We sought to identify potential hypothalamic mediators of GnRH activity in the house finch. The GnIH–NPY axis is a good candidate for mediating energetic signals on the reproductive axis (Davies and Deviche, 2014), with GnIH-ir and NPY-ir affected by 4 weeks of food restriction in Abert's towhees (Davies et al., 2015a). However, we found no change in hypothalamic GnIH expression in response to food restriction, which provides no evidence that GnIH is involved in mediating energy-related signals in house finches. Likewise, we found no evidence for the involvement of NPY.As birds were killed after 7 weeks of food restriction, we cannot, however, exclude that GnIH and/or NPY mediate faster acting and temporary effects of energetic status on GnRH cells that were not detected under the current conditions.

CORT, energy homeostasis and the HPG axis. Glucocorticoids, such as CORT, have been negatively related to body condition in multiple wild birds, including house finches (Duckworth et al., 2001), and food restriction increases plasma CORT in other species (Lynn et al., 2010; Pérez-Rodríguez et al., 2006). A role for glucocorticoids in suppressing HPG axis activity is also well studied in avian species (Deviche et al., 2012; Kwok et al., 2007; Lynn et al., 2010; Wingfield et al., 1982). We hypothesized that an increase in plasma CORT during food restriction contributes to inhibition of the HPG axis. However, we found no effect of food

restriction on plasma CORT and the data therefore do not support a role for this hormone in the observed changes in HPG axis activity resulting from energetic deficit.

Metabolic versus perceptual pathway. Whether food availability acts as a proximate cue to affect breeding through direct metabolic effects (availability of energy) or through indirect perceptual effects (visual and tactile pathways) remains a matter of debate (Hahn et al., 2005). Avian testes in vitro respond directly to metabolic stress (McGuire et al., 2013), and in mammals, the administration of glucose (Ohkura et al., 2000) and fatty acids (Garrel et al., 2011) can alter LH secretion. Generally consistent with these observations, in the European starling, decreased access to food affected gonadal maturation only when birds also lost body mass (Meijer, 1991; Dawson, 1986), implicating the importance of a metabolic pathway in influencing the HPG axis. By contrast, a perceptual pathway (visual cues) appears to participate in food availability-mediated effects on the HPG axis in the spotted antbird, *Hylophylax n. naevioides* (Hau et al., 2000). Whether this is also the case in house finches is not known. Indeed, food restriction in the present study resulted in energetic deficit as indicated by decreased fat and muscle stores. However, food-restricted birds may also have been exposed to decreased visual and tactile cues (associated with less food in their bowl) than *ad libitum*-fed birds. Further studies are necessary to clarify the pathway(s) by which food availability affects the HPG axis in this and other avian species. Additionally, there is a need for studies investigating the effects of food availability on female birds, which, because of a higher energy investment in reproduction, are likely to be even more sensitive to fluctuations in energy homeostasis (Ball and Ketterson, 2008; Caro, 2012; Caro et al., 2009; Farner and Follett, 1979).

Conclusions. The energetic condition of house finches influences testicular development and function. These effects may result from inhibition of the entire HPG axis through lower GnRH release or be directly regulated at the testicular level. However, we found no evidence that changes in the sensitivity of the pituitary gland or gonads to GnRH or LH, respectively, during food restriction underlie the observed difference in plasma testosterone. The present data also do not provide evidence that food restriction alters the activity of the HPA axis and, therefore, that CORT is responsible for the observed difference in plasma testosterone. If energetic signals

primarily affect the HPG axis through changes in GnRH activity, the GnIH– NPY axis does not appear to modulate this activity long-term. Further understanding of the mechanisms by which energetic homeostasis affects the HPG axis in avian species will benefit from investigations aimed at identifying the level(s) of integration on the HPG axis as well as the metabolic factors and hormones involved.

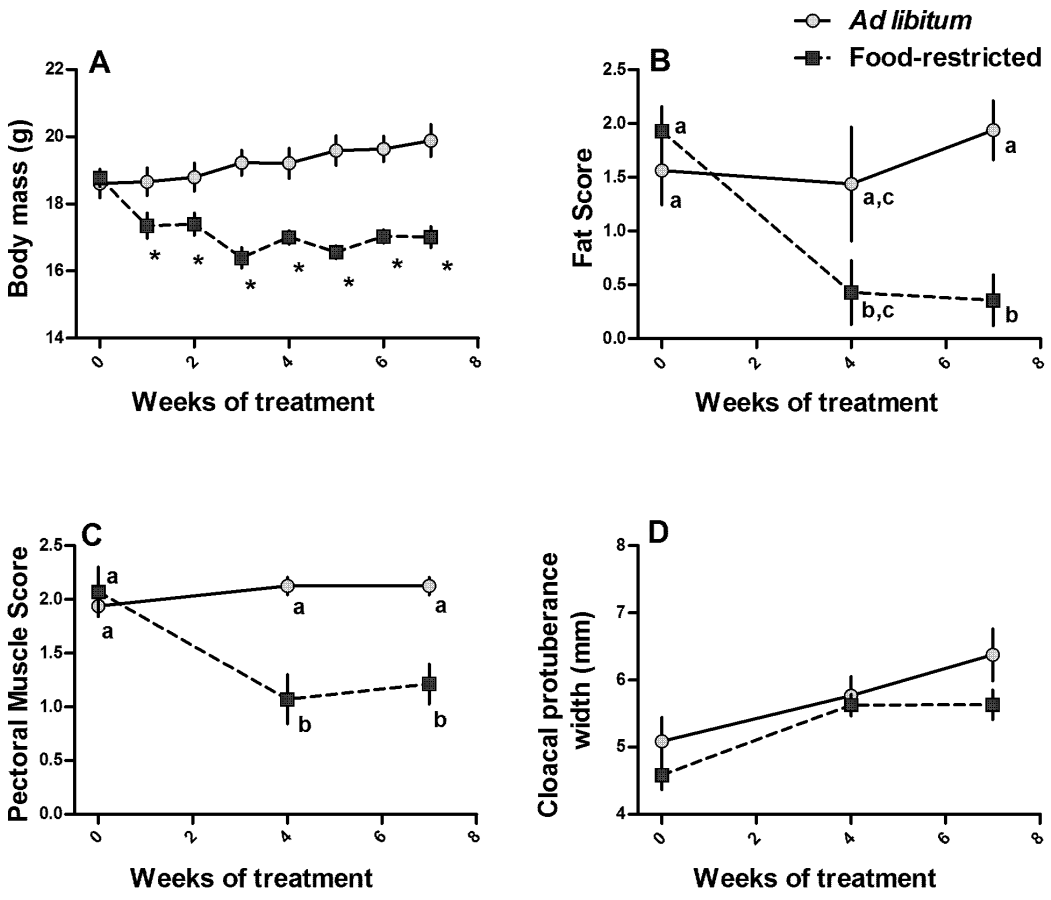

Figure 2.1. Food restriction negatively affects body mass (A), furcular fat (B) and pectoral muscle (C), but does not affect CP width (D), in male house finches, *Haemorhous mexicanus*. Birds were either fed *ad libitum* (N = 8, light circles) or food-restricted (N = 7, dark squares) and photostimulated at time 0. Data are plotted as means ± SEM. Points with identical letters are not statistically different (P > 0.05, SNK tests). An asterisk indicates that within a timepoint, the two groups are statistically different.

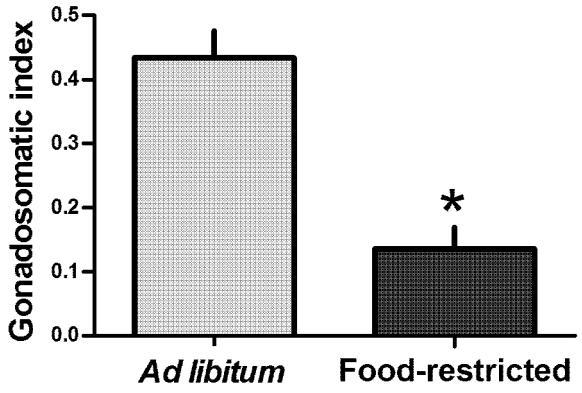

Figure 2.2. Gonadosomatic index (testis mass as a percentage of body mass) is lower in food-restricted male house finches, *Haemorhous mexicanus.* Birds were either fed *ad libitum* (n = 8, light bar) or food-restricted (N = 7, dark bar) and photostimulated for 7 weeks. Data are plotted as means ± SEM, and the asterisk denotes a significant difference between the groups ($P < 0.05$; Student's t-test).

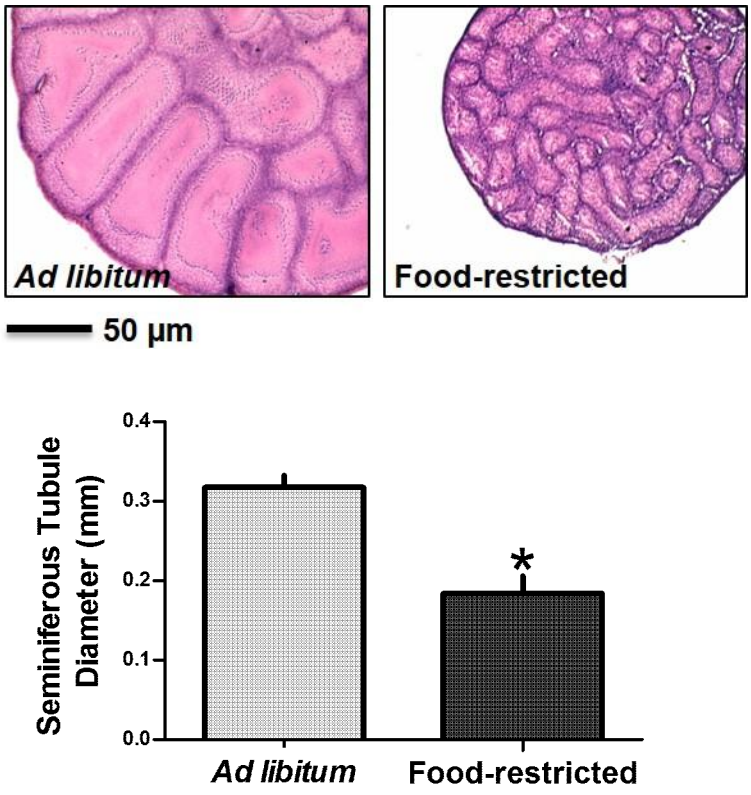

Figure 2.3. The diameter of ST of the testes is smaller in food-restricted male house finches, *Haemorhous mexicanus*. Birds were either fed *ad libitum* (N = 8, first panel, light bar) or food-restricted (n = 7, second panel, dark bar) and photostimulated for 7 weeks. Data are plotted as means ± SEM, and the asterisk denotes a significant difference between the groups ($P < 0.05$; Student's t-test).

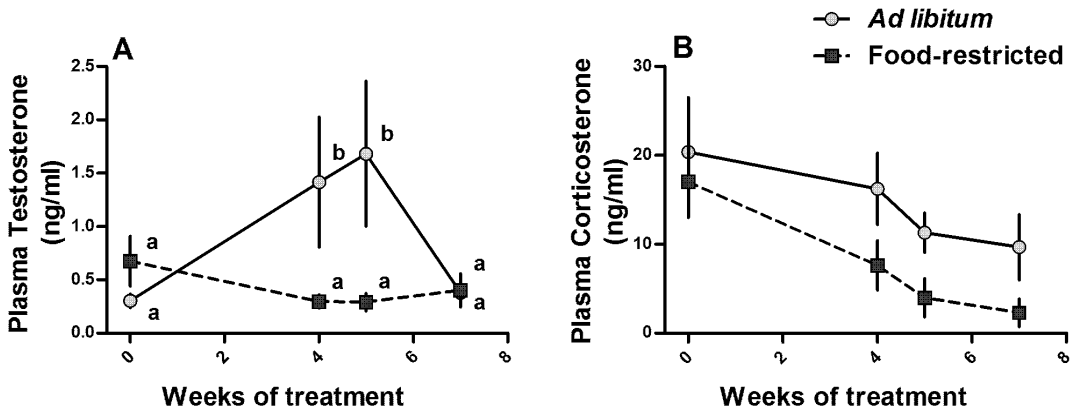

Figure 2.4. Food restriction suppresses the long day-induced increase in baseline plasma T in male house finches, *Haemorhous mexicanus,* but does not affect baseline plasma CORT. Birds were either fed *ad libitum* (N = 8, light circles) or food-restricted (N = 7, dark squares) and photostimulated at time 0. Data are displayed as means ± SEM Means with identical letters are not statistically different (P > 0.05, SNK tests).

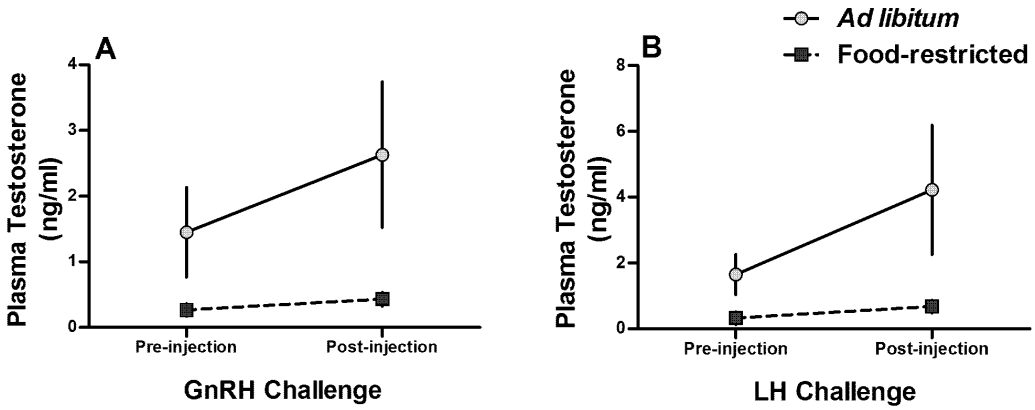

Figure 2.5. The plasma T increase that occurs as a result of GnRH or LH challenge is not affected by food availability in male house finches, *Haemorhous mexicanus.* Birds were either fed *ad libitum* (N = 8, light circles) or food-restricted (N = 8, dark squares) and exposed to a GnRH (A) or LH (B) challenge. Data are shown as means ± SEM

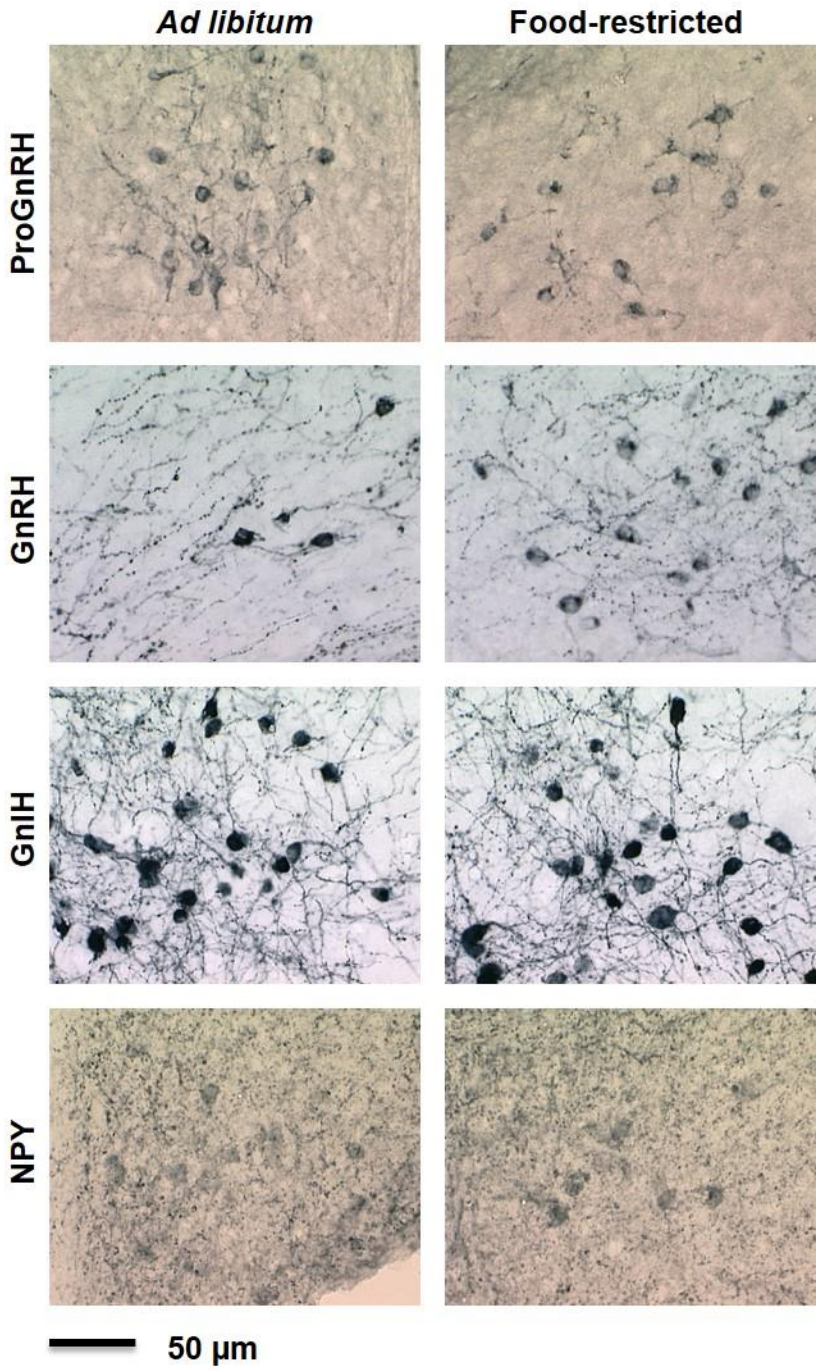

Figure 2.6. Representative photomicrograph of hypothalamic brain sections illustrating the pattern of GnRH-I, proGnRH, GnIH and NPY immunoreactivity in male house finches, *Haemorhous mexicanus.* Birds were either fed *ad libitum* (left panel) or food-restricted (right panel) and photostimulated for 7 weeks.

Table 2.1. Immunocytochemical analysis of proGnRH, GnRH, GnIH and NPY in house finches, *Haemorhous mexicanus.* Birds were either fed *ad libitum* or food-restricted and photostimulated for 7 weeks. Cell count data were analyzed by Mann-Whitney U tests. Data are given as median and interquartile range (IQR). Cell area, cell optical density, and fiber density was analyzed by Student's t-tests. Data are presented as means ± SEM

		Ad libitum Median (IQR)	**Food-restricted** Median (IQR)	U	P	
GnRH	Perikaryon number	96 (65)	135 (30)	8	**0.021**	
ProGnRH	Perikaryon number	92 (119)	98 (46)	26	0.870	
GnIH	Perikaryon number	233 (124)	269 (242)	25	0.779	
NPY	Perikaryon number	128 (72)	118 (40)	21	0.710	
		Ad libitum Mean (SEM)	**Food-restricted** Mean (SEM)	T	DF	P
GnRH	Cell area (μm^2)	88.70 (4.020)	83.02 (2.63)	1.15	13	0.272
	Cell optical density	0.400 (0.041)	0.330 (0.036)	1.27	13	0.226
	Fiber density in ME	0.450 (0.022)	0.445 (0.035)	0.12	12	0.906
GnIH	Cell area (μm^2)	79.10 (4.860)	78.10 (3.67)	0.17	13	0.869
	Cell optical density	0.734 (0.095)	0.753 (0.051)	0.42	13	0.681
	Fiber density in ME	0.112 (0.012)	0.107 (0.013)	0.26	12	0.800
	Fiber density in POA	0.116 (0.003)	0.123 (0.004)	1.33	13	0.208

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CHAPTER 3

THE ROLE OF GONADOTROPIN-RELEASING HORMONE IN ENERGY-DEPENDENT GONADAL GROWTH

Abstract

Seasonal activation of the vertebrate hypothalamic-pituitary-gonadal (HPG) axis and gonadal development is initiated by gonadotropin-releasing hormone-I (GnRH) release from the hypothalamus. In photoperiodic species, the consistent annual change in photoperiod is the primary environmental signal affecting GnRH cell activity, including changes in the synthesis and secretion of this neuropeptide. Non-photoperiodic environmental cues such as energy availability also influence the HPG axis activity, but the mechanisms mediating this influence, in particular on the GnRH system, are not well understood. Understanding how the neuroendocrine system integrates environmental information is critical to determine the plasticity and adaptability of physiological responses in changing environments. The primary objective of this study was to investigate GnRH-mediated changes in HPG axis activity and gonadal development in response to energy availability in a wild bird. I hypothesized that negative energy balance inhibits HPG axis activity by affecting GnRH secretion. Moderate food restriction for several weeks in male house finches, *Haemorhous mexicanus,* decreased body condition and inhibited photoinduced testicular growth compared to birds fed *ad libitum*. Food restriction did not affect plasma luteinizing hormone (LH; a correlate of GnRH release) or plasma testosterone, but it enhanced the plasma LH response to an N-methyl-D-aspartate (NMDA) injection. Thus, food restriction may decrease photoinduced HPG axis activation by acting centrally, in particular by attenuating the release of accumulated GnRH stores.

Introduction

Seasonal reproductive development in vertebrates is controlled through activation of the hypothalamic-pituitary-gonadal axis (HPG). Environmental signals (in birds, primarily long days) stimulate gonadotropin-releasing hormone-I (GnRH) secretion from the hypothalamus (Dawson et al., 2001; Follett et al., 1977). GnRH stimulates the anterior pituitary gland to secrete luteinizing

hormone (LH) and follicle-stimulating hormone (FSH) (Hattori et al., 1986; Sharp et al., 1990). LH and FSH then act on the gonads to increase steroid hormone production and secretion, gonadal growth, and gametogenesis (Deviche et al., 2005; Kirby and Froman, 2000). Steroid hormones, in turn, modulate HPG axis activity via negative feedback on the hypothalamus and pituitary gland (Deviche et al., 2006). The stimulating effects of GnRH are opposed by gonadotropin-inhibitory hormone (GnIH), which decreases GnRH and/or gonadotropin release in response to photoperiod and other environmental signals (Tsutsui et al., 2012).

The mechanisms by which photoperiod regulates the activity of the avian HPG axis have been extensively studied (Dawson, 2014; Yoshimura, 2013). In response to long days, many avian species that are strict seasonal breeders undergo a process of photostimulation during which the HPG axis is activated. Photostimulation is usually followed by photorefractoriness, during which continued long day exposure, instead of having stimulatory effects, now exerts the opposite action, thereby decreasing HPG axis activity and causing regression of the reproductive system. In these species, photosensitivity and, therefore, the ability of long days to again stimulate the HPG axis, is reinstated after sufficient exposure to short days (Stevenson et al., 2012b). Each level of the HPG axis is under some degree of independent regulation (Schaper et al., 2012; Stevenson et al., 2013; Williams, 2012b), but photoperiod regulates HPG axis activity primarily by altering GnRH synthesis and secretion (Ball, 1993; Joseph et al., 2013; Nicholls et al., 1988). GnRH synthesis can be investigated by measuring the expression of its precursor peptide, proGnRH (Parry et al., 1997) or GnRH gene expression. GnRH secretion is not easily measured directly, but plasma LH can be used as an indirect measure of this secretion (Ball, 1993). N-methyl-D-aspartate (NMDA) is a neuroexcitatory amino acid glutamate analog which stimulates GnRH release (Deviche et al., 2008; Iremonger et al., 2010), and the plasma LH increase that occurs in response to a NMDA injection can be used as indicator of stored, releasable GnRH (Meddle et al., 1999; Stevenson et al., 2012b). Photostimulation is associated with elevated GnRH synthesis and release, photorefractoriness with a decline in GnRH release followed by a decline in synthesis, and photosensitivity with renewed synthesis (Bentley et al., 2013; Dawson and Goldsmith, 1997; Foster et al., 1987; Stevenson et al., 2009; Stevenson et al.,

2012b). The difference, therefore, between photosensitivity and photostimulation involves changes in GnRH transport and release.

In contrast to species that are photorefractory, birds that breed in a more opportunistic fashion, as well as most mammals, respond to photoperiod only with changes in GnRH release and no apparent changes in GnRH synthesis (MacDougall-Shackleton et al., 2009; Stevenson et al., 2012b). This mechanism presumably allows more flexibility, making it possible to respond rapidly to stimulatory cues by increasing GnRH release from already available stores.

The hypothalamic GnRH system also responds to non-photoperiodic environmental signals, but whether changes in GnRH release and/or synthesis are involved in this response is not entirely clear. Brain GnRH-immunoreactivity (ir) changes independently of photoperiod in equatorial rufous-collared sparrows, *Zonotrichia capensis* (Moore et al., 2006), and in response to social signals in European starlings, *Sturnus vulgaris* (Stevenson and Ball, 2009) and ring-necked doves, *Streptopelia capicola* (Mantei et al., 2008). The significance of these findings is, however, ambiguous because an increase in brain GnRH-ir may reflect either an increase in synthesis that outpaces the rate of secretion or decreased secretion and/or transport of the peptide. For example, in the opportunistic rufous-winged sparrow, *Peucaea carpalis,* monsoon-related factors influence GnRH-ir and proGnRH-ir once gonads are developed (Small et al., 2007), indicating changes in both synthesis and release of GnRH. By contrast, in another opportunistic species, the zebra finch, *Taeniopygia guttata,* short-term fasting does not affect hypothalamic GnRH-ir or GnRH mRNA (Lynn et al., 2015), suggesting that this manipulation influences neither the synthesis nor the secretion of GnRH. Elucidating how the neuroendocrine system integrates information related to environmental factors and responds to these factors is crucial for understanding the capacity of organisms to cope with environmental changes through plasticity and/or adaptation of the HPG axis (Wingfield, 2014).

Photoperiod is the primary proximate environmental signal that most middle and high latitude birds use to regulate seasonal changes in HPG axis activity. Reliance on photoperiod presumably evolved because of its reliability to predict seasonal increases in food supply and other optimal environmental conditions (Dawson and Sharp, 2007). Reproductive success, and

ultimately fitness, is maximized by synchronizing breeding, and in particular, chick-rearing, with peaks in local food supply (Daan et al., 1990, Lack, 1968; Perrins, 1970). These peaks can vary inter-annually and in relation to the consistent annual photoperiodic cycle. Therefore, the ability to monitor and respond to increased food availability and to one's energy balance by altering HPG axis activity has the potential to enhance reproductive success (Visser et al., 1998). The use of food availability as a supplementary cue is evidenced in populations of free-living birds in which the timing of breeding varies inter-annually and between territories in relation to food supply (Caro et al., 2006; Harris, 1969; Korpimaki, 1987; Nager and van Noordwijk, 1995; Perrins and McCleery, 1989; Solonen, 2004), and through studies in which lay date is advanced by food supplementation (Davies and Deviche, 2014). However, the mechanisms by which food availability influences the timing of reproduction through changes in HPG axis activity and gonadal development remain poorly understood.

Food deprivation in domestic birds can affect all levels of the HPG axis including the hypothalamus (Ciccone et al., 2007; Kobayashi et al., 2002; Tanabe et al., 1991). By contrast, studies on this subject and involving moderate food restriction similar to what birds are exposed to naturally remain rare and have produced inconsistent results (Davies et al., 2015; Dawson, 1986; Hahn, 1995). In Chapter 2, I found that food restriction inhibits photo-induced gonadal development in male house finches, *Haemorhous mexicanus*, and also increases GnRH-ir without affecting proGnRH-ir. These results suggest in this species that the inhibitory influence of food restriction on the HPG axis involves an inhibition of GnRH secretion (Foster et al., 1988; Lee et al., 1990). This mechanism may be adaptive: if HPG axis plasticity in response to local environmental conditions is important in the early stages of breeding, even in a strictly seasonal breeder, then altering GnRH secretion in response to these conditions may offer increased flexibility with respect to the onset of breeding.

The primary objective of this study was to comprehensively investigate GnRH-mediated changes in HPG axis activity and gonadal development in response to food availability. I hypothesized that food availability affects HPG axis activity by regulating GnRH secretion. I investigated hypothalamic GnRH release and the capacity of the hypothalamus to release stored

GnRH in food-restricted male house finches. I used plasma LH as a correlate of GnRH release and used the plasma LH response to a NMDA injection to determine the responsiveness of the hypothalamus to release GnRH. I also examined responsiveness of the HPG axis at the level of the pituitary gland by measuring LH secretion in response GnRH injection. My previous study suggested that food restriction does not alter the pituitary gland responsiveness to GnRH (Chapter 2) and this allowed me to confirm that in the present study I was in fact measuring differences in responsiveness at the hypothalamic level. If food availability affects GnRH release without affecting production, I predicted that initial plasma LH would be lower in food-restricted birds than in *ad libitum*-fed birds, and a NMDA injection to these birds would increase plasma LH to the same extent as in *ad libitum*-fed birds.

Methods

All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee. All necessary permits to capture animals were obtained from the US Fish and Wildlife Service and the Arizona Game and Fish Department.

Capture and initial conditions. Adult male house finches (N=20) were caught in Tempe, AZ, USA (33.41 N, -111.91 W; elevation: 360 m) between 31 January and 8 February 2015, at which time they were naturally exposed to non-stimulating (11L: 13D) photoperiod and had undeveloped testes (Hamner, 1968). Birds were caught using food-baited traps, sexed based on plumage coloration, and aged based on plumage characteristics (Pyle, 1997). Only after-second year (i.e., hatched in 2013 or earlier) males were selected. Birds were transported to Arizona State University Animal Care Facilities, placed in visually isolated, individual cages at 25° C, and kept on a natural photoperiod (11L:13D; lights on at 7:30 AM). They initially received sunflower seeds *ad libitum*. The diet was gradually (over 10 days) changed to Mazuri small bird breeding diet (PMI Nutrition International, Richmond, IN, USA) and then remained constant throughout the study.

Food Restriction and Photostimulation. The daily food consumption of each bird was measured over the course of 1 week. On 28 February 2018 (day 1), birds were randomly divided into 2 groups (N = 10): (1) *ad libitum* food availability (AL; = controls) and (2) food-restricted (FR).

Food-restricted birds received a daily ration of food equal to 70% of their *ad libitum* food intake (Chapter 2) until the end of the study whereas control birds continued to receive food *ad libitum*. At this time, all birds were transferred to a moderately stimulatory day length (13L: 11D; lights on at 6 AM) for the remainder of the study (6 weeks). House finches regain photosensitivity by the end of October (Hamner, 1966) and were thus photosensitive at the time of exposure to a longer photoperiod.

Morphology. All birds were weighed daily to the nearest 0.1 g beginning on the day prior to photostimulation and dietary manipulation (27 February 2018: day 0) and continuing for the remainder of the study. Body fat reserves, muscle stores, and cloacal protuberance width were determined on day 0 and at the middle (3 weeks) and end (6 weeks) of the study. The amount of furcular fat was visually estimated using a scale of 0–5 according to Helms and Drury (1960). As the pectoral muscles contain the largest store of proteins in birds, their size was estimated using a scale of 0–3, with 0 for concave pectoral muscles and a prominent keel and 3 for convex pectoral muscles that protrude above the keel (Salvante et al., 2007). Cloacal protuberance width (± 0.1 mm) was measured using digital calipers.

Blood Sampling and Hormone Challenges. The effect of food restriction on the plasma LH response to a NMDA or a GnRH injection was investigated after 3-4 weeks of photostimulation. An initial blood sample (100 µl; time 0: T0) was taken from the jugular vein of each finch into a heparinized microsyringe and immediately placed on ice. Each bird then received an intramuscular injection of either 1.2 mg NMDA (Sigma Chemical Co., MO, USA) or 1.25 µg GnRH-I (Sigma Chemical Co., MO, USA) dissolved in 50 µl sterile saline solution. After an injection, finches were returned to their cage and they were bled again (100 µl) 20 minutes later (time 20: T20). The dose and sampling time for the NMDA injection is based on previous studies which found a stimulatory effect of NMDA on plasma LH (Deviche et al., 2008; Meddle et al., 1999). The dose and sampling time for the GnRH injection is based on my previous experiment in house finches, which found a stimulatory effect of GnRH on plasma T 30 minutes after injection (Chapter 2). A shorter sampling time (20 minutes) was used here as samples were used to measure plasma LH and not T. Each bird received the opposite treatment 1 week later,

with the weekly sequence of injections divided equally among each group and the daily sequence randomized across all birds. All samples were collected between 9:00 and 11:00 AM. They were centrifuged within 3 hours of collection, and plasma was collected and stored at -80°C until assayed.

Additional blood samples for plasma LH and T determination were collected on day -1 and after 6 weeks of the treatment. At each time, blood (150 µl) was taken and plasma was stored as described above. Samples obtained during weeks 3 and 4 of treatment and before injection were also used to analyze unstimulated plasma LH throughout the study.

Euthanasia and Testis measurement. After 6 weeks of photostimulation and dietary manipulation, and 2 weeks after the last injection, birds received an intramuscular injection of 400 µl anesthetic solution (0.9% NaCl containing 20 mg/ml xylacine and 100 mg/ml ketamine). To preserve the brain for potential future immunocytochemical analysis, birds were perfused transcardially with 35 ml wash solution (0.9% NaCl and 0.1% $NaNO_2$ in 0.1 M phosphate buffer, PB) followed by 35 ml of fixative (4% paraformaldehyde and 0.1% $NaNO_2$ in 0.1 M PB). The testes were removed, rinsed in saline, and weighed to the nearest 0.01 mg. Gonadosomatic index (GSI) was calculated as testis mass as a percentage of body mass.

Plasma LH and T Assays

Luteinizing hormone (LH). I used a validated radioimmunoassay (Sharp et al., 1987) to measure plasma LH, with slight modifications. This radioimmunoassay has been used to quantify plasma LH in many avian species (Ciccone et al., 2007; Davies et al., 2015; Deviche et al., 2012; Fraley et al., 2013; Meddle et al., 2002), including house finches (Salvante et al., 2013). Briefly, the assay reaction volume was 60 µL, comprised of 20 µL of plasma sample or standard, 20 µL of primary rabbit LH antibody and 20 µL of I^{125}-labelled LH. The primary antibody was precipitated to separate free and bound I^{125} label using 20 µL of donkey anti-rabbit precipitating serum and 20 µL of non- immune rabbit serum. All samples were assayed in duplicate in a single assay. The intra-assay coefficient of variation was 4.89% and the minimum detectable concentration was 0.15 ng/ml.

Testosterone (T). A validated (Deviche and Cortez, 2005) commercial enzyme-linked immunoassay (Enzo Life Sciences, Farmingdale, NY, USA) was used to measure plasma T following the manufacturer's instructions. Plasma was diluted 15x in assay buffer containing 1 µl displacement reagent per 99 µl plasma. Samples were assayed in duplicate with all samples from each bird on a single assay plate. Each assay plate included a complete standard curve. The assay sensitivity was 4.81 pg/ml and the intra-assay coefficient of variation was 2.9% (N=39 samples).

Statistical Analyses. Effects of the dietary manipulation on body mass, morphological characteristics, and plasma hormones were analyzed using two-way repeated measures analysis of variance (ANOVA), with time (number of days) as the within-subject factor and food availability as the between-subjects factor. Effect of the dietary manipulation on testis mass was analyzed using a Student's t-test. Effects of GnRH or NMDA injection on plasma LH were analyzed using two-way repeated measures ANOVA with time (T0 vs. T20) as the within-subject factor and food availability as the between-subjects factor. For ordinal scale data (fat and muscle scores), data were ranked before proceeding with analyses. Data sets that were not normally distributed or homoscedastic (Shapiro-Wilk test and Levene's test, respectively) were either natural log- (plasma LH) or square root-transformed (plasma T) prior to analysis. For data sets that did not display sphericity (body mass, baseline plasma LH), according to Mauchly's spericity test, degrees of freedom were deflated using a Ɛ- derived Greenhouse-Geiser correction. When a statistically significant treatment x time interaction was detected using ANOVA, Fisher's Least Significant Difference (LSD) tests were used to perform pair-wise comparisons. Pearson's correlations were used to examine relationships between dependent variables. With repeated data, the change in parameter from initial values in response to food manipulation (day 0) or in response to injection (T0) were first calculated and used in correlation analysis. Data were analyzed using SPSS (version 24; IBM, Armonk, NY, USA). The significance level of all statistical tests was set at $P = 0.05$.

Three birds (1 FR and 2 AL) died during the experiment, resulting in the absence of data for 2 birds after 3 weeks and 3 birds after 4 weeks. Additionally, I was unable to collect a T20

blood sample after NMDA injection to one bird, and to obtain enough blood to measure baseline LH for one bird at day 0 and another at the end of the study. I estimated missing values using multiple imputation (MI) and the NORM program (http://sites.stat.psu.edu/~jls/misoftwa.html; Schafer, 1999). Multiple imputation relies on more plausible assumptions than other approaches to coping with missing data (e.g., case deletion or replacement with group means), properly accounts for uncertainty about missing values (leading to appropriate standard errors), and retains original sample sizes (Little and Rubin, 2002).

Results

Body Mass. Body mass was affected by food availability ($F_{1,15} = 11.37$, $P = 0.004$), time ($F_{3.11,46.64} = 14.08$, $P < 0.001$), and the interaction between food availability and time ($F_{3.11,46.64} = 23.67$, $P < 0.001$; Fig. 3.1A). *Ad libitum*-fed and food-restricted birds had similar body mass at the start of the dietary manipulation (LSD tests, $P > 0.05$), and AL birds experienced minor fluctuations in body mass, while FR birds lost mass within the first week of food restriction and then maintained lower body mass than AL birds for the duration of the study (LSD tests, $P < 0.002$).

Furcular fat scores were affected by food availability ($F_{1,18} = 15.60$, $P = 0.001$) and there was a food availability x time interaction ($F_{2,36} = 15.49$, $P < 0.001$; Fig. 3.1B), with no effect of time alone ($F_{2,36} = 2.00$, $P = 0.15$). *Ad libitum*-fed birds had more furcular fat 6 weeks into the experiment than at the start (LSD tests, $P < 0.001$), whereas FR birds lost fat stores after 6 weeks of food restriction and had less fat than AL birds 3 and 6 weeks after treatment onset (LSD tests, $P < 0.01$).

Pectoral muscle size was affected by food availability ($F_{1,18} = 18.49$, $P < 0.001$) and there was a food availability x time interaction ($F_{2,36} = 22.90$, $P < 0.001$; Fig. 3.1C), with no effect of time alone ($F_{2,36} = 2.65$, $P = 0.08$). Pectoral muscle size increased in AL birds after 3 weeks of dietary manipulation (LSD tests, $P < 0.02$) but decreased after 3 weeks of treatment in FR birds (LSD tests, $P < 0.02$), with smaller pectoral muscles in these birds compared with AL birds at 3 weeks (LSD tests, $P < 0.001$).

Cloacal Protuberance. Cloacal protuberance (CP) width differed between food treatment groups over the course of the study ($F_{2,36} = 5.55$, $P = 0.008$; Fig. 3.1D). It increased in AL birds after 3 weeks of exposure to long days, remaining at this size after 6 weeks (LSD tests, $P < 0.002$). In FR birds, CP width was not affected by long day exposure (LSD tests, $P > 0.13$), and was smaller in FR birds as compared to AL birds after 3 weeks of dietary manipulation (LSD tests, $P = 0.04$). There was a main effect of time ($F_{2,36} = 12.69$, $P < 0.001$) and no effect of food availability alone ($F_{1,18} = 1.47$, $P = 0.076$).

Plasma T. Baseline plasma T decreased during the period of dietary manipulation ($F_{1,18} = 8.50$, $P = 0.009$), but was unaffected by food availability ($F_{1,18} = 0.96$, $P = 0.34$), and there was no food availability x time interaction ($F_{1,18} = 0.43$, $P = 0.52$; Fig. 3.2B).

Plasma LH. Baseline plasma LH changed over time in both AL and FR birds ($F_{1.90,34.14} = 9.39$, $P = 0.001$), increasing above initial levels after 3 and 4 weeks before declining after 6 weeks (LSD tests, $P < 0.02$). There was a marginal effect of treatment, with baseline plasma LH lower overall in FR as compared to AL birds, but this difference did not reach significance ($F_{1,18} = 4.02$, $P = 0.06$). There was no interaction between time and food availability on baseline plasma LH ($F_{1.90,34.14} = 0.73$, $P = 0.48$; Fig. 3.2A).

Plasma LH increased in response to GnRH challenge ($F_{1,18} = 199.50$, $P < 0.001$), but this increase was unaffected by food availability ($F_{1,18} = 0.18$, $P = 0.68$), and there was no interaction between the effect of GnRH challenge and food availability on plasma LH ($F_{1,18} = 1.55$, $P = 0.23$; Fig. 3.3A). Considering the fold increase in LH following GnRH challenge, there was no effect of treatment ($T_{18} = -1.24$, $P = 0.23$; Fig. 3.3C).

Plasma LH increased in response to NMDA challenge ($F_{1,18} = 131.63$, $P < 0.001$). There was no main effect of food availability on plasma LH ($F_{1,18} = 1.13$, $P = 0.30$). There was a significant interaction between the effects of food availability and NMDA challenge on plasma LH ($F_{1,18} = 4.69$, $P = 0.044$; Fig. 3.3B). Plasma LH in FR birds did not differ significantly from plasma LH in AL birds prior to (LSD tests, $P = 0.093$) or after NMDA-challenge (LSD tests, $P = 0.97$), but the fold increase in plasma LH after NMDA injection was greater in FR birds (6X) compared to AL birds (3X; $T_{18} = -2.17$, $P = 0.04$; Fig. 3.3D).

Testis Size. Food-restricted birds had a lower GSI than AL birds ($T_{13.4} = 4.58$, $P < 0.001$; Fig. 3.4).

Testis size and function in relation to body condition. Overall among both food treatment groups, GSI was positively related to the change in (Δ) body mass, fat, and muscle at multiple time points (e.g.: Δ body mass at 6 weeks and GSI: $r_{17} = 0.62$, $P = 0.008$; Fig. 3.5A). These relationships were not present within AL or FR birds only. Plasma T was not correlated either with any measure of body condition or with GSI.

Plasma LH in relation to body condition and testis size. Within FR birds, there were multiple positive correlations between Δ baseline plasma LH and Δ body mass, with the strongest correlation observed at 6 weeks ($r_9 = 0.78$, $P = 0.018$; Fig. 3.5B). There were no correlations between plasma LH and body mass in AL birds, or when AL and FR groups were combined. There was a positive correlation between Δ baseline plasma LH at 3 weeks and GSI ($r_{10} = 0.64$, $P = 0.047$; Fig. 3.5C) that was not present in AL birds, or among both groups combined.

With the two groups combined, there was a negative correlation between Δ muscle at 3 weeks and NMDA-induced Δ plasma LH ($r_{20} = -0.46$, $P = 0.04$; Fig. 3.6A). Within FR birds there was a marginally significant negative correlation between Δ muscle at 3 weeks and NMDA-induced Δ plasma LH ($r_{10} = -0.62$, $P = 0.056$) but no relationship in AL birds. There were no relationships overall between NMDA-induced Δ plasma LH and Δ body mass, fat, or GSI.

There were no relationships between GnRH-induced Δ plasma LH and any measure of body condition or GSI.

Relationships between baseline plasma LH and challenge-induced plasma LH. Overall among both treatment groups, there was a positive correlation between initial plasma LH and max plasma LH in response to NMDA ($r_{20} = 0.57$, $P = 0.009$).This relationship was also present in FR birds ($r_{10} = 0.77$, $P = 0.009$), and was almost significant in AL birds ($r_{10} = 0.60$, $P = 0.065$). In FR birds, there was a positive correlation between initial plasma LH and NMDA-induced Δ plasma LH ($r_{10} = 0.66$, $P = 0.036$; Fig. 3.6B). This relationship was not present overall or within AL birds.

There were no relationships between baseline plasma LH and GnRH-induced Δ plasma LH.

Discussion

I tested the hypothesis that food availability affects photo-induced HPG axis activity and gonadal growth by regulating GnRH secretion. Baseline GnRH secretion was estimated by measuring plasma LH in intact birds and I predicted that initial plasma LH would be lower in FR than in AL birds. I determined the potential to secrete GnRH by measuring the plasma LH response to an NMDA challenge and predicted that in response to this challenge, plasma LH would increase to a similar level in AL and FR birds, i.e., that in relative terms it would increase more in FR than in AL finches. Food restriction decreased body condition and resulted in smaller testes and diminished CP growth, but had no effect on baseline plasma T or LH. However, plasma LH increased more in FR than AL birds in response to a NMDA challenge. These results are consistent with the hypothesis that food restriction did not alter basal GnRH secretion but enhanced the capacity to secrete GnRH in response to pharmacological stimulation.

Testis development and function under food restriction. The inhibition of testicular development under food restriction is consistent with the findings in my previous study (Chapter 2). Smaller testes in the FR than AL birds likely were associated with lower levels of spermatogenesis, as I found smaller seminiferous tubules in FR house finches previously (Chapter 2). Testis mass was positively associated with changes in body mass, muscle, and fat among all birds. This could indicate that the decline in body condition under food restriction was responsible for the group difference in testis size. However, this relationship is not also apparent within individual treatment groups, especially FR birds, suggesting that differences in testis sizes reflect an effect of food restriction that is not secondary to a change in body condition.

Seasonal testicular growth is stimulated primarily by FSH, but additionally by LH and T (Deviche et al., 2011). I found no effect, however, of 6 weeks of food restriction on plasma T, or any relationship between plasma T and testis mass. Testosterone was lowered by 3-4 weeks of food restriction in my previous study, but only transiently during photostimulation, with differences disappearing after 6 weeks (Chapter 2). Multiple types of food restriction lower plasma T in avian

species (Perez-Rodriguez et al., 2006; Lynn et al., 2010; Lynn et al., 2015). Even in free-living house finches, body condition is positively related to plasma T (Duckworth et al., 2001). It is, therefore, possible that I missed the time period over which food restriction influences plasma T. Supporting this hypothesis, I found no evidence in the present study for photoinduced CP growth, a T-dependent trait (Deviche and Cortez, 2005), in FR birds, and no relationship between plasma T at 6 weeks and any measure of body condition.

Baseline plasma LH under food restriction. Baseline plasma LH increased in response to photostimulation but was not influenced by food restriction. If food availability controls gonadal development by affecting GnRH and subsequently gonadotropin release and gonadal stimulation, I predicted that smaller testes in FR than AL birds would be associated with a parallel difference in plasma LH and T, but this was not the case. This finding is surprising given the positive relationship in FR finches between plasma LH midway through the experiment and GSI. The lack of an effect of food restriction on baseline LH may be related to particularities of the present experimental design. For example, transient differences after 1 or 2 weeks of photostimulation/dietary manipulation would not have been detected under the sampling protocol. A similar photostimulation and dietary manipulation, however, resulted in differences detectable after 4 but not 2 weeks in Abert's Towhees, *Pipilo aberti* (Davies et al., 2015). In the few studies that measured both plasma LH and gonadal growth in wild birds under food restriction, parallel changes actually do not appear common. The decline in plasma LH in Abert's Towhees occurred with no detectable differences in gonadal growth (Davies et al., 2015) and in food-restricted red crossbills, *Loxia curvirostra,* held on long days, testis growth was significantly inhibited but plasma LH was not significantly lower than in *ad libitum*-fed birds (Hahn et al., 1995).

The relationship present between declining body mass in FR birds and lower plasma LH suggests that low body condition associated with food restriction does have an effect, but only among birds that are in poor body condition. Supporting this idea, food supplementation of a preferred food source did not affect body condition but increased plasma LH in the opportunistically breeding pine siskin, *Spinus pinus* (Watts and Hahn, 2012). Taken together, the relationships between body mass and plasma LH, between body mass and GSI, and between

plasma LH and GSI, demonstrate a potential pathway by which low body condition lowers plasma LH (and potentially FSH), which then inhibits testicular development.

LH responsiveness under GnRH and NMDA challenge. My previous study found no differential responsiveness of the pituitary gland to GnRH under food restriction, as measured by plasma T, and co-occurring with no change in LH-induced plasma T (Chapter 2). In the present study, there was no difference in GnRH-stimulated LH release, confirming that food restriction does not attenuate the LH responsiveness to GnRH. Consistent with previous studies, baseline plasma LH in the present study, therefore, can serve as an indicator of GnRH release, and the LH response to NMDA reveals the potential of hypothalamic GnRH to secrete this neuropeptide. In this context, I can conclude that food restriction did not have a measurable effect on basal GnRH release, but that basal GnRH release may be inhibited by decreases in body mass associated with food restriction.

Food restriction resulted in an enhanced response to NMDA injection, with a greater increase in plasma LH from initial levels in FR than AL finches. Seasonally breeding birds have varied LH responses to NMDA in relation to photoperiodic state, with the largest response occurring during photosensitivity, a moderate response under photostimulation, and barely any response under photorefractoriness (Dawson, 2005; Deviche et al., 2008). The switch between photosensitivity and photostimulation is associated with a marked increase in the release of GnRH synthesized under short days (Stevenson et al., 2012b). If photostimulatory conditions in the present study were not sufficient to override the inhibitory effect of food restriction on basal GnRH release, then FR birds would have had larger stores of GnRH that they were capable of releasing in response to NMDA stimulation. Although the proxy for basal GnRH release, baseline plasma LH, was unaltered by food restriction, the negative relationship between NMDA-induced LH release and body condition (specifically muscle stores) provides some evidence that birds in a negative energetic state have more releasable GnRH. In fact, GnRH-ir, as measured in my previous study, was also higher in FR than in AL birds (Chapter 2).

Multiple types of evidence, primarily from mammals, demonstrate how food availability might modify GnRH release. Food availability appears to primarily affect GnRH activity in the

median eminence, the region of its release (Temple and Rissman, 2000). It affects both thyroid hormones (Costa-e-Sousa, 2012; Darras et al., 1995; Herwig et al., 2009) and hypothalamic deiodinase expression (Herwig et al., 2009), both of which influence photoinduced morphological changes in glial cells that surround GnRH terminals in the median eminence and regulate its release (Yamamura et al., 2004; Yoshimura et al., 2003). Recent evidence actually links regulation of gonadal growth by food availability to altered glial cell activity in proximity to GnRH nerve terminals (Steinman et al., 2012). Gonadotropin-inhibitory hormone (GnIH) activity may also play a role in regulating GnRH release under food restriction, as its activity under some circumstances relates to feeding (Clark et al., 2012; Davies et al., 2015; Fraley et al. 2013) and in European starlings has been shown to modulate the effect of other non-photic factors to GnRH cells (Calisi et al., 2011). I did, however, find no change in GnIH-ir in previously FR house finches (Chapter 2). In birds, NMDA acts primarily to stimulate GnRH nerve terminals (Deviche et al., 2008; Meddle and Follet, 1997) and its stimulatory effect on plasma LH, and presumably GnRH release, may consist in overriding these mechanisms inhibiting GnRH release under food restriction.

Conclusion. This is one of the first studies investigating the regulation of GnRH release by food availability in a wild bird. Food restriction inhibited photoinduced gonadal development and this inhibition may involve attenuated photoinduced GnRH release. I propose during food restriction that low body condition decreases basal GnRH release, thereby elevating GnRH stores in nerve terminals and resulting in enhanced GnRH and, therefore, LH secretion during pharmacological stimulation. In birds that naturally experience fluctuating and unpredictable environmental conditions, such plasticity in HPG axis activity is crucial for making decisions about allocating energy towards reproduction or survival. Continued investigation into the central and peripheral mechanisms by which animals integrate energetic information will help in understanding the plasticity of breeding responses and ultimately how populations succeed or fail in adjusting to environmental changes.

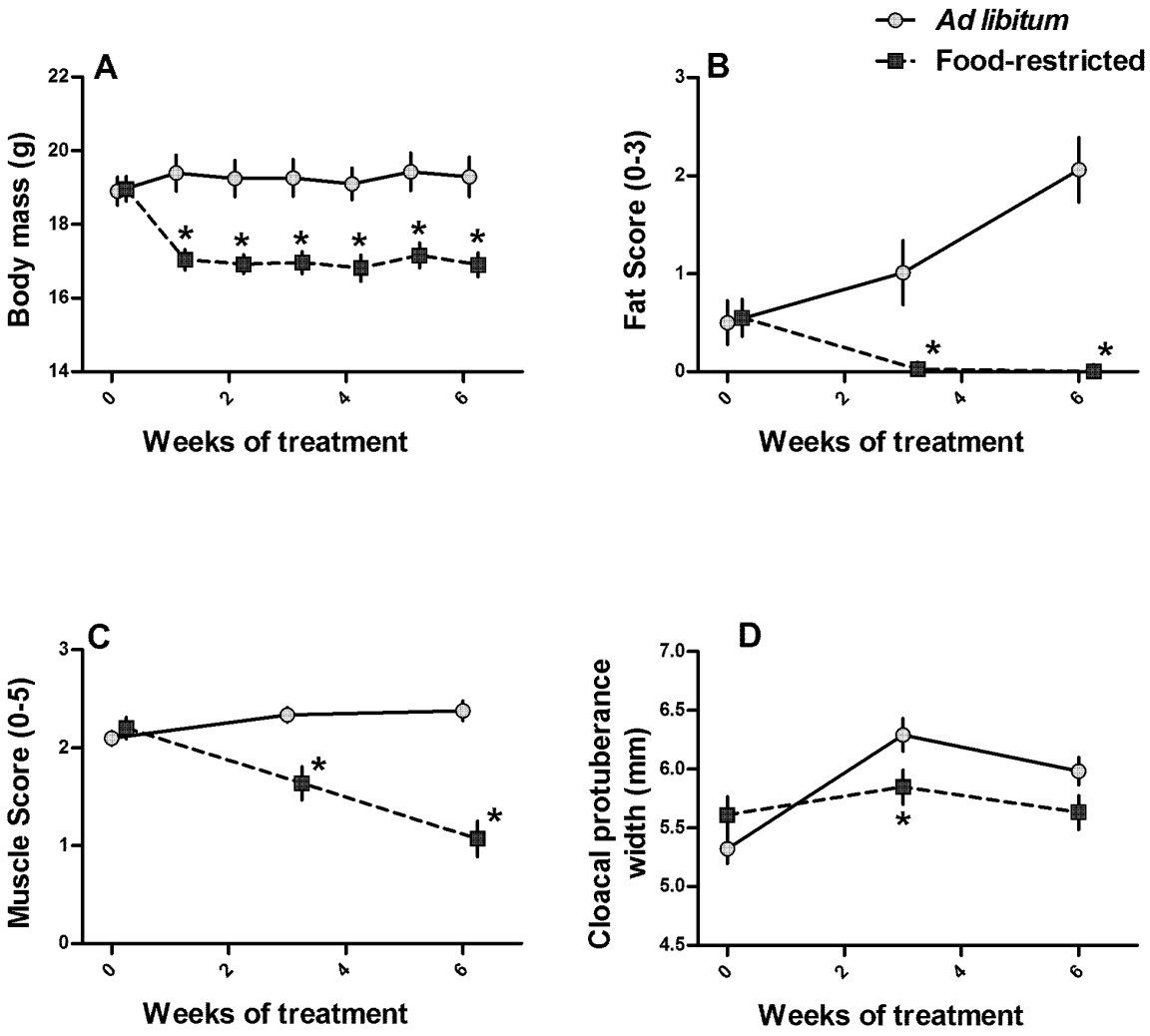

Figure 3.1. Effects of food restriction on body condition and cloacal protuberance width in male house finches, *Haemorhous mexicanus*. Body mass (A) was reduced by food restriction after 1 week and remained lower than *ad libitum*-fed birds. Both Furcular fat score (B) and pectoral muscle score (C) were reduced by 3 weeks of food restriction and remained lower than control birds. Cloacal protuberance width (D) increased in response to long day exposure (beginning at day 0), but did not increase in size in food-restricted birds, and was smaller after 3 weeks compared to *ad libitum*-fed birds. Data are plotted as means ± SEM. An asterisk (*) indicates a significant difference between treatment groups ($P<0.05$, LSD tests). For visual clarity, some points have been separated along the horizontal axis.

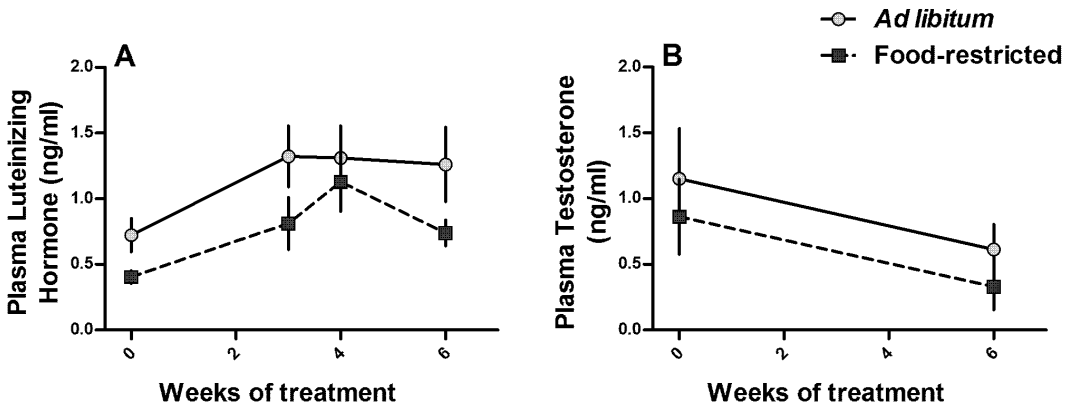

Figure 3.2. Baseline plasma luteinizing hormone (LH) and testosterone (T) are unaffected by food restriction in male house finches, *Haemorhous mexicanus*. Plasma LH (A) changed over the duration of the study, initially increasing in response to photostimulation, but this response was consistent between food-restricted and *ad libitum*-fed birds. Plasma T (B) decreased over the 6 weeks of the study, but similarly in both food-restricted and *ad libitum*-fed birds. Data are plotted as means ± SEM.

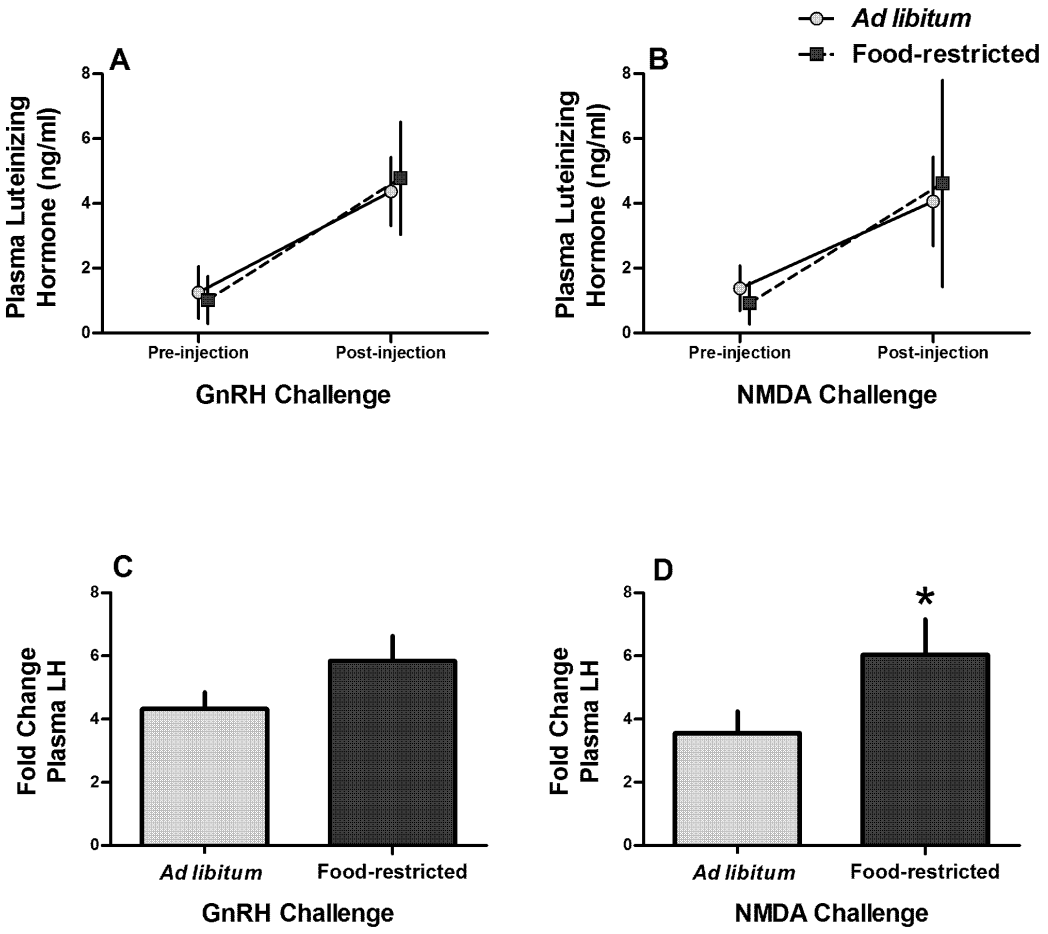

Figure 3.3. Food restriction differentially affects the increase in plasma luteinizing hormone (LH) that occurs in response to a gonadotropin-releasing hormone (GnRH) or N-methyl-D-aspartate (NMDA) challenge in male house finches, *Haemorhous mexicanus*. GnRH challenge (A) increased plasma LH similarly in food-restricted birds as compared to *ad libitum*-fed birds, with the percent change in LH (C) being similar between groups. Food restriction enhanced (B) the increase in plasma LH that occurred in response to a NMDA challenge with food-restricted birds having a greater percent change (D) than *ad libitum*-fed birds. Data are shown as means ± SEM, and the asterisk denotes a significant difference between the groups ($P < 0.05$; Student's t-test).

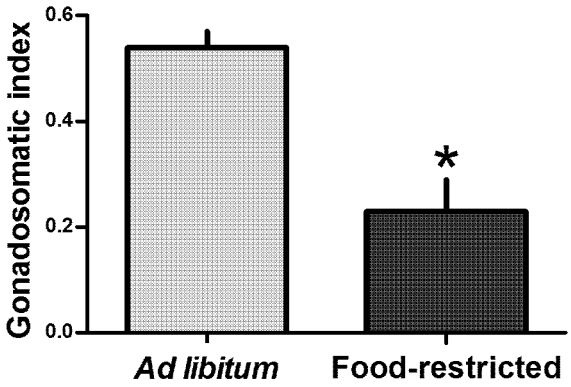

Figure 3.4. Food restriction for 6 weeks reduces testis mass in photostimulated male house finches, *Haemorhous mexicanus*. Gonadosomatic index (testis mass as a percentage of body mass) was lower in food-restricted birds as compared to birds fed *ad libitum.* Data is plotted as means ± SEM, and the asterisk denotes a significant difference between the groups ($P < 0.05$; Student's t-test).

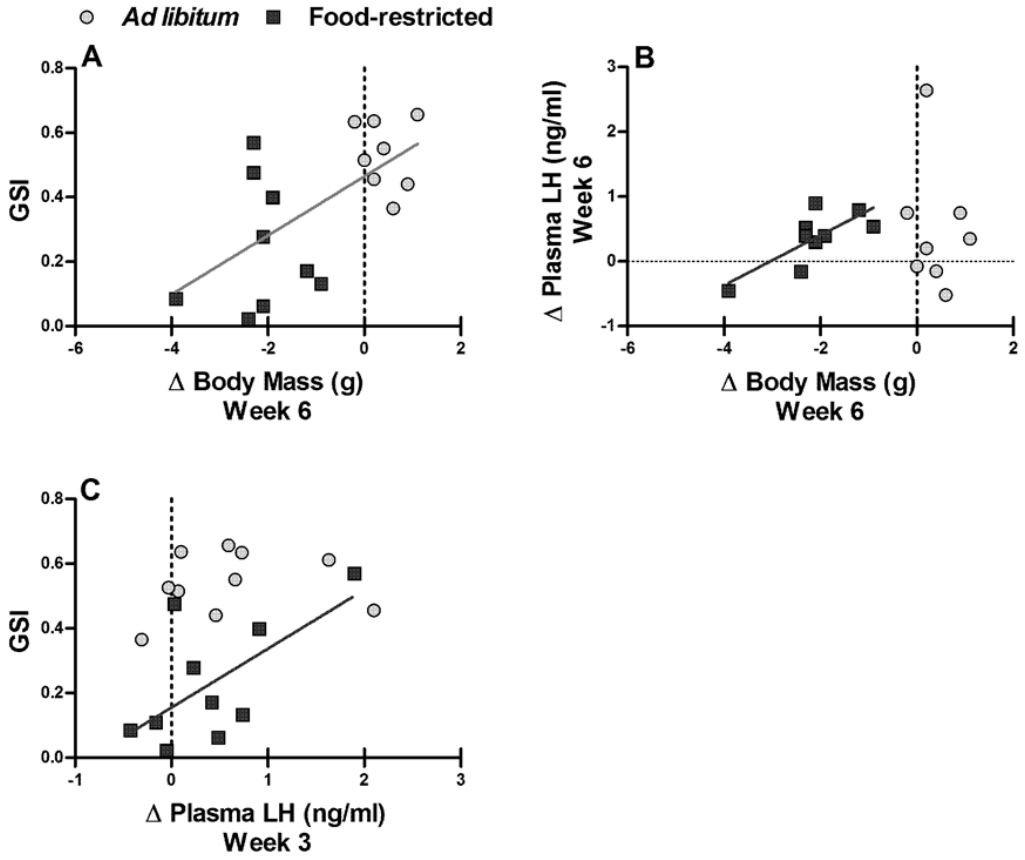

Figure 3.5. Relationships between body mass, plasma luteinizing hormone (LH), and plasma testosterone (T) differ among food-restricted and *ad libitum*-fed male house finches, *Haemorhous mexicanus*. Among both treatment groups, (A) Δ body mass between week 6 and the experiment start date was positively correlated with gonadosomatic index (GSI; testis mass as a percentage of body mass; Pearson's correlation: $r_{17} = 0.62$, $P = 0.008$) but this relationship was not present within either group. Within food-restricted birds only, (B) Δ body mass between week 6 and the experiment start date was positively correlated with the Δ plasma LH during this time (Pearson's correlation: $r_9 = 0.78$, $P = 0.018$) and (C) the Δ plasma LH between week 3 and the experimental start date was positively correlated with GSI (Pearson's correlation: $r_{10} = 0.64$, $P = 0.047$).

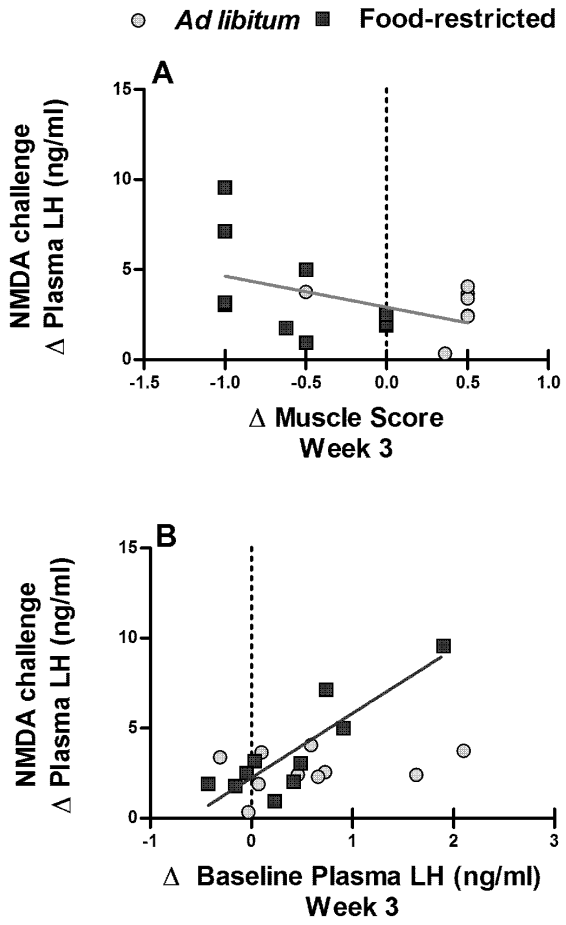

Figure 3.6. The increase in plasma luteinizing hormone (LH) in response to N-methyl-D-aspartate (NMDA) challenge is negatively related to muscle size and positively related to baseline plasma LH in male house finches, *Haemorhous mexicanus*. A negative correlation between (A) the Δ muscle score between week 3 and the experimental start date and the increase in plasma LH after an NMDA injection was present across all birds (Pearson's correlation: $r_{20} = -0.46$, $P = 0.040$). This relationship was nearly significant in food-restricted birds ($r_{10} = -0.62$, $P = 0.056$) but there was no relationship in *ad libitum*-fed birds. A positive correlation between the Δ plasma LH between week 3 and the experiment start date, and the increase in plasma LH after an NMDA injection was present in food-restricted birds (Pearson's correlation: $r_{10} = 0.66$, $P = 0.036$) but not within *ad libitum*-fed birds.

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CHAPTER 4

METABOLIC REGULATION OF GONADAL FUNCTION

Abstract

Reproductive success requires that individuals acquire sufficient energy resources. Altering energy homeostasis through restricting food availability or increasing energy expenditure (e.g., locomotion and thermoregulation) inhibits reproductive development in multiple avian species, but the nature of the energy-related signal mediating this effect is unclear. To investigate this question, I examined reproductive and metabolic physiology in male house finches in breeding condition under 1) moderate food restriction (FR), and 2) high temperature (HT), in which birds were exposed to a high ambient temperature cycle (37.8° C day, 29.4° C night) compared to a control group (CT; 29.4° C day, 21.1° C night). I hypothesized that either FR or HT inhibits reproductive development through lowering available metabolic fuel, specifically plasma glucose (GLU) and free fatty acids (FFA). Following FR for 4 weeks, finches lost body mass and experienced a 90% reduction in testis mass compared to CT birds. Food restriction did not affect plasma GLU. Plasma FFA, however, were higher after 3 days of FR, potentially indicating increased FFA utilization. In a separate group of finches, 4 weeks of exposure to HT resulted in reduced body mass and voluntary food consumption relative to CT birds. Testis mass decreased by 70% in HT birds, but this treatment did not influence plasma GLU or FFA. Both FR and HT birds expressed less testicular 17β-hydroxysteroid dehydrogenase (17β-HSD) mRNA than controls and across all the birds, this expression correlated positively to testis mass. Testicular expression of other testicular genes measured was unaffected by FR or HT. These studies are among the first to highlight the potential role of metabolic fuel in mediating inhibitory effects of FR on the reproductive system and to demonstrate a negative effect of HT on reproductive development in a wild bird. Further studies are needed to clarify the role of metabolic mediators and their involvement under various conditions of energy availability and demand.

Introduction

Food availability constitutes the ultimate factor responsible for the evolution of seasonal breeding patterns and a proximate factor that synchronizes the timing of photoperiodic

reproductive responses with local and inter-annual variation in food supply (Bronson, 1989; Lack, 1968; Thomas et al., 2001). The intimate link between reproduction and food availability is the availability of energy (Drent and Daan, 1980; Wade et al., 1996). Reproduction is energetically expensive (Meijer and Drent, 1999; Ricklefs, 1974) and may, therefore, be constrained by energy availability (Drent and Daan, 1980; Meijer and Drent, 1999; Nager, 1997; Perrins, 1970). If such an energetic tradeoff exists, then changes in either energy intake or energy expenditure should affect reproduction and metabolic signals should mediate these effects (Schneider et al., 2004). There is extensive evidence relating changes in food availability to the timing of reproduction (Ruffino et al., 2014), hypothalamic-pituitary-gonadal (HPG) axis activity, and gonadal development in avian species, both in natural and experimental conditions (Dawson et al., 1986; Davies et al., 2015; Hahn et al., 1995; Meijer, 1991; Perfito et al., 2008; Schoech et al., 2004). In some cases, these reproductive effects seem to be directly related to energy homeostasis. For example, in European starlings, *Sturnus vulgaris,* food restriction inhibits testicular development (Meijer, 1991) only when resulting in lower body mass (Dawson, 1986). Beyond body condition, however, indicators of energy balance and metabolism are rarely measured. The precise role, therefore, of metabolic factors in regulating reproduction in response to food availability remains poorly defined.

In addition to food availability, ambient temperature is a proximate cue that some avian species use to fine-tune HPG axis activity and gonadal development. Warmer temperatures are associated with earlier or longer reproduction and greater HPG axis activity in some birds, but these effects can be species-, population-, and sex-specific (Dawson, 2005, 2018; Gao et al., 2018; Halupka and Halupka, 2017; Perfito et al., 2005; Schaper et al., 2012; Silverin et al., 2008; Wegge and Rolstad, 2017; Whelan et al., 2017; Wingfield et al., 1997, 2003).Temperature may influence the timing of food availability or the food abundance, and thus the energy supply. Supporting this hypothesis, cold temperatures in later winter and spring preserve food hoards in Canadian Jays, *Perisoreus canadensis*, and are associated with larger broods (Whelan et al., 2017). Alternatively or in addition, temperature may exert reproductive effects by altering energy expenditure. For example, warmer spring temperatures are likely to positively affect reproduction

in birds when these temperatures decrease thermoregulatory costs. Consistent with this observation, most studies to date demonstrating an impact of moderately warm temperatures on HPG axis activity and gonadal development have found positive effects (Jones, 1986; Lewis and Farner, 1973; Perfito et al., 2005; Silverin et al., 2008; Wingfield et al., 2003). High temperatures approaching or exceeding the upper limit of the thermoneutral zone (TNZ), however, can have negative effects. In free-ranging avian populations, heat waves result in a decline in abundance and species richness, which may in part be due to a decline in reproduction (Albright et al., 2011). In domestic species, heat stress is associated with a decrease in laying rates, gonadal growth, and gonadal steroid production (Ma et al., 2014; Rozenboim et al., 2004). When exposed to high temperatures, birds primarily use evaporative water loss to maintain constant body temperature (Albright et al., 2017). However, this mechanism is metabolically costly and itself produces heat (Smith et al., 2015). Birds also tend to eat less when exposed to high temperatures, which may reduce metabolic heat production (Chowdhury et al., 2012; Geraert et al., 1996; Ma et al., 2014). Extreme heat, therefore, imposes a tradeoff between maintaining temperature homeostasis and acquiring and maintaining sufficient energy for reproduction.

The idea that the physiological mechanisms that control reproduction are inextricably linked to those that control energy balance, and that the primary signal mediating this interaction is the availability of metabolic fuel for oxidation (i.e., glucose (GLU), free fatty acids (FFA), and amino acids), is the basis of the “metabolic hypothesis” (Schneider, 2004; Wade et al., 1996). This hypothesis leads to predictions about how fluctuating metabolic conditions influence the activity of the HPG axis. Either restricting food availability (i.e., decreasing energy intake) or increasing energy expenditure (e.g., increasing thermoregulatory demands) is predicted to decrease HPG axis activity by lowering the supply of metabolic fuel for oxidation (Schneider, 2004; Wade et al., 1996). The metabolic hypothesis has substantial support in mammals, but has not been adequately tested in avian species. Metabolism, and in particular the role of glucose metabolism, differs between birds and mammals, with birds apparently less reliant on glucose for energy than mammals (Scanes, 2015). Sensitivity of the HPG axis to the availability of particular fuel types may, therefore, also differ in birds and mammals.

Changes in metabolic fuels have been linked to reproduction and energy balance in domestic birds. In some cases, plasma GLU changes in response to food availability (Scanes and Braun, 2013), high temperatures (Chowdhury et al., 2012; Ma et al., 2014), and during different stages of the breeding/nonbreeding season (Gayathri, 2004; Scanes, 2015). Lipid metabolism is also altered by food availability (Scanes, 2015) and high temperatures (Chowdhury et al., 2012). Under negative energy balance, glycogenolysis and lipolysis are stimulated, resulting in energy store mobilization. Avian studies that directly relate changes in metabolic fuels under energetic challenges to reproduction, however, are rare, especially in wild birds. Without this knowledge, it is unclear whether food- or temperature-related effects on the HPG axis are due to metabolic fuel availability, and if so, whether the HPG axis responds to changes in one fuel type in particular.

Environmental information is generally thought to converge on the HPG axis through affecting gonadotropin-releasing hormone (GnRH) release from the hypothalamus (Ball, 1993), but recent evidence suggests that the gonads can respond directly to energy-related factors. Avian gonads possess glucocorticoid receptors (GR), and plasma testosterone (T) decreases in response to acute stress in wild rufous-winged sparrows, *Peucaea carpalis,* in the absence of a decline in plasma luteinizing hormone (LH; Deviche et al., 2010). Both corticosterone (CORT) and metabolic stress (GLU and FFA metabolism) decrease T release by LH/follicle-stimulating hormone (FSH)-stimulated testes *in vitro* (McGuire et al., 2013). These inhibitory effects may be mediated through affecting gonadal gonadotropin-inhibitory hormone (GnIH; Bentley et al., 2008; McGuire et al., 2013), a neuropeptide which exerts inhibitory effects through actions in the brain to decrease GnRH and/or gonadotropin release (Tsutsui et al., 2007), but has recently been shown to also be expressed by the gonads (Tsutsui et al., 2010). Heat shock proteins (HSPs) are molecular chaperones that contribute to maintaining cellular homeostasis in multiple tissues under fluctuating environmental conditions, including changes in temperature (Rajaei-Sharifabadi et al., 2017) and food availability (Liew et al., 2003). Expression of HSPs in the gonads can, therefore, serve as an indicator of the physiological response to cellular stress.

The primary objective of this study was to investigate how alterations in energy homeostasis resulting either from food restriction (FR) or from exposure to high temperatures

(HT) affect the HPG axis activity. I quantified energy homeostasis through measuring body condition, food intake, plasma metabolites (GLU and FFA), and key behaviors that indicate activity levels and motivation to feed or drink, and related changes in energy homeostasis to HPG axis activity (plasma T and testis size). I hypothesized that FR and HT constitute an energetic challenge and inhibit gonadal development and function by lowering available plasma GLU and FFA. I also predicted in food-restricted and heat-challenged birds that lower plasma GLU and initially increased plasma FFA (as triglycerides are mobilized from lipid stores and oxidized), but ultimately lower plasma FFA (as lipid stores are exhausted), would be associated with smaller testes and lower plasma T relative to control birds.

The second objective of this study was to investigate the integration of energetic signals by gonads. I hypothesized that changes in gonadal hormone receptors and enzymes involved in steroid synthesis fine-tune responses to fluctuating energetic conditions. To address this question, I measured the expression of key gonadal genes under FR and HT via real-time PCR on isolated RNA.

Methods

All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee. All necessary permits to capture animals were obtained from the US Fish and Wildlife Service and the Arizona Game and Fish Department.

Capture and initial conditions. Adult male house finches (N=36) were caught in Tempe, AZ, USA (33.41 N, -111.91 W; elevation: 360 m) during the breeding season, between 22 April and 2 May 2016. Birds at this time are naturally photostimulated and males have developed testes (Hamner, 1968). I caught birds using food-baited traps, and sexed and aged them based on plumage characteristics (Pyle, 1997). Only after-second year (i.e., hatched in 2014 or earlier) males were retained. I transported birds to Arizona State University Animal Care Facilities, randomly assigned them to one of two temperature- and photoperiod-controlled rooms, and placed them in individual cages that were visually isolated from each other. All birds were kept on a natural photoperiod (13L: 11D; lights on at 6 AM) for the duration of the study. The initial temperature was held on a semi-natural cycle (29.4° C day, 21.1° C night) similar to the average

temperatures experienced by wild birds in April/May, with the nightly temperature being within the house finch thermoneutral zone (20-37°; Dawson et al., 1985; Weathers, 1981). All birds initially received sunflower seeds and water *ad libitum.* Over the course of 10 days, the diet was gradually changed to Mazuri small bird breeding diet (PMI Nutrition International, Richmond, IN, USA) and food bowls were covered such that only the bird's head fit into the bowl to minimize food spillage. Once all birds were accustomed to consuming the Mazuri pellet diet, their individual daily food consumption was measured over the course of 1 week.

Experimental Treatments: Food Restriction and High Temperature. Approximately 3 weeks after finches were brought into captivity, they were randomly assigned to one of three treatment groups (N=12): (1) food restriction (FR), (2) high temperature (HT), and (3) a control group (CT). Birds assigned to the FR and CT group were placed in one room and those assigned to the HT group were placed in the other. Control and FR birds remained exposed to the semi-natural temperature cycle (29.4° C day, 21.1° C night). Food-restricted birds received a daily ration equal to 70% of their individual *ad libitum* food intake (Chapter 2) whereas CT and HT birds continued to receive food *ad libitum*. Birds in the HT group were exposed to an 8.4° C higher daily temperature cycle (37.8° C day, 29.4° C night). It took approximately 3 hours after lights on for the control chamber to heat from 21.1° C to 29.4° C, and for the hot chamber to heat from 29.4° C to 36.7° C, and an additional 2 hours for the hot chamber to reach its maximum of 37.8° C (Fig. 4.1). Both chambers cooled down after lights off to their nighttime temperature within 2 hours.

Morphology and Food Intake. All birds were weighed daily to the nearest 0.1 g and individual food intake was measured to the nearest 0.1 g. At 5 times throughout the study (prior to treatments (day 0) and after 3, 7, 17, and 27 days of treatment), fat stores, muscle stores, and cloacal protuberance (CP) width were measured. The amount of furcular fat was visually estimated using a scale of 0–5 (0 = no visible fat; 5 = bulging fat; Helms and Drury, 1960). As the pectoral muscles are the largest store of protein in birds, their size was estimated using a scale of 0–3, with 0 for concave pectoral muscles and a prominent keel and 3 for convex pectoral muscles

that protrude above the keel (Salvante et al., 2007). Cloacal protuberance width (± 0.1 mm) was measured using digital calipers.

Plasma collection. Blood samples were obtained at the same times (day 0, 3, 7, 17, and 27) as morphological measurements. At each time, blood (100 µl) was taken from the jugular vein of each finch into a heparinized microsyringe and immediately placed in a microcentrifuge tube on ice. Blood was centrifuged within 3 h, and plasma was collected and stored at −80°C until assayed. All samples were collected between 8:00 h and 10:30 h. I measured plasma T and GLU in all samples, and plasma FFA in samples collected on days 0, 3, and 27.

Plasma assays

Testosterone. A validated (Deviche and Cortez, 2005) commercial enzyme-linked immunoassay (Enzo Life Sciences, Farmingdale, NY, USA) was used to measure plasma T. Plasma was diluted 8x in assay buffer containing 1 µl displacement reagent per 99 µl plasma. Samples were assayed in duplicate with all samples from each bird on a single plate, but with samples randomized on each plate, and with birds from the 3 treatments randomized across 5 plates. Each plate included a complete standard curve. Three additional house finch plasma samples were used as an internal control across the 5 plates. The assay sensitivity was 17.2 pg/ml and the inter- and intra-assay coefficients of variation were 10.5% (N=3 samples assayed on each plate) and 2.4% (N=119 samples), respectively.

Metabolites. I measured plasma GLU and FFA using colorimetric assay kits (GLU: Cayman Chemical Co., Ann Arbor, MI, USA; FFA: BioVision, Milpitas, CA, USA). Instructions outlined by the manufacturer were followed. Samples were assayed in duplicate with all samples from each bird on a single plate, but with samples randomized on each plate, and with birds from the 3 treatments randomized across 5 plates. To measure plasma GLU, I diluted plasma 8x in assay buffer before dispensing into assay wells. Three additional house finch plasma samples were used as an internal control across the 3 plates. The average inter- and intra-assay coefficients of variation for the glucose assay were 1.2% and 2.4% respectively, and the assay sensitivity was 0.23 mg/dl. To measure plasma FFA, I diluted plasma 5x in assay buffer before

dispensing into assay wells. The average inter- and intra-assay coefficients of variation for the FFA assay were 13.4% and 4.7%, respectively, and the assay sensitivity was 2.0 µM.

Behavior. I used digital video cameras to record the behavior of each finch at 3 points throughout the study: prior to treatments - week 0, and after 1 and 3 weeks of treatment. At each time point, birds were divided between 2 days so that recordings took place within a similar time window (1 to 5 PM). Video cameras were installed in front of cages to record 3 birds at a time and for 40 consecutive minutes. The sequence of recordings between the days and the time of day that birds were videotaped were randomized.

The last 30 minutes of each 40 minute-long video were analyzed. The frequency of 4 behaviors was counted using Cowlog 2.0 software (Hänninen and Pastell, 2009): locomotor activity (number of hops/30 min), feeding frequency (number of times head entered food bowl/30 min), drinking activity (number of times head entered water bowl/30 min), and bill wipes (number of times bill was brushed against perch or surface of the cage/30 min), which can indicate aggressive behavior (Wingfield, 2005). Video analyses were independently performed by 3 investigators who were not aware of the group to which a bird belonged. Results for each of the 4 behaviors in 6 birds were normalized across the 3 investigators using the data from one investigator as an arbitrary calibrator.

Testis collection and qPCR. After 27 days of treatment, birds received an intramuscular injection of 400 µl anesthetic solution (0.9% NaCl containing 20 mg/ml xylacine and 100 mg/ml ketamine) and were euthanized by decapitation. Testes were removed, rinsed in distilled water, and weighed to the nearest 0.01 mg. Testes were then placed in RNALater (Invitrogen, Carlsbad, CA, USA) and stored at −80°C until assayed. Gonadosomatic index (GSI) was calculated as testis mass as a percentage of body mass.

The expression of 9 genes hypothesized to influence testicular function was analyzed by qPCR. These included the gonadotropin receptors (LHR and FSHR), receptors activated by CORT (glucocorticoid receptor (GR) and mineralcorticoid receptor (MR)), two steroidogenic enzymes (steroidogenic acute regulatory protein (StAR) and 17β-hydrogysteroid dehydrogenase (17β-HSD)), a potential inhibitor of T release (GnIH), and two HSPs that may be critical for

functionality of steroid receptors (Pratt 1993; Sapolsky et al., 2000; Kojika et al., 1996), especially during stress (HSP60 and HSP70).

Total RNA was extracted and purified from 20 mg of testis tissue using RNeasy mini kit (Qiagen, Valencia, CA, USA). Homogenization was carried out using the Digital Sonifier 250 (Branson, Danbury, CT, USA). RNA was quantified using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE, USA) and quality was determined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Following quantification, 1 ug RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The resulting cDNA product was used as a template for quantitative real-time PCR to measure gene expression. Quantitative PCR reactions were carried out following manufacturer's instructions for PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Carlsbad, CA, USA) and ran on an ABI 7900HT thermocycler (ThermoFisher) using a 384 well format, with samples run in triplicate. Primers were designed for each gene from previously published or predicted sequences of the Zebra Finch genome using Primer-Blast (Table 4.1). Primer sets were purchased from Invitrogen (Carlsbad, CA, USA), and optimized for purity and specificity. Melt curves were analyzed prior to use and primers were not used if secondary structure or dimerization was found in no-template controls. The program qBase was used to determine by geNORM algorithm the best normalizing genes from a set of 3: RPL4, HPRT, and GAPDH. The geometric mean of RPL4 and HPRT was selected as the best normalization factor. RPL4 and HPRT have also been shown to be among the most stable testicular housekeeping genes in passerines (Zinzow-Kramer et al., 2014). Each plate included a standard curve to calculate efficiency.

Raw fluorescent data were analyzed using the ABI 7900HT thermocycler system software and cycle thresholds were obtained using this software. Expression values for each gene were calculated as fold difference = $(E_{GOI})^{\Delta Ct\ GOI}/(E_{Norm})^{\Delta Ct\ Norm}$, where GOI is the gene of interest, Norm is the normalization factor calculated from the reference genes, E is the average PCR efficiency as calculated by a standard curve, and ΔCt is the cycle threshold difference between the treatment (FR or HT) and control (CT) group. Final data (gonadal gene expression) for display and statistical analysis are fold change, which was calculated by dividing the

expression value of each gene for each bird by the average expression of this gene in the CT group.

Statistical Analyses. Effects of treatment on GSI and gonadal gene expression were analyzed via one-way ANOVA, followed by Dunnett's test for comparisons with CT group only. Outliers were identified as any data point more than 1.5 interquartile ranges below the first quartile or above the third quartile, and removed. As a result of this analysis, one CT plasma sample was removed from the analysis of plasma FFA, GLU, and T; in addition, one CT sample, 1 FR sample, and between 1-3 HT samples were removed from the expression analysis of each gonadal gene. Data sets that were not normally distributed or homoscedastic, according to the Shapiro-Wilk test and Levene's test, respectively, were transformed prior to analysis to meet these assumptions, using either a square root (MR, GR) or natural log transformation (FSH, LH, 17β-HSD, StAR, GnIH, HSP60, and HSP70).

Effects of treatment on repeated data (morphological characteristics, food intake, plasma T and metabolites, and behavior) were analyzed using two-way repeated measures ANOVA, with time as the within-subject factor and treatment (control, food restriction, high temperature) as the between-subjects factor. Four birds died over the course of the experiment in the FR group, resulting in the absence of data for 1 bird after 3 days and 3 additional birds after 7 days. Additionally, I was unable to measure either plasma T or metabolites in several samples. This resulted in 9 missing measurements for each morphological characteristic (body mass, CP) out of 180 total, and 17 missing plasma measurements out of 468 total. I estimated missing values for interval scale data only using multiple imputation (MI) and the NORM program (http://sites.stat.psu.edu/~jls/misoftwa.html; Schafer, 1999). Multiple imputation relies on more plausible assumptions than other approaches to dealing with missing data (e.g., case deletion or replacement with group means). It also properly accounts for uncertainty about missing values (leading to appropriate standard errors) and retains original sample sizes (Little and Rubin, 2002).

Data sets that were collected on an ordinal scale (fat and muscle scores, behavior) were ranked before proceeding with the analysis. For interval scale data sets that were not normally distributed or homoscedastic, according to the Shapiro-Wilk test and Levene's test, respectively,

transformations were unable to meet these assumptions, and the ANOVA was performed on ranked values. This included plasma T, GLU, and FFA. For data sets that did not display sphericity, according to Mauchly's spericity test, degrees of freedom were deflated using a Ɛ-derived Greenhouse-Geiser correction. This included body mass and food consumption. When a statistically significant treatment x time interaction was detected using ANOVA, Fisher's Least Significant Difference (LSD) tests were used to perform pair-wise comparisons. Because there was high individual variation in gene expression, effect sizes (n_p^2) for results were calculated in cases of significance and considered small if $n_p^2 > 0.0099$, medium if $n_p^2 > 0.0588$, and large if $n_p^2 > 0.1379$ (Cohen, 1988).

To examine relationships between dependent variables, Spearman's correlations were used, as many relationships appeared nonlinear. I examined relationships within metabolic variables (body mass, plasma FFA and GLU, food intake, feeding frequency, and locomotor activity), within reproductive variables (GSI, plasma T, and gonadal gene expression), and then between metabolic and reproductive variables. Relationships were examined within individual treatment groups as well as with all individuals included. With repeated data used in correlations, the change in parameter value between each time point and pre-treatment levels (day 0) was first calculated. Data were analyzed using SPSS (version 24; IBM, Armonk, NY, USA). The significance level of all statistical tests was set at P = 0.05.

Results

Body Condition. Body mass changed over time ($F_{2.21,72.92} = 33.81$, $P < 0.001$) and there was a treatment x time interaction ($F_{4.42, 72.92} = 12.17$, $P < 0.001$; Fig.4.2A), but no effect of treatment alone ($F_{2,33} = 2.26$, $P = 0.12$). Food-restricted and HT birds had similar mass to CT birds at the start of the treatment (LSD tests, $P > 0.05$). Control birds maintained roughly the same body mass throughout the study (LSD tests, $P > 0.05$), but FR birds lost mass within the first 7 days of treatment, and maintained lower body mass than CT birds for the duration of the experiment (LSD tests, $P < 0.05$). Heat-stressed birds loss body mass within 7 days of treatment and roughly maintained lower body mass throughout the study (LSD tests, $P < 0.05$), but mass did not differ from that of CT birds at any time (LSD tests $P > 0.05$).

Furcular fat was affected by treatment ($F_{2,29} = 6.20$, $P = 0.002$), time ($F_{4,116} = 7.67$, $P < 0.001$), and the interaction between treatment and time ($F_{8, 116} = 3.94$, $P < 0.001$; Fig.4.2B). Control birds and HT birds maintained roughly the same amount of fat throughout the experiment (LSD tests, $P > 0.05$), whereas FR birds lost fat stores within the first 3 days of the treatment and had less fat than CT birds from 3 days of treatment to the end of the study (LSD tests, $P < 0.05$).

Muscle score was affected by treatment ($F_{2,29} = 10.59$, $P < 0.001$) and time ($F_{4,116} = 5.01$, $P = 0.001$), and there was a treatment x time interaction ($F_{8,116} = 3.56$, $P = 0.001$; Fig. 4.2C). Control birds maintained the same amount of muscle throughout the experiment (LSD tests, $P > 0.05$), whereas FR birds lost muscle after 17 days of treatment but had less muscle than CT birds after 3 days and throughout the 27 days of treatment (LSD tests, $P < 0.05$). Heat-stressed birds maintained roughly the same amount of muscle throughout the experiment (LSD tests, $P > 0.05$).

Food intake. Food intake was affected by treatment ($F_{1,33} = 581.99$, $P < 0.001$) and time ($F_{4,132} = 212.02$, $P < 0.001$), and there was a treatment x time interaction ($F_{8,132} = 15.67$, $P < 0.001$; Fig. 4.2D). Food-restricted birds ate all food available, which was approximately 70% of each bird's *ad libitum* ration. Both CT and HT birds voluntarily ate less over the course of the study (LSD tests, $P < 0.001$), but HT birds consumed less food than CT birds at every time point measured (LSD tests, $P < 0.001$).

Reproductive Parameters. There was a significant effect of treatment on GSI ($F_{2,31} = 5.02$, $P = 0.01$; Fig. 4.3C), which was lower in FR birds (Dunnett's test, $P = 0.014$) and in HT birds (Dunnett's test, $P = 0.04$) than in CT birds. Plasma T changed significantly over the course of the study ($F_{4,128} = 3.93$, $P = 0.005$; Fig. 4.3B), but was not affected by treatment ($F_{2,32} = 0.74$, $P = 0.48$), and there was no treatment x time interaction ($F_{8,128} = 1.12$, $P = 0.36$). Overall, plasma T decreased after 7 days, increased after 17 days, and decreased after 27 days similar to beginning levels (LSD tests, $P < 0.05$). Cloacal protuberance width significantly decreased over the course of the study ($F_{4,132} = 3.93$, $P = 0.005$), but was not affected by treatment ($F_{2,33} = 2.08$, $P = 2.10$), and there was no treatment x time interaction ($F_{8,132} = 1.67$, $P = 0.11$; Fig. 4.3A).

Plasma Metabolites. Plasma GLU changed significantly over the course of the study ($F_{4,128}$ = 6.81, P < 0.001; Fig. 4.4A), but was not affected by treatment ($F_{2,32}$ = 0.091, P = 0.91), and there was no treatment x time interaction ($F_{8,128}$ = 0.91, P = 0.48). Amongst the treatment groups, plasma GLU increased after 3 days and remained higher than initial levels for the duration of the study (LSD tests, P < 0.01).

Plasma FFA changed over time ($F_{2,66}$ = 7.29, P = 0.001) and there was a treatment x time interaction ($F_{4,66}$ = 3.47, P = 0.01; Fig. 4.4B). Plasma FFA were similar among treatment groups at the start of the experiment (LSD tests, P > 0.05), but in CT birds and HT birds, their concentration decreased after 3 days and rose after 27 days (remaining lower than initial levels in CT birds but not in HT birds; LSD tests, P < 0.05). Food-restricted birds, on the other hand, maintained consistent plasma FFA throughout the experiment, and so plasma FFA at day 3 was higher in FR birds compared to CT birds (LSD tests, P < 0.05).

Behavior. There was a significant treatment x time interaction for locomotor activity ($F_{4,58}$ = 3.44, P = 0.01; Fig. 4.5A). Birds across treatment groups had similar activity at the beginning of the study, but after 3 weeks of treatment, FR birds were more active than CT birds (LSD tests, P < 0.05). Heat-stressed birds were less active than initially after 1 week of treatment, but not significantly less so than CT birds at any time (LSD tests, P < 0.05).

Bill wiping activity decreased in all treatment groups over the course of the study ($F_{2,58}$ = 3.70, P = 0.031; Fig. 4.5B) but there was no effect of treatment ($F_{2,29}$ = 0.37, P = 0.70) nor was there a treatment x time interaction ($F_{4,58}$ = 1.58, P = 0.20).

Feeding frequency decreased over time across treatment groups ($F_{2,58}$ = 3.92, P = 0.03), and overall was different among the treatment groups ($F_{2,29}$ = 5.43, P = 0.01; Fig. 4.5C), with the trend of lower feeding frequency over time in HT birds, but the treatment x time effect was not significant ($F_{4,58}$ = 2.20, P = 0.08).

There was no effect of treatment ($F_{2,29}$ = 2.13, P = 0.14), time ($F_{2,58}$ = 1.57, P = 0.22), or a time x treatment interaction ($F_{4,58}$ = 0.26, P = 0.90) on drinking activity (Fig. 4.5D).

Gene expression. There generally was considerable individual variation in expression of the genes under study (see error bars, Fig. 4.6). Treatment affected the gene expression of 17β-

HSD ($F_{2,22}$ = 12.29, P < 0.001, n_p^2 = 0.55; Fig. 4.6), with food-restricted birds (Dunnett's test, P = 0.001) and HT birds (Dunnett's test, P = 0.001) having lower gene expression than CT birds.

Treatment had a marginally significant effect on LHR ($F_{2,21}$ = 0.65, P = 0.054, n_p^2 = 0.06), with the trend of lower LHR in HT birds compared to CT birds. Treatment did not affect the gene expression of FSHR ($F_{2,19}$ = 0.34, P = 0.71), StAR ($F_{2,25}$ = 0.73, P = 0.49), GnIH ($F_{2,22}$ = 0.84, P = 0.45), HSP60 ($F_{2,25}$ = 0.43, P = 0.96), HSP70 ($F_{2,24}$ = 0.35, P = 0.71), GR ($F_{2,22}$ = 0.60, P = 0.56), or MR ($F_{2,23.}$ = 2.19, P = 0.14).

Relationships between variables

Within metabolic parameters. There were multiple relationships within metabolic variables, some of which depended on the treatment group (Table 4.2). Considering all of the experimental birds, there were negative correlations between plasma GLU and FFA, between plasma GLU and feeding frequency, between plasma FFA and body mass, and between body mass and locomotor activity, as well as a positive correlation between plasma FFA and feeding frequency. Within HT birds, the positive correlation between plasma FFA and feeding frequency was present, as well as a positive correlation between plasma GLU and body mass. There were no correlations among metabolic parameters within CT or FR birds.

Within reproductive parameters. There were multiple relationships within reproductive variables, some of which depended on the treatment group (Table 4.3; Fig. 4.7). Considering all of the experimental birds, GSI correlated positively with plasma T, 17β-HSD, GR, and HSP60 gene expression. Plasma T was positively correlated with GR and HSP60 gene expression, and negatively correlated with LHR gene expression. There was an overall positive correlation between LHR and FSHR gene expression. Gene expression of 17β-HSD was positively correlated with GR and HSP60 gene expression, GR and MR gene expression were correlated with each other, as well as each with HSP60 gene expression. Finally, there was a negative correlation between GnIH and HSP60 gene expression.

Within HT birds, the correlations between GSI and plasma T, and 17β-HSD and GR gene expression were present, as well as an additional positive correlation between GSI and MR gene expression. The positive correlations between plasma T and GR gene expression, and between

GR and MR gene expression were present as well. Within FR birds, the positive correlations between LHR and FSHR gene expression, between GR and MR gene expression, and between 17β-HSD and HSP60 were present. There were no correlations among reproductive parameters within CT birds.

***Between reproductive and metabolic parameters*.** There were several relationships between reproductive and metabolic variables, most of which depended on the treatment group (Table 4.4; Fig. 4.8). Across all birds, there were positive correlations between GSI and body mass, 17β-HSD gene expression and body mass, and between 17β-HSD gene expression and food intake. Within HT birds, GSI was positively related to body mass, and 17β-HSD gene expression was also positively related to both food intake and drinking activity, and StAR gene expression was positively related to food intake and feeding activity, but negatively related to locomotor activity. Within FR birds, and StAR gene expression was negatively related to both feeding and drinking frequency. Within control birds, plasma T was positively related to body mass, and MR gene expression was positively related to feeding frequency. Plasma GLU and FFA were not related to any reproductive parameter.

Discussion

I investigated how changes in energy homeostasis resulting either from food restriction or from high temperature affect reproductive physiology, and the role of fuel availability in mediating these effects. To this aim, I compared a control (CT) group of male house finches that was fed *ad libitum* and exposed to a thermoneutral temperature cycle with (a) a food restriction (FR) group which experienced a 30% food restriction while kept on a thermoneutral temperature cycle, and (b) a high temperature (HT) group which was fed *ad libitum* while experiencing a 8.3°C warmer temperature cycle. Measures of energy homeostasis and gonadal function were collected multiple times over a 4 week period. Food restriction and high temperature influenced energy balance, as shown by FR birds losing body mass, fat, and muscle, and increasing locomotor activity, and HR birds eating less and also losing muscle mass relative to CT birds. Only in FR birds, however, were plasma metabolites altered, with evidence of increased FFA utilization early during the food

restriction period. Testicular growth and function were inhibited in both treatment groups, accompanied by a decline in gonadal 17β-HSD gene expression.

Alterations in energy homeostasis

Food restriction. The present method of food restriction produced similar changes in body condition as found previously (Chapter 2). These changes resulted, at least in part, from a decrease in fat reserves and pectoral muscle size. Additionally, locomotor activity was higher in FR than CT birds, a common effect of this manipulation (Fokidis et al., 2011; Krause et al., 2017). Increased activity under FR may occur through elevated CORT and is likely associated with increased foraging behavior (Fokidis et al., 2011). I had predicted that changes in body condition and behavior during food restriction would be accompanied by changes in plasma GLU and/or FFA, but found no effect of this treatment on plasma GLU.

Plasma GLU in birds has traditionally been considered to remain relatively constant regardless of diet or feeding frequency (Klasing, 1998; Polakof et al., 2011). In many wild birds, however, plasma GLU changes during the daily cycle, being higher during the day than during the night (Downs et al., 2010; Jenni and Jenni-Eiermann, 1996), which likely reflects daily changes in feeding and/or temperature (Pollock, 2002). Furthermore, there is evidence in some situations that food availability or type affects plasma GLU. For example, in vesper sparrows, *Pooecetes gramineus*, 10 hours of fasting decreases plasma GLU (Swain, 1987). On the other hand, in red-winged blackbirds, *Agelaius phoeniceus,* and common grackles, *Quiscalus quiscula,* plasma GLU increases within an hour of a carbohydrate meal, but this increase in European starlings depends on the meal composition in carbohydrates (del Rio et al., 1988). Overall, there are few studies investigating effects of chronic, moderate changes in food availability on plasma GLU. It appears that at least in house finches, glycemia is not affected by chronic mild food restriction.

Plasma GLU increased across groups after 3 days and then remained elevated. This effect may have resulted from dietary adjustments or from stress associated with the early phases of experimentation. Indeed, transfer from sunflower seeds to a pellet diet was completed only 1 week prior to the beginning of experimental manipulations. In addition, birds had been handled minimally until these manipulations began, whereas from day 0 on, they were handled

daily. Handling may have initiated a stress response, which is often associated with glycogenolysis, protein catabolism, lipid mobilization, gluconeogenesis, and increased feeding. Corticosterone contributes to these changes: it acts to increase energy availability, especially GLU (Sapolsky et al., 2000), by elevating plasma GLU (Dallman and Bhatnagar, 2011). Plasma GLU after both 7 and 27 days was negatively related among all birds to feeding frequency at 21 days, indicating that changes in plasma GLU, again potentially through CORT, might mediate hunger and feeding behavior. Consistent with this hypothesis, pharmacological blockade of GLU utilization stimulates feeding in mammals (Smith and Epstein, 1969), but has the opposite effect in white-crowned sparrows, *Zonotrichia leucophrys* (Boswell et al., 1995). Absent additional information, it is unknown whether GLU has species-specific effects on feeding or the behavioral influence of GLU utilization blockade differs in birds and mammals.

Food restriction altered plasma FFA. Plasma FFA in CT birds decreased after 3 days. As is the case for GLU (see above), this decrease may have resulted from the recent diet change or from the stress associated with increased handling. In FR birds, however, plasma FFA did not decrease and remained constant throughout the study. Higher plasma FFA in FR birds, accompanied by the decline in furcular fat, presumably reflects lipid mobilization and use, and is what I predicted to happen under initial or moderate food restriction. To cope with energetic challenge, birds first utilize glycogen stores, then mobilize fatty acids from lipid stores, and if lipid stores become exhausted, break down muscle for gluconeogenesis (Jenni-Eiermann et al., 2002; Landys et al., 2004; Sapolsky et al., 2000; Scanes, 2015). In accordance with this, plasma FFA after 3 days were negatively related to plasma GLU after 3 days and body mass after 7 days across all birds. Reciprocal utilization of energetic fuel type thus occurred under this potentially stressful acclimation period, and enhanced FFA utilization predicted a future decline in body condition. A similar relationship was found in migratory white-crowned sparrows. In this species, an increase in plasma triglycerides, which indicates decreased FFA utilization, correlates positively to mass change (Cerasale and Guglielmo, 2006). Further evidence that plasma FFA indicated a lower energy state in the house finches used in this study is the existence of a positive relationship between plasma FFA and feeding frequency.

High Temperature. Finches exposed to the high temperature cycle experienced minor changes in body condition, including a decline in body mass and pectoral muscle size but not fat stores. This result is notable considering that HT birds were exposed to a temperature exceeding the upper limit of their TNZ by less than 1° C only for approximately 8 hours a day. The observed negative changes in body condition in HT finches could be either direct, i.e., result from the metabolic costs of thermoregulation or indirect, i.e., result from decreased food consumption and feeding frequency. The present results are consistent with correlative evidence in house finches indicating that ambient temperature correlates negatively with foraging effort (Shochat et al., 2004).

In the chicken, high temperatures decrease food intake and body mass but the latter decrease is not entirely explained by a decrease in food intake (Gereart et al., 1996; Habashy et al., 2017). High temperatures decrease feeding efficiency and lower the proportion of energy stored as protein versus fat (Gereart et al., 1996). The observation in the present study that high temperatures reduced muscle scores but not fat reserves is consistent with these findings, and suggests that heat exerts indirect metabolic effects, i.e., effects that do not directly follow changes in food intake. Consistent with this conclusion, HT finches decreased their food intake below that of FR birds, yet exhibited milder metabolic changes. High temperature altered neither plasma GLU nor plasma FFA, which were similar to those of CT birds. Acute (hours) HT in chickens lowers plasma triglycerides, but raises plasma GLU (Chowdhury et al., 2012; Xie et al., 2015). These metabolites, however, are unaltered under chronic (days) HT whereas markers of tissue damage increase (Xie et al. 2015). Whether high temperatures caused tissue damage in the present study is unknown.

Alterations in gonadal function

Food Restriction. Testes were smaller in FR and HT than in CT birds. Maximum testis size in free-ranging house finches occurs around May and testicular regression begins in early July, as birds become photorefractory, and is completed by August (Hamner, 1968). In captivity, however, this timing can change, with regression occurring earlier than in the wild depending on the photoperiodic regime (Hamner, 1968). In the present study, finches were captured in late

April/early May, held under constant long photoperiod, and euthanized for testis measurement in late June. The observed decrease in CP width and plasma T suggests that birds became photrefractory during the study, which was presumably associated with a decrease in testis mass in CT birds.

I previously reported inhibited testicular growth under FR in photostimulated house finches (Chapter 2). Thus, it appears in this species that food restriction inhibits photoinduced gonadal growth and, as shown here, may accelerate gonadal regression during photorefractoriness. These results are consistent with the effects of food availability generally reported in opportunistic species. In particular, food restriction inhibits photostimulated gonadal growth in red crossbills, *Loxia curvirostra* (Hahn, 1995) and zebra finches, *Taeniopygia guttata* (Perfito et al., 2008), whereas enhanced food (quantity, quality) stimulates gonadal growth in photostimulated spotted antbirds, *Hylophylax naevioides*, and to a lesser extent in spotted antbirds held on a constant photoperiod (O'Brien and Hau, 2005). Most avian species show some gonadal response to photoperiod, but gonadal development in opportunistic species is thought to be relatively more reliant on non-photic cues than in species that primarily rely on a predictable cycle of seasonal changes in photoperiod (Dawson, 2008). In these photoperiod-dependent breeders, changes in food availability does, in fact, seem to influence male gonadal function less than in opportunistic breeders. For example, a food restriction regime similar to that used here did not alter testis growth in photostimulated Abert's towhees, *Pipilo aberti* (Davies et al., 2015). In the European starling, food restriction lowered testis mass under constant photoperiod when this treatment also lowered body mass (Meijer, 1991), but it did not affect photostimulated testicular growth when the treatment did not decrease body mass (Dawson, 1986). Consistent with this finding, I observed a positive relationship between body mass at the middle of the study and testis mass at the time of euthanasia. Taken together, these results suggest complex interactions between food availability, energy homeostasis, and photoperiod, which might vary with breeding strategy and other species-specific factors.

Food restriction did not influence plasma T, as was the case in my previous study (Chapter 2), but plasma T was lower in the present study than in photostimulated finches

(Chapter 2). This difference may reflect inter-assay differences in hormone concentrations, but might indicate that T production and/or release is less affected by food availability when levels are already low under partial gonadal regression. Across all birds in this study, plasma T was related to testis mass, but neither were related to FSHR or LHR gene expression. Elevated plasma T can in some circumstances stimulate gonadal development (house finch: Deviche et al., 2006) and slow gonadal regression (Brown and Follett, 1977). Binding of FSH to FSHR generally stimulates spermatogenesis, and most testicular tissue is devoted to spermatogenesis (Deviche et al., 2011). Therefore, the absence of a decline in FSHR expression under food restriction when testis mass is reduced, and the absence of a relationship between testis mass and FSHR expression is surprising. However, plasma FSH was not measured and may have been lower in FR than CT finches. Similarly, LH acts to stimulate plasma androgen production by binding to gonadal LHR (Kirby and Froman, 2000). With no effect of food restriction on plasma T, it is not necessarily surprising that LHR expression was unaffected by this treatment. Furthermore, FSHR and LHR expressions were positively related overall as well as in FR birds alone, further implying coordination between their downstream effects (gonadal growth and plasma T release, respectively).

Food restriction influenced (decreased) the expression of only one gonadal gene measured (17β-HSD). 17β-HSD converts androstenedione to testosterone, the final step in T synthesis. By contrast, the expression of StAR, a transport protein that is critical to the early stages of steroid hormone production, was unaltered by food restriction. FSHR, LHR, and GnIH gene expressions were likewise not influenced by food restriction. As mentioned above (Results), the absence of detectable changes in the expression of these genes may be a consequence of high individual variation and small sample sizes. Alternatively, food restriction may have a gene-specific effect on a downstream enzyme responsible for T production. Consistent with this proposition, in zebra finches, fasting exerts gene-specific gonadal effects: it reduced plasma T and expression of StAR and LHR, but not 17β-HSD (Lynn et al., 2015). It is, however, unclear in the present study why the decline in17β-HSD expression under food restriction was not associated with decreased plasma T, either overall or within individuals.

High Temperature. High temperature exposure decreased testis mass but not plasma T. Studies on free-living and captive wild birds generally find a positive association between temperature and gonad development (Jones, 1986; Lewis and Farner, 1973; Perfito et al., 2004, 2005; Silverin et al., 2008; Wingfield et al., 2003), but the "cold" temperatures to which birds were exposed in these studies were nearly always outside the bird's TNZ while the "warm" temperatures were within it. Although extremely high temperatures negatively affect wild bird populations (Abright et al., 2007; 2017), to my knowledge, there have been few field studies investigating these effects on reproduction and no captive studies investigating gonadal responses to temperatures beyond the upper limit of a wild bird's TNZ. In domestic poultry, exposure to temperatures beyond the TNZ can reduce ovarian growth (Ma et al, 2014; Rozenboim et al., 2004; Wilson et al., 1972) and steroid production (Rozenboim et al., 2004).

The overall positive relationship between plasma T and testis mass was also found only within HT birds, but the lack of an overall effect of HT on plasma T indicates some independence between temperature-related effects on gonad size versus androgen release. Indeed, cold temperature inhibits gonadal growth without affecting total androgens in a population of song sparrows, *Melospiza melodia* (Perfito et al., 2005) or plasma LH in white-crowned sparrows (Wingfield et al., 2003). Both gonad size and plasma LH, however, were lower under cold temperatures in great tits, *Parus major* (Silverin et al., 2008). Coordination of temperature-related effects on both gonadal size and function may depend on the extent/direction of thermal challenge or may be population or species-specific.

As was the case following food restriction, high temperature significantly reduced the expression of only one gonadal gene measured, 17β-HSD. To my knowledge, this is the first study to measure gonadal gene expression in response to high temperatures in any vertebrate. The lack of effect on expression of other genes measured here may relate to relatively small sample sizes and/or high variability in the expression of these genes. The strong inhibition of 17β-HSD gene expression in both HT and FR birds, as well as the positive relationship between 17β-HSD and testis mass in HT birds, suggests that this enzyme plays a key role in mediating gonadal growth in response to various environmental stressors, including temperature exceeding

the species' TNZ. It remains to be determined whether it also plays a role under cold temperature-mediated inhibition of gonadal growth. As temperatures rise, and in some areas, to highs exceeding the upper TNZ limit of many avian species, it is becoming increasingly important to understand the consequences of these increases on reproductive physiology and behavior.

Interaction between energy homeostasis and gonadal function. The mammalian reproductive system responds to metabolic fuel availability (Dupont et al., 2014; Schneider et al., 2012), and *in vitro*, avian gonads respond to GLU or FFA oxidation (McGuire et al., 2013). Based on these observations, I hypothesized that the primary signal mediating the interaction between reproduction and energy balance is the availability of metabolic fuel for oxidation, and predicted that altered gonadal growth and steroid release would be related to plasma GLU and/or FFA under FR or HT. This was, however, not the case: Plasma GLU was unaltered under either energetic challenge, and it was not related either to plasma T or to testis size. Furthermore, plasma FFA were only altered upon 3 days of FR, but were not related to plasma T or testis mass in this group or among all birds. These results do not support the hypothesis that the avian HPG axis responds directly to metabolic fuel availability, but do not exclude that such a response occurs and depends upon complex mechanisms. For example, the avian HPG axis activity is influenced by a suite of metabolic hormones, including CORT and leptin, and by metabolic signals that exert effects indirectly, through neural circuits involving NPY, AgRP, and GnIH (Davies et al., 2014). These "secondary" neural mediators communicate information related to "primary" sensory stimuli (available fuel) to the HPG axis and may themselves alter this primary signaling (Schneider, 2004). A comprehensive understanding of these pathways requires extensive experimentation involving pathway activity identification and manipulation. Examination of factors downstream of fuel oxidation might prove particularly useful: GLU and FFA oxidation are interrelated through ATP, the final energy currency for all cells. Evidence from the mammalian literature points to components of this final pathway to ATP formation, or ATP itself, as ultimately responsible for regulating HPG axis activity (Schneider et al., 2012).

Although this study did not provide evidence for a direct role of fuel availability on gonadal growth and function, I did find positive relationships between gene expression of

steroidogenic enzymes and feeding and locomotor activity. 17β-HSD, the enzyme whose expression decreased in FR and HT groups, was positively related overall to food consumption, and in HT birds, also to drinking activity. Birds primarily use evaporative water loss to maintain body temperature under high temperatures, and thus adequate replacement of water to sustain this cooling mechanism may have been important in maintaining steroidogenesis. Furthermore, StAR gene expression was not influenced by food restriction or temperature, but positive relationships in HT birds between StAR expression and both food intake as well as feeding behavior, plus a negative relationship between StAR and locomotor activity, suggest that positive energy balance (i.e., ingesting more and expending less) may influence the expression of this enzyme under HT. The negative relationship within FR birds between StAR and feeding and drinking behavior is likely related to overall negative effects of increased activity under food restriction, for example spending more time “foraging” for food that is not available.

A seemingly paradoxical finding was the positive relationship between GR gene expression and both testis size and 17β-HSD expression. At baseline plasma levels, CORT acts primarily through MR to regulate metabolism, activity levels, and feeding behavior (Landys et al. 2004). By contrast, GR activation primarily occurs during stress, i.e., when plasma CORT is elevated above baseline (Lattin et al., 2014), and generally has negative effects on gonadal steroidogenesis (Wingfield and Sapolsky, 2003), gonadal growth (Hull et al., 2007), and Leydig cell function (Hardy et al., 2005). One explanation for the positive relationship between GR and gonadal function is that this might not also represent increased CORT activity on GR. In the chronically stressed house sparrow, *Passer domesticus*, gonadal GR expression increases but this increase is not associated with changes in baseline CORT or testis mass (Lattin et al., 2014). Alternatively, GR activation may influence steroidogenesis and testis growth/maintenance positively. Indeed, in mammals glucocorticoids are required at specific stages of spermatogenesis (Whirledge and Cidlowski, 2013). Glucocorticoids can also stimulate steroidogenic enzyme activity, including 17β-HSD, in rat and human ovarian cells at certain stages in the ovarian cycle (Michael and Cook, 1994). Thus, glucocorticoids may affect gonadal growth and function positively also in birds.

Heat shock protein gene expression in various tissues increases in response to high temperatures (Ma et al., 2014; Mehaisen et al., 2017; Rajaei-Sharifabadi et al., 2017; Xie et al., 2014) and food restriction (Liew et al., 2003), thereby contributing to protecting cell functions during stress. In wild-caught house finches, circulating HSP60 and HSP70 are higher during the hotter months as compared to cooler months (Hill et al., 2013) of the year. Here I found no effect of FR or HT on HSP60 or HSP70 gene expression, but HSP60 gene expression was positively related to GR and MR gene expression. Binding of HSPs and notably HSP70 to steroid receptors, is critical for the stabilization and functioning of these receptors (Kojika et al., 1996; Pratt 1993; Sapolsky et al., 2000). Furthermore, if proper GR activation is necessary for testicular function at this stage of the seasonal cycle, during which gonads are developed, as hypothesized above, a positive relationship between the gene expression of HSP60 and 17β-HSD and testis size would be predicted, as was found in the present study. HSP60 has been implicated in mammalian spermatogenesis (Meinhardt et al., 2002). Interestingly, I also observed a negative relationship between GnIH and HSP60 gene expressions. Gonadal GnIH is upregulated in response to a variety of stressors and may inhibit steroidogenesis (Bentley et al., 2017), but the precise role of gonadal GnIH in mediating gonadal responses to stress requires further examination as the existence of this gonadal GnIH system has only recently been discovered (Bentley et al., 2008). To my knowledge, no link has been shown between HSPs and GnIH.

Conclusion. The hypothesis that negative energy homeostasis inhibits reproductive development and function by lowering available energetic fuel was tested under two situations of altered energy homeostasis. Food availability and temperature altered energy balance and reproductive physiology. Changes in body condition and locomotor activity occurred under food restriction, with utilization of fatty acids from fat stores occurring in the early stages of food restriction, reflecting negative energy homeostasis. Changes in body condition and food intake occurred under high temperatures, but with no change in plasma metabolites. Availability of plasma glucose was resistant to any change in energy homeostasis. Testis mass and testicular gene expression of 17β-HSD declined under either food restriction or high temperature, with the decline in 17β-HSD expression potentially mediated by lower GR and HSP60 activity. This is one

of the first studies examining how high temperatures affect energy homeostasis and reproductive physiology in a wild bird. A further understanding of how food availability and temperature affect reproductive physiology in wild birds, their interactional effects, and the role of metabolism, will require studies to continue to identify and to manipulate the metabolic and hormonal pathways affected. This information is crucial for understanding changes in reproduction and natural population dynamics in environments that are continually subject to change through both natural and anthropogenic processes.

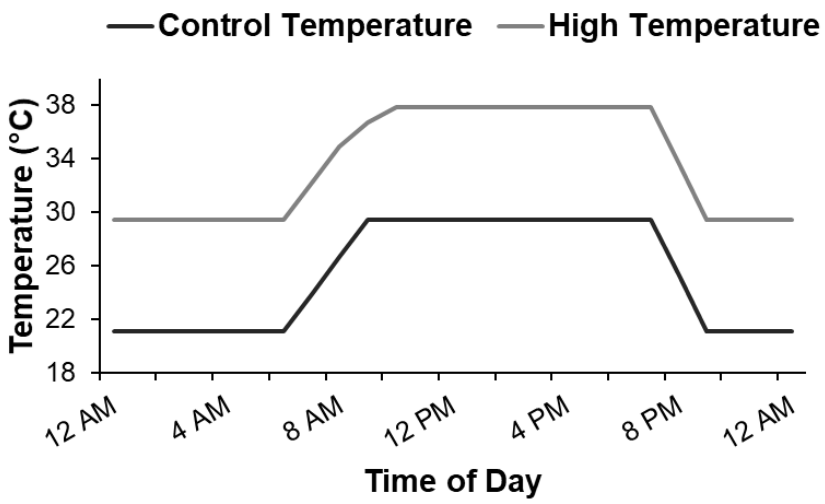

Figure 4.1. Temperature cycles experienced by captive male house finches*, Haemorhous mexicanus*. All birds were kept on the control temperature cycle from capture until experimental start date, at which time birds in the high temperature group (HT) were switched to the "High Temperature" cycle and birds in the control (CT) and food restriction (FR) groups remained on the "Control Temperature" cycle. Temperatures began to ramp up at lights on (6 AM) and began to ramp down at lights off (7 PM).

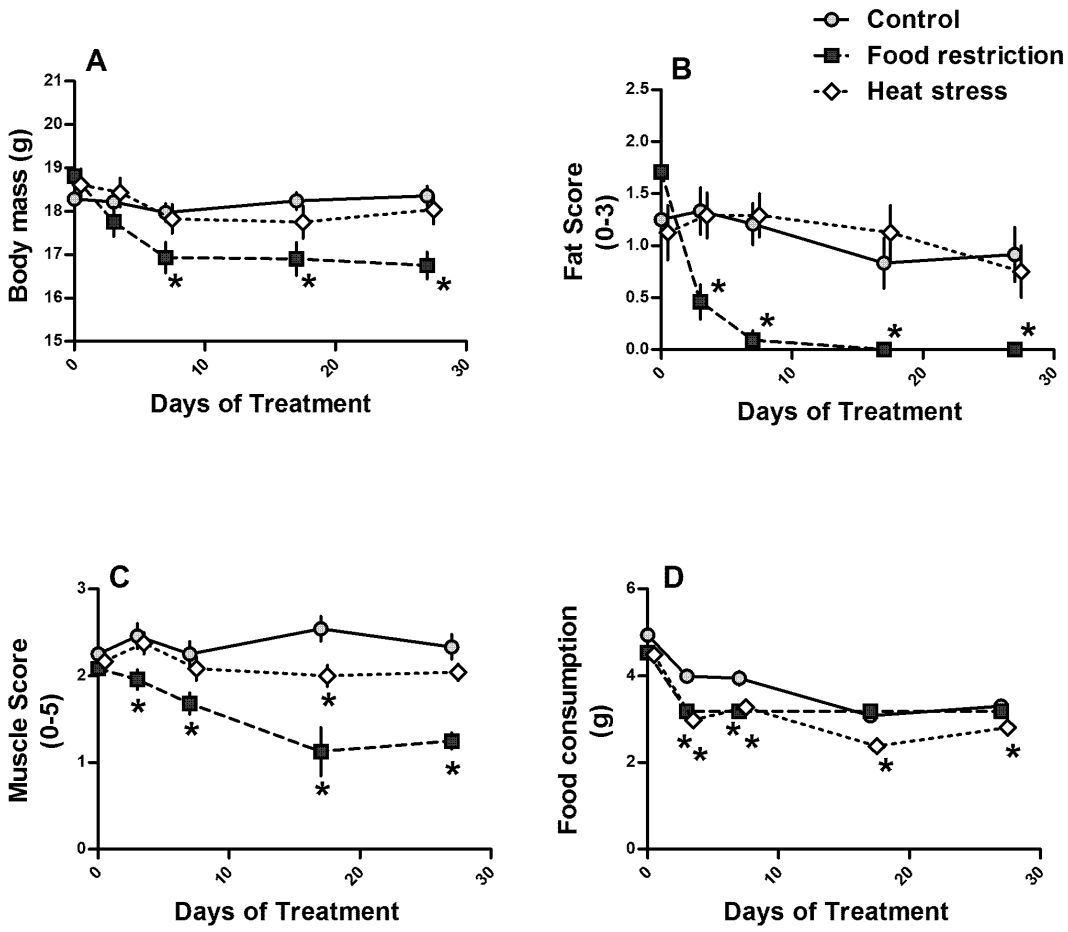

Figure 4.2. Effects of food restriction and high temperature on body condition and food consumption in male house finches, *Haemorhous mexicanus*. Body mass (A) was reduced by food restriction (FR) after 7 days and remained lower than control (CT) birds, and was reduced under high temperatures (HT) but not to levels lower than CT birds. Furcular fat score (B) was reduced by FR after 3 days but unaffected by HT. Pectoral muscle score (C) was reduced by 3 days of FR and remained lower than CT birds, and was reduced by HT after 17 days. Food consumption (D) was experimentally reduced by food restriction to approximately 70% of initial *ad libitum* intake, and was reduced after 3 days of HT remaining lower than CT birds. Control birds decreased food consumption over the course of the study. Data are plotted as means ± SEM. An asterisk (*) indicates a significant difference as compared to the control group ($P<0.05$, LSD tests). For visual clarity, points have been separated along the horizontal axis.

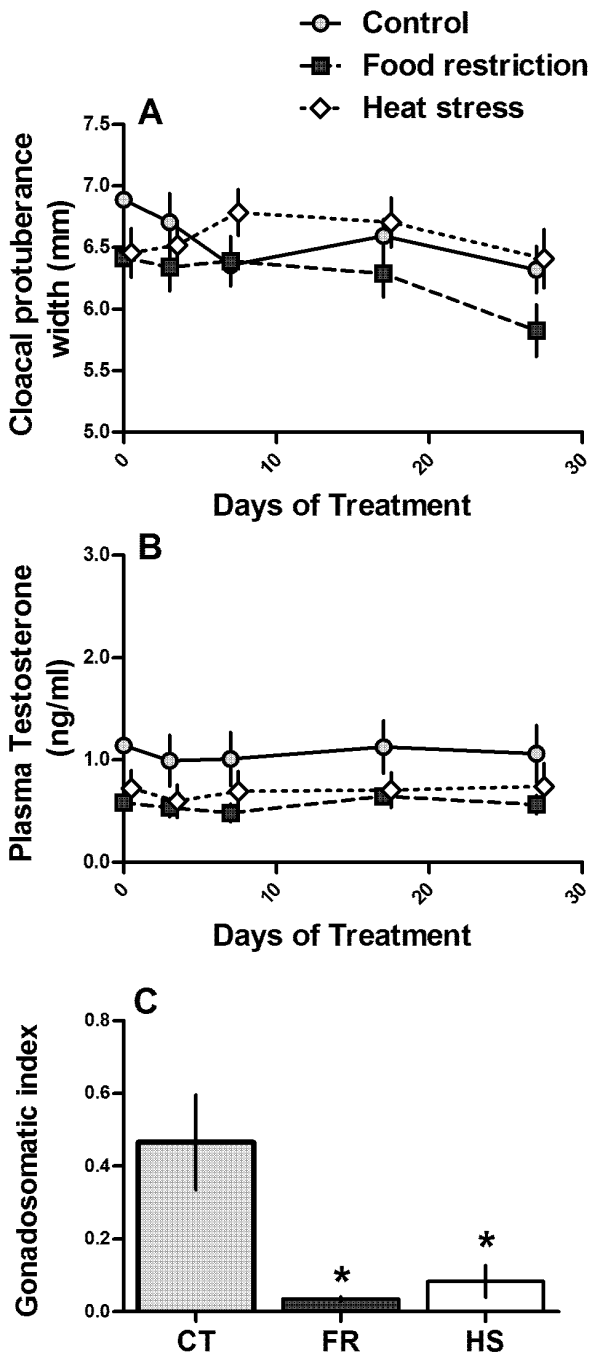

Figure 4.3. Food restriction or high temperature reduces testis mass but has no effect on cloacal protuberance width or plasma testosterone in male house finches, *Haemorhous mexicanus*. Cloacal protuberance width (A) and plasma testosterone (B) changed over the course of the study but were not affected by food or heat treatment. Gonadosomatic index (C), calculated as testis mass as a percentage of body mass, was lower under food restriction and high temperature. Data are plotted as means ± SEM. An asterisk (*) indicates a significant difference as compared to the control group ($P<0.05$, Dunnett's test). For visual clarity, points have been separated along the horizontal axis.

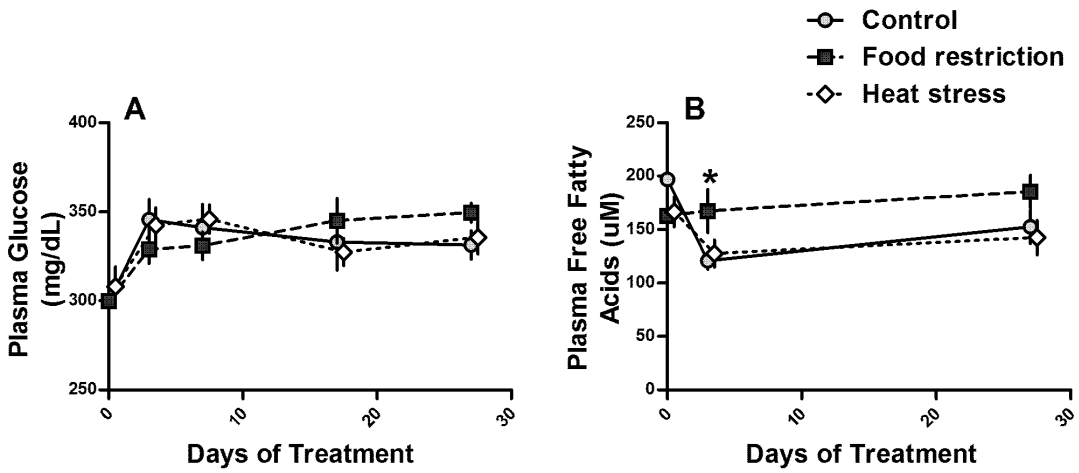

Figure 4.4. Plasma glucose is unaffected by food availability or temperature but plasma free fatty acids are modulated by food availability in male house finches, *Haemorhous mexicanus*. Plasma glucose (A) was unaffected by food or heat treatment, but increased after 3 days in all groups and remained at that level. Plasma free fatty acids (B) changed over time in birds in both the control and high temperature group, but remained constant in food restricted birds, with this group having higher levels after 3 days than control birds. Data are plotted as means ± SEM. An asterisk (*) indicates a significant difference as compared to the control group ($P<0.05$, LSD tests). For visual clarity, points have been separated along the horizontal axis.

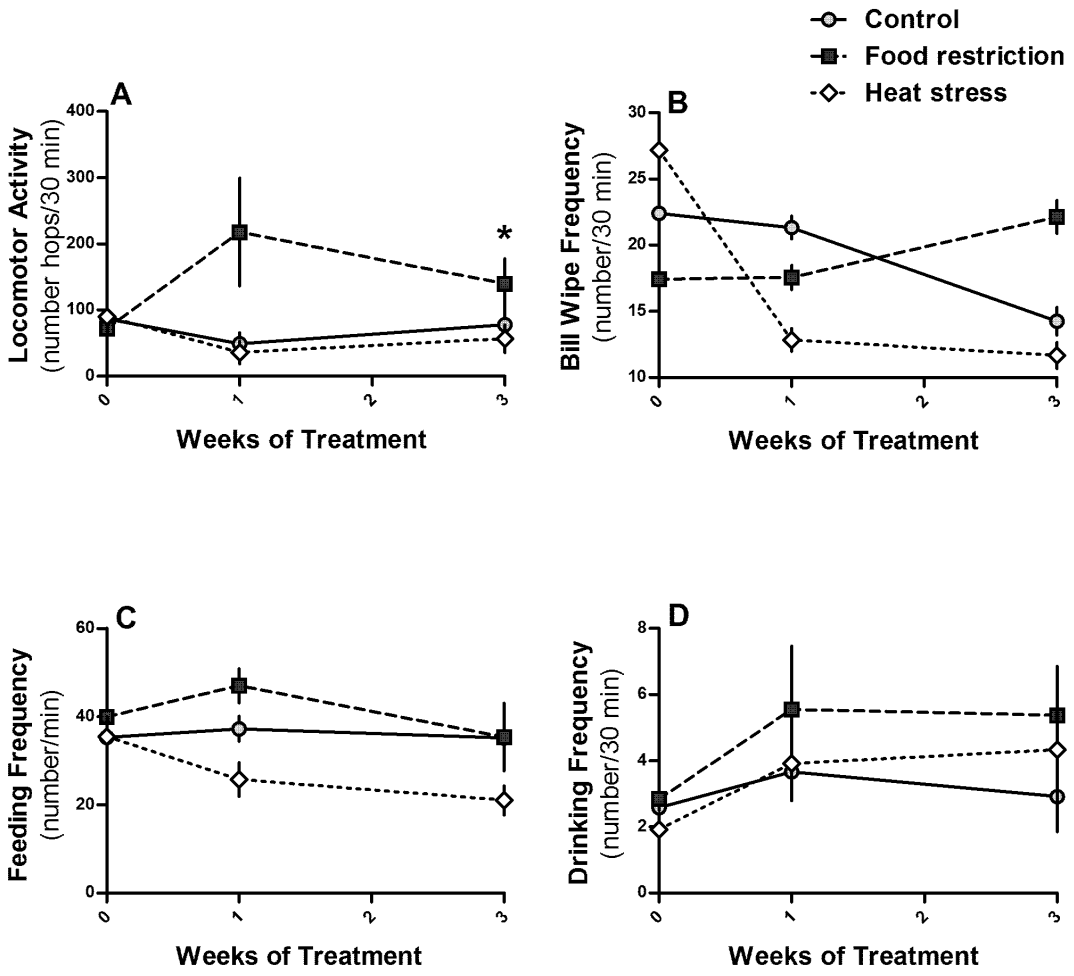

Figure 4.5. Food restriction increases locomotor activity in male house finches, *Haemorhous mexicanus*. Behaviors were measured per bird per 30 minute period. (A) Locomotor activity (# of hops) increased after 3 weeks of food restriction but was unaffected by high temperature, relative to control birds, (B) bill wiping decreased over time overall, but was unaffected by treatment, (C) feeding activity (# times the head entered food bowl) decreased over time overall and was different among treatment groups, being lower under high temperature, with no significant interaction, and (D) drinking activity (# times the head entered water bowl) did not vary with time or amongst treatment groups. An asterisk (*) indicates a significant difference as compared to the control group ($P<0.05$, LSD tests).

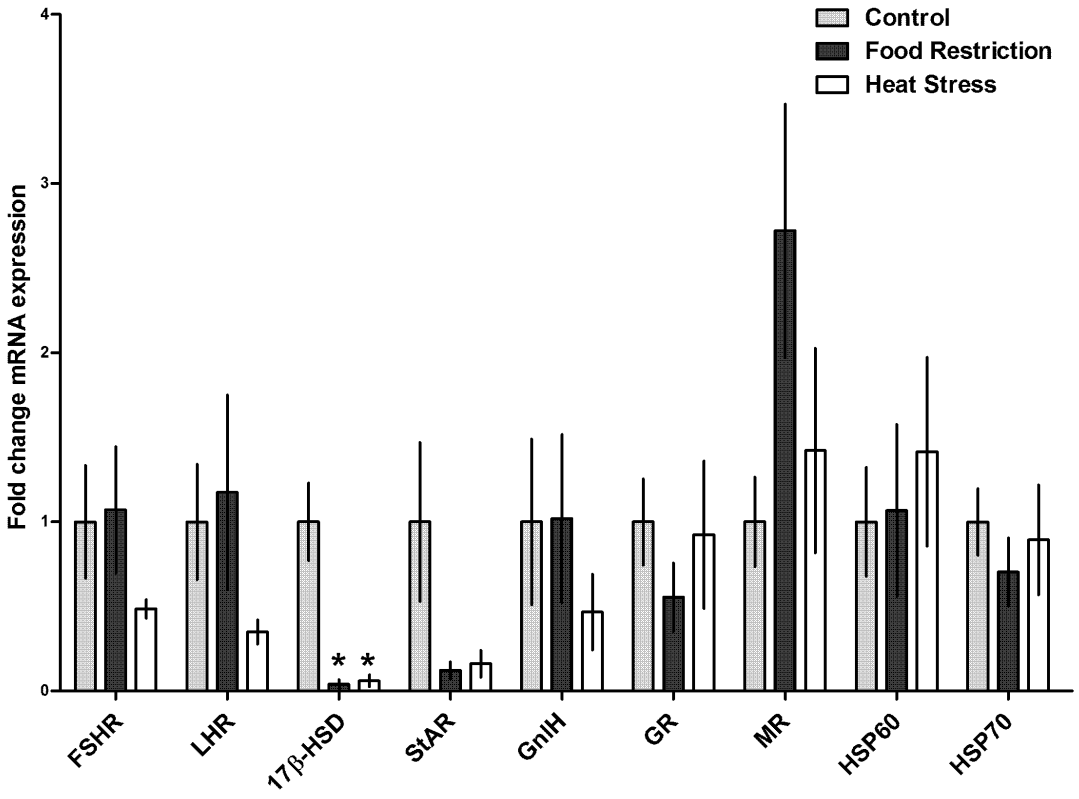

Figure 4.6. Gonadal mRNA expression of 17β-HSD is reduced under food restriction and high temperature in male house finches, *Haemorhous mexicanus*. Food availability or temperature did not affect gonadal mRNA expression of any other measured genes. An asterisk (*) indicates a significant difference as compared to the control group (P = 0.001, Dunnett's test).

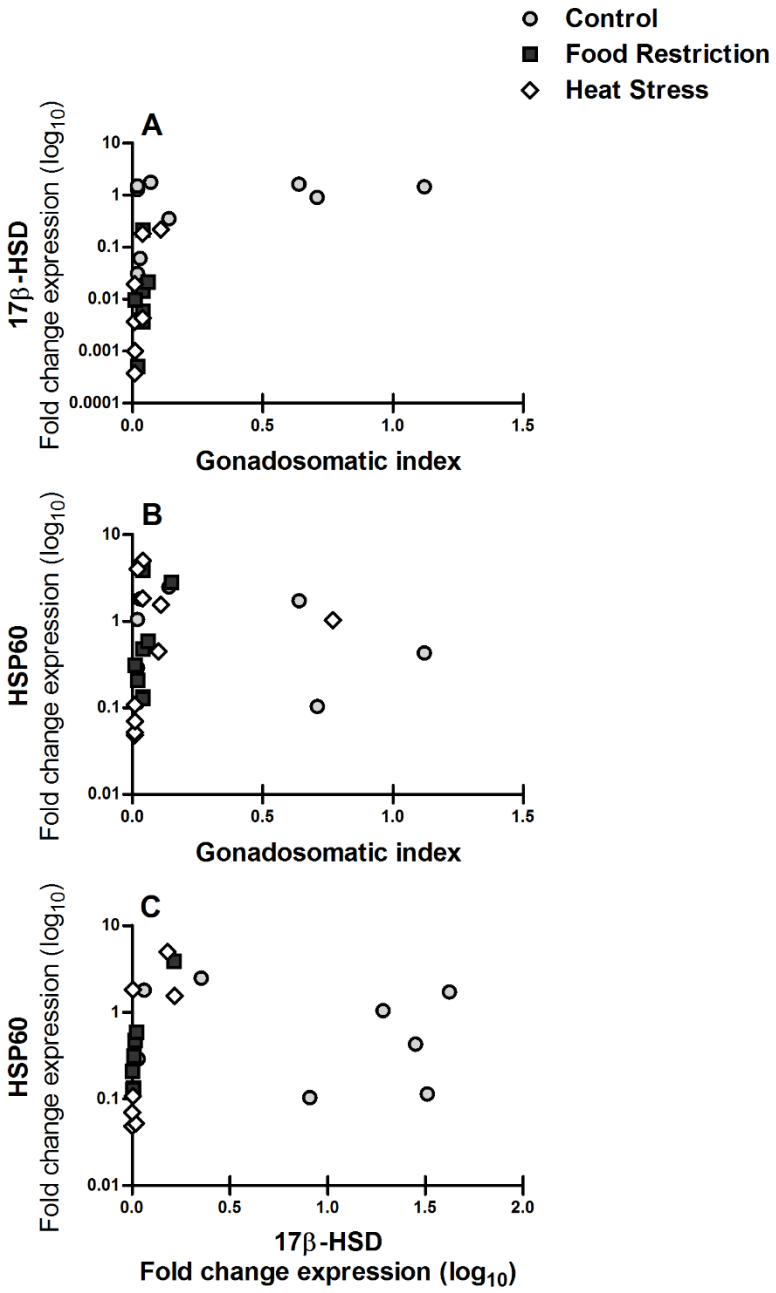

Figure 4.7. Positive overall relationships among testis size and testicular gene expression are present in house finches, *Haemorhous mexicanus*. (A) A positive relationship between gonadosomatic index (GSI; testis mass as a percentage of body mass) and testicular 17β-HSD expression is present overall (Spearman's correlation: $\rho = 0.65$, $P < 0.01$) and within birds exposed to high temperatures ($\rho = 0.86$, $P < 0.05$). (B) A positive relationship between GSI and testicular HSP60 expression is present overall ($\rho = 0.46$, $P < 0.05$). (C) A positive relationship between testicular 17β-HSD and HSP60 expression is present overall ($\rho = 0.44$, $P < 0.05$) and within food-restricted birds ($\rho = 0.89$, $P < 0.01$).

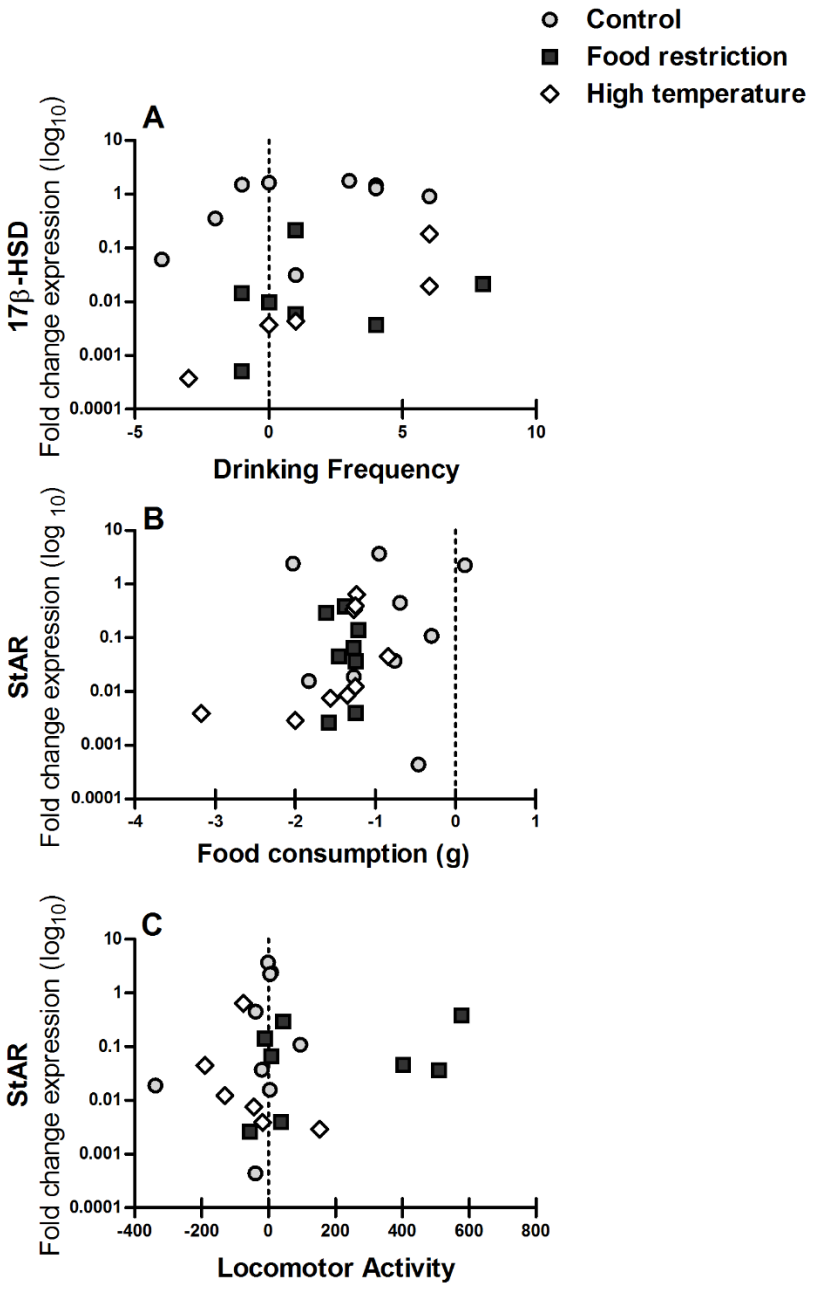

Figure 4.8. Relationships between steroidogenic enzyme expression and behaviors are present in house finches, *Haemorhous mexicanus,* exposed to high temperatures. In the high temperature group, there was a (A) positive relationship between 17β-HSD gonadal gene expression and drinking frequency (Spearman's correlation: $\rho = 0.97$, $P < 0.01$), a (B) positive relationship between StAR gonadal gene expression and food consumption ($\rho = 0.90$, $P < 0.001$), and a (C) negative relationship between StAR gonadal gene expression and locomotor activity ($\rho = -0.83$, $P < 0.05$), but no relationships were present within the control or food restriction group.

Gene	Forward Primer	Reverse Primer	Bp	GeneBank Accession #
FSHR	ACCGAGCTGAGATTTGTCCT	CACGAGGTTGTTGGCTTTCT	183	XM_002196132.2
LHR	AACATATCCCAGCCACTGCT	TTGGAGTCATCGAGCATTTCG	141	XM_012572265.1
17B-HSD	CAGAGCATTGCTTTATTGCTTGA	GCCCAATGACTGCATCAAACTT	97	XM_002190567
StAR	TGACTGGGAAGGTACATCCT	GTGCACAGGAGTTACATAGGG	235	NM_001076686.2
GnIH	TCCAAAGGTATCGCACAGGC	AACAGGCAGTGACTTCCCAA	100	XM_002198944.3
GR	CTCTCTACAATTCCCAAGGAGGG	GCCAACATTTCTGGGAACTCA	248	XM_002192952.2
MR	TTGACTCCATGCATGATCTTGTT	CTTGGGTCCTTCATGGGTGT	246	XM_012573310.1
HSP60	ACGAGGATCAGAGAATTGGCATT	CCCAAGCATTGCATCGTAGC	152	XM_002192300.3
HSP70	CTGCCAAGAACCAGGTAGCA	CCCTCATTCACCACACGGAA	133	XM_002200592.3
RPL4	ATGAGAAGCCAGGAAATCCA	CAGTGGGTTCTTCTTCAGGACT	81	XM_012578030.1
HPRT	CAATGAATACTTCAGAGATTTGAACC	TGAACTCTGCTTTCATGCTTTG	85	XM_002190239.2
GAPDH	GGAACATCCCGAAGCGGTAA	GCCGATACGGCCAAATCCAT	61	NM_001198610.1

Table 4.1. Primer sequences, accession numbers, and anticipated size of amplified products.

	Body Mass	Plasma GLU	Plasma FFA	Feeding Frequency	Locomotor Activity
Body Mass		CT: 0.032 (17D, 7D) FR: 0.15 f(17D, 7D) **HT: 0.87***(17D,7D)**	CT: 0.07 (3D, 7D) FR: -0.16 (3D, 7D) HT: -0.24 (3D, 7D)	CT: -0.16 (21D, 27D) FR: 0.49 (21D, 27D) HT: -0.15 (21D, 27D)	CT: -0.33 (21D, 17D) FR: -0.12 (21D, 17D) HT: -0.50 (21D, 17D)
Plasma GLU	0.24 (7D, 17D)		CT: -0.21 (3D, 7D) FR: -0.22 (3D, 7D) HT: -0.46 (3D, 7D)	CT: -0.75** (21D, 27D) FR: -0.43 (21D, 27D) HT: -0.22 (21D, 27D)	CT: 0.06 (21D, 27D) FR: 0.03 (21D, 27D) HT: -0.01 (21D, 27D)
Plasma FFA	**-0.41* (7D, 3D)**	**-0.38* (7D,3D)**		CT: 0.43 (21D, 27D) FR: 0.54 (21D, 27D) **HT: 0.69* (21D, 27D)**	CT: -0.45 (21D, 27D) FR: 0.20 (21D, 27D) HT: 0.25 (21D, 27D)
Feeding Frequency	-0.15 (27D, 21D)	**-0.40** (27, 21D)**	**0.57*** (27D, 21D)**		CT: -0.48 (21D, 21D) FR: 0.09 (21D, 21D) HT: -0.19 (21D, 21D)
Locomotor Activity	**-0.48** (17D, 21D)**	-0.01 (27D, 21D)	0.00 (27D, 21D)	-0.25 (21D, 21D)	

Table 4.2. Relationships within metabolic parameters. Values given are Spearman's correlation coefficients (ρ). Values below the crosses are relationships overall among all birds in all groups and values above the crosses are separated by treatment group: CT = Control, FR = food restriction, HT = high temperature. In parentheses () are the days used in comparison. All days were analyzed, but those with strongest correlation within a group are presented. Bolded values are significant with * for P<0.05, ** for P<0.01, and *** for P<0.001.

	GSI	T	LHR	FSHR	StAR	17β-HSD	GnIH	GR	MR	HSP60	HSP70
GSI		CT: 0.55 FR: 0.52 **HT: 0.77****	CT: -0.16 FR: 0.37 HT: -0.20	CT:-0.11 FR: 0.25 HT: 0.60	CT: 0.61 FR:-0.29 HT: 0.10	CT: 0.25 FR: 0.61 **HT: 0.86***	CT:-0.07 FR:-0.64 HT:-0.62	CT: 0.67 FR: 0.14 **HT: 0.72***	CT:-0.25 FR: 0.31 **HT: 0.80****	CT: 0.08 FR: 0.60 HT: 0.55	CT: 0.59 FR:-0.07 HT: 0.12
T	**0.45****		CT: -0.46 FR: -0.37 HT: -0.25	CT: -0.41 FR: -0.46 HT: 0.60	CT: 0.53 FR: 0.43 HT:-0.03	CT:-0.20 FR: 0.11 HT: 0.29	CT:-0.10 FR: 0.14 HT:-0.41	CT: 0.54 FR: 0.29 **HT: 0.75***	CT:-0.20 FR: 0.24 HT: **0.83****	CT: 0.50 FR: 0.14 HT: 0.29	CT:-0.24 FR: 0.05 HT: 0.20
LHR	-1.0	**-0.50***		CT: 0.77 **FR: 0.94**** HT:-0.70	CT:-0.39 FR:-0.49 HT: 0.21	CT:-0.57 FR:-0.26 HT: 0.21	CT:0.49 FR:-0.50 HT: 0.02	CT:-0.37 FR:-0.30 HT:-0.50	CT: 0.20 FR: 0.26 **HT:-0.68***	CT:-0.37 FR: 0.62 HT:-0.57	CT:-0.09 FR: 0.62 HT: 0.48
FSHR	0.11	-0.32	0.75***		CT:-0.05 FR:-0.71 HT:-0.80	CT: 0.02 FR:-0.03 HT: 0.00	CT: 0.32 FR:-0.37 HT: 0.00	CT:-0.77 FR: 0.03 HT: 0.60	CT: 0.39 FR:-0.04 HT: 0.50	CT:-0.36 FR:-0.07 HT: 0.70	CT:-0.07 FR:-0.21 HT: 0.40
StAR	-0.37	0.29	-0.13	-0.30		CT: 0.50 FR:-0.75 HT: 0.36	CT: 0.24 FR: 0.46 HT: 0.14	CT: 0.00 FR: 0.14 HT: 0.17	CT:-0.04 FR:-0.21 HT:-0.14	CT: 0.17 FR:-0.36 HT: 0.38	CT: 0.17 FR:-0.26 HT:-0.26
17β-HSD	**0.65****	0.00	0.08	0.14	0.29		CT:-0.26 FR:-0.43 HT:-0.49	CT: 0.00 FR: 0.43 HT: 0.18	CT:-0.07 FR: 0.71 HT: 0.43	CT:-0.14 **FR: 0.89**** HT: 0.64	CT: 0.52 FR: 0.28 HT:-0.14
GnIH	-0.37	-0.16	0.22	0.03	0.19	-0.33		CT:-0.66 FR:-0.31 HT:-0.10	CT: 0.31 FR:-0.32 HT:-0.57	CT:-0.43 FR:-0.68 HT:-0.21	CT:-0.43 FR: 0.36 HT:-0.12
GR	**0.61****	**0.46***	-0.14	0.07	-0.06	**0.48***	-0.37		CT:-0.26 **FR: 0.86*** **HT: 0.78***	CT: 0.57 FR: 0.64 HT: 0.48	CT: 0.54 FR: 0.29 HT: 0.02
MR	0.37	0.22	-0.25	0.24	-0.10	0.12	-0.07	**0.46***		CT: 0.21 FR: 0.56 **HT: 0.67***	CT: 0.31 FR:-0.10 HT:-0.07
HSP60	**0.46***	0.31	0.26	0.12	0.12	**0.44***	**-0.44***	0.58**	0.57**		CT: 0.14 FR:-0.10 HT:-0.17
HSP70	0.27	0.09	-0.02	-0.28	0.07	0.27	0.22	0.20	-0.6	0.02	

Table 4.3. Relationships between testis size, plasma testosterone, and testicular gene expression. Values given are Spearman's correlation coefficients (ρ). Values below crosses are relationships overall among all birds in all groups and values above the crosses are separated by treatment group: CT = control, FR = food restriction, HT = high temperature. Bolded values are significant with * for P<0.05, ** for P<0.01, and *** for P<0.001.

	GSI	Plasma T	17β-HSD	StAR
Body Mass				
All	**0.37* (17D)**	0.17 (27D, 27D)	**0.50* (27D)**	0.16 (27D)
CT	-0.08 (17D)	**0.66* (27D, 27D)**	-0.21 (27D)	0.14 (27D)
FR	-0.04 (17D)	0.17 (27D, 27D)	-0.14 (27D)	-0.01 (27D)
HT	**0.68* (17D)**	0.05 (27D, 27D)	0.46 (27D)	0.35 (27D)
Food intake				
All	0.11 (27D)	-0.03 (27D, 27D)	**0.58** (3D)**	0.29 (3D)
CT	-0.65 (27D)	-0.26 (27D, 27D)	0.05 (17D)	-0.03 (3D)
FR	0.19 (27D)	0.21 (27D, 27D)	FR: -0.11 (17D)	-0.08 (3D)
HT	0.27 (27D)	**0.60*** (27D, 27D)	**0.79* (17D)**	**0.90*** (3D)**
Feeding Frequency				
All	0.23 (21D)	-0.07 (27D, 21D)	0.26 (21D)	0.01 (7D)
CT	-0.01 (21D)	-0.19 (27D, 21D)	0.36 (21D)	-0.13 (7D)
FR	**0.90* (21D)**	0.41 (27D, 21D)	-0.80 (21D)	**-0.95*** (7D)**
HT	0.32 (21D)	0.44 (27D, 21D)	0.68 (21D)	**0.94** (7D)**
Drinking Frequency				
All	0.01 (21D)	-0.02 (27D, 21D)	0.22 (7D)	-0.26 (7D)
CT	-0.22 (21D)	-0.18 (27D, 21D)	0.23 (7D)	-0.08 (7D)
FR	0.22 (21D)	0.09 (27D, 21D)	0.29 (7D**)**	**-0.57* (7D)**
HT	0.08 (21D)	0.08 (27D, 21D)	**0.97** (7D)**	0.20 (7D)

Table 4.4. Relationships between reproductive and metabolic parameters. Values given are Spearman's correlation coefficients (ρ). "All" includes birds from all groups, CT = control, FR = food restriction, and HT = high temperature. In parentheses () are the days in analysis for the repeated measure that are presented. All days were analyzed, but those with strongest correlation within a group are presented. Bolded values are significant with * for P<0.05, ** for P<0.01, and *** for P<0.001.

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CHAPTER 5

CONCLUSIONS

Multilevel HPG axis integration of non-photic cues occurs in a seasonally breeding bird

Gonadal development and function. My dissertation work demonstrates important roles of both food availability and temperature on the reproductive system of a photoperiodic songbird. Gonadal size and function were consistently negatively affected both by food restriction and high temperatures during (Chapters 2, 3) and after (Chapter 4) reproductive development. The house finch, *Haemorhous mexicanus,* is photoperiodic, exhibiting clear neuroendocrine and gonadal responses to the annual change in photoperiod (Cho et al., 1998; Hamner, 1966). Opportunistic species are the primary models that have been used thus far in studying the effects of non-photic cues on reproduction, likely because these cues are better indicators of favorable breeding conditions under unpredictable environments than is photoperiod (Dawson, 2008). The inhibitory effects of food restriction and thermal stress on gonadal development and function in house finches are consistent with those found in opportunistic species (Hahn, 1995; O'Brien and Hau, 2005; Perfito et al., 2008). The fact that these negative consequences occur in more photoperiodic species suggests that the ability of the hypothalamic-pituitary-gonadal (HPG) axis to integrate a suite of environmental information to influence reproductive development is conserved across avian species with diverse breeding patterns, even if the relative sensitivity to various environmental cues might vary among species. Both non-photic cues I examined decreased body condition. My studies do not demonstrate that this decrease was responsible for the inhibition of gonadal development under food restriction and high temperatures. However, the results, in particular correlations between body condition and gonadal development, suggest that this was the case. Further work should attempt to disentangle energetic and non-energetic effects of environmental cues, the results of which I predict would highlight the critical role of energy homeostasis for reproductive function across avian species.

Taken together, the morphological and hormonal data reveal inhibition of both endocrine and exocrine testicular functions during under food restriction (Chapter 2), but indicate that a

direct relationship between testis size and plasma testosterone (T) is not universal across time and experimental treatments. Gonads were smaller in experimental than control finches after several weeks of food restriction or high temperature under multiple photoperiodic treatments, with smaller seminiferous tubules at least in food-restricted birds. If these results are applicable to free living birds, they indicate that a chronic energy challenge could negatively impact sperm production (Deviche et al., 2011) and potentially fertility of male house finches. In contrast, the hormonal response (plasma T) to the above manipulations was variable, with lower plasma T occurring only transiently in food-restricted, photostimulated house finches (Chapter 2). Multiple factors may account for the independence of testis size and plasma T under negative environmental conditions. Perhaps there is an energetic tradeoff even within the reproductive system, by which energy is preferentially directed away from maintaining or growing the gonads (especially the cells and structures involved in spermatogenesis), so that energy is available for the production of testosterone. Of course the ultimate purpose of enhancing HPG axis activity and gonadal growth and function for a male bird is to successfully father offspring. Early during the breeding season, this success requires establishing a territory and finding a mate, behaviors of which are in part T-dependent (Riters and Alger, 2011). Perhaps maintaining T production under negative environmental conditions allows the bird to perform early breeding behaviors, and then, if conditions improve, develop gonadal exocrine structures and proceed with mating. Alternatively, the energetic cost of maintaining T production and T-related behaviors may be smaller than the cost of maintaining/developing testes. There is little information available on the relative costs of maintaining large gonads versus high plasma T, but both do entail energetic costs (Deviche and Cortez, 2005; Wright and Cuthill, 1989). Testing of these hypotheses would require comparing these energetic costs, analysis of testicular morphology under different energetic states, and examining the overall costs and benefits of prioritizing different reproductive functions at different stages of the breeding season.

Within the gonads, one steroidogenic enzyme, 17β-hydroxysteroid dehydrogenase, showed a strong response to food restriction and high temperature, in which gonadal mass was reduced. This observation suggests a crucial role for this enzyme in mediating gonadal growth

during environmental challenges. This is one of the first studies, to my knowledge, investigating gonadal gene expression in response to energetic stress in a wild bird. Zebra finches, *Taeniopygia guttata,* show different changes in gonadal gene expression under short-term fasting, but the difficulty in comparing acute versus chronic energy challenge and in comparing a photoperiodic species with a more opportunistic one, necessitates more data on this subject. Uncovering how the steps preceding T production and testis growth are altered is critical for understanding how endocrine, morphological, and ultimately behavioral traits are mechanistically regulated by the environment, and subsequently for predicting how multiple environmental factors interact to affect breeding success.

Neuroendocrine integration. In mammals, energy deficits influence the activity of the HPG axis by acting primarily on the gonadotropin-releasing hormone (GnRH) system (Wade et al., 1996). The same presumably applies to birds but there is little research to resolve this. In Chapters 2 and 3, I show that the GnRH system is affected by food availability. Food-restricted house finches have a greater number of hypothalamic GnRH-ir cells than *ad libitum-fed* birds, with no differences in the number of cells containing proGnRH-ir (Chapter 2). These results, combined with an increased capacity to release stored GnRH (Chapter 3), suggest that food restriction inhibits the secretion, but not production, of GnRH. This mechanism to regulate GnRH cell function by a non-photic cue is the same as that demonstrated in several opportunistic species for GnRH regulation by photoperiod (MacDougall-Shackleton et al., 2009; Stevenson et al., 2012b). The regulation of GnRH release alone is thought to allow the animal to be more flexible and able to respond rapidly to stimulatory cues by increasing GnRH release from readily available stores. Perhaps independently regulating GnRH production versus secretion in response to different environmental cues is a way of prioritizing these cues and ultimately in determining the breeding strategy of an animal.

My studies provide no evidence for a role of gonadotropin-inhibitory hormone (GnIH) or neuropeptide Y (NPY) in regulating GnRH or gonadotropin release under food restriction, nor for GnIH to regulate gonadal growth or function peripherally. Other studies, however, demonstrate roles for these and other neuropeptides in the regulation of energy balance and reproduction

(Kriegsfield et al., 2015; McGuire et al., 2013). The further mapping of these neuroendocrine circuits in birds, investigations of their modulation by energetic information, and exploring the diversification of these circuits across species, will help in understanding the plasticity and evolutionary context of neuroendocrine responses in integrating environmental information.

High temperatures alter metabolic and reproductive physiology

My studies are among the first to determine the effect of high ambient temperatures, beyond thermoneutrality, concurrently on metabolism and reproduction in a non-domestic avian species. House finches are native to the desert southwest and during the breeding season, temperatures routinely exceed the high temperature treatment used in this study, yet captive finches had significantly reduced testis size and steroidogenic activity. Heat waves can result in a decline in avian species and abundance (Albright et al., 2011). However, it is unclear if this decline results from decreased food availability or foraging effort (Becker et al., 1997; Shochat et al., 2004), increased need for limited water sources (Albright et al., 2017; McKechnie and Wolf, 2010), or increased thermoregulatory costs that direct energy away from other processes such as growth of nestlings or reproduction of adults (Cunningham et al., 2013; Lusk et al., 2001). House finches in my study had unlimited access to food and water, but an energetic cost was apparent through lower body mass and protein stores. I cannot determine whether this cost was due to the energetic requirements of thermoregulation or resulted from lowering the bird's motivation to feed. Combined with evidence from domestic birds, it appears that there are at least some energetic costs of thermoregulation at high temperatures that are not associated with lower food intake (Gereart et al., 1996; Habashy et al., 2017). Average ambient temperatures in the coming decades are predicted to rise, especially in desert environments and in urban areas, and it is important to understand the consequences of this rise on reproductive physiology and behavior. Small passerines, especially, have limited abilities to cope with extreme temperatures (Albright et al., 2017; McNab, 1970) and it is likely that elevated ambient temperatures will have particularly severe consequences especially in species not already inhabiting hot environments.

A complex link between metabolic and reproductive physiology

My dissertation work supports the idea that the physiological mechanisms that control reproduction are inextricably linked to those that control energy balance – similar mechanisms inhibited reproductive physiology under two situations of altered energy balance (Chapter 4). The hypothesis that the primary mediating signal in this interaction is the availability of metabolic fuel, however, was not supported by my work. This hypothesis forms the basis of the "metabolic hypothesis" which has extensive support in mammals (Schneider, 2004; Wade et al., 1996). Birds may use other metabolic information such as protein or specific nutrients in signaling to the reproductive system. My work does indicate the ability to monitor energy homeostasis and adjust HPG axis activity. Expression of testicular steroidogenic enzymes was related to feeding, drinking, and locomotor activity (Chapter 4). The specific mediators for this information, however, were not elucidated by this study.

It is important to point out that my results do not exclude the possibility that food restriction and high temperatures inhibit reproductive physiology through non-energetic mechanisms. The sensory processing of food availability and temperature might affect brain circuits independent of alterations in energy homeostasis. For example, in the spotted antbird, *Hylophylax n. naevioides,* visual signals related to food appear to affect HPG axis activity (Hau et al., 2000). If other species use similar signals, I predict that their effects could be detected early, before any change in energy homeostasis has taken place. Further research that untangles the role and relative weights of non-energetic and energetic effects of environmental cues would certainly prove insightful.

Physiological studies inform population-level responses and adaptability

Seasonal changes in the timing, averages, and peaks of both food availability and temperature are expected in response to global climate change and urbanization (Meehl and Tebaldi, 2004; Parmesan and Yohe, 2003). In some habitats, changes in the emergence of food resources are already occurring and negatively impacting avian populations (Both et al., 2006; Visser et al., 1998). Temporal shifts in these and other environmental variables may not occur in tandem (Lof et al., 2012), potentially further complicating an animal's ability to integrate environmental information and time breeding at the most favorable time. My studies, while

physiological in nature, provide important foundations for investigations into adaptive responses of breeding in energetically variable environments. This work is essential in predicting how natural populations may respond to shifts in food availability and temperature. Scientifically sound predictions will ultimately assist in policy decisions related to mitigating climate change and the conservation of avian species.

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