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Food availability, energetic constraints and reproductive development in a wild seasonally breeding songbird

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Summary

1. In many organisms, food availability is a proximate cue that synchronizes seasonal development of the reproductive system with optimal environmental conditions. Growth of the gonads and secondary sexual characteristics is orchestrated by the hypothalamic–pituitary–gonadal (HPG) axis. However, our understanding of the physiological mechanisms by which food availability modulates activity of the HPG axis is limited.

2. It is thought that many factors, including energetic status, modulate seasonal reproductive activation. We tested the hypothesis that food availability modulates the activity of the HPG axis in a songbird. Specifically, we food-restricted captive adult male Abert’s Towhees Melozone aberti for 2 or 4 weeks during photoinduced reproductive development. A third group (control) received ad libitum food throughout. We measured multiple aspects of the reproductive system including endocrine activity of all three levels of the HPG axis [i.e. hypothalamic gonadotropin-releasing hormone-I (GnRH-I), plasma luteinizing hormone (LH) and testosterone (T)], and gonad morphology. Furthermore, because gonadotropin-inhibitory hormone (GnIH) and neuropeptide Y (NPY; a potent orexigenic peptide) potentially integrate information on food availability into seasonal reproductive development, we also measured the brain levels of these peptides.

3. At the hypothalamic level, we detected no effect of food restriction on immunoreactive (ir) GnRH-I, but the duration of food restriction was inversely related to the size of ir-GnIH perikarya. Furthermore, the number of ir-NPY cells was higher in food-restricted than control birds. Food restriction did not influence photoinduced testicular growth, but decreased plasma LH and T, and width of the cloacal protuberance, an androgen-sensitive secondary sexual characteristic. Returning birds to ad libitum food availability had no effect on plasma LH or T, but caused the cloacal protuberance to rapidly increase in size to that of ad libitum-fed birds.

4. Our results support the tenet that food availability modulates photoinduced reproductive activation. However, they also suggest that this modulation is complex and depends upon the level of the HPG axis considered. At the hypothalamic level, our results are consistent with a role for the GnIH and NPY systems in integrating information on energetic status. There also appears to be a role for endocrine function at the anterior pituitary gland and testicular levels in modulating reproductive development in the light of energetic status and independently of testicular growth.

Key-words: gonad development, gonadotropin-inhibitory hormone, gonadotropin-releasing hormone, hypothalamic–pituitary–gonadal axis, luteinizing hormone, neuropeptide Y, reproductive physiology, seasonal reproduction, testosterone

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Introduction

To maximize reproductive success, organisms often temporally synchronize their breeding period with optimal environmental conditions (Baker 1938; Wingfield & Kenagy 1986; Lindström 1999; Lourdais et al. 2002). This synchronization has been demonstrated in a variety of taxa (Munro, Scott & Lam 1990; Boyd 1991; Nager & van Noordwijk 1995; Olsson & Shine 1998; Olive, Lewis & Beardall 2000). In many adult organisms, the reproductive system is regressed outside the breeding period and must recrudesce before the start of the next breeding period. In these organisms, reproductive recrudescence (i.e. regrowth of the gonads and secondary sexual characteristics) is, therefore, an important determinant of the breeding period. This is the case in most birds, which generally exhibit distinct breeding and non-breeding life-history stages that are characterized by dramatic changes in reproductive physiology, morphology and behaviour (Williams 2012; Davies & Deviche 2014).

To correctly time reproductive development, organisms use proximate environmental cues to predict favourable conditions (Ims 1990), and one such cue in birds is the annual change in day length (photoperiod). The annual cycle of photoperiod is constant between years at a given location and can predict favourable conditions. Accordingly, birds use photoperiod as the ‘initial predictive cue’ that stimulates the hypothalamic–pituitary–gonadal (HPG) axis to begin reproductive development (Wingfield 1980; Dawson et al. 2001) and the mechanism by which increasing vernal photoperiod influences the avian HPG axis has been studied extensively. Light is detected by deep brain photoreceptors, and the circadian system is used to measure the length of this light signal (Follett, King & Meddle 1998; Sharp 2005). Information from these photoreceptors is relayed via the pars tuberalis (PT) of the pituitary gland to increase the production and secretion of the neuropeptide gonadotropin-releasing hormone-I (GnRH-I; King & Millar 1982; Sharp & Ciccone 2005). Gonadotropin-releasing hormone-I is the primary secretagogue of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary gland (Kuenzel 2000). These hormones stimulate development of the gonads and secretion of the sex steroids testosterone (T) and oestradiol (E2) in males and females, respectively (Murton & Westwood 1977), and these steroids, in turn, stimulate development of secondary sexual organs and promote the expression of reproductive behaviours.

In contrast to photoperiod, many environmental variables often vary from year to year at a given location. Birds may modulate the activity of their reproductive system in the light of supplementary environmental cues, including ambient temperature (Wingfield et al. 2003; Schaper et al. 2012b), rainfall (Hau et al. 2004; Small, Sharp & Deviche 2007), social interactions (Wingfield & Wada 1989; Maney, Goode & Ball 2007; Small et al. 2008a; Stevenson et al. 2008) and food availability (Lack 1968; Hau, Wikelski & Wingfield 2000; Hahn et al. 2005; O’Brien & Hau 2005). Although considerable information is available about the effects of day length on the HPG axis, much less is known about the physiological mechanisms by which these non-photic environmental factors influence reproductive activity and most studies to date have focused primarily on the pituitary gland. Food availability, in particular, is thought to modulate reproductive activity partly through an individual’s energetic status. Accordingly, within the window of opportunity for breeding determined by day length, a bird’s energetic status is predicted to constrain the timing of reproductive development (Drent & Daan 1980; Wingfield & Kenagy 1986; Meijer & Drent 1999; Hahn et al. 2005). Thus, birds in good energetic status can begin this development shortly after stimulation by sufficiently long days, whereas birds in poor energetic status delay development until they have acquired sufficient energy stores. Various experimental and correlative approaches, including food supplementation (Schoech 1996, 2009; Scheuerlein & Gwinner 2002; Harrison et al. 2010) and natural variation in the abundance of wild food sources (Ligon 1974; Hahn 1998), found that increased food availability is associated with earlier seasonal breeding in wild birds (reviewed by Davies & Deviche 2014). Captive studies on the effect of food restriction or deprivation on the avian reproductive system have, however, yielded inconsistent results (Dawson 1986; Meijer 1991; Hahn 1995; Perfilio et al. 2008).

A candidate neuropeptide that may integrate information on food availability and fine-tune seasonal reproductive development is gonadotropin-inhibitory hormone (GnIH; Tsutsui et al. 2000) and its links with hypothalamic cells that produce neuropeptide Y (NPY; McConn et al. 2014). Across vertebrates, GnIH opposes the effect of GnRH on the HPG axis (Greives et al. 2008) and inhibits its reproductive function by acting on hypothalamic GnRH neurons, anterior pituitary gland gonadotropes and gonads (Tsutsui et al. 2010, 2012, 2013; Clarke 2011; Clarke & Parkington 2013; Davies & Deviche 2014). In addition, GnIH has an orexigenic effect in birds and mammals (Tachibana et al. 2005, 2008; Clarke et al. 2012; Clarke & Parkington 2013; Tsutsui et al. 2013; McConn et al. 2014). In mammals, GnIH neurons project to hypothalamic regions that regulate appetite and energetic status (Qi, Oldfield & Clarke 2009; Ubuka et al. 2009). These regions contain NPY-producing neurons, and the orexigenic action of GnIH may, therefore, be mediated by these neurons (Clarke et al. 2009). Neuropeptide Y is among the most potent endogenous orexigenic factors (Boswell 2001; Hill, Elmquist & Elias 2008; Pralong 2010), and, in mammals, NPY-producing cells integrate information on energetic status via both hormones and metabolites (Marty, Dallaporta & Thorens 2007). Indeed, reciprocal projections from NPY cells to GnIH cells and evidence from mammalian studies suggest that GnIH cells modulate their activity in response to energetic status (Klingerman et al. 2011). Therefore, the GnIH–NPY system appears to...
simultaneously regulate the activity of the HPG axis and food intake in response to energetic status and may play a role in the modulation of reproductive development by food availability (Davies & Deviche 2014).

Here, we investigated whether food availability modulates reproductive development in a seasonally breeding songbird and examined potential neuroendocrine mechanisms mediating this modulation. Specifically, we hypothesized that energetic status influences reproductive development. To test this hypothesis, we compared hypothalamic levels of GnRH, plasma LH and T, testicular development and cloacal protuberance width of adult male Abert’s Towhees Melozone aberti (Baird) subjected to various food availability regimes during photoinduced gonadal development. We predicted that birds with access to ad libitum food and, hence, in good energetic status would undergo reproductive activation at a faster rate than food-restricted birds in poor energetic status. We also aimed to shed light on the physiological mechanism(s) by which food availability modulates the HPG axis and, for this, quantified hypothalamic levels of GnRH and NPY in response to food availability. To our knowledge, this study is the first in a wild bird species to examine the effects of food restriction on the endocrine regulation of reproductive development at all levels of the HPG axis simultaneously. A better understanding of the physiological mechanisms by which food availability modulates seasonal reproductive activation will improve our understanding of how variation in this environmental cue is transduced into annual variation in the timing of breeding periods.

Materials and methods

BIRD CAPTURE AND HOUSING

During January 2011, we caught 24 adult male Abert’s Towhees from Robbins Butte Wildlife Area, Maricopa Co., AZ (altitude: 249 m; latitude: 33°19’N; longitude: 112°38’W), using mist nets and conspecific song playback. We determined sex and age using behaviour (singing and aggressive response to conspecific playback and conspecific song playback. We determined sex and age using

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confirm that the body mass of each bird was reduced and stabilized at the target reduction.

From each bird, we collected a blood sample to quantify plasma LH and T immediately prior to (i) the switch to food restriction (defined as week 0), (ii) the switch back to ad libitum food (week 2) and (iii) the end of the study (week 4; Fig 1). We collected blood (c. 200 μL) from the right jugular vein using a heparinized syringe within 2 min of removing a bird from its cage. The sample was then placed on ice and centrifuged within 1 h. We harvested plasma using a Hamilton glass syringe and froze aliquots at −80 °C until assayed. Immediately following collection of each blood sample, we quantified the furcular fat stores and size of the pectoral muscles of all the birds to estimate their energetic condition. We visually estimated the amount of furcular fat by assigning a score of 0–5 (a score of 0 representing no fat, 5 representing bulging fat deposits; Helms & Drury 1960). Furthermore, because the pectoral muscles in birds are the largest store of protein and muscle protein can be converted into energy via gluconeogenesis, we also estimated the size of the pectoral muscles on a scale ranging from 0 to 3 (adapted from Gosler et al. 2008b) and stored at 4°C.

To investigate the effect of food availability on the central control of reproduction and testicular development, at the end of the study, we collected the brain and testes of each bird. Following deep anaesthesia induced by intramuscular injection (250 μL into each pectoral muscle) of a ketamine/xylazine cocktail (ketamine: 8 mg per 0.5 mL (160 mg kg⁻¹); xylazine: 160 μg per 0.5 mL (3.2 mg kg⁻¹)) dissolved in 0.9% NaCl solution, we transcardially perfused birds with 35 mL of wash solution (0.9% NaCl and 0.1% NaNO₃ in 0.1 M phosphate buffer, PB), followed by 35 mL of fixative (4% paraformaldehyde and 0.1% NaNO₃ in 0.1 M PB). We then decapitated birds, exposed the brain and placed heads in 10×10×10 cm gelatin-embedded vials (Strader & Buntin 2001). Pre-absorption of the NPY antiserum has been established in the chicken (Kuenzel & McMurtry 1988) and Ring Dove Streptopelia risoria (Strader & Buntin 2001). Pre-absorption of the NPY antiserum with human/rat NPY (H-6375; Bachem, Torrance, CA, USA) before application to Aetar’s Towhee brain sections abolished the staining. Following two washes in 0.1 M PB for 30 min, we serially exposed sections to 0.36% H₂O₂, washed them 3×5 min in 0.1 M PB, blocked background immunoreactivity for 1 h and incubated sections overnight in primary antiserum. We then washed sections three times for 10 min in 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 three times for 10 min in 0.1% PBT, incubated for 1 h in avidin–biotin complex (ABC Vectastain Elite kit; Vector Laboratories, Burlingame, CA, USA), washed three times for 15 min in 0.1% PBT, incubated in Vector SG peroxidase chromagen for 2 min and washed twice for 5 min in 0.1 M PB. After mounting on glass microscope slides, we allowed immunolabelled sections to dry at room temperature for 24 h before dehydrating through a graded ethanol series, clearing in xylene and coverslipping using Permount mounting medium (Fisher Scientific, Pittsburgh, PA, USA).

**GNRH, GnIH and NPY Immunocytochemistry**

We immunostained brain sections for GnRH, GnIH and NPY in two assays per peptide, with sections from randomly selected birds in each assay. Previous studies have determined the distribution of these peptides in the avian brain. Gonadotropin-inhibitory hormone-I is primarily synthesized in the pre-optic area (POA; Parry et al. 1997; Dawson & Goldsmith 1997), and GnIH is synthesized solely in the paraventricular nucleus (PVN; Tsutsui et al. 2000; Osugi et al. 2004; Tsutsui et al. 2010). Neuropeptide Y-producing neurons are widely distributed throughout the avian brain, but the only cell population that responds to energetic status is located in the infundibular nucleus (IN; Kuenzel & McMurtry 1988; Boswell 2001; Boswell, Li & Takeuchi 2002). Therefore, from each bird, we randomly selected sections covering the entire POA, PVN and IN for GnRH, GnIH and NPY, respectively; Fig. 2). For GnIH and NPY, we stained an average of 10 sections per bird and for GnRH, an average of six sections per bird. Since sections were randomly selected from each bird, we assumed that this sampling design gives an unbiased estimate of the number of immunoreactive cells for a given bird and, therefore, calculated the median number of cells per section for each bird.

The GnRH and GnIH staining protocols have been previously published and validated in our laboratory (Small et al. 2008b). The specificity of the NPY antiserum has been established in the chicken (Kuenzel & McMurtry 1988) and Ring Dove Streptopelia risoria (Strader & Buntin 2001). Pre-absorption of the NPY antiserum with human/rat NPY (H-6375; Bachem, Torrance, CA, USA) before application to Aetar’s Towhee brain sections abolished the staining. Following two washes in 0.1 M PB for 30 min, we serially exposed sections to 0.36% H₂O₂, washed them 3×5 min in 0.1 M PB, blocked background immunoreactivity for 1 h and incubated sections overnight in primary antiserum. We then washed sections three times for 10 min in 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 three times for 10 min in 0.1% PBT, incubated for 1 h in avidin–biotin complex (ABC Vectastain Elite kit; Vector Laboratories, Burlingame, CA, USA), washed three times for 15 min in 0.1% PBT, incubated in Vector SG peroxidase chromagen for 2 min and washed twice for 5 min in 0.1 M PB. After mounting on glass microscope slides, we allowed immunolabelled sections to dry at room temperature for 24 h before dehydrating through a graded ethanol series, clearing in xylene and coverslipping using Permount mounting medium (Fisher Scientific, Pittsburgh, PA, USA).

**GNRH**

We used anti-cGnRH-I rabbit polyclonal antiserum (6DL3/L4 prepared by P.J. Sharp) at a dilution of 1:10 000 in 0.3% PBT. To block non-specific sites, we used normal rabbit serum (Vector Laboratories, Inc.; 1:200 in 0.3% PBT), and we used biotinylated rabbit anti-sheep IgG (Vector Laboratories, Inc.; 1:200 in 0.3% PBT) as a secondary antibody.

**GnIH**

We used anti-Japanese Quail GnIH antiserum (Tsutsui et al. 2000) at a dilution of 1:5000 in 0.3% PBT. We used normal horse serum (Vector Laboratories; 1:30 in 0.3% PBT) to block non-specific sites and biotinylated mouse/rabbit IgG (Vector Laboratories; 1:100 in 0.3% PBT) as a secondary antibody.

**NPY**

We used anti-human/rat NPY antiserum (T-4070; Bachem) at a dilution of 1:2000 in 0.3% PBT. We used normal goat serum (Vector Laboratories; 1:30 in 0.3% PBT) to block non-specific sites and biotinylated rabbit IgG (Vector Laboratories; 1:100 in 0.3% PBT) as a secondary antibody.
IMMUNOCYTOCHEMISTRY DATA COLLECTION

All data were collected without knowledge of the treatment group. For each bird, we counted the number of cells immunoreactive for GnRH-I, GnIH and NPY present in each immunostained section. We quantified the area and optical density of GnRH and GnIH immunolabelled perikarya using digital photographs taken at 400× magnification with an Olympus DEI-750D digital camera mounted on an Olympus BX60 light microscope (Olympus Optical Co., Ltd., Tokyo, Japan). Due to the dense network of NPY-ir fibres in the IN (Fig. 2), perikaryon area and optical density could not be accurately quantified for this peptide. Light intensity, aperture diameter and camera shutter speed were standardized for all image captures. We photographed five randomly selected perikarya from each section. Only perikarya for which the entire perimeter was unobstructed and clearly visible were used; perikarya with overlapping structures, such as other perikarya, were not analysed. Digitized images were analysed using Image-Pro Plus (Media Cybernetics, LP, Silver Spring, MD, USA) by manually outlining each perikaryon and then determining the immunolabelled area and optical density (arbitrary units: 0 = no staining, complete light transmission; and 1 = complete staining saturation, no light transmission) of each. All images were standardized for individual variations in background immunolabelling using Image-Pro’s background correction function.

To determine the density of GnRH-I-ir and GnIH-ir fibres in the median eminence (ME), we took pictures from two sections per brain. We corrected for background staining of each image as described above. On the resulting image, we used Image-Pro Plus to measure the optical density of five areas of interest (AOI, 65 × 65 μm each) per brain section. Areas of interest were evenly spaced from left to right along the ventral edge of the ME. We calculated an average optical density for each section, then an average for each bird.

TESTICULAR MORPHOLOGY

We sectioned testes at a thickness of 30 μm using a cryostat at –21 °C and stored sections in 0.1 M PB until mounting on glass microscope slides later the same day. After allowing sections to dry at room temperature for 24 h, we rehydrated them through a graded ethanol series before staining with haematoxylin (S212A; Poly Scientific, Bay Shore, NY, USA) for 3 min. We then rinsed the sections for 5 min under running tap water before destaining by dipping them in acid ethanol ten times. Following another 2 min rinse with tap water, we stained sections with eosin (S176, Poly Scientific) for 30 s, dehydrated them through a graded ethanol series, cleared them in xylene and coverslipped using Permount.

Vernal reproductive development in many seasonally breeding birds involves a marked increase in testis size caused by increases in the length and diameter of seminiferous tubules. Seminiferous tubule diameter is, therefore, a sensitive indicator of testicular exocrine function (Amann 1986; Jenkins, Ross & Young 2007).
We randomly selected eight sections from each bird (four from each testis) and, using Image-Pro Plus, measured the shortest diameter of 10 seminiferous tubules per section randomly selected using a grid overlaid on the image. These measurements were used to calculate an average seminiferous tubule diameter for each bird. We also recorded when spermatozoa were present in the seminiferous tubules.

**HORMONE ASSAYS**

To measure plasma LH, we used the radioimmunoassay described previously (Sharp, Dunn & Talbot 1987), with slight modifications. 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 125I-labelled LH. The primary antibody was precipitated to separate free and bound 125I label using 20 µL of donkey anti-rabbit precipitating serum and 20 µL of non-immune rabbit serum. All samples were measured in duplicate in a single assay. The intra-assay coefficient of variation was 3.6% and the minimum detectable dose was 0.2 ng mL⁻¹. This radioimmunoassay has been used extensively to quantify plasma LH in many avian species (Lal et al. 1990; Lea, Talbot & Sharpe 1991; Malecki et al. 1998; Ciccone, Dunn & Sharpe 2007; Schaper et al. 2012a; Fraley et al. 2013), including multiple Emberizidae sparrows (Meddle et al. 2002; Deviche, Sabo & Sharpe 2008; Deviche et al. 2012a,b; Wingfield et al. 2012).

To quantify plasma T, we used competitive enzyme-linked immunoassay kits, according to the manufacturer’s instructions after 8x dilution in assay buffer containing steroid displacement reagent (Enzo Life Sciences, Ann Arbor, MI, USA). This assay has been validated for Albert’s Towhee in our laboratory (Fokidis, Orchikin & Deviche 2009). We assayed samples in duplicate and randomly assigned them to assay plates, except that all samples collected from any given individual were assayed on the same plate. Each plate included a complete standard curve. The average assay sensitivity was 9.4 pg mL⁻¹. The average intra- and inter-assay coefficients of variation were 6.9% and 3.7%, respectively (n = 2 plates; 66 samples). The primary antibody used in this assay has <5% cross-reactivity with 17ß-oestradiol, 5α-dihydrotestosterone (DHT), corticosterone and progesterone (manufacturer’s specifications).

**STATISTICAL ANALYSES**

To analyse the effects of food availability (ad libitum, reinstated ad libitum or restricted) on body mass, furcular fat score, pectoral muscle score, cloacal protuberance width, and plasma LH and T, we used a two-way analysis of variance with repeated measures (RMANOVA). The sphericity assumption was tested using Mauchly’s test, and if this was violated, we used the Greenhouse-Geisser correction. To analyse the effect of food availability on the number of GnRH, GnIH and NPY cells, we used Kruskal-Wallis test followed by Dunn’s pairwise comparison. Gonadotropin-releasing hormone-I and GnIH measures (median number of ir cells, cell body area and optical density, and optical density of fibres in the ME), and seminiferous tubule diameter were compared using one-way ANOVA. We performed all statistical analyses using PASW version 18.0 (Chicago, Illinois, USA) with an alpha of 0.05 on untransformed data, with the exception of cloacal protuberance width data that were log-transformed to attain normality. Post hoc comparisons for ANOVAS were performed using Tukey’s honestly significant difference (HSD) test. Data analysed using parametric methods are presented as means ± standard error of the mean (SEM), and data analysed using nonparametric methods are presented as medians ± interquartile range (IQR). All graphs depict untransformed data.

**RESULTS**

**BODY MASS**

Body mass was significantly affected by food availability ($F_{2,19} = 32.68$, $P < 0.0001$), time ($F_{2,38} = 93.34$, $P < 0.0001$) and the interaction between these factors ($F_{4,38} = 39.12$, $P < 0.0001$; Fig. 3). Body mass of ad libitum-fed birds was similar throughout the experiment (Tukey HSD, $P ≥ 0.05$). Two weeks of food restriction caused body mass to decrease in both the reinstated ad libitum and the food-restricted groups (Tukey HSD, $P ≤ 0.05$). Body mass did not decrease further in food-restricted birds, but returning birds to ad libitum food availability for 2 weeks resulted in a body mass increase to levels similar to those at the beginning of the study (Tukey HSD, $P < 0.05$).

**FAT SCORE**

Furcular fat scores were significantly affected by food availability ($F_{2,19} = 10.78$, $P = 0.001$), time ($F_{2,38} = 74.56$, $P < 0.0001$) and the interaction between these factors ($F_{4,38} = 15.25$, $P < 0.0001$; Fig. 3). Fat scores of birds exposed to ad libitum food availability decreased over the 4-week study (Tukey HSD, $P < 0.05$). Two weeks of food restriction caused fat scores to further decrease in both the reinstated ad libitum and the food-restricted groups (Tukey HSD, $P < 0.05$). Fat scores did not decrease further in birds maintained on restricted food availability for another 2 weeks, but returning birds to ad libitum food availability for 2 weeks caused fat score to increase to levels similar to those at the beginning of the study (Tukey HSD, $P < 0.05$).

**PECTORAL MUSCLE SCORE**

Pectoral muscle scores were significantly affected by food availability ($F_{2,19} = 10.94$, $P = 0.001$), time ($F_{2,38} = 57.08$, $P < 0.0001$) and the interaction between these factors ($F_{4,38} = 24.72$, $P < 0.0001$; Fig. 3). Pectoral muscle scores of birds exposed to ad libitum food availability were similar throughout the experiment (Tukey HSD, $P < 0.05$). Two weeks of food restriction caused pectoral muscle score to decrease in both the reinstated ad libitum and the food-restricted groups (Tukey HSD, $P < 0.05$). Pectoral muscle score did not decrease further in birds maintained on restricted food availability, but returning birds to ad libitum food availability for 2 weeks caused it to increase to levels similar to those at the beginning of the study (Tukey HSD, $P < 0.05$).

**GnRH**

We found no effect of food availability treatment on GnRH cells, including the number of GnRH-I-ir perikarya per section ($H = 0.54$, 2 d.f., $P = 0.76$), the perikaryon
Constraints on reproductive development

GnIH

GnIH-ir perikaryon area was influenced by the experimental treatments ($F_{2,21} = 3.67, P = 0.045$). Ad libitum birds had greater GnIH-ir perikaryon area than food-restricted birds (Tukey HSD, $P < 0.05$). However, there was no effect of food availability on the number of GnIH-ir cells ($H = 0.87, 2$ d.f., $P = 0.65$), the optical density of GnIH-ir perikaryon ($F_{2,21} = 3.37, P = 0.056$) or the density of ME GnIH-ir fibres ($F_{2,21} = 0.70, P = 0.51$; Fig. 4).

NPY

The number of NPY-ir cells was a function of food availability treatment ($H = 7.56, 2$ d.f., $P = 0.023$; Fig. 4), with food-restricted birds having more NPY-ir cells than ad libitum or reinstated ad libitum birds (Dunn’s method, $P < 0.05$).

PLASMA LUTEINIZING HORMONE

There was no overall effect of food availability treatment on plasma LH ($F_{2,19} = 0.29, P = 0.75$), nor was there an overall effect of time ($F_{5,28.6} = 0.25, P = 0.72$). However, there was an interaction between treatment and time ($F_{5,28.6} = 3.39, P = 0.031$; Fig. 5). Post hoc tests revealed that plasma LH did not change over time in either the ad libitum or the reinstated ad libitum birds (Tukey HSD, $P’ > 0.05$), but plasma LH of food-restricted birds decreased between weeks 0 and 4 (Tukey HSD, $P < 0.05$).

TESTICULAR PHYSIOLOGY AND MORPHOLOGY

There was an effect of food availability treatment on plasma T ($F_{2,19} = 10.97, P = 0.001$), but there was no effect of time ($F_{2,38} = 0.042, P = 0.96$) or an interaction between these factors ($F_{4,38} = 1.33, P = 0.28$; Fig. 5). Post hoc tests revealed that plasma T of the ad libitum-fed birds was higher than both reinstated ad libitum and food-restricted groups (Tukey HSD, $P < 0.05$), and the latter two groups had similar plasma T (Tukey HSD, $P > 0.05$). Plasma T in the reinstated ad libitum and food-restricted groups did not differ at any point (Tukey HSD, $P > 0.05$). At the time of sacrifice, there was no effect of treatment on paired testis width ($F_{2,21} = 2.21, P = 0.14$; Table 1) or seminiferous tubule diameter ($F_{2,21} = 0.61, P = 0.56$; Table 1). Furthermore, spermatozoa were present in the seminiferous tubules of all birds.

CLOACAL PROTUBERANCE

Cloacal protuberance width was significantly affected by food availability ($F_{2,19} = 7.96, P = 0.003$), time ($F_{2,38} = 8.13, P = 0.001$) and the interaction between these factors ($F_{4,38} = 13.68, P < 0.001$; Fig. 5). Cloacal protuberance width of birds exposed to ad libitum food availability increased during the first 2 weeks of the experiment.
Tukey HSD, \( P < 0.05 \)), but did not increase further during the last 2 weeks. Two weeks of food restriction caused cloacal protuberance width to decrease in both the reinstated ad libitum and the food-restricted groups (Tukey HSD, \( P < 0.05 \)). Cloacal protuberance width did not decrease further in birds maintained on restricted food availability, but returning birds to ad libitum food availability for 2 weeks caused cloacal protuberance width to increase to levels similar to those of the ad libitum birds.

**Fig. 4.** The number, area and optical density of perikarya and optical density of fibres in the median eminence (ME) immunolabelled for gonadotropin-releasing hormone-I (GnRH-I), gonadotropin-inhibitory hormone (GnIH) and neuropeptide Y (NPY) of adult male Abert’s Towhees Melozone aberti following food availability treatment. Birds were exposed to ad libitum food availability for 4 weeks (\( n = 6 \); ‘ad libitum’), 2 weeks of food restriction (70% of ad libitum consumption) followed by 2 weeks of ad libitum food (\( n = 8 \); ‘reinstated ad libitum’) or restricted food availability for 4 weeks (\( n = 8 \); ‘food-restricted’). Superscript letters indicate significant differences between the groups (\( P < 0.05 \); Tukey honestly significant difference test). AU, arbitrary units. Data presented are means (±SEM), with the exception of the number of cells, which are presented as medians and interquartile range.

**Fig. 5.** Plasma luteinizing hormone, plasma testosterone and cloacal protuberance width were modulated by food availability in adult male Abert’s Towhees Melozone aberti. Plasma luteinizing hormone (a) decreased between weeks 0 and 4 in food-restricted birds. Plasma testosterone (b) was lower in birds exposed to two (reinstated ad libitum) or four (restricted) weeks of food restriction (70% of ad libitum consumption) compared to control birds with ad libitum food availability. Cloacal protuberance width (c) was reduced by food restriction and returning birds to ad libitum food availability increased it to a size similar to those of the control group. Points with identical letters are not significantly different (\( P > 0.05 \); Tukey honestly significant difference test). For details of study design, see Fig. 1 and the Materials and methods section. Data points are means ± SEM. For visual clarity, points have been separated along the horizontal axis.
Table 1. Photoinduced testicular development of adult male Abert’s Towhees Melozone aberti was not affected by food availability. Towhees were exposed to either ad libitum food availability for 4 weeks (‘ad libitum’), 2 weeks of food restriction (70% of ad libitum consumption) followed by 2 weeks of ad libitum food (‘reinstated ad libitum’) or restricted food availability for 4 weeks (‘food-restricted’) before we collected testes. Data presented are means (± SEM)

| Food availability treatment group | Ad libitum | Reinstated ad libitum | Food-restricted | Statistics P-value |
|----------------------------------|------------|-----------------------|-----------------|-------------------|
| Paired testis mass (mg)           | 348.8 (±18.1) | 357.0 (±30.1) | 294.7 (±17.0) | 0.14              |
| Seminiferous tubule diameter (µm)| 489.1 (±17.1) | 479.1 (±14.2) | 462.9 (±18.4) | 0.56              |

Discussion

It has long been recognized that food availability plays a crucial proximate role in the development of gonads and secondary sexual characteristics of seasonally breeding birds (Lack 1968; Hahn et al. 2005; Williams 2012), but the mechanism mediating this role remains unclear. Food availability is thought to modulate reproductive development partly via energetic status. Limited food availability may constrain this development because birds lack the necessary energy stores for tissue growth and hormone production (Drent & Daan 1980; Wingfield & Kenagy 1986; Meijer & Drent 1999; Hahn et al. 2005). Studies in controlled laboratory conditions aimed at testing whether food availability modulates reproductive development through its effects on energetic status must, therefore, ensure that food availability treatments produce a disparity in the energetic condition. To that end, we measured body mass and energy stores (as estimated by furcular fat stores and pectoral muscle size). All of these parameters were, indeed, reduced by the food restriction regime, indicating that food-restricted birds were in lower energetic condition than control birds. Furthermore, returning birds to ad libitum food availability caused all of these parameters to increase to levels similar to those at the beginning of the study. Therefore, the food availability treatments resulted in three groups of birds that differed in the duration that they experienced reduced energetic status.

TESTICULAR GROWTH AND ENDOCRINE ACTIVITY

Despite differences in energetic status, Abert’s Towhees in the three experimental groups had similar testis masses and seminiferous tubule diameters. Furthermore, the seminiferous tubules of all towhees contained spermatozoa. If testis growth and spermatogenesis are energetically expensive processes, we would have expected the energetic constraint imposed by food restriction to reduce photoinduced testis growth. As we detected no such reduction, our observations are consistent with the proposition that testis growth in the Abert’s Towhee may not be particularly energetically demanding. This conclusion is consistent with results of a study by Caro & Visser (2009) in which Great Tits Parus major exposed to ambient temperatures of 8 °C or 22 °C during photoperiodically induced reproductive development had different basal metabolic rates, but showed similar testicular growth. We should point out that neither our study nor that of Caro & Visser (2009) can exclude the possibility that testis growth is energetically demanding, but when energy is limited, birds continue to allocate energy to this process at the cost of other processes. The energetic costs of testis growth and maintenance are notoriously difficult to quantify (Vézina & Salvante 2010). Estimates of the metabolic cost of testis growth based solely on tissue energy content are consistent with the proposition that this process is energetically inexpensive (Walsberg 1983). However, as others have pointed out (Vézina & Salvante 2010), these estimates fail to account for the costs of tissue synthesis, maintenance and function. Indeed, the potential costs of testicular function, particularly increased T production and secretion, are likely to be high.

Testosterone plays a key role in regulating life-history trade-offs in male vertebrates and promotes investment in sexual traits, which generally comes at a cost to somatic maintenance. Elevated plasma T during breeding promotes expression of male reproductive behaviours, such as singing, courtship, territorial aggression and mate guarding (Foerster et al. 2002; Hau 2007; Kurvers et al. 2008). These behaviours by themselves confer energetic costs (Oberweger & Goller 2001; Ward, Speakman & Slater 2003; Ward & Slater 2005; Hasselquist & Bensch 2008), which are likely compounded by indirect costs through reduction in the time spent at rest and for self-maintenance behaviours (Lynn et al. 2000) and foraging (Thomas et al. 2003). Behaviours that are stimulated by T also increase predation risk (Schmidt & Belinsky 2013) and decrease survival (Reed et al. 2006). Furthermore, investment in T-dependent sexual traits generally comes at a cost to somatic maintenance, such as immune performance (Hau 2007). We suggest, therefore, that our finding that plasma T was higher in birds with ad libitum food compared to birds that had experienced a period of food restriction is consistent with T-mediated reproductive trade-offs. Specifically, when energy is limited, T production and secretion may be suppressed to avoid the costs of T-mediated male reproductive behaviours. In support of this suggestion, adult male Zebra Finches Taeniopygia guttata subjected to short-term (4–10 h) food deprivation decreased plasma T, singing rate and courtship behaviour towards females.
HYPOTHALAMIC AND ANTERIOR PITUITARY GLAND ENDOCRINE ACTIVITY

The mechanism(s) by which energetic status affects T production is/are unclear, but potentially involves modulation at multiple levels of the HPG axis. This mechanism may involve reduced gonadotropin production and/or secretion. We found that plasma LH did not change over the study in either the ad libitum-fed birds or the birds subjected to 2 weeks of ad libitum food access suggests, however, that there may be lasting (i.e. on the order of weeks) effects of poor energetic status on T production and secretion.

THE GNIH–NPY SYSTEM

Alternatively, food availability may modulate the production and secretion of gonadotropins through effects on the GnIH–NPY system. There is evidence in seasonally breeding birds that GnIH controls reproductive activation through inhibitory actions on gonadotropes (Bentley et al. 2003; Calisi, Rizzo & Bentley 2008; Small et al. 2008b; Perfito et al. 2011). Furthermore, via their interactions with NPY cells in the IN, GnIH cells may modulate this inhibitory influence in response to energetic status (Clarke et al. 2009; Klingerman et al. 2011). In the present study, the area of GnIH-ir perikarya was inversely related to the amount of time that birds experienced negative energetic status. In addition, food-restricted birds had more IN NPY-ir perikarya than ad libitum-fed birds. These observations are consistent with the idea that negative energetic status was associated with upregulation of the NPY system that, in turn, promoted the release of GnIH. Enhanced GnIH secretion in food-restricted birds may account for the reduced LH secretion and consequently plasma T in these birds.

Another neuropeptide whose activity is related to energetic status is GnRH-II, a second form of GnRH that is highly conserved across almost all vertebrates (Schneider & Rissman 2008). In the Musk Shrew Suncus murinus mid-brain levels of GnRH-II show the opposite response to negative energetic status as compared to that of NPY: GnRH-II content declined in response to food restriction and increased after refeeding (Kauffman et al. 2006). Crucially, these energetically aware GnRH-II cells were associated with NPY-ir fibres and, therefore, appear to work with NPY cells to coordinate the reproductive system response to energetic status (Bojkowska et al. 2008). Although, to our knowledge, experimental studies of the response of GnRH-II to negative energetic status in birds are lacking, the available evidence in birds is consistent with a role similar to that in mammals (Maney, Richardson & Wingfield 1997; Stevenson & MacDougall-Shackleton 2005; Stevenson et al. 2008; Perfito et al. 2011). In particular, GnRH-II cells in the White-crowned Sparrow Zonotrichia leucophrys possess putative GnIH receptors (Bentley et al. 2006). Thus, GnRH-II may contribute to the reproductive system response to negative energetic status of birds. We suggest, therefore, that improving our...
understanding of the interactions between GnIH, NPY, GnRH-II and their role in integrating information on energetic status into the reproductive system may shed light on the physiological mechanisms by which food availability influences the HPG axis.

**GONADAL GNIH**

Finally, food availability may affect plasma T through gonadally produced GnIH. Avian testes express this peptide and its receptors, and experimental evidence supports an inhibitory role for GnIH in the control of T secretion (Bentley et al. 2009; McGuire & Bentley 2010; Tsutsui et al. 2012), including in response to signals of metabolic stress (McGuire, Koh & Bentley 2013). Overall, the effects of food availability on T production are potentially mediated at multiple levels of the HPG axis. Further research is needed, however, to shed light on the specific mechanism(s) involved.

**SECONDARY SEXUAL CHARACTERISTICS**

In contrast to the long-term effects on T secretion, cloacal protuberance width appears to be highly sensitive to changes in energetic status. Indeed, 2 weeks of food restriction caused this organ to decrease in size. Furthermore, returning food-restricted birds to ad libitum food availability for 2 weeks caused cloacal protuberance width to rapidly increase and reach a size similar to that of ad libitum-fed birds. This increase, despite no associated change in plasma T, suggests that factors other than plasma T regulate development of the cloacal protuberance and that these factors are responsive to energetic status. The T metabolite DHT stimulates growth of the cloacal protuberance (Tramontin, Wingfield & Brenowitz 2003; Owen-Ashley, Hasselquist & Wingfield 2004). We are aware of just one study that has examined plasma DHT in birds experiencing food restriction. In male Garden Warblers *Sylvia borin* caught during their spring migration, during which they also develop their gonads, food restriction reduced body mass but had no effect on plasma DHT (Bauchinger, Van’t Hof & Biebach 2008). Therefore, further research is needed to examine whether DHT is also responsive to food availability.

**Conclusion**

Many birds use food availability as a proximate environmental cue to optimally time seasonal reproductive development. However, despite appreciating the importance of this cue for decades, the physiological mechanisms by which information on food availability is integrated into the HPG axis remain unclear. We hypothesized that seasonal reproductive activation is constrained by energetic status. Indeed, we found that energetic status modulates activity of the HPG axis in male Abert’s Towhees; however, the response is complex and appears to vary with the level of the HPG axis considered. Our results are consistent with a role for the GnIH–NPY system in integrating information on energetic status at the level of the hypothalamus. However, this does not appear to involve a modulation of the amount of hypothalamic GnRH-I. There also appears to be a role for anterior pituitary gland and/or testicular endocrine function in modulating reproductive development in the light of energetic status. Despite no evidence that energetic status modulated testicular growth, plasma LH and T were reduced in response to poor energetic status. A further illustration of the complexity by which energetic status affects the reproductive system is that the cloacal protuberance – but not LH or T secretion – was responsive to returning food-restricted birds to ad libitum food availability. Future research is warranted to elucidate the relative importance of the hypothalamus and the gonads in integrating information on energetic status.

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**Data accessibility**

Data are permanently deposited and available in the NSF LTER Network data base (link: https://caplter.asu.edu/data/data-catalog/?id=613).

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