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RESEARCH ARTICLE

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.

KEY WORDS: Luteinizing hormone, Gonadal development, Gonadotropin-releasing hormone, Hypothalamic–pituitary–gonadal axis, Seasonal reproduction, Testosterone

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 photoperiod 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).

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List of symbols and abbreviations

CORT	corticosterone
FSH	follicle-stimulating hormone
GnIH	gonadotropin-inhibitory hormone
GnRH	gonadotropin-releasing hormone-I
HPA	hypothalamic–pituitary–adrenal
HPG	hypothalamic–pituitary–gonadal
ir	immunoreactivity
LH	luteinizing hormone
ME	median eminence
NPY	neuropeptide-Y
POA	preoptic area
proGnRH	gonadotropin-releasing hormone pro-peptide
PVN	paraventricular nucleus
TrSM	tractus septomesencephalicus

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., 2015b). 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.

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. 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. 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. 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). Seminiferous tubule diameter was also smaller in food-restricted birds than in *ad libitum*-fed birds (t_{13}=5.13, P<0.001; Fig. 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. 4A). Plasma testosterone did not change in response to photostimulation in

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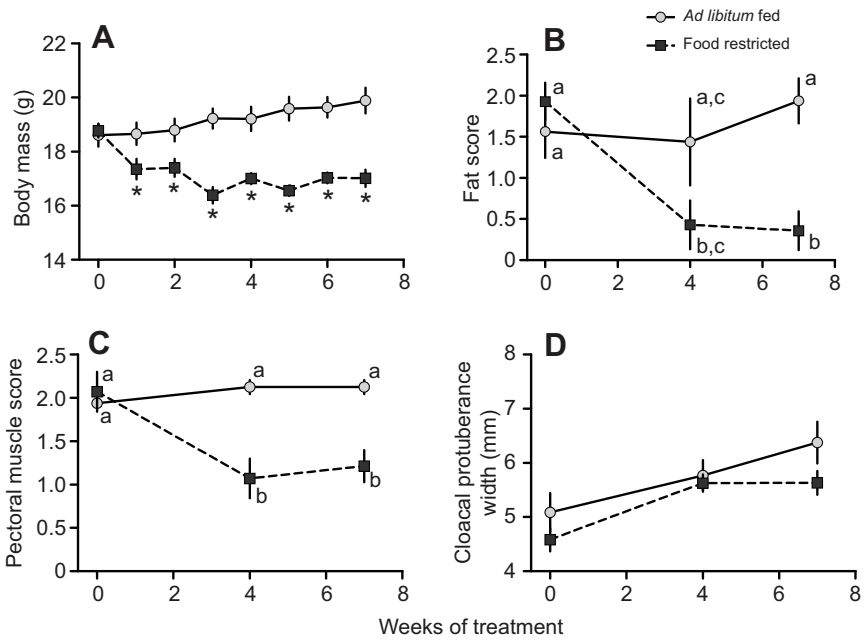

Fig. 1. Effects of food availability on male house finches, *Haemorhous mexicanus*. Food restriction negatively affects body mass (A), furcular fat (B) and pectoral muscle (C), but does not affect cloacal protuberance width (D). House finches were either *ad libitum* fed (N=8) or food restricted (N=7) and photostimulated at time 0. Data are plotted as means±s.e.m. Means with identical letters are not statistically different (P>0.05, SNK tests). An asterisk indicates that for a given time point, the two groups are statistically different.

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. 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−β=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. 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−β=0.23) of the ANOVA to detect group differences.

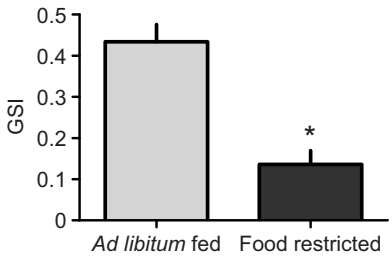

Fig. 2. Gonadosomatic index is lower in food-restricted male house finches. Birds were either *ad libitum* fed (N=8) or food restricted (N=7) and photostimulated for 7 weeks. Gonadosomatic index (GSI) was calculated as testis mass as a percentage of body mass. Data are plotted as means+s.e.m., and the asterisk denotes a significant difference between the groups (P<0.05, Student's t-test).

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. 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

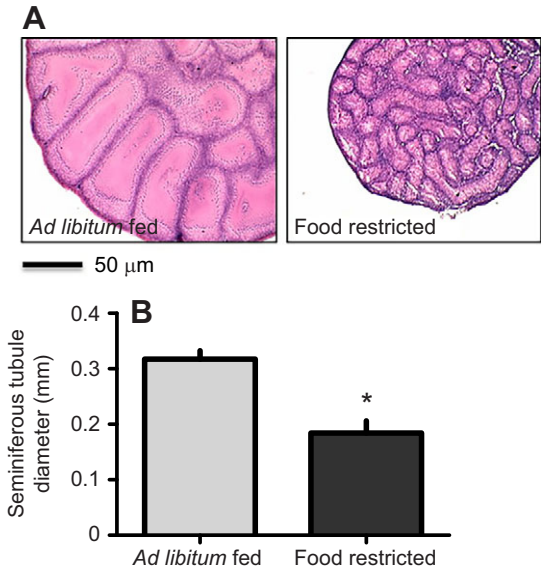

Fig. 3. The diameter of the seminiferous tubules is smaller in food-restricted male house finches. Birds were either *ad libitum* fed (N=8) or food restricted (N=7) and photostimulated for 7 weeks. (A) Hematoxylin and eosin-stained sections of testis showing the seminiferous tubules. (B) Data are plotted as means+s.e.m., and the asterisk denotes a significant difference between the groups (P<0.05, Student's t-test).

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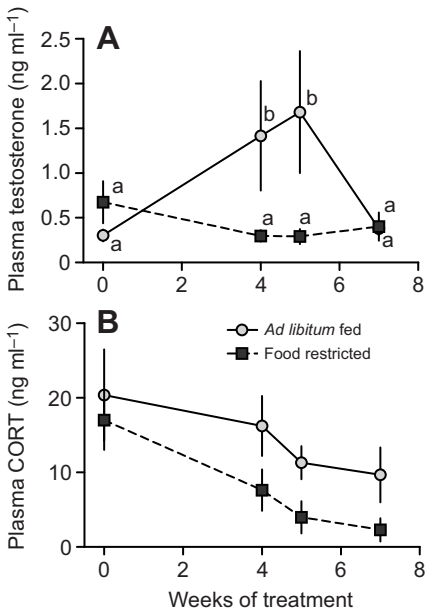

Fig. 4. Food restriction suppresses the long day-induced increase in baseline plasma testosterone but does not affect baseline plasma corticosterone. Male house finches were either *ad libitum* fed (*N*=8) or food restricted (*N*=7) and photostimulated at time 0, and plasma testosterone (A) and corticosterone (CORT; B) levels were measured. Data are displayed as means±s.e.m. Means with identical letters are not statistically different (*P*>0.05, SNK tests).

experimental groups (*U*=8, *P*=0.02; Fig. 6, Table 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

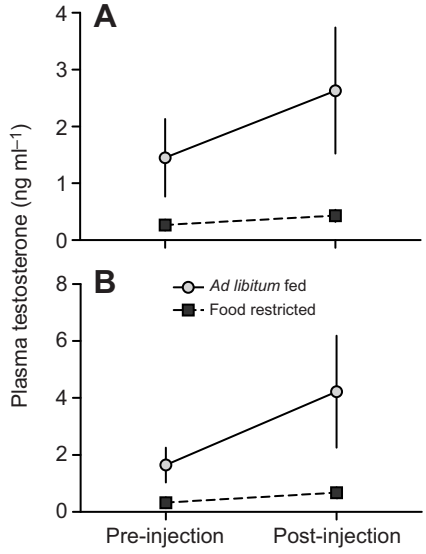

Fig. 5. Food availability does not affect the gonadotropin-releasing hormone- or luteinizing hormone-induced increase in plasma testosterone. Male house finches were either *ad libitum* fed (*N*=8) or food restricted (*N*=8) and exposed to a gonadotropin-releasing hormone (GnRH; A) or luteinizing hormone (LH; B) challenge. Data are shown as means±s.e.m.

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.

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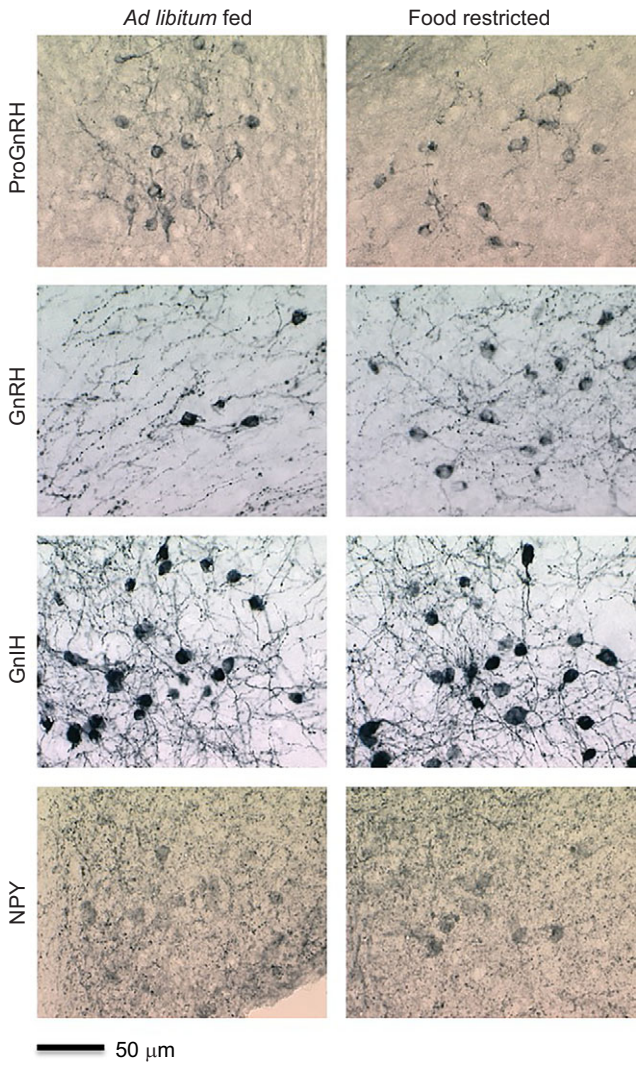

Fig. 6. Effect of food restriction on brain neuropeptide immunoreactivity in male house finches. Birds were either *ad libitum* fed (left panel) or food restricted (right panel) and photostimulated for 7 weeks. Representative photomicrographs of hypothalamic brain sections are shown for GnRH pro-peptide (proGnRH), GnRH, gonadotropin-inhibitory hormone (GnIH) and neuropeptide Y (NPY) immunoreactivity.

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

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Table 1. Immunocytochemical analysis of GnRH, proGnRH, GnIH and NPY in house finches, *Haemorhous mexicanus*

A. Cell count data

	Ad libitum fed	Food restricted	*U*	*P*
GnRH	96 (65)	135 (30)	8	**0.021**
ProGnRH	92 (119)	98 (46)	26	0.870
GnIH	233 (124)	269 (242)	25	0.779
NPY	128 (72)	118 (40)	21	0.710

B. Cell area, cell optical density and fiber density data

	Ad libitum fed	Food restricted	*t*	d.f.	*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

Birds were either *ad libitum* fed or food restricted and photostimulated for 7 weeks. (A) Cell count data (perikaryon number) are given as median and interquartile range (IQR) and were analyzed by Mann–Whitney *U*-tests. (B) Cell area, cell optical density and fiber density data are presented as means±s.e.m. and were analyzed by Student's *t*-tests. Optical density and fiber density values are in arbitrary units: 0=no staining, 1=complete saturation. ME, median eminence; POA, preoptic area. Bold indicates a significant difference.

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., 2012), 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

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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.

MATERIALS AND 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^{-1}) or freshly prepared ovine LH solution (Batch AFP5551B; The National Peptide and Hormone Program, Harbor-UCLA Medical Center, Torrance, CA, USA; 1 mg kg^{-1}) 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^{-1} xylacine and 100 mg ml^{-1} ketamine). Birds were perfused transcardially with 35 ml wash solution (0.9% NaCl and 0.1% $NaNO_2$ in 0.1 mol l^{-1} phosphate buffer, PB) followed by 35 ml of fixative (4% paraformaldehyde and 0.1% $NaNO_2$ in 0.1 mol l^{-1} 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^{-1} 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,

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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. Photographs 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^{-1} and the inter- and intra-assay coefficients of variation were 3.2% (N=3 samples 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^{-1} 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^{-1} PB, incubated in 0.36% hydrogen peroxide, washed 3 times for 5 min in 0.1 mol l^{-1} 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^{-1} 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^{-1} 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; Dutlow et 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 solutions were 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 photographs 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 photographs. 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 photographs 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 an 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.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.V. and P.D. conceived and designed the experiments. S.V. and B.V. performed food restrictions and collected blood samples. S.V. did hormone assays and statistical analyses. S.V. and E.C. did immunocytochemical and histological procedures and analyses. K.T. provided the GnIH antibody. S.V. and P.D. drafted the manuscript.

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