Investigating the Influence of Food on Reproductive Physiology and Gonad Growth: Urbanization as a Natural Experiment

by

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ABSTRACT

For animals that experience annual cycles of gonad development, the seasonal timing (phenology) of gonad growth is a major adaptation to local environmental conditions. To optimally time seasonal gonad growth, animals use environmental cues that forecast future conditions. The availability of food is one such environmental cue. Although the importance of food availability has been appreciated for decades, the physiological mechanisms underlying the modulation of seasonal gonad growth by this environmental factor remain poorly understood.

Urbanization is characterized by profound environmental changes, and urban animals must adjust to an environment vastly different from that of their non-urban conspecifics. Evidence suggests that birds adjust to urban areas by advancing the timing of seasonal breeding and gonad development, compared to their non-urban conspecifics. A leading hypothesis to account for this phenomenon is that food availability is elevated in urban areas, which improves the energetic status of urban birds and enables them to initiate gonad development earlier than their non-urban conspecifics. However, this hypothesis remains largely untested.

My dissertation dovetailed comparative studies and experimental approaches conducted in field and captive settings to examine the physiological mechanisms by which food availability modulates gonad growth and to investigate whether elevated food availability in urban areas advances the phenology of gonad growth in urban birds. My captive study demonstrated that energetic status modulates reproductive hormone secretion, but not gonad growth. By contrast, free-ranging urban and non-urban birds did not differ in energetic status or plasma levels of reproductive hormones either in years in which urban birds had advanced phenology of gonad growth or in a year that had no habitat-related disparity in seasonal gonad growth. Therefore, my dissertation

provides no support for the hypothesis that urban birds begin seasonal gonad growth because they are in better energetic status and increase the secretion of reproductive hormones earlier than non-urban birds. My studies do suggest, however, that the phenology of key food items and the endocrine responsiveness of the reproductive system may contribute to habitat-related disparities in the phenology of gonad growth.

DEDICATION

To my parents, Susan and Graham, who sparked my love of nature and provided unwavering support.

To my wife, Emily, because without her love and encouragement I would not have embarked on this adventure nor would I have made it through.

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CHAPTER 1

INTRODUCTION

Almost all vertebrates live in a seasonally changing environment and synchronize breeding to coincide with optimal local environmental conditions (Baker, 1938; Boyd, 1991; Lindström, 1999; Lourdais et al., 2002; Munro et al., 1990; Olive et al., 2000; Wingfield and Kenagy, 1986). Many vertebrates undergo annual cycles of gonad development in which the gonads must develop before breeding can commence (Davies and Deviche, 2014; Murton and Westwood, 1977). Hence, the seasonal timing (phenology) of gonad development is a fundamental determinant of the breeding period. To optimally time seasonal gonad growth, vertebrates use environmental cues that forecast future conditions. Most vertebrates use the circannual cycle of day length (photoperiod) as the cue to initiate gonad development (Dawson et al., 2001). At any given location, environmental conditions vary from year-to-year, so vertebrates also use 'supplementary' environmental cues, such as ambient temperature (Perfito et al., 2004), precipitation (Small et al., 2008a), and food availability (Davies and Deviche, 2014; Hahn et al., 2005), to fine-tune the rate of development to local conditions in a given year. There is, therefore, considerable variation in the phenology of gonad development between years, between individuals in a population, and between populations. However, the physiological mechanisms underlying the modulation of gonad development phenology to local conditions remains poorly understood.

A burgeoning body of research demonstrates that birds breeding in urbanized areas begin the breeding period earlier than do their nearby non-urban conspecifics (for reviews see: Chamberlain et al., 2009; Deviche and Davies, 2014). A limited number of studies also suggest that the advanced breeding phenology of urban birds is associated with earlier gonad development (Deviche et al., 2010; Partecke et al., 2005). Since urban

and non-urban bird populations experience almost identical annual cycles of natural photoperiod, comparisons of urban and non-urban bird populations may be an effective way to better understand the ecological and physiological mechanisms underlying variation in gonad development phenology. Despite this opportunity, to my knowledge, only a single study has simultaneously compared gonad development and reproductive endocrine activity in urban versus non-urban birds (Partecke et al., 2005).

It is hypothesized that human provided food (i.e., bird feeders and waste food) and/or increases in net primary productivity (Buyantuyev and Wu, 2009; Imhoff et al., 2004) and arthropod abundance (Cook and Faeth, 2006) in urban areas increase food availability for urban birds and that this disparity drives the advanced gonad development of urban birds (Chamberlain et al., 2009; Robb et al., 2008a; Robb et al., 2008b; Schoech and Bowman, 2001). A key assumption of this hypothesis is that elevated food availability improves the energetic status of urban birds and enables them to initiate gonad development as early as photoperiod permits (Drent and Daan, 1980; Hahn et al., 2005; Meijer and Drent, 1999). However, the physiological mechanisms underlying the modulation of gonad development phenology by energetic status are poorly understood in birds.

1.1. Study Species

The Abert's Towhee (*Melozone aberti*) is a large (40 – 55 g) sparrow of the Emberizidae family. This species is common in riparian woods and marshes of the Sonoran Desert, and throughout the Phoenix Metropolitan area, particularly in suburban yards (Rosenberg et al., 1991). Adults are sedentary, form life-long pair bonds, and hold a permanent territory (1.5 - 2 ha; Rosenberg et al., 1991). The sedentary nature of towhees suggests that, once established, adults are unlikely to leave their territory and habitat-

associated physiological differences likely result from local environmental conditions. Abert's Towhees consume a variety of food types including arthropods, seeds, and vegetation, but insects dominate the diet in all seasons (Rosenberg et al., 1991). In urban areas, towhees will also consume a wide variety of human-provided food (pers. obs.).

1.2. Dissertation Overview

The aim of my dissertation was to investigate the influence of food availability on the physiological control of seasonal gonad development. This dissertation capitalized on the putative disparity in food availability between urban and desert habitats in Phoenix, AZ to examine the physiological mechanisms by which food availability affects the hypothalamo-pituitary-gonadal (HPG) axis, and simultaneously improve our understanding of the influence of land use type on the physiology of birds.

My studies tested the hypothesis that urbanization of Phoenix, AZ is associated with advanced phenology of gonad development owing to elevated food availability. To test this hypothesis I addressed the following two questions:

1) How does urbanization influence seasonal reproductive physiology?

In Chapter 2, I examined whether the phenology of gonad development in urban and desert Abert's Towhees is associated with the seasonal rise in gonadal endocrine activity. To accomplish this goal, I measured testis size and plasma testosterone secretion of free-ranging towhees.

In Chapter 3, I assessed whether the phenology of gonad development in urban and desert Abert's Towhees is associated with hypothalamic levels of neuropeptides key to gonad development and the responsiveness of reproductive endocrine glands.

In Chapter 4, I took a global view and compared the breeding phenology of urban birds versus their non-urban conspecifics in cities around the world and explored

whether differences in breeding phenology are related to changes in environmental conditions associated with urbanization. To accomplish this goal, I used a meta-analytical approach and tested whether the difference in breeding phenology between urban and non-urban birds is related to seasonal changes in the strength of the urban heat island and the phenology of plant growing seasons.

2) How does energetic status influence the neuroendocrine control of reproduction?

In Chapter 5, I experimentally tested whether energetic status modulates the phenology of the rise in reproductive hormone secretion and gonad development. To accomplish this goal, I manipulated food availability of captive towhees to induce a disparity in energy stores and body mass, and measured levels of key reproductive hormones in the hypothalamus and gonads.

In Chapter 6, I experimentally tested whether neuropeptide Y simultaneously coordinates reproductive endocrine activity and food intake. To accomplish this goal, I quantified the gonadal endocrine and feeding behavior responses to centrally administered NPY.

CHAPTER 2

ADVANCED SEASONAL REPRODUCTIVE DEVELOPMENT IN AN URBAN BIRD IS NOT MIRRORED IN THE UNDERLYING REPRODUCTIVE PHYSIOLOGY

Urban animals inhabit an environment considerably different than do their non-urban conspecifics, and to persist urban animals must respond and adapt to these novel environmental conditions. The timing of seasonal reproductive development (i.e, growth of gonads and secondary sex organs) is a fundamental determinant of the breeding period and is frequently advanced in urban bird populations. However, the underlying mechanism(s) by which birds adjust the timing of reproductive development to urban areas remain(s) largely unknown. Here, I compared the timing of reproductive development in urban and non-urban Abert's Towhees (*Melozone aberti*) in Phoenix, Arizona, and examined whether energetic status constrains the timing of development. I found that male Abert's Towhees adjust the timing of their reproductive development to urban areas of Phoenix, Arizona USA by advancing the initiation of development compared to their non-urban, desert conspecifics. Despite this habitat-related disparity in the timing of reproductive development, urban and non-urban towhees had similar body condition, fat stores, and immune performance. Thus, multiple lines of evidence provide no support for the hypothesis that energetic constraints are responsible for the earlier reproductive development of urban male Abert's Towhees. Furthermore, I also investigated whether this advanced reproductive development is associated with an earlier rise in endocrine activity of the HPG axis, as measured by plasma testosterone (T) levels. Testosterone is a key hormone for vertebrate male reproductive function and spermatogenesis, but plasma levels of this hormone did not mirror the difference in reproductive development. These findings suggest that future research aimed at elucidating the mechanism(s) underlying the advanced reproductive development of

urban bird populations may benefit from measuring not only levels of plasma reproductive hormones, but also hormone receptor densities and downstream effects.

1. Introduction

Urbanization profoundly alters ecosystems and produces environments that differ considerably from non-urban areas. Urban areas are characterized by a high proportion of impervious surface (i.e., buildings, roads, etc.), human density (Heil et al., 2007; Nakwa et al., 2008), noise (Halfwerk and Slabbekoorn, 2013), artificial light (Gaston et al., 2013; Longcore and Rich, 2004), vehicular traffic (Benítez-López et al., 2010), habitat fragmentation (Delaney et al., 2010), toxins (Hofer et al., 2010), and feral animals (Van Heezik et al., 2010). Furthermore, urbanization modifies primary productivity (Buyantuyev and Wu, 2009; Imhoff et al., 2004), food abundance (Cook and Faeth, 2006; Cowie and Hinsley, 1988; Faeth et al., 2005; Robb et al., 2008a), and ambient temperature (Imhoff et al., 2010; Zhang et al., 2010). For urban animal populations to persist, animals must respond and adjust to these modified environmental conditions. As urban spaces are the most rapidly expanding habitat type worldwide (Grimm et al., 2008), the potential impact of urbanization on biodiversity is considerable and there is an urgent need to understand the mechanisms responsible for adjustment to these new habitats.

Many animals have distinct seasonal breeding periods that are synchronized with optimal environmental conditions to maximize fitness (Both et al., 2006; Charmantier et al., 2008; Visser et al., 2006; Williams, 2012). In many vertebrates, including most birds, the transition from the non-breeding to the breeding life history stage is associated with extensive physiological and morphological changes, such as increases in the plasma

concentrations of key reproductive hormones and gonad volume, respectively (Murton and Westwood, 1977; Williams, 2012). Reproductive development, in particular growth of the gonads and secondary sex organs, is, therefore, a fundamental determinant of the breeding period, and it may be advantageous for urban birds to adjust the timing of reproductive development to local environmental conditions. Indeed, a consistent effect of urbanization on bird populations is an advancement of the timing of seasonal reproductive development (Deviche et al., 2010; reviewed by Deviche and Davies, 2014; Partecke et al., 2005). Although this phenomenon appears to be widespread and consistent, the underlying mechanism(s) remain(s) largely unknown.

Reproductive development is controlled by the activity of the hypothalamo-pituitary-gonadal (HPG) axis. This axis begins with the hypothalamus, the site of production of gonadotropin-releasing hormone-I (GnRH-I; Sharp and Ciccone, 2005). GnRH-I is the primary releasing factor that stimulates the release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland (Kuenzel, 2000). Gonadotropins initiate gonad development, gametogenesis, secretion of the sex steroids testosterone (T) and estradiol (E_2) in males and females, respectively, and expression of reproductive behaviors (Deviche et al., 2010; Murton and Westwood, 1977). Modulation of the HPG axis activity, through changes in hormone secretion, hormone carrier protein concentrations, and hormone receptor densities, has the potential to modulate the timing and/or rate of reproductive development.

Activity of the HPG axis is determined by information provided by a suite of environmental cues that can predict future conditions (Dawson et al., 2001; Dawson, 2008). In seasonally breeding birds, the annual change in day length (photoperiod) is

the initial predictive cue used to begin reproductive development. However, the timing of change in this cue is constant from year to year, so birds also use a host of supplementary cues, such as ambient temperature (Schaper et al., 2012b) and food availability (Hahn et al., 2005), to fine-tune development to a given year's conditions. Urbanization potentially modifies some or all of these non-photic cues. In particular, urbanization potentially creates differences in food availability between urban and non-urban areas. For example, in Phoenix (Arizona, USA), where the present study was conducted, urbanization is associated with higher plant density and an earlier seasonal growth of plants (Buyantuyev and Wu, 2009; Buyantuyev and Wu, 2010), as well as increased arthropod abundance (Cook and Faeth, 2006).

Understanding the mechanism by which food availability influences seasonal reproductive development is complicated by the fact that this factor may provide environmental information (i.e., an abundance of food could signal optimal conditions via physiological pathways independent of energetics) as well as influence the energetic status (via effects on food consumption). Within the window of opportunity for reproductive development governed by day length, a bird's energetic status is thought to constrain the timing of reproductive development (Drent and Daan, 1980; Hahn et al., 2005; Meijer and Drent, 1999; Wingfield and Kenagy, 1986). Life history theory posits that when resources are limited there is a resource allocation trade-off between reproduction and self-maintenance, in which allocation into reproduction comes at a cost to somatic processes, such as immune function (Stearns, 1989; Zera and Harshman, 2001). If urbanization affects the food availability (see above), this may lead to differences in energetic status between urban birds and their non-urban conspecifics, and, in turn, a disparity in reproductive development, energy stores, and investment into somatic processes such as immunity between urban and non-urban populations.

I compared the timing of reproductive development in urban and non-urban Abert's Towhees (*Melozone aberti*) in Phoenix, Arizona. I predicted that urban towhees will develop their testes and cloacal protuberance (CP, a T-dependent secondary sexual characteristic) earlier than do non-urban conspecifics. Furthermore, I compared endocrine activity of the HPG axis in these populations and predicted that plasma T, a hormone essential for reproductive development and male reproductive function (Brown and Follett, 1977; Deviche et al., 2010; Dornas et al., 2008; Penfold et al., 2001), would increase earlier in the spring in urban than non-urban birds. If urbanization increases food availability, I also predicted that urban towhees will have greater endogenous fat stores, be in better body condition (i.e., body mass corrected for body size), and have higher immune function compared to non-urban birds.

2. Methods

2.1. Study species

Abert's Towhees (*Melozone aberti*) are common in riparian woods and marshes of the Sonoran Desert and also throughout the Phoenix Metropolitan area, particularly in urban yards (Rosenberg et al., 1991). They consume a variety of foods including arthropods and seeds, but the available evidence suggests that arthropods dominate the diet in all seasons, making up 73% of the diet in winter and 96% in summer (Rosenberg et al., 1991). In urban areas, this species will also consume a wide variety of human-provided food (S. Davies, pers. obs.). Abert's Towhees are sedentary, form life-long pair bonds, and hold a permanent territory (1.5 - 2 ha; Rosenberg et al., 1991). Captive studies indicate that males are photoperiodic and develop their reproductive system in response to increasing day length (S. Davies, unpublished data). Free-ranging towhees can have multiple broods in a given breeding season and active nests have been found from

February to September (Tweit and Finch, 1994). Most broods are attempted during spring, however, and the number of active nests and CP width increase substantially during March and peak in April (Tweit and Finch, 1994). Breeding during the summer is dependent on monsoon rainfall (Tweit and Finch, 1994), suggesting that, in addition to day length, this species modulate its reproductive activity based on the use of supplementary environmental cues.

2.2. Study sites

To investigate the effect of urban areas on vernal development of the reproductive system, I compared adult male Abert's Towhees from five urban and four Sonoran Desert localities in Maricopa County, Arizona (Davies et al., 2013). Urban localities were distributed throughout the Phoenix metropolitan area: in the cities of Phoenix (356 m above sea level [m.a.s.l.]; latitude: 33°25'N; longitude: 112°04'W), Gilbert (242 m.a.s.l.; latitude: 33°21'N; longitude: 111°44'W), and Tempe, and included the Arizona State University Tempe campus, residential housing, city parks, and riparian areas adjacent to the Salt River (357 m.a.s.l.; latitude: 33°26'N; longitude: 111°56'W). Desert localities were Robbins Butte Wildlife Area (247 m.a.s.l.; latitude: 33°19'N; longitude: 112°38'W), Powers Butte Wildlife Area (242 m.a.s.l.; latitude: 33°18'N; longitude: 112°43'W), and the confluence of the Agua Fria and Gila Rivers (278 m.a.s.l.; latitude: 33°23'N; longitude: 112°21'W). On average, these desert study sites were 9 km from the nearest urban area and 61 km from urban study sites. These desert locations border the Gila River and the vegetation is characteristic of the Sonoran Desert, including mesquite (*Prosopis* spp.), palo verde (*Parkinsonia* spp.), saltbush (*Atriplex* spp.), creosote (*Larrea tridentata*), white bursage (*Ambrosia dumosa*), and willows

(*Salix* spp.). These areas also contain dense thickets of invasive salt cedar (*Tamarix* spp.) and, in the case of the Robbins Butte and Powers Butte areas, retired agricultural lands.

2.3. Land use and land cover

To classify the land use and land cover (LULC) around my study sites, I obtained LULC data within a 1 km radius of each study site from the Central Arizona-Phoenix Long-Term Ecological Research program database (Li et al. in review). Briefly, this database uses high spatial resolution, 4 band aerial photography from the National Agricultural Imagery Program (NAIP) and classifies LULC to a resolution of 1 m using object-based image analysis (OBIA). Each meter was classified as one of the following 12 LULC types: building, road, soil, tree, grass, shrub, cropland, fallow, lake, canal, swimming pool, or seasonal river. These were then grouped into categories of 'impervious surface' (i.e., building and road), 'vegetation' (i.e., tree, grass, shrub, cropland, and fallow), and 'open water' (i.e., lake, canal, swimming pool, and seasonal river).

2.4. Bird capture and blood collection

All experimental procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and conducted under appropriate scientific collecting permits issued by the Arizona Game and Fish Department and the US Fish and Wildlife Service. I caught adult male Abert's Towhees using a mist net and playback of conspecific song and 'squeal duets,' a vocalization used by both males and females presumably to strengthen pair bonds (Tweit and Finch, 1994). The time of capture ranged from 05:45 AM to 11:30 AM (mean capture time: urban = 07:41 AM; desert = 07:46 AM PST). The study was conducted between January and May in both

2011 and 2012. I determined sex and age by behavior (singing and territorial aggression only in males; Tweit and Finch, 1994), skull pneumatization, and morphology (developed CP only in adult males, incubation patch only in adult females; Pyle, 1997). To quantify plasma T, I collected a blood sample (maximum ~300 µl) from the right jugular vein using a heparinized 0.3 cc syringe with a 29.5 gauge needle. In many vertebrate species, including multiple species of birds, the stress of capture and handling causes a rapid decrease in plasma T (Deviche et al., 2012b). Therefore, I collected samples within 3 min of capture to represent initial (i.e., pre-stress) values. Samples were placed on ice until centrifuged for 10 min at 10,000 rpm later the same day (within 8 h) and the plasma was harvested using a Hamilton glass syringe. I stored samples at -80°C until assay.

2.5. Morphometrics

Following blood collection, I measured body mass (± 0.5 g), wing chord (± 0.5 mm), and CP width (± 0.1 mm). To quantify fat stores, I visually inspected 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 and Drury, 1960). Following an injection of analgesic (Meloxicam; 0.1 mg/kg) into the left pectoral muscle, I measured left testis length and width (± 0.5 mm) via unilateral laparotomy. Briefly, the bird was restrained to a working surface and the feathers on the left flank were dampened with 70% alcohol to move them out of the surgical field. I swabbed the surgical field with betadine and then topically applied lidocaine anesthetic (Akorn, Lake Forest, IL, USA) before making a small incision between the last two ribs to expose the left testis. Testis length and width were measured by positioning the tips of forceps at each end of the testis. Volume of the testis was calculated from the formula for an ovoid sphere: $V = 4 / 3\ \pi a^2 b$, where V is volume,

a is the radius of the testis at its widest point and *b* is half the long axis. All fat scores and testis measurements were made by the same observer (SD). After the procedure, I closed the incision using cyanoacrylate adhesive (3M, St. Paul, MN, USA) and applied a topical antibiotic (Alpharma, Baltimore, MD, USA). Each bird then received a uniquely numbered aluminum US Geological Survey leg band and was released at the capture site.

2.6. Innate immunity assay

To assess immune performance, I quantified lytic and agglutination capacity using the hemoagglutination-hemolysis assay. I modified a previously described protocol (Matson et al., 2005) that quantifies the most dilute plasma concentration that can lyse and agglutinate foreign red blood cells, and has been used in Abert's Towhees (Butler et al., 2013). This assay is an ideal candidate to test habitat-related intraspecific differences in immune performance because it does not rely on a single, antigen-specific response in individuals that may have been exposed to different antigens. I serially diluted 30 µl of each plasma sample from 1:2 to 1:2048 with phosphate-buffered saline (PBS) along rows of round bottom 96-well plates. As a negative control, the final well of each row contained only PBS. I then added 10 µl of 50% heparinized whole sheep blood diluted 1:100 (HemoStat Laboratories, Dixon, CA; SBH050) to each well. After gently vortexing each plate, I covered the plates with parafilm and incubated them at 39°C for 90 min. I then tilted plates for 20 min at room temperature before scanning them using a flatbed scanner at 600 dots per inch to quantify agglutination. Following a 70-min incubation at room temperature, I rescanned each plate to quantify lysis. Plate images were independently visually scored, without knowledge of the sample identity (Matson et al., 2005). Scores obtained independently by two observers were repeatable (determined via the intraclass correlation coefficient; Lessells and Boag, 1987) for both lysis ($F_{62,62}$ = 7.26,

r^2 = 0.86, P = <0.0001) and agglutination ($F_{62,62}$ = 39.2, r^2 = 0.98, P = <0.0001). Thus, average values were used in the analysis. Lysis and agglutination were scored as the lowest plasma concentration at which RBCs were ruptured and at which a compact pellet of RBCs formed, respectively (Matson et al., 2005).

2.7. Testosterone assay

I quantified plasma T using a commercial competitive enzyme-linked immunoassay, according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY, USA). This assay has previously been validated in the Abert's Towhee (Fokidis et al., 2011). Before assay, plasma was diluted 8× with assay buffer containing steroid displacement reagent (Enzo Life Sciences; designed to eliminate interference of binding globulins with antibody binding in the assay) at a concentration equal to 1% of raw plasma volume. Samples were assayed in duplicate and randomly assigned to assay plates, with a standard curve on each plate. The average detection limit was 21.1 pg/ml. The average intra- and inter- assay coefficient of variation were 6.1% and 17.3%, respectively (n = 3 plates; 94 samples).

2.8. Statistical analysis

To test whether LULC differed between my study sites, I calculated the proportion of each LULC classification in the 1 km radius area surrounding each study site. I then arcsine transformed these values and used independent samples Student's t-tests to examine whether sites differed in the amount of impervious surface, vegetation, and open water. To test if body condition differed between the two habitats and/or changed over the course of spring, I used an ANCOVA to compare the residuals of a reduced major axis regression between body mass and wing chord. The ANCOVA model

included habitat (urban vs. desert) as a fixed factor and day of year (Day 1 = January 1st) as a covariate. I analyzed agglutination capacity using a similar ANCOVA model. Because furcular fat score was an ordinal variable and lytic capacity deviated from normality, I analyzed these data using a generalized linear model with a Poisson distribution. Testis volume, plasma T, and CP width data were log transformed prior to analysis to attain normality. To test whether urban and desert birds differ in vernal reproductive development, I used ANCOVA with testis volume, plasma T, or CP width as dependent variables, habitat as a fixed factor, and day of year as a covariate. The interaction between habitat and the covariate was non-significant for all ANCOVA tests, demonstrating homogeneity of regression slopes. I also included year (2011 vs. 2012) as a fixed factor in the full models, but found no effect of this factor in any of the tests and so removed it from the models. Linear regression analysis found no diurnal pattern in plasma T (r^2 = 0.01, P = 0.45), so time of capture was not included in the model. For all statistical analyses I used PASW version 20.0 (SPSS Inc., Chicago, Illinois, USA) with an alpha of 0.05. Data are presented as means ± standard errors of the mean (SEM) and all graphs depict untransformed data.

3. Results

3.1. Land Use and Land Cover

Compared to desert sites, urban sites had a higher proportion of impervious surface (urban: 37.4 ± 6.1 %, desert: 0.9 ± 0.8 %; t_7 = 7.96, P < 0.0001) and a lower proportion of vegetation (urban: 23.9 ± 4.8 %, desert: 35.7 ± 2.0 %; t_7 = -2.63, P = 0.034). However, the proportion of open water was similar between the two study site types (urban: 7.7 ± 3.7 %, desert: 14.1 ± 3.7 %; t_7 = -1.21, P = 0.26). This difference persisted when swimming pools were excluded from the analysis (urban: 7.6 ± 3.7 %,

desert: 14.1 ± 3.7 %; t_7 = -1.23, P = 0.26). The desert sites had a higher – although not significantly different – proportion of open water due to the presence of river beds, which were classified as seasonal river. When river beds were removed from the analysis, there was still no detectable difference in the proportion of open water (urban: 7.6 ± 3.7 %, desert: 0.1 ± 0.2 %; t_7 = -1.79, P = 0.12).

3.2. Reproductive development

Testis volume increased over the course of spring ($F_{1,76}$ = 143.52, P < 0.0001), and the slopes of the regression lines were similar between the two habitats ($F_{1,75}$ = 0.10, P = 0.32; Fig. 1), indicating that the rate of testicular development did not differ between urban and non-urban birds. However, the intercept of the regression line with the horizontal axis (sampling date) was less for urban than desert birds ($F_{1,76}$ = 69.01, P < 0.0001), indicating that urban birds began testicular development earlier than did non-urban birds. Similarly, CP width increased over the course of spring ($F_{1,76}$ = 87.84, P < 0.0001; Fig. 1) and the slopes of the regression lines were similar for birds sampled in the two habitats ($F_{1,75}$ = 2.39, P = 0.13). As for testis volumes, the intercept of the regression line was less for urban than rural birds ($F_{1,79}$ = 34.92, P < 0.0001), indicating that urban birds developed CPs earlier than did non-urban birds. Plasma T increased over the course of the spring ($F_{1,77}$ = 9.39, P = 0.003), but did not differ between urban and desert birds ($F_{1,77}$ = 0.02, P = 0.899; Fig. 1).

3.3. Further characterization of reproductive development

To test whether the relationships between testis volume, CP width, and plasma T differed between urban and non-urban birds, I used ANCOVAs with habitat as a fixed factor. However, habitat was not a significant factor in any of the models (testis volume

vs. CP width: $F_{1,76}$ = 0.002, P = 0.97; testis volume vs. plasma T: $F_{1,76}$ = 1.47, P = 0.23; plasma T vs. CP width: $F_{1,76}$ = 2.56, P = 0.11), indicating that the two groups were homogenous. I, therefore, combined data from the two habitats. Linear regression revealed that CP width was positively related to testis volume (r^2 = 0.59, P < 0.0001; Fig. 2). However, plasma T was related to neither CP width (r^2 = 0.01, P = 0.502) nor testis volume (r^2 = 0.04, P = 0.079 ; Fig. 2).

3.4. Energetic status

Furcular fat score was similar between urban and desert birds (χ^2_1 = 0.24, P = 0.63) and did not change over the course of spring (χ^2_1 = 0.76, P = 0.38; Fig. 3). Body condition was also similar between habitats ($F_{1,76}$ = 2.178, P = 0.144) and did not change over the course of spring ($F_{1,76}$ = 0.68, P = 0.795; Fig. 3).

3.5. Immune performance

Lytic capacity was similar between habitats (χ^2_1 = 1.97, P = 0.16) and did not change over the course of spring (χ^2_1 = 0.02, P = 0.89; Fig. 4). Agglutination capacity was also similar between habitats ($F_{1,56}$ = 0.598, P = 0.442), but there was a (non-significant) trend toward an increase in agglutination capacity over the course of spring ($F_{1,56}$ = 3.826, P = 0.055; Fig. 4).

4. Discussion

The objective of my study was twofold. The first objective was to compare the timing of vernal reproductive development of a bird species inhabiting urban and non-urban localities in a desert ecoregion. Urbanization of Phoenix is generally associated with higher plant density, an earlier seasonal growth of plants (Buyantuyev and Wu,

2009; Buyantuyev and Wu, 2010), and increased arthropod abundance (Cook and Faeth, 2006). I found, however, that the proportion of vegetation at my urban sites was lower than that of desert sites, which likely reflects the fact that Abert's Towhees in desert areas inhabit riparian areas. Since food availability is an important environmental cue that birds use to time reproductive development, I predicted that urban Abert's Towhees would develop their testes and CP earlier than desert conspecifics. Indeed, my results are consistent with this prediction. However, this potential disparity in food availability for Abert's Towhees may influence reproductive development via energetic constraints. This hypothesis posits that the timing of reproductive development – within the window of opportunity created by environmental cues – is limited by a bird's energetic status. Hence, I compared fat stores, body condition, and immune performance between urban and desert towhee populations. The second objective was to examine a potential mechanism by which this habitat-specific disparity in reproductive development, if present, may arise. I also predicted that advanced reproductive development will be associated with an earlier increase in endocrine activity of the HPG axis, as measured by plasma T. I found, however, that this was not the case and there was no association between the timing of reproductive development and the increase in plasma T. I also quantitatively demonstrated that, compared to the desert study sites, the urban study sites have more impervious surface and less vegetation. The two study site types did not, however, differ in the amount of open water.

4.1. Reproductive development

The pattern of seasonal reproductive development observed here (i.e., development coincident with increasing day length) is consistent with the Abert's Towhee being photoperiodic and using increasing day length as the initial predictive cue

to initiate reproductive development. This proposition is also supported by the results of captive studies in which towhees developed their testes in response to long artificial photoperiod (S. Davies, unpublished data). Urban male Abert's Towhees developed their reproductive system, as assessed by testis volume and CP width, earlier than desert conspecifics. This observation is consistent with previous studies on other urban species of birds (Deviche et al., 2010; Partecke et al., 2005; Schoech and Bowman, 2003). Assuming that the testes are functional (i.e., produce sperm) at approximately half maximum size (Gwinner, 1986; Jenkins et al., 2007; Partecke et al., 2005; Young et al., 2001), the results suggest that urban male Abert's Towhees are capable of breeding approximately 29 days earlier than their desert conspecifics. Although I did not measure lay date and further research is, therefore, necessary to demonstrate that the two populations differ in their actual onset of breeding, the present observations are consistent with the proposition that earlier gonad development plays a role in advancing the breeding phenology of urban bird populations (Chamberlain et al., 2009; Deviche and Davies, 2014; Partecke et al., 2005). Whether differences in the timing of reproductive development and/or onset of breeding in urban birds are adaptive remains debated. Indeed, the few studies that have examined reproductive success in urban versus non-urban birds suggest that it is highly variable and additional research is needed on this topic (Beck and Heinsohn, 2006a; Chamberlain et al., 2009; Newhouse et al., 2008; Richner, 1989; Rollinson and Jones, 2002b; Stracey and Robinson, 2012).

4.2. Energetic constraints

To test whether reproductive development is constrained by a bird's energetic status, I examined whether urban and desert Abert's towhee populations differed in energy stores, body condition, and immune performance. If desert towhee populations

develop their reproductive system later than their urban conspecifics due to an energetic constraint, I predicted corresponding disparities in fat stores and body condition between the two populations. However, this was not the case. Furthermore, neither fat stores nor body condition changed over the course of the spring. Life history theory predicts that when resources are limited there is a resource allocation trade-off between reproduction and self-maintenance (Stearns, 1989), in which allocation to reproduction comes at a cost to somatic processes, such as immune function (Zera and Harshman, 2001). If a disparity in energetic status plays a role in the difference in timing of reproductive development between urban and desert towhee populations, I, therefore, also predicted population differences in immune performance. Again, this was not the case. Therefore, multiple lines of evidence provide no support for the hypothesis that energetic constraints limit reproductive development of non-urban Abert's Towhees compared to their urban conspecifics. This conclusion is consistent with a study of Florida Scrub-jays (*Aphelocoma coerulescens*) in which urban females bred earlier than their non-urban conspecifics and had higher plasma protein levels, but did not differ in body condition, total body lipids, or plasma calcium (Schoech and Bowman, 2003). Similarly, urban male and female European Blackbirds (*Turdus merula*) developed their gonads earlier than non-urban conspecifics, but body mass did not differ between the populations and fat stores were actually lower in the urban blackbirds (Partecke et al., 2005). Taken together, these results provide little support for the hypothesis that the delay in reproductive development observed in non-urban birds results from these birds being energetically constrained.

4.3. Supplementary environmental cues

My findings are, however, consistent with the hypothesis that differences in information provided by environmental cues in urban versus desert areas account for the advanced timing of reproductive development in urban birds. Most birds prioritize the use of environmental cues in a hierarchical fashion, with the circannual change in day length serving as the initial predictive cue that creates a temporal window of opportunity for seasonal reproductive development. Urban and desert sites in the present study are at similar latitudes and the advancement in reproductive development of urban relative to desert towhees, therefore, is not due to differences in natural day length. Within the above temporal window, supplementary environmental cues, such as food availability and ambient temperature, fine-tune the precise timing of reproductive development. As it is unlikely that urban and desert towhees differ with respect to the nature of environmental cues used to time reproductive development, I suggest that it is the strength and/or timing of these cues that influences this development.

A wealth of studies indicate that supplementary environmental cues, such as artificial light, ambient temperature, plant growing seasons, and food supply, differ between urban and non-urban areas. Artificial light is a ubiquitous characteristic of urban areas (Gaston et al., 2013; Longcore and Rich, 2004) and may play a role in the advancement in the timing of seasonal reproduction, such as observed in the current study. For example, Blue Tits (*Cyanistes caeruleus*) in Vienna, Austria with territories exposed to street light layed, on average, 1.5 days earlier than Blue Tits in territories without street light (Kempenaers et al., 2010). This difference may result from direct stimulation of the HPG axis, whereby artificial light directly activates hypothalamic encephalic receptors and leads to a false perception of longer day lengths earlier in the season. However, as yet, there is limited evidence for such a direct effect and findings

from studies in controlled laboratory conditions are contradictory (Deviche and Davies, 2014; Spoelstra and Visser, 2014). A study on male European Blackbirds, in which captive blackbirds were exposed to light intensity similar to those experienced by urban blackbirds at night, found that artificial light at night advanced the timing of gonad development and rise in plasma T (Dominoni et al., 2013). By contrast, a similar study in Western Scrub-jays (*Aphelocoma californica*) did not detect an effect on plasma T in males and found that artificial light at night depressed plasma LH in males and E_2 in females (Schoech et al., 2013). Furthermore, the spectral sensitivity of avian hypothalamic encephalic receptors suggests that direct activation of the HPG axis by urban light is unlikely (Deviche and Davies, 2014). However, further studies are necessary to elucidate whether artificial lights in urban areas directly stimulate the HPG axis and cause the earlier reproductive development observed here or, instead, whether artificial lights exert indirect effects on reproductive development (e.g., by changing behavior; Deviche and Davies, 2014; Spoelstra and Visser, 2014).

The process of urbanization, particularly the replacement of vegetation and evaporative soil surfaces with impervious, low-albedo paving and buildings, causes urban areas, including Phoenix (Baker et al., 2002; Brazel et al., 2007; Buyantuyev and Wu, 2010), to be warmer than non-urban areas, creating 'urban heat islands' (Imhoff et al., 2010; Peng et al., 2011; Zhang et al., 2010). The seasonal growth of many plants and the emergence of many arthropods are temperature-dependent (van Asch and Visser, 2007). Accordingly, evidence suggests that the urban heat island effect advances the timing of the vernal increase in plant growth in urban areas (Buyantuyev and Wu, 2009; Buyantuyev and Wu, 2012; Imhoff et al., 2004). Compelling evidence covering long periods and large geographical areas, demonstrates that warmer spring temperatures are associated with earlier seasonal breeding in birds (Charmantier et al., 2008; Crick et al.,

1997; McCleery and Perrins, 1998; Visser et al., 2006; Williams, 2012). Studies in controlled laboratory settings also support the hypothesis that ambient temperature plays a role in the timing of reproductive development (Schaper et al., 2012b; Visser et al., 2009). It is conceivable, therefore, that the elevated ambient temperatures in urban areas advance reproductive development of urban birds. However, the average increase in urban ambient temperature is relatively small (i.e., just a few degrees; Imhoff et al., 2010; Zhang et al., 2010) and I are not aware of any evidence that these small increases in temperature by themselves suffice to cause differences in the phenology of reproductive development of birds (Deviche and Davies, 2014). I are also not aware of studies with sufficient temporal resolution to test whether urban areas are associated with advances in the phenology of arthropod emergence, but long-term studies of caterpillar phenology demonstrate that the peak in caterpillar abundance is highly synchronized with tree phenology (van Asch and Visser, 2007; Visser et al., 2006). If the timing of arthropod emergence parallels the advanced plant growing season in urban areas, I predict that arthropod abundance will increase earlier in the year in urban areas compared to non-urban areas. A wealth of research demonstrates that bird populations synchronize breeding with the peak in food availability (Cresswell and Mccleery, 2003; Visser et al., 2006), and birds may use the timing of plants and/or arthropods as environmental cues to optimally time reproduction to local conditions. I, therefore, suggest that the advances in reproductive development of urban male Abert's Towhees are associated with advances in the timing of plant growth, which itself may cause early arthropod emergence. Human-provided food, such as from feeders and discarded food waste, available in urban areas may constitute an additional environmental cue, giving birds the perception that local food availability peaks earlier in urban areas compared to non-urban areas (Amrhein, 2014; Deviche and Davies, 2014; Robb et al., 2008a).

4.4. Physiological mechanisms

To shed light on the physiological mechanism responsible for modulating reproductive development to urban areas, I tested the hypothesis that the advanced reproductive development of male Abert's Towhees in Phoenix is associated with an earlier increase in endocrine activity of the HPG axis. Testosterone is essential for male reproductive function and spermatogenesis (Brown and Follett, 1977; Deviche et al., 2010; Dornas et al., 2008; Penfold et al., 2001). Furthermore, in seasonally breeding birds, plasma T either parallels plasma LH (Deviche et al., 2010; Deviche et al., 2006; Fowler et al., 1994; Groscolas et al., 1986; Mauget et al., 1994) or follows the seasonal peak in plasma LH (Degen et al., 1994; Deviche and Sharp, 2001; Röhss and Silverin, 1983). Therefore, plasma T is a good candidate to assess endocrine activity of the HPG axis and compare the physiological control of reproductive development between the two towhee populations. Despite the substantial difference in the timing of reproductive development between urban and desert male Abert's Towhees, plasma T was similar between the populations and was not correlated with testis volume. This finding is consistent with those in other studies comparing plasma concentrations of reproductive hormones in urban and non-urban bird populations that differ in reproductive development and/or laying dates. For example, despite developing testes 20 days earlier than non-urban European Blackbirds, urban male blackbirds had lower plasma LH and T (Partecke et al., 2005). Similarly, urban female European Blackbirds, which developed follicles 28 days earlier than non-urban blackbirds, and Florida Scrub-jays, which initiated first clutches 20 days earlier than non-urban jays, had similar plasma LH and E_2 to their corresponding non-urban populations (Partecke et al., 2005; Schoech and Bowman, 2003). Thus, the lack of association between reproductive development (i.e., testis volume and CP width) and endocrine activity of urban and non-urban bird

populations, such as I report here, is apparently widespread. Although few studies simultaneously examine reproductive development, endocrine activity, and lay date, the available evidence suggests that a lack of association between these parameters is also widespread. For example, variation in the laying date of the first egg of male-female pairs of Great Tits (*Parus major*) caused by experimental ambient temperature treatments in controlled conditions, was not correlated with plasma LH level and, at best, was only weakly correlated with gonad size (Schaper et al., 2012a). Similarly, despite a habitat-related difference in lay date of one month between two wild Blue Tit populations, seasonal profiles of plasma LH and T and testes development differed by less than two weeks (Caro et al., 2006). The apparent lack of association between plasma LH or T and testis development may be accounted for by differences in the effects of photoperiod on plasma LH versus plasma FSH. In the White-crowned Sparrow (*Zonotrichia leucophrys*) and the Great Tit, plasma LH and, in turn, plasma T peak substantially before the testes reach their maximum development, and levels of these hormones fall at the time of breeding (Silverin, 1984; Wingfield and Farner, 1978). In the Great Tit, plasma FSH, on the other hand, increases later than plasma LH and reaches it peaks around the time of breeding (Silverin et al., 1997). The available evidence, therefore, suggests that, although plasma levels of reproductive hormones are broadly indicative of the timing of breeding in birds, these levels do not reflect fine-scale (i.e., less than a month) differences in breeding between populations. Future research on avian reproductive ecology – including populations in urban areas – may benefit from measuring not only plasma levels of reproductive hormones, but also hormone receptor densities in target tissues and factors downstream of hormone binding. In support of this, studies on the endocrine control of behavior in birds have found that steroid receptor density often better predicts

the occurrence of steroid-dependent behaviors than do plasma hormone levels (Ball and Balthazart, 2008; Horton et al., 2014; Ketterson et al., 2009; Rosvall et al., 2012).

4.5. Conclusions

A burgeoning body of research demonstrates that birds adjust to urban areas by breeding earlier than their non-urban conspecifics. However, the underlying cause of this difference is unclear. I found that urban male Abert's Towhees developed their reproductive system earlier than do their desert conspecifics. These results add to the mounting evidence indicating that urban birds develop their reproductive system earlier than do their nearby non-urban conspecifics. My results suggest that urban and non-urban towhees are in similar energetic status and that the earlier reproductive development of urban birds is potentially due to differences in the timing and/or strength of information provided by environmental cues in urban versus desert areas. I, therefore, encourage future research to focus on potential environmental cues that differ between urban and non-urban areas. Prominent candidates include the timing of seasonal increase of plants and food supply. The physiological mechanism responsible for this difference in timing of reproductive development remains unclear, but my results suggest that future studies may find it illuminating to compare not only the plasma levels of reproductive hormones, but also hormone receptor densities in target tissues and factors downstream of hormone binding in urban and non-urban birds.

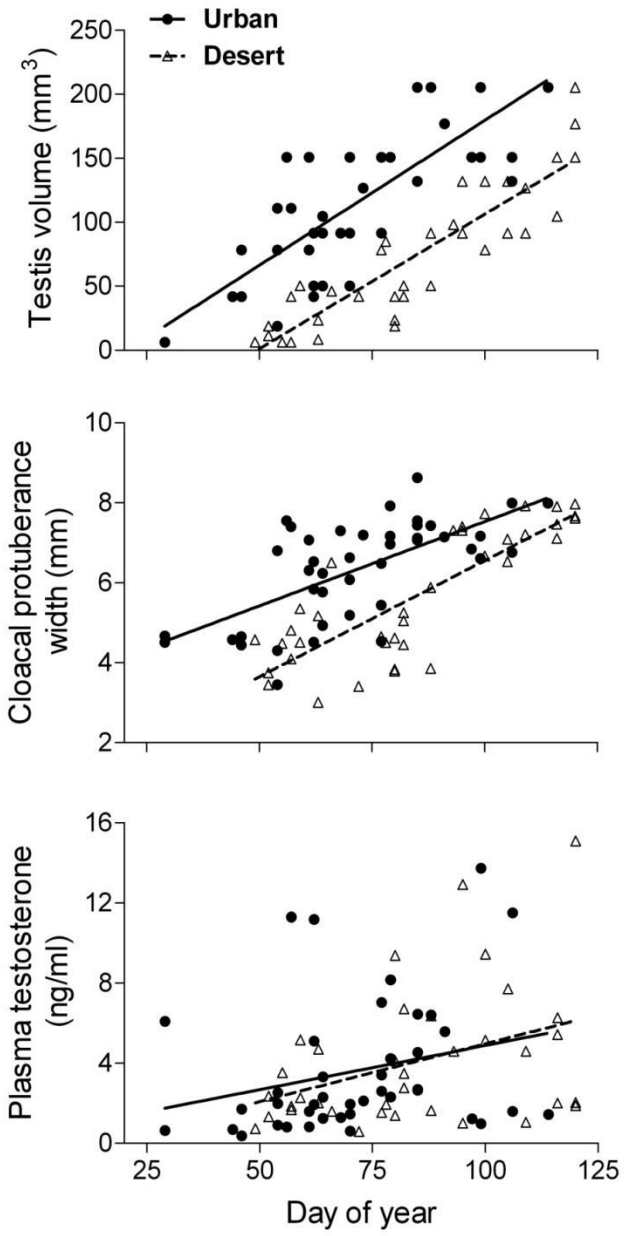

Fig. 1. The vernal development of testis volume and cloacal protuberance width is advanced in free-ranging Abert's Towhees (*Melozone aberti*) inhabiting urban (filled circles) localities in Phoenix, AZ, USA compared to their non-urban, desert conspecifics (open triangles). Plasma testosterone concentration, however, was similar in towhees from these two populations. Each point represents one individual. On the horizontal axes, 1 = January 1st.

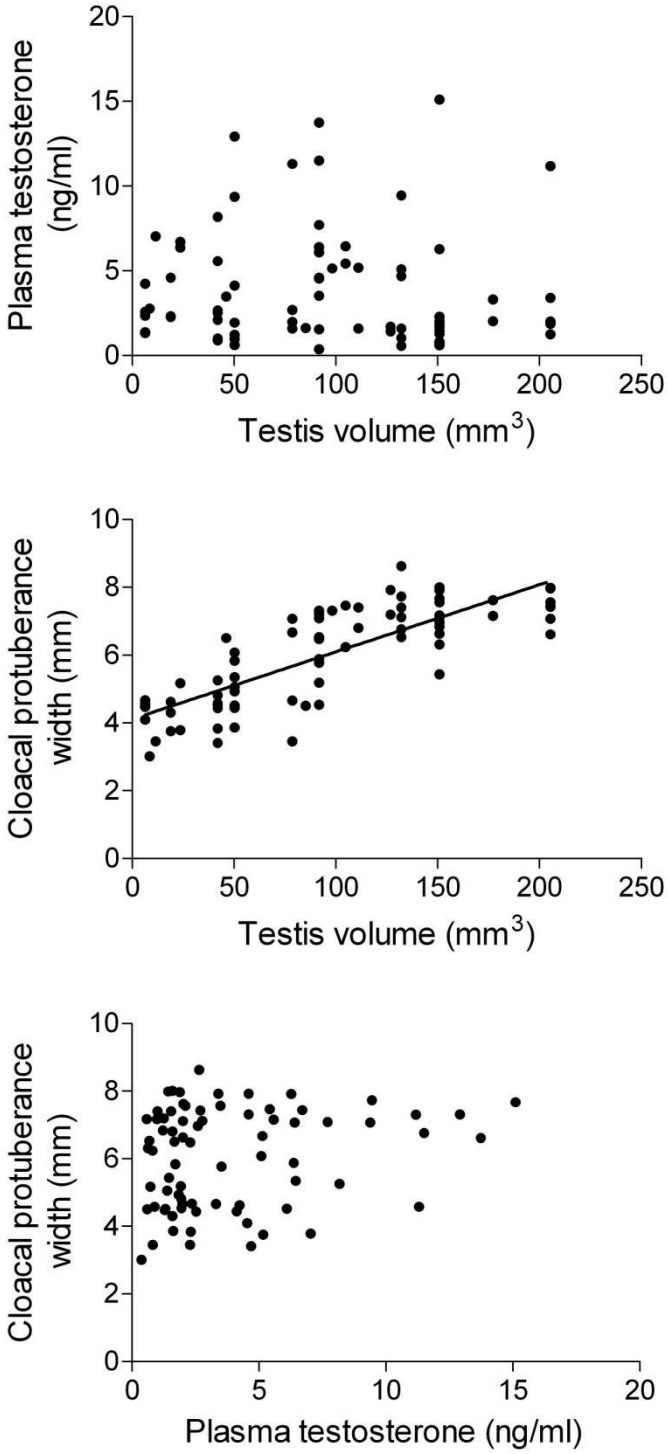

Fig. 2. Relationships between testis volume, cloacal protuberance width, and plasma testosterone in free-ranging Abert's Towhees (*Melozone aberti*). Testis volume was not related to plasma testosterone, but was related to cloacal protuberance width. There was also no relationship between plasma testosterone and cloacal protuberance width. Each point represents one individual. For the day of year axis, 1 = January 1st.

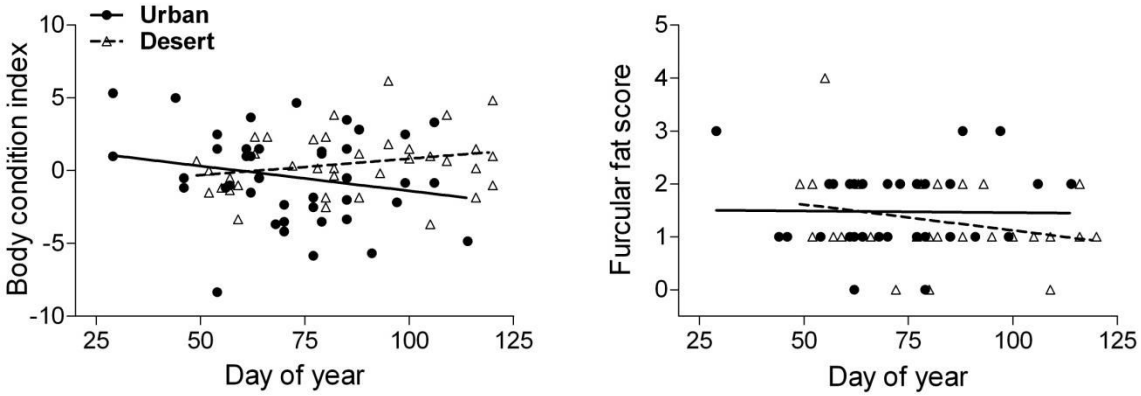

Fig. 3. Body condition and furcular fat stores are similar in free-ranging Abert's Towhees (*Melozone aberti*) inhabiting urban (filled circles) and non-urban, desert (open triangles) localities in and around Phoenix, AZ, USA. Each point represents one individual. On the horizontal axis, 1 = January 1st. Note that the trend lines are not significant.

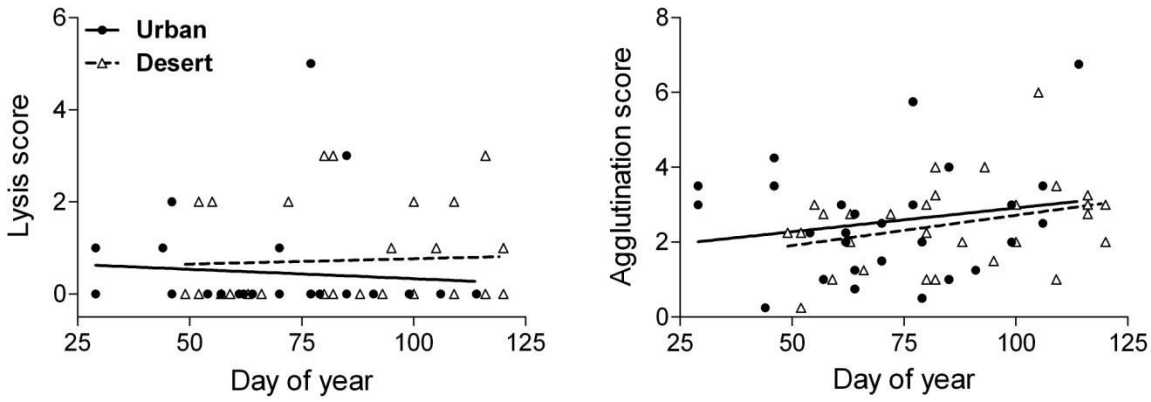

Fig. 4. Immune performance, as measured by lysis and agglutination capacities, is similar in free-ranging Abert's Towhees (*Melozone aberti*) inhabiting urban (filled circles) and non-urban, desert (open triangles) localities in and around Phoenix, AZ, USA. Each point represents one individual. On the horizontal axis, 1 = January 1st. Note that the trend lines are not significant.

CHAPTER 3

THE ECOLOGICAL AND PHYSIOLOGICAL CAUSES OF VARIATION IN THE PHENOLOGY OF GONAD GROWTH IN AN URBAN AND DESERT SONGBIRD

For many animals, the seasonal timing (phenology) of gonad cycles is an important adaptation to local environmental conditions. The available evidence suggests that birds adjust to urban areas by advancing the phenology of vernal gonad growth. Comparisons of urban birds against their corresponding non-urban conspecifics, therefore, provide 'natural experiments' that may shed light on the causes of variation in gonad growth phenology. To test whether the habitat-related disparity in gonad growth phenology is due to greater food abundance in urban areas or, alternatively, a habitat-related difference in the phenology of key food types, I compared ground arthropod biomass in urban and desert areas, the phenology of new plant growth (the phenology of leaf foliage progression), and the energetic status (i.e., body condition and fat stores) and hypothalamic levels of neuropeptide Y (NPY, a neuropeptide that potentially links food abundance to reproductive endocrine activity) of urban and desert Abert's Towhees, *Melozone aberti*. To shed light on the physiological mechanism underlying variation in gonad growth phenology, I compared the (neuro)endocrine activity of the reproductive system at the hypothalamic (gonadotropin -releasing hormone [GnRH] and -inhibitory hormone [GnIH]) and gonadal (baseline plasma testosterone; T) levels, as well as the morphology dependent on these processes (paired testes mass, seminiferous tubule diameter, and cloacal protuberance width). I also compared the phenology of gonad growth in three years that differ in winter precipitation levels. There was no habitat-associated difference in ground arthropod biomass, but, compared to desert areas, the phenology of leaf foliage progression was advanced in urban areas and exhibited a lack of seasonal change. In three years that differed in the habitat-related disparity in gonad

growth and winter precipitation levels, energetic status did not differ between the two populations at any time. This finding provides no support for the hypothesis that greater food abundance in urban areas drives the disparity in gonad growth phenology between urban and desert Abert's Towhees. My results are consistent, however, with the hypothesis that differences in the predictability and magnitude of change in the availability of key food sources between urban and desert areas contributes to the observed habitat-related disparity in inter-annual variability in gonad growth of Abert's Towhees. I found no difference in any measure of baseline reproductive endocrine activity, suggesting that this does not determine habitat-related variability in gonad growth phenology. I did find, however, that desert – but not urban – towhees had a marked plasma T response to GnRH challenge, which suggests that responsiveness of the anterior pituitary gland and/or gonads contributes to the difference in gonad growth phenology between urban and desert towhees.

1. Introduction

Phenotypic plasticity, the ability of an organism to change its phenotype in response to environmental changes, is a crucial adaptation to seasonal environments (Baker, 1938; Charmantier et al., 2008; Lourdais et al., 2002; Miner et al., 2005; Wingfield and Kenagy, 1986). A major seasonal phenotypic change for many animals is the cycle of gonad growth (Bubenik et al., 1997; Callard et al., 1978; Dawson, 1983; Itoh et al., 1990; Palmer et al., 1988). Cycles of gonad growth are broadly governed by the seasonal timing (phenology) of the rise in reproductive hormone secretion (Deviche et al., 2010; Williams, 2012; Wingfield and Kenagy, 1986), and it is often assumed that inter-individual and interannual variation in the phenology of hormone secretion

underlies inter-individual and interannual variation in the timing of gonad growth. However, large intra-individual differences in plasma levels of reproductive hormones at a given time mean that they rarely reflect fine-scale (i.e., less than a month) differences in the phenology of gonad cycles (Caro et al., 2006; Schaper et al., 2012a; Schaper et al., 2012b; Williams, 2008). The underlying cause of inter-individual and interannual variation in the phenology of gonad growth, therefore, remains unclear.

In many vertebrates, including most birds, gonad growth results from an endocrine cascade initiated by neuroendocrine responses to environmental cues (Dawson et al., 2001; Dawson, 2008; Williams, 2012; Wingfield, 1980; Wingfield, 2008). Endocrine activity of the hypothalamo-pituitary-gonadal (HPG) axis is stimulated by a photoinduced increase in secretion of gonadotropin-releasing hormone (GnRH) (Dawson and Goldsmith, 1997; King and Millar, 1982; Sharp, 2005; Sharp and Ciccone, 2005). This hormone stimulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland, which, in turn, stimulate gonad growth and gametogenesis (Kuenzel, 2000). In males, the testes then secrete testosterone (T), which is essential for spermatogenesis (Brown and Follett, 1977; Dornas et al., 2008; Penfold et al., 2001) and promotes male reproductive behaviors, such as courtship, territorial aggression, and mate guarding (Foerster et al., 2002; Hau, 2007; Kurvers et al., 2008). Gonad growth is also modulated by a hypothalamic neuropeptide, gonadotropin-inhibitory hormone (GnIH; Tsutsui et al., 2000), which opposes the actions of GnRH. Specifically, GnIH inhibits gonad growth by acting on hypothalamic GnRH cells, anterior pituitary gland gonadotropes, and gonads (Clarke, 2011; Clarke and Parkington, 2013; Davies and Deviche, 2014; Tsutsui et al., 2013). Within the window of reproductive development set by photoperiod, these processes are fine-tuned to local environmental conditions by supplementary cues, such as ambient

temperature (Caro et al., 2013; Schaper et al., 2012b; Wingfield et al., 2003), the abundance of food (Davies and Deviche, 2014; Hahn et al., 2005; but see Perfito et al., 2008), and/or the timing of the seasonal availability of particular food types (Grieco et al., 2002; Hau et al., 2000; Visser et al., 2006; Visser et al., 2012; Watts and Hahn, 2012).

Urbanization is associated with complex modifications of environmental conditions: some environmental factors increase, some are unchanged, and others undergo changes in their seasonality. Urbanization does not affect natural photoperiod. However, urban areas are characterized by increases in the proportion of impervious surface (i.e., buildings, roads, etc.) and the density of humans (Heil et al., 2007). Associated with these changes, compared to corresponding non-urban areas, urban areas have higher levels of artificial light (Gaston et al., 2013; Longcore and Rich, 2004), noise (Halfwerk and Slabbekoorn, 2013; Nemeth and Brumm, 2010), and the number of novel plant and animal species (McKinney, 2008; Van Heezik et al., 2010). Urbanization also modifies the seasonality of a suite of environmental variables, such as primary productivity (Buyantuyev and Wu, 2009; Imhoff et al., 2004), food abundance (Cook and Faeth, 2006; Cowie and Hinsley, 1988; Faeth et al., 2005; Robb et al., 2008a), and ambient temperature (Imhoff et al., 2010; Zhang et al., 2010). For urban animal populations to persist, they must respond and adjust to these modified environmental conditions.

Birds are one of the best studied taxa in urban ecology and the evidence to date suggests that one of the adjustments that birds make to persist in urban areas is an advancement in the phenology of seasonal gonad growth, relative to their non-urban conspecifics (Deviche et al., 2010; Deviche and Davies, 2014; Partecke et al., 2005). Accordingly, my multi-year study of male Abert's Towhees, *Melozone aberti*, found that

these birds advance the phenology of their vernal gonad growth in urban areas of Phoenix, Arizona compared to desert towhees (S. Davies, unpublished). However, this advancement was not mirrored by an advancement in the phenology of the vernal increase in plasma levels of T (Chapter 2). The physiological mechanism underlying the earlier gonad growth in urban birds, therefore, remains unclear. Hence, the first aim of this study was to compare activity of the reproductive system in adult male Abert's Towhees from urban and desert locations. As my previous study found no difference in gonadal endocrine function, I examined whether the timing of gonad growth of male Abert's Towhees reflects a divergence in endocrine activity upstream of the gonads. To this end, I measured hypothalamic levels of neuropeptides that ultimately modulate gonad growth (i.e., GnRH and GnIH). Furthermore, I also investigated whether the endocrine responsiveness of the anterior pituitary gland and/or the gonads is related to the phenology of vernal gonad growth. For this, birds were exposed to a GnRH challenge, which involved measuring the increase in plasma T in response to an injection of GnRH (Deviche et al., 2012a; Jawor et al., 2006). This technique has the potential to be insightful in terms of the physiological control of seasonal gonad growth because it minimizes intra-individual variation in hormone levels (McGlothlin et al., 2010), is individually repeatable (Jawor et al., 2007), and the birds' responsiveness to GnRH challenge varies as a function of their breeding stage (Jawor et al., 2006).

The ecological driver of the advanced gonad growth phenology of urban birds is likewise unclear. Urbanization appears to modify many supplementary environmental cues that birds use to time gonad growth. A particularly compelling candidate environmental factor that is both modified by urbanization and plays an important role in the phenology of gonad growth in birds is food availability (reviewed by Deviche and Davies, 2014). Food availability potentially influences the phenology of gonad growth via

two distinct pathways (Davies and Deviche, 2014; Deviche and Davies, 2014; Hahn et al., 2005). In the first, the overall abundance of food modulates the energetic status of birds. In turn, birds in good energetic condition can begin gonad growth shortly after stimulation by sufficiently long photoperiod, whereas birds in poor energetic condition delay gonad growth until they have acquired sufficient energy stores. In Phoenix, urbanization is associated with increased arthropod abundance (Cook and Faeth, 2006), which may increase food abundance for towhees. Following this, it is predicted that urban birds will be in better energetic status and can begin gonad development as early as photoperiod allows. The second hypothesis by which food availability potentially influences the phenology of gonad growth posits that the phenology of seasonally available key food types, such as growing green vegetation and arthropods, acts as an environmental cue forecasting the occurrence of optimal conditions (Davies and Deviche, 2014; Deviche and Davies, 2014; Hahn et al., 2005). Crucially, this pathway is hypothesized to operate via perceptual pathways independent of energetics (Hau et al., 2000). The growing season of plants in urban areas of Phoenix begins earlier than that of desert areas (Buyantuyev and Wu, 2010), which likely advances the availability of growing green vegetation and potentially also advances the phenology of arthropods that are synchronized with plant growth. The second aim of this study was to shed light on the ecological driver of the timing of gonad growth in urban and desert birds. Specifically, I aimed to test whether food abundance and the phenology of plant growth differ between urban and desert areas. To that end, I compared the phenology of leaf foliage progression and ground arthropod dry biomass in urban and desert locations.

Information on food availability may be integrated into gonad development via GnIH and its links with hypothalamic cells that produce neuropeptide Y (NPY). GnIH-secreting cells receive projections from NPY-secreting cells, which integrate information

on food availability via both hormones and metabolites (Marty et al., 2007). The GnIH-NPY system has the potential to regulate gonad growth in response to food availability and may contribute to the modulation of gonad growth by local food abundance (Davies and Deviche, 2014). Therefore, I also compared hypothalamic levels of NPY in urban and non-urban birds.

2. Methods

2.1. Study species and sites

The Abert's Towhee, *Melozone aberti*, is an appropriate species for this study because it is common in non-urban areas of the Sonoran Desert and also throughout the Phoenix Metropolitan area (Arizona, USA), particularly in urban yards (Rosenberg et al., 1991). This species consumes a variety of food types, including arthropods and seeds (Rosenberg et al., 1991). Furthermore, Abert's Towhees are sedentary, form life-long pair bonds, and hold a permanent territory (1.5 - 2 ha; Rosenberg et al., 1991). The study was conducted at three desert and four urban sites (Davies et al., 2013). Desert localities were Robbins Butte Wildlife Area (247 m above sea level [m.a.s.l.]; latitude: 33°19'N; longitude: 112°38'W), Powers Butte Wildlife Area (242 m.a.s.l.; latitude: 33°18'N; longitude: 112°43'W), and the confluence of the Agua Fria and Gila Rivers (278 m.a.s.l.; latitude: 33°23'N; longitude: 112°21'W). These desert locations all have low human population densities, but differ in proximity to urban development. Robbins Butte and Powers Butte Wildlife Areas are 5 – 10 km and 14 km, respectively, southwest of fringe suburban developments of the city of Buckeye. The site at the confluence of the Agua Fria and Gila Rivers is 3.5 km south of fringe suburban developments of the city of Avondale. All desert sites border the Gila River and vegetation is characteristic of upland

Sonoran Desert, including mesquite (*Prosopis* spp.), palo verde (*Parkinsonia* spp.), saltbush (*Atriplex* spp.), creosote (*Larrea tridentata*), white bursage (*Ambrosia dumosa*), and willows (*Salix* spp.). These areas also contain thickets of invasive salt cedar (*Tamarix* spp.) and, in the case of Robbins Butte Wildlife Area, retired agricultural lands. Urban localities were distributed throughout the Phoenix metropolitan area: in the cities of Phoenix (356 m.a.s.l.; latitude: 33°25'N; longitude: 112°04'W) and Tempe (357 m.a.s.l.; latitude: 33°26'N; longitude: 111°56'W), and included residential housing, city parks, and riparian areas adjacent to the Salt River. All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and conducted under appropriate scientific collecting permits issued by the Arizona Game and Fish Department and the US Fish and Wildlife Service.

2.2. Bird capture, blood collection, and morphometrics

Between March 14th and April 5th 2013, I caught adult male Abert's towhees using a mist net and song playback. Playback consisted of conspecific song and 'squeal duets', a vocalization used by males and females presumably to strengthen pair bonds (Tweit and Finch, 1994). I determined sex using behavior (singing and territorial aggression in males only), and the presence of a developed cloacal protuberance (CP, an androgen-sensitive secondary sexual characteristic). All birds had fully pneumatized skulls, indicating that they were adults. Within 3 minutes of capture, I bled all birds (i.e., birds from both studies, see below) from the right jugular vein using a heparinized syringe with a 29-gauge needle. Blood samples (~100 μl) were immediately placed on ice until centrifuged later the same day (within 6 h) and the plasma harvested using a Hamilton glass syringe. Plasma samples were then frozen at -80°C until they were assayed. I also measured body mass (± 0.5 g), wing chord (± 0.5 mm), CP width (± 0.1 mm, using

digital calipers), and fat stores of birds in both studies. Fat stores were quantified by assigning a score of 0 – 5 (0 representing no fat, 5 representing bulging fat deposits; Helms & Drury 1960) to the amount of fat visible in the furcular region.

2.3. Plant and arthropod phenology

To examine whether plant phenology differs between the two habitats, I took measurements of leaf foliage progression from three desert and four urban localities every three weeks. To compare the two habitats, I scored leaf foliage progression of three tree species that were common to both habitats: mesquite (*Prosopis* spp.), palo verde (*Parkinsonia* spp.), and salt cedar (*Tamarix* spp.). Measurements were taken from one 100 meter transect at each locality and I scored all trees within 10 m either side of the transect. Leaf progression score represented a qualitative six-point scale of leaf foliage progression (1 = no leaves or leaf buds, 2 = buds closed, 3 = buds splitting slightly, 4 = buds split and leaves visible, 5 = leaves open [on distal end of branches], 6 = leaves open over majority of the tree; adapted from (Perfito et al., 2004). I used these data to calculate median scores for each site then for each habitat.

The Abert's Towhee forages primarily on the ground (e.g., over 70% of foraging observations by Rosenberg et al. (1991) were on the ground). Furthermore, although this species consumes a variety of food types, arthropods dominate the diet in all seasons (Rosenberg et al., 1991). Therefore, to test whether food availability for Abert's Towhees differs between the two habitats, I quantified ground arthropod biomass using pitfall trap as in Cook and Faeth (2006). Pitfall traps consisted of two empty plastic cups (volume: 500 ml; height: 12 cm; diameter at opening: 9.5 cm), one inside the other and buried in the ground with the top of the cups slightly (~5 mm) below the surface of the soil. I placed 10 traps in each site (spaced 10 meters apart), along the same 100 meter

transects as used for the tree phenology measurements. Traps were opened on the same day that tree phenology was scored and were left open for 72 hours. After 72 hours, I collected the contents of each trap by emptying its content into individual resealable plastic bags. Traps were then cleaned of all debris and blocked using caps between sampling periods. Bags containing trap contents were frozen later the same day at -20°C until analysis. To quantify arthropod biomass in each trap, I sorted all arthropods, eliminated debris (e.g., sand and soil), and transferred the arthropods from a given trap into a plastic cup. Cup contents were dried in individual containers in an oven at 60°C for 48 hours and until mass was constant over 12 hours. The entire arthropod contents of each trap was then weighed to the nearest milligram, and an average dry arthropod biomass was calculated for each site at each sampling period.

2.4. Study 1 – The central control of reproduction and gonad development

The objective of study 1 was to test whether urban and desert Abert's Towhees (*n* = 8 per habitat) differ in the amount of brain GnRH, GnIH, and NPY. Within 5 min of capture, each bird was deeply anesthetized by intramuscular injection of a ketamine and xylazine cocktail. Birds were then transcardially perfused with 35 ml of wash solution (0.9% NaCl and 0.1% $NaNO_2$ in 0.1 M phosphate buffer, PB), followed by 35 ml of fixative (4% paraformaldehyde and 0.1% $NaNO_2$ in 0.1 M PB). Birds were then decapitated, the skulls opened to expose the brain, and the heads stored in fixative on ice until they were refrigerated later the same day and stored overnight at 4°C. Testes were also collected and stored in fixative on ice. Later the same day, I removed all extra connective tissue from the testes and then weighed them to the nearest 0.1 mg. The day after perfusion, brains were dissected out of the skull and post-fixed overnight (in 4% paraformaldehyde and 0.1% $NaNO_2$ in 0.1 M phosphate buffer [PB]). Brains and testes

were gelatin-embedded and cryoprotected according to a modification of a previously published protocol (Saldanha et al., 1994) and stored at −80°C until sectioned.

Using the stereotaxic atlas of the canary brain (Stokes et al., 1974) as a reference, I coronally sectioned brains (30 μm thick sections) using a cryostat at −20°C. Sections were divided into four parallel series by systematically alternating between four wells containing cryoprotectant solution (Watson et al., 1986). One series was used for each assay (GnRH-I, GnIH, and NPY) and the fourth series was kept as a backup. Sections were stored in cryoprotectant at −20 ° C until immunolabeled.

2.5. Immunocytochemistry

I labeled brain sections for GnRH, GnIH, and NPY immunoreactivity in three assays per peptide. Each assay included sections from 4 – 6 randomly selected birds. In the case of GnRH and GnIH, the protocols have previously been validated in the Deviche laboratory (Small et al., 2008a). The specificity of the NPY antibody has been established in a variety of avian species (Kuenzel and McMurtry, 1988; Strader and Buntin, 2001). Preabsorption of the NPY antibody with human/rat NPY (Bachem, Torrance, CA, USA, at a concentration of 1 μM, Strader and Buntin, 2001) before application to towhee brain sections abolished staining. The distribution of these three peptides in the avian brain has been described in previous studies. GnRH-I is synthesized in the preoptic area (POA) (Dawson and Goldsmith, 1997; Parry et al., 1997) and the lateral hypothalamus (LHy) (Silver et al., 1992). GnIH is synthesized solely in the paraventricular nucleus (PVN) (Osugi et al., 2004; Tsutsui et al., 2010). NPY-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) (Boswell, 2001; Boswell et al., 2002; Kuenzel and McMurtry, 1988). I used the tractus septomesencephalicus (TrSM) as an anatomical

landmark to identify the POA, approximately corresponding to level A2.8 of the canary, *Serinus canaria,* atlas (Stokes et al. 1974). Following two 30 min washes in 0.1 M PB, I serially exposed sections to 0.36% H_2O_2, three 5 min washes in 0.1 M PB, blocked background immunoreactivity for 1 h (see below), and incubated overnight in primary antibody (see below). I 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 (see below), 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, I allowed immunolabeled 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, Pittsburg, PA, USA).

For GnRH, I used anti-cGnRH-I antiserum (6DL31/4 prepared by P.J. Sharp) at a dilution of 1: 10,000 in 0.3% PBT. To block non-specific sites, I used normal rabbit serum (Vector Laboratories, Inc.; 1: 200 in 0.3% PBT), and I used biotinylated rabbit anti-sheep IgG (Vector Laboratories, Inc.; 1: 200 in 0.3% PBT) as a secondary antibody. In the case of GnIH, I used anti-quail GnIH antiserum (Tsutsui et al., 2000) at a dilution of 1: 5,000 in 0.3% PBT. I 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. For NPY, I used anti-human/rat NPY antiserum (Bachem, Torrance, CA, USA) at a dilution of 1:10,000 in 0.3% PBT. To block non-specific sites, I used normal goat serum (Vector Laboratories; 1: 30 in 0.3% PBT),

and biotinylated rabbit IgG (Vector Laboratories; 1: 100 in 0.3% PBT) as a secondary antibody.

2.6. Immunocytochemistry data collection

All immunocytochemistry data were collected without knowledge of a bird's capture location. For each bird, I counted the number of cells immunoreactive for GnRH-I, GnIH, and NPY present in each immunostained section. I analyzed sections across the whole region where each peptide is produced (i.e., POA, LHy, IN, and PVN; an average of 15 sections per bird for GnRH and GnIH, and 9 sections for NPY). I then multiplied the number of immunoreactive cells by four to estimate the total number of cells for each bird. I 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 fibers in the IN, perikaryon area and optical density could not be accurately quantified for this NPY-ir perikarya. Light intensity, aperture diameter, and camera shutter speed were standardized for all image captures. I 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 analyzed). Digitized images were analyzed 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). 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, GnIH-ir, and NPY-ir fibers in the median eminence (ME), I took images from two sections per brain. I corrected for background staining of each image as described above. On the resulting image, I used Image-pro Plus to measure the optical density of five areas of interest (AOI, 65 × 65 μm each) per brain section. AOIs were evenly spaced from left to right along the ventral edge of the ME. I calculated an average optical density for each section, and then an average for each bird.

2.7. Testicular morphology

I sectioned testes at a thickness of 30 μm using a cryostat at – 21° C and stored sections in 0.1 M phosphate buffer until mounting on glass microscope slides later the same day. After allowing sections to dry at room temperature for 24 h, I rehydrated them through a graded ethanol series before staining with hematoxylin (S212A, Poly Scientific, Bay Shore, NY, USA) for 3 min. I 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, I stained sections with eosin (S176, Poly Scientific, Bay Shore, NY, USA) for 30 s, dehydrated them through a graded ethanol series, cleared them in xylene, and coverslipped slides 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 et al., 2007). I randomly selected eight sections from each bird (four from each testis) and, using Image Pro, measured the shortest diameter of 10 seminiferous tubules per section that were randomly selected using a grid overlaid on the image. These measurements were used to calculate an average seminiferous tubule diameter for each bird.

2.8. Habitat and inter-annual variation in gonad development and precipitation

To investigate the inter-annual variation in gonad growth between urban and desert birds, I compared cloacal protuberance width (a proxy for paired testis volume; see results) of birds in the current study (2013) with birds sampled in a previous study (2011 and 2012; S. Davies, unpublished data) during same period (i.e., March 14th to April 5th). To better understand the ecological driver of inter-annual variation in gonad growth, I also compared precipitation at the study sites. Desert plant phenology, as measured by satellite imagery, is dependent on cumulative precipitation during the preceding 3 months (Buyantuyev and Wu, 2012). Therefore, I calculated the cumulative precipitation during January to March at three desert and five urban weather stations closest to my study sites. Precipitation data are available online from the Flood Control District of Maricopa County, AZ (http://www.fcd.maricopa.gov/).

2.9. Study 2 – GnRH challenge

The objective of study 2 was to determine whether sensitivity of the anterior pituitary gland and/or the gonads to a GnRH challenge differs between urban and desert Abert's Towhees. Within 2 minutes of the initial bleed described above, I randomly assigned birds (n = 8 per treatment per habitat; i.e., total sample size = 32) to receive an intravenous injection via the jugular vein of either synthetic chicken GnRH-I (Sigma Chemical Co., MO, USA; at a dose equal to ≃25 μg/kg) in 0.1 ml saline solution (0.9% NaCl) or 0.1 ml saline (control). I collected a second blood sample (~100 μl; as described above) from each bird 20 min after the injection to determine post-injection plasma T. Between the injection and the post-injection bleeds, birds were held in individual cloth bags. I selected the GnRH dose and the time between injection and post-injection blood samples based on their effectiveness to stimulated LH secretion in Cassin's Sparrows,

Peucaea cassinii (Deviche et al. 2012). All birds received a uniquely numbered aluminum tarsal band from the U.S. Geological Survey and were released at the capture site.

2.10. Hormone assays

I quantified plasma T concentration using commercial competitive enzyme-linked immunoassays (Enzo Life Sciences, Ann Arbor, MI, USA). Samples were diluted 8× using assay buffer containing steroid displacement reagent. This assay has been validated for Abert's Towhee in the Deviche laboratory by demonstrating parallelism of a standard curve with a curve produced by serial plasma dilution (2- to 32-fold; Fokidis et al. 2009). I assayed samples in duplicate and randomly assigned them to assay plates, except that the two samples collected from any given individual were assayed on the same plate. A separate standard curve was included on each plate, taken from the same stock standard solution. Average assay sensitivity was 1.7 pg/ml. The average intra- and inter- assay coefficient of variation were 6.8% and 2.1%, respectively.

2.11. Statistical analysis

I compared the phenology of leaf foliage progression and arthropod dry biomass between the two habitats (i.e., urban vs. desert) using repeated measures ANOVA (rmANOVA) with sampling period as the within-subjects factor. In the case of leaf phenology, data were ranked before analysis (Conover and Iman 1981) and are presented as medians (± interquartile range; IQR). To analyze relationships between habitat and body mass, wing chord, body condition, furcular fat score, cloacal protuberance width, and (log transformed) initial plasma T I combined data from birds caught in study 1 and study 2. To test if body mass, wing chord, and body condition differed between the two

habitats, I used Student's t-tests. Body condition was calculated as the residuals of a linear regression between body mass and wing chord. I used a Mann-Whitney rank-sum test to investigate differences in furcular fat scores as a function of the habitat. Cloacal protuberance width data were not normally distributed and so were analyzed using the Mann-Whitney rank-sum test. To test whether urban and desert towhees differ in paired testis mass and seminiferous tubule diameter, I used Student's t-tests.

I tested whether cloacal protuberance widths of birds in the current study (2013) differed from those of birds sampled in a previous study (2011 and 2012) using a two-way ANOVA with habitat and year (i.e., 2011 and 2012 vs. 2013) as fixed factors. I analyzed cumulative precipitation during January to March at three desert and five urban weather stations using a rmANOVA.

To analyze whether the number of brain cells immunoreactive for GnRH, GnIH, and NPY differed in urban and non-urban birds, I used Mann-Whitney rank-sum test. For the remaining measures of GnRH, GnIH, and NPY (GnRH and GnIH: cell body area and optical density, and optical density of fibers in the median eminence [ME]; NPY: density of fibers in the ME), I used Student's t-tests. In the case of GnHI cell body optical density and density of NPY-ir fibers in the ME, I used Mann-Whitney rank-sum test because these data were not normally distributed. To examine the interrelationships between the number of cells immunoreactive for GnRH, GnIH, and NPY, and the relationships of these variables with baseline plasma T, I used Spearman's rank correlations.

To analyze the plasma T response (post-challenge plasma T (ng/ml) – initial plasma T (ng/ml)) to GnRH challenge, I used a two-way ANOVA with habitat and treatment (GnRH vs. saline) as fixed factors. Data were tested for normality using the Shapiro-Wilk test and equal variances using Levene's test. I performed all statistical

analyses using SPSS version 19 (Chicago, Illinois, USA) with $\alpha = 0.05$. Post-hoc comparisons were performed using Tukey's honestly significant difference (HSD) test.

3. Results

3.1. Plant and arthropod phenology

There was no overall change in plant phenology over the course of the study ($F_{3,21} = 0.08$, $P = 0.97$). However, there was a significant effect of habitat ($F_{1,7} = 57.84$, $P < 0.001$) and the interaction of this factor with time ($F_{3,21} = 6.74$, $P = 0.002$; Fig. 5). Post hoc analysis showed that the median urban tree phenology score did not change over the course of the study. The median tree phenology score of desert sites, on the other hand, was lower at the beginning of the study and increased over course of the spring to similar levels as urban trees. Ground arthropod dry biomass increased over the study period ($F_{3,21} = 4.49$, $P = 0.014$; Fig. 5). However, there was no difference between urban and desert sites ($F_{1,7} = 0.077$, $P = 0.79$), and no interaction between habitat and time ($F_{3,21} = 0.99$, $P = 0.43$; Fig. 5).

3.2. Bird capture time and date

All birds were caught between 06:28 AM and 10:13 AM (mean capture time: urban = 07:49 AM; desert = 07:53 AM). Capture time was similar between urban and desert birds in study 1 ($t_{14} = -0.56$, $P = 0.58$), between the four treatment groups in study 2 ($F_{3,28} = 0.32$, $P = 0.81$), and when birds from both experiments are combined ($t_{46} = 0.19$, $P = 0.85$). Likewise, the dates that birds were caught were similar between urban and desert birds in study 1 (mean date: urban = March 22nd; desert = March 24th; $t_{14} = -0.36$, $P = 0.73$), between the four treatment groups in study 2 (urban GnRH = March 24th, urban control = March 23rd; desert GnRH = March 23rd, desert control = March

22nd; $F_{3,28}$ = 0.14, P = 0.93), and when birds from both study are combined (urban = March 23rd; desert = March 22nd; t_{46} = -0.20, P = 0.85).

3.3. Morphometrics

Urban and desert birds from both studies did not differ with respect to body mass (mean (± SEM): urban = 47.8 (± 0.33) g; desert = 48.1 (± 0.58) g; t_{46} = -0.50, P = 0.62) or wing chord (mean (± SEM): urban = 92.0 (± 0.33) mm; desert = 91.3 (± 0.54) mm; t_{46} = 1.15, P = 0.26). Body condition was likewise similar between urban and desert birds (t_{46} = -1.03, P = 0.31). Furthermore, urban and desert towhees had similar furcular fat scores (median (± IQR): urban = 1 (± 0); desert = 1 (± 0); U = 288.0, n_1 = 24, n_2 = 24, P = 0.99) and cloacal protuberance widths (median (± IQR): urban: 7.28 (± 0.62) mm; desert: 7.24 (± 0.9) mm; U = 280.0, n_1 = 24, n_2 = 24, P = 0.88).

3.4. Study 1 – The central control of reproduction

GnRH, GnIH, and NPY

Urban and desert birds did not differ in the number of GnRH-ir cells in the POA (U = 40.0, n_1 = 8, n_2 = 8, P = 0.44) or the LHy (U = 35.5, n_1 = 8, n_2 = 8, P = 0.72; Table 1). Likewise, the total number of GnRH-ir cells (i.e., POA plus LHy) was similar between birds from the two habitats (U = 39.0, n_1 = 8, n_2 = 8, P = 0.50; Table 1). Urban and desert towhees also had similar POA GnRH-ir cell body area (t_{14} = -1.91, P = 0.077) and optical density (t_{14} = -0.33, P = 0.75), and optical density of GnRH-ir fibers in the ME (t_{14} = -1.07, P = 0.30; Table 1).

Urban and desert birds had a similar number of GnIH-ir cells in the PVN (U = 36.0, n_1 = 8, n_2 = 8, P = 0.72; Table 1). Furthermore, urban and desert birds had similar GnIH-ir cell body area (t_{14} = -0.32, P = 0.76) and optical density (U = 38.0, n_1 = 8, n_2 = 8,

P = 0.57), and optical density of GnIH-ir fibers in the ME (t_{14} = 0.47, P = 0.65; Table 1). Likewise, the number of NPY-ir cells in the IN (U = 60.0, n_1 = 8, n_2 = 8, P = 0.44) and the optical density of NPY-ir fibers in the ME (U = 32.0, n_1 = 8, n_2 = 8, P = 0.96; Table 1) were similar in urban and desert towhees.

The number of GnIH-ir cells in the PVN was related to the total number of GnRH-ir cells (r^2 = 0.59, P = 0.017), the number of GnRH-ir cells in the POA (r^2 = 0.53, P = 0.035), and the number of GnRH-ir cells in the LHy (r^2 = 0.56, P = 0.025; Fig. 6). There was no association between the number of cells immunoreactive for GnIH and NPY (r^2 = 0.003, P = 0.99; Fig. 6).

3.5. Gonad growth, testosterone secretion, and associations with the central control of reproduction

Urban and desert birds had similar testis masses (t_{14} = 0.88, P = 0.39) and seminiferous tubule diameters (t_{14} = 1.94, P = 0.07; Table 2). Initial plasma T of towhees in study 1 was also similar between the two habitats ($F_{1,15}$ = 0.01, P = 0.94; Table 2). Initial plasma T was not related to testis mass (r^2 = 0.21, P = 0.45) or cloacal protuberance width (r^2 = 0.18, P = 0.67), but testis mass was positively related to cloacal protuberance width (r^2 = 0.88, P < 0.0001; Fig. 7).

The cloacal protuberance width differed between birds in the current study versus birds in my previous study ($F_{1,71}$ = 11.63, P = 0.001). Furthermore, there was a difference between birds from the two habitats ($F_{1,71}$ = 6.60, P = 0.012), and a two-way interaction between the study year and habitat ($F_{1,71}$ = 10.34, P = 0.002; Fig. 8). Cloacal protuberance width of urban towhees did not differ between the two studies (Tukey's HSD: P > 0.05; Fig. 8). By contrast, desert birds had larger cloacal protuberance in the current than the previous study (Tukey's HSD: P < 0.001; Fig. 8).

To better understand the ecological driver of this inter-annual variation, I also compared the cumulative precipitation during January to March. Cumulative precipitation during this period differed between the two habitats ($F_{1,15}$ = 15.42, P = 0.008) and between years ($F_{1,15}$ = 218.17, P < 0.001), and there was an interaction between these factors ($F_{1,15}$ = 53.16, P < 0.001; Fig. 9). Precipitation did not differ between urban and desert locations in 2011 and 2012 (Tukey's HSD: P's = 0.64; Fig. 9). However, precipitation was higher during 2013 than 2011 and 2012, and also higher in the urban than desert locations (Tukey's HSD: P < 0.001; Fig. 9).

To better understand the relationships between the central control of reproduction and initial plasma T, I combined initial plasma T data from urban and desert birds. The total number of GnRH-ir cells was not related to baseline plasma T (r^2 = 0.47, P = 0.065; Fig. 10). However, when I examined the association between baseline plasma T and the number of GnRH-ir cells in the POA and the LHy separately, this revealed a positive association between baseline plasma T and the number of cells in the POA (r^2 = 0.50, P = 0.05), but not the LHy (r^2 = 0.41, P = 0.12; Fig. 10). There was also no association between baseline plasma T and the number of GnIH-ir cells in the PVN (r^2 = 0.21, P = 0.44) or the number of NPY-ir cells in the IN (r^2 = 0.01, P = 0.84; data not shown).

3.6. Study 2 – GnRH challenge

Initial plasma T was similar in birds from urban and desert habitats ($F_{1,28}$ = 0.74, P = 0.40) and in birds from the two treatment groups ($F_{1,28}$ = 0.18, P = 0.67; Table 3). Furthermore, there was no interaction between habitat and treatment group on baseline plasma T ($F_{1,28}$ = 0.59, P = 0.45). The plasma T response to GnRH challenge was effected by treatment ($F_{1,28}$ = 13.47, P = 0.001) and the interaction between treatment and time

($F_{1,28}$ = 63.11, P < 0.0001). Specifically, injection of GnRH elicited an increase in plasma T (Tukey's HSD: P < 0.001), whereas saline injection caused a decrease in plasma T (Tukey's HSD: P < 0.001).

There was no overall effect of habitat on the plasma T response to treatment ($F_{1,28}$ = 1.27, P = 0.27). However, there was an effect of treatment ($F_{1,28}$ = 26.86, P < 0.0001) and the interaction between treatment and habitat ($F_{1,28}$ = 5.59, P = 0.025; Fig. 11). This difference resulted from a greater plasma T response to GnRH challenge of desert towhees compared to that of urban towhees (Tukey's HSD: P = 0.021; Fig. 11). Desert towhees differed in their plasma T response to GnRH challenge compared to saline injection (Tukey's HSD: P < 0.001). By contrast, the plasma T responses of urban towhees to GnRH challenge and to saline injection did not differ (Tukey's HSD: P = 0.056). The response to saline injection was similar in birds from the two habitats (Tukey's HSD: P = 0.389).

4. Discussion

The phenology of gonad growth is an important phenotypic change that determines the breeding period for many species of animals (Bubenik et al., 1997; Callard et al., 1978; Dawson, 1983; Itoh et al., 1990; Palmer et al., 1988). However, the underlying cause of fine-scale variation in the phenology of gonad growth remains unclear. The available evidence suggests that birds adjust to urban areas by advancing the phenology of vernal gonad growth (Deviche et al., 2010; Deviche and Davies, 2014; Partecke et al., 2005). Comparisons of urban birds against their corresponding non-urban conspecifics, therefore, provide 'natural experiments' that may shed light on the physiological causes of variation in gonad growth phenology. To that end, I compared activity of the reproductive system in adult male Abert's Towhees from urban and desert

locations. Considering that my previous study on Abert's Towhees from the same locations as the current study found that urban males advanced the phenology of vernal gonad growth, relative to desert males (S. Davies, unpublished), my finding that these populations did not differ in any measure of baseline reproductive (neuro)endocrine activity is surprising. Specifically, I found no difference in any measure of the central control of reproduction (i.e., hypothalamic GnRH and GnIH), in any measure of gonad growth or baseline endocrine activity (i.e., paired testes mass, seminiferous tubule diameter, baseline plasma T), or in a secondary sexual characteristic (i.e., cloacal protuberance width). Interestingly, the inter-annual comparison of cloacal protuberance width, which is tightly linked to paired testes mass, indicates that, in the three years considered here, there is limited inter-annual variation in the phenology of gonad growth in urban towhees. By contrast, in nearby populations of desert towhees, there is considerable inter-annual variation, whereby desert towhees advanced the phenology of gonad growth in the current year relative to previous years. This finding has important implications for both the ecological and physiological causes of variation in the phenology of gonad growth in urban and non-urban birds.

4.1. Ecological control of gonad growth phenology

The first implication of my finding that desert – but not urban – towhees exhibit inter-annual variation in the phenology of gonad growth concerns the hypothesis that greater food abundance in urban areas contributes to the advanced phenology of gonad growth seen in urban birds (Deviche and Davies, 2014). Under this hypothesis, urban birds are expected to be in better energetic status, which enables them to initiate gonad growth as early as photoperiod allows. In contradiction to this hypothesis, however, I found that ground arthropod biomass did not differ between urban and desert locations.

This finding suggests that food abundance for urban versus desert Abert's Towhees is unlikely to have differed. Accordingly, multiple measures of energetic status (i.e., body condition and fat stores) and hypothalamic levels of NPY, a neuropeptide that, in mammalian models, links food availability to endocrine activity of the HPG axis, were similar in urban and desert towhees. When I also consider that the phenology of gonad growth (as indicated by cloacal protuberance width) was advanced only in the previous study, but energetic status did not differ between the two populations in either study, there is little evidence to support the hypothesis that greater food abundance in urban areas drives the disparity in gonad growth phenology between urban and desert Abert's Towhees.

An alternative hypothesis to explain the observed inter-annual variation in the phenology of gonad growth between urban and desert towhees is that the inter-annual variation in the phenology of key food types differs between urban and desert locations (Deviche and Davies, 2014). Under this hypothesis, the overall abundance of food is similar between urban and desert areas, and, hence, I would not expect to see a habitat-related difference in energetic status of towhees. However, the phenology of key seasonally available food sources, such as growing green vegetation and arthropods, which birds may use as a supplementary cue to optimally time gonad growth, may be more consistent from year-to-year in urban areas, relative to desert areas. In support of this proposition, studies of a variety of songbird species demonstrate that the presence and/or consumption of preferred food types accelerates gonad growth. For example, providing green vegetation in captivity advanced gonad growth in male Atlantic Canaries (*Seirus canaria*; Voigt et al., 2007), Common Redpolls (*Carduelis flammea*; Hahn et al., 2005), and female White-crowned Sparrows (*Zonotrichia leucophrys*; Ettinger and King, 1981). Similarly, gonad growth was accelerated in captive male Pine Siskins

(*Spinus pinus*) and Spotted Antbirds (*Hylophylax naevioides*) by providing them with their preferred diet of seeds and live arthropods, respectively (Hau et al., 2000; Watts and Hahn, 2012). Furthermore, the ecology of the Sonoran Desert suggests that potentially there is considerable variation in the phenology of seasonally available food types both inter-annually and between urban and desert areas. In arid regions like the Sonoran Desert, the phenology of vernal plant growth is highly dependent on winter precipitation (Bowers and Dimmitt, 1994; Noy-Meir, 1973; Schwinning et al., 2004). Levels of winter precipitation are highly variable and unpredictable from year to year, and, therefore, so too is the phenology of vernal plant growth. In years with high levels of winter precipitation, the plant growing season is advanced, whereas in years with low levels of precipitation, the plant growing season is delayed (Bowers and Dimmitt, 1994; Noy-Meir, 1973). By contrast, due to anthropogenic resource input (in this case, principally irrigation water), the phenology of vernal plant growth in urban areas of Phoenix is decoupled from precipitation (Buyantuyev and Wu, 2012). Consequently, urbanization of Phoenix is associated with reduced inter- and intra- annual variability in plant growth phenology, relative to desert areas. This is in accordance with my finding that the growing season of three tree species in urban areas had already begun (as indicated by the presence of green leaves) at the start of my study and exhibited no seasonal change in leaf phenology. These tree species in desert areas, on the other hand, were inactive at the start of my study and demonstrated a marked seasonal increase in leaf phenology. Reduced inter-annual variability in urban plant growth phenology is also in accordance with my inter-annual comparison of the phenology of gonad growth in Abert's Towhees. Like plant growing seasons, urban towhees also appear to have limited inter-annual variability in the timing of gonad growth. In particular, the limited year-to-year variability in gonad growth phenology is in the face of considerable variability in

winter precipitation. By contrast, the inter-annual variability of gonad growth phenology in desert towhees corresponds with the variation in winter precipitation. In my previous study, winter precipitation was low and the phenology of gonad growth in desert birds was delayed, whereas, in the current study, precipitation was higher and the phenology of gonad growth was advanced. Since precipitation during the current study was high, relative to the three years considered here, I suggest that the phenology of trees in desert areas was relatively early, which may account for lack of habitat-related difference in gonad growth phenology of towhees.

A wealth of research demonstrates that bird populations synchronize the breeding period with the peak in arthropod, especially caterpillar, availability (Cresswell and Mccleery, 2003; Visser et al., 2006). Long-term studies of caterpillar phenology demonstrate that the peak in caterpillar availability is synchronized with tree phenology (van Asch and Visser, 2007; Visser et al., 2006). I, therefore, predict that the phenology of arthropod availability in urban areas will parallel the phenology of urban plants and have limited inter-annual variability. The phenology of arthropod availability in desert areas, on the other hand, will exhibit greater inter-annual variability. If birds use the timing of growing green vegetation and/or arthropods as environmental cues to optimally time gonad development to local conditions, this may account for the disparity in inter-annual variability seen between urban and desert Abert's Towhee populations. Specifically, in years with high winter precipitation levels, the phenology of plants and arthropods in desert areas will be relatively early and Abert's Towhees in these areas will begin gonad development relatively early. In years with low winter precipitation levels, the phenology of plants, arthropods, and, in turn, gonad growth will be delayed. In contrast, the phenology of urban plants, arthropods, and Abert's Towhee gonad growth is independent of precipitation patterns and is relatively constant from year-to-year. I

suggest that future comparative studies of gonad growth phenology of urban and non-urban birds may find it illuminating to also compare the phenology of plants and potential food sources, and to study these factors over multiple years with variable environmental conditions.

4.2. Physiological control of gonad growth phenology

My hypothesized disparity in the seasonality of supplementary environmental cues between urban and desert areas may also account for the observed habitat-related difference in responsiveness of the anterior pituitary gland and/or gonads to GnRH challenge. Desert towhees inhabit an environment with an unpredictable and marked seasonal change in growing green vegetation (and possibly also arthropod availability), whereas urban birds inhabit an environment with a more predictable and limited change in these factors (Bowers and Dimmitt, 1994; Buyantuyev and Wu, 2012; Noy-Meir, 1973). I found that GnRH challenge elicited a marked increase in plasma T in desert, but not urban, towhees. This raises the intriguing possibility that, in response to the habitat-related disparity in the predictability and magnitude of change in plant and arthropod phenology, Abert's Towhees have adjusted the responsiveness of the anterior pituitary gland and/or gonads to environmental stimuli that would naturally elicit an increase in GnRH secretion. Evidence suggests that a range of supplementary environmental cues, such as the availability of preferred food types (Hahn et al., 2005; Hau et al., 2000), precipitation (Small et al., 2008a), and song (Small et al., 2008b; Wingfield and Wada, 1989), can rapidly elicit an increase in endocrine activity of the HPG axis. It may be beneficial for desert towhees to rapidly respond to the unpredictable and marked seasonal increase in plant and arthropod phenology and initiate gonad growth. By contrast, the more predictable and less marked seasonal increase in urban plant and

arthropod phenology may mean that it is less important for urban birds to respond as rapidly. It may be fruitful to compare the response to GnRH challenge of urban and non-urban birds at multiple times during vernal gonad growth while quantifying the phenology of plants and arthropods.

The second implication of my finding that desert, but not urban, towhees exhibit inter-annual variation in the phenology of gonad growth relates to the proposition that variation in baseline plasma T levels underlies variation in the phenology of gonad growth. Considering that baseline plasma T was similar between urban and desert towhees in both the current study and the previous study, whereas cloacal protuberance width varied considerably between years in the desert population, there is little evidence that variation in baseline levels of this hormone underlies variation in the phenology of gonad growth. This finding accords with other studies that have compared plasma concentrations of reproductive hormones in populations of urban and non-urban birds with disparities in gonad growth and/or lay dates. For instance, plasma LH and T were lower in male urban European Blackbirds compared to non-urban blackbirds, despite the urban population developing gonads 20 days earlier than their non-urban counterparts (Partecke et al., 2005). Likewise, urban female European Blackbirds and Florida Scrub-jays had similar plasma LH and E_2 to their non-urban conspecifics, but developed follicles 28 and 20 days earlier than their respective non-urban counterparts (Partecke et al., 2005; Schoech and Bowman, 2003). Overall, therefore, the available evidence suggests that variation in gonad growth phenology of urban and non-urban birds is not determined by variation in the phenology of LH, estradiol, or T secretion.

4.3. Conclusions

Evidence available to date suggests that birds adjust to urban areas by advancing the phenology of gonad growth. However, the ecological and physiological causes of this phenomenon are poorly understood. The findings of the current study demonstrate that the phenology of gonad growth is not always advanced in urban birds, relative to their desert conspecifics, in all years. In three years that differed in the habitat-related disparity in gonad growth, energetic status did not differ between the two populations at any time. This finding provides no support for the hypothesis that greater food abundance in urban areas driver the disparity in gonad growth phenology between urban and desert Abert's Towhees. My results are consistent however, with the hypothesis that differences in the predictability and magnitude of change in supplementary environmental cues, particularly the availability of key food sources, between urban and desert areas contributes to the observed habitat-related disparity in inter-annual variability in gonad growth of Abert's Towhees. The physiological mechanism responsible for this habitat-related variability in gonad growth phenology of urban and desert Abert's Towhees remains unclear, but my findings do not support the notion that it is not determined by variation in the phenology of baseline endocrine activity of the HPG axis. My study does, however, raise the intriguing possibility that responsiveness of the anterior pituitary gland and/or gonads contributes to the difference in gonad growth phenology between urban and desert towhees.

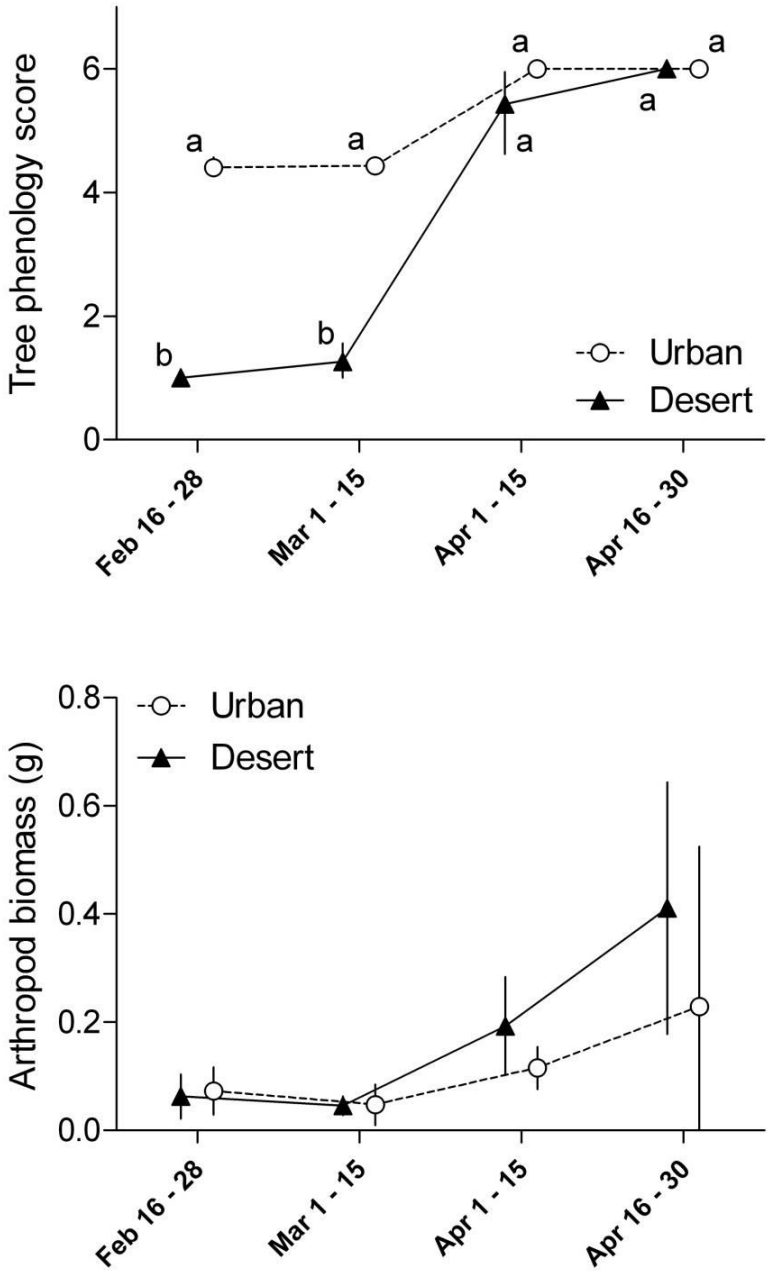

Fig. 5. The phenology of tree leaf foliage progression, but not ground arthropod abundance, differed between urban and desert study sites. Tree leaf foliage progression of three species (mesquite, *Prosopis* spp.; palo verde, *Parkinsonia* spp.; and salt cedar, *Tamarix* spp.) common to both habitats was scored on a scale of 0 – 6. Ground arthropods were collected in empty pitfall traps and then dried before weighing to calculate dry biomass. The tree phenology panel depicts medians (± IQR), whereas the arthropod biomass panel depicts means (± SEM). Points with identical letters are not significantly different ($P > 0.05$; Tukey HSD test). Both variables were measured along the same 100 m transects (4 urban sites; 3 desert sites) and collected at the same time, but datasets have been offset on the horizontal axis for visual clarity.

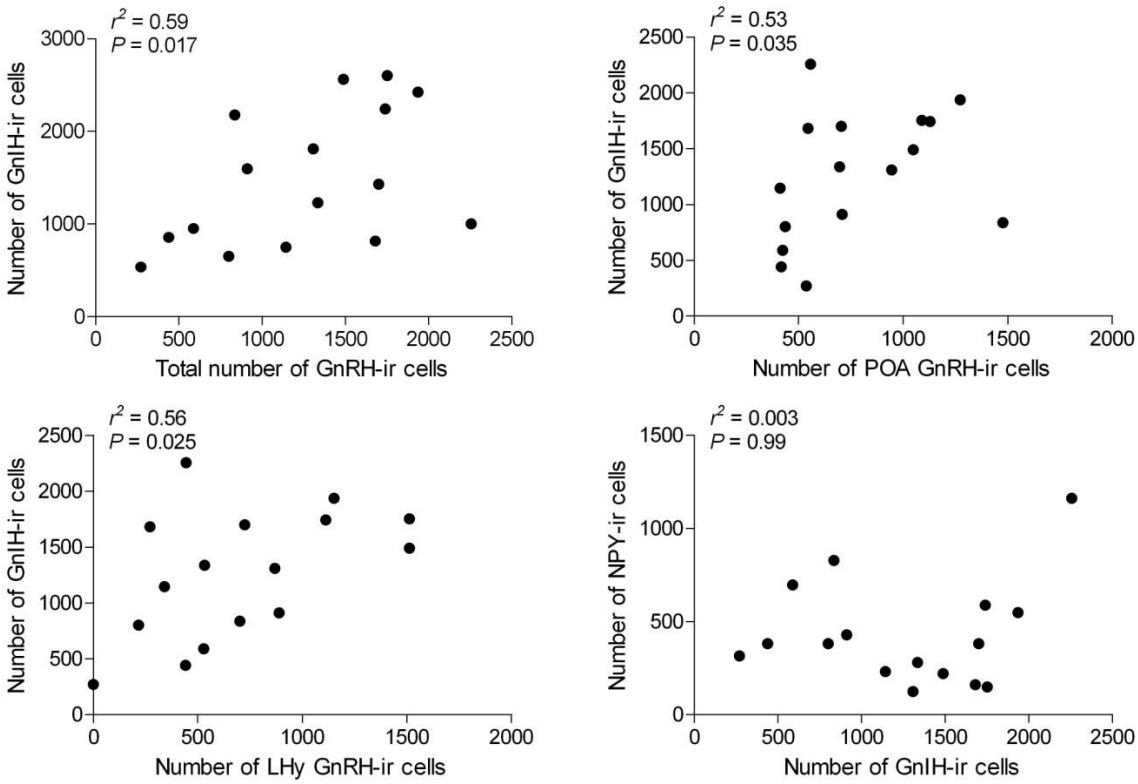

Fig. 6. The number of cells immunoreactive (ir) for gonadotropin-inhibitory hormone (GnIH) is related to the number of ir-gonadotropin-releasing hormone (GnRH) cells, but not ir-neuropeptide Y (NPY) cells, in free-ranging adult male Abert's Towhees, *Melozone aberti*. Statistics are the results of Spearman rank correlations. The number of GnRH-ir cells in the preoptic area (POA) and the lateral hypothalamus (LHy) were counted, and total GnRH-ir is the combination of these two locations. Gonadotropin-inhibitory hormone-ir cells in the paraventricular nucleus and NPY-ir cells in the infundibular nucleus were counted. Each point represents one individual.

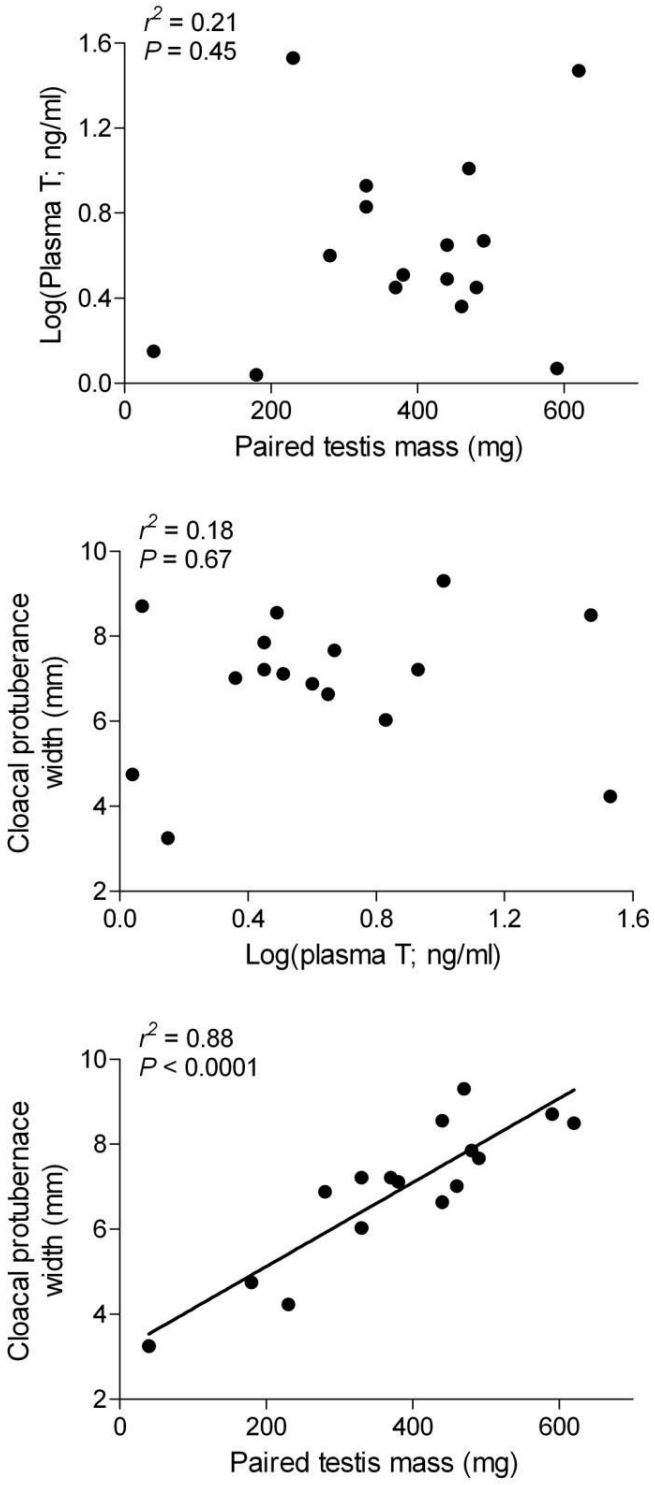

Fig. 7. Pearson correlations between baseline plasma testosterone (T (ng/ml); log transformed), paired testis mass, and cloacal protuberance width in free-ranging adult male Abert's Towhees, *Melozone aberti*. Each point represents one individual.

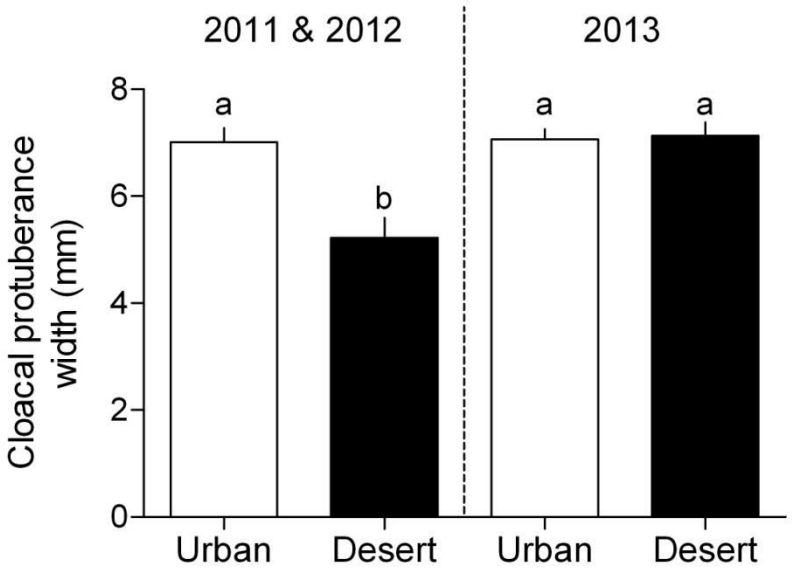

Fig. 8. Desert, but not urban, adult male Abert's Towhees, *Melozone aberti*, advanced the timing of seasonal reproductive development in 2013 compared to 2011 & 2012. The cloacal protuberance width of urban towhees in the current study (2013) was similar to that of birds from the same population sampled in a previous study (2011 & 2012). By contrast, cloacal protuberance width of desert towhees was larger in the current study compared to that of birds from the same population sampled in a previous study. Data points are means ± SEM, and points with identical letters are not significantly different ($P > 0.05$; Tukey HSD test).

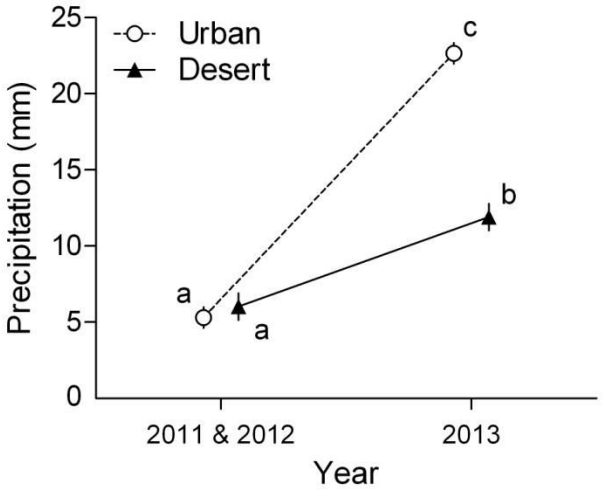

Fig. 9. In the current study (2013), precipitation was higher compared to the previous two years (2011 and 2012) and was higher in urban compared to desert locations. I compared the cumulative precipitation of three desert and five urban weather stations during January to March. Points are means and error bars show SEM, and points with identical letters are not statistically different. For visual clarity, points have been offset on the horizontal axis.

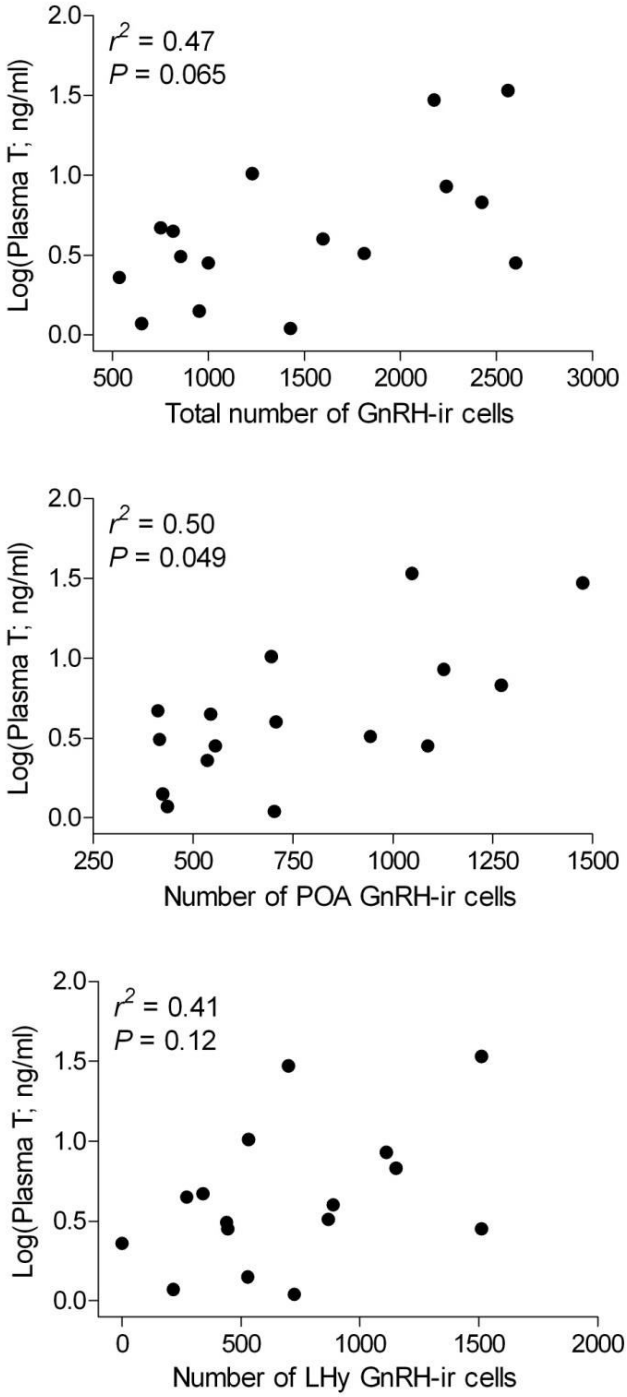

Fig. 10. Spearman rank correlations between baseline plasma testosterone (T (ng/ml); log transformed) and the number of cells immunoreactive (ir) for gonadotropin-releasing hormone (GnRH) in free-ranging adult male Abert's Towhees, *Melozone aberti*. The number of GnRH-ir cells was counted in the preoptic area (POA) and the lateral hypothalamus (LHy), and total GnRH-ir is the combination of these two locations. Each point represents one individual. Note that the scales of the horizontal axes differ between panels.

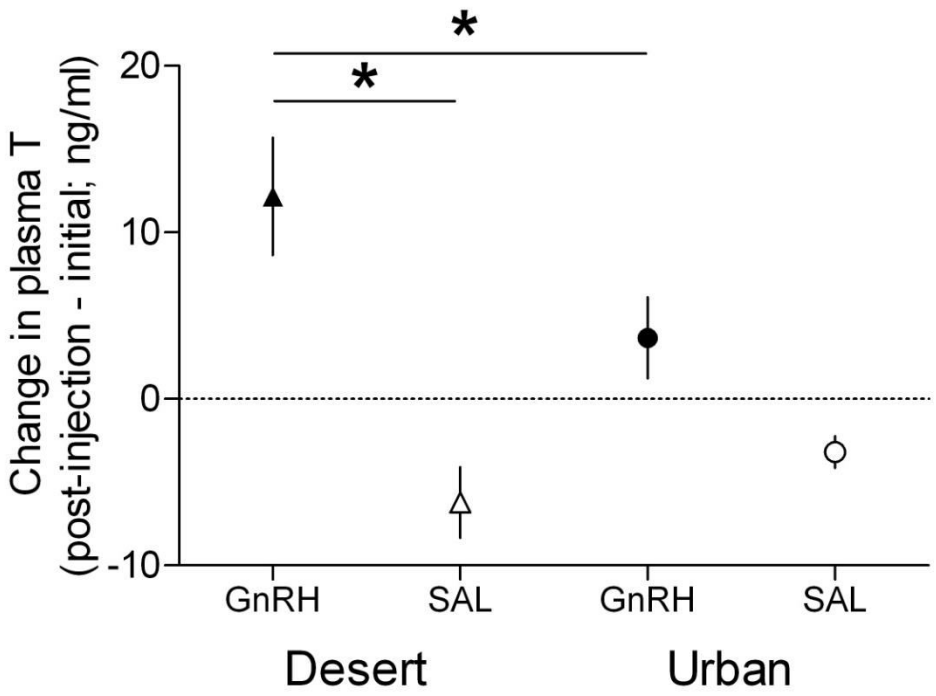

Fig. 11. Desert Abert's Towhees, *Melozone aberti*, have a larger plasma testosterone (T) response to GnRH challenge than urban towhees. Free-ranging adult males (n = 8 per group) were bled within 3 min of capture, received an intravenous injection of gonadotropin-releasing hormone (GnRH) or vehicle (saline; SAL), and were bled again 20 min later (post-injection). Points are means (calculated as post-challenge plasma T (ng/ml) – initial plasma T (ng/ml)) and error bars show SEM. Asterisks denote significant differences between groups ($P < 0.05$; Tukey HSD test).

Table 1. The number, area, and optical density of cell bodies, and optical density of fibers in the median eminence (ME), immunolabeled for three neuropeptides (gonadotropin-releasing hormone-I (GnRH), gonadotropin-inhibitory hormone (GnIH), and neuropeptide Y (NPY)) of free-ranging urban (n = 8) and desert (n = 8) adult male Abert's Towhees, *Melozone aberti*. The total number of cells immunoreactive for GnRH was calculated as the number in the preoptic area (POA) plus the number in the lateral hypothalamus (LHy). Unless indicated otherwise, data presented are means (± SEM) and *P*-value statistics are the results of student's t-tests. A superscript letter indicates that data presented are medians (± IQR) and *P*-value statistics are the results of Mann Whitney U tests. AU = arbitrary units.

		Habitat		Statistics
		Urban	Desert	*P*-value
GnRH	Number of cells in the POA[a]	624 (402)	843.5 (612)	0.44
	Number of cells in the LHy[a]	582 (714)	616 (504)	0.72
	Total number of cells[a]	1142 (1226)	1520 (1232)	0.50
	Cell body area (μm²)	73.2 (± 5.2)	85.2 (± 3.5)	0.077
	Cell body optical density (AU)	0.31 (± 0.02)	0.32 (± 0.03)	0.75
	ME fiber optical density (AU)	0.20 (± 0.016)	0.21 (± 0.017)	0.30
GnIH	Number of cells[a]	1316 (1014)	1322 (928)	0.72
	Cell body area (μm²)	87.9 (± 4.8)	91.1 (± 9.1)	0.76
	Cell body optical density (AU) [a]	0.28 (0.34)	0.35 (0.15)	0.57
	ME fiber optical density (AU)	0.104 (±0.015)	0.094 (± 0.017)	0.65
NPY	Number of cells[a]	348 (178)	484 (548)	0.44
	ME fiber optical density (AU) [a]	0.32 (0.07)	0.33 (0.09)	0.96

[a] data are presented as medians and interquartile range.

Table 2. Vernal testicular recrudescence and testosterone secretion of free-ranging adult male Abert's Towhees, *Melozone aberti*, did not differ between urban and desert populations (n = 8 birds per habitat). The *P*-value statistics are the results of student's t-tests. Data presented are means (± SEM).

	Habitat		Statistics
	Urban	Desert	*P*-value
Paired testis mass (mg)	415.6 (± 38.7)	349.5 (± 64.5)	0.39
Seminiferous tubule diameter (μm)	397.8 (± 23.4)	328.4 (± 27.0)	0.07
Plasma testosterone (ng/ml)	7.5 (± 3.4)	7.6 (± 3.8)	0.94

Table 3. Plasma testosterone levels (ng/ml) of free-ranging adult male Abert's Towhees, *Melozone aberti*, bled within 3 min of capture (initial) and 20 min after (post-injection) an injection of either gonadotropin-releasing hormone-I or saline. Data presented are means (± SEM) and n = 8 per group.

	Habitat			
	Urban		Desert	
Treatment	Initial	Post-injection	Initial	Post-injection
Saline	5.0 (± 1.2)	1.8 (± 0.5)	8.6 (± 2.5)	2.4 (± 0.5)
GnRH	7.4 (± 3.4)	11.1 (± 1.8)	7.0 (± 2.5)	19.2 (± 3.0)

CHAPTER 4

PLASTICITY IN THE SEASONAL BREEDING OF URBAN BIRDS IS RELATED TO THE SEASONALITY OF THE URBAN HEAT ISLAND EFFECT

Phenotypic plasticity in seasonal timing (i.e., phenology) of breeding can assist in fine-tuning reproduction to local environmental conditions. Urban areas are expanding at an unprecedented rate and this expansion dramatically alters local environmental conditions. A major challenge for organisms is to adjust their breeding phenology to these novel environmental conditions. Compared to their non-urban conspecifics, urban birds advance their phenology of seasonal breeding. However, the role of phenotypic plasticity and the environmental driver(s) of this adjustment are largely unknown. To address this question, I use meta-analysis and meta-regression with effect sizes calculated from the difference in days between the population breeding initiation date in urban and the corresponding conspecific non-urban bird populations. I show for the first time, to my knowledge, that the adjustment of breeding phenology by urban birds is not geographically uniform; instead, there is a latitudinal gradient whereby the urbanization-associated advancement of breeding phenology decreases with increasing latitude. To explain the relationship between latitude and breeding phenology, I show for the first time, to my knowledge, that birds breeding in cities with a seasonally consistent urban heat island effect and plant growing season adjust their breeding schedule more than birds breeding in cities with strong seasonality in the urban heat island effect and plant growing season. My results show that although urbanization is associated with ecological homogenization, whereby ecological processes of geographically distinct urban areas are more similar to each other than to their corresponding non-urban areas, plasticity in the seasonal timing of a crucial life history event of urban birds is not uniform across cities and is an exception to the homogenization caused by urbanization.

1. Introduction

Phenotypic plasticity, the ability to alter the expression of a genotype in response to environmental conditions, is an important mechanism by which individuals can rapidly adapt to environmental changes (Charmantier et al., 2008). Earth's rapid urbanization continues and the decades ahead are projected to see the largest and fastest period of urban expansion in human history (Fragkias et al., 2013). Urbanization is characterized by profound environmental changes, and urban animals must adjust to an environment vastly different from that of their non-urban conspecifics. Correctly timing the phenology of life history events is a crucial adaptation to local environmental conditions (Miner et al., 2005).

In seasonally breeding animals such as birds, optimally timing reproduction each year has a strong effect on reproductive success (Baker, 1938). As a result, birds use the seasonal profile of ambient temperature and potentially also plant growth to synchronize breeding with the availability of food for their young to maximize fitness (Charmantier et al., 2008; Visser et al., 2006). Moreover, within a breeding season, the overwinter survival rate of offspring is higher in the first than in later breeding events and this rate declines steeply when breeding is delayed (Visser et al., 2006). To understand how animal populations can adjust to current and projected urbanization it is, therefore, critical to assess the role of phenotypic plasticity in the timing of breeding. Here, I use a meta-analysis approach to compare the reproductive phenology of urban bird populations to that of non-urban conspecific populations.

2. Methods

2.1. Literature search

I performed a literature search using the *ISI Web of Knowledge*, *Science Direct*, and *PubMed* with the search terms: 'bird* OR avian' and 'urban* OR garden* OR yard* OR city' as topics, and 'breed* OR reproduct* OR nest* OR incubat*' as title. The database was last searched on March 20th 2014. I then read the titles and online abstract of all resulting publications. Publications that did not fit the inclusion criteria (see below) were excluded. Publications that appeared to fit my criteria were read in full to verify that they did indeed meet my inclusion criteria. Using the articles retrieved by the database search above, I also identified further articles by backward- and forward-searching for articles cited in these papers or for articles citing these papers, respectively. I cannot exclude the possibility that some relevant studies were not identified in my literature search. However, I assume that my search protocol yielded an unbiased sample of the effect sizes available in the published literature.

2.2. Inclusion criteria

A study was included in my analysis if it met two criteria. First, the study must have involved a paired comparison of both urban and non-urban populations of the same species of wild bird during the same breeding season. Second, the study must have quantified a trait considered to be associated with the timing of breeding in birds (e.g., lay date, start of incubation, or hatch date). Accordingly, I identified 23 independent studies that encompass 18 species (16 passerines, 1 falcon, 1 owl) and 20 cities across 4 continents, ranging in latitude from 60.1° N to 35.3 ° S (Appendix A). Four temporal measures of breeding phenology were included in my analysis: dates of first egg laying, clutch initiation, first clutch completion, and hatch. As the population mean date at

which the first egg is laid (lay date) is an important determinant of fitness and can be quantified reliably, when a study measured lay date and other traits, I included only the estimates of lay date. The results of my study are consistent when averaging to obtain one data point from each type of breeding phenology measure, indicating that the type of breeding phenology measure does not markedly affect the results. Furthermore, the difference in phenology between urban and non-urban birds is consistent when averaging to obtain one data point from each species or each family, indicating that neither the inclusion of multiple studies of a given species nor possible phylogenetic artifacts bias the results. Multiple tests also suggest that any potential bias towards publishing studies yielding statistically significant results does not markedly affect my results (Borenstein et al., 2011).

2.3. Effect Size Computation

Most studies did not quantitatively define their urban or non-urban areas. Furthermore, the definition of each land use type varied between studies. For instance, some authors define areas dominated by residential houses as 'urban', whereas others defined this as 'suburban' (in the later case the term urban was used to refer to areas with a high proportion of impervious surface, such as business districts and city center). Similarly, areas defined as 'non-urban' included areas with limited land use change, such as national parks and preserves, as well as areas dominated by agriculture. Therefore, I adopted broad classifications of 'urban' or 'non-urban', with 'urban' encompassing city centers and suburban areas, and 'non-urban' encompassing agricultural areas and national preserves.

The effect size for a study was calculated from the difference in days between the population breeding initiation date in the urban and the corresponding non-urban

populations. Where possible, I obtained means, standard deviation, and sample sizes of all urban and non-urban parameter estimates. However, in some cases this information was unavailable so I attempted a number of approaches. First, I attempted to contact the author to obtain the full dataset. Second, I calculated effect sizes from the overall statistics (i.e., *t* or *F*). When a study subdivided within either urban or non-urban areas, I calculated weighted mean and pooled standard deviation for broader land classification as either 'urban' or 'non-urban'. If multiple years were included in a study, where possible I calculated weighted mean and pooled standard deviation from the data provided or entered the overall statistics (i.e., *t* or *F*).

2.4. Meta-Analysis Statistical Methods

All analyses were carried out using Comprehensive Meta Analysis 2.2 (Biostat Inc., Englewood, NJ, USA), using standard meta-analysis procedures and the random-effects model. I estimated standardized effect size using Hedges' *g*, which corrects for potential overestimation of effect size in studies with small sample sizes (Borenstein et al., 2011). Hedges' *g* is the most appropriate effect size statistic for my study because it compares a continuous dependent variable (i.e., population mean breeding phenology trait) across an independent categorical variable (i.e., urban or non-urban area) (Borenstein et al., 2011). Effect sizes were calculated so that a positive value indicates that the phenology of the non-urban population was advanced relative to the urban population, whereas a negative value indicates that the urban population was advanced. The null hypothesis was an effect size of zero, which I tested (using Comprehensive Meta Analysis 2.2) by inspecting the 95% confidence interval of the mean weighted effect size to see whether it included zero.

To test the relationships between breeding phenology and the urban heat island effect and the vegetation cover of a city, I used data from Peng et al. (2011). Briefly, these authors calculated the urban heat island by comparing the daytime land surface temperature of urban and corresponding non-urban areas. The difference in vegetation cover between urban and corresponding non-urban areas was calculated using the enhanced vegetation index (EVI). Both parameters were quantified using satellite imagery with a spatial resolution of 1 km. The seasonal amplitude of these parameters was calculated as the difference between summer and winter in the heat island effect or vegetation cover. Where possible, I selected data for the city in my analysis. If a particular city was not available, I used data from a substitute city. However, I only used a substitute city if it met the following criteria. First, the populations of the target city and the substitute city were within an order of magnitude. Second, the cities were within 3 degrees latitude. Third, the distance between the cities was less than 500 km. Fourth, the difference in altitude between the cities was less than 500 m.

2.5. Publication bias

I used two methods to quantitatively assess, and correct for, potential publication bias. First, I used the rank correlation to test the hypothesis that the effect size of a study is associated with its sample size (Begg and Mazumdar, 1994). Second, I estimated the number of putative 'missing' studies using the 'trim and fill' method (Duval and Tweedie, 2000), under the random effects model. I then used estimations of effect sizes from these 'missing' studies to recalculate the mean effect size and 95% confidence interval.

3. Results

In my meta-analysis, the dependent variable was the standardized measure of the magnitude of the statistical effect from each study (Hedges' *g*; hereafter effect size), calculated from the difference in days between the population breeding initiation date in the urban and the corresponding non-urban populations (Borenstein et al., 2011). I found that urban birds breed earlier than corresponding non-urban conspecifics (g = -0.52 [95% confidence interval (CI): -0.75 to -0.28], P = <0.0001). Specifically, the breeding phenology of urban bird populations is advanced by an average of 8 days (95% CI: -10.65 to -5.28). However, urbanization does not affect bird populations uniformly. Instead, the breeding phenology effect size is related to city latitude (Q = 16.3, df = 1, P = <0.0001; Fig. 12A): on average, the magnitude of the difference in phenology (in days) between urban and non-urban populations decreases by 0.6 days for every degree increase in city latitude (Q = 30.5, df = 1, P = <0.0001; Fig. 12D). It has been shown that urban bird populations lay earlier than their non-urban conspecifics (Chamberlain et al., 2009; Deviche and Davies, 2014), but the present study is the first to identify an association between the magnitude of the difference between pairs of urban and non-urban populations and the city latitude.

4. Discussion

To explain the relationship between latitude and breeding phenology, I propose two non-mutually exclusive hypotheses. First, one or several environmental features of urban areas that advance avian breeding phenology decrease in magnitude with decreasing latitude. Second, plasticity in the ability to adjust the timing of breeding increases as latitude decreases. According to the first hypothesis, one or several features of urban areas advance(s) avian breeding phenology and the strength of this feature is

inversely related to latitude. Urban and non-urban areas differ in ambient temperature and duration of plant growing seasons in a latitude-related manner and, therefore, these factors are prominent candidates. In general, urban areas are warmer than corresponding non-urban areas (urban heat island effect; Grimm et al., 2008)). However, the strength of this effect varies geographically and seasonally (Imhoff et al., 2010; Peng et al., 2011; Zhang et al., 2010). Indeed, the strongest urban heat island effect occurs in high latitude cities and the weakest occurs in low latitude cities (Imhoff et al., 2010; Peng et al., 2011; Zhang et al., 2010). Furthermore, in high latitude cities the urban heat island effect is stronger during the summer than the winter (Peng et al., 2011) whereas in low latitude cities this effect is relatively weak and consistent throughout the year. Supporting the hypothesis that the latitudinal trend in urban heat island effect plays a role in advancing the breeding phenology of urban birds, the difference in breeding phenology between urban and non-urban populations decreases as the seasonal amplitude of the urban heat island effect increases (effect size: $Q = 16.1$, $df = 1$, $P < 0.00001$; days: $Q = 25.4$, $df = 1$, $P < 0.00001$; Fig. 12B and E). The latitudinal trend in the seasonal amplitude of the urban heat island effect is also mirrored in the phenology of plant growth (Imhoff et al., 2004; Peng et al., 2011). Seasonal plant growth can be used as a proxy for the peak in food availability with which birds attempt to synchronize breeding (van Asch and Visser, 2007). Cities with a large seasonal change in the strength of the urban heat island effect also have a large seasonal change in plant growth and so have distinct plant growing seasons (Peng et al., 2011). By contrast, cities with a small and seasonally consistent urban heat island effect have a long and seasonally consistent plant growing season (Peng et al., 2011). In further support of my hypothesis that one or several environmental features of urban areas advance avian breeding phenology, the difference in breeding phenology between urban and non-urban populations increases as

the seasonality of vegetation growth decreases (effect size: $Q = 13.8$, $df = 1$, $P = 0.0002$; days: $Q = 12.6$, $df = 1$, $P = 0.0004$; Fig 12C and F). Therefore, low latitude cities appear to create a relatively aseasonal environment, whereas high latitude cities amplify seasonality. I show for the first time, to my knowledge, that the latitudinal gradient of seasonality in ambient temperature and plant growth of cities correlates with the degree to which birds adjust their breeding phenology. I suggest that this is because the timing of optimal conditions for breeding – particularly the peak in food availability – is advanced more in low latitude cities compared to high latitude cities, which may account for the present observations.

A second hypothesis to explain the relationship between latitude and breeding phenology is that the timing of breeding is more plastic in low than high latitude-breeding birds. In support of this hypothesis, bird populations that breed at low latitudes appear to have more flexible breeding schedules than higher latitude populations (Schoech and Hahn, 2007). This difference presumably results from low latitude populations being more responsive than high latitude populations to environmental cues, such as food availability and ambient temperature, which are used to fine-tune breeding phenology (Schoech and Hahn, 2007). I emphasize that my hypotheses are not mutually exclusive and future research is needed to determine their relative importance. The second hypothesis, if correct, has important practical implications. For example, a considerable proportion of human households provide food for wild birds (Robb et al., 2008a) and this practice is probably the most widespread wildlife management activity (Amrhein, 2014; Jones, 2011). Many species in my analysis use human-provided food. Following the second hypothesis, the effect of this practice on urban bird populations may vary latitudinally and human feeding of wild birds may accordingly exert a stronger effect on the timing of breeding in low- than high-latitude urban bird populations.

Others have noted that the advancement of breeding phenology of urban bird populations likely reduces the synchrony of reproductive events with their conspecific non-urban populations (Partecke et al., 2005), thereby potentially reducing the rate of genetic exchange among populations and ultimately leading to genetic isolation over time (Hendry and Day, 2005). My results raise important new implications for this hypothesis because birds breeding in cities with a seasonally consistent heat island effect (i.e., at lower latitudes) are more likely to undergo genetic differentiation than birds breeding in cities with a seasonally amplified heat island effect (i.e., at higher latitudes).

I found that bird populations adjust to urban areas by advancing their breeding phenology compared to their non-urban conspecifics and report for the first time, to my knowledge, that this effect is not uniform across cities. Indeed, I show that the degree of advancement is related to the strength and seasonal amplitude of the urban heat island effect and plant growing season of a given city. The results are also consistent with the hypothesis that low latitude bird populations are more responsive than high latitude populations to environmental factors that cause advancement of their breeding schedule. One implication of the present findings is that the practice of providing food for wild birds may exert a stronger effect on the reproductive adjustment to urbanization in low than high latitude cities. More generally, changes in ambient temperature and plant growing seasons caused by the urban heat island effect echo similar changes caused by global climate change. Ambient conditions in urban areas may, therefore, represent forerunners of future climates and studies in these areas may be valuable in understanding and assessing the mechanisms by which animal populations adjust to ongoing global climate change (Grimm et al., 2008).

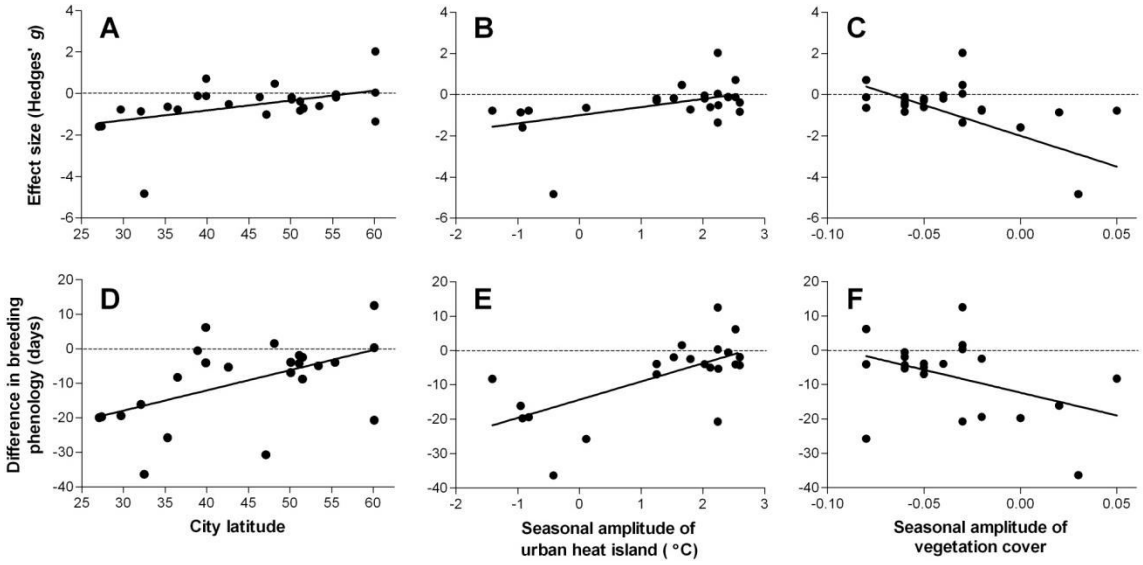

Fig. 12. Plasticity in the timing of breeding of urban bird populations is greater in cities at low latitude and with a seasonally consistent urban heat island effect and plant growing season. Meta-regression shows that the difference in breeding phenology between urban bird populations and their non-urban conspecific populations (in either effect sizes or days) is related to city latitude (A and D), the seasonal amplitude of the urban heat island effect (B and E), and the seasonal amplitude of vegetation cover (C and F). The effect size for a study was calculated from the difference in days between the population breeding initiation date in the urban and the corresponding non-urban populations. A positive value indicates that the phenology of the non-urban population was advanced relative to the urban population, whereas a negative value indicates that the urban population was advanced. Seasonal amplitude of the urban heat island effect and vegetation cover were calculated as the difference between summer and winter daytime surface urban heat island effect (°C) and enhanced vegetation index, respectively, between urban and corresponding non-urban areas (data from Peng et al. (2011)). Each point represents one study.

CHAPTER 5

FOOD AVAILABILITY, ENERGETIC CONSTRAINTS, AND REPRODUCTIVE DEVELOPMENT IN A WILD BIRD

In many organisms, food availability is a proximate cue that synchronizes seasonal development of the reproductive system (recrudescence) with optimal environmental conditions. Reproductive recrudescence is orchestrated by the hypothalamo-pituitary-gonadal (HPG) axis. However, the physiological mechanisms by which food availability modulates activity of the HPG axis are poorly understood. It is thought that the timing of recrudescence is modulated by an individual's energetic status.

I tested this hypothesis by examining whether food availability modulates the activity of the HPG axis. Specifically, I food-restricted captive adult male Abert's Towhees (*Melozone aberti*) for two or four weeks during photoinduced reproductive recrudescence. A third group (control) received *ad libitum* food throughout. I measured multiple aspects of the reproductive system that play a crucial role in recrudescence, including: hypothalamic gonadotropin-releasing hormone-I (GnRH-I), plasma testosterone (T), and reproductive morphology. Furthermore, because gonadotropin-inhibitory hormone (GnIH) and neuropeptide Y (NPY) potentially integrate information on food availability into seasonal reproductive recrudescence, I also measured the brain levels of these peptides.

At the hypothalamic level, I detected no effect of food restriction on immunoreactive (ir) gonadotropin-releasing hormone-I (GnRH-I), but the duration of food restriction was inversely related to the size of ir-gonadotropin-inhibitory hormone (GnIH) perikarya. Furthermore, the number of ir-neuropeptide Y (NPY; a potent orexigenic peptide) cells was higher in food-restricted than control birds. Food

restriction did not influence photoinduced testicular growth, but decreased plasma T and the width of the cloacal protuberance, an androgen-sensitive secondary sexual characteristic. Returning birds to ad libitum food availability had no further effect on plasma T, but caused the cloacal protuberance to rapidly increase in size and reach a size similar to ad libitum-fed birds.

My results support the tenet that food availability modulates reproductive recrudescence. However, they also suggest that this modulation is complex and depends upon the level of the HPG axis considered. At the hypothalamic level, my results are consistent with a role for the GnIH and NPY in integrating information on energetic status. At the gonadal level, there also appears to be a role for testicular endocrine function in modulating reproductive recrudescence in light of energetic status and independently of testicular growth.

1. Introduction

To maximize reproductive success, organisms often temporally synchronize their breeding period with optimal environmental conditions (Baker, 1938; Lindström, 1999; Lourdais et al., 2002; Wingfield and Kenagy, 1986). This synchronization has been demonstrated in a variety of taxa (Boyd, 1991; Munro et al., 1990; Nager and van Noordwijk, 1995; Olive et al., 2000; Olsson and Shine, 1998). In many organisms, the reproductive system is regressed outside the breeding period and must recrudesce before the start of the next breeding period. Reproductive recrudescence 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 behavior (Davies and Deviche, 2014; Williams, 2012).

To correctly time reproductive recrudescence, organisms use proximate environmental cues that can predict favorable conditions (Ims, 1990). A main such cue in birds is the annual change in day length (photoperiod). The annual cycle in photoperiod is constant between years at a given location and can predict favorable conditions. Accordingly, birds use photoperiod as the "initial predictive cue" that stimulates the hypothalamo-pituitary-gonadal (HPG) axis to begin reproductive recrudescence (Dawson et al., 2001; Wingfield, 1980). The mechanism by which increasing vernal photoperiod influences the avian HPG axis has been studied extensively. This influence begins at the hypothalamus level (Sharp, 2005), where light increases the production and secretion of the neuropeptide gonadotropin-releasing hormone-I (GnRH-I; King and Millar, 1982; Sharp and Ciccone, 2005). GnRH-I is the primary secretogue 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 estradiol (E_2) in males and females, respectively (Murton and Westwood, 1977), and these steroids in turn stimulate development of secondary sexual organs and promote the expression of characteristic reproductive behaviors.

In contrast to photoperiod, many environmental variables often vary between years at a given location. Birds adjust to this variation by modulating the activity of their reproductive system in light of information from supplementary environmental cues, including ambient temperature (Schaper et al., 2012b; Wingfield et al., 2003), rainfall (Hau et al., 2004; Small et al., 2007), and food availability (Hahn et al., 2005; Lack, 1968). But while considerable information is available about the effects of day length on the HPG axis, little is known about the physiological mechanisms by which these non-photic environmental factors influence reproductive recrudescence. Food availability, in

particular, is thought to modulate reproductive recrudescence, 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 recrudescence (Drent and Daan, 1980; Hahn et al., 2005; Meijer and Drent, 1999; Wingfield and Kenagy, 1986). Thus, birds in good energetic condition can begin reproductive recrudescence shortly after stimulation by sufficiently long days, whereas birds in poor energetic condition delay recrudescence until they have acquired sufficient energy stores. Various experimental and correlative approaches, including food supplementation (Harrison et al., 2010; Scheuerlein and Gwinner, 2002; Schoech, 1996; Schoech, 2009) and natural variation in the abundance of wild food sources (Hahn, 1998; Ligon, 1974), found that increased food availability is associated with earlier seasonal breeding in wild birds (reviewed by Davies and Deviche, 2014). Captive studies on the effect of food restriction or deprivation on avian reproductive recrudescence, however, have yielded inconsistent results (Dawson, 1986; Hahn, 1995; Meijer, 1991; but see Perfito et al., 2008). Furthermore, investigations into the physiological mechanisms by which food availability affects reproductive recrudescence have generally focused on the pituitary gland and, overall, these mechanisms remain poorly understood.

A candidate mechanism to integrate information on food availability and fine-tune seasonal reproductive recrudescence is the neuropeptide gonadotropin-inhibitory hormone (GnIH; Tsutsui et al., 2000) and its links with hypothalamic cells that produce neuropeptide Y (NPY). GnIH opposes the effect of GnRH on the HPG axis across vertebrates (Greives et al., 2008) and inhibits reproductive function by acting on hypothalamic GnRH neurons, anterior pituitary gland gonadotropes, and gonads (Clarke, 2011; Clarke and Parkington, 2013; Davies and Deviche, 2014; Tsutsui et al., 2013). In addition, GnIH has an orexigenic effect in birds and mammals (Clarke et al.,

2012; Clarke and Parkington, 2013; Tsutsui et al., 2013). In mammals, GnIH neurons project to hypothalamic regions that regulate appetite and energetic status (Qi et al., 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 et al., 2008; Pralong, 2010) and, in mammals, NPY-producing cells integrate information on energetic status via both hormones and metabolites (Marty et al., 2007). Furthermore, there appears to be reciprocal projections from NPY cells to GnIH cells, and evidence from mammalian studies suggests 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 recrudescence by food availability (Davies and Deviche, 2014).

I investigated whether food availability modulates reproductive development in a seasonally breeding songbird and examined potential neuroendocrine mechanisms mediating this modulation. Specifically, I hypothesized that energetic status influences reproductive recrudescence. To test this hypothesis, I compared hypothalamic levels of GnRH, testicular development, plasma T, and cloacal protuberance width of adult male Abert's Towhees (*Melozone aberti*) subjected to various food availability regimes during photoinduced gonadal development. I predicted that birds with access to *ad libitum* food and, hence, in good energetic condition would undergo reproductive recrudescence at a faster rate than food-restricted birds in poor energetic condition. I 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, GnIH, and NPY in response to food availability. To my knowledge, this study is the first in a wild bird species to

examine the effects of food restriction on the endocrine regulation of reproductive recrudescence at the hypothalamic and the gonadal levels. A better understanding of the physiological mechanisms by which food availability modulates reproductive recrudescence will improve our understanding of how variation in this crucial environmental cue is transduced into annual variation in the timing of breeding periods.

2. Methods

2.1. Capture and housing of wild birds

During January 2011, I 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 mistnets and conspecific song playback. I determined sex and age using behavior (singing and aggressive response to conspecific playback in males only), skull pneumatization, and wing chord (wing chord ≥ 92 mm = male; Pyle, 1997). I transported birds to Arizona State University and randomly divided them between two environmental chambers, both maintained at 23°C (± 1°C) with a short light cycle (9 L: 15D; lights on at 06:00). I individually housed birds in 76 x 46 x 46 cm cages with opaque barriers on three sides of each cage and provided *ad libitum* food (Mazuri small bird maintenance diet) and water. To permit individual identification, I marked each bird with a numbered aluminum tarsal band. After a one-week acclimation period in captivity, I increased the photoperiod to 14 L (lights on at 06:00) to induce reproductive development. To measure the average daily *ad libitum* food consumption of each individual over two weeks, I provided a pre-weighed amount of food shortly after the lights went on and weighed the remaining food (including spillage on the cage bottom) 24 hours later. To minimize spillage, I partially covered food dishes with tape, allowing only a small opening to access food.

2.2. Experimental protocol

Following the two week *ad libitum* food consumption period, I randomly assigned birds to one of three groups (n = 8 birds per group): (1) *ad libitum* food, (2) restricted food availability for four weeks (see more below) or (3) two weeks of food restriction followed by two weeks of *ad libitum* food (Fig. 13). Two birds in the control group died during the study, reducing this group size to six.

To ensure that the food restriction regime caused a marked and consistent reduction in body mass, I food-restricted birds with the goal of reducing a bird's body mass to 85% of its *ad libitum* mass. This target body mass is comparable to that used by Lal et al. (1990) in similar studies on cockerels (~83% of *ad libitum* mass; Lal et al., 1990) and on European Starlings (Sturnus vulgaris, ~80% of *ad libitum* mass; Meijer, 1991). To reach this target weight and based on a separate pilot study on 6 towhees, experimental birds received 70% of their individual average *ad libitum* food consumption. I weighed all birds daily immediately prior to dispensing the daily food ration. If a bird's mass decreased below its 85% target mass, this bird was immediately fed the difference between its current mass and its target mass (Morrison et al., 2002). By using this individually customized, mildly flexible restriction regime, I could control food availability and concurrently confirm that the body mass of each bird was reduced and stabilized at the target reduction.

From each bird, I collected a blood sample (see below) to quantify plasma T immediately prior to (1) the switch to food restriction (defined as day 0), (2) the switch back to *ad libitum* food (day 14), and (3) the end of the study (day 28; Fig 13). I collected blood (~200 μl) from the right jugular vein using a heparinized syringe within two minutes of removing a bird from its cage. The sample was then placed on ice and

centrifuged within 1 h. I harvested plasma using a Hamilton glass syringe and froze aliquots at -80°C until assayed. Immediately following collection of each blood sample, I quantified the furcular fat stores and size of the pectoral muscles of all the birds to estimate their energetic condition. I 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 and 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, I also estimated the size of the pectoral muscles on a scale ranging from 0 – 3 (adapted from Gosler, 1991; 0 representing concave pectoral muscles and a prominent keel, 3 representing convex pectoral muscles that protruded above the keel; Salvante et al., 2007). At this time, I also measured the width of the cloacal protuberance (± 0.1 mm; using digital calipers), an androgen-sensitive secondary sexual characteristic. I staggered the collection of blood samples and morphometrics over two consecutive days by randomly assigning towhees to one of four groups, with 6 birds in each group. I collected blood samples for any given group always in the same order beginning at 09:00 AM, and noted the time that each blood sample was taken.

2.3. Perfusion and tissue collection

To investigate the effect of food availability on the central control of reproduction and testicular development, on day 29 I collected the brain and testes of each bird. Following deep anesthesia induced by intramuscular injection (250 µl into each pectoral muscle) of a ketamine/xylazine cocktail (ketamine: 8 mg/0.5 ml [160 mg/kg]; xylazine: 160 µg/0.5 ml [3.2 mg/kg] dissolved in 0.9% NaCl solution), I transcardially perfused birds with 35 ml of wash solution (0.9% NaCl and 0.1% $NaNO_2$ in 0.1 M phosphate buffer, PB), followed by 35 ml of fixative (4% paraformaldehyde and 0.1% $NaNO_2$ in 0.1

M PB). I then decapitated birds, exposed the brain, and placed heads in fixative overnight at 4° C. I also collected the testes and removed all extra connective tissue before weighing to the nearest 0.1 mg and postfixing as described for brains. The day after perfusion, I dissected brains out of the skull and postfixed overnight (4% paraformaldehyde and 0.1% $NaNO_2$ in 0.1 M PB). Brains and testes were gelatin-embedded and cryoprotected according to a modification of a previously published protocol (Saldanha et al., 1994; Small et al., 2008a), and stored at – 80° C until sectioned.

I coronally sectioned brains at a thickness of 30 μm using a cryostat at –21°C, with the stereotaxic atlas of the canary (*Serinus canaria*) brain as a reference (Stokes et al., 1974). Sections were divided into four parallel series by systematically alternating between four separate six-well Falcon plates containing cryoprotectant solution (Watson et al., 1986) and stored in cryoprotectant at – 20° C until immunolabeled. One series was used for each assay (cGnRH-I, GnIH, or NPY).

2.4. GnRH, GnIH, and NPY immunocytochemistry

I 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. GnRH-I is primarily synthesized in the preoptic area (POA; Dawson and Goldsmith, 1997; Parry et al., 1997) and GnIH is synthesized solely in the paraventricular nucleus (PVN; Osugi et al., 2004; Tsutsui et al., 2010). NPY-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; Boswell, 2001; Boswell et al., 2002; Kuenzel and McMurtry, 1988). Therefore, from each bird I randomly selected sections covering the

entire POA, PVN, and IN for GnRH, GnIH, and NPY, respectively; Fig. 14). For GnIH and NPY, I stained an average of 10 sections per bird and for GnRH an average of 6 sections per bird. Since sections were randomly selected from each bird, I 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 the Deviche laboratory (Small et al., 2008a). The specificity of the NPY antiserum has been established in the chicken (Kuenzel and McMurtry, 1988) and Ring Dove (*Streptopelia risoria*; Strader and Buntin, 2001). Preabsorption of the NPY antiserum with human/rat NPY (Bachem, Torrance, CA, USA) before application to towhee brain sections abolished the staining. Following two washes in 0.1 M phosphate buffer (PB) for 30 min, I serially exposed sections to 0.36% H_2O_2, washed them 3 x 5 min in 0.1 M PB, blocked background immunoreactivity for 1 h (see below), and incubated sections overnight in primary antiserum (see below). I 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 (see below), 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, I 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, Pittsburg, PA, USA).

GnRH. I used anti-cGnRH-I antiserum (6DL31/4 prepared by P.J. Sharp) at a dilution of 1: 10,000 in 0.3% PBT. To block non-specific sites, I used normal rabbit serum (Vector Laboratories, Inc.; 1: 200 in 0.3% PBT), and I used biotinylated rabbit anti-sheep IgG (Vector Laboratories, Inc.; 1: 200 in 0.3% PBT) as a secondary antibody.

GnIH. I used anti-quail GnIH antiserum (Tsutsui et al., 2000) at a dilution of 1: 5,000 in 0.3% PBT. I 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. I used anti-human/rat NPY antiserum (Bachem, Torrance, CA, USA) at a dilution of 1:10,000 in 0.3% PBT. I 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.

2.5. Immunocytochemistry data collection

All data were collected without knowledge of the treatment group. For each bird, I counted the number of cells immunoreactive for GnRH-I, GnIH, and NPY present in each immunostained section. I 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 fibers in the IN (Fig. 14), 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. I 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 analyzed. Digitized images were analyzed 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 fibers in the median eminence (ME), I took images from two sections per brain. I corrected for background staining of each image as described above. On the resulting image, I used Image-pro Plus to measure the optical density of five areas of interest (AOI, 65 × 65 μm each) per brain section. AOIs were evenly spaced from left to right along the ventral edge of the ME. I calculated an average optical density for each section, then an average for each bird.

2.6. Testicular morphology

I sectioned testes at a thickness of 30 μm using a cryostat at – 21° C and stored sections in 0.1 M phosphate buffer until mounting on glass microscope slides later the same day. After allowing sections to dry at room temperature for 24 h, I rehydrated them through a graded ethanol series before staining with hematoxylin (S212A, Poly Scientific, Bay Shore, NY, USA) for 3 min. I 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, I stained sections with eosin (S176, Poly Scientific, Bay Shore, NY, USA) 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 et al., 2007). I randomly selected eight sections from each bird (four from each testis) and, using Image Pro, 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. I also recorded when spermatozoa were present in the seminiferous tubules.

2.7. Testosterone assay

To quantify plasma testosterone, I 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). This assay has been validated for Abert's Towhee in the Deviche laboratory (Fokidis et al., 2009). I 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. Average assay sensitivity was 9.4 pg/ml. 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 less than 5 % crossreactivity with 17β-estradiol, 5α-dihydrotestosterone, corticosterone, and progesterone (manufacturer's specifications).

2.8. Statistical analyses

To analyze 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 testosterone I used a two-way analysis of variance with repeated

measures (rmANOVA). The sphericity assumption was verified using Mauchly's test. To analyze the effect of food availability on the number of GnRH, GnIH, and NPY cells, I used Kruskal-Wallis test followed by Dunn's pairwise comparison. GnRH and GnIH measures (median number of ir-cells, cell body area and optical density, and optical density of fibers in the ME), and seminiferous tubule diameter I used one-way ANOVA. I 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 analyzed using parametric methods are presented as means ± standard error of the mean (SEM) and data analyzed using non-parametric methods are presented as medians ± interquartile range (IQR). All graphs depict untransformed data.

3. Results

3.1. 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. 15). Body mass of *ad libitum*-fed birds was similar throughout the experiment (Tukey HSD, $P \geq 0.05$). Two weeks of food restriction caused body mass to decrease in both the reinstated *ad libitum* and the food-restricted groups (Tukey HSD, $P \leq 0.05$). Body mass did not decrease further in birds maintained on restricted food availability, but returning birds to *ad libitum* food availability for two weeks resulted in a body mass increase to levels similar to those at the beginning of the study (Tukey HSD, $P < 0.05$).

3.2. 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. 15). Fat scores of birds exposed to *ad libitum* food availability decreased over the four 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 two weeks, but returning birds to *ad libitum* food availability for two weeks caused fat score to increase to levels similar to those at the beginning of the study (Tukey HSD, $P < 0.05$).

3.3. Pectoral muscle score

Pectoral muscle scores were significantly affected by food availability ($F_{2, 19} = 10.94$, $P < 0.001$), time ($F_{2, 38} = 57.08$, $P < 0.001$), and the interaction between these factors ($F_{4, 38} = 24.72$, $P < 0.001$; Fig. 15). 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 two weeks caused it to increase to levels similar to those at the beginning of the study (Tukey HSD, $P < 0.05$).

3.4. GnRH

I 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

area ($F_{2, 21}$ = 0.24, P = 0.79) or optical density ($F_{2, 21}$ = 2.07, P = 0.15), or the optical density of ME GnRH-I -ir fibers ($F_{2, 21}$ = 0.40, P = 0.68; Table 4).

3.5. 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 perikarya ($F_{2, 21}$ = 3.37, P = 0.056) or the density of ME GnIH-ir fibers ($F_{2, 21}$ = 0.70, P = 0.51; Table 4).

3.6. NPY

The number of NPY-ir cells was a function of food availability treatment (H = 7.56, 2 *d.f.*, P = 0.023; Fig. 16), with food-restricted birds having more NPY-ir cells than *ad libitum* or reinstated *ad libitum* birds (Dunn's method, $P < 0.05$).

3.7. Testicular physiology and morphology

There was a significant overall 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. 17). Post hoc tests revealed that the three groups had similar plasma T at the start of the study, but plasma T was higher in *ad libitum*-fed birds at weeks two and four (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 weight ($F_{2, 21}$ = 2.21, P = 0.14; Table 5) or seminiferous tubule diameter ($F_{2, 21}$ = 0.61, P =

0.56; Table 5). Furthermore, spermatozoa were present in the seminiferous tubules of all birds.

3.8. 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. 17). Cloacal protuberance width of birds exposed to *ad libitum* food availability increased during the first two weeks of the experiment (Tukey HSD, P < 0.05), but did not increase further during the last two 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 two weeks caused cloacal protuberance width to increase to levels similar to those of the *ad libitum* birds.

4. Discussion

It has long been recognized that food availability plays a crucial proximate role in the reproductive recrudescence of seasonally breeding birds (Hahn et al., 2005; Lack, 1968; Williams, 2012), but the mechanism mediating this role remains unclear. Food availability is thought to modulate reproductive recrudescence partly via energetic status. Limited food availability may constrain reproductive recrudescence because birds lack the necessary endogenous energy stores (Drent and Daan, 1980; Hahn et al., 2005; Meijer and Drent, 1999; Wingfield and Kenagy, 1986). By contrast, greater food availability lifts this constraint because birds are able to obtain sufficient endogenous energy stores for developing reproductive tissues. Studies in controlled laboratory

conditions aimed at testing whether food availability modulates reproductive recrudescence through its effects on energetic status must, therefore, ensure that food availability treatments produce a disparity in the energetic condition of birds. To that end, I 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.

Despite considerable differences in energetic status, 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, I would have expected the energetic constraint imposed by the food restriction regime to reduce photoinduced testis growth. As I detected no such reduction, my observations are consistent with the proposition that testis growth in the Abert's Towhee may not be particularly energetically demanding. Such a finding is consistent with results of a study by Caro and Visser (2009) in which Great Tits (*Parus major*) exposed to ambient temperatures of 8°C or 22°C during photoperiodically induced reproductive recrudescence had different basal metabolic rates, but showed similar testicular growth. I should point out that neither my study nor that of Caro and Visser (2009) can exclude the possibility that testis growth is energetically demanding, but even 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 and 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 and 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 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 characteristic male reproductive behaviors, such as singing, courtship, territorial aggression, and mate guarding (Foerster et al., 2002; Hau, 2007; Kurvers et al., 2008). These behaviors by themselves confer energetic costs (Hasselquist and Bensch, 2008; Oberweger and Goller, 2001; Ward et al., 2003; Ward and Slater, 2005), which are likely compounded by indirect costs through reduction in the time spent at rest and for self-maintenance behaviors (Lynn et al., 2000) and foraging (Thomas et al., 2003). Behaviors that are stimulated by T also increase predation risk (Schmidt and 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). I suggest, therefore, that my 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 behaviors. In support of this suggestion, adult male Zebra Finches (*Taeniopygia guttata*) subjected to short term (4 – 10 hours) food deprivation decreased plasma T, singing rate, and courtship behavior toward

females (Lynn et al., 2010). If the hypothesis that T production and secretion are suppressed during periods of energy constraint to avoid the costs associated with T-mediated male reproductive behaviors is correct, such a system would allow the reproductive morphology of birds to develop at approximately the correct time (as determined by photoperiod), but the characteristic male reproductive behaviors necessary for breeding may be inhibited due to low plasma T. Once food availability, and thus energetic status, is suitable, plasma T might increase, causing the expression of male reproductive behaviors and the commencement of breeding. However, my finding that plasma T was still low after the reinstated *ad libitum* group had two weeks of *ad libitum* food access suggests that there may be long-term (i.e., on the order of weeks) effects of poor energetic status on T secretion.

The mechanism by which food availability affects T production is unclear. This mechanism may involve reduced gonadotropins production and/or secretion. Food availability manipulations of wild and domesticated avian species generally show that food restriction decreases the production and secretion of LH and FSH, and reduces the sensitivity of the pituitary gonadotrope to GnRH (Tanabe et al., 1981; Lal et al., 1990; Kobayashi et al., 2002; Hoshino et al., 1988; Hahn, 1995; but see Perfito et al., 2008). Decreased production and secretion of gonadotropins may itself result from impairment of the GnRH system. In laying hens food restriction appears to reduce the releasable store of GnRH-I in the ME (Bruggeman et al., 1998). Furthermore, providing laying hens with *ad libitum* food after a period of food restriction increased hypothalamic GnRH-I mRNA (Ciccone et al., 2007). In cockerels, food restriction decreased the *in vitro* baseline and depolarization-induced release of GnRH-I from isolated mediobasal hypothalami (Lal et al., 1990). Taken together, these studies in chickens suggest that food restriction inhibits production of GnRH-I mRNA and release of the peptide in this

species. However, I found no effect of food restriction on the hypothalamic GnRH system of towhees or on the amount of their ME GnRH-I-ir. It is possible that changes in GnRH-I expression similar to those observed in chickens occurred in food-restricted towhees, but these changes are not reflected in changes in hypothalamic levels of the peptide. Future studies that simultaneously measure the production, storage, and secretion of GnRH are warranted to shed light on whether results in domesticated species apply to free-ranging birds.

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 recrudescence through inhibitory actions on gonadotropes (Bentley et al., 2003; Perfito et al., 2011; Small et al., 2008a). Furthermore, via its 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 food restriction. In addition, food-restricted birds had more IN NPY-ir perikarya than *ad libitum*-fed birds. These observations are consistent with the idea that food restriction was associated with increased activity of the NPY system that, in turn, promoted the release of GnIH. Enhanced GnIH secretion may have reduced gonadotropin secretion and consequently plasma T. I suggest, therefore, that improving our understanding of the interactions between GnIH, NPY, 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.

Finally, food availability may affect plasma T through gonadally produced GnIH. This peptide and its receptor are expressed in the avian testis, and experimental evidence

supports a functional role for GnIH in the control of gonadal endocrine function. The gonadotropin-stimulated secretion of T by cultured House Sparrow (*Passer domesticus*) testes is reduced by application of GnIH (McGuire and Bentley, 2010). In addition, recent research suggests that gonadal GnIH activity during reproductive recrudescence responds to metabolic signals. In an *in vitro* experiment on cultured testes collected from European Starlings prior to breeding, administration of corticosterone, the main glucocorticoid in birds, increased GnIH expression and decreased the gonadotropin-stimulated increase in T secretion (McGuire et al., 2013). Furthermore, using the same *in vitro* system, McGuire et al. (2013) found that metabolic stress, induced by inhibiting glucose and fatty acid metabolism, decreases T production. Overall, the effects of food availability on T production are potentially mediated at multiple levels of the HPG axis. Further research is, however, needed to shed light on the specific mechanism(s) involved.

In contrast to the long-term effects on T secretion, cloacal protuberance width appears to be highly sensitive to changes in energetic status. Indeed, two weeks of food restriction caused this organ to decrease in size. Furthermore, returning food-restricted birds to *ad libitum* food availability for an additional two weeks caused cloacal protuberance width to rapidly increase and reach a size similar to *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 5α-dihydrotestosterone (DHT) stimulates growth of the cloacal protuberance (Owen-Ashley et al., 2004; Tramontin et al., 2003). I am 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 et al., 2008). Therefore, further research is needed to examine whether DHT is also responsive to food availability.

4.1. Conclusion

Many birds use food availability as an important proximate environmental cue to optimally time seasonal reproductive recrudescence. 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. I hypothesized that reproductive recrudescence is constrained by energetic status. Indeed, I 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. My 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 in the amount of GnRH-I peptide in the hypothalamus. There also appears to be a role for testicular endocrine function in modulating reproductive recrudescence in light of energetic status. Despite no evidence that testicular growth is modulated by energetic status, the secretion of T is 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 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.

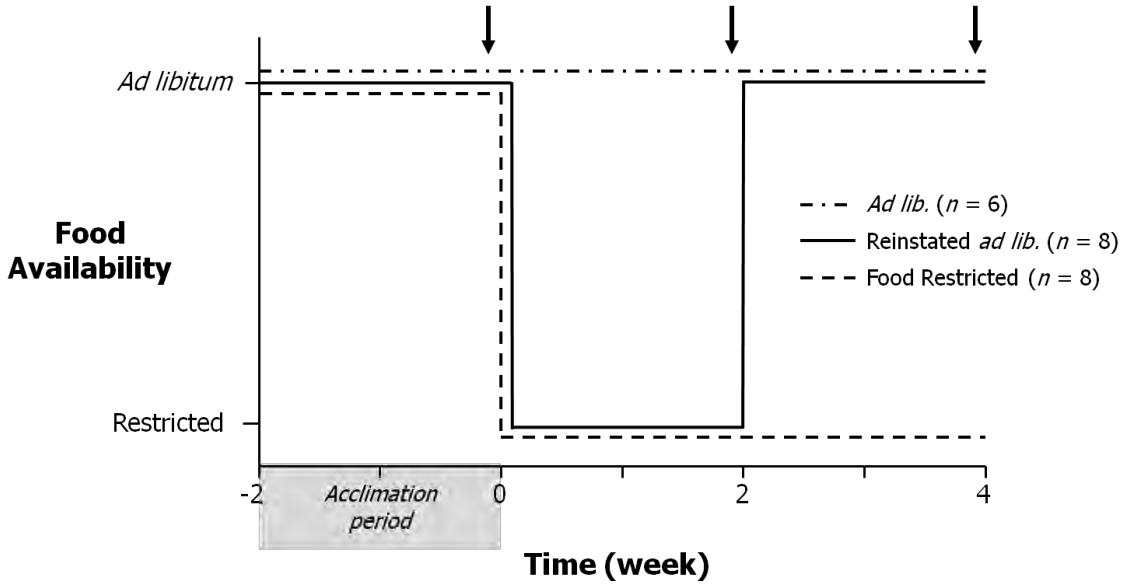

Fig. 13. Schematic representation of the food availability regime. During the acclimation period, I quantified the daily food consumption of each bird. Food-restricted birds received 70% of their individual *ad libitum* consumption. Photoperiod for the duration of the experiment shown in the schematic was 14 L: 10 D throughout. Arrows indicate the collection of blood samples and morphometric data. Following the third collection period, I also collected the brain and testes from each bird.

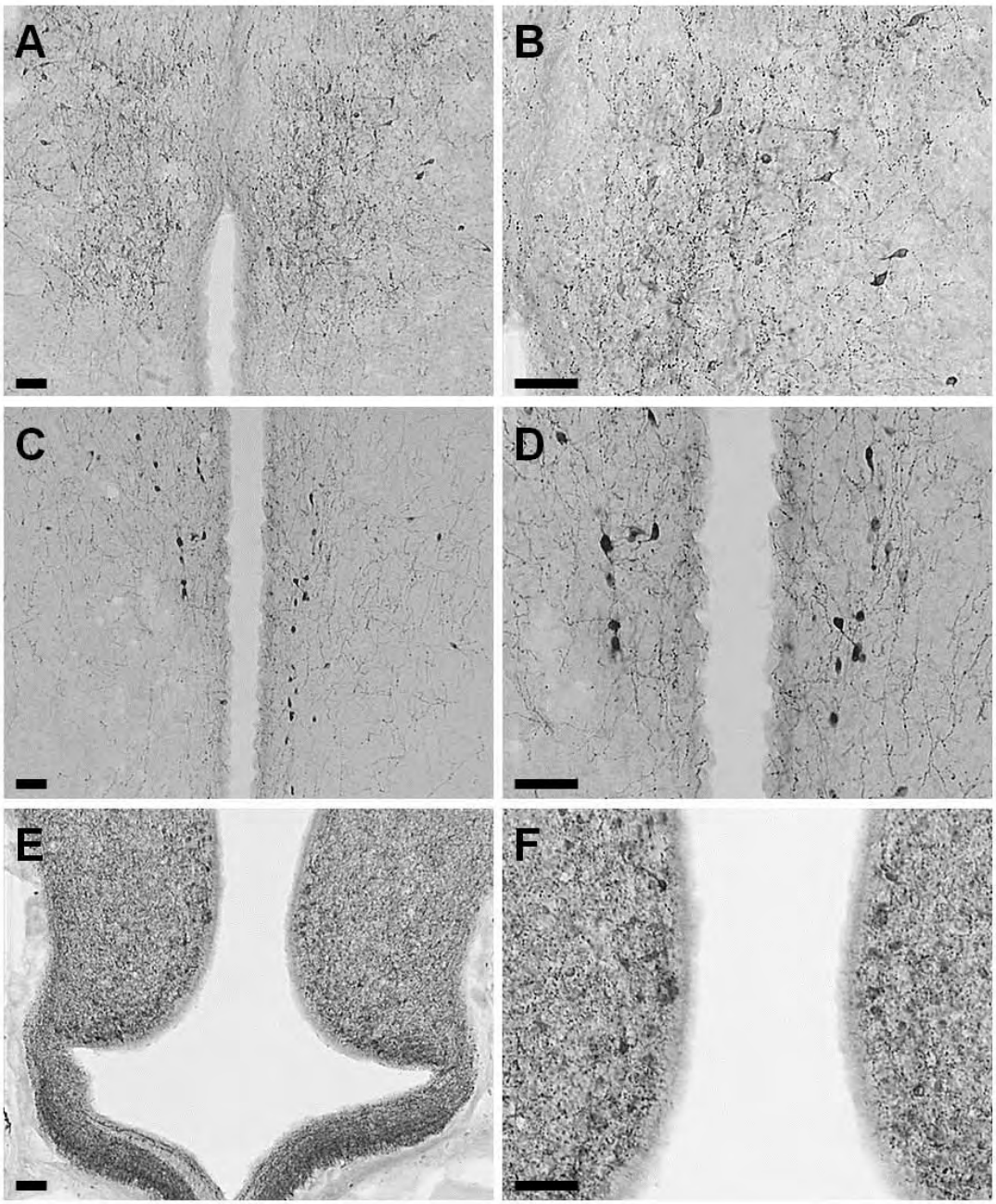

Fig. 14. Representative photomicrographs of immunolabelled coronal brain sections of male Abert's Towhees (*Melozone aberti*) under lower magnification (left column) and higher magnification (right column). A and B immunoreactive gonadotropin-releasing hormone-I (GnRH-I) perikarya in the preoptic area. C and D immunoreactive gonadotropin-inhibitory hormone (GnIH) perikarya in the paraventricular nucleus. E and F immunoreactive neuropeptide Y (NPY) perikarya in the infundibular nucleus. Scale bars = 50 μm.

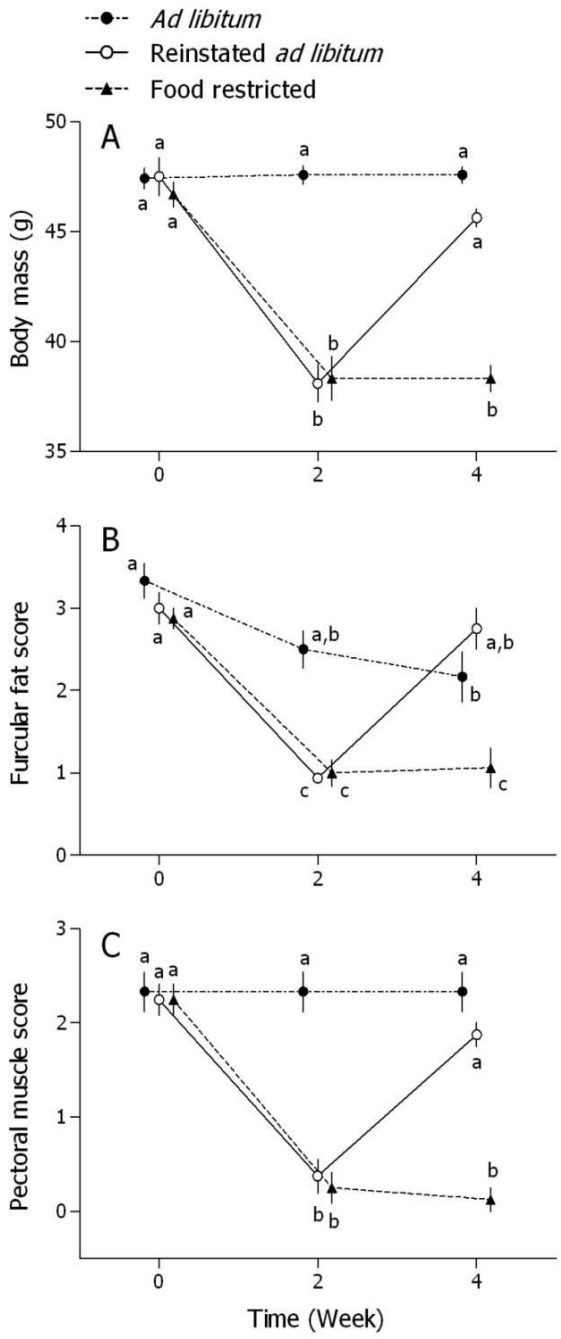

Fig. 15. Body mass, furcular fat, and pectoral muscles are modulated by food availability in adult male Abert's Towhees (*Melozone aberti*). Body mass (A), furcular fat score (B), and pectoral muscle score (C) were reduced following two weeks of food restriction (i.e., 70% of *ad libitum* consumption), and returning birds to *ad libitum* availability increased these parameters to levels similar to those of the control group. For details of study design and sample sizes, see Fig. 1 and the methods section. Data points are means ± SEM, and points with identical letters are not significantly different ($P > 0.05$; Tukey HSD test). For visual clarity, points have been separated along the horizontal axis.

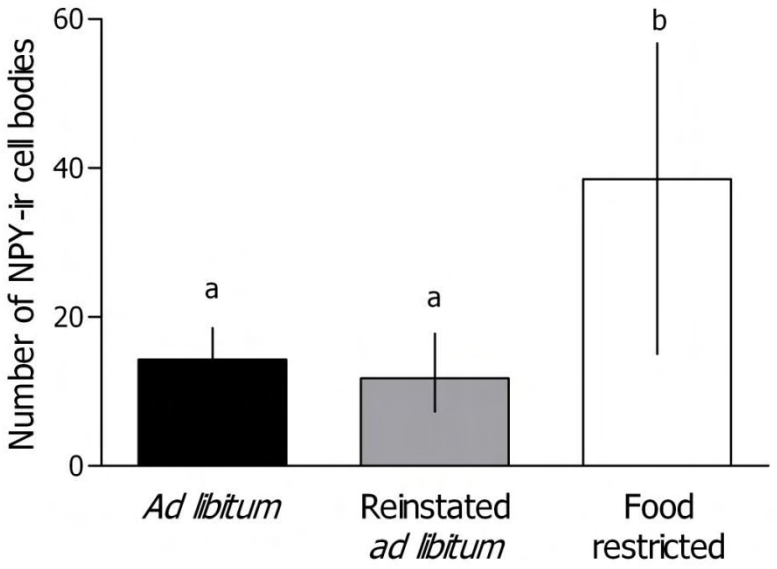

Fig. 16. Food restriction increased the number of neuropeptide Y immunoreactive (NPY-ir) cell bodies in the infundibular nucleus of adult male Abert's Towhees (*Melozone aberti*). Birds were exposed to either *ad libitum* food availability for four weeks (n = 6; "*Ad libitum*"), two weeks of food restriction (70% of *ad libitum* consumption) followed by two weeks of *ad libitum* food (n = 8; "reinstated *ad libitum*"), or restricted food availability for four weeks (n = 8; "food-restricted"). Data are medians ± IQR, and bars with identical letters are not significantly different ($P > 0.05$; Dunn's method).

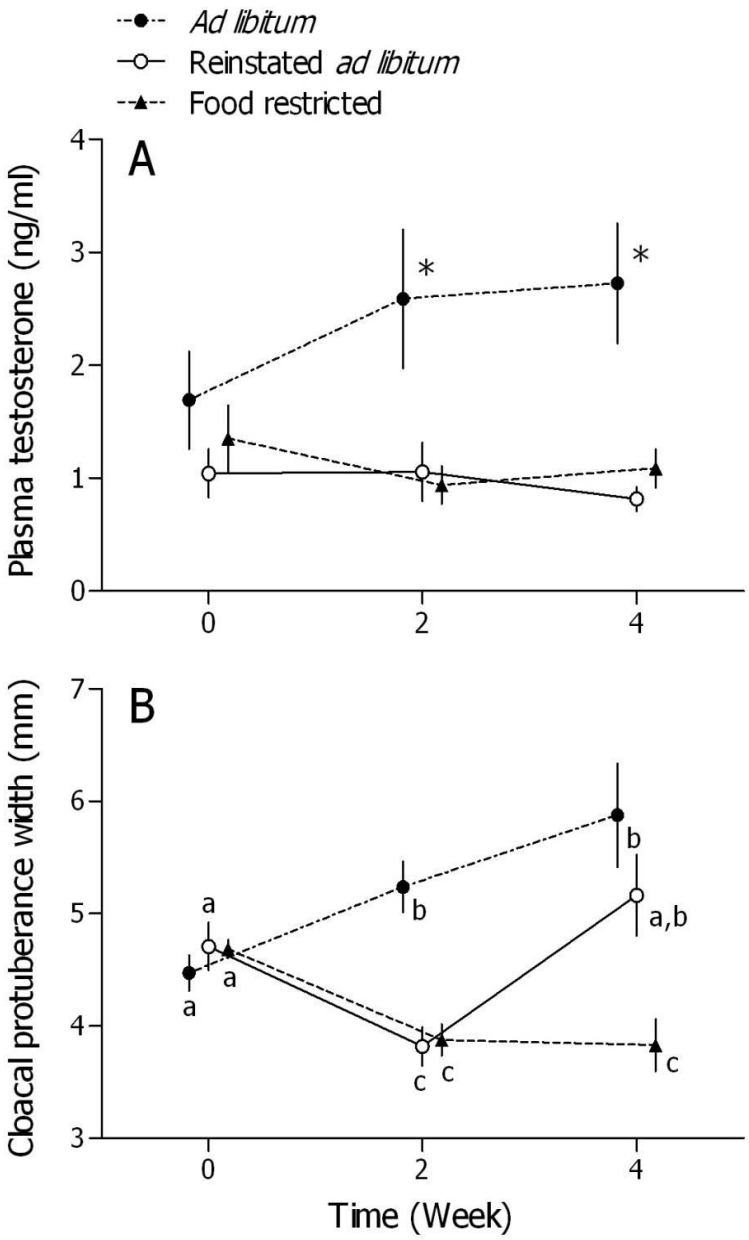

Fig. 17. Plasma testosterone and cloacal protuberance width are modulated by food availability in adult male Abert's Towhees (*Melozone aberti*). Plasma testosterone (A) was lower in birds exposed to two (reinstated *ad libitum*) or four (restricted) weeks of food restriction (70% of *ad libitum* consumption) compared to control birds with *ad libitum* food availability. An asterisk denotes a significant difference between groups within each time point. Cloacal protuberance width (B) 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 HSD test). For details of study design, see Fig. 1 and the methods section. Data points are means ± SEM. For visual clarity, points have been separated along the horizontal axis.

Table 4. The number, area, and optical density of cell bodies and optical density of fibers in the median eminence (ME) immunolabeled for gonadotropin-releasing hormone-I (GnRH-I) and gonadotropin-inhibitory hormone (GnIH) of adult male Abert's Towhees (*Melozone aberti*) following food availability treatment. Birds were exposed to *ad libitum* food availability for four weeks (n = 6; "*Ad libitum*"), two weeks of food restriction (70% of *ad libitum* consumption) followed by two weeks of *ad libitum* food (n = 8; "reinstated *ad libitum*"), or restricted food availability for four weeks (n = 8; "food-restricted"). When the overall analysis was significant, superscript letters indicate significant differences between the groups ($P \leq 0.05$; Tukey HSD test). AU = arbitrary units. Unless indicated otherwise, data presented are means (± SEM).

		Food availability treatment group			Statistics
		Ad libitum	Reinstated *ad libitum*	Food-restricted	*P*-value
GnRH	Number of cells/section[a]	15.5 (13.0)	14.4 (3.3)	14.8 (16.6)	0.76
	Cell body area (μm²)	62.1 (±3.1)	62.0 (±2.6)	59.7 (±2.7)	0.79
	Cell body optical density (AU)	0.27 (±0.01)	0.25 (±0.01)	0.29 (±0.02)	0.15
	ME fiber optical density (AU)	0.19 (±0.04)	0.20 (±0.03)	0.16 (±0.03)	0.68
GnIH	Number of cells/section[a]	39.5 (±20.0)	48.0 (±33.8)	31.0 (±39.8)	0.65
	Cell body area (μm²)	68.2 (±3.2)[a]	60.5 (±2.5)[a,b]	59.2 (±1.6)[b]	0.045
	Cell body optical density (AU)	0.48 (±0.03)	0.45 (±0.03)	0.39 (±0.02)	0.056
	ME fiber optical density (AU)	0.15 (±0.01)	0.15 (±0.01)	0.16 (±0.01)	0.51

[a] data are presented as medians and interquartile range.

Table 5. Testicular recrudescence of adult male Abert's Towhees (*Melozone aberti*) was not affected by food availability. Towhees were exposed to either *ad libitum* food availability for four weeks ("*Ad libitum*"), two weeks of food restriction (70% of *ad libitum* consumption) followed by two weeks of *ad libitum* food ("reinstated *ad libitum*"), or restricted food availability for four weeks ("food-restricted") before I collected testes. Data presented are means (± SEM).

	Food availability treatment group			Statistics
	Ad libitum	Reinstated *ad libitum*	Food-restricted	*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

CHAPTER 6

CENTRAL NEUROPEPTIDE Y ADMINISTRATION STIMULATES FEEDING BEHAVIOR WITHOUT INFLUENCING PLASMA TESTOSTERONE IN A SONGBIRD

In mammalian and avian model species, neuropeptide Y (NPY) simultaneously promotes feeding behavior and suppresses the secretion of reproductive hormones, thereby modulating the resource allocation trade-off between investing into essential somatic processes or into the reproductive system. Investigations into this dual role of NPY in birds have focused on domesticated species and, to my knowledge, no study has examined this role in songbirds. I determined whether NPY treatment acutely regulates feeding behavior and activity of the reproductive system in a male songbird, the Abert's Towhees (*Melozone aberti*). Intracerebroventricular (ICV) administration of NPY promoted behaviors associated with feeding (latency to initiate pecking in the food bowl, number of feeding bouts following treatment, and number of pecks into the food bowl during each feeding bout), and it stimulated hopping and drinking behavior. By contrast, I found no effect of NPY treatment on plasma testosterone secretion. These results suggest that, unlike in mammalian and other avian model species, in male Abert's Towhees NPY stimulates feeding behavior without concurrently influencing T secretion.

1. Introduction

The ability to optimally regulate energetic status is essential not only for survival but also for reproduction. Life history theory posits that there is a resource allocation trade-off between the energetic requirements for survival and reproduction (Stearns, 1989; Zera and Harshman, 2001). When energy is limited, reproductive activity, including the secretion of reproductive hormones, is curbed in favor of essential somatic processes, and behavior is redirected to rectify energetic imbalance (Hill et al., 2008;

Schneider et al., 2013). Once energy requirements are met, reproductive activity can resume. An important behavior that regulates energetic status is feeding. In rodent and domesticated avian species, neuropeptide Y (NPY) stimulates feeding behavior in response to changes in energetic status (Boswell, 2001; Marty et al., 2007; Mobbs et al., 2005; Pralong, 2010), and concurrently modulates the secretion of reproductive hormones (see below; Hill et al., 2008; Minami and Sarkar, 1992; Pralong, 2010). This peptide, therefore, is potentially an important orchestrator of the trade-off between survival and reproduction.

The NPY gene and the stimulatory effect of the peptide on feeding behavior appear to be conserved across vertebrates (Mercer et al., 2011). The available evidence suggests that during periods of energy limitation, decreased plasma concentrations of leptin and insulin (Schwartz et al., 1991; Schwartz et al., 1996), and increased ghrelin and glucocorticoids (Nakazato et al., 2001; Ponsalle et al., 1992), stimulate NPY production and secretion in cell bodies in the arcuate nucleus of the hypothalamus (Chronwall et al., 1985; Kalra et al., 1999). In turn, these NPY cells stimulate feeding via projections to the paraventricular nucleus of the hypothalamus (PVN; Chronwall et al., 1985; Kalra et al., 1999). However, the majority of studies on the role of NPY in feeding behavior regulation have used rodents and domesticated avian species (Clarke, 2008; Kuenzel et al., 1987). To my knowledge, just one study has investigated this topic in a wild caught bird species, the migratory White-crowned Sparrow (Zonotrichia leucophrys gambelii, Richardson et al., 1995). In preparation for seasonal migration, this species undergoes a period of premigratory fattening, which is characterized by marked increases in feeding behavior and body mass. It is unclear whether NPY plays a similar role in sedentary (i.e., non-migratory) birds that do not experience marked seasonal changes in feeding behavior

and body mass. Hence, the first objective of my study was to examine whether NPY acutely regulates feeding behavior in a sedentary bird.

In addition to its role in feeding behavior, evidence from mammalian species suggests that NPY modulates the activity of the reproductive system (Hill et al., 2008; Minami and Sarkar, 1992; Pralong, 2010). Supporting this hypothesis, cells that produce gonadotropin-releasing hormone (GnRH), the neurohormone essential for initiating reproductive development (Kuenzel, 2000; Sharp and Ciccone, 2005), interact with NPY cells. NPY neurons contact cell bodies in the preoptic area of the hypothalamus that produce GnRH (Kuenzel, 2000), and influence the release of GnRH from the median eminence (ME, Contijoch et al., 1993). In addition, there are interactions between NPY neurons and neurons that produce gonadotropin-inhibitory hormone (GnIH), a neurohormone that acts in opposition to GnRH (Clarke and Parkington, 2013; Greives et al., 2008; Tsutsui et al., 2013). In the sheep, NPY neurons receive projections from GnIH neurons (Clarke et al., 2009). Furthermore, in the chicken, the PVN, the main site of GnIH synthesis (Tsutsui et al., 2012), contains a dense network of NPY neurons (Kuenzel, 2000), and NPY content in this area responds to energetic status (Zhou et al., 2005). Neuropeptide Y is, therefore, a potential neuromodulatory link between energetic status and the reproductive system. However, there are few experimental investigations on this topic in birds and, to my knowledge, none in a seasonally breeding, wild-caught bird species. Hence, the second objective of my study was to determine whether NPY modulates the activity of the reproductive system in such a species.

2. Methods

2.1. Animals and housing conditions

All procedures were approved by the Institutional Animal Care and Use Committee at Arizona State University. The studies described here used adult male Abert's Towhees (*Melozone aberti*). This songbird is appropriate for this study because it is sedentary and its body mass is seasonally consistent (S. Davies, unpublished data). Male towhees also undergo reproductive development in captivity in response to photostimulation (S. Davies, unpublished data). Furthermore, in captivity Abert's Towhees readily consume a uniform pellet diet (Mazuri small bird maintenance diet, PMI Nutrition International, Richmond, IN, USA), making quantification of feeding behavior tractable.

Using mist nets and conspecific song playback, I caught 10 adult male Abert's Towhees between Oct 30th and Nov 4th 2013 from Robbins Butte Wildlife Area, Maricopa County, Arizona, USA (247 m above sea level; latitude: 33°19'N; longitude: 112°38'W). To permit individual identification, each bird received a uniquely numbered aluminum leg band. I transferred towhees to Arizona State University animal care facilities and housed them in individual cages (60 cm × 40 cm × 40 cm) with opaque barriers on two sides, maintained them at a constant temperature of 23 °C (±1 °C), and provided *ad libitum* food and water. Initially, birds were maintained on a 9L:15D light cycle, but to induce gonadal development I increased photoperiod in two steps: the first, on Nov 5th, was increased to 14.5L:9.5D, and the second, on Dec 3rd, was increased to 16L:8D. One bird died before the start of the experiment.

2.2. Intracerebroventricular cannulation

To centrally administer NPY, on Jan 14th and 15th 2014 each bird was chronically implanted with a cannula aimed at the right lateral ventricle. Prior to implantation, I anaesthetized each bird using a cocktail of ketamine (~160 mg/kg) and xylazine (~3.2 mg/kg), then removed the feathers from the top of the head to the nape and cleaned the area with povidone-iodine. I then fixed its head in a stereotaxic frame and secured it anteriorly at the ventral surface of the beak and posteriorly at the external auditory meatus. The beak was then pointed down at 45°, and I made a ~2 cm long incision in the skin to expose the skull. Using a 25 gauge hypodermic needle, I made a small hole (~2 mm diameter) in the skull 1 mm lateral to the mid-sagittal sinus. I then inserted a 4 mm long, 26 gauge steel cannula guide (Plastics One, Roanoke, VA, C315G; containing a 4 mm long implanting cannula to keep the guide cannula free of obstructions) 3 mm ventral to the surface of the brain. I used the stereotaxic atlas of the canary, *Serinus canaria*, brain (Stokes et al., 1974) and thionin-stained Abert's Towhee brain sections to estimate where cannulas should be implanted. The guide was then affixed to the skull with cranioplastic cement. Once the cement was dry, I replaced the implanting cannula with a 28 gauge internal dummy cannula (Plastics One, C315DC). I then closed the incision with cyanoacrylate adhesive (3M, St. Paul, MN, USA), applied a topical antibiotic (Alpharma, Baltimore, MD, USA) to the incision site, and returned the bird to its home cage. All birds were allowed to recover for at least 7 days before the start of the experiments (described below). To confirm cannula placement in the lateral ventricle, at the end of the experiments I injected 5 µl of India ink into the cannula. Ten minutes after the injection, birds were euthanized and their brains excised and then frozen on dry ice. I sectioned brains in a cryostat at -18 °C and determined the location of the ink by scoring

whether ink was visible (1) ipsilaterally, (2) contralaterally, and/or (3) in the third ventricle.

2.3. Infusates and injections

The NPY infusate consisted of porcine NPY (Sigma-Aldrich, St. Louis, MO, USA, Cat. No. N3266) dissolved in 0.1 M phosphate buffer (PB) vehicle and diluted, again using PB vehicle, to final concentrations of 0.0625 μg/μl or 0.25 μg/μl. These NPY doses were selected based on their ability to increase food intake of male White-crowned Sparrows (Richardson et al., 1995) and Ring Doves (*Streptopelia risoria*; Strader and Buntin, 2001) following ICV administration. The working solutions of NPY and PB vehicle were frozen as aliquots, stored at -20°C, and thawed as needed for injections.

For ICV injections, birds were removed from their home cage and held in the experimenter's hand. The internal dummy cannula was removed and replaced with a 33 gauge, 4.5 mm infusion cannula (Plastics One, VA, USA) attached via polyethylene tubing to a 10 μl Hamilton syringe. The infusate (4 μl) was injected over ~30 s and the cannula held in place for 1 min following injection. I then reinstalled the dummy cannula and returned birds to their cage. To obviate any masking effect of the early morning hyperphagia on food intake, I administered all injections at least 5 hours after the lights in the room were turned on.

2.4. Experiment 1: the effect of ICV NPY injection on feeding behavior

The goal of this experiment was to determine the behavioral response to ICV injection of NPY. Between Jan 21st and 28th 2014, each bird was removed from its home cage and within 1 min received an ICV injection of either vehicle (control) or 0.25 μg/μl NPY (i.e., 1 μg/bird). Birds did not receive the low NPY dose in this experiment because

cannulas are patent only for a limited period and using birds in both experiments precluded administering both doses. The treatment was randomly assigned to each bird. Immediately following the injection, I returned the bird to its home cage and recorded behavior using a high-definition video camera positioned in the center of the room. Similar to previous studies on this topic in birds (Kuenzel et al., 1987; Richardson et al., 1995; Strader and Buntin, 2001), behavior was recorded for a total of 120 min. I separated each trial into four 30 min intervals to examine the temporal profile of the behavioral response to NPY administration. For each interval, I quantified (1) the number of feeding bouts, (2) the number of pecks into the food bowl, (3) the number of drinks from the water bowl, and (4) the number of hops. In the videos, birds clearly could be seen picking up, manipulating, and then eating food pellets. To feed, a bird must perch on the rim of the food bowl, lean into the bowl, and then peck. Therefore, although I did not quantify food intake *per se*, I are confident that the above measures reflect actual food consumption (da Silva et al., 2008). I defined a feeding bout as any occasion that a bird pecked into the food bowl and demarcated the beginning and end as the hopping on to- and off of- the food bowl, respectively. I also quantified the latency to feed (defined as the first peck into the food bowl) after return to the cage. Four days after receiving its first injection, each bird received the opposite treatment and thus served as its own control. To obviate any confounding effect of a diurnal pattern of food intake, I administered first and second treatments at approximately the same time of day for a given bird. Indeed, the difference (± standard error of the mean; SEM) in the time of day between the first treatment and the second for a given bird was on average 37 min (± 13).

2.5. Experiment 2: the effect of ICV NPY injection on plasma testosterone

The goal of this experiment was to test whether ICV injection of NPY acutely modulates testosterone secretion. Between Jan 30th and Feb 5th 2014, each bird was removed from its home cage and a blood sample taken within 1 min to quantify initial plasma testosterone. All blood samples (volume ~125 µl) were collected from the right jugular vein using a heparinized syringe and immediately placed on ice, and the plasma was harvested and frozen at -80 °C within 20 min. Within 1 min of taking the first blood sample, I randomly assigned the bird to receive an ICV injection of either vehicle (control), 0.25 µg NPY, or 1 µg NPY. Each bird received all treatments, with 2 days separating consecutive injections to a given bird. To prevent an effect of treatment order, I randomized the order of treatments for each bird. Thus, each bird served as its own control. The injection method was the same as described above. Immediately following the injection, I returned the bird to its home cage. Sixty min after the injection, I collected a second blood sample (as described above) to quantify post-NPY administration plasma testosterone. The delay between NPY administration and the post-injection blood sample collection was based on the effect of central NPY administration on plasma insulin 30 to 60 min after NPY treatment to chickens (Kuenzel and McMurtry, 1988). Furthermore, central NPY administration decreases plasma luteinizing hormone (LH) in rodents as rapidly as 15 min after treatment and for at least 60 min (Kerkerian et al., 1985; McDonald et al., 1985; Pinilla et al., 2007). To obviate any confounding effect of a diurnal pattern of testosterone secretion, I administered the three treatments to a given bird at approximately the same time of day. Indeed, the difference (± SEM) in the time of day between the three treatments for a given bird was on average 36 min (± 7).

2.6. Hormone assay

I quantified plasma testosterone using validated commercial competitive enzyme-linked immunoassay kits following the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY, USA, Fokidis et al., 2011). All samples were diluted 10× in assay buffer before assay and were assayed on the same day. I randomly assigned samples to assay plates, but assayed all samples from a given bird on the same plate. The average inter- and intra-assay coefficients of variation were 6.2% and 3.8%, respectively, and the assay sensitivity was 0.94 pg/ml.

2.7. Statistical analysis

To test whether NPY administration affected the latency to feed, relative to vehicle treatment, I used a repeated measures ANOVA (rmANOVA) with treatment (i.e., vehicle vs. NPY) as the within-subject factor. I tested whether the temporal profile of behavior differed between treatments using a rmANOVA of ranked data (Conover and Iman, 1981), with time interval and treatment as within-subject factors. I tested the acute effect of NPY administration on plasma testosterone using a rmANOVA with time (i.e., initial vs. post-injection) and treatment (i.e., vehicle, 0.25 μg NPY, or 1 μg/μl NPY) as within-subject factors. Data for rmANOVA met the sphericity assumption (Mauchly's test). Post hoc comparisons were made using Tukey's HSD test, where appropriate. All statistical analyses were performed using SPSS 21 (SAS, Chicago, IL, USA), with $\alpha = 0.05$. All graphs depict untransformed data.

3. Results

3.1. Cannula placement

In all nine birds, ink was visible in the ipsilateral and contralateral lateral ventricles as well as in the third ventricle, indicating that infusates were successfully administered into the ventricular system.

3.2. Behavior

Central administration of NPY decreased the latency to peck in the food bowl ($F_{1,8}$ = 8.59, P = 0.019; Fig. 18). The number of feeding bouts did not differ between the four time intervals ($F_{3,6}$ = 4.48, P = 0.056; Fig. 19). Neuropeptide Y administration increased the number of bouts relative to vehicle treatment ($F_{1,8}$ = 24.34, P = 0.001) and there was an interaction between treatment and time interval ($F_{3,6}$ = 5.76, P = 0.034; Fig. 19). Post hoc comparisons revealed that NPY elevated the number of feeding bouts by the second interval and it remained at this level for the third and fourth intervals. Furthermore, the number of pecks into the food bowl per feeding bout was higher in NPY- than vehicle-treated birds ($F_{1,8}$ = 6.29, P = 0.036) and differed between time interval ($F_{3,6}$ = 8.27, P = 0.015; Fig. 19). There was, however, no treatment x time interval interaction ($F_{3,6}$ = 1.24, P = 0.38).

NPY administration increased the number of hops compared to vehicle administration ($F_{1,8}$ = 9.20, P = 0.016; Fig. 20) and this number varied between time intervals ($F_{3,6}$ = 5.43, P = 0.038). However, the interaction between treatment and time interval was not significant ($F_{3,6}$ = 0.96, P = 0.47). The number of drinks was also higher following administration of NPY compared to vehicle ($F_{1,8}$ = 11.42, P = 0.01) and differed between time interval ($F_{3,6}$ = 14.37, P = 0.004; Fig. 20). However, there was likewise no treatment x time interval interaction ($F_{3,6}$ = 2.28, P = 0.18).

3.3. Plasma testosterone

There was no overall difference between initial and post-injection plasma testosterone ($F_{1,8}$ = 1.48, P = 0.26: Fig. 21). Furthermore, there was no effect of any of the treatments ($F_{2,16}$ =1.06, P = 0.37) or a time x treatment interaction ($F_{2,16}$ = 2.10, P = 0.16) on plasma testosterone.

4. Discussion

Neuropeptide Y has a potent orexigenic effect across vertebrates (Mercer et al., 2011). Among birds, however, almost all research on this topic has focused on domesticated species (Boswell, 2001; Davies and Deviche, 2014), and just one study has investigated a wild caught species. In the migratory White-crowned Sparrow, NPY may play an important role during the premigratory fattening period (Richardson et al., 1995). I am not aware of any study investigating whether NPY administration influences feeding behavior in a sedentary species. My findings that ICV injection of NPY rapidly promotes the expression of feeding behavior in the sedentary male Abert's Towhee support such an effect of NPY also in non-migratory birds.

Compared to vehicle treated birds, NPY treated birds began feeding sooner after being returned to their cage. Neuropeptide Y treatment also rapidly increased the number of feeding bouts and the number of pecks into the food bowl during each feeding bout. My results are, therefore, consistent with the hypothesis that NPY stimulates feeding behavior and, in turn, food intake. The robust stimulatory effect of central NPY administration on feeding behavior of the Abert's Towhee agrees with findings in other avian species. Central injection of NPY rapidly increases food intake in broiler chicks (Kuenzel et al., 1987), Ring Doves (Strader and Buntin, 2001), and White-crowned

Sparrows (Richardson et al., 1995). My results also suggest that the stimulatory effects of NPY on feeding behavior are not exhibited only in the context of premigratory fattening.

In addition to stimulating feeding behavior, NPY treatment to towhees rapidly increased their locomotor behavior. This finding is consistent with results obtained in rodents (DiBona, 2002; Tecott and Heberlein, 1998). For example, NPY administration decreased resting behavior in Syrian hamsters (*Mesocricetus auratus*; Kulkosky et al., 1988) and locomotor behavior is decreased in NPY knockout mice (Bannon et al., 2000), as well as in mice that lack NPY receptor subtypes (Edelsbrunner et al., 2009; Pedrazzini et al., 1998). Furthermore, in rodents NPY suppresses anxiety, fear, and responsiveness to stressful stimuli, such as restraint (Bannon et al., 2000; Li et al., 2002; Thorsell and Heilig, 2002). Neuropeptide Y, therefore, concurrently stimulates hunger, reduces fear, anxiety, and sensitivity to stress, and promotes locomotor behavior (Tecott and Heberlein, 1998). These behavioral changes promote foraging and food acquisition – even during adverse environmental conditions – with the ultimate outcome being to rectify energetic imbalance. Although few studies have measured the behavioral responses to NPY administration in wild birds, if this peptide has similar effects in birds as in mammals, it may regulate energetic status in natural settings. This regulation may be particularly important following events that disrupt food intake, such as nighttime (in diurnal species) or storms, or that incur unusually high energy expenditure, such as low ambient temperature or migration.

NPY is also an important neuromodulator of the reproductive system in birds and mammals (Hill et al., 2008; Minami and Sarkar, 1992; Pralong, 2010). It is surprising, therefore, that I found no effect of central NPY administration on plasma testosterone in adult male Abert's Towhees. However, evidence from mammalian studies suggests that the effect of NPY treatment on the reproductive system is complex and depends upon the

sex steroid milieu. The available data, mostly from female rats, indicate that central administration of NPY stimulates the release of LH in sex steroid-primed animals (Minami and Sarkar, 1992). By contrast, NPY administration inhibits LH release in ovariectomized rats (Kalra and Crowley, 1984; Kerkerian et al., 1985; McDonald et al., 1985), rabbits (Khorram et al., 1987), and monkeys (Kaynard et al., 1990). In male rats, NPY administration stimulates plasma LH when administered to castrated or intact animals (Pinilla et al., 2007). The limited evidence from birds also mirrors the view that the influence of NPY on the reproductive system is complex. Contijoch (1993) reported a stimulatory effect of NPY treatment on LH release *in vitro* from ME tissue extracted from hens during their natural preovulatory LH surge, but no effect was detected before this surge.

I suggest two potential explanations to account for the lack of effect of central NPY administration on plasma testosterone observed here. First, NPY indeed has no influence on the reproductive system of Abert's Towhees in breeding condition. Accordingly, in this species NPY stimulates feeding behavior but does not influence gonadal steroid secretion. Similarly, food intake of male layer chicks is stimulated by central administration of GnIH, but this treatment has no effect on plasma testosterone (Tachibana et al., 2005). Crucially, the effect of GnIH treatment on food intake is hypothesized to be exerted by NPY (Tachibana et al., 2005). If GnIH does indeed increase food intake via effects on NPY cells, the results of the present study are consistent with those of Tachibana et al. (2005).

The second explanation is that the NPY doses and/or the time between administration and the post-injection blood sample collection were unsuitable to detect an effect of the experimental treatment. The doses of NPY used here increased food intake of broiler chicks (Kuenzel et al., 1987), White-crowned Sparrows (Richardson et

al., 1995), and Ring Doves (Strader and Buntin, 2001). Considering that the doses I selected were similar to those used in previous avian studies and that these doses increased feeding behavior in Abert's Towhees, I believe it is unlikely that the doses were unsuitable. In terms of the time between administration and the post-injection blood samples, to my knowledge this is the first study in intact birds examining the effect of central NPY administration on plasma testosterone. Significant effects of central NPY administration on feeding behavior in avian species are generally measured 30 to 60 min after treatment (Kuenzel et al., 1987; Richardson et al., 1995; Strader and Buntin, 2001). Furthermore, in broiler chicks an effect of central NPY administration on plasma insulin was observed in a similar time frame (Kuenzel and McMurtry, 1988). More broadly, studies in rodents observed effects of central NPY administration on plasma LH that begun as rapidly as 15 min after treatment and persisted for at least 60 min (Kerkerian et al., 1985; McDonald et al., 1985; Pinilla et al., 2007). The time between administration and the post-injection blood sample collection in my study is, therefore, consistent with previous avian and mammalian studies aimed at investigating changes in feeding behavior and plasma hormone levels. However, a study by Kalra and Crowley (1984) in female rats observed a rapid (i.e., within 10 min) effect of central NPY treatment on plasma LH, but this effect had dissipated after 60 min. A similar situation may have occurred in the present study. Future studies in wild caught avian species could, therefore, benefit from adopting a finer temporal scale of sampling.

4.1. Conclusion

Life history theory predicts that there is a resource allocation trade-off between the requirements for survival and for reproduction. Feeding behavior is an important component of this trade-off. Evidence from mammalian and avian model species

suggests that NPY simultaneously coordinates feeding behavior and activity of the reproductive system, and, hence, functions in the resource allocation trade-off. However, to my knowledge, no study has examined whether NPY exerts this role in wild caught birds. To address this question, I examined whether NPY administration acutely regulates feeding behavior and activity of the reproductive system in male Abert's Towhees. I found that ICV administration of NPY rapidly promotes behaviors necessary for foraging (locomotion) and feeding (the latency to peck in the food bowl, the number of feeding bouts, and the number of pecks into the food bowl during each feeding bout). By contrast, I found no effect of NPY treatment on plasma testosterone secretion. These results suggest that, unlike in mammalian and other avian model species, in male Abert's Towhees NPY stimulates feeding behavior without also influencing gonadal steroid secretion.

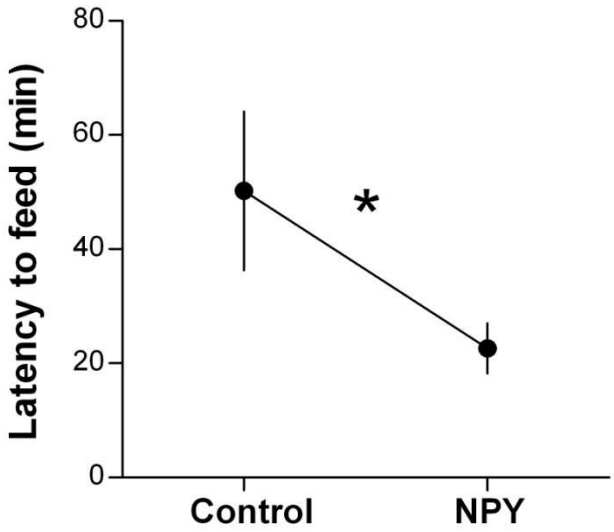

Fig. 18. Neuropeptide Y (NPY) rapidly decreased the latency to feed in adult male Abert's Towhees (*Melozone aberti*). The latency to feed (defined as the time taken to the first peck into the food bowl) was recorded beginning immediately after returning a bird to its home cage following an intracerebroventricular (ICV) injection of either vehicle or 1 µg NPY. I used a crossover design where each bird (n = 9) received both treatments, in a randomized order. Data points are means ± SEM. An asterisk denotes a statistically significant difference between treatments (P < 0.05; Tukey's HSD test).

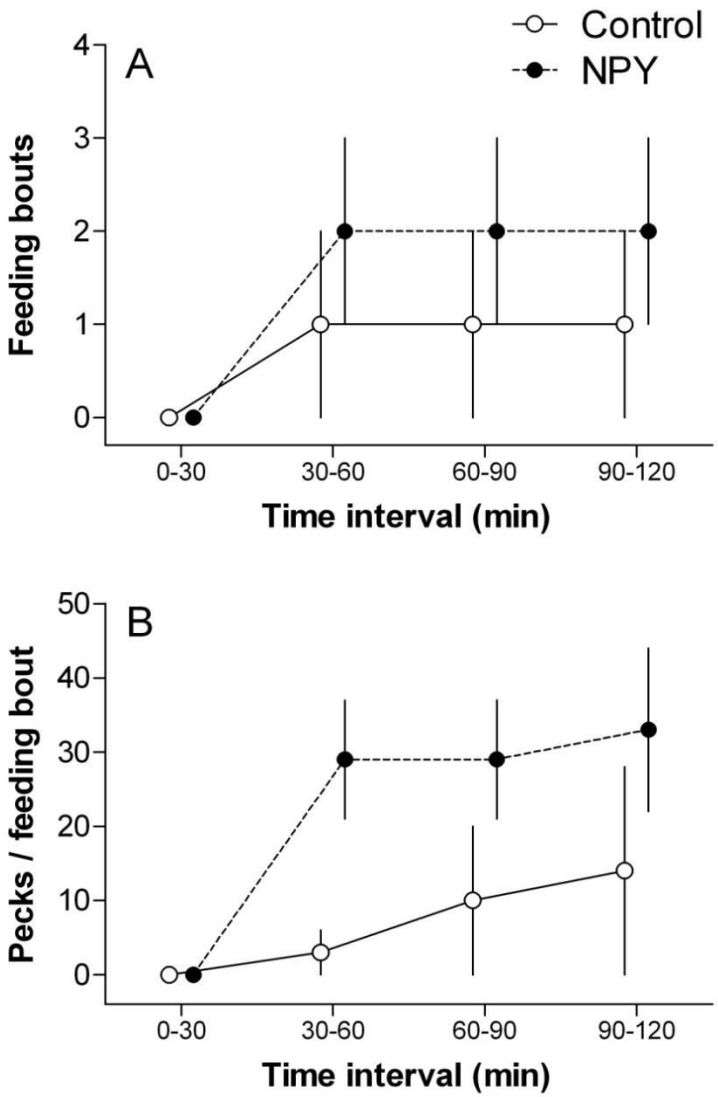

Fig. 19. Neuropeptide Y (NPY) rapidly promoted feeding behavior in adult male Abert's Towhees (*Melozone aberti*). The number of feeding bouts (A) and the number of pecks per feeding bout (B) were recorded beginning immediately after returning a bird to its home cage following an intracerebroventricular (ICV) injection of either vehicle or 1 μg NPY. I used a crossover design where each bird (n = 9) received both treatments, in a randomized order. Data points are medians ± median absolute deviation.

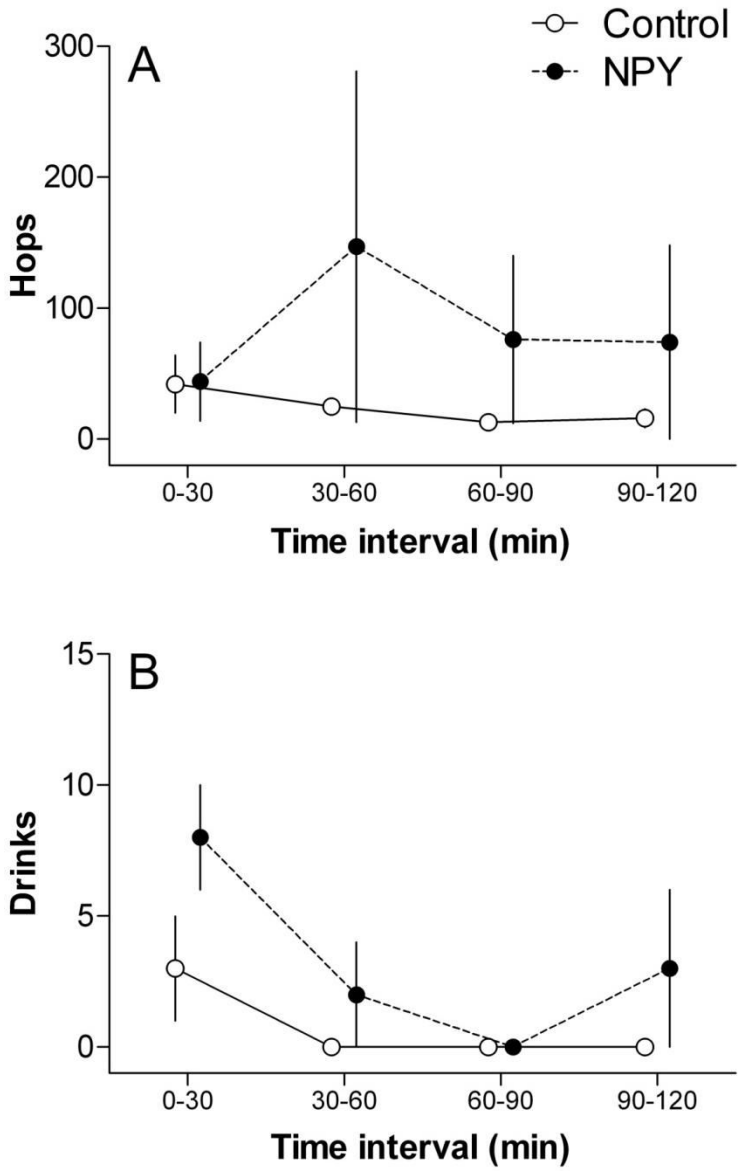

Fig. 20. Neuropeptide Y (NPY) rapidly promotes locomotor and drinking behavior in adult male Abert's Towhees (*Melozone aberti*). The number of hops (A) and drinks from the water bowl (B) were recorded beginning immediately after returning a bird to its home cage following an intracerebroventricular (ICV) injection of either vehicle or 1 μg NPY. I used a crossover design where each bird (n = 9) received both treatments, in a randomized order. Data points are medians ± median absolute deviation.

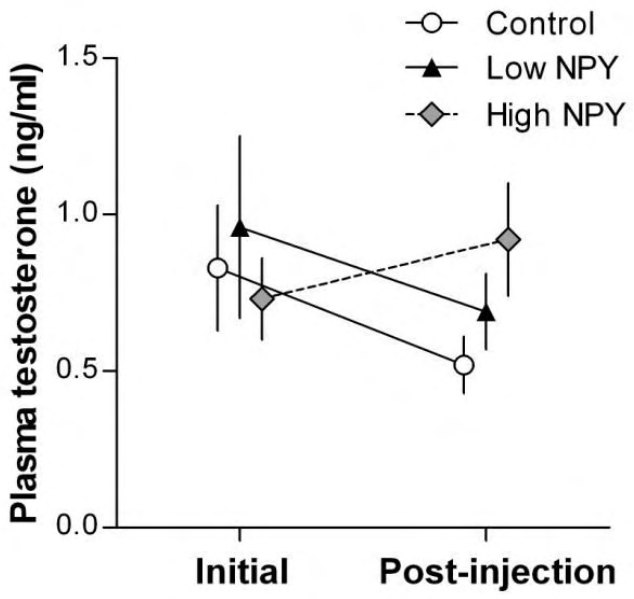

Fig. 21. Plasma testosterone of adult male Abert's Towhees (*Melozone aberti*) is not affected by intracerebroventricular (ICV) injection of NPY. Plasma testosterone was measured within 1 min of a bird being removed from its home cage (initial) and 60 min after injection (post-injection). I used a crossover design where each bird (n = 9) received vehicle (control), 0.25 μg NPY (Low NPY), and 1 μg NPY (High NPY) in a randomized order and with 2 days between each treatment. Data points are means ± SEM. For visual clarity, points have been offset along the horizontal axis.

CHAPTER 7

CONCLUSIONS

My dissertation research revealed disparities in the phenology of gonad development between birds breeding in geographically close urban and non-urban areas that were not related to body condition, energy stores, or baseline plasma levels of a key reproductive hormone, testosterone (T; Chapter 2). Crucially, however, this habitat-related disparity occurred only in some of the years compared during my dissertation (Chapter 3). My research, therefore, suggests that unlike desert Abert's Towhees, which appear to depend on winter precipitation levels to determine the phenology of gonad development, urban towhees have limited interannual variation in the phenology of gonad development and are independent of winter precipitation levels. In accordance with this proposition, my meta-analysis comparing the breeding phenology of urban birds versus their non-urban conspecifics in cities around the world suggests that the adjustment of breeding schedules to urban areas is a function of the seasonality of environmental variables (Chapter 4). My captive experiment (Chapter 5) revealed that energetic status does indeed modulate avian gonadal endocrine function, but not gonad growth. Furthermore, although neuropeptide Y (NPY) stimulates feeding behavior, it does not influence gonadal endocrine function (Chapter 6), which provides no support for the hypothesis that this peptide modulates the phenology of gonad development in light of food intake in Abert's Towhees.

1.1. Contributions to understanding avian breeding in an urbanizing world

Although a burgeoning body of research demonstrates that birds adjust to urban areas by breeding earlier than their corresponding non-urban conspecifics (for reviews see Chamberlain et al., 2009; Deviche and Davies, 2014) and developing their gonads

earlier (Deviche et al., 2010; Partecke et al., 2005), the ecological and physiological mechanisms underlying the adjustment of gonad development to urban areas remain(s) equivocal. It is hypothesized that the ecological driver of this disparity in gonad development is greater food availability for urban birds compared to non-urban birds (Chamberlain et al., 2009; Robb et al., 2008a; Robb et al., 2008b; Schoech and Bowman, 2001). Further, elevated food availability improves the energetic status of urban birds and enables them to initiate the seasonal rise in reproductive endocrine activity and gonad development earlier than non-urban birds. However, this hypothesis has not been tested empirically.

In chapter 2, I demonstrated that urban Abert's Towhees initiate gonad development earlier than do desert towhees. This disparity was not, however, associated with a difference in energetic status, as indicated by fat stores and body condition. Furthermore, innate immune performance was similar between the two populations, providing no evidence for a resource allocation trade-off between reproduction and essential somatic processes. Taken together, these findings do not support the proposition that earlier gonad development of urban birds is driven by improvements in energetic status due to greater food availability in urban areas. I should point out that I cannot exclude the possibility that food availability does indeed contribute to the habitat-associated disparity in the phenology of gonad development; however, such a role would have to act via pathways independent of energetic status, such as the perception of increasing food availability.

Despite the habitat-related difference in the phenology of gonad development, baseline plasma levels of testosterone were similar between the two populations. This finding is important because it contradicts the suggestion that advanced gonad development of urban birds is driven simply by an earlier increase in endocrine activity

of the reproductive system. I suggest, therefore, that future research on avian reproductive ecology may benefit from examining not only plasma levels of reproductive hormones, but also hormone receptor densities in target tissues and factors downstream of hormone binding. In support of this, studies on the endocrine control of behavior in birds have found that steroid receptor density often better predicts the occurrence of steroid-dependent behaviors than do plasma hormone levels (Ball and Balthazart, 2008; Horton et al., 2014; Ketterson et al., 2009; Rosvall et al., 2012).

In Chapter 3, I examined whether the phenology of gonad development is determined by hypothalamic levels of reproductive neuropeptides or the endocrine responsiveness of the anterior pituitary gland and/or testes. I found that the two populations did not differ in hypothalamic levels of key reproductive neuropeptides. This result was unsurprising, however, considering that during this year urban and desert Abert's Towhees had similar gonad development phenologies. Although this finding was unexpected, it was arguably the most illuminating aspect of this study. In particular, when the results of Chapters 2 are considered in the light of Chapter 3, there are a number of crucial implications. First, in both Chapters 2 and 3 the two populations had similar energetic status and baseline plasma testosterone levels, despite a considerable difference in the phenology of gonad development only in Chapter 2. Taken together, the findings of Chapters 2 and 3 provide further support for the suggestion that the adjustment of avian gonad growth phenology to urban areas involves a mechanism that is independent of either energetic status or baseline reproductive endocrine activity. Second, a multi-year comparison of cloacal protuberance width, which is tightly linked to testis mass, showed that urban towhees did not adjust the phenology of gonad development in three years that differed in levels of winter precipitation (Chapter 3). By contrast, desert towhees advanced the phenology of gonad development in the year with

substantially higher winter precipitation levels. This is consistent with the idea that urban areas are 'aseasonal' habitats with limited inter- and intra- annual variability in the phenology of plant growth that is independent of winter precipitation levels. On the other hand, the phenology of plant growth in desert areas is dependent on winter precipitation and hence is unpredictable from year to year and undergoes marked seasonal changes. To my knowledge, there is no long-term comparative study of urban and non-urban populations of an avian species. My dissertation has demonstrated that the conclusions from comparative urban avian ecology studies are highly dependent on the year considered. I suggest, therefore, that considerable progress in our understanding of both fundamental avian reproductive ecology and the adjustments birds make to breeding in urban areas could be made through long-term studies simultaneously comparing the interannual variation in the phenology of plant growth, food availability, and gonad growth.

In line with my suggestion that urban towhees have adjusted to a relatively aseasonal environment, I found that the marked responsiveness of the anterior pituitary gland and/or testes to GnRH challenge observed in desert towhees is absent in urban towhees. Desert towhees inhabit an environment with an unpredictable and marked seasonal change in growing green vegetation (and possibly also arthropod availability), whereas urban birds inhabit an environment with a more predictable and limited change in these factors (Bowers and Dimmitt, 1994; Buyantuyev and Wu, 2012; Noy-Meir, 1973). This raises the intriguing possibility that, in response to the habitat-related disparity in the predictability and magnitude of change in plant and/or arthropod phenology, Abert's Towhees have adjusted the responsiveness of the anterior pituitary gland and/or gonads to environmental stimuli that would naturally elicit an increase in GnRH secretion. It may be beneficial for desert towhees to rapidly respond to the

unpredictable and marked seasonal increase in plant and arthropod phenology and initiate gonad growth. By contrast, the more predictable and less marked seasonal increase in urban plant and arthropod phenology may mean that it is less important for urban birds to respond as rapidly. Future comparisons of avian gonad development in urban and non-urban areas should quantify not only whether the overall food availability differs between the two areas, but also whether the phenology of these food sources differs between urban and non-urban areas.

My dissertation has demonstrated that changes to the phenology of seasonal gonad growth and potentially also endocrine responsiveness of the HPG axis are involved in the adjustment to urban areas by male Abert's Towhees. However, it is unclear to what extent this adjustment reflects phenotypic plasticity or genetic divergence. Seasonal changes in the activity of the HPG axis and the size of the gonads are prime examples of phenotypic plasticity (Miner et al., 2005), and shifts in the timing of the seasonal increase in these traits by urban birds is thought to involve a major role for plasticity (Partecke et al., 2004; Partecke et al., 2005; Yeh and Price, 2004). However, there is the potential for genetic changes in addition to plastic changes. First, as Evans et al. (2010) point out, although plastic changes may adjust the phenotype to urban areas, it may be more costly to do so without genetic change, and directional selection for extreme phenotypes may generate genetic divergence from the non-urban population (i.e., 'genetic assimilation'; Wuddington, 1961). A second possibility for genetic changes in urban bird populations is immigrant selection (Lomolino, 1984). Under this hypothesis, some individuals of the non-urban source population may be genetically pre-adapted to urban areas and, therefore, may be the only individuals capable of colonizing and inhabiting urban areas. Surprisingly few studies have examined the effect of urban areas on genetic divergence and the evidence available to

date is inconsistent (for review see Delaney et al., 2010). Furthermore, genetic differentiation over small geographic scales is generally not expected in highly mobile organisms like birds because of gene flow from surrounding areas. Overall, therefore, there is a high likelihood that adjustment to urban areas by male Abert's Towhees involves plasticity in the timing of the seasonal increase in gonad growth and endocrine responsiveness of the HPG; however, further investigations are required to determine whether genetic divergence also contributes.

1.2. Contributions to the physiological control of seasonal gonad development

A central tenet of avian reproductive ecology is that food availability is a crucial supplementary environmental cue. It is believed that this cue modulates the phenology of gonad development and breeding in birds via effects on energetic status. Despite appreciating this for decades, the underlying physiological mechanisms that mediate the influence of energetic status on the avian reproductive system remain unclear. My dissertation addressed this deficit in understanding and supported the hypothesis that energetic status modulates avian reproductive function (Chapter 5). However, my findings suggest that this modulation is complex and depends upon the level of the hypothalamo-pituitary-gonadal (HPG) axis considered. In particular, I detected no effect of energetic status on hypothalamic levels of gonadotropin-releasing hormone (GnRH), but my results are consistent with a role for gonadotropin-inhibitory hormone (GnIH) and NPY in integrating information on energetic status. Chapter 6, however, demonstrated that although NPY stimulates feeding behavior it had no influence on gonadal endocrine function. Thus, the role of NPY in orchestrating food intake and reproductive function requires further investigation.

Furthermore, Chapter 5 suggested that energetic status modulates the endocrine function of the testes. By contrast, and somewhat surprisingly, I detected no effect of energetic status on testicular growth. To account for the disparity between testosterone secretion and gonad development, I hypothesize that gonad development may not be energetically expensive, and, thus, differences in gonad growth in response to experimentally modulated energetic status would not be expected. When energy is limited, however, T production may be suppressed to avoid the costs of T-mediated male reproductive behaviors, such as singing, aggression, and territoriality. The mechanism by which energetic status modulates testicular endocrine function is unclear and little research has explored this pathway in birds. Hence, future research in this area may prove fruitful.

Overall, chapter 5 demonstrates that energetic status can indeed modulate reproductive function, potentially via effects on gonadal endocrine function. However, considering Chapters 2 and 3 demonstrated that disparities in the phenology of gonad development between urban and desert Abert's Towhees was not explained by differences in baseline gonadal endocrine function, it appears that the adjustment of gonad growth phenology to urban areas is not driven by improvements in energetic status due to greater food availability in urban areas. Instead, I suggest that birds may use the phenology of plant growth and/or the availability of key food sources as a perceptual cue to adjust the phenology of gonad development to local conditions.

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APPENDIX A

TABLE OF STUDIES COMPARING THE BREEDING PHENOLOGY OF URBAN AND NON-URBAN BIRD POPULATIONS

Studies comparing the breeding phenology of urban and non-urban bird populations. Effect sizes (Hedges' *g*) and their 95% confidence interval (CI) were calculated so that a positive value indicates that the phenology of the non-urban population was advanced relative to the urban population, whereas a negative value indicates that the urban population was advanced.

Species	**Family**	**City**	**City Latitude**	**Mean annual ambient temperature (C°)**	**Parameter**	**Effect size**	**Upper 95% CI**	**Lower 95% CI**	**Reference**
Acadian flycatcher (*Empidonax virescens*)	Tyrannidae	Columbus, Ohio USA	39.9	11	Lay date	0.72	1.09	0.35	(Shustack and Rodewald, 2010)
Australian Magpie (*Gymnorhina tibicen*)	Cracticidae	Brisbane, Australia	-27.4	20	Clutch initiation	-1.59	-1.05	-2.13	(Rollinson and Jones, 2002a)
Blue Tit (*Cyanistes caeruleus*)	Paridae	Ghent, Belgium	51.1	17	Lay date	-0.77	-0.29	-1.25	(Dhondt et al., 1984)
Blue Tit (*Cyanistes caeruleus*)	Paridae	Lodz, Poland	51.5	11	Lay date	-0.38	-0.28	-0.49	(Marciniak et al., 2007)
Blue Tit (*Cyanistes caeruleus*)	Paridae	Annaba, Algeria	36.5	8	Lay date	-0.72	-0.45	-0.99	(Brahmia et al., 2013)
Blue Tit (*Cyanistes caeruleus*)	Paridae	Uusimaa, Finland	60.1	5	Lay date	2.03	2.23	1.82	Solonen, unpublished
Carrion Crow (*Corvus corone*)	Corvidae	Lusanne, Switzerland	46.3	10	Hatch date	-0.17	0.15	-0.49	(Richner, 1989)
Cooper's Hawk (*Accipiter cooperii*)	Accipitridae	Tucson, Arizona, USA	32.1	20	Hatch date	-0.86	-0.47	-1.26	(Boal and Mannan, 1999)
Dark-eyed Junco (*Junco hyemalis*)	Emberizidae	San Diego, California, USA	32.5	17	Lay date	-4.83	-4.06	-5.60	Atwell *et al.* (in press)

Eurasian Blackbird (*Turdus merula*)	Turdidae	Czech Republic and Slovakia (national nestling ringing datasets)	48.1	8	Clutch initiation	-0.29	-0.17	-0.41	(Najmanová and Adamík, 2009)
Eurasian Magpie (*Pica pica*)	Corvidae	Sheffield, UK	53.4	10	Lay date	-0.52	-0.31	-0.72	(Eden, 1985)
Eurasian Magpie (*Pica pica*)	Corvidae	Sofia, Bulgaria	42.6	11	Lay date	-0.61	-0.15	-1.06	(Antonov and Atanasova, 2003)
Eurasian Starling (*Sturnus vulgaris*)	Sturnidae	UK (national network of nest record cards)	55.4	8	Lay date	-0.04	0.08	-0.16	(Siriwardena and Crick, 2002)
Eurasian Starling (*Sturnus vulgaris*)	Sturnidae	Rennes, France	48.1	11	Lay date	0.47	0.86	0.09	(Mennechez and Clergeau, 2006)
Florida Scrub-jay (*Aphelocoma coerulescens*)	Corvidae	Lake Placid, Florida USA	27.3	22.2	Lay date	-1.60	-1.06	-2.14	(Schoech and Bowman, 2003)
Great Tit (*Parus major*)	Paridae	Ghent, Belgium	51.1	11	Lay date	-0.83	-0.74	-0.92	(Dhondt et al., 1984)
Great Tit (*Parus major*)	Paridae	Cardiff, UK (Urban); Wytham woods, Oxfordshire, UK (rural)	51.5	5	Lay date	0.05	0.17	-0.07	(Cowie and Hinsley, 1987)

Great Tit (*Parus major*)	Paridae	Uusimaa, Finland	60.1	11.5	Lay date	-0.70	-0.28	-1.11	Solonen, unpublished
House Sparrow (*Passer domesticus*)	Passeridae	UK (national network of nest record cards)	55.4	10	Lay date	-1.01	-0.56	-1.47	(Crick and Siriwardena, 2002)
House Sparrow (*Passer domesticus*)	Passeridae	Szentgal, Hungary	47.1	8	Lay date	-0.18	-0.07	-0.30	(Seress et al., 2012)
House Wren (*Troglodytes aedon*)	Troglodytidae	Washington, D.C. – Baltimore, Maryland metropolitan area, USA	38.9	14	Lay date	-0.12	0.58	-0.81	(Newhouse et al., 2008)
Northern Cardinal (*Cardinalis cardinalis*)	Cardinalidea	Columbus, Ohio USA	39.9	11	Clutch initiation	-0.12	-0.04	-0.20	(Shustack and Rodewald, 2010)
Northern Mockingbird (*Mimus polyglottos*)	Mimidae	Gainesville, Florida USA	29.7	20	First clutch completion date	-0.77	-0.51	-1.03	(Stracey and Robinson, 2012)
Song Thrush (*Turdus philomelos*)	Turdidae	Czech Republic and Slovakia (national nestling ringing datasets)	33.4	8	Clutch initiation	-0.19	-0.03	-0.34	(Najmanová and Adamík, 2009)
Tawny Owl (*Strix aluco*)	Strigidae	Uusimaa, Finland	60.1	5	Hatching date	-1.35	-0.95	-1.76	(Solonen, 2014)
White-winged Chough (*Corcorax melanorhamphos*)	Corcoracidae	Canberra, Australia	-35.3	12	Clutch initiation	-0.64	-0.26	-1.02	(Beck and Heinsohn, 2006b)

APPENDIX B

APPROVAL DOCUMENTATION FROM UNIVERSITY INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 315
Tempe, Arizona 85287-6111
Phone: (480) 965-4387 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number:	**12-1225R**
Protocol Title:	**Investigating the Influence of Food on Reproductive Physiology: Urbanization as a Natural Experiment**
Principal Investigator:	**Pierre Deviche**
Date of Action:	**11/7/2011**

The animal protocol review was considered by the Committee and the following decisions were made:

- ☐ The original protocol was APPROVED as presented.
- ☒ The revised protocol was APPROVED as presented.
- ☐ The protocol was APPROVED with RESTRICTIONS or CHANGES as noted below. The project can only be pursued, subject to your acceptance of these restriction or changes. If you are not agreeable, contact the IACUC Chairperson immediately.
- ☐ The Committee requests CLARIFICATIONS or CHANGES in the protocol as described in the attached memorandum. The protocol will be considered when these issues are clarified and the revised protocol is submitted.
- ☐ The protocol was approved, subject to the approval of a WAIVER of provisions of NIH policy as noted below. Waivers require written approval from the granting agencies.
- ☐ The protocol was DISAPPROVED for reasons outlined in the attached memorandum.
- ☐ The Committee requests you to contact _____ to discuss this proposal.
- ☐ A copy of this correspondence has been sent to the Vice President for Research.
- ☐ Amendment was approved as written.

Documentation of Level III Training will need to be provided to the IACUC office before the participant can perform procedures independently. For more information on Level III requirements see https://researchintegrity.asu.edu/training/animals/levelthree

Total # of Animals: **148** **Pain Level:** **C-18, D-130** **Species:** **Birds (Abert's Towhee)**
Approval Period: **11/7/2011 – 11/6/2014**

Signature: C. Miller for D. Murphy Date: 11/7/11
IACUC Chair or Designee

Original: Principal Investigator
Cc: IACUC Office
IACUC Chair