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Prior Experience with Photostimulation Enhances Photo-Induced Reproductive Response in Female House Finches: A Potential Basis for Age-Related Increase in Reproductive Output

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ABSTRACT

In many vertebrates parental age is related to reproductive output with older individuals often performing better (e.g., advanced timing, more offspring) than younger ones. First-year birds differ from older birds in that they lack previous experience with the reproductively-stimulatory effects of long day lengths (photostimulation). The goal of this study was to examine whether this age-related increase in reproductive output can be attributed, at least in part, to previous experience with photostimulation in a photoperiodic bird, the female house finch (*Carpodacus mexicanus*). Specifically, we investigated whether previous experience with photostimulation influences the early stages of reproductive development by quantifying plasma luteinizing hormone (LH), plasma vitellogenin, ovarian follicle size, and immunoreactivity of hypothalamic gonadotropin-releasing hormone (GnRH-I) and vasoactive intestinal polypeptide (VIP). By differentially manipulating photoperiod, we generated two groups of first-year female finches: a photo-experienced group that had been through one photoperiodically-induced cycle of gonadal development and regression, and a photo-naïve group exposed to long days since hatch. Both groups were then transferred from long to short days for nine weeks, to ensure full photoperiodic responsiveness, and then photostimulated for four weeks and exposed to conspecific or heterospecific male song starting 90 minutes before sacrifice. Following photostimulation, although photo-experienced and photo-naïve groups exhibited similar surges in plasma LH concentrations, circulating vitellogenin levels increased in photo-experienced, but not in photo-naïve birds. After four weeks of photostimulation, egg yolk deposition was observed in two of six photo-experienced birds but in none of the photo-naïve birds.

After four weeks of photostimulation and exposure to conspecific or heterospecific male song, more GnRH-I-ir cells were seen in the septo-preoptic hypothalamus of photo-experienced than of photo-naïve birds. In contrast, there were no differences between the photo-experienced and photo-naïve birds, irrespective of the song type they were exposed to, in numbers of visible VIP-ir cells in the mediobasal hypothalamus. Our results demonstrate that previous photo-experience enhances some of the early stages of photo-induced reproductive development, and that the reproductive neuroendocrine system of photo-experienced, photoperiodic birds is primed to respond rapidly to reproductively-stimulatory environmental and social cues.

KEYWORDS

Bird song, gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), parental age, photoperiodic history, seasonal breeding, reproduction, vasoactive intestinal polypeptide (VIP), vitellogenin

INTRODUCTION

In most iteroparous animals reproductive output is related to parental age with older individuals performing better than younger ones (Clutton-Brock, 1988; Stearns, 1992). This is most apparent when comparing first-time and reproductively-experienced breeders. For example, in second-year, male European starlings (*Sturnus vulgaris*) testicular maturation is three to four weeks earlier and testicular regression approximately two weeks later than in the same birds in their first year (Dawson, 2003). This results in a longer period of spermatogenic activity and presumably in an earlier seasonal increase in circulating testosterone levels in the older birds. This age-related difference in reproductive function is likely to contribute to the earlier establishment of territories and occupation of nesting cavities by reproductively-experienced males than by first-year males (Feare, 1984). An age-related difference in reproductive function is also seen in female birds, with reproductively-experienced females generally initiating egg production earlier and subsequently laying more, and sometimes larger eggs, and producing more fledglings than first-year females (Saether, 1990; Fowler, 1995). Furthermore, the reproductive output of individual females improves between their first and second breeding attempts (Newton et al., 1981; Hannan and Cooke, 1987; Forslund and Pärt, 1995; Newton and Rothery, 1998).

These observations can be explained by two hypotheses (Forslund and Pärt, 1995). The “constraint” hypothesis suggests that individuals breeding for the first time are limited by deficiencies in general life skills, by a slow development of the reproductive system (i.e., age per se), or by lack of breeding experience needed to perfect

reproductive behaviors and “prime” the reproductive system to develop more rapidly. This hypothesis predicts that age-related differences in reproductive output are due to the inability of first-year females to match older females’ reproductive physiology and/or behaviours. The “restraint” hypothesis predicts that the resources an individual can afford to allocate to reproduction should increase with age as a consequence of an increase in the rate of the seasonal maturation of the reproductive system, experience of reproductive behavior, and improved life skills. According to this hypothesis, age-related differences in reproductive output are due to first-year females investing fewer resources into their first reproductive attempt than birds that are two years or older. Both of these hypotheses predict that the reproductive potential of first-year females must be limited by factors that do not limit the reproductive potential of second-year and older females.

Reproduction in seasonally breeding animals is scheduled to coincide with favorable environmental requirements, such as an ample food supply or nest site availability, to produce offspring (Perrins, 1970). For most temperate zone species, optimal conditions for breeding vary somewhat predictably with season (Wingfield et al., 1992), and consequently, the most reliable cue for initiating the breeding season is the annual cycle of changes in day length (i.e., photoperiod) (Wingfield, 1980, 1983). Temperate-zone birds hatched in the spring and summer are unresponsive to the reproductively-stimulatory effects of long photoperiods (i.e., photorefractory), which prevents premature development of the reproductive system (Farner et al., 1983; Williams et al., 1987a, 1987b, 1989; McNaughton et al., 1992). Exposure to short day lengths during fall and winter dissipates photorefractoriness resulting in the hypothalamus becoming

responsive to reproductively-stimulatory photoperiodic and social cues. Increasing daylength in spring accelerates reproductive development (Farner et al., 1983; Follett, 1984; Nicholls et al., 1988) by stimulating the release of gonadotropin-releasing hormone (GnRH-I) from the hypothalamus and the consequent increase in secretion of luteinizing hormone (LH) and follicle-stimulating hormone from the anterior pituitary (Sharp and Ciccone, 2005). In females, a photo-induced increase in gonadotropin secretion stimulates ovarian development and production of 17ß-estradiol (E2) and progesterone (Williams, 1998). Elevated circulating E2 stimulates the liver to synthesize and secrete very-low density lipoprotein (VLDLy), the yolk lipid precursor, and vitellogenin, the yolk protein precursor, which are then taken up by developing ovarian follicles (Bergink et al., 1974; Deeley et al., 1975; Stifani et al., 1988; Walzem, 1996; Williams, 1998).

Exposure of photo-sensitive birds to long day lengths also increases the secretion of vasoactive intestinal polypeptide (VIP) from the hypothalamus (Mauro et al., 1992; Chaiseha et al., 1998) to stimulate the production and release of prolactin from the anterior pituitary gland (Mauro et al., 1989; El Halawani et al., 1996; Tong et al., 1997, 1998). Increased plasma prolactin plays a role in the onset and maintenance of incubation and parental care (Haywood, 1993; Sockman et al., 2006; Angelier and Chastel, 2009) and in the onset of photorefractoriness, gonadal regression and postnuptial molt at the end of the breeding season (Farner et al., 1983; Nicholls et al., 1988; El Halawani et al., 1997; Dawson and Sharp, 1998; Kuenzel, 2003).

A major difference between first-year and older birds is that older birds have had previous experience with photostimulation (photo-experienced) and at least one cycle of

129 photo-induced gonadal development and regression, whereas birds breeding for the
130 first time are at that moment experiencing photostimulation for the first time (photo-
131 naïve).

132 The goal of this study was to examine whether prior experience with photostimulation
133 affects early reproductive development and responses to reproductive cues and thus
134 contributes to the age-related differences observed in reproductive output. Using a
135 photoperiodic bird, the female house finch (*Carpodacus mexicanus*), we investigated
136 whether previous photoexperience, rather than age, per se, influences the rate at which
137 stimulatory environmental cues are integrated into the neuroendocrine signaling
138 pathways that regulate the early stages of photo-induced reproductive development. We
139 designed the experiment based on a similar study examining the contribution of
140 photoperiodic experience to age-related differences in early reproductive development
141 in female European starlings (Sockman et al., 2004). In that study all birds were initially
142 exposed to a short daylength (8h L: 16h D) for 12 weeks to ensure they were fully
143 photosensitive. The photo-naïve group was maintained on short days for an additional
144 20 weeks while the photo-experienced group was transferred to long days for 12 weeks
145 and then back to short days for 8 weeks to induce photosensitivity for the second time.
146 Both groups were then photostimulated. The initial photo-induced increase in plasma
147 LH in the photo-naïve group was 3-fold less than the increase in the photo-experienced
148 group, while the photo-induced increase in plasma vitellogenin in the photo-naïve group
149 was more rapid than in the photo-experienced group. It is possible that the differences
150 in these photoinduced responses may have been a consequence of prolonged
151 photosensitivity of the photo-naïve group. This may have 1) desensitized the pituitary of

the photo-naïve group to GnRH, thus dampening the LH response to photostimulation, and 2) increased hepatic storage of vitellogenin, resulting in the more rapid increase in circulating vitellogenin levels after two weeks of photostimulation (Sockman et al., 2004). We designed the present study to avoid these possible problems by maintaining a photo-naïve group in a reproductively quiescent state from hatch by exposure to long days to maintain photorefractoriness. After the induction of photosensitivity by exposure to short days, the first photoperiodic response of the photo-naive group was, therefore, more physiologically comparable to the photo-experienced group than in the earlier starling study. As conspecific song and availability of mates are “supplementary” cues that female songbirds use to fine-tune the timing of early reproductive development (Wingfield, 1980, 1983), we housed all females with males, and during the last day of the study, isolated the females and exposed them to conspecific male song in an effort to maximize reproductive development, using heterospecific male song as a control.

We predicted that after photostimulation and exposure to conspecific male song, photo-experienced females would have higher circulating LH and vitellogenin levels, larger or more developed reproductive organs, and more immunocytochemically visible hypothalamic GnRH and VIP neurons than photo-naïve birds. If these predictions are correct, they would be consistent with the view that second year and older finches lay earlier than first year finches, in part, because of their photoperiodic history rather than age per se.

MATERIALS AND METHODS

Animals and housing

We captured house finches between June and July of 2006 in Chapel Hill, North Carolina (35.91˚N 79.05˚W) and transferred them into large, outdoor flight cages at the University of North Carolina at Chapel Hill, NC, where we conducted the study. This study was approved by the University's Institutional Animal Care and Use committee (protocol 07-260). For the entire study we provided the birds with food (Daily Maintenance, Roudybush; Woodland, CA) and water ad libitum. We identified hatch-year birds (i.e., new fledglings) by the presence of feather tufts on the head and new, unworn wing feathers (Hill, 2002). On 24 July 2006, we moved all hatch-year birds into large indoor cages on a photoperiod (16h L: 8h D, referred to as long days) that maintained them in a non-reproductive, photorefractory state (Nicholls et al., 1988). Following completion of their annual molt, we identified males and females by the presence or absence, respectively, of yellow plumage on the head and later confirmed *post mortem.*

Photoperiod manipulation

On 21 November 2006 (week 0), we randomly assigned and transferred two females and one male to each of ten light-proof, foam-lined, sound-attenuation chambers located together in one room. Each chamber had a cage with three perches, an air intake and fan-driven exhaust, and a fluorescent light that maintained the chamber-specific photoperiod. We changed the photoperiod to 8h L: 16h D (referred to as short days) in five chambers in order to begin the process of instating sensitivity to

194 reproductive stimuli (Fig. 1). On 16 January 2007 (week 8), we changed the photoperiod
195 in these five chambers to 16h L: 8h D, driving these birds first through a reproductive-
196 like state, and then into a non-reproductive (photorefractory) state (Nicholls et al., 1988)
197 (hereafter referred to as the photo-experienced group) (Fig. 1). Throughout this time, we
198 maintained the original 16h L: 8h D photoperiod in the other five chambers, thereby
199 maintaining the non-reproductive (photorefractory) status of these birds (Nicholls et al.,
200 1988; Williams et al., 1989) (hereafter referred to as the photo-naïve group) (Fig. 1). We
201 spatially interspersed the replicate chambers of both treatments to control for location
202 effects. We exposed the birds to this long-day photoperiod until all of the birds in the
203 photo-experienced group initiated molt, resulting in the photo-experienced group's being
204 exposed to long days for 17 weeks, and the photo-naïve group for 25 weeks. On 16
205 May 2007 (week 25), we moved each triplet group of birds into each of ten cages
206 located together in one room and changed the photoperiod in the room to 8h L: 16h D,
207 thereby beginning the process of instating sensitivity to reproductive stimuli for the first
208 time in the photo-naïve group and for the second time in the photo-experienced group
209 (Fig. 1). On 18 July 2007 (week 34), after exposing all birds to the 8h L: 16h D
210 photoperiod for 9 weeks, we changed the photoperiod in the room to 16h L: 8h D,
211 driving the photo-naïve birds into a reproductive-like state for the first time, and the
212 photo-experienced birds for the second time (Fig. 1).

213 **Body mass measurements and blood sampling**

214 Starting on 21 November 2006 (week 0), we measured the body mass of each bird
215 once every week during the 38-week photoperiod manipulation. We took a blood
216 sample (~150 µl) from each bird at pre-determined time points throughout the study

(Fig. 1) to measure temporal variation in circulating levels of LH and vitellogenin. We centrifuged the blood samples to separate the plasma and stored the plasma samples at -20˚C until analysis. Only the data for female house finches will be presented here. Some mortality occurred during the 38-week photoperiod manipulation (see Fig. 2).

Song exposure, sacrifice and tissue collection

On the afternoon of 13 August 2007, at week 38 of the study, we weighed seven females (n = 2 photo-experienced; n = 5 photo-naïve) from five cages, moved them individually into each of seven light-proof, sound attenuation chambers (58 x 41 x 36 cm, Industrial Acoustics Company, New York, NY, USA) located together in one room, and isolated the birds for one full day. Each chamber was equipped with a cage containing two perches, a food cup, and a water bottle; a fan-driven ventilation system; a light that we used to maintain the 16h L: 8h D photoperiod within the chamber; and a speaker (Pioneer Corp. TS-G1040R, Tokyo, Japan). We powered the speakers by a daisy chain of four mono-block amplifiers interfaced with a computer.

Beginning 1 h after the onset of the photophase on 15 August 2007, we exposed one bird to a song set recorded from either male house finches or male northern cardinals (*Cardinalis cardinalis*) (see 'Song recordings used for playback') for 30 min through the chamber's speaker (hereafter termed song treatment). We played the song at approximately 80 dB at 5 cm from the speaker to approximate the amplitude of songs that a free-living bird would experience from a nearby male. Each female heard a unique set of songs from a unique set of male singers (i.e., no male's song was played

to more than one female). We staggered exposure to the song treatment by 30 min between females.

At 90 min after the onset of the song treatment, we weighed the birds, and after taking a blood sample (~150 µl) from a brachial vein, rapidly decapitated them and removed their brains. Using previously described protocols (Sockman and Salvante, 2008), we halved each brain using a mid line sagittal cut, fixed one hemisphere (5% acrolein for 4.5 hours; alternating left and right hemispheres from successive birds), and stored the fixed hemispheres at -80˚C after cryoprotection in 30% sucrose. We recorded the color and diameter of the three largest ovarian follicles from each bird.

We repeated these procedures for the remaining females (n = 4 photo-experienced; n = 4 photo-naïve) after moving them individually into each of 8 light- and sound-proof chambers on the afternoon of 15 August 2007. By balancing the song treatment levels between subjects from the same photoperiod experience group, we generated four female treatment groups: (1) photo-experienced, conspecific song (n = 3); (2) photo-experienced, heterospecific song (n = 3); (3) photo-naïve, conspecific song (n = 5); and (4) photo-naïve, heterospecific song (n = 4).

Song recordings used for playbacks

We recorded the songs used for playback from free-living male house finches and Northern cardinals in the area surrounding the UNC-Chapel Hill campus using a short-shotgun microphone (Sennheiser ME-66/K6, Wedemark, Germany), connected to a digital recorder (Marandtz PMD 660, Mahwah, NJ, USA) set to record uncompressed files sampled at 44.1 kHz. We then selected two songs from each of 24 male house

finches and 24 male cardinals using Raven software (v.1.2.1, Cornell Lab of Ornithology). We matched conspecific and heterospecific songs based on individual song duration and created eight sets of duration-matched house finch and northern cardinal songs composed of six songs (two songs from three different males) arranged in a random order such that all six songs were repeated the same number of times within the 30 minutes. All song sets were 30 minutes long and contained a total of 15 minutes of song and 15 minutes of silence.

GnRH and VIP immunocytochemistry and quantification

We sectioned the fixed brain hemispheres in the sagittal plane at 40 µm on a cryostat and performed immunocytochemistry (ICC) for GnRH on every third section as previously described by Sockman and colleagues (Sockman et al., 2004). As part of another study, we initially labeled the tissue for ZENK immunoreactivity using a different chromogen. We quenched the tissue with 0.5% H_2O_2 before incubating with a 1:10000 dilution of GnRH primary antibody (HU60 bleed H, provided by H.F. Urbanski, Division of Neuroscience, Oregon Regional Primate Center, Beaverton, Oregon). The details of the GnRH antibody have been described previously (Urbanski, 1992). The rabbit-raised GnRH antibody recognizes intact, but not fragmented, forms of GnRH-I and GnRH-II found in birds (Sharp et al.,1990; Sharp and Ciccone, 2005). We processed all of the tissue in two ICC batches. Given the uneven mortality between treatment groups, we counterbalanced the four photoexperience-song treatment groups as much as possible within each ICC batch.

We performed ICC for VIP on an alternate set of every third section, as previously described for the transcription factor ZENK (Sockman et al., 2002) except we incubated the sections with VIP primary antibody (Immunostar, Hudson, WI, USA) at 1:10,000 dilution for 48 hours at 4˚C. We processed all of the sections in two ICC batches, counterbalancing the four photoexperience-song treatment groups within each batch.

We conducted all quantification procedures blind to the experimental condition of each subject. Using a Leica DM4000 digital research microscope, we summed the number of GnRH-immunoreactive (GnRH-ir) cells with visible nuclei in the septo-preoptic area between the anterior commissure and the supraoptic decussation of every third-cut section (one or two sections were quantified per subject) under 200x magnification and Köhler illumination. While GnRH-ir cell bodies were not seen in both sections from some birds, GnRH-ir fibers were always present. Although the GnRH antibody recognizes both GnRH-I and -II, only GnRH-I and not GnRH-II cell bodies are present in the septo-preoptic area (Millam et al., 1993; van Gils et al., 1993; Sharp, 2005). Previous studies have found that this region is innervated by central photoreceptors (Saldanha et al., 1994, 2001) and responds to photostimulation with increased fos-like immunoreactivity (Meddle and Follett, 1995, 1997; Millam et al., 2003) and increased GnRH-immunoreactivity (Dawson and Goldsmith, 1997; Péczely and Kovács, 2000; Sockman et al., 2004; Teriuyama and Beck, 2000). We quantified VIP-immunoreactivity (VIP-ir) in every third section of tissue medially from the medial edge of the occipitomesencephalic tract under 400x magnification and Köhler illumination. We counted the number of VIP-ir cell bodies with visible nuclei in four sections through the infundibular nuclear complex (INF) and the ventromedial nucleus (VMN). Previous studies have shown that these

areas of the hypothalamus contain dense concentrations of VIP-ir cells and fibers (Yamada et al., 1982; Péczely and Kiss, 1988; Mauro et al., 1989, 1992).

LH and vitellogenin assays

We assayed plasma LH concentrations using a micromodification (Caro et al., 2006) of a homologous chicken LH radioimmunoassay (Sharp et al., 1987) using LH antiserum 1/8 at 1:24000 dilution and LH, code AE1a run 4, as iodinated label and standard. The sensitivity of the assay was 0.45 ng/ml at 80% displacement and 1.55 ng/ml at 50% displacement of the iodinated label from the LH antibody. All samples were analyzed in one assay.

Plasma samples were assayed for vitellogenin using the zinc method developed for the domestic hen (Zinc kit – Wako Chemicals, Virginia, USA) (Mitchell and Carlisle, 1991) and validated for passerines (Williams and Christians, 1997). This method measures total plasma zinc, and then separates the zinc bound to serum albumen from that bound to vitellogenin and very-low density lipoprotein (VLDL) by depletion of vitellogenin and VLDL from the plasma sample by precipitation with dextran sulfate. The depleted plasma sample is then assayed for zinc. Vitellogenic zinc is equal to the difference between total and depleted zinc; VLDL accounts for only 2% of total plasma zinc (Mitchell and Carlisle 1991). The concentration of vitellogenic zinc is proportional to the plasma concentration of plasma vitellogenin (Mitchell and Carlisle, 1991). Intra- and inter-assay coefficients of variation determined for a laying hen plasma pool were 3% (n = 15 sample replicates) and 7% (N = 16 assays), respectively.

Statistical analyses

Our data consisted of a combination of fixed (e.g., photoexperience, week) and hierarchically-structured random (e.g., individual nested within triplet) effects, each of which may differ from the others in its correlation structure. In addition, some mortality occurred during the 38-week study, rendering our dataset unbalanced. Therefore, we analyzed these data in a mixed, multilevel modeling framework using the software Stata IC 10.0 for the Macintosh (Stata Corporation, College Station, TX), which readily accommodates unbalanced, hierarchically-structured combinations of fixed and random effects (Burton et al., 1998; Goldstein et al., 2002; Rabe-Hesketh and Skrondal, 2005). We used Stata's command for multilevel mixed-effects linear regression but, for the GnRH-ir and VIP-ir cell count data, we instead used the command for multilevel mixed-effects Poisson regression because count data tend to follow Poisson distributions. These models estimated parameters with restricted maximum likelihood and used z-tests to test the null hypothesis that a coefficient equaled zero. For more information on the rationale for and approach to mixed, multilevel modeling frameworks, see Sockman et al. (2008).

For analyses of GnRH-ir and VIP-ir, we counted the number of GnRH-ir or VIP-ir cell bodies and used photoexperience, song treatment, and their interaction as fixed factors, with observation (individual bird) nested within triplet as a random intercept and as a random coefficient for song treatment. For analyses of body mass and circulating LH and vitellogenin levels, we used photoexperience, week and their interaction as fixed factors and nested observation (the individual bird's measurement that week) within female as a random coefficient for week and nested female within triplet as a random

intercept. For ovarian follicle size, we used photoexperience, female body mass, follicle order (from most to least developed) and the interaction between photoexperience and follicle order as fixed factors and nested observation (the individual bird's measurement of an individual follicle) within female as a random coefficient for follicle order and nested female within triplet as a random intercept. As female body mass differed between photoexperience groups at the end of the study, it was included as a covariate to control for differences in ovarian follicle size due to body mass alone (Sockman et al., 2004). For comparison of circulating LH levels in the two groups during the different rounds of photostimulation (photo-experienced: first and second rounds of photostimulation; photo-naïve: first round of photostimulation), we used photoexperience, number of weeks exposed to long days, their interaction and the interaction between photoexperience and round of photostimulation (i.e., first or second) as fixed factors and nested observation (the individual bird's measurement that week) within female as a random coefficient for week and nested female within triplet as a random intercept. For comparison of circulating vitellogenin levels in the two groups during their first rounds of photostimulation, we used photoexperience, number of weeks exposed to long days and their interaction as fixed factors and nested observation (the individual bird's measurement that week) within female as a random coefficient for week and nested female within triplet as a random intercept. The nesting structure we used for random effects follows the approach recommended by Schielzeth and Forstmeier (2009).

RESULTS

Body mass

At the start of the study the two groups of female house finches had the same body mass (week 0: $p > 0.5$; Fig. 2), but this changed during the initial 8 week photoperiodic treatment period (weeks 2-8: photoperiodic treatment x week: $z = -2.05$, $p < 0.05$; Fig. 2). While the body mass of birds that had experienced changing photoperiod did not change during the 8 week exposure to short days (weeks 2-8: week: $p > 0.4$), it increased in photo-naïve females retained on long days (weeks 2-8: week: $z = 2.38$, $p < 0.02$) (Fig. 2). However, body mass did not differ between photo-experienced and photo-naïve females during the following photoperiodic treatment period when both groups were exposed to long days (weeks 9-12: all $p > 0.1$; weeks 9-25: all $p > 0.2$) and subsequently exposed to short days (weeks 26-34: all $p > 0.1$) (Fig. 2). After both groups were returned to long days, photoperiodic treatment affected body mass over the last four weeks of the study (weeks 35-38: photoperiodic treatment x week: $z = 2.31$, $p < 0.03$; Fig. 2). During this period, the body mass of photo-experienced females increased (weeks 35-38: week: $z = 2.49$, $p < 0.02$) while that of photo-naïve females, which were being photostimulated for the first time, did not change (weeks 35-38: week: $p > 0.7$; Fig. 2).

GnRH and VIP immunoreactivity

Photoperiodic experience influenced the way in which song treatment affected GnRH-ir in the hypothalamic septo-preoptic area of female house finches (photoexperience x song treatment: $z = 3.73$, $p < 0.001$; Fig. 3). Within the photo-experienced group,

391 females exposed to conspecific song had more GnRH-ir cells than females exposed to
392 heterospecific song (song treatment: $z = 3.35$, $p < 0.001$; Fig. 3). The opposite was true
393 for the photo-naïve group; females exposed to conspecific song had fewer GnRH-ir
394 cells than females exposed to heterospecific song (song treatment: $z = -5.17$, $p < 0.001$;
395 Fig. 3). Furthermore, within the group of females exposed to conspecific song, the
396 photo-experienced females had more GnRH-ir cells than photo-naïve females
397 (photoexperience: $z = 3.95$, $p < 0.001$; Fig. 3). Again, the opposite was true for the
398 females exposed to heterospecific song; photo-experienced females had fewer GnRH-ir
399 cells than photo-naïve females (photoexperience: $z = -2.08$, $p < 0.04$; Fig. 3).

400 Neither photoexperience nor song treatment nor their interaction influenced the number
401 of VIP-ir cells in the INF (Fig. 4a) or the VMN (Fig. 4b) of the hypothalamus (all $p > 0.3$).
402 Even when song treatment and the interaction between photoexperience and song
403 treatment were removed from the model, VIP-ir cell count in the INF and VMN were not
404 related to photoexperience (both $p > 0.1$).

405 **Plasma LH and vitellogenin**

406 At the beginning of the study and at the end of the 8-week short day photoexperience
407 treatment, both groups of females had similar, low levels of circulating LH (weeks 0 and
408 8: both $p > 0.5$; Fig. 5). Photoexperience determined the way circulating LH levels
409 changed during the four weeks immediately following photoexperience treatment
410 (weeks 9-12: photoexperience x week: $z = -10.34$, $p < 0.001$; Fig. 5). During this time
411 plasma LH levels remained low in photo-naïve females, who maintained their non-
412 reproductive state (weeks 9-12: week: $p > 0.8$; Fig. 5). In contrast, plasma LH levels in

413 photo-experienced females photostimulated for the first time increased almost 15-fold
414 after one week of exposure to long days and declined to levels that were still 5-fold
415 higher than those of photo-naïve females after four weeks on long days (weeks 9-12:
416 week: $z = -10.55$, $p < 0.001$; Fig. 5). Plasma LH levels were low in both groups at week
417 25 (photoexperience: $p > 0.3$) and week 34 (photoexperience: $p > 0.7$) of the study,
418 when both groups of females were in a non-reproductive state (Fig. 5).

419 During the last four weeks of the study, both groups were photostimulated, and
420 circulating LH levels in both groups increased almost 15-fold after exposure to long
421 days for one week, and then declined approximately 3-fold by the end of the study
422 (weeks 35-38: week: $z = 7.51$, $p < 0.001$; photoexperience and photoexperience x
423 week: $p > 0.9$; Fig. 5). This photo-induced surge in LH was similar to the LH surge
424 observed in photo-experienced females undergoing photo-induced early reproductive
425 development for the first time (photo-experienced weeks 9-12 vs. photo-experienced
426 weeks 35-38 vs. photo-naïve weeks 35-38: weeks exposed to long days: $z = -7.76$, $p <$
427 0.001; photoexperience and photoexperience x weeks exposed to long days (1 through
428 4): $p > 0.5$, photoexperience x round of photostimulation: $p > 0.8$; Fig. 5).

429 Both groups of females had similar, low levels of circulating vitellogenin at the beginning
430 of the study (Week 0: $z = -1.53$, $p > 0.1$) and at the end of the 8-week photoperiodic
431 treatment (Week 8: $z = 1.00$, $p > 0.3$) (Fig. 6). Plasma vitellogenin levels also did not
432 differ between the groups in the four weeks following photoperiodic treatment, despite
433 the fact that photo-experienced females were undergoing photostimulation for the first
434 time and the photo-naïve females remained in a non-reproductive state (Weeks 9-12: all
435 $p > 0.3$; Fig. 6). There was, however, one photo-experienced female that had elevated

vitellogenin levels after one week of exposure to reproductively-stimulatory long days, but her vitellogenin levels were undetectable in the following week (Fig. 6). Similar to plasma LH, circulating vitellogenin levels were low in both groups at week 25 (photoexperience: $p > 0.6$) and week 34 (photoexperience: $p > 0.2$) of the study, when both groups of females were in a non-reproductive state (Fig. 6).

When both groups were returned to long days, photoexperience affected how plasma vitellogenin changed over the last four weeks of the study (weeks 35-38: photoexperience x week: $z = 2.00$, $p < 0.05$; Fig. 6). Photo-experienced females undergoing photo-induced early reproductive development for the second time exhibited an increase in circulating vitellogenin levels over the last four weeks of the study (Weeks 35-38: week: $z = 2.18$, $p < 0.03$; Fig. 6). In contrast, the vitellogenin levels of females in the photo-naïve group, who were undergoing photostimulation for the first time, did not change during this time (Weeks 35-38: week: $p > 0.5$; Fig. 6). This pattern was similar to that of photo-experienced females during their first round of photostimulation (photo-experienced weeks 9-12 vs. photo-naïve weeks 35-38: photoexperience: $p > 0.1$; weeks exposed to long days: $p > 0.8$; interaction $p > 0.1$; Fig. 6).

The effect of photoexperience on changes in vitellogenin levels was also apparent within individual females. The number of times photo-experienced females were photostimulated affected how vitellogenin changed over the first four weeks on long days (photo-experienced: weeks 9-12 vs. weeks 35-38: round of photostimulation: $z = -2.05$, $p < 0.05$; number of weeks exposed to long days: $z = -2.06$, $p < 0.05$; interaction: $z = 2.06$, $p < 0.04$; Fig. 6). As mentioned above, vitellogenin levels were relatively stable

and low while photo-experienced females were undergoing photostimulation for the first time and, in contrast, increased steadily during the second round of photostimulation.

Follicular development

Photo-experienced females undergoing photo-induced early reproductive development for the second time had larger ovarian follicles than photo-naïve females being photostimulated for the first time (photoexperience: $z = 1.97$, $p < 0.05$; Fig. 7a). The photo-experienced group exhibited large inter-individual variation in ovarian follicle diameter that can be explained by variation in female body mass (body mass: $z = 3.91$, $p < 0.001$). When ovarian follicle diameters were adjusted for body mass, the marked inter-individual variation in the follicle diameter of photo-experienced females decreased (Fig. 7b). By the end of the study, following four weeks of photostimulation, two of the six photo-experienced females had yellow ovarian follicles that had begun to take up yolk, and one of these females laid an egg on the last day of the study. In contrast, none of the nine photo-naïve females had any yellow, yolky follicles.

DISCUSSION

When we exposed female house finches to reproductively-stimulatory long day lengths, we found that females with previous experience with photostimulation (photo-experienced females) had greater increases in body mass, more GnRH-ir cells in the septo-preoptic hypothalamus , greater increases in plasma vitellogenin levels and more pronounced growth and development of ovarian follicles than age-matched females that were photostimulated for the first time (photo-naïve females). In contrast, we did not

observe an effect of prior experience with photostimulation on photo-induced circulating LH levels nor VIP immunoreactivity in the hypothalamic INF or VMN . Additionally, photoexperience influenced the effects of song exposure on GnRH-ir in the hypothalamic septo-preoptic area, with conspecific song elevating GnRH-ir cell count in photo-experienced but reducing GnRH-ir cell count in photo-naïve females. Our results suggest that previous photoexperience sensitizes the neuroendocrine system to the reproductively-stimulatory effects of increasing photoperiod and changes the way the neuroendocrine system responds to the supplementary cue of male song. These effects of photoexperience may be responsible, at least in part, for the age-related advance in the early stages of reproductive development.

Body mass

The photo-induced increase in the body mass of photo-experienced female house finches observed over the last four weeks of the study is consistent with the increase in body mass associated with early reproductive development and egg production. Sockman and colleagues observed similar photoexperience-dependent patterns of changes in body mass in female European starlings (Sockman et al., 2004). The one gram difference in body mass between photo-experienced and photo-naïve females in our study was likely due to the additional mass of the newly re-grown ovary, the larger and more developed ovarian follicles of photo-experienced females, the yolk deposited into the largest of the follicles in two of the photo-experienced females, and the recently re-grown oviduct of the photo-experienced female that laid an egg on the last day of the study (Vézina and Salvante, 2010). Egg-producing female passerines of similar size to house finches display similar gains in body mass above non-breeding values (e.g.,

503 great tits, *Parus major* (Silverin, 1978); pied flycatchers, *Ficedula hypoleuca* (Ojanen,
504 1983); zebra finches, *Taeniopygia guttata* (Salvante et al., 2010)).

505 **VIP**

506 We did not find an effect of photoexperience on VIP-ir in the INF or the VMN of the
507 hypothalamus following four weeks of concurrent photostimulation. Similarly, compared
508 to levels measured while exposed to short days, previously photo-naïve, male European
509 starlings showed no change in basal hypothalamic VIP levels in response to
510 photostimulation (measured every two weeks through week 8, then every four weeks
511 through week 24 of exposure to long days) (Dawson et al., 2002). It is possible that the
512 four weeks of photostimulation in our study was long enough for any photoexperience-
513 related differences in the timing of up-regulation of VIP expression to disappear. Only
514 two weeks of photostimulation was enough to trigger a significant increase in VIP-ir cell
515 count in the INF of turkeys in their second reproductive season (Mauro et al., 1989,
516 1992). However, as VIP and prolactin play regulatory roles later in the breeding season
517 during incubation and chick rearing, it is also possible that four weeks of
518 photostimulation was not sufficient to detect significant photo-induced increases in VIP
519 or photoexperience-related differences in VIP expression. VIP-ir cell count in the INF of
520 the reproductively-experienced turkeys mentioned above, continued to increase after 10
521 days of photostimulation through egg laying, incubation and into photorefractoriness
522 (Mauro et al., 1989, 1992). Similarly, while ten days of exposure to a 16h L: 8h D
523 photoperiod was sufficient to stimulate an increase in nascent VIP mRNA in the
524 hypothalamus of female turkeys, significant increases in both steady-state cytoplasmic
525 VIP mRNA levels and VIP levels in the median eminence were not apparent until the

526 birds laid their first eggs (Chaiseha et al., 1998). Therefore, we may have measured
527 VIP-ir too late to observe any influence that photoexperience may have had on the
528 timing of the onset of VIP expression, but too early to detect any photoexperience-
529 related differences in the extent of VIP expression. In contrast, the photo-experienced
530 male house finches in the present study had more VIP-ir cells in the INF than the photo-
531 naïve males (unpublished data; K.G. Salvante, R.A. Aldredge, K.W. Sockman). This sex
532 difference supports previous research showing early up-regulation of reproductive
533 hormones in male birds (Caro et al., 2006), in contrast to more fine-tuned
534 synchronization between local breeding schedules (i.e., timing of egg production and
535 laying) and the up-regulation of reproductive hormones in female birds (Caro et al.,
536 2009).

537 **GnRH**

538 The elevated photo- and conspecific song-induced GnRH expression observed in
539 photo-experienced females suggests that previous experience with photostimulation
540 “primes” the brain to increase its responsiveness to socially-relevant environmental
541 stimuli during subsequent breeding seasons. In photostimulated European starlings
542 prior photoexperience increases the responsiveness of specific brain regions involved in
543 song perception (Sockman and Ball, 2009). Similarly, photorefractory adult European
544 starlings with prior photoexperience, and thus prior exposure to elevated levels of
545 GnRH, exhibited a larger LH response following exogenous GnRH administration than
546 photorefractory juveniles that have not been previously exposed to elevated GnRH
547 levels (McNaughton et al., 1995). Moreover, there is evidence in mammals that GnRH
548 up-regulates its own receptors (Clayton, 1989). Therefore, if the priming effect of GnRH

549 on its own receptors persists until the next breeding season, the age-related advance in
550 laying date observed in many birds (Saether, 1990; Fowler, 1995) may be due, at least
551 in part, to previous experience with photostimulation and the associated exposure to
552 elevated GnRH levels.

553 While the priming effect of previous exposure to elevated GnRH explains the patterns of
554 GnRH-ir observed in photo-experienced versus photo-naïve females and females
555 exposed to conspecific song, it does not explain the other effects of photoexperience
556 and song treatment on GnRH-ir. Why did photo-naïve females exposed to
557 heterospecific song have more GnRH-ir cells than photo-experienced birds exposed to
558 heterospecific song? During the four-week period of simultaneous photostimulation of
559 the photo-experienced and photo-naïve females (weeks 35-38), both groups of females
560 were exposed to conspecific song from the co-housed male house finches. As the
561 photo-experienced females exhibited more advanced early reproductive development at
562 the end of the study than the photo-naïve females, the photo-experienced females were
563 temporally closer to having to choose a mate, and thus may have been more sensitive
564 to changes in social cues conveying information about the availability or quality of
565 potential mates. Therefore, it is possible that the removal of the more socially-relevant
566 conspecific song and/or the introduction of the less socially-relevant heterospecific song
567 as the females' only auditory signal triggered the down-regulation of GnRH levels within
568 the septo-preoptic area of the more reproductively-advanced photo-experienced
569 females compared to the photo-naïve females. European starling females exposed to
570 one week of preferred long-bout conspecific male song and then subsequently exposed
571 to a 30 minute song stimulus of less-preferred, short-bout, conspecific song exhibited a

decrease in expression of the immediate early gene ZENK (the avian homolog of and an acronym for zif-268, egr-1, NGFI-A, and Krox-24) in the auditory telencephalon compared to females that were exposed to a 30 minute song stimulus of preferred long-bout conspecific song (Sockman et al., 2005). The decrease in GnRH and ZENK activity in these instances, respectively, may represent a decrease in attraction to or preference for the secondary auditory signals to which these females were exposed (Sockman, 2007).

Circulating LH

Following nine weeks on short photoperiods, exposure to long day lengths for only one week induced a similar surge in circulating LH levels in both photo-naïve females undergoing photostimulation for the first time and photo-experienced females undergoing photostimulation for the second time. This surge was similar to the LH surge observed in photo-experienced females undergoing photostimulation for the first time at the beginning of this study and in photo-experienced European starlings undergoing photostimulation for the second time (Sockman et al., 2004). Photo-experienced European starlings undergoing photostimulation for the first time also had similar levels of circulating LH after one week on long days as all of the photostimulated females in our study (Sockman et al., 2004). Interestingly, the marked photo-induced LH surge was absent in photo-naïve starlings exposed to reproductively-stimulatory long days for the first time (Sockman et al., 2004). That study suggested that the lack of LH response may have been due to desensitization of the pituitary to GnRH by negative feedback of chronic, low-level gonadal steroid activity associated with the prolonged time (32 weeks) that photo-naïve females spent on a 8h L: 16h D photoperiod. In contrast, our photo-

naïve females only spent nine weeks exposed to short day lengths and did respond to photostimulation with a surge in LH. Therefore, the lack of an effect of photoexperience on the LH response to photostimluation suggests that LH may not play a direct role in the physiological mechanisms underlying age-related variation in early reproductive development. However, as the up-regulation of LH receptors is hormone-dependent (Piquette et al., 1991; Segaloff et al., 1991; You et al., 2000; Johnson and Bridgham, 2001; Johnson and Woods, 2009), if the expression of these regulatory hormones is dependent on photoexperience, then LH activity may also vary with photoexperience and potentially contribute to the age-related variation observed in early reproductive development.

Circulating vitellogenin and follicular development

Photoexperience influenced both circulating levels of the egg yolk precursor, vitellogenin, and the timing of yolk deposition into developing ovarian follicles. In passerine birds, the onset of vitellogenin production is tightly coupled with the onset of follicular yolk deposition (Challenger et al., 2001; Salvante and Williams, 2002). As both groups of females had relatively stable and low vitellogenin levels during the first four weeks of their first round of photostimulation, this relationship suggests that neither group had begun follicular yolk deposition during this time. This, together with the elevated plasma vitellogenin levels and the presence of an egg and yolky follicles in photo-experienced females, but not in age-matched photo-naïve females by the end of the study, is consistent with the hypothesis that photoexperience influences the age-related advancement of egg production and laying date.

One major difference between the two groups at the end of the study was that the photo-experienced females had previously been exposed to elevated circulating levels of E2 during their first experience with photostimulation and gonadal development. Primary exposure of the avian liver to E2 induces genomic changes to the regulatory sites of the genes coding for vitellogenin and apoVLDL-II, the VLDLy-specific surface protein, including demethylation of the E2-receptor complex binding site at the 5' end of the vitellogenin gene and changes to the chromatin of the vitellogenin and apoVLDL-II genes resulting in nuclease-hypersensitive sites (Wilks et al., 1982; Burch and Weintraub, 1983; Kok et al., 1985). These and other E2-induced genomic changes may contribute to the earlier induction and more rapid synthesis of vitellogenin and apoVLDL-II mRNA and circulating vitellogenin and VLDLy following secondary estrogen exposure (Bergink et al., 1973, 1974; Jost et al., 1978; Codina-Salada et al., 1983; Wang and Williams, 1983; Jost et al., 1986). Previous exposure to elevated levels of E2 may also contribute to the advance in egg formation in photo-experienced females via the stimulatory effect that E2 has on the synthesis of its own receptors (Sutherland and Baulieu, 1976; Cidlowski and Muldoon, 1978) and DNA polymerase activity (Sutherland et al., 1977) in the avian oviduct. Secondary estrogen administration has also been associated with rapid increases in both nuclear binding of progesterone receptor (Boyd-Leinen et al., 1984) and ovalbumin mRNA transcription (Swaneck et al., 1979) in the avian oviduct. If these priming effects of E2 persist until the next breeding season, they could contribute to the advancement of oviduct growth and development, egg formation and laying date in photo-exerienced females.

We have found that female house finches with prior photoexperience exhibited advanced early reproductive development in comparison with age-matched birds with no prior experience with reproductively-stimulatory long days. Yet, our results do not favor one or the other of Forslung and Pärt's (1995) "constraint" or "restraint" hypotheses. The hormonal mechanisms underlying these differences, including the potential priming effects of GnRH and E2, suggest that first-time breeders may indeed be constrained by their lack of previous exposure to these reproductive hormones, thus supporting Forslund and Pärt's "constraint" hypothesis (Forslund and Pärt, 1995). However, our results do not disprove Forslund and Pärt's "restraint" hypothesis, as we do not know whether first-year females have decided to invest fewer resources into their first breeding attempt to offset the lower reproductive potential associated with initiating early reproductive development and egg laying later than in future breeding attempts. While the lower plasma vitellogenin levels, decreased neural responsiveness to socially-relevant environmental stimuli, and delayed early reproductive development we observed in photo-naïve females photostimulated for the first time may reflect physiological and neural constraints, they may also be components of the mechanisms underlying the females' decision to invest fewer resources into their first reproductive attempt. Regardless of whether the constraint, restraint or both hypotheses are true, our results suggest that photoexperience, and not age, per se, may, at least in part, explain the advancement in laying date and enhanced reproductive output observed in older seasonally-breeding birds compared to first-year females (Saether, 1990; Fowler, 1995).

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FIGURE LEGENDS

Figure 1. Experimental design. The photoperiod treatment is depicted at the top of the figure as white bands for long days (16 L: 8 D) and as black bands for short days (8 L: 16 D). At Week 0 we exposed all females to a 16 L: 8 D photoperiod. From Weeks 1 to 8, we exposed the females in the photo-experienced group (Experienced; ■) to short days, and we maintained the females in the photo-naïve group (Naïve; □) on long days. We exposed all females to long days from Weeks 9 thru 25, to short days from Weeks 26 to 34, and then finally to long days for the last four weeks (Weeks 35-38) of the study. Downward arrows indicate the weeks during which we took a blood sample from each bird.

Figure 2. Body mass. Body mass (mean ± SEM) of photo-experienced (Experienced ■) and photo-naïve (Naïve; □) female house finches throughout the study. See Figure 1 legend for description of photoperiod treatments. Sample sizes, with number of triplicate groups and number of females in parentheses, are listed above the corresponding weeks for each photoexperience group. We compared the body masses of the photoexperience groups at the beginning of the study (Week 0), throughout the photoperiod treatment (Weeks 2-8), during the first four weeks following photoperiod treatment (Weeks 9-12), throughout the common long-day exposure (Weeks 9-25), during the common short-day exposure (Weeks 26-34), and throughout the final common long-day exposure (Weeks 35-38).

Figure 3. Gonadotropin releasing hormone immunoreactivity. Number of gonadotropin releasing hormone immunoreactive (GnRH-ir) cells (mean ± SEM) in the hypothalamic septo-preoptic area of photo-experienced and photo-naïve female house finches following exposure to either conspecific male house finch song (gray columns) or heterospecific male northern cardinal song (white columns). Sample sizes (number of independent females) are shown at the bases of the columns corresponding to each photoexperience-song treatment group.

Figure 4. Vasoactive intestinal polypeptide immunoreactivity. Number of vasoactive intestinal polypeptide immunoreactive (VIP-ir) cells (mean ± SEM) in a) the infundibular nuclear complex (INF) and b) the ventromedial nucleus (VMN) of the hypothalamus of photo-experienced and photo-naïve female house finches following exposure to either conspecific male house finch song (gray columns) or heterospecific male northern cardinal song (white columns). Sample sizes (number of independent females) are shown at the bases of the columns corresponding to each photoexperience-song treatment group.

Figure 5. Luteinizing hormone. Plasma luteinizing hormone (LH) levels (mean ± SEM) in photo-experienced (Experienced; ■) and photo-naïve (Naïve; □) female house finches throughout the study. See Figure 1 legend for description of photoperiod treatments. Sample sizes, with number of triplicate groups and number of females in parentheses, are listed above the corresponding weeks for each photoexperience group. We compared the circulating LH levels of the photoexperience groups at the beginning of the study (Week 0), at the end of the

photoperiod treatment (Week 8), during the first four weeks following photoperiod treatment (Weeks 9-12), at the end of the common long-day exposure (Week 25), at the end of the common short-day exposure (Week 34), and throughout the final common long-day exposure (Weeks 35-38).

Figure 6. Vitellogenin. Plasma vitellogenin levels (mean ± SEM) in photo-experienced (Experienced; ■) and photo-naïve (Naïve; □) female house finches throughout the study. See Figure 1 legend for description of photoperiod treatments. Sample sizes, with number of triplicate groups and number of individuals in parentheses, are listed above the corresponding weeks for each photoexperience group. We compared the circulating vitellogenin levels of the photoexperience groups at the beginning of the study (Week 0), at the end of the photoperiod treatment (Week 8), during the first four weeks following photoperiod treatment (Weeks 9-12), at the end of the common long-day exposure (Week 25), at the end of the common short-day exposure (Week 34), and throughout the final common long-day exposure (Weeks 35-38).

Figure 7. Ovarian follicle size. Effect of photoexperience on the average diameter (mean ± SEM) of the three largest ovarian follicles a) before and b) after adjustment for body mass, following four weeks of photostimulation. Sample sizes (number of independent females) are shown at the base of each column.

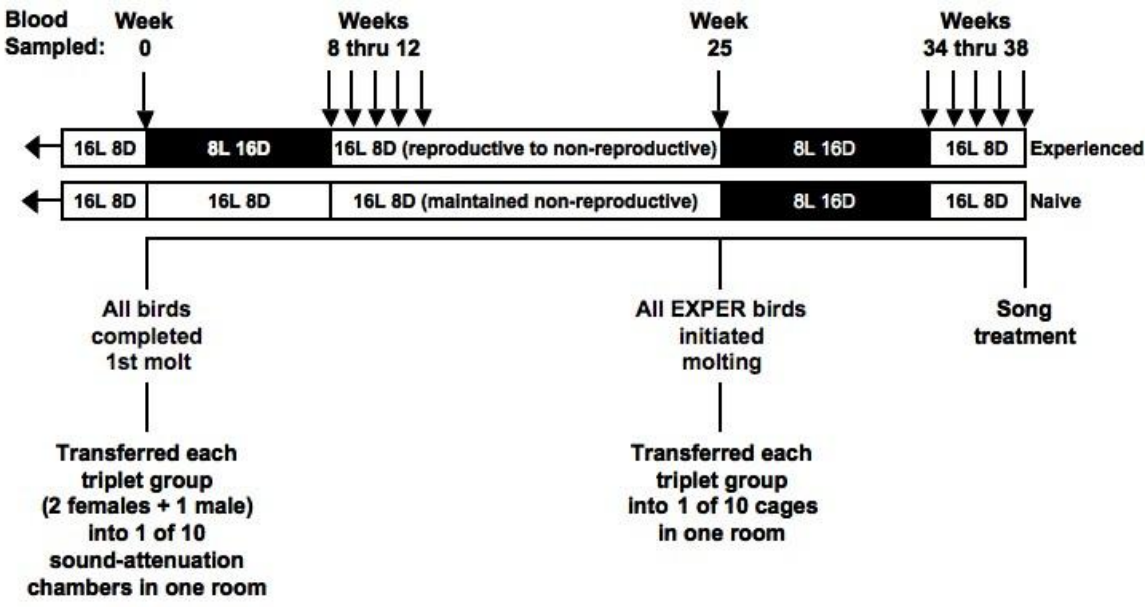
Blood Sampled:
Week 0
Weeks 8 thru 12
Week 25
Weeks 34 thru 38
16L 8D
8L 16D
16L 8D (reproductive to non-reproductive)
8L 16D
16L 8D
Experienced
16L 8D
16L 8D
16L 8D (maintained non-reproductive)
8L 16D
16L 8D
Naive
All birds completed 1st molt
All EXPER birds initiated molting
Song treatment
Transferred each triplet group (2 females + 1 male) into 1 of 10 sound-attenuation chambers in one room
Transferred each triplet group into 1 of 10 cages in one room

Figure 1.

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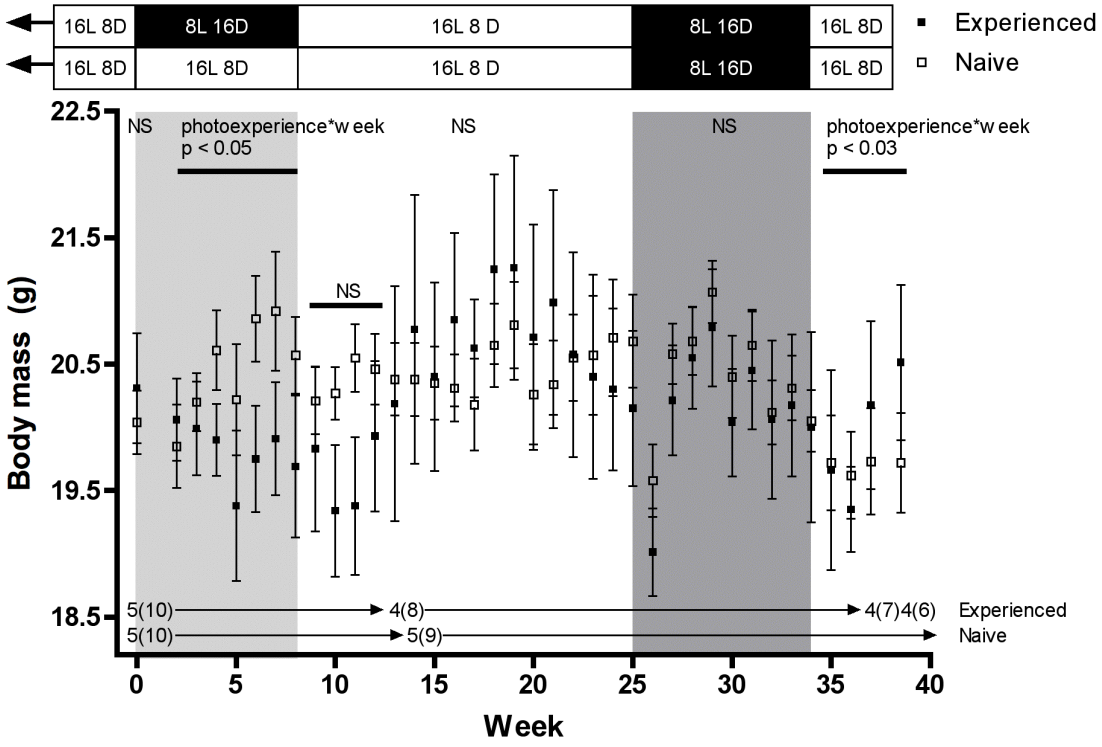

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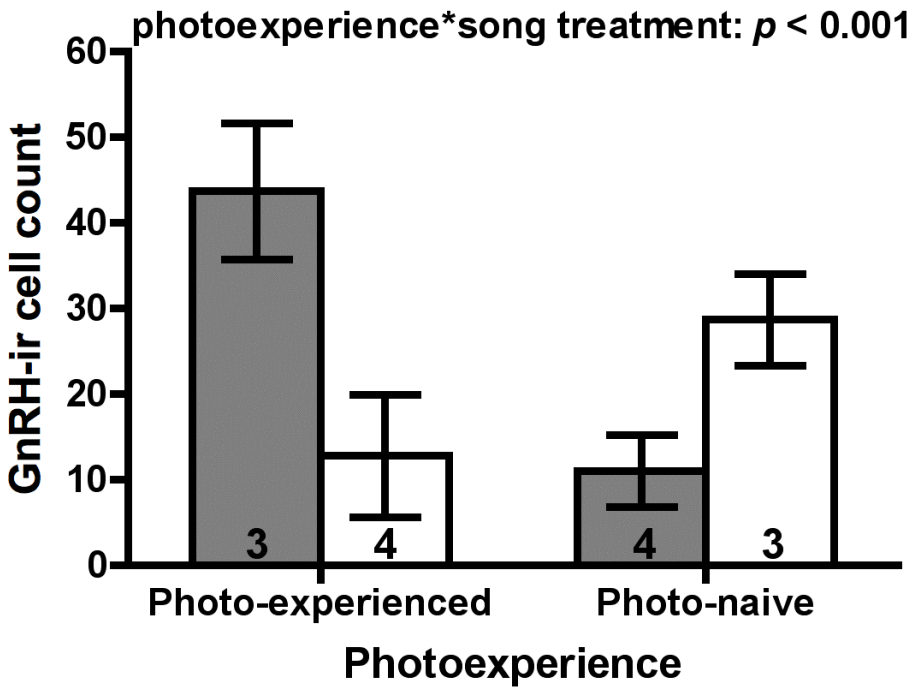
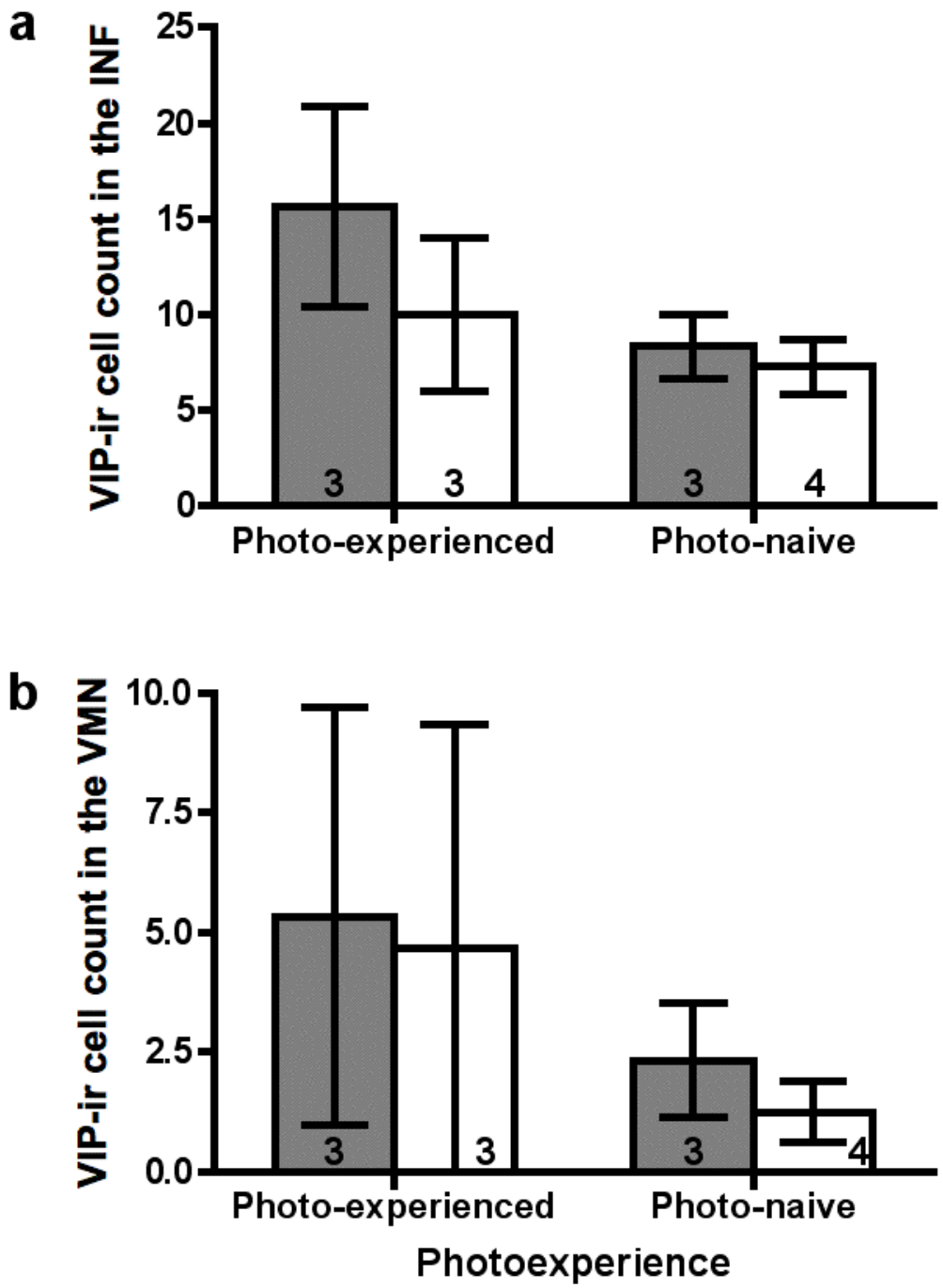

photoexperience*song treatment: p < 0.001
GnRH-ir cell count
60
50
40
30
20
10
0
3
4
4
3
Photo-experienced
Photo-naive
Photoexperience

Figure 3.



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1018 Figure 4.

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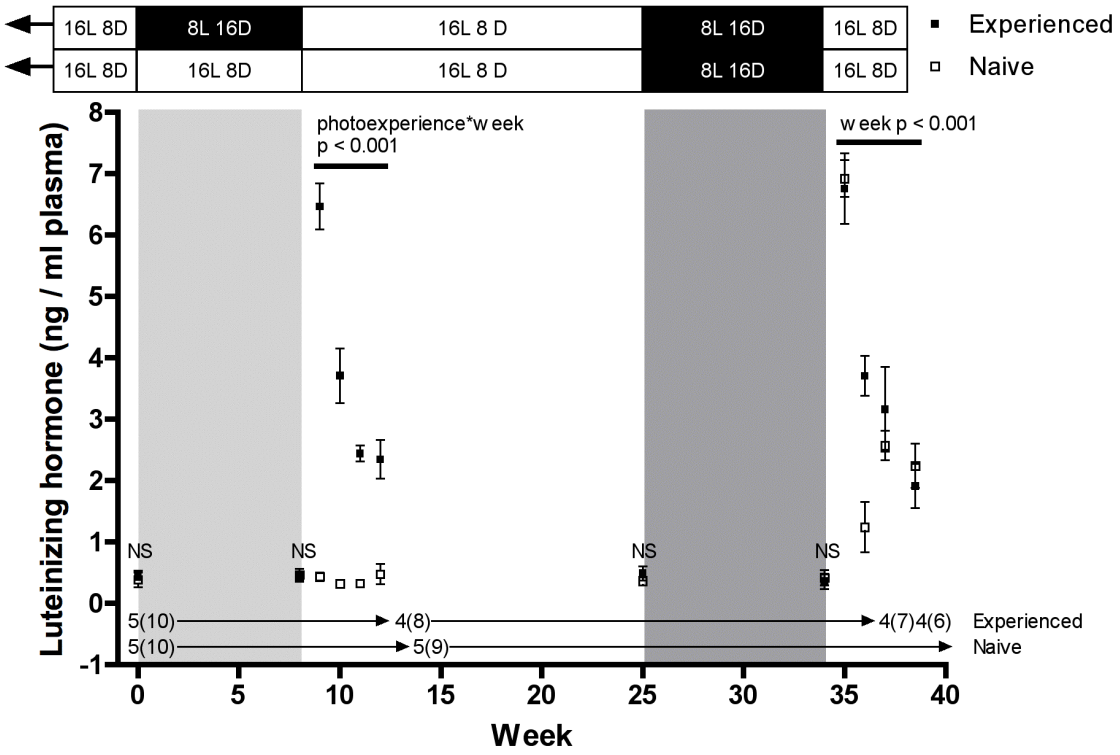

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1022 Figure 5.

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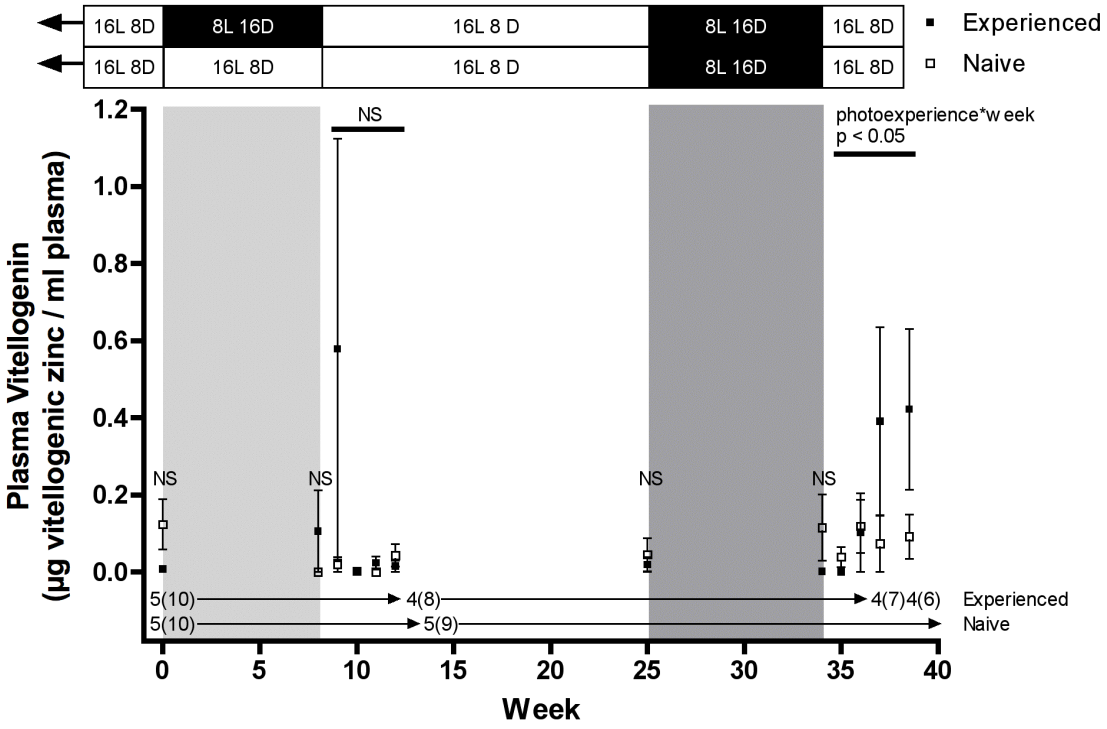

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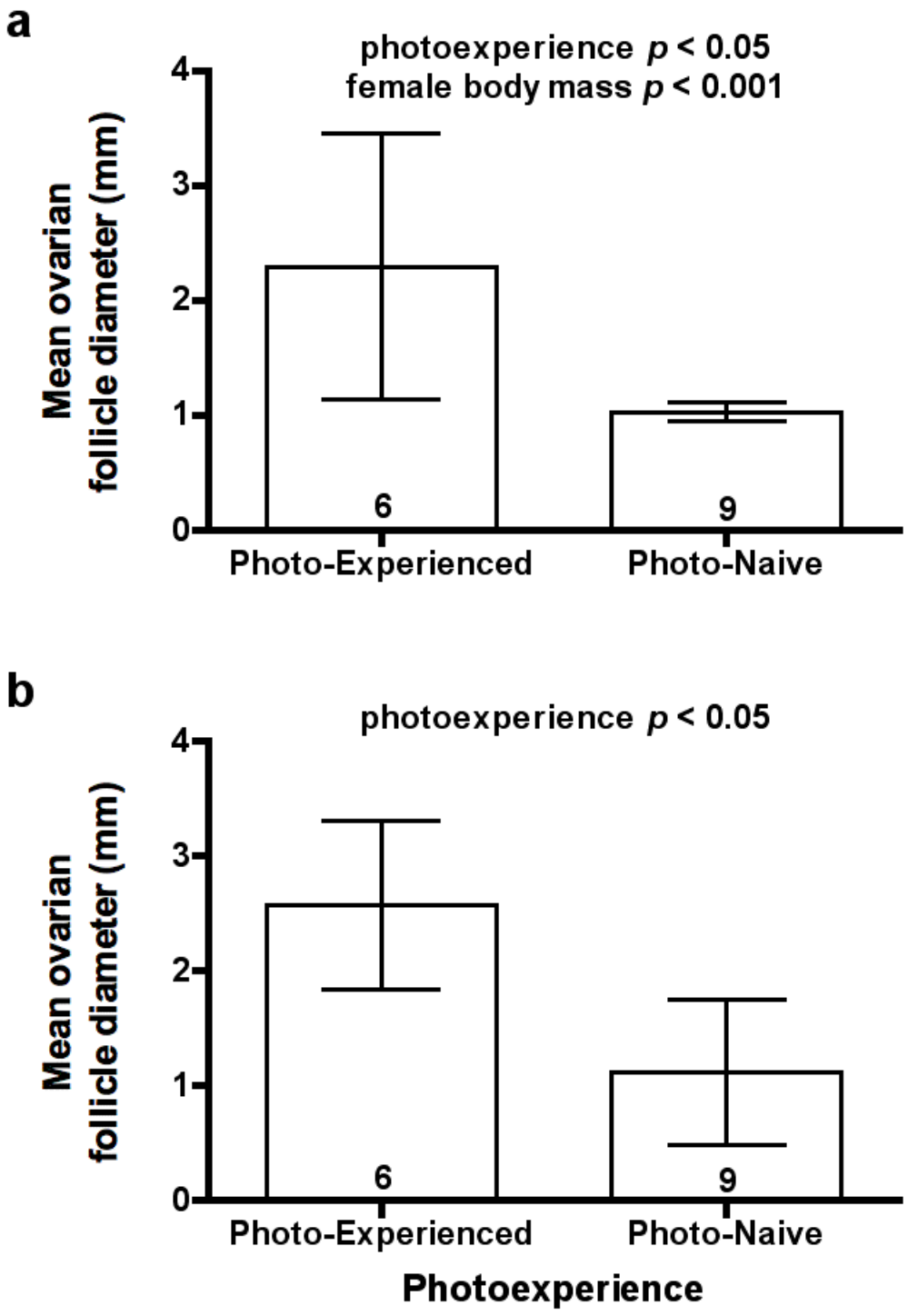

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1029 Figure 7.