Social Cues Regulate Reciprocal Switching of Hypothalamic Dio2/Dio3 and the Transition Into Final Follicle Maturation in European Starlings (*Sturnus vulgaris*)

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With final maturation of ovarian follicles, birds are committed to a major energetic investment: egg laying. Follicles develop in a 2-step process: 1) initial development of regressed follicles stimulated by long days and 2) yolk incorporation into hierarchical follicles, ovulation, and oviposition. We know little about how females transduce environmental cues into neuroendocrine signals regulating the second step. The present study measures gene expression in tissues within the hypothalamo-pituitary-gonadal axis. Females were housed in seminatural enclosures experiencing natural changes in photoperiod and environmental cues (eg, temperature, rainfall, etc), without males or with constant access to males (January to April). By April, females with males had begun to lay eggs, whereas those without males had not. In a second study, females without males for 3.5 months were then given access to males for 7 days. Restricting male access completely inhibited final follicle maturation, whereas 7-day male access stimulated full vitellogenesis and follicle maturation. Few gene expression changes were attributable to constant male access (January to March), but naïve females given 7-day male access had increased type 2 deiodinase (DIO2) and decreased DIO3 synthesis in the hypothalamus, potentially influencing local thyroid hormone metabolism, increased expression of LH receptor and aromatase in follicles and vitellogenin in liver. Our data suggest that initial follicle development may be more heavily influenced by photoperiod, but the second step (final maturation) is sensitive to other cues such as social interactions. This is the first demonstration of a social effect on the Dio2/Dio3 system, previously thought only responsive to photoperiod cues. **(*Endocrinology* 156: 694–706, 2015)**

The "decision" by female birds to initiate egg laying is a pivotal physiological step in the timing of reproduction and has major fitness consequences (1, 2). Once rapid final maturation of follicles begins, including the incorporation of yolk into developing follicles, the females are committed to a major energetic investment that results in egg laying. Ecologists have made significant progress understanding the environmental variables (eg, food, temperature) that influence the onset of laying in the breeding season. They have correlated decades' worth of "reproductive timing" data, usually using lay date as the metric for the beginning of the breeding season in the field (3–6), with long-term changes in environmental cues such as temperature and food availability, as well as using experimental work demonstrating advancement or delay of lay dates in free-living birds (7, 8). Despite the ecological data, physiologists are far behind in understanding the mechanisms regulating the important physiological transition that underlies clutch initiation. In fact, most of the experimental work investigating how avian species respond to environmental cues such as day length has largely excluded females from consideration (9, 10). This is not un-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received June 5, 2014. Accepted December 4, 2014.
First Published Online December 9, 2014

Abbreviations: DIO2, type 2 deiodinase; E2, estradiol; *FSH-r*, FSH receptor; GnIH, gonadotropin inhibitory hormone; GOI, gene of interest; HPG, hypothalamo-pituitary-gonadal; *LH-r*, LH receptor; LR8, low density lipoprotein receptor relative with 8 ligand binding repeats; RFID, radio-frequency identification; SW, small white; VLDL, very low-density lipoprotein; VTG, vitellogenin.

common for animal research in general (11), and although exciting new studies are beginning to uncover the genetic mechanisms underlying the perception of long days by the brain in males (12, 13), it is unclear whether these same mechanisms operate in females, or whether they operate over a similar time frame. Given that there is now substantial evidence that climate change can affect timing of reproduction specifically in females (gleaned by measuring lay dates in the field) (3, 4, 14, 15), it is critical that we understand how this important event is controlled (16).

For seasonal breeders, increasing photoperiod stimulates the reproductive axis by causing increased GnRH synthesis and secretion to the anterior pituitary gland. The mechanisms by which photoreceptive cells, likely to be in the hypothalamus (12, 17–20), interact with and stimulate GnRH neurons are not fully understood, but a model for GnRH release has been proposed from studies in male quail (21). The first change in gene expression after a single long day in quail was increased expression of the β-subunit of TSH in the pars tuberalis of the pituitary at 14 hours after dawn, followed 4 hours later by increased expression of type 2 deiodinase (DIO2) and decreased expression of DIO3 in the medial basal hypothalamus (13). The combined change in expression putatively causes a local increase in the active thyroid hormone metabolite, T_3. Some studies have shown that injections of T_3 cause increased LH secretion and gonadal growth in male quail (23) and maintain reproductive activation in mammals (24). Taken together, these studies suggest that for long-day breeders an increase of T_3 locally in the hypothalamus causes increased GnRH release, which induces gonadotropin release from the anterior pituitary gland.

In males, photostimulation with long-day lengths is often sufficient for full activation of the reproductive axis, including complete growth of the testes, maturation of gametes, and stimulation of sexual behaviors such as song (25). Females respond to photostimulation in a very different way in the laboratory than do males. For example, photostimulation appears to activate the hypothalamus and pituitary, but ovarian follicles often only show partial development (26–30). For follicles to become competent to ovulate and produce fertile eggs, they increase estradiol secretion to activate vitellogenesis in the liver (31, 32), and follicles incorporate 2 yolk precursors, vitellogenin (VTG) and very low-density lipoprotein (VLDL)y (33, 34) through the VTG/VLDL receptor, LR8 (low density lipoprotein receptor relative with 8 ligand binding repeats) (35, 36). Many photoperiodic females in the laboratory begin development of small white (SW) follicles (1–2 mm in diameter) that protrude from the ovarian stromal tissue but that never increase any further in size or begin to incorporate yolk. One hypothesis is that although long days serve to activate GnRH and gonadotropin secretion, either the stimulation is less pronounced or is shorter lived in females and/or that a set of stimuli other than day length is responsible for activation of final follicle maturation (37, 38). One stimulus that has been known for decades to influence ovarian follicle development is male song (39, 40).

In this study, we attempt to uncover the specific mechanisms that regulate reproductive maturation in a wild songbird female (*Sturnus vulgaris*), including both the initial stimulation by long days (to SW follicles) as well as the important shift to final rapid maturation of follicles that immediately precedes egg laying. We examine changes in expression of key genes known to be important for hypothalamo-pituitary-gonadal (HPG) axis regulation and consider changes along the entire HPG within the same individual. Specifically, in the hypothalamus we measure expression of 2 of the neuropeptides known to play critical roles in regulating gonadotropin secretion, GnRH and gonadotropin inhibitory hormone, GnIH, as well as the thyroid metabolizing enzymes DIO2 and DIO3. In the anterior pituitary, we measure expression of the β-subunit for one of the gonadotropins that activate follicle growth and development, FSH, and in the ovary we measure LH and FSH receptor expression. To characterize ovarian activation, we measure genes that play important roles in the production of yolk and its incorporation into follicles, namely the aromatase enzyme that produces estradiol from the ovary, a yolk precursor, VTG, and the receptor in the follicle that is responsible for taking up yolk precursors from circulation (LR8). We also measure circulating levels of both yolk precursors, VTG and VLDLy. We characterize these changes in 2 ways: first we compare gene expression over time in females housed in a semi-natural environment with or without access to males, and second, we compare gene expression as follicles mature regardless of social housing.

Materials and Methods

Animals

All procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals, and with the approval of the Animal Care and Use Committee of the University of California, Berkeley. Juvenile European starlings (*S. vulgaris*) were captured in the late summer and fall and transported to the Field Station for the Study of Behavior, Ecology and Reproduction at the University of California, Berkeley. Females were housed in multiple outdoor semi-natural aviaries ($12 \times 6 \times 3.5$ m) in mixed sex groups in the fall (Figure 1, event A) until January, when birds were randomly assigned to treatments (Figure 1, event B). Females were either placed in aviaries with other female conspecifics (2011 n = 15

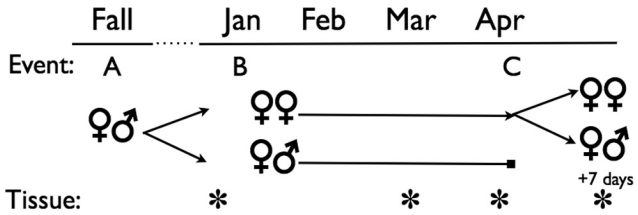

Figure 1. Females were housed in mixed-sex aviaries in the fall (A) and then randomly assigned to treatment groups (B), either same sex or mixed sex aviaries. Birds were housed in seminatural outdoor aviaries and exposed to natural changes in day length and climate until early April when females in mixed sex aviaries had begun egg laying. A subset of females housed in same sex aviaries was assigned to either mixed sex or same sex aviaries for +7 days (C). Tissue was collected just before event B, in March, just before event C and after +7 days (* symbols).

and 2012 n = 14) or with randomly assigned conspecific males (2011 n = 21 and 2012 n = 6). All birds experienced natural weather and changes in photoperiod (9 h light-15 h dark in December to 13 h light-11 h dark in April; 37.9°N, 122.3°W). Food (Purina Layena pellets) and water were available ad libitum and all aviaries were equipped with nest boxes (18"Height × 6.5"Width × 6.5"Length). Birds could see and hear conspecifics in neighboring aviaries. Males actively courted females in their own and in neighboring aviaries but could not make physical contact with those in neighboring aviaries.

Experimental protocol

We collected tissue from females housed with males in January just before transfer to treatment groups (Figure 1, * symbols; baseline n = 15 females), then again in mid-March and during the first week of April from aviaries with males present (n = 5 March, n = 18 April). Tissue was collected from females housed without males on the same dates (n = 8 March, n = 11 April). In aviaries that contained both sexes, birds paired and displayed breeding behavior. Nest-building behavior was observed in both treatment groups. Females housed with males had started to lay eggs by the first week of April in both 2011 and 2012. After the first week of April sampling point, we used a cohort of females that had not been housed with males (naïve females) and gave them access to males for the first time for 7 days (Figure 1, event C; +7 d males present; n = 12) while at the same time kept a second group of females that had not been housed with males for 7 days (+7 d no males present, n = 6) as controls.

We measured nest-building behavior within each nest box at least once each week by scoring nest completion (0 = empty/bottom of the box obvious, 0.25 = bottom of the nest box just covered with plant material, 0.50 = bottom of the box completely covered no grass ring around nest cup, 0.75 = nest full of nesting material with an unfinished swirled grass ring around the nest cup, and 1.00 = nest complete with swirled grass ring around nest cup).

Glass passive integrated transponder tags (2 × 12 mm; Cyntag, Inc) were attached to plastic leg bands (Avinet, Inc). Radiofrequency identification (RFID) transponders and antennae (41) were attached to each nest box to measure nest visitation and to verify the identity of nest box occupants. In 2012, in addition to behavioral observations, RFID data were collected for 31 daylight hours over 3 days. RFID were programmed to collect data at 500-ms intervals with 1-s pauses, between 6 AM and 9 PM hours for the last 3 days of the +7-day time period. In 2011, nest occupants and identity of pairs were identified by behavioral observations using color band identification.

Tissue collection/RNA extraction

At each sampling point, birds were quickly captured and euthanized by decapitation between 9 AM and 12 PM after deep anesthesia with isoflurane. Brains, gonads and livers were extracted and rapidly frozen on dry ice, transported to the laboratory and stored at −80°C. Brains were cut into 40-μm sections on a cryostat and 3-mm tissue punches (Harris Uni-core, Electron Microscopy Sciences no. 69036) centered along the midline and ventral edge of each section were collected from alternate sections starting at the tractus septomesencephalicus through the hypothalamus until the emergence of the median eminence. Punches were immediately added to PureZOL (Bio-Rad no. 73206880), homogenized, and frozen at −80°C until RNA extraction. Gonads and liver tissue were stored at −80°C until RNA extraction. Anterior pituitary glands were left in the skull and immersed in RNA Later (Sigma R0901) for 24–48 hours at 4°C, then extracted from the skull under a dissecting microscope and frozen at −80°C until RNA extraction. Total RNA was extracted from tissue using a PureZOL RNA isolation reagent according to manufacturer's instructions. To eliminate DNA contamination, total RNA was deoxyribonuclease treated with DNA-free (Ambion no. AM1906) and then reverse transcribed (brain punches 1-μg RNA, anterior pituitary 500 ng, follicles 500 ng, and liver 500 ng) using iScript cDNA synthesis kit (Bio-Rad no. 170–8891) according to manufacturer's instructions.

Quantitative PCR

We used 7.5-μL Bio-Rad SsoAdvanced SYBR Green Supermix (no. 172–5261) in a 15-μL reaction volume with 5μM primers and 3.75-μL cDNA (dilution for brain 1:25, pituitary 1:10, liver 1:100, follicles 1:25) on an Applied Biosystems StepOne machine. Quantitative PCR primers were designed using European starling sequences for each gene of interest (GOI) (for primer sequences, please see Supplemental Table 1) using Primer3 software (42). Published *Gallus* sequences were used to design primers for control genes. Nontemplate controls were included for each primer pair to check for formation of primer-dimers, and if present, always amplified at least 10 cycles later compared with template-containing samples. Specificity was analyzed by running a melt curve analysis, and cycle thresholds and efficiency values were calculated using PCR Miner (22) on raw data. GOI's were normalized using multiple control genes (*GAPDH*, *HPRT*, *18S*, and *BACT*) by dividing the GOI by the composite normalization factor of all controls (43). We calculated fold change above minimum as normalized GOI expression/normalized minimum group mean.

Plasma yolk precursor analysis

Plasma samples were assayed for vitellogenic zinc (zinc kit; Wako Chemicals) and total triglycerides (glycerol reagents A and B; Sigma) as indexes of the yolk precursors VTG and yolk-targeted VLDLy, respectively, following Mitchell and Carlisle (31) and as previously described for passerines including the European starling (33, 66). VTG was assayed on 5 96-well plates and intra- and interassay coefficient of variation (%) was 4.8% and

4.3%, respectively. VLDL was measured in 2 plates, and values for a laying hen plasma pool were 14.7 and 13.3 mmol l^{-1}.

Statistical analysis

The number of total visits, the number of individuals visiting each nest box, and the proportion of total visits for each female for each nest box (ie, the proportion of visits/female/nest box = average [number of visits/female/total visits by all individuals/nest box]) were compared between aviary types using a Mann-Whitney *U* test. The mean of nest completion was calculated by taking the average of all the nest boxes in each social treatment for the month. Differences between means were tested using a repeated measures ANOVA with treatment as a between subjects factor and time as a within subjects factor for data collected from January through April. Because the introduction of males to previously naïve females (+7 d males present) was not a repeated measure, we compared that group separately with its control (+7 d no males) using a Student's *t* test.

We used a two-way ANOVA with Tukey's post hoc comparisons with test for pairwise differences in gene expression among groups (2 treatment groups: males vs no males present, and 3 time points: baseline, March, and April). After the April time point when females that had males for the entire experiment had started to lay eggs, we gave males to a subset of females from the no males present group for 7 days. We treated this comparison separately, because the groups are not equivalent treatments to the previous 3 sampling points. To test differences between these 2 groups, we used Student's *t* tests. We also tested for differences among groups based on follicle sizes, regardless of treatment (categorized using the largest follicle present as <0.5, 1–2, 2–4, 4–6.5, 6.5–9, 9–11, or 11–12 mm) within follicle tissue, or among individuals at different follicle stages for expression in the brain and in the liver. When testing gene expression in follicles over time (LH receptor [*LH-r*], FSH receptor [*FSH-r*], *aromatase*, and *LR8*), we first calculated the average expression of the GOI over multiple follicle types within each individual. Data were log-transformed to equalize variance when necessary.

Results

Nest box attendance and nest-building behavior with social treatment

Birds visited nest boxes at similar rates in aviaries with or without males present (Mann-Whitney *U*, $P = .48$, median visits 177.0 and 112.5 for aviaries with or without males, respectively). Nest boxes were visited by a greater total number of different individuals in aviaries without males than in aviaries with males (Mann-Whitney *U*, $P = .002$) (Supplemental Figure 1). The proportion of visits for each individual was smaller in aviaries without males present than in male/female aviaries (Mann-Whitney *U*, $P = .002$) (Supplemental Figure 1). In aviaries with males, boxes were frequented by members of a pair, and the median proportion of visits between each individual was 0.50. Nest boxes in aviaries with no males were visited by a greater number of different birds (4–6 different females on average), and the proportion of visits for each female 0.25.

Females housed with males built nests sooner than females housed with other females (repeated measures ANOVA, males vs no males $F_{(1,42)} = 12.59$, $P = .001$, time × social treatment interaction $F_{(3,126)} = 13.35$, $P = .001$) (Figure 2A). When females were able to form pairs with males, nests were significantly closer to completion (than when females were housed only with other females) by March and continued to be closer to completion in April. Nest building did not change significantly in aviaries without males during the entire sampling period. After males were introduced to previously naïve females for 7 days, nests were closer to completion compared with the female-only aviary during the same time period ($t_{(16)} = 2.27$, $P = .037$) (Figure 2B).

Ovarian follicle development and vitellogenesis with photostimulation and social treatment

Ovarian follicle sized increased between baseline and March, although follicles in March were only 1%–2% of their final volume (Figure 3). In females with constant access to males, follicle size increased markedly (1000-fold) between March and April as they initiated yolking or vitellogenesis (time: $F_{(2,52)} = 64.74$, $P < .001$, social treatment: $F_{(1,52)} = 8.72$, $P = .005$) and were much larger than in females without access to males in April (post hoc comparisons $P < .05$). In contrast, follicles stopped growing in March in females without access to males (interaction: $F_{(1,52)} = 5.11$, $P = .03$). Critically, females housed without access to males did not enter into the final maturation stage of development, and no preovulatory follicles formed in April. Twelve of 15 females housed with constant access to males had begun yolking follicles at the same time point in April. When males were introduced to naïve females for the first time, follicles were larger than controls after 7 days ($t_{(16)} = 5.00$, $P < .001$) (Figure 3, inset). Nine of the 10 females developed a preovulatory

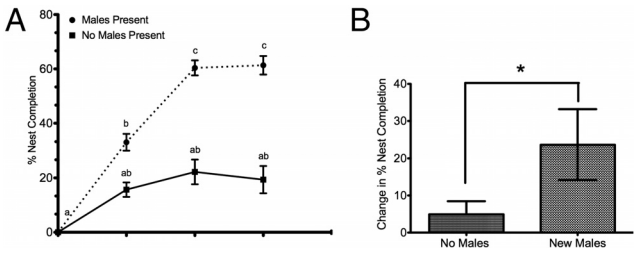

Figure 2. Females with males present in the aviary completed nests more quickly than females housed without males present (A). When males were added into aviaries with previously deprived females, nest building increased to a greater degree than in females housed without males during the same time period (B). Means ± SEM. Unique letters or asterisk indicate significant different means tested with post hoc comparisons.

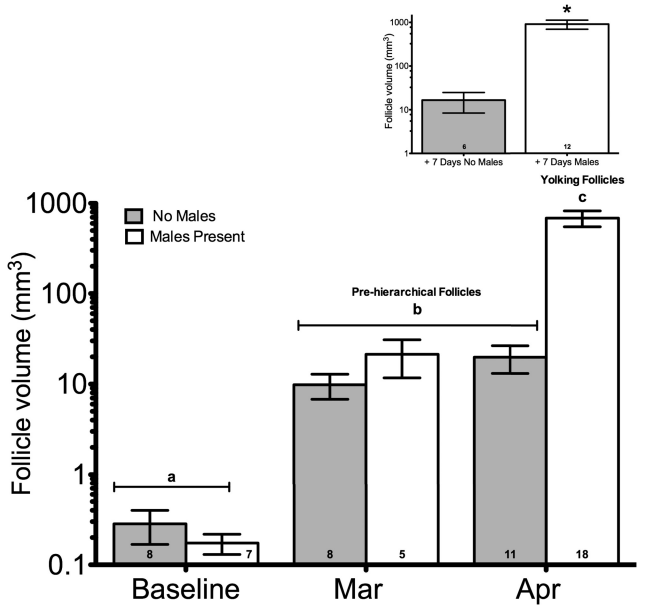

Figure 3. Volume of the largest developing follicle in females pretreatment during short day lengths (baseline, December/January) and during March and April after random assignment into aviaries either containing only females (no males) or aviaries with males (males present). Once females in the males present group began to lay eggs (April), females that had been deprived of males were either given access to males for 7 days (+7 d males) or remained in aviaries with other females (+7 d no males). Mean ± SEM, unique letters or asterisk indicate significantly different means tested with post hoc comparisons.

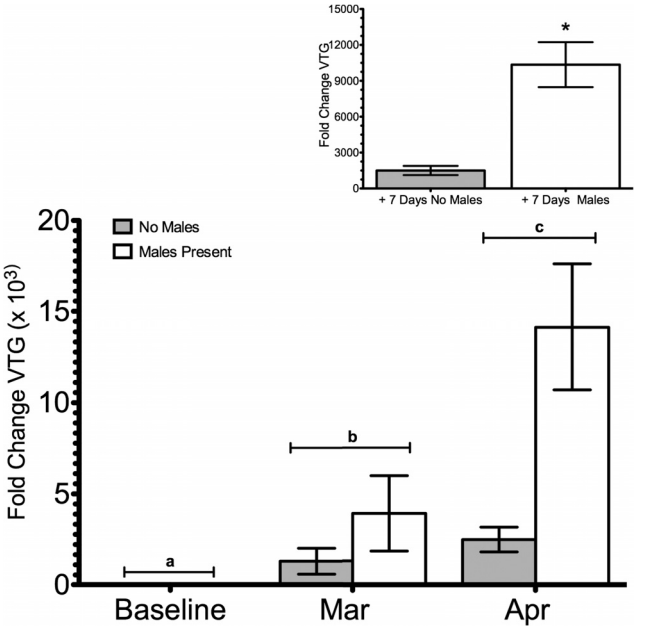

Figure 4. Fold change ($\times 10^3$) in VTG mRNA expression in liver tissue in females during short day lengths (baseline, December/January) and during March and April after random assignment to aviaries either containing only females (no males present) or aviaries with males (males present). Once females in the males present group began to lay eggs (April), females that had been deprived of males were either given access to males for 7 days (inset; +7 d males) or remained in aviaries with other females (inset; +7 d no males). Mean ± SEM, unique letters or asterisk indicate significantly different means.

hierarchy within 7 days when exposed to males, whereas none of the control females (+7 d no males) progressed past the small yellow follicle stage over that same 7-day time period.

In the liver, VTG mRNA expression increased markedly over time ($F_{(2,46)} = 6.16$, $P = .004$) (Figure 4) and was higher overall with physical access to a mate ($F_{(1,46)} = 5.10$, $P = .03$, interaction: $F_{(1,46)} = 2.06$, $P = .16$), largely because of much higher expression in females with constant access to males in April. VTG expression was also higher in naïve females given males for 7 days compared with controls ($t_{(16)} = 3.81$, $P = .002$) (Figure 4, inset).

Hypothalamic and pituitary gene expression changes with photostimulation and social treatment

In contrast to follicle size, hypothalamic GnRH expression increased significantly above baseline in March and April in both social treatment groups (time: $F_{(2,41)} = 10.39$, $P < .001$, social treatment: $F_{(1,41)} = 0.29$, $P = .59$, interaction: $F_{(1,41)} = 0.51$, $P = .61$) (Figure 5A), and GnRH expression was similar between females with or without males +7 days ($t_{(10)} = 1.50$, $P = .16$) (Figure 5F). Hypothalamic GnIH expression increased above baseline in March and then declined in April back to baseline (time: $F_{(2,41)} = 10,40$, $P < .001$, post hoc comparisons $P < .05$) (Figure 5B) but was not affected by constant access to males (social treatment: $F_{(1,41)} = 0.04$, $P = .83$, interaction: $F_{(1,41)} = 0.68$, $P = .51$). When previously naïve females were given males for 7 days, GnIH did not change significantly compared with controls ($t_{(10)} = 0.72$, $P = .49$) (Figure 5G), although variation increases in females given males. Hypothalamic *DIO2* showed a similar expression pattern to GnRH. It increased above baseline after March in both social treatment groups and stayed elevated in April (time: $F_{(2,38)} = 17.07$, $P < .001$, interaction: $F_{(1,38)} = 1.98$, $P = .15$) (Figure 5C) but was not different between females with constant access to males and females without males ($F_{(1,38)} = 1.04$, $P = .32$). When previously naïve females were given males, *DIO2* expression increased compared with females without males sampled on the same day ($t_{(9)} = 2.79$, $P = .02$) (Figure 5H). *DIO3* expression did not change over time or with constant access to males (time, $F_{(2,32)} = 1.30$, $P = .29$, social treatment, $F_{(1,32)} = 1.37$, $P = .25$, interaction: $F_{(1,32)} = 2.96$, $P = .07$) (Figure 5D) but was lower in naïve females given males for 7 days compared with controls ($t_{(9)} = 2.56$, $P = .03$) (Figure 5I). Like *GnRH* and *DIO2*, *FSHb* in the anterior pituitary increased over time ($F_{(2,35)} = 4.39$, $P = .02$) (Figure 5E) but was not affected by constant access to

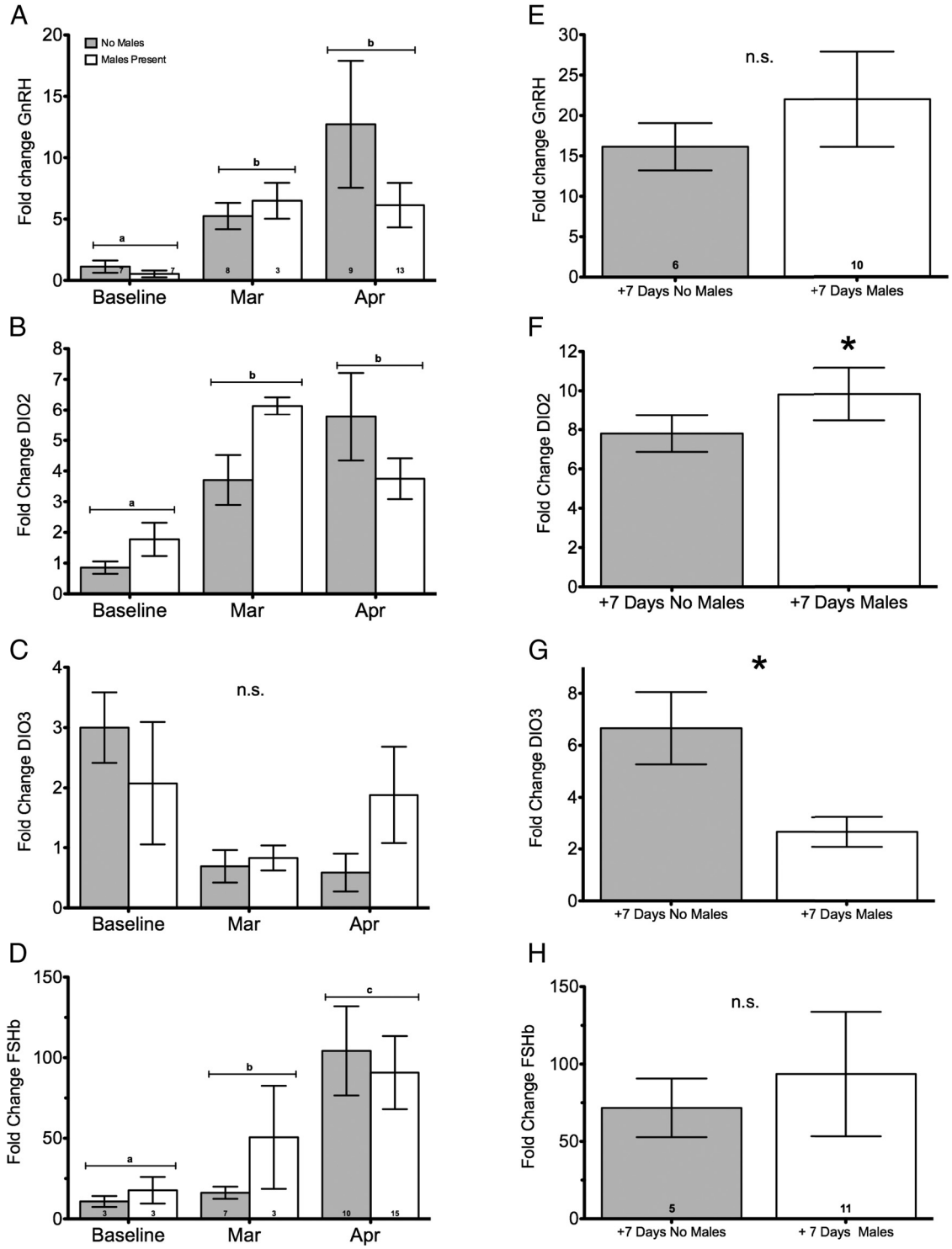

Figure 5. Fold change in mRNA expression of GnRH (A and E), DIO2 (B and F), or DIO3 (C and G) in hypothalamic tissue punches, and fold change of FSHb (D and H) in anterior pituitary tissue. Left panels show changes in females over time housed with our without males between Jan and April, and right panels show means for females housed with males for 7 days. Mean ± SEM, unique letters or asterisk indicate significantly different means tested with post hoc comparisons.

males (social treatment: $F_{(1,35)} = 0.70$, $P = .41$, interaction: $F_{(1,35)} = 0.43$, $P = .65$). *FSHb* expression was similar to controls in naïve females given males for 7 days to controls ($t_{(14)} = 0.80$, $P = .43$) (Figure 5J).

Gene expression in developing follicles in response to photostimulation and social treatment

LH-r expression was similar to baseline in March and increased above baseline in April (time: $F_{(2,50)} = 22.21$,

$P < .001$, Tukey's post hoc $P < .05$) (Figure 6A) and was similar between females with constant access to males and females without males (social treatment: $F_{(1,50)} = 2.88$, $P = .10$, interaction: $F_{(1,50)} = 0.13$, $P = .72$). However, naïve females given males for 7 days had higher expression of *LH-r* compared with controls ($t_{(16)} = 2.28$, $P = .03$) (Figure 6E). *FSH-r* mRNA expression in April was significantly higher than baseline, and expression was intermediate in March (time: $F_{(2,49)} = 9.67$, $P < .001$, Tukey's post hoc $P < .05$) (Figure 6B). *FSH-r* expression was not affected by constant access to males (social treatment: $F_{(1,49)} = 0.57$, $P = .81$, interaction: $F_{(1,49)} = 1.91$, $P =$

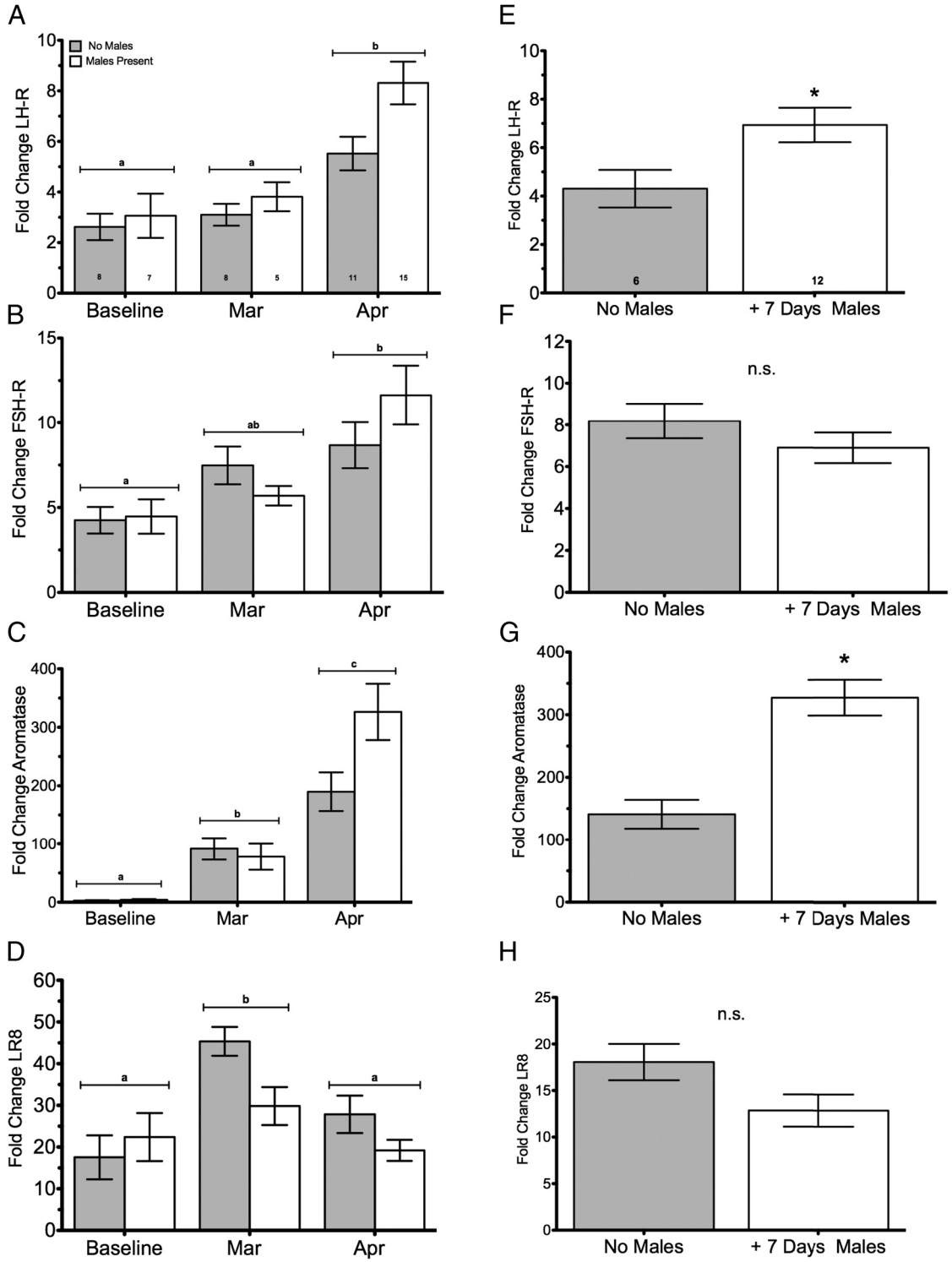

Figure 6. Average fold change in mRNA expression of LH-R (A and E), FSH-R (B and F), aromatase (C and G), and LR8 (D and H) in ovarian follicles. Mean ± SEM, unique letters or asterisk indicate significantly different means tested with post hoc comparisons.

.17), nor exposure to males for 7 days compared with controls ($t_{(16)} = 1.23$, $P = .24$) (Figure 6F). Aromatase mRNA expression closely resembled that of *LH-r* expression (Figure 6C). It increased from baseline to March and from March to April (time: $F_{(2,50)} = 115.2$, $P < .001$, Tukey's post hoc $P < .05$) but was not affected by constant access to males ($F_{(1,50)} = 0.43$, $P = .51$, interaction: $F_{(1,50)} = 0.83$, $P = .37$). However, *aromatase* expression was significantly elevated in females given males for the first time (+7 d males present) compared with controls ($t_{(16)} = 4.17$, $P = .001$) (Figure 6G). The mRNA expression of the yolk uptake receptor (*LR8*) increased from baseline to March and back to baseline in April (time: $F_{(2,50)} = 4.95$, $P = .01$) (Figure 6D). Females without males had significantly more LR8 expression overall (social treatment: $F_{(1,50)} = 7.67$, $P = .007$, interaction: $F_{(1,50)} = 0.56$, $P = .46$) largely from higher expression in March and April compared with females with males. *LR8* expression was similar in females given males for 7 days compared with controls ($t_{(16)} = 1.84$, $P = .08$) (Figure 6H).

Overall changes in gene expression as follicles mature (regardless of social treatment)

LH-r mRNA expression in the follicles increased as follicles matured ($F_{(5,161)} = 14.76$, $P < .001$) (Figure 7B). LH-r expression increased as follicles increased to greater than 4 mm and remained elevated until follicles reached maximal size (12 mm). FSH-r mRNA expression peaked when follicles reached 2–6 mm and then decreased to baseline expression levels ($F_{(5,161)} = 4.99$, $P < .001$) (Figure 7C). Aromatase expression increased steadily as follicles matured ($F_{(5,160)} = 58.45$, $P < .001$) (Figure 7D), with the lowest expression in regressed follicles and increasing until follicles reached maximal size. Lastly, LR8 mRNA expression was highest in 1- to 2-mm follicles and decreased as follicles increased in size ($F_{(5,162)} = 19.68$, $P < .001$) (Figure 7E).

VTG mRNA expression in the liver steadily increased in females as they progressed from regressed to maximal sized follicles ($F_{(5,63)} = 41.78$, $P < .001$) (Figure 8A). Plasma VTG increased above baseline when follicles reached 4 mm and stayed elevated until follicles reached maximal size ($F_{(5,40)} = 19.99$, $P < .001$) (Figure 8B). Plasma VLDLy increased above baseline when follicles reached 6 mm and stayed elevated until follicles reached maximal size ($F_{(5,40)} = 13.66$, $P < .001$) (Figure 8C).

In the hypothalamus, both *GnRH* and *DIO2* expression increased significantly as follicles developed from fully regressed (fold change: 0.88 ± 0.30 and 1.31 ± 0.33, respectively) to follicles that were greater than 1 mm in diameter (fold change: 10.27 ± 2.26 and 5.27 ± 0.97, respectively), and stayed similarly elevated for the rest of follicle development (range of fold change: 7.49–19.86 and 4.88–7.39, respectively; $F_{(5,62)} = 6.410$, $P < .001$ and $F_{(5,58)} = 9.133$, $P < .001$, respectively) (data not shown). Neither GnIH nor DIO3 (data not shown) expression changed with follicle development ($F_{(5,62)} = 1.28$, $P = .28$ and $F_{(5,62)} = 1.111$, $P < .37$).

Discussion

Our data clearly demonstrate differential timing of development or "switching on" of different levels of the HPG axis, with central parts of the axis (hypothalamus and pituitary) being activated relatively early (March) and peripheral parts of the axis related to growth (in the ovary) being switched on relatively late, either immediately before or coincident with onset of egg formation (April). Furthermore, these data suggest that the effect of supplementary/synchronizing cues (in this case, access to a mate) that are involved in the final activation of the ovary/liver may only be obvious after the initial activation of the ovary and liver has occurred. In support of this idea, 1) hypothalamic GnRH, GnIH, DIO2, and pituitary FSH mRNA increased over time, were significantly elevated in March, before onset of ovarian development, and showed no change in response to constant male presence (Figure 5); 2) in contrast, LH-r and FSH-r in the ovary did not increase until April (Figure 6); 3) VTG mRNA was slightly elevated in March (but to very low levels compared with April) and was significantly higher in females with access to males (Figure 4); and 4) plasma yolk precursor levels (Figure 8) were elevated only immediately before, or coincident with, rapid yolk development of ovarian follicles (as shown previously for this species) (33, 44). The changes in LH-r, FSH-r, and VTG mRNA are consistent with a lack of ovarian "competence" to respond to elevated circulating gonadotropin levels and lack of ovarian steroidogenesis (no substantial estradiol (E2)-induced VTG synthesis), until April, just before onset of laying. Furthermore, ovarian competence to respond to gonadotropins appears to depend on the "supplemental cue" of male presence.

What is it about the presence of males that so profoundly influences the transition into the final stage of follicle maturation and oviposition? Females in aviaries without males could see and hear males in neighboring aviaries and sometimes interacted with them through the thin wire fencing between aviaries, but none of the females housed without males began incorporating yolk into follicles or had developed preovulatory follicles. In contrast, on the exact same day, females housed with males had begun ovulating, and oviposition occurred in neighboring aviaries. We have known for a long time that the behavior

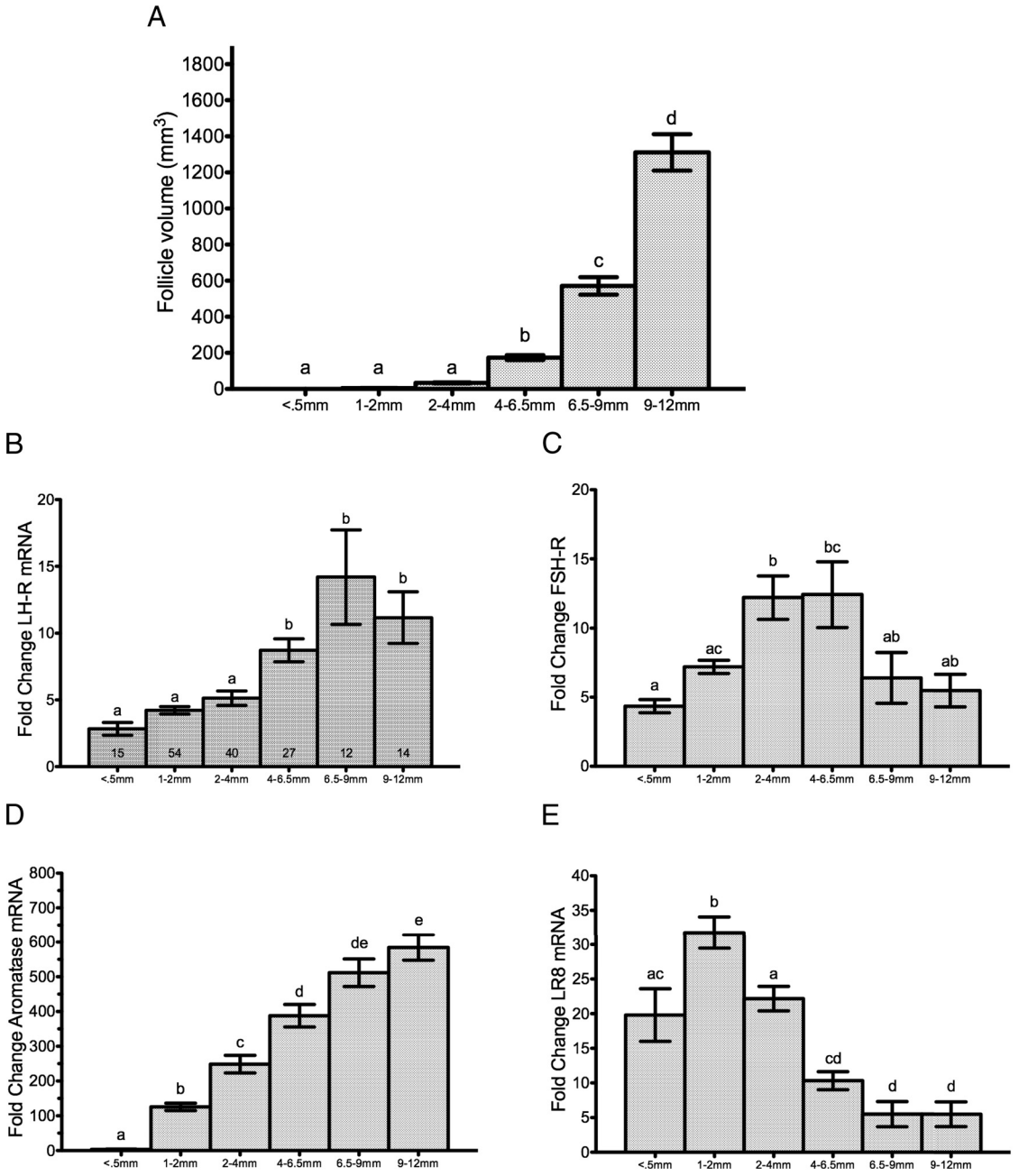

Figure 7. Follicles were categorized by diameter into 6 groups. Follicles less than 2 mm in diameter were either fully regressed or SW, less than 4 mm were small yellow (SY), but more than 4 mm could be any number of F1–F4, so volume reflects approach to ovulation more accurately (A). Fold change in mRNA expression of LH-R (B), FSH-R (C), aromatase (D), and LR8 (E) using the same categories regardless of social housing. Mean ± SEM, unique letters indicate significantly different means tested with post hoc comparisons.

of males can influence the physiology and behavior of females and vice versa (reviewed in Refs. 45–47), but only recently have we started to consider how the timing of these interactions come into play in determining how potent a stimulus they provide is. Cheng et al (48), Cheng and Zuo (49), Cheng (50, 51), and Cheng and coworkers (52) have shown in a series of elegant experiments that the female's own vocalization (both hearing her own vocalization as well as proprioception of the display that goes along with it) plays an important role in final follicle maturation in ring doves (*Streptopelia risoria*). In addition, they have identified direct projections from auditory nuclei to preoptic-anterior hypothalamic known to contain GnRH neurons (49). When auditory nuclei are stimulated by the female's own call, LH secretion is enhanced compared with hearing male calls or another female's call. The results from the present study agree with these findings and suggest that something other than hearing and seeing male courtship is required for starling females to complete follicle development.

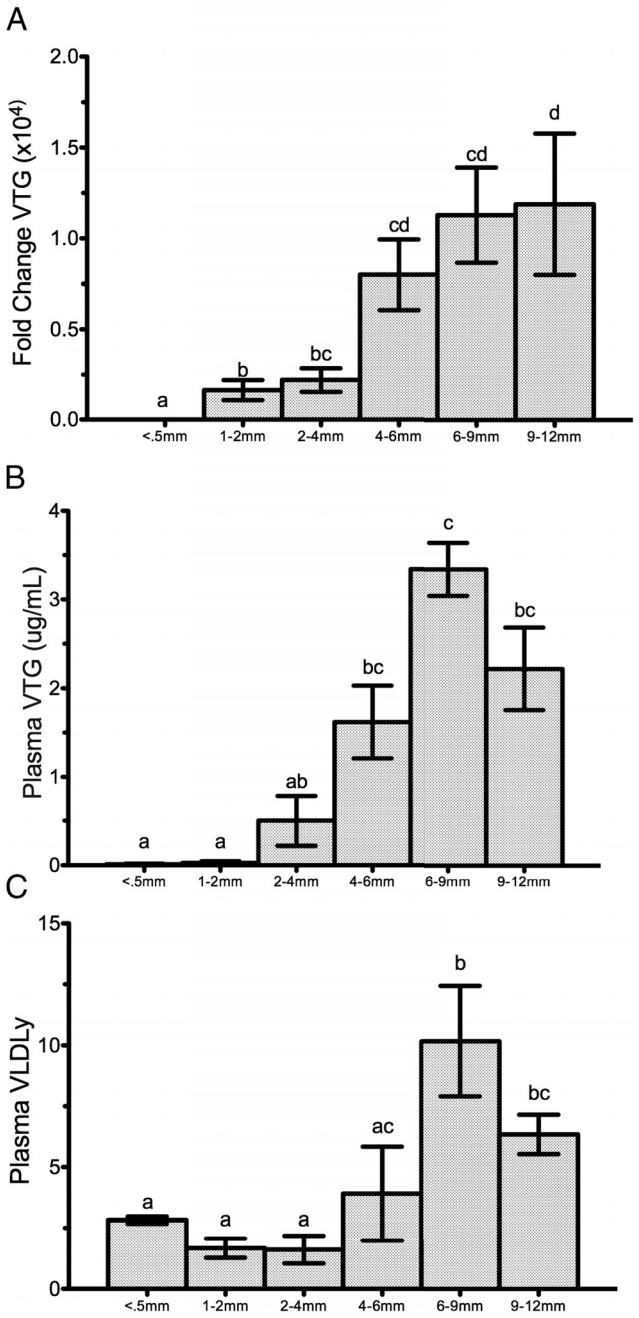

Figure 8. Fold change in mRNA expression of VTG in liver (A), VTG in plasma (B), and plasma VLDLy (C) in females categorized using the diameter of her largest ovarian follicle regardless of social housing treatment. Mean ± SEM, unique letters indicate significantly different means tested with post hoc comparisons.

In our study, the presence of a male also changed the behavioral interactions of and among females. We saw almost no aggression among females housed without males, because females would often rest together in groups of 3–4 on top of and nearby nest boxes. In contrast, females housed with males formed pairs, defended nest boxes aggressively, and were able to exclude conspecifics (Supplemental Figure 1). Both males and females showed aggressive and sexual behaviors in male/female aviaries, and males were introduced to naïve females, pairs formed quickly, and began defending nest boxes and quickly building nests. Females housed with males also had nests that were closer to completion than females housed without males (Figure 2). Even though females in aviaries without males could hear song and see male displays, we likely interfered with the normal progression of courtship, because starling males normally collect and display green plants to females as part of courtship and nest building (56, 57). Most of the work investigating behavioral effects on ovarian follicle development in songbirds considers only effects of male song. Our data suggest that like ring doves, some component of the dynamic interaction between male and female is necessary for final follicle maturation. One caveat to our findings is that by removing individuals (baseline, March, and April) or adding males to naïve females (+7 d), we likely also altered social hierarchies within the aviaries. This disruption would have been experienced by females in both social treatments. During the 3 time points between baseline and April, females with or without access to a mate would both have experienced social disruption, whereas naïve females (+7 d) with or without access to a mate would have experienced both social disruption, and only females with access to a mate for the first time would have experienced physical courtship by males.

Gene expression changes and social treatment

Between January and early April, lengthening days (from 9 h light-15 h dark to 13 h light-11 h dark) stimulated up-regulation of many of the key hypothalamic genes important for photoperiodic activation of the HPG axis, but the degree of stimulation was not affected by the supplemental cue of male presence as far as we could determine with our sampling schedule. The changes in mRNA expression that we measured with a relatively acute exposure to males (7 d) were not always consistent with changes over prolonged access to male partners (3 mo). For example, both GnRH and GnIH expressions increased early with prolonged access to males (between baseline and March) (Figure 5, A and B) but were not affected with acute exposure (Figure 5, F and G), whereas DIO2 increased with prolonged access to males (between baseline and March) (Figure 5C) and also with acute exposure (Figure 5H). We suggest that this might reflect different regulation of the underlying photoperiodic machinery, namely expression of peptides and proteins like GnRH, GnIH, and FSHb that may be tonically synthesized over this time period and/or stored for release vs relatively rapid activation of the DIO2/DIO3 enzyme system. When naïve females were given males for 7 days, DIO2 increased

and DIO3 decreased significantly, ie, there was reciprocal switching between the 2 genes. Based on the model proposed in quail (21), this would result in an increase of local concentrations of T_3 and lead to an increase in GnRH secretion (GnRH transcription remains unchanged) with exposure to males. DIO3 expression did not change significantly over time with constant exposure to males but decreased expression with acute male exposure (Figure 5, D and I). Although there was a consistent decrease in expression between baseline and March, this difference was not significant. Our sample sizes are small in March, so this could be a result of low power/small sample size and fairly large variation in our baseline samples. DIO3 mRNA expression was also unchanged over a broader time scale in a different study of the same species at the same location (58). A sustained inverse relationship between DIO2 and DIO3 may not be required for physiological effects. Stevenson and Prendergast (59) showed that although *DIO2* expression did not change with prolonged exposure to an inhibitory photoperiod, DIO3 expression was increased and was coincident with a downregulation of the reproductive axis. The present study is the first time that a social stimulus has been shown to influence this putative DIO-dependent mechanism for GnRH release that has previously been considered to be responsive solely to changes in photoperiod.

Tobari et al (60) recently showed a social effect on GnIH expression. Acute exposure (1 h) of the reverse scenario to the present study (females shown to males) increased GnIH mRNA expression, likely through activation of noradrenergic receptors. In our experiment, GnIH expression increased with lengthening photoperiod, but we did not find effects of male exposure to females on GnIH expression. The variation in GnIH expression increased with acute exposure to males (Figure 5G), even though the mean values were not different. The direction of change is at least in agreement with findings in quail. It is possible that a combination of photoperiod and social cues combine to regulate GnRH release via a common mechanism; how these different cues (and perhaps others) are integrated into a final common pathway remains to be determined.

Within the ovary during the transition between SW follicles and preovulatory, yolking follicles, a complex relay of gene expression and cell signaling within individual follicles occurs and produces growth and increased sex steroid production and secretion. Circulating E2 signals the liver to produce yolk precursors, VTG, and yolk-targeted VLDLy. A yolk-specific receptor (LR8) then is responsible for taking up yolk precursors into preovulatory follicles. The dynamics of gene expression within the ovary have been well studied in the domestic chicken (*Gallus gallus*), but we know less about gene expression dynamics in wild, seasonally breeding females. In the present study, all of the genes that we measured in the ovary changed expression of mRNA over time but were similar between females with or without access to males (Figure 6, A–D). VTG expression was activated above baseline by March and then showed an enormous increase between March and April, especially in females with constant access to males (1300- to 2500- and 3900- to 14 000-fold change in females without or with constant access to males, respectively). When previously naïve females were given access to males for 7 days, they quickly began final follicle maturation, and LH-r, aromatase, and VTG expression increased compared with controls. Expression of FSHb mRNA in the anterior pituitary was similar between treatments, and we unfortunately do not have the sequence for LHb in starlings.

Gene expression as follicles mature (regardless of social treatment)

As follicles grew from completely regressed to SW follicles (regardless of social treatment), the first genes to increase mRNA expression were aromatase and the yolk receptor LR8 (Figure 7, D and E). Our finding that LR8 mRNA expression was highest in SW follicles and declined as follicles developed is consistent with expression in zebra finches (*Taeniopygia guttata*) (61). Aromatase expression steadily increased for the duration of follicle maturation until follicles were nearing ovulation. This finding is different from that in chickens, where aromatase and E2 content in vitro declines as follicles mature (62, 63). FSH-r expression was elevated when follicles were slightly larger (2–6.5 mm) and declined as follicles grew (6.5–12 mm). It is difficult to directly compare these findings with the research in *G. gallus* for 2 reasons. The first is that we measure expression in the entire follicle and do not separate theca and granulosa layers. The second is that typically in studies with chickens, tissue is collected from birds that have been laying for a consistent period of time, and follicles are collected a known number of hours before ovulation, so do not represent the first preovulatory hierarchy, but rather a cohort are sampled within a long bout of laying. In general, however, the pattern of change in FSH-r mRNA that we find here are consistent with results in chickens (53). Bahr and Johnson (54) showed binding of FSH declines as follicles develop, suggesting that the mature protein for FSH-r is also declining. In the present study, LH-r expression increased last, after follicles reached 4 mm in diameter and remained elevated until follicles were nearing ovulation. In chickens, LH-r mRNA (53) and LH-r binding increase as follicles mature, and the ability of LH to induce adenylate cyclase activity also in-

creases (54). To our knowledge, the dynamics in gene expression within individual follicles that we measure here are the first for any wild passerine.

The change in VTG mRNA expression in the liver as follicles develop is enormous (a 10 000-fold change from regressed to yolking follicles). VTG expression increased as soon as follicles had started growing (Figure 8A) and before circulating levels of VTG increased in plasma (Figure 8B). A similar change in plasma VTG has been shown before in starlings (44), and at a population level, circulating VLDLy increases as females begin periods of egg laying (55). In the present study, circulating VLDLy concentration did not increase significantly until the largest follicle was at least 6 mm in diameter, but changes in plasma VLDLy and VTG were in parallel.

Conclusions

In the same way that we are familiar with thinking about photosensitivity of hypothalamic components of the HPG axis (ie, the stimulatory effects of long days are only possible when individuals have reached a photosensitive state), our data suggest that the ovary may have an analogous but delayed period of sensitivity during which it is competent to respond to stimulatory supplemental cues, in this case social signals, or male presence. It is tempting to speculate that reciprocal switching of Dio2/Dio3 in the hypothalamus in response to social stimulation caused an increase of circulating LH, followed by increased expression of LH-r and aromatase in the ovary. There is currently no starling LH assay available, so we were unable to measure circulating levels of LH to confirm this effect. However, it is possible that the effects that we observed could also be explained by increased GnRH release via activation of the Dio2/Dio3 system, which then caused ovarian stimulation, either with or without a prerequisite of ovarian competence. Future studies are needed to discriminate distinct temporal mechanisms of social stimulation of ovarian function.

Acknowledgments

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This work was supported by National Science Foundation Grant IOS 1122044 (to G.E.B.) and Natural Sciences and Engineering Research Council of Canada Discovery and Accelerator grants (to T.D.W.).

Disclosure Summary: The authors have nothing to disclose.

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