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Changes in processes downstream of the hypothalamus are associated with seasonal follicle development in a songbird, the dark-eyed junco (*Junco hyemalis*)

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ABSTRACT

Mechanisms related to seasonal reproductive timing in vertebrates have received far more study in males than in females, despite the fact that female timing decisions dictate when rearing of offspring will occur. Production and release of gonadotropin-releasing hormone (GnRH) by the hypothalamus stimulates the pituitary to secrete gonadotropins, initiating the beginning stages of gonadal recrudescence and production of the sex steroids, testosterone and estradiol, which are necessary to prime the liver for secretion of yolk precursors in breeding female birds. While stimulation by the hypothalamus can occur during the pre-breeding period, egg development itself is likely regulated downstream of the hypothalamus. We used GnRH challenges to examine variation in breeding-stage-specific patterns of pituitary and ovarian responsiveness in free-living female dark-eyed juncos (*Junco hyemalis*) and also examined the ovary and liver for variation in mRNA expression of candidate genes. Baseline LH levels increased during the transition from pre-breeding to egg-development, however no significant difference was observed in post-GnRH injection levels for LH or sex steroids (testosterone and estradiol). Interestingly, a stage by time-point interaction was observed, with post-GnRH LH levels increasing over baseline during the pre-breeding stage, but not during the egg-development stage. We observed a decrease in liver mRNA expression of estradiol receptor-alpha, and glucocorticoid and mineralocorticoid receptors and a decrease in glucocorticoid receptor expression levels in the ovary. A decline in FSH receptor expression across stages was also observed in the ovary. Combined, our data suggest seasonal variation in female's sensitivity to signals of HPG activity and energetic or stress signals. These data provide additional insight into the physiological mechanisms regulating onset of clutch initiation.

1. Introduction

Most temperate-zone animals breed seasonally to ensure that offspring are born at an optimal time for rearing and survival (Baker, 1938; Murton and Westwood, 1977). Before young arrive, parents must undergo extensive physiological and behavioral preparation for breeding. Often, preparatory events must be initiated well in advance of seasonal increases in food availability (Gibb, 1950).

In most seasonally breeding species, environmental cues are perceived and transduced at the level of the hypothalamus, where gonadotropin-releasing hormone (GnRH) neurons trigger an endocrine cascade resulting in gonadal activation (Dawson, 2008; Jacobs and Wingfield, 2000). More specifically, GnRH triggers secretion of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary. LH stimulates the synthesis and release of steroids such as testosterone (T) and estradiol (E_2) from the gonads (Adkins-Regan, 2008; Wingfield, 2012). These hormones are then transported to target tissues where they bind to receptors, thus shaping physiological, morphological, and behavioral traits. While the above sequence is fairly well established, particularly in males, it is less well known how changes in sensitivity of tissues downstream of the hypothalamus may influence the final transition leading to female clutch initiation in oviparous species (Caro, 2012). This investigation seeks to document whether changes in sensitivities at one or more

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levels downstream of the hypothalamus are observed as females transition from a non-breeding status in early spring into the onset of egg development.

Here, we asked whether female pituitary and gonadal hormonal responsiveness to stimulation with gonadotropin-releasing hormone (GnRH) changes from pre-breeding to egg-development. In addition, we also assessed whether expression of key genes in the ovary and liver are important for responding to endocrine signal changes during the transition into a reproductively mature state. The ovary is the site of follicular maturation and the liver is necessary for the production of yolk-precursors utilized in the developing follicle (Williams, 2012). Specifically, we asked whether expression for reproductive hormone receptors on the ovary (*LHR*, *FSHR*) and the liver (*ERα*) increase during the transition to breeding maturity, which would likely increase responsiveness to these hormones following stimulation with GnRH. Finally, we also assessed whether expression of the two main receptors for corticosterone (CORT), mineralocorticoid receptor (*MR*), which binds CORT with high affinity, and the low-affinity glucocorticoid receptor (*GR*), (De Kloet et al., 1998; Funder, 1997), display increases in expression during the transition to breeding. Reproduction is energetically expensive, and glucocorticoid hormones, which play an essential role in metabolism and energy regulation (Landys et al., 2006; Sapolsky et al., 2000) may play an integral role in the ability of the ovary and liver to be responsive to a seasonal increase in baseline circulating glucocorticoids leading up to reproduction (Romero, 2002). Alternatively, an increase in expression of these hormone receptors, particularly the low-affinity GR, may also increase the sensitivity of these tissues to stress-induced levels of CORT (Abraham et al., 2013; Bambino and Hsueh, 1981; Denari and Ceballos, 2006; Hsueh and Erickson, 1978; Kwok et al., 2007; Lattin et al., 2011), which may enable a delayed investment in and maturation of reproductive tissues when conditions are sub-optimal (Lattin et al., 2016; Wingfield et al., 1998).

2. Materials and methods

2.1. Study species

The dark-eyed junco (*Junco hyemalis*) is a seasonally breeding songbird commonly found throughout North America (Nolan, 2002). Dark-eyed juncos are an excellent model organism because they have been studied extensively, are abundant, and are relatively easy to capture. Juncos are ground-nesters with clutches ranging between 3 and 5 eggs (mean and median = 4) per nest attempt (Nolan, 2002). Our field site was located in the Black Hills National Forest, South Dakota, USA, where juncos are observed to lay eggs between May and June.

2.2. Trapping and handling

All birds were captured passively in continuously monitored seed-baited mist-nets or walk-in traps that were maintained for ~4 weeks prior to and up to the onset of clutch initiation in this population (4/20/2009-5/15/2009 and 4/17/2016-5/14/2016). Morphometric measures such as body mass (to nearest 0.1 g), tarsus length (to nearest 0.1 mm), wing length, tail length, and presence/absence of a brood patch were recorded for all individuals. All individuals received a unique numbered metal band and color band combination to enable later identification. Females were differentiated from males by plumage differences during the very early breeding season and by the presence of a brood patch (female) or an enlarged cloacal protuberance (male) thereafter.

2.3. Breeding stage assignment

In 2009, females were assigned to either the pre-breeding stage or egg development stage by visual assessment; females were examined for the presence of a brood patch and pronounced abdominal swelling indicative of egg development (Cain and Ketterson, 2012). Birds were also assigned to either the pre-breeding or egg development stage by backdating when the date of first egg was known. Each of the females assigned to the "egg development" stage also demonstrated a markedly larger body mass than average and/or were developing or displayed a full brood patch (CBB unpublished data). Nests were located for a subset of the sampled females, and in all cases, backdating supported the breeding stage assignments (CBB unpublished data).

In 2016 we used measures of circulating levels of very-low-density lipoprotein (VLDL) in addition to visual assessment to categorize females into groups comparable to the 2009 "pre-breeding" and "egg development" stages (Lamarre et al., 2017). During this time period leading up to breeding, the ovary possesses a large number of pre-vitellogenic "white" follicles, and VLDL levels are low. During the egg development stage yolk-precursor proteins, including VLDL, are at elevated levels in circulation (Williams, 2012).

2.4. Very-low-density lipoprotein (VLDL) assay and validation

To confirm the use of VLDL as a marker of egg development in our study species, in 2016 we captured birds from off the study site and collected blood and ovaries to enable visual categorization of reproductive stage. Briefly, a total of 24 birds were collected at one of two time points; one roughly 2–3 weeks prior to predicted clutch initiation in this population, and a second point when some females in the population had begun to build nests and initiated clutches. A small blood sample was collected for measurement of VLDL. Birds were then euthanized, and we characterized the ovary as either containing all small white follicles or displaying a clear follicular hierarchy. Of the twenty-four females where we were able to compare circulating VLDL levels with visual inspection of the ovary, the assignment method (assay methods and categorization methods described below) correctly assigned individuals 95.8% of the time (i.e. 23 of the 24 were correctly assigned) (see Supplemental Fig. 1).

2.5. Administration of GnRH

All females received an injection in their pectoral muscle of a solution containing 1.25 μg chicken GnRH (American Peptide product #54-8-23, Sunnyvale, CA, USA) dissolved in 50 μL phosphate buffer solution (Bergeon Burns et al., 2014; Jawor et al., 2007, 2006). This dose is capable of fully activating the HPG axis in the dark-eyed junco (Jawor et al., 2006). Pre- and post-injection blood samples were collected as described below for measurement of relevant hormones.

2.6. Pituitary responsiveness to GnRH injection

2.6.1. Time course of pituitary LH response to GnRH

Previous work from our group have demonstrated that testosterone levels peak 30 min following an intramuscular GnRH injection (Jawor et al., 2006). However, LH levels are predicted to be elevated in circulation much sooner than sex steroid levels (e.g., 2 min following intrajugular injection of GnRH in rufous-winged sparrows (Deviche et al., 2010)). In order to ascertain better information regarding the time-course of LH response to GnRH and document the effectiveness of GnRH to elevate LH 15 min post-injection, in 2009, pre-breeding females received an injection of GnRH and had a blood sample collected at 5, 15 or 30 min post-injection (individuals randomized for each time point, n = 6 for each time point) (Fig. 1).

There were significant main effects of both the treatment groups (5, 15, or 30 min latency; $F_{2,96} = 10.8$, $p < 0.0001$) and GnRH time point (pre- or post-GnRH injection; $F_{1,50} = 30.4$, $p < 0.0001$) on LH, as well as a significant interaction between treatment group and GnRH time point ($F_{2,50} = 11.6$, $p < 0.0001$). Post-hoc tests revealed that LH was significantly elevated from pre-injection levels in birds measured at 5 min ($p < 0.001$; Pre-injection: 1.7 ± 0.53 ng/mL, n = 6; Post-

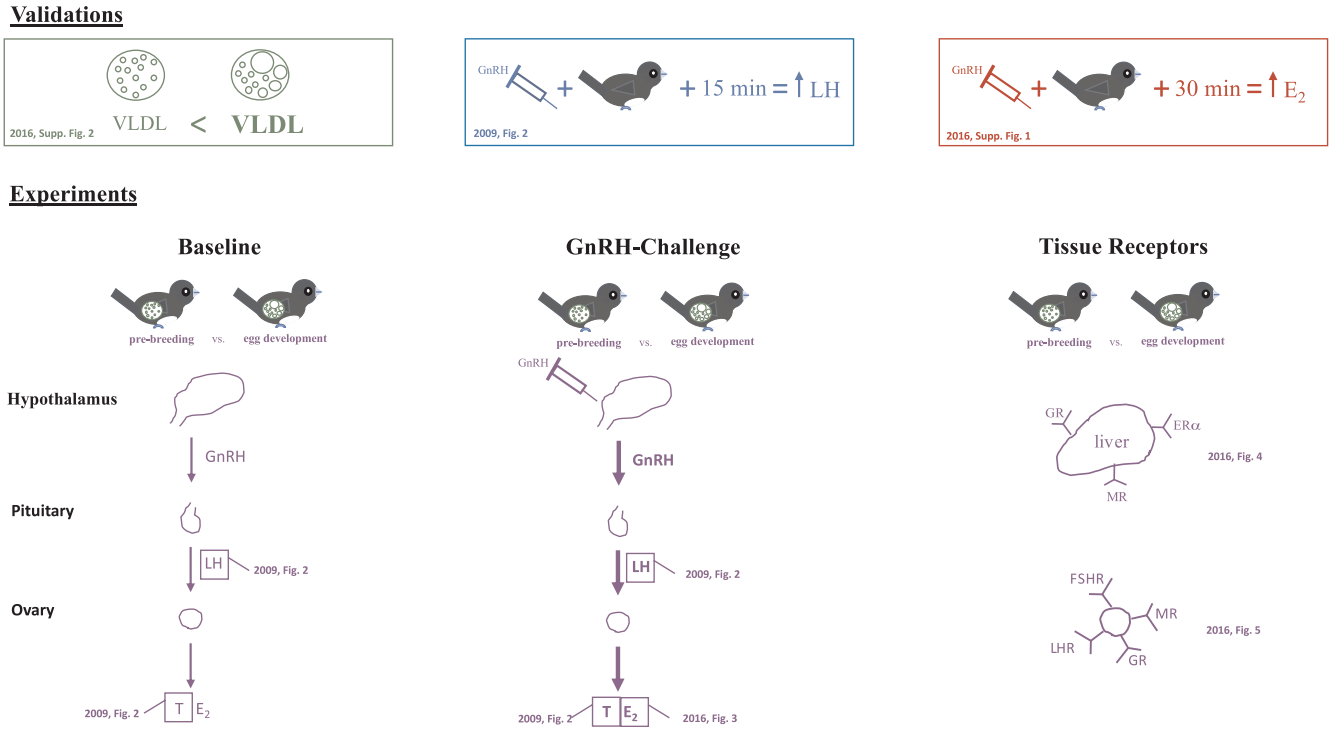

Fig. 1. Schematic outlining methods undertaken. First, we validated the usefulness of measurements of blood very-low-density lipoprotein (VLDL) to categorize females in the egg-development stage. Prior to wide-scale sampling, we sought to confirm that gonadotropin-releasing hormone (GnRH) injection stimulates an elevation in luteinizing hormone (LH) levels within 15 min and an increase in estrogen (E_2) levels within 30 min. Next, we asked whether females alter baseline and GnRH-induced hormone levels (LH, E_2, and testosterone [T]) as they transition from a pre-breeding stage into an egg development stage. Finally, we compared mRNA expression for candidate genes in liver and ovarian tissues to gauge sensitivity to reproductive hormones (luteinizing hormone receptor [*LHR*], follicle-stimulating hormone receptor [*FSHR*], estrogen receptor alpha [*ERα*]) or signals of increased metabolic demand and/or stress (glucocorticoid receptor [*GR*] and mineralocorticoid receptor [*MR*]).

injection: 4.8 ± 0.98 ng/mL, n = 6) and at 15 min (p = 0.001; Pre-injection: 1.4 ± 0.19 ng/mL, n = 40; Post-injection: 2.2 ± 0.21 ng/mL, n = 40) min post-challenge, but did not differ from pre-injection levels in birds measured at 30 min post-challenge (p = 1.000; Pre-injection: 0.9 ± 0.24 ng/mL, n = 6; Post-injection: 0.9 ± 0.20 ng/mL, n = 6) (see Supplemental Fig. 2).

2.6.2. Breeding stage and LH responsiveness to GnRH

Immediately after capture an initial blood sample was collected followed by an injection with GnRH. As the optimal time for sampling was not yet known, after the six individuals were collected at each of the three time points for the time-course investigation (see above), for the remainder of the 2009 field season all additional individuals were sampled at 15 min post-GnRH injection and assayed for LH. In total, a blood sample was collected at 15 min post-injection from 35 females categorized in the pre-breeding stage and 13 females categorized in the egg-development stage.

2.7. Gonadal responsiveness to GnRH injection

2.7.1. Breeding stage and testosterone responsiveness to GnRH

Testosterone was assessed from plasma collected prior to GnRH injection as well as in plasma collected at the 15-minute time point following injection with GnRH (see above) when sufficient plasma (> 30 μL) remained following the LH assay. We were able to assay post-GnRH testosterone from 23 pre-breeding females and from 15 females in the egg-development group in 2009. Note: due to logistical constraints and limitations on blood volume we were permitted to collect, blood samples were only collected at 15-minutes post-injection.

2.7.2. Validation of estrogen response to GnRH

To date, while many studies have investigated the ability of GnRH to induce secretion of testosterone in female songbirds, here we sought to confirm the ability of GnRH injections to stimulate an increase in estrogen levels (Fig. 1). To this end, we sampled a small number of females captured from off the main study site. Our estrogen assay required a relatively large volume of plasma (> 100 μL), thus females only had one blood sample collected (i.e. no individual had both baseline and post-GnRH injection samples). For this validation we compared females that were either 1) sampled for circulating E_2 without receiving an injection of GnRH (baseline) or 2) were sampled 30 min after receiving a GnRH injection (post-GnRH) (Jawor et al., 2006). Females were sampled for E_2 levels during the pre-breeding stage (Baseline: n = 6; Post-GnRH: n = 6) or the egg-development stage (Baseline: n = 5; Post-GnRH: n = 3).

There was a main effect of GnRH injection on circulating levels of estrogen; females injected with GnRH had higher plasma estrogen compared with females that did not receive an injection ($F_{1,18}$ = 8.054, p = 0.016). There was also a significant effect of stage; females during the egg-development stage had estrogen levels higher than those of females sampled during the pre-breeding period ($F_{1,18}$ = 7.036, p = 0.011). There was no significant interaction detected between breeding stage and injection (p > 0.1) (see Supplementary Fig. 3).

2.7.3. Breeding stage and estrogen responsiveness to GnRH

In 2016, females received a GnRH injection and had a single blood sample taken 30 min later to measure E_2 levels. Females were categorized as either in the pre-breeding (n = 35) or in the egg-development stage (n = 15) based on circulating VLDL levels, as described below. All blood samples were stored on ice until centrifugation to separate red blood cells from plasma. Plasma samples were stored at

−20 °C until further analysis.

2.8. VLDL and hormone assay methods

2.8.1. Very-low-density lipoprotein (VLDL)

We followed previously established protocols for measuring plasma VLDL originally developed in the domestic hen (Mitchell and Carlisle, 1991) and later validated for use in passerines (Challenger et al., 2001; Williams and Christians, 1997; Williams and Martyniuk, 2000). To ascertain an unbiased cut-off for baseline level of circulating VLDL in the female plasma we measured VLDL in five male birds caught during the pre-breeding period (Vanderkist et al., 2000; Mitchell and Carlisle, 1991; Williams and Christians, 1997). The highest value of VLDL observed in the sampled males was 0.96 mg/mL. As established previously (Vanderkist et al., 2000), a 'non-breeding' cut-off was determined by doubling the value observed in males. Thus, females with values of less 1.92 mg/ml were categorized as pre-breeding (n = 43) and females with values greater than 1.92 mg/ml were categorized as in the egg-development stage (n = 23). No plasma was available from 2009 for VLDL assays. A plasma pool from non-breeders (Sigma-Aldrich, P3266-1 mL) was used for calculation of inter- and intra-assay variation. The intra-plate variation ranged from 0.6% to 4.8% (3 plates: 1: 4.8%, 2: 0.6%, 3: 3.5%) and inter-plate variation was 3.0%.

2.8.2. Luteinizing hormone (LH) assay

Plasma LH was measured using the heterologous radioimmunoassay (RIA) that has been utilized extensively in songbirds (Follett et al., 1975, 1972; Sharp et al., 1987; Wingfield et al., 1991), including dark-eyed juncos (Bergeon Burns et al., 2014; Greives et al., 2016; Jawor et al., 2007, 2006; Rosvall et al., 2013). This RIA employed a double-antibody, post-precipitation process with antisera raised against purified chicken LH. Each sample was run in duplicate (20 μL each) on a single assay. The minimum detectable concentration was 0.078 ng/mL and intra-assay variation was 10.2 ± 1.3%.

2.8.3. Testosterone (T) assay

Plasma testosterone was assayed using an enzyme immunoassay (EIA) kit (Testosterone; ADI-901-065; Enzo Life Sciences, Farmingdale, NY, USA) as described previously (Clotfelter et al., 2004). Hormones were doubly extracted using diethyl ether, dried under nitrogen gas, and approximately 2000 cpm of ^{3}H-T was added to each sample (40 μL) for determination of sample recovery. Extracts were then re-suspended in 50 μL ethanol and diluted with assay buffer (to a volume of 350 μL). Each sample was plated in duplicate (100 μL per well) following the manufacturer's guidelines. Concentrations of testosterone were determined using a four-parameter logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories, Inc.). Average recovery of ^{3}H-T after extraction was 90%, and T concentrations were corrected to reflect incomplete recovery. Intra-plate variations ranged from 5.0% to 13.2% (two plates: 1: 5.0% and 2: 13.2%) and inter-plate variation was 13.2%.

2.8.4. Estradiol (E_2) assay

Plasma estradiol (E_2) was assayed using an EIA kit (17β–estradiol high sensitivity; ADI-900-174; Enzo Life Sciences, Farmingdale, NY, USA). This kit has previously been validated for use in songbirds (Gall et al., 2013; Wilcoxen et al., 2015). When available, 100 μL of plasma was used during extraction. Nineteen samples out of 94 had volumes smaller than 100 μL; the dilution factors for these samples were adjusted accordingly. Hormones were triply extracted using diethyl ether, dried under nitrogen gas, and reconstituted in assay buffer (260 μL). Each sample was plated in duplicate (100 μL per well) following the manufacturer's guidelines. Concentrations of E_2 were calculated using a four-parameter logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories, Inc.). Intra-plate variation ranged from 3.0% to 8.8% (three plates: 1: 4.2%, 2: 3.0%, 3: 8.8%) and inter-plate variation was 5.4%. The assay detection limit is 14.0 pg/ml. Samples that were below detection limit of the assay (n = 6 in pre-breeding stage, n = 4 in the egg-development stage) were removed from subsequent analysis.

2.9. Breeding stage and ovarian and liver candidate gene expression

2.9.1. Tissue processing and molecular methods

To assess mRNA expression of candidate genes in the liver and ovary, a subset of females was collected during 2016. Birds were collected at two time points: pre-breeding stage: April 25–27, 2016, n = 14; VLDL = 1.4 ± 0.09 mg/mL) and again later during the "early breeding season," the period of time when the first known eggs of the focal study population were being matured and laid (May 12–14, 2016; n = 8; VLDL = 3.4 ± 0.83 mg/mL). Individuals were euthanized by an overdose of isoflurane followed by rapid decapitation. Ovaries and livers were collected, rapidly frozen on powdered dry ice, and stored at −80 °C until RNA extraction. During late breeding collection, follicles from ovaries that had entered a hierarchy were collected and stored; these follicles were analyzed separately from the remainder of the ovary (n = 3 ovaries with hierarchical follicles).

Total RNA was extracted from tissue (ovary; theca and granulosa layers of follicles; partial section of liver) using a RNAzol®RT isolation reagent (Sigma Aldrich, St. Louis, MO) according to the manufacturer's instructions. Following spectrophotometry to quantify total RNA concentration and optical density, 1 μg was treated with TURBO DNAse and removal reagents as per manufacturer's guidelines (Life Technologies, Carlsbad, CA). After again determining RNA concentration, we then reverse transcribed 500 ng of RNA (ovary, follicles, or liver) using qScript reverse transcriptase and oligo(DT) primers (Quanta Biosciences, Beverly, MA) in a total reaction volume of 5 μL. To verify that samples were not contaminated with DNA, we ran a portion of our samples without reverse transcriptase.

The resulting cDNA was used as a template for quantitative real-time PCR (qPCR) to measure relative abundance of mRNA expression of estrogen receptor α (*ERα*), glucocorticoid receptor (*GR*), and mineralocorticoid receptor (*MR*) in the liver, as well as follicle-stimulating hormone receptor (*FSHR*), luteinizing hormone receptor (*LHR*), *GR*, and *MR* in the ovary. We also quantified expression of *PPIA* and *RPL4* reference genes (Zinzow-Kramer et al., 2014) for normalization of the expression of each gene of interest. We performed qPCR using a Stratagene Mx3000P machine equipped with MxPro software (v.4.10, Agilent, Santa Clara, CA, USA). Reactions (10 μL) were run in triplicate using 5 μL Perfecta SYBR green low ROX (Quanta Biosciences, no. 95056-100), 3 μL cDNA (diluted 1:40), and primers at a concentration of 0.3 μM, including no template controls (see Supplementary material for primers). Thermocycling conditions for *LHR*, *ERα*, *PPIA*, and *RPL4* reactions were as follows: 10 min at 95 °C, 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min. Thermocycling conditions for *GR*, *MR*, and *FSHR* reactions were as follows: 10 min at 95 °C, 40 cycles of 95 °C for 30 s, 65 °C for 1 min, and 72 °C for 1 min. A final melting phase of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s was run for each primer to confirm single-product specificity of each sample.

A pooled sample of cDNA made for each tissue group (10 μL per liver and 15 μL per ovary sample from the first 10 females collected) was run on every qPCR plate, serving as a calibrator to which each individual sample was compared. An average of the two reference genes (*PPIA* and *RPL4*) was calculated for each individual by tissue type. We used the $2^{-\Delta\Delta Ct}$ method of quantification (Livak and Schmittgen, 2001), in which the abundance of each gene of interest is expressed as the fold change expression relative to a pooled standard, normalized by the expression of the reference genes (*PPIA* and *RPL4*). We assessed amplification efficiencies for each gene using a 5-point standard dilution curve in MxPro (efficiencies ranged from 89.0% to 98.7%). See Supplemental Table 1 for primer sequence information.

2.10. Statistical analyses

All statistical analyses were performed using R v3.2.3 (R Core Team, 2014) and effects were considered significant at $\alpha \leq 0.05$.

2.10.1. Breeding stage response of LH and T to GnRH challenge

Baseline and 15 min post-GnRH LH and T levels (in separate models) were analyzed using a linear mixed-effects model, with breeding stage and blood sample time point (pre- or post-challenge) as fixed effects and the individual ID as a random effect to control for repeated measures from the same individual. A breeding stage*time point interaction was included to ask whether tendency to elevate LH or T in response to GnRH varied by stage of breeding. Tukey's post-hoc analyses explored the breeding stage*time point interactions, identifying pairwise differences between initial and post-GnRH challenge LH or T within each breeding stage.

2.10.2. Breeding stage response of E_2 to GnRH challenge

To assess whether ovarian estrogen secretion in response to an injection with GnRH varies during the transition from pre-breeding to egg-development, we used a Welch's two sample *t*-test (unequal variances between groups) and tested for differences between GnRH-induced E_2 levels across breeding stages.

2.10.3. Ovarian and liver gene expression

A linear model was performed with collection period as a fixed effect to test for differences in candidate gene expression between sampling time points on each tissue. Values are provided as means ± SEM.

3. Results

3.1. Breeding stage and LH responsiveness to GnRH

There was a significant main effect of time point (pre-injection or 15 min post injection) ($F_{1,51} = 13.6$, $p < 0.001$) and of breeding stage (pre-breeding or egg development) ($F_{1,40} = 11.1$, $p < 0.010$) on LH levels. There was also a significant interaction between time point and breeding stage ($F_{1,51} = 4.3$, $p = 0.044$) on LH levels. Post-hoc pairwise comparisons within each breeding stage revealed that following a GnRH challenge, females significantly elevated LH during the pre-breeding ($t = -5.53$, $df = 52$, $p < 0.0001$; pre-injection: 1.0 ± 0.15 ng/mL; post-injection: 2.0 ± 0.21 ng/mL), but did not increase LH from initial levels during egg development ($t = -0.95$, $df = 52$, $p = 0.778$; pre-injection: 2.8 ± 0.54 ng/mL; post-injection: 3.1 ± 0.54 ng/mL). Baseline LH levels significantly increased from pre-breeding to egg development ($t = -3.855$, $df = 53$, $p = 0.002$). Post-GnRH challenge LH levels did not significantly differ across breeding stages ($t = -2.35$, $df = 53$, $p = 0.100$) (Fig. 2).

3.2. Breeding stage and testosterone responsiveness to GnRH

There was a significant main effect of time point (pre-injection or 15 min post injection), with testosterone levels elevated post-injection ($F_{1,32} = 28.7$, $p < 0.0001$), but no main effect of breeding stage (pre-breeding or egg development) ($F_{1,32} = 0.03$, $p = 0.875$) on testosterone levels. There was no significant interaction between time point and breeding stage ($F_{1,32} = 0.12$, $p = 0.731$) on T levels. (Fig. 2).

3.3. Breeding stage and estrogen responsiveness to GnRH

Due to limitations on blood volume that could be collected, and the volume of plasma needed for the assay, baseline levels were not able to be obtained. Estradiol levels 30 min following a GnRH injection did not differ between pre-breeding females and females in the egg development stage (pre-breeding: 43.6 ± 4.33 pg/mL; egg development: 64.2 ± 11.67 pg/mL; $t = -1.65$, $df = 18$, $p = 0.116$; Fig. 3).

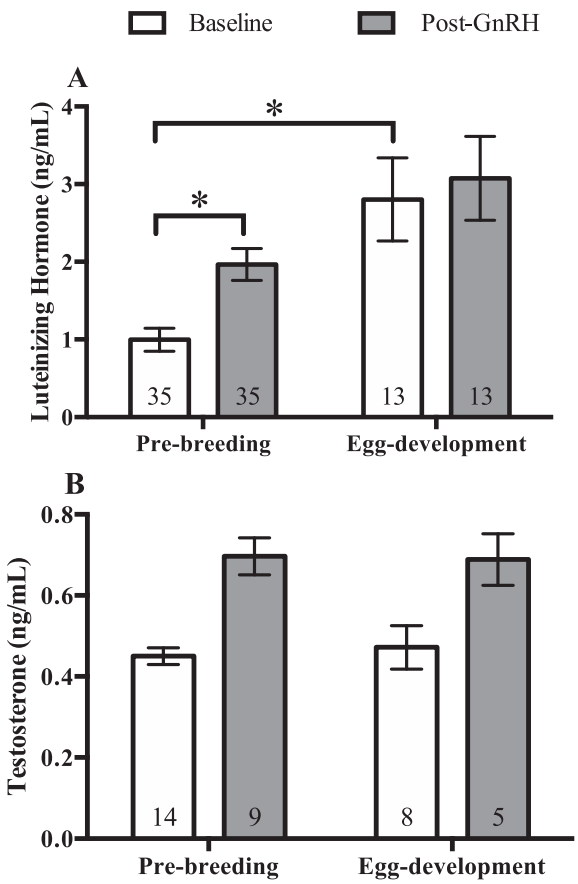

Fig. 2. Both a) luteinizing hormone and b) testosterone readily respond to a gonadotropin-releasing hormone (GnRH) challenge in the early breeding season in the female dark-eyed junco (*Junco hyemalis*). Figures show means ± SEM. Significant differences between breeding stages and time points are denoted with asterisks (*).

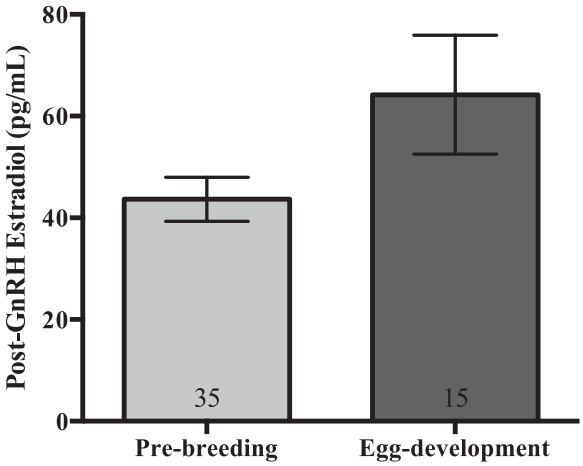

Fig. 3. Circulating levels of estrogen following an injection with gonadotropin-releasing hormone (GnRH) were not different for females captured and injected during the pre-breeding stage versus the egg-development stage. Figure shows mean ± SEM. Significant differences between breeding stages are denoted with asterisks (*).

3.4. Breeding stage and ovarian and liver candidate gene expression

In the liver, *GR*, *MR*, and *ERα* mRNA expression levels were significantly higher during the early pre-breeding sampling period, as compared to the later sampling period when clutches were being

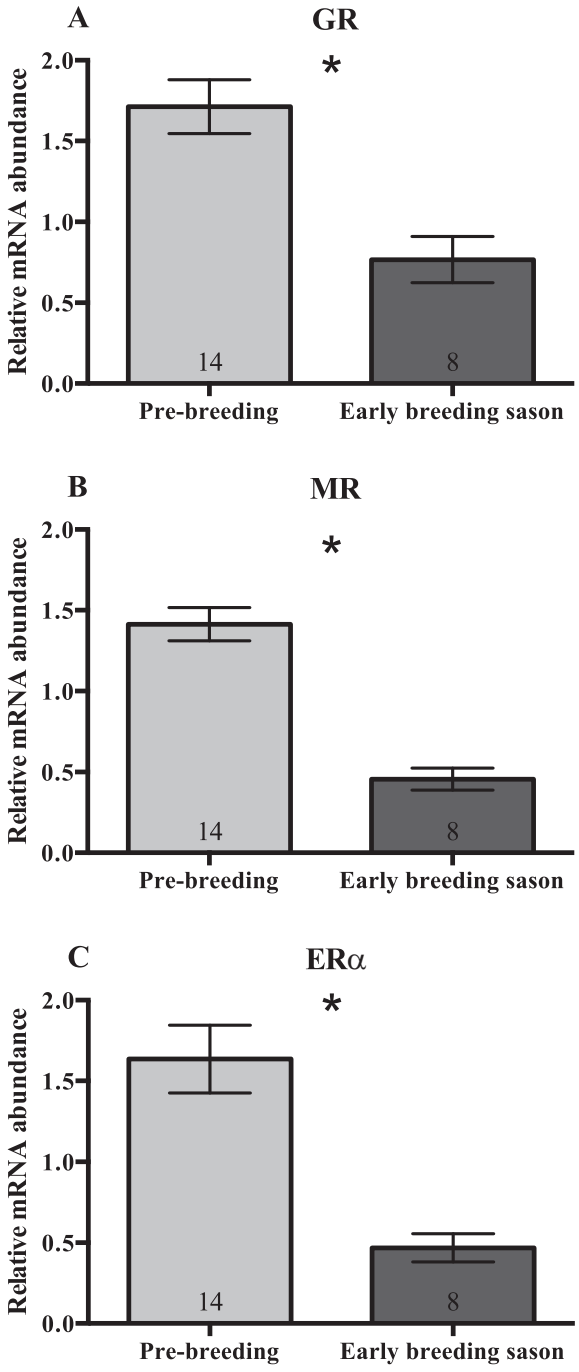

Fig. 4. mRNA expression of candidate genes in the liver are higher in the pre-breeding sampling period than in the early-breeding season for female dark-eyed juncos (*Junco hyemalis*). Liver gene expression is shown across breeding stages for **(A)** glucocorticoid receptor (*GR*), **(B)** mineralocorticoid receptor (*MR*), and **(C)** estrogen receptor alpha (*ERα*). Gene expression is unit-less and quantified on a $\log_2$-fold change relative to an arbitrary calibrator. Figures show means ± SEM. Significant differences between breeding stages are denoted with asterisks (*).

initiated (*GR*: $t = -3.85$, $F_{1,20} = 14.72$, $p = 0.001$; pre-breeding: $\bar{X} = 1.7 \pm 0.17$; early-breeding season: $\bar{X} = 0.8 \pm 0.14$; *MR*: $t = -6.56$, $F_{1,20} = 43.03$, $p < 0.0001$; pre-breeding: $\bar{X} = 1.4 \pm 0.10$; early-breeding season: $\bar{X} = 0.5 \pm 0.07$; *ERα*: $t = -4.07$, $F_{1,20} = 16.53$, $p < 0.001$; pre-breeding: $\bar{X} = 1.6 \pm 0.21$; early-breeding season: $\bar{X} = 0.5 \pm 0.09$; Fig. 4).

In the ovary, both *FSHR* and *GR* mRNA expression levels were higher during the early sampling period, as compared to the later sampling period when clutches were being initiated (*FSHR*: $t = -3.36$, $F_{2,22} = 9.14$, $p = 0.003$; pre-breeding sampling ovary: $\bar{X} = 2.0 \pm 0.27$; later sampling ovary: $\bar{X} = 0.8 \pm 0.17$; *GR*: $t = -2.73$, $F_{2,22} = 5.67$, $p = 0.012$; pre-breeding sampling ovary: $\bar{X} = 0.9 \pm 0.14$; later sampling ovary: $\bar{X} = 0.4 \pm 0.05$; Fig. 5). Similarly, *FSHR* and *GR* mRNA expression levels were higher during the pre-recruiting stage, compared to maturing follicles collected during the later sampling period (*FSHR*: $t = -3.42$, $F_{2,22} = 9.14$, $p = 0.003$, $\bar{X} = 0.2 \pm 0.10$; *GR*: $t = -2.60$, $F_{2,22} = 5.67$, $p = 0.016$, $\bar{X} = 0.2 \pm 0.09$; Fig. 5).

The mRNA expression levels of *LHR* in the ovary did not significantly differ between the two sampling periods (*LHR*: $t = 0.093$, $F_{2,22} = 16.13$, $p = 0.926$; pre-breeding sampling ovary: $\bar{X} = 0.1 \pm 0.03$; early-breeding season ovary: $\bar{X} = 0.2 \pm 0.07$; Fig. 5). However, *LHR* mRNA expression levels were elevated in maturing follicles compared with ovaries collected during the pre-breeding sampling periods ($t = 5.52$, $F_{2,22} = 16.13$, $p < 0.0001$, $\bar{X} = 3.4 \pm 1.75$; Fig. 5). There were no differences observed in *MR* mRNA expression between the two sampling periods (*MR*: $t = -1.48$, $F_{2,22} = 1.18$, $p = 0.154$; pre-breeding sampling ovary: $\bar{X} = 2.0 \pm 0.35$; early-breeding season ovary: $\bar{X} = 1.2 \pm 0.35$; Fig. 5).

4. Discussion

Despite knowledge that females play the critical role in determining onset of reproduction (Ball and Ketterson, 2008; Caro et al., 2009), research on the mechanisms regulating seasonal reproduction in songbirds has continued to focus more on males than on females (Caro, 2012; Dawson, 2015, 2003). Here, we focused on female birds during the time period from an early reproductive stage prior to rapid yolking of follicles, to full reproductive status when follicles were yolking. While we did not observe a difference in the LH levels secreted by the pituitary in response to a single injection of GnRH during this transition, we did observe increases in circulating baseline LH and a no change in post-GnRH LH over baseline during this transition; stimulated estradiol levels were similar across stages. Further, we observed a decrease in liver mRNA expression of estradiol receptor-alpha, glucocorticoid receptor, and mineralocorticoid receptor, and a decrease ovarian mRNA expression in glucocorticoid receptor and follicle-stimulating hormone receptor.

4.1. Pituitary transition to egg-laying

We found that, as expected, baseline LH levels increase during the transition into egg-development, suggesting increased hypothalamic GnRH release (Williams, 2012; Williams et al., 2004). Similar to white-crowned sparrows, exogenous GnRH was capable of increasing LH levels in females during the pre-breeding stage (Wingfield et al., 1979). Luteinizing hormone levels in blood samples collected 15 min following injection with exogenous GnRH however did not significantly differ between females sampled prior to breeding and those sampled during the egg-development stages. Further, females in the egg-development stage did not significantly elevate LH levels over baseline in response to a GnRH injection. The failure to elevate LH over baseline following injection with GnRH during the egg development stage may likely be due to a 'ceiling effect' in which we were unable to detect an elevation post GnRH-injection compared with the already elevated baseline levels of LH. Another consideration, however, is that the pituitary response to GnRH may have differed at an earlier time point following stimulation, as opposed to 15 min post-injection. Post-GnRH LH levels at 5 min showed the greatest response to exogenous GnRH, and testosterone levels were elevated in response to GnRH already by 15 min post injection. Therefore, it is possible that some individual variation may have been lost at the 15 min post-injection time point, which is when LH measurements were taken for seasonal comparisons. Of the three

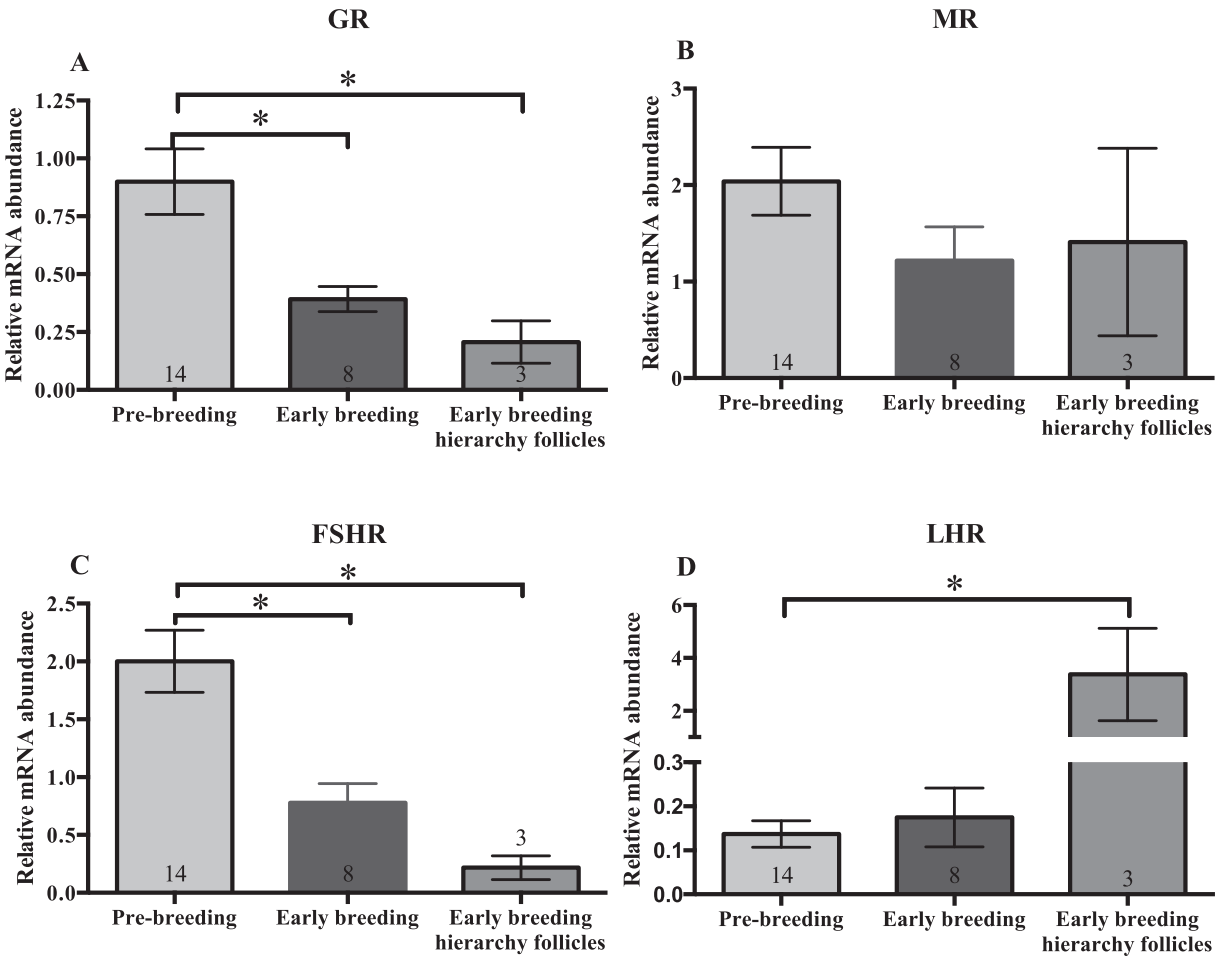

Fig. 5. mRNA expression in the ovary is higher for glucocorticoid receptor (*GR*) and follicle-stimulating hormone receptor (*FSHR*) in the pre-breeding season compared to the early-breeding season, when female dark-eyed juncos (*Junco hyemalis*) enter rapid follicle growth. Ovary gene expression is shown across breeding stages for **(A)** *GR*, **(B)** mineralocorticoid receptor (*MR*), **(C)** *FSHR*, and **(D)** luteinizing hormone receptor (*LHR*). During the early-breeding season, all hierarchical follicles were separated from the ovary and follicle layers and were combined into one tissue sample; this tissue was analyzed separately from the remainder of ovarian tissue. Gene expression is unit-less and quantified on a $\log_2$-fold change relative to an arbitrary calibrator. Figures show means ± SEM. Significant differences between breeding stages are denoted with asterisks (*).

time points we sampled following an intramuscular injection with GnRH, we observed the greatest levels of LH at the 5 min post-injection time point, with levels still significantly elevated but lower at the 15 min time point and levels back to baseline at the 30 min time point. It is still possible that LH peaks even sooner than 5 min; using an intrajugular injection, Deviche et al. (2010) observed peak LH at 2 min post-injection. Thus, future work aimed at investigating LH responsiveness to GnRH stimulation should utilize a much shorter post-injection sampling time point to measure LH, such as 5 min post-GnRH.

4.2. Gonadal hormones during transition to egg-laying

Downstream of the pituitary, we assessed the transition in basal (testosterone) and GnRH-induced gonadal responsiveness prior to and during egg development. We observed significant increases in testosterone (T) levels following a GnRH injection during both the pre-breeding and egg development stages. However, neither baseline nor post-GnRH T significantly differed across the two breeding stages. It is interesting to note that although baseline LH rose during the transition, circulating baseline levels of T did not rise. One explanation for this lack of rise in baseline T may be due to its role as the precursor for the synthesis of estrogen. Unfortunately, we were unable to fully assess whether or not estrogen levels rose along with rising LH levels as we were only able to obtain a single post-GnRH blood sample from each individual. We also may have lacked statistical power to detect a significant change in baseline testosterone levels; we only had enough plasma from 8 individuals to assay baseline testosterone.

Similar to our findings, female Northern cardinals (*Cardinalis cardinalis*) have been shown to elevate T in response to GnRH prior to egg development, during the non-breeding season (i.e., "reproductive quiescence") (DeVries et al., 2011), presumably also stimulated via increased LH release. In contrast to our findings, in a study on dark-eyed juncos breeding in Virginia, where T was measured at 30 min after a GnRH challenge, T significantly rose only during the egg development stage, but not during the earlier pre-breeding stage (Jawor et al., 2007). The breeding environment in South Dakota is thought to be more extreme than in Virginia (Bergeon Burns et al., 2014), which could result in a delay in laying despite physiological readiness to breed in South Dakota. An alternative, but not mutually exclusive explanation for this apparent difference between subspecies in a female junco's capacity to elevate T in response to GnRH during pre-breeding, is a potential difference between subspecies in sensitivity to the same supplementary cues, such as temperature, food availability, and social cues. In white-crowned sparrows (*Zonotrichia leucophrys*), subspecies experiencing less predictable environments appear to be more sensitive to temperature cues, perhaps to fine-tune timing of breeding. This sensitivity to temperature is reflected in ovarian development, however, and not in seasonal circulating LH levels (Wingfield et al., 2003; Wingfield et al.,

1997; Wingfield et al., 1996).

In the current study, GnRH-induced estradiol (E_2) levels did not significantly differ across breeding stages (pre-recruiting and rapid follicle growth). This is concordant with no observed difference in GnRH-induced testosterone levels in this study across these stages. In the dark-eyed junco, post-injection T and E_2 levels were positively correlated when pooled E_2 samples were used (Rosvall et al., 2013). Therefore, T and E_2 levels could be co-regulated and therefore co-evolve due to shared dependence on upstream stimulation of the HPG axis (Rosvall et al., 2013). Interestingly, while max-induced sex steroid levels did not vary across stages, LH levels were higher in response to GnRH in the egg-development stage compared with the pre-breeding stage (Fig. 2). Thus, additional mechanisms, including, but not limited to, seasonal changes in receptor density (see below) may explain the lack of increase in sex steroid hormones across this transition even while the gonadotropin hormone LH response to GnRH was marginally elevated. Unlike testosterone, all estradiol levels reported in this study were from blood samples obtained after GnRH-injection (i.e. no baseline blood sample was collected), which did not allow us to look at an individual's response to GnRH stimulation. Future work should be done in larger birds where more plasma can be taken safely to look at an individual female's ability to respond to exogenous GnRH across the breeding season.

While we were unable to measure baseline levels of estradiol due to a limitation in plasma volume, we were able to collect pilot data from unique individuals to assess the ability of the ovary to respond to GnRH across the two breeding stages and compare that with circulating (baseline) levels of E_2 at the same time points (see Supplementary Fig. 3). Although our sample size was quite small, we observed an effect of breeding stage on baseline E_2 levels in our control individuals as well as an increase in E_2 from pre-breeding to egg-development in individuals that had received an injection of GnRH. Similarly, a previous study in European starlings (*Sturnus vulgaris*) found that circulating E_2 remains at low levels until the stage of rapid yolk development, and reaches maximal levels when birds have complete follicle hierarchies (Williams et al., 2004). Maintaining low levels of circulating E_2 until initiation of rapid follicle growth could be a beneficial mechanism to minimize negative effects from elevated sex steroid levels over long periods of time.

4.3. Changes in abundance of transcripts for hormone receptors in liver and gonad as females transition to egg-laying

Rapid follicle growth occurs quickly in preparation for ovulation in birds (Bêty et al., 2003; Drent and Daan, 1980; Rowe et al., 1994; Williams, 2012). To successfully yolk developing follicles, the ovaries must respond appropriately to endocrine signals leading to secretion of estrogens, and the liver must be responsive to estrogen signals that initiate production and secretion of yolk-precursors. However, these tissues may also appropriately adjust sensitivity to HPG axis stimulation via the two receptors for glucocorticoids, MR and GR, which may enable the ovary and liver to be responsive to signals of increased metabolism and/or stress. Thus, we assessed mRNA expression of receptors for gonadotropins and glucocorticoids in the ovary and liver at two time points; one time point ~15 days prior to the first egg laid in the population with the second time point collected just as laying had begun. In the liver, all genes (*GR*, *MR*, and *ERα*) were expressed at higher levels in the period prior to follicular development compared with those sampled during the early breeding season in our population. MR has a higher affinity for CORT and is therefore thought to mediate responses to lower levels of circulating CORT. Both GR and MR receptors influence the response to elevated CORT levels during an acute stressor (Hu et al., 2008; Landys et al., 2006). One interpretation of our observed higher *GR* and *MR* expression during the pre-breeding period would be to allow the liver to be sensitive to stressors, so that yolk precursor synthesis would not be activated until the female is experiencing a relatively non-stressful environment, or until sensitivity has decreased. However, an alternative interpretation might be that high expression of *GR* and *MR* transcripts is indicative of future GR and MR receptor abundance (which would be likely during rapid follicle growth). If that were the case, perhaps the liver is getting primed for this energetically expensive task. To more clearly delineate these possible alternatives, it will be necessary to compare mature receptor levels in these tissues at these times points.

We also observed elevated levels of *GR* mRNA in the ovary during the pre-breeding period. A particularly interesting implication of potentially higher sensitivity to glucocorticoid (CORT) levels is the knowledge that CORT can influence gonadal steroidogenesis by suppressing transcription and translation of steroidogenic enzymes (Hu et al., 2008) without affecting LH secretion from the pituitary (Deviche et al., 2014, 2012; McGuire et al., 2013; Wingfield et al., 1982). That is, if the ovary is more sensitive to CORT at certain stages it may be less likely to synthesize estradiol regardless of the level of stimulation it is receiving from the pituitary. In the European starling, the gonads of both sexes are able to directly detect cues of stress and respond to these cues in a season-specific manner by modulating sex steroid secretion (McGuire et al., 2013). Production of ovarian steroid hormones is essential for initiation of vitellogenesis by the liver (Johnson and Woods, 2007). Sex steroid hormones are also important for increases in the liver synthesis of yolk-targeted VLDL (VLDLy) (Mitchell and Carlisle, 1991; Wallace, 1985; Walzem, 1996; Williams and Martyniuk, 2000) via activation of the ERα receptor. A complex relay of gene expression and cell signaling within the follicles occurs during the transition to rapid follicle growth and results in increased sex steroid production and secretion. High expression levels of *ERα* that we observed in the early collection period may be indicative of the liver preparing for the ovary's signal that it is time to start increasing production of yolk precursors. Unfortunately, we were unable to assess circulating levels of CORT in the current study due to limited plasma volume.

While ovarian *GR* expression levels were elevated during pre-breeding compared to the period of time of follicle development in the population, ovarian *MR* expression did not change across the two breeding stages. MR has a 6 to 10-fold higher affinity for CORT than GR, suggesting a two-tier system for CORT binding under baseline and stress-induced concentrations of hormone (De Kloet et al., 1998, 1990; Reul and de Kloet, 1985). Due to GR's lower affinity for binding CORT, it is likely more important for reacting to stress-induced activation of the HPA-axis, which may explain why this receptor is differentially expressed across the two breeding stages.

Vertebrates display seasonal changes in circulating (unstressed and stressed) glucocorticoid concentrations and in many species, including birds, glucocorticoid concentrations are commonly elevated during the breeding season (Romero, 2002). One explanation for changes in GR across the season is the ovary's requirement for energy mobilization as it gears up for an energetically expensive process. In the early stages of gonadal recrudescence individuals face higher metabolic costs (~20% increase in resting metabolic rate) (Vézina and Salvante, 2010; Vézina and Williams, 2005, 2003) and supplementary cues are important in making the decision to delay or advance the onset of breeding (Ball, 1993; Dawson, 2008; Hinde and Steel, 1976; Wingfield and Sapolsky, 2003). Once an individual is committed to breeding, supplementary cues become less influential (Ball, 1993; Dawson, 2008; Hinde and Steel, 1976; Wingfield and Sapolsky, 2003), and the gonads become capable of dampening responsiveness to stress hormones and metabolic stress (McGuire et al., 2013). Modulation of the response to stress is likely more important in the critical sensitive period prior to onset of full gonadal recrudescence because the gonads are not yet maximally stimulated by components of the HPG axis, such as gonadotropins (LH and FSH). Therefore, the ovary should be sensitive to stressors during the pre-breeding period, so that an individual avoids initiating egg development too early and risks a mistimed breeding attempt.

FSHR was highly expressed in the ovary during the pre-breeding

period, but showed lower levels during rapid follicle growth. Follicle-stimulating hormone receptor mRNA in the granulosa layer is the earliest marker of selection of follicle recruitment (Woods and Johnson, 2005). Increasing FSH responsiveness is thought to lead to expression of luteinizing hormone receptor mRNA. Similar to our findings, previous research in the European starling showed elevated *FSHR* expression when follicles were developing, followed by a decline as follicles continued to grow (Perfito et al., 2014), which is consistent with results in chickens (Zhang et al., 1997). Previous work has also demonstrated a decline in FSH binding as follicles develop, suggesting that the mature protein for FSHR is similarly declining (Bahr and Johnson, 1984).

Expression of *LHR* did not change across the two breeding stages in our population. In contrast to our findings, expression of *LHR* increased dramatically prior to and leading to development of follicles nearing ovulation in the European starling (Perfito et al., 2014). However, that study did not separate out follicles, and instead measured the entire ovary intact. In our study, we separated the yolking follicles from the rest of the ovary. The ovary without these developing follicles did not show an increase in *LHR* mRNA expression, while the follicles that had undergone significant growth and yolking and entered a hierarchy had a comparatively large elevation in *LHR* mRNA expression. However, the number of ovaries where we were able to measure hierarchical follicles was small, suggesting caution in interpretation. In chickens, *LHR* mRNA and LHR binding continue to increase as follicles mature (Zhang et al., 1997). However, *LHR* transcript (mRNA) abundance may not impact actual sensitivity to LH (Bergeon Burns et al., 2014) because it is indicative of future LHR abundance and not necessarily of current receptor abundance. It is possible that the ovary and liver maintain high mRNA expression levels of key genes in the period prior to rapid follicle growth but that these mRNA products are not translated until environmental cues signal appropriate timing. It is also important to note that we cannot assume transcript abundance always reflects actual protein abundance, and sensitivity can be affected by variables other than receptor density (Bergeon Burns et al., 2014).

4.4. In summary

We found that (1) both the pituitary and gonads are responsive to activation of the HPG axis via exogenous GnRH, (2) circulating LH levels are higher during rapid follicle growth, (3) GnRH-induced levels of T and E_2 do not change over the transition into breeding, and (4) mRNA expression suggests that both the liver and ovary change their responsiveness to stress hormones and reproductive hormones during this critical time period. Prior to developing a follicle hierarchy, both the pituitary and gonads show evidence of sensitivity to upstream stimulation by the HPG-axis. Therefore, it is possible that the communication between the ovary and liver is a potential mechanism regulating onset of egg development. Gonadotropin-inhibitory hormone (GnIH) is another potential player, as it has been shown to act not just at the level of the hypothalamus, but also directly on the pituitary and gonad (Ubuka and Bentley, 2009). Future research should focus on identifying if the stage of initial cell proliferation is determining subsequent steroidogenic and/or yolk uptake capacity during rapid follicle growth (Williams, 2012).

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Competing Interests

The authors declare no competing or financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.10.012.

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