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How experimental competition changes ovarian gene activity in free-living birds: Implications for steroidogenesis, maternal effects, and beyond

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ABSTRACT

The ovary plays an important role in mediating both a female's response to her social environment and communicating it to her developing offspring via maternal effects. Past work has focused on how ovarian hormones respond to competition, but we know little about how the broader ovarian transcriptomic landscape changes, either during or after competition, giving us a narrow perspective on how socially induced phenotypes arise. Here, we experimentally generated social competition among wild, cavity-nesting female birds (tree swallows, *Tachycineta bicolor*), a species in which females lack a socially induced rise in circulating testosterone but they nevertheless increase allocation to eggs. After territory settlement, we reduced availability of nesting cavities, generating heightened competition; within 24 h we reversed the manipulation, causing aggressive interactions to subside. We measured ovarian transcriptomic responses at the peak of competition and 48 h later, along with date-matched controls. Network analyses indicated that competing females experienced an immediate and temporary decrease in the expression of genes involved in the early stages of steroidogenesis, and this was moderately correlated with plasma testosterone; however, two days after competition had ended, there was a marked increase in the expression of genes involved in the final stages of steroidogenesis, including HSD17B1. Gene networks related to the cell cycle, muscle performance, and extracellular matrix organization also displayed altered activity. Although the functional consequences of these findings are unclear, they shed light on socially responsive ovarian genomic mechanisms that could potentially exert lasting effects on behavior, reproduction, and maternal effects.

1. Introduction

Social animals often compete for limited resources necessary for reproduction, and a female's behavioral and physiological response to competition can greatly influence her fitness (Rosvall, 2011; Stockley and Bro-Jørgensen, 2011). The ovary may play a particularly important role in mediating these responses because it regulates gamete development and hormone production. Ovulation is widely recognized as being sensitive to the social environment as it can be suppressed by the presence of a dominant female (Abbott, 1987; Faulkes et al., 1990) or, conversely, it can be accelerated by social stimulation (Darling, 1938; Evans et al., 2009; Waas et al., 2005). Social behaviors are also mediated by gonadally-derived hormones (Albert et al., 1992; Goymann et al., 2008; Wingfield et al., 1990), which can additionally shape the next generation in potentially adaptive ways via maternal effects (Groothuis et al., 2005; Schwabl, 1993). Thus, the ovary plays an important role in mediating both a female's response to her own social environment and communicating it to her developing offspring.

Ovarian function is largely regulated by the hypothalamo-pituitary-gonadal (HPG) axis, which is activated when external stimuli, like competitive interactions, trigger secretion of gonadotropin-releasing hormone (GnRH) from the brain, initiating a cascade of events that culminate in ovarian production of sex steroids that then pass into the female circulation and the oocyte (Adkins-Regan, 2005). Competition-

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related research has historically focused on circulating levels of the sex steroid testosterone (T). For example, work in male vertebrates suggests that T elevates in circulation after social challenges to promote aggressive phenotypes (i.e., challenge hypothesis; Archer, 2006; Hirschenhauser and Oliveira, 2006; Wingfield et al., 1990). In females, T does stimulate aggression, but plasma T levels rarely elevate in response to social challenges, with T secretion frequently remaining unchanged or even decreasing after social competition (reviewed in Rosvall et al., 2020). Likewise, socially induced allocation of hormones to the oocyte have focused on T, and this phenomenon is particularly well studied in birds (reviewed in Bentz et al., 2016; Groothuis et al., 2005). Females engaging in aggressive interactions tend to allocate more T to egg yolks (Bentz et al., 2013; Eising et al., 2008; Hargitai et al., 2009; Mazuc et al., 2003) and offspring exposed to elevated yolk T display traits that are potentially adaptive in the context of competition, like faster growth (Eising et al., 2001; Navara et al., 2005; Schwabl, 1996) and increased aggression (Bentz et al., 2021a; Eising et al., 2006; Partecke and Schwabl, 2008). Despite receiving less attention, other gonadal hormones, like estradiol and progesterone, are also socially sensitive in circulation (Albert et al., 1992; Goymann et al., 2008; Merritt et al., 2018) and have been measured in egg yolks (Groothuis et al., 2005; Lipar et al., 1999; Schwabl, 1993). Extra-gonadal steroids, like corticosterone, can also be socially induced (Deviche et al., 2014; Potticary and Duckworth, 2020; Pinxten et al., 2004), and can negatively influence gonadal production of both circulating and yolk steroids (Henriksen et al., 2011). Thus, a more holistic approach is needed to better understand the role these and other potentially interacting hormonal pathways play in regulating socially induced phenotypes.

Genomic approaches offer one way to identify suites of behaviorally relevant genes and pathways, and such efforts have helped to provide a more mechanistic and unbiased understanding of animal behavior (Zuk and Balenger, 2014). However, past efforts to discover the genomic mechanisms underlying responses to competition have focused on the brain (Alaux et al., 2009; Bentz et al., 2021b; Mukai et al., 2009; Rittschof et al., 2014; Sanogo et al., 2012), despite gonads also being rich sources of phenotypic variation (Rosvall et al., 2016), even when accounting for standardized upstream signaling along the HPG axis (George and Rosvall, 2018; Jawor et al., 2007). Candidate gene analyses indicate genomic mechanisms play an important role in regulating gonadal hormones in both the female circulation and the oocyte. For example, declines in circulating T across the breeding season occur alongside declines in the expression of ovarian steroidogenic enzymes (Bentz et al., 2019a) and yolk T correlates with ovarian aromatase expression during egg laying (Egbert et al., 2013). The role of ovarian genes in maternal effects is further highlighted in the evidence showing there is a heritable component to yolk T concentrations (Okuliarová et al., 2011; Tschirren et al., 2009). However, we still know little about how the social environment impacts the transcriptomic landscape of the ovary.

Moreover, few studies explore whether and how the effects of competition outlast the competitive event itself, at least in adults. There is some evidence from neurogenomic studies suggesting the gene regulatory effects of competition can last on the scale of hours (Bukhari et al., 2017; Shpigler et al., 2017) and days (Bentz et al., 2021b). Comparable lasting effects in the ovary would be particularly relevant for maternal effects. The maternal transfer of hormones is thought to occur during egg formation, with sex steroids passively diffusing into the rapidly accumulating yolk directly from the steroid-producing cells that surround the growing follicle (Okuliarová et al., 2010), allowing yolk steroids to increase without needing a concomitant rise in circulation (Groothuis and Schwabl, 2008; Moore and Johnston, 2008). However, this presents a relatively short window for social interactions to influence the transfer of hormones. Egg production is also a physiologically demanding process (Williams, 2005), which would deter females from being overly aggressive during this time, although egg laying typically occurs after territories are already established. It remains unclear if competition during early territory establishment, occurring *before* the short period of yolking, can act to prime the ovary for later steroid uptake (e.g., via retention, selective transport, or increased production; Groothuis and Schwabl, 2008; Moore and Johnston, 2008; Navara et al., 2006). At the time of early territory establishment, a female's follicles would still be small and undeveloped. The granulosa cells of prehierarchical follicles are relatively incompetent to produce progesterone, but the thecal cells are fully capable of producing androgens and estrogens (Tilly et al., 1991; Rangel and Gutierrez, 2014). If competition can exert lasting genomic effects on the ovary, this would hint at a potential mechanism allowing earlier social interactions (during territory establishment) to shape maternal effects.

Here, we experimentally generated a period of heightened competition during early territory establishment, and we analyzed the immediate and longer lasting effects on the ovarian transcriptome. Our experiment used free-living tree swallows (*Tachycineta bicolor*), which are obligate secondary cavity-nesting birds, meaning they cannot excavate their own cavities. In this system, a female's reproductive success hinges on her ability to aggressively compete for a nesting cavity (Rosvall, 2008) and females, more so than males, aggressively defend cavities against intruders (Lipshutz and Rosvall, 2021). There is also strong experimental and correlational evidence that tree swallows increase yolk T allocation in response to social interactions (Bentz et al., 2013; Whittingham and Schwabl, 2002). Using a population of free-living tree swallows breeding in artificial cavities (nest boxes), we experimentally increased competition by reducing nest box availability after initial territory settlement. We then returned boxes within 24 h to reverse the experiment. We collected females during the peak of competition or 48 h after the end of the competitive period, along with date-matched controls. We measured transcriptomic responses in the ovary and used gene co-expression network analyses to identify pathways that were differentially regulated during and/or after competition.

We have previously published on the hormonal (George et al., 2021) and neurogenomic changes (Bentz et al., 2021b) in the birds from this experiment. Rates of physical aggression, including grappling fights, significantly increased in response to the manipulation of available nest boxes, but subsided to control-like levels after boxes were returned to their initial positions (Fig. 1A). Using behavioral observations and radio-frequency identification (RFID) devices at nest boxes, Bentz et al. (2021b) confirmed that focal females were engaged in these interactions and did not lose box ownership or mates during the experimental period. George et al. (2021) showed that plasma T *decreased* in response to aggressive interactions during peak competition, using an expanded dataset that also included the females in the current study (Day 0: $F = 7.58$, $p = 0.015$; Fig. 1B). There was no significant difference in plasma T two days after competition ended (Day 2: $F = 0.03$, $p = 0.867$; Bentz et al., 2021b; Fig. 1B). The females in this population are physiologically capable of elevating plasma T in response to an exogenous GnRH challenge at this breeding stage (George and Rosvall, 2018). Furthermore, females in this study had lower methylation on the GnRH receptor in the brain on Day 2, suggesting that this top-down regulator of the HPG axis may be poised for *more* transcriptional activity in the days following competition (Bentz et al., 2021b). Here we extend this past work with a new inquiry on how the social environment shapes the ovarian transcriptome.

2. Methods

2.1. Study population

We monitored breeding tree swallows located at several sites near Bloomington, Indiana (39°9 N, 86°31 W) between March and April 2019. In earlier years, birds were captured in their nest boxes and given a unique numbered USGS aluminum band on one leg and a plastic-colored band on the other leg. Each plastic leg band has a personal integrated transponder (PIT) tag (2.3 mm tag with an EM4102

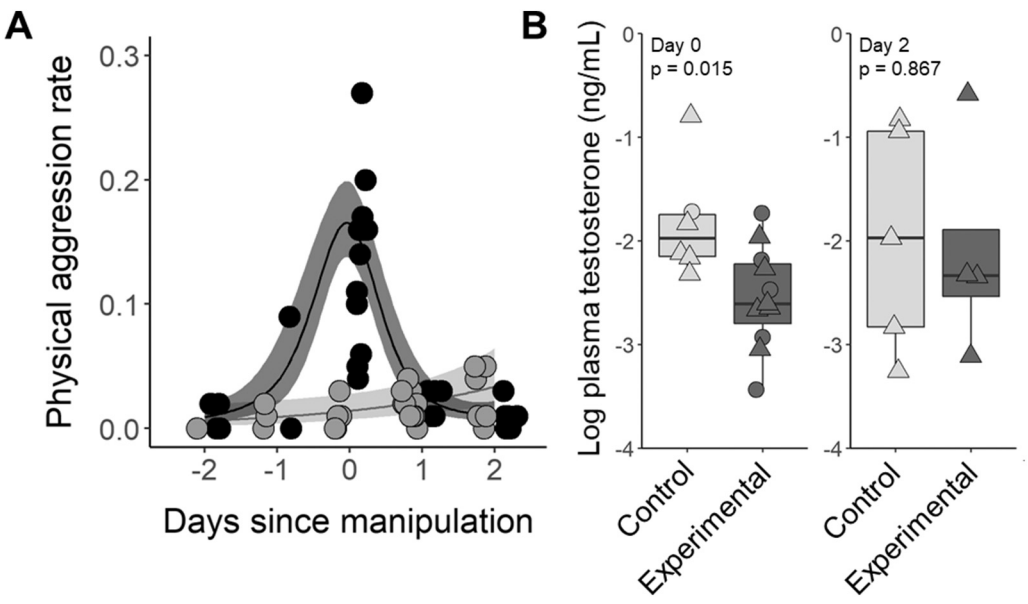

Fig. 1. Effects of the experimental manipulation on aggression and plasma testosterone. (A) Smooth function for physical aggression rate (minutes in which physical aggression was observed per minutes observed per boxes occupied during daily observations). Circles represent individual observations and lines are the smooth function. Dark gray represents experimental sites and light gray represents control sites. Shading corresponds to 95% confidence intervals. Reprinted from Bentz et al. (2021b) with permission. (B) Log-transformed plasma testosterone in control and experimental females on Day 0 and Day 2. Boxplots depict the median count (horizontal line) bounded by the upper and lower quartile of plasma testosterone for each treatment, and whiskers represent 1.5 interquartile ranges. Triangles represent the subset of females used in the current study. Data from Bentz et al. (2021b) and George et al. (2021).

transponder from Eccel Technologies, UK) that transmits a unique identifying number when activated by a RFID reader/antenna (designs from Bridge et al., 2019; Bridge and Bonter, 2011) attached to a copper coil antenna located on nest box entrances. RFID readers were programmed to scan every 500 ms to detect any PIT tags near the antenna (i.e., at the nest entrance), and then recorded date/time and tag identity. We estimated counts of unique PIT tags to determine box ownership (defined as the female with >50% of the counts each day).

This study was approved by the Bloomington Institutional Animal Care and Use Committee under protocol #18–004, as well as all relevant federal, state, and local permits.

2.2. Experimental manipulation of competition

As previously published in Bentz et al. (2021b), we temporarily induced competition at experimental sites by reducing nest box availability after territory establishment (i.e., once nest ownership could be confidently confirmed). In the evening, after birds had departed to roosting sites, all unclaimed boxes and 50% of claimed boxes were removed from experimental sites. The remaining 'focal' boxes were claimed by stable, known residents. New boxes were erected to accommodate only half of the displaced females and were evenly dispersed ~20 m between two focal boxes, but otherwise 100 m away from other focal or newly placed boxes. This placement was intended to facilitate interactions between focal females and conspecifics (displaced and/or non-territory-holding females) competing for the nearby new boxes. The next morning (Day 0), 2 h after females returned from roosting, we collected roughly half of the focal females (those whose boxes we did not remove) at each experimental site. We also collected females from control sites, at which no box removal occurred. Control sites were > 300 m from experimental sites, which is beyond the tree swallow home range (McCarty and Winkler, 1999). In the afternoon of Day 0, after collections were complete, all boxes were returned to their initial positions. Then, 48 h after the experimental manipulation (Day 2), we collected the remaining focal females that had previously experienced competition, as well as a second set of date-matched controls at undisturbed control sites. Females collected on Day 2 were all box owners on Day 0, confirmed with RFID devices and observations (Bentz et al., 2021b).

2.3. Sample collection

Focal females were captured in their nestboxes (via nestbox trap) and immediately euthanized with an overdose of isoflurane, followed by decapitation. Trunk blood was collected and kept on ice, centrifuged to isolate plasma, and plasma was stored at -20 °C. Tissues, including ovaries, were immediately dissected, frozen on powdered dry ice in the field, and transferred to -80 °C in the lab. All ovarian follicles were undeveloped and white, suggesting females were not yet reproductively active. Furthermore, nesting is quite synchronous in this system, and across all sites, females had completed ≤50% of nest construction at the time of the experiment. The earliest first egg date occurred ~2wks after the experiment ended, indicating no potential confound with reproduction per se. Our final sample size was 20 females collected across Day 0 (experimental, $n = 6$; control, $n = 5$) and Day 2 (experimental, $n = 4$; control, $n = 5$). Collections occurred between 0800 and 1330 h (average 1052 h ± 22 min) from April 16–18.

2.4. RNA sequencing

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), and resuspended in water. Quality and quantity of RNA was analyzed with a TapeStation 2200 (Agilent Technologies, Santa Clara, CA). Total RNA was used for cDNA library construction using a TruSeq Stranded mRNA kit (Illumina). Sequencing was performed using an Illumina NextSeq 500 Kit with a 75 bp sequencing module to generate paired-end reads. The resulting reads were cleaned using Trimmomatic v.0.36 (Bolger et al., 2014) and mapped to the reference tree swallow transcriptome (Bentz et al., 2019b) using Bowtie2 v.2.3.4.3 (Langmead and Salzberg, 2012). Results were filtered to only included reads mapped in proper pairs, sorted, and indexed using Samtools v.1.9 (Li et al., 2009). Approx. 24.9 million read pairs per sample were mapped to the entire transcriptome accounting for ~91% (range 89–92%) of the total trimmed read pairs. When mapped against a high-quality protein-only subset of the transcriptome, approx. 10.3 million read pairs per sample were mapped with high confidence, which account for ~38% of the total trimmed read pairs (Table S1). By design, the high-quality subset maps directly to a specific gene. RNA-seq data have been deposited into NCBI's Gene Expression Omnibus (GSE184993).

2.5. Identification of differentially expressed genes

Differential gene expression and normalized values were determined using DESeq2 v.1.16.1 (Love et al., 2014) in R/Bioconductor. Transcripts with <10 counts in >10% of the samples were filtered out (n = 12,447 transcripts retained). P-values were corrected using Benjamini-Hochberg corrections and FDR ≤ 0.10 were considered differentially expressed (default value in DESeq2). Due to the nature of biological variation in the wild and our relatively small sample sizes, we provide SE for our log2 fold change calculations. Differentially expressed genes (DEGs) were assessed for enrichment of biological process Gene Ontology (GO) terms using PANTHER (Mi et al., 2018) with a Fisher's Exact test and a cut-off of FDR ≤ 0.05. GO terms were further summarized with REVIGO (http://revigo.irb.hr/), which clusters GO terms based on semantic similarity (similarity threshold = 0.7).

2.6. Construction of weighted gene co-expression networks

Genes do not act alone, instead performing their functions in shared pathways and networks (Sinha et al., 2020), so we performed a weighted gene co-expression network analysis (WGCNA) to determine how whole networks of putatively co-regulated genes, rather than individual genes, respond to the manipulation (Langfelder and Horvath, 2008). Using the normalized counts from DESeq2, we filtered out genes with low counts (<10 norm counts in >10% of the samples) and low variability (50% least variable genes) (n = 6201 genes retained). An adjacency matrix was calculated based on pair-wise Pearson correlation coefficients for a signed network including all pair-wise comparisons of the genes across all samples (n = 20). A soft threshold power (β) = 9 was chosen in accordance with scale-free topology (Fig. S1). Modules were identified using average linkage hierarchical clustering with the topological overlap dissimilarity as the distance measure. Minimum module size was 30 and Dynamic Tree Cut was used to merge highly similar modules (threshold of 0.15) (Fig. S2). Genes not assigned to a module were classified to the colour gray.

2.7. Finding modules of interest

Expression levels for each module were summarized by the first principal component (module eigengene), which we used to test for an association between modules and traits of interest (i.e., treatment on Day 0 and Day 2, and plasma T levels as published in George et al., 2021) using a Pearson correlation test. We additionally included field site and time of capture to account for these potentially confounding variables. We assessed each module for enrichment of biological process GO terms using PANTHER (Mi et al., 2018). We only assessed genes with a significant (p < 0.05) module membership (MM; the correlation between the gene expression profile and module eigengene). For trait-associated modules, we additionally narrowed the subset we assessed to genes with a significant (p < 0.05) trait-based gene significance (GS; the absolute value of the correlation between the gene and trait) for the trait(s) of interest.

We identified intramodular hub genes in trait-associated modules, as these genes are potential drivers of the patterns highlighted in these modules. We defined intramodular hub genes as those with a high trait-based GS (> |0.20|) and high MM (> 0.60) with a threshold of p < 0.05 for both (Horvath and Dong, 2008). Intramodular hub genes were visualized in Cytoscape (Shannon et al., 2003). Genes are represented as nodes and interactions between nodes are edges, which are defined by intramodular connection strengths (weights) obtained from WGCNA. We only show the top 1% strongest interactions and those that are supported by evidence obtained from the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) online database (https://string-db.org/) (Szklarczyk et al., 2019).

3. Results

3.1. Differentially expressed genes in the ovary

To understand the broad scope of ovarian responses to competition, including but not limited to potential changes in ovarian sex hormone production, we performed RNA-seq with ovarian tissues and compared females collected at experimental and control sites at both time periods. There were n = 92 DEGs between control and experimental females at the peak of competition (Day 0) and n = 108 DEG two days after competition had subsided (Day 2; Fig. S3). Three DEGs were present in contrasts on both days (FAM179A-like, PEBP4-like, and LYRM1; for all DEGs see Table S2).

The most significant DEG on Day 0 was the hedgehog signaling gene Indian Hedgehog Homolog (IHH) (log2 fold change = 2.18; Fig. S3). No significantly enriched functional annotations were identified for DEGs on Day 0. The most significant DEG on Day 2 was 17-beta-hydroxysteroid dehydrogenase 13 (HSD17B13) (log2 fold change = −2.49; Fig. S3), which is involved in lipoprotein metabolism. DEGs on Day 2 were enriched for the gene ontology (GO) biological process 'cellular component morphogenesis' (GO:0032989; n = 13 genes, FDR = 0.047).

3.2. Trait-associated modules in ovarian gene networks

WGCNA constructed 13 modules, not including the gray module of "unassigned" genes (Fig. 2A; Table S3). Trait-associated modules are discussed below in accordance with expression patterns. For functional enrichment results for all modules, including those not associated with a trait, see Table S4.

3.2.1. Modules that elevated during and declined after competition

Three modules (cyan, light cyan, and midnight blue) were positively associated with the treatment on Day 0 and negatively associated on Day 2 (Fig. 2A). Several intramodular hub genes in these modules were differentially expressed on Day 0 (cyan, n = 4 genes; midnight blue, n = 8) and Day 2 (cyan, n = 2; midnight blue, n = 1; light cyan, n = 2) (Fig. 2B), including IHH in the midnight blue module which was the most strongly DEG on Day 0. There were no enriched biological processes for the light cyan or midnight blue modules at either timepoint; however, for the cyan module, genes negatively associated with the treatment on Day 2 were significantly enriched for processes related to the cell cycle (e.g., double-strand break repair via break-induced replication, FDR < 0.001; mitotic DNA replication, FDR = 0.01) (Table S4; Fig. S4A,B). While no enriched processes were found for Day 0 cyan genes, there was considerable overlap between cyan module hub genes on Day 0 and Day 2, with 46.3% of the genes being similar (19 of 41 genes). A network of cyan Day 2 intramodular hub genes identified L-Myc-1 proto-oncogene (MYCL) as the most highly connected hub gene (Fig. S4B).

3.2.2. Modules that declined during and elevated after competition

One module (purple) was negatively associated with the treatment on Day 0 and positively associated on Day 2 (Fig. 2A). Within this module, no significantly enriched biological processes were identified. However, numerous intramodular hub genes were differentially expressed on either Day 0 (n = 5 genes) or Day 2 (n = 9), including Estrogen Related Receptor Beta (ESRRB) which was upregulated two days after competition had ended (Fig. 2B). Additionally, Low Density Lipoprotein-related Protein 2 (*LRP2; also known as megalin) was an intramodular hub gene* (Table S3). The purple module was also associated with time of day, suggesting expression of these genes decreased as the day progressed (Fig. 2A).

3.2.3. Modules that only elevated after competition

Two modules (magenta and blue) were positively associated with the treatment on Day 2 but did not significantly respond to competition

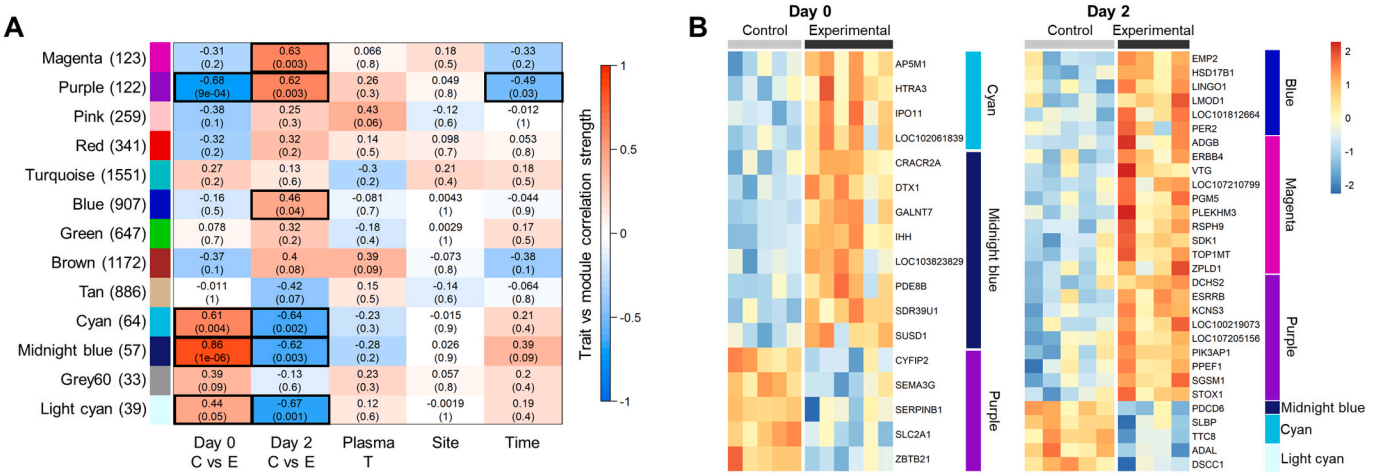

Fig. 2. Module-trait relationships determined by Pearson correlation tests in a WGCNA. (A) Thirteen modules were constructed from genes in all 20 samples across 2 treatment groups and 2 timepoints. Statistical associations are presented for treatment (control [C] and experimental [E]) at the peak of competition (Day 0) and two days later (Day 2), plasma testosterone (T), field site, and time of day females were collected. Correlation coefficients are shown with associated *p*-values in parentheses. *P*-values <0.05 are denoted with a black box. (B) Heatmaps depicting expression levels of differentially expressed intramodular hub genes in modules associated with the treatment. Blue denotes down regulated genes and red denotes up regulated genes. Expression is scaled across replicates.

when it was at its peak (Figs. 2A; 3A), indicating these modules represent the delayed effects of earlier competition. Numerous intramodular hub genes in both modules were differentially expressed two days after competition subsided (blue, $n = 6$ genes; magenta, $n = 10$) (Fig. 2B). Within the magenta module, vitellogenin (VTG) was a differentially expressed intramodular hub gene (Fig. 2B; Table S3), suggesting this yolk precursor plays a role in the lingering effects of competition. While the magenta module did not have enriched biological processes, the blue module was functionally enriched for many processes, including extracellular matrix organization (FDR < 0.001), cell-cell adhesion (FDR <0.001), muscle processes (muscle contraction, FDR < 0.001; myoblast fusion, FDR = 0.002), and regulation of cell communication (FDR = 0.003) (Fig. 3B; Table S4). A network of blue intramodular hub genes identified Neuropilin and Tolloid-like 2 (NETO2), a gene involved in the regulation of cell communication, as the most highly connected hub gene, followed by Transforming Growth Factor Beta Induced (TGFBI), a gene involved in extracellular matrix organization (Fig. 3B). Two of the DEG in the blue module were highlighted in the network, including the steroidogenic HSD17B1, which was highly connected to steroid 17-alpha-hydroxylase/17,20 lyase (CYP17A1), and Leiomodin 1

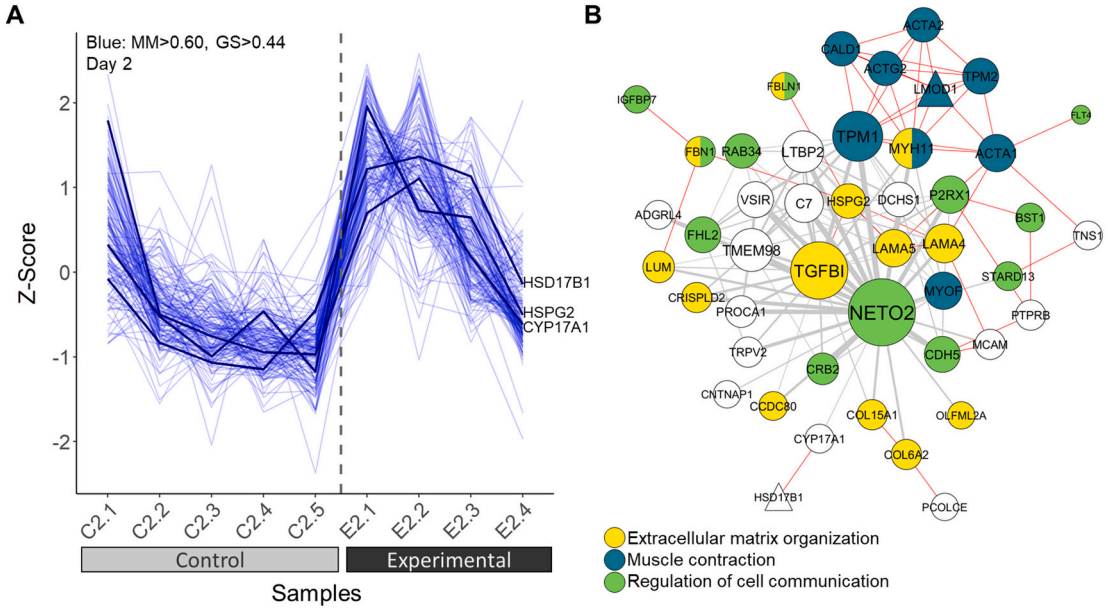

Fig. 3. Expression profile and network diagram of blue module genes. (A) Expression profile of intramodular hub genes, where each line represents one gene. All genes have a high module membership (MM > 0.6; $p < 0.05$) and high gene significance (GS > 0.44; p < 0.05) with the treatment on Day 2 across control (C2, light gray) and experimental (E2, dark gray) ovaries. For each gene, *Z*-score = (x-μ)/σ, where μ and σ are the average expression and standard deviation of a gene across all samples, respectively, and x is the expression of the gene in a specific sample; the y axis therefore represents relative expression levels. Samples are arranged in descending order of median *Z*-score within each treatment. Dark blue lines highlight 3 genes of interest (17β-Hydroxysteroid dehydrogenase 1, HSD17B1; perlecan, HSPG2; steroid 17-alpha-hydroxylase/17,20 lyase, CYP17A1). (B) Network diagram of intramodular hub genes in the blue module based on connection strengths (weights) from WGCNA. Nodes represent genes; size is scaled by number of connections, triangular shape denotes differential expression, and colour indicates enrichment of Gene Ontology Biological Processes. Edges represent connections between genes; width is scaled by weight. Only genes with strong connections (top 1% of all weights in the blue module; weight cut off >0.27) and/or interactions confirmed by STRING (indicated with red edges) are shown.

(LMOD1), a gene involved in smooth muscle contraction (Fig. 3A,B).

3.2.4. Modules related to testosterone

Only one module (pink) demonstrated a near-significant ($p = 0.06$) positive correlation ($r = 0.43$) with plasma T (Fig. 2A). Genes with significant MM were enriched in steroid-related processes (response to steroid hormone, FDR < 0.001; steroid metabolic process, FDR < 0.001; steroid biosynthetic process, FDR = 0.009), sodium ion transport (sodium ion transport, FDR < 0.001; cellular sodium ion homeostasis), nutrient-related processes (insulin receptor signaling pathway, FDR; glucose metabolic process, FDR; response to nutrient levels, FDR = 0.001), and response to glucocorticoid (FDR < 0.001) and cAMP (FDR = 0.049) (Table S4). While the pink module was not significantly related to treatment on Day 0 ($p = 0.10$; Fig. 2A), 40 of the 196 pink module genes with high MM (>0.60) also had a high negative GS on Day 0 (GS -0.45 to −0.70; all $p < 0.05$), meaning they showed evidence of initial downregulation in response to competition. Critically, these genes included three highly connected steroidogenic genes (Steroidogenic Acute Regulatory Protein, STAR; Cytochrome P450 Family 11 Subfamily A Member 1, CYP11A1 [also known as P450scc], and adrenodoxin, ADX) (Fig. 4A). More broadly, the 40 genes that strongly declined in experimental females on Day 0 were involved in several biological processes. Over 50% of the genes in response to cAMP and sodium ion transport had high MM and a high negative GS on Day 0, while over 33% of the genes in processes related to steroidogenesis and nutrient levels similarly had high MM and GS (Fig. 4B; Fig. S5). STAR was notably a member of a number of these biological processes, including steroid biosynthetic/metabolic processes and response to glucocorticoid, nutrient levels, and cAMP (Fig. 4B).

4. Discussion

Experimental manipulation of competition among free-living female songbirds induced immediate and longer lasting genomic responses in the ovary. We previously showed that females at experimental sites experienced a temporary increase in physically aggressive interactions compared to controls (Bentz et al., 2021b), and this occurred alongside an immediate, but temporary decrease in circulating T (George et al., 2021). Here, focused on the ovary, new network analyses indicated that competition induced a temporary downregulation of gene networks involved in steroidogenesis, highlighting several key genes in early, rate-limiting steps. However, two days after competition had ended, there was a marked increase in the expression of genes involved in the final stages of sex steroid biosynthesis. Ovaries also showed longer lasting effects on genes involved in cell cycle processes, smooth muscle performance, and extracellular matrix organization. Although the nature of our sampling meant we could not measure the functional consequences of these transcriptomic changes, the affected pathways shed light on the full scope of social responsiveness in the ovary, both during and after social competition. Moreover, we find that roughly half of ovarian gene networks have divergent responses immediately during vs. two days after competition, highlighting that future work on ovarian responses to the social environment should carefully consider temporal effects.

4.1. Immediate effects of competition on hormone-related gene networks

Although many male vertebrates respond to competitive interactions with the release of gonadal T (Archer, 2006; Hirschenhauser and Oliveira, 2006; Wingfield et al., 1990), these same patterns do not always apply to females (Rosvall et al., 2020), despite the fact that T is a natural and important part of female reproductive physiology (Staub and DeBeer, 1997) and this hormone has clear behavioral effects on females (Goymann and Wingfield, 2014; Ketterson et al., 2005). In tree swallows, there is evidence that exogenous T increases aggression (Rosvall, 2013), but T levels naturally decline shortly after aggressive interactions (George et al., 2021). Here, using WGCNA, we find evidence from the pink module that links ovarian gene networks with plasma T levels. One of the most significant biological processes enriched in this module was steroid biosynthesis and it contained several highly connected steroidogenic genes (STAR, ADX, and CYP11A1 [also known as P450scc]). Expression of these three genes was significantly negatively associated with the treatment on Day 0, indicating they concurrently decreased in females at experimental sites during a period of intense aggressive interactions. STAR facilitates the delivery of cholesterol to ADX and CYP11A1, which catalyze its conversion to pregnenolone, the precursor to all steroid hormones (Strushkevich et al., 2011) and the first rate-

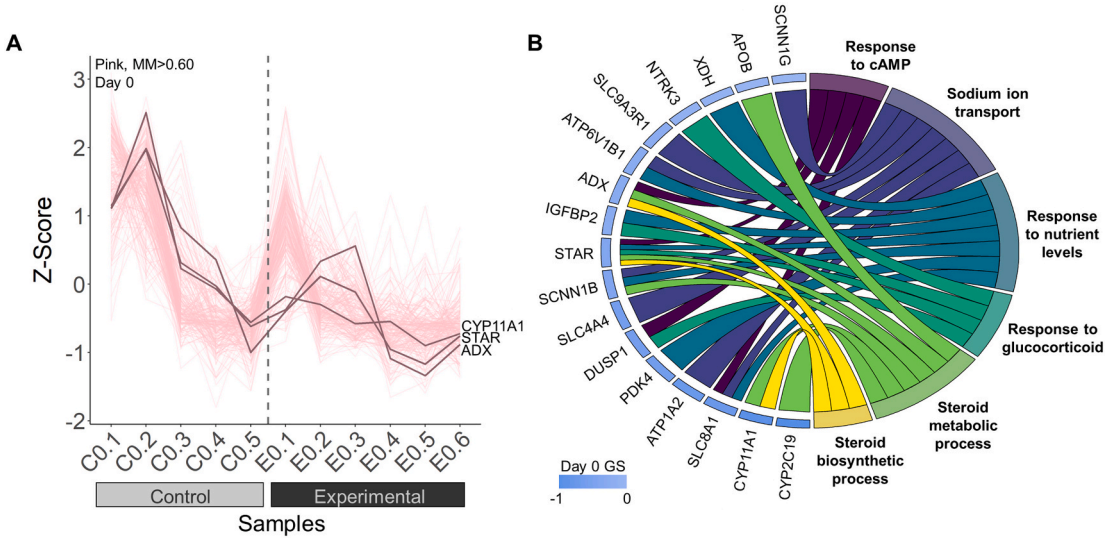

Fig. 4. Expression patterns and biological process enrichment in pink module genes. (A) Expression profile of pink module genes with high module membership (MM > 0.6) on Day 0 for control (C0, light gray) and experimental (E0, dark gray) ovaries. For each gene, *Z*-score = (x-μ)/σ, where μ and σ are the average expression and standard deviation of a gene across all samples, respectively, and x is the expression of the gene in a specific sample; the y axis therefore represents relative expression levels. Samples are arranged in descending order of median Z-score within each treatment. Dark pink lines highlight 3 steroidogenic genes that have high gene significance (GS > |0.50|; $p < 0.05$) with the treatment on Day 0 (cholesterol side-chain cleavage enzymes, CYP11A1; steroidogenic acute regulatory protein, STAR; adrenodoxin, ADX). (B) GOChord plot of genes in the pink module with high MM (>0.60) and gene significance (GS > |0.20|, p < 0.05) on Day 0. Genes are linked to their assigned pathway via colored ribbons and ordered according to the observed GS on Day 0 (blue, negative association with treatment on Day 0).

limiting step of steroidogenesis (Jamnongjit and Hammes, 2006; Stocco and Clark, 1996). Beyond the reported correlation of these pink module genes with T levels, we do not yet know how these findings may relate to other gonadal steroids. Progesterone secretion by granulosa cells would be limited at this follicular stage, but estradiol or androgenic precursors may have been affected (Tilly et al., 1991; Rangel and Gutierrez, 2014). Regardless, these data suggest that ovaries rapidly respond to competition via down-regulation of steroidogenesis.

The pink module was also enriched for other processes, including response to glucocorticoid and nutrient levels, that may provide some insight into how social interactions influence steroidogenesis. Glucocorticoid levels, for example, can elevate in response to a social challenge (Deviche et al., 2014; Potticary and Duckworth, 2020; Pinxten et al., 2004), which can directly suppress STAR (Lynn et al., 2015; Martin and Tremblay, 2008; Payne et al., 1992). Fasting can also elevate glucocorticoids and decrease T levels (Lynn et al., 2015), and reproductive suppression can even occur as a result of a temporary nutrient depletion from increased activity (Souza et al., 2007; Wade and Jones, 2004). While we did not measure glucocorticoid levels, glucocorticoid processes were differentially regulated in the hypothalamus of the females in this study (Bentz et al., 2021b). Specifically, socially challenged females had lower methylation of the glucocorticoid receptor, suggesting that glucocorticoid signaling or negative feedback could be affected at this time. Thus, one possibility is that peak competition may induce a stress response and/or a negative energy balance, triggering a temporary suppression of ovarian sex steroidogenesis – a key question for future hormonal testing.

4.2. Enduring effects of competition on the ovary

Ovarian steroidogenic gene expression was heightened days later among experimental females, despite earlier declines along this pathway. Compared to controls, experimental females had significantly higher expression of HSD17B1 on Day 2 after competition. HSD17B enzymes can act on many substrates, so their function is partly defined by tissue expression (Mindnich et al., 2004). Isoforms expressed in the gonads regulate sex steroid production (Rebourcet et al., 2020), and HSD17B1 was identified as one of the most ovary specific HSD17Bs in the tree swallow transcriptome (Bentz et al., 2019b). While HSD17B1 synthesizes both T and estradiol in mammalian species (Nokelainen et al., 1996; Payne and Hales, 2004), isoform functionality also varies among taxonomic groups and mRNA expression in pre-ovulatory follicles of songbirds is positively correlated with yolk T in GnRH-injected birds (Egbert et al., 2013). Further, HSD17B1 was not correlated with aromatase gene expression in our dataset. Thus, it can be suggested that androgens are the likely steroidogenic product. HSD17B1 was also an intramodular hub in the blue module and was highly connected to another blue hub gene, CYP17A1, which together convert pregnenolone to dehydroepiandrosterone, as a part of the preferred steroidogenic pathway in immature follicles (Lee and Bahr, 1994; Lee et al., 1998). While we did not find that experimental females had elevated circulating T two days after competition, this does not necessarily preclude them from producing eggs with elevated T (Groothuis and Schwabl, 2008; Moore and Johnston, 2008; Navara et al., 2006). Past work demonstrates that tree swallows can increase yolk T in response to aggressive interactions (Bentz et al., 2013; Whittingham and Schwabl, 2002), despite a lack of socially induced circulating T (George et al., 2021). Heightened expression of these ovarian steroidogenic enzymes, taken together with the previous finding that the hypothalamic GnRH receptor may be poised for more transcriptional activity in the days following competition (Bentz et al., 2021b), suggests that females may have a greater ability to produce androgens well after competition.

Beyond these shifts along the steroidogenic pathway, other gene regulatory changes shed light on additional, lasting effects of competition. The most significant biological process in the blue module was extracellular matrix organization, the genes of which increased in tandem with HSD17B1 and CYP17A1 in experimental females in the days after competition had ended. The extracellular matrix is a dynamic structure that not only provides physical scaffolding for cellular components but can also bind and sequester various factors (Frantz et al., 2010). For example, heparan sulfate proteoglycan 2 (HSPG2; also known as perlecan) was a highly connected gene in the blue module network and is a major constituent of the follicular extracellular matrix. HSPG2 is robustly expressed at the fetal-maternal interface (Rohde et al., 1998) and, in birds, is localized in the extracellular matrix of thecal cells – the site of T and estradiol production – where it can bind lipoproteins destined for later uptake in the oocyte (Hummel et al., 2004, 2007). Furthermore, HSPG2 is the most likely estrogen-binding protein during follicular development, when pre-ovulatory follicles have a higher estradiol concentration than serum (Bentov et al., 2016). Thus, HSPG2 can act as a transient holding compartment for steroids, but here we show that this process may also be socially sensitive and correlated with steroidogenic gene expression.

Past researchers have postulated that follicular sequestration or selective uptake of sex steroids is a potential mechanism mediating maternal effects (Groothuis and Schwabl, 2008; Moore and Johnston, 2008; Navara et al., 2006), and our gene network results provide some initial support that competition could jointly regulate steroid production and sequestration days after these social interactions. In addition to the results highlighted in the blue module, the purple module was also positively associated with the experiment on Day 2 and a *notable endocytic* receptor for protein-bound androgens and estrogens (LRP2; Hammes et al., 2005) was an intramodular hub. Nevertheless, one important caveat is that, due to the nature of our sampling design, we could not assess yolk deposition directly, although evidence suggests that this species increases yolk T allocation in response to heightened social interactions (Bentz et al., 2013; Whittingham and Schwabl, 2002). Thus, these lasting genomic effects suggest that pre-hierarchical follicles can integrate social information in ways that may shape later maternal effects, an intriguing idea in need of further study.

Competition also affected gene networks that may relate to follicle development. IHH, an intramodular hub in the midnight blue module and the most significant upregulated differentially expressed gene during the peak of competition, plays a key role in hedgehog signaling and follicle development (Shen et al., 2020). Cell cycle- and muscle-related processes were enriched in the cyan and blue modules, respectively, and these processes are connected to follicle development, ovarian growth, and smooth muscle functioning during gonadal differentiation (Shen et al., 2020; Yin et al., 2019). Also, two days after competition ended, VTG, a primary yolk precursor, was significantly upregulated. Yolk precursors are not produced in advance of yolk deposition and all females in this study had small, white follicles, but the onset of yolk precursor production can occur rapidly (Challenger et al., 2001), suggesting these females who were socially challenged in the past few days may be better prepared for rapid yolk deposition in the future. Darling (1938) suggested that social stimulation can accelerate and synchronize the rate at which females are physiologically ready to reproduce. European starlings (*Sturnus vulgaris*), for example, breed earlier and more synchronously at higher breeding densities (Evans et al., 2009), and winners of territorial disputes will also advance their breeding schedules (Helm et al., 2006). Thus, it is possible that the genomic responses to social interactions found here, in females that have successfully defended their territories, could drive physiological changes that accelerate reproductive timing.

4.3. Conclusions

We identified hundreds of socially responsive genes and processes in the ovary, both during peak competition and days after the period of heightened aggression, revealing the full scope of transcriptomic responses that may occur in the ovary as females prepare to breed, although future work is needed to directly explore these functional

connections. Ultimately, these data shed light on how the social environment shapes female behavioral endocrinology, with implications for mechanisms that could be critical to female behavior and adaptive transgenerational plasticity.

CRediT authorship contribution statement

A.B.B., K.A.R., and E.M.G. designed research; A.B.B., K.A.R., E.M.G., and T.A.E. performed research; A.B.B., D.B.R., and A.B. analyzed data; A.B.B. and K.A.R. wrote the paper, and all authors provided feedback.

Declaration of competing interest

All authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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Data deposition

RNA-sequencing datasets can be obtained from the Gene Expression Omnibus database (GSE184993).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2022.105171.

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