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Maternal social environment shapes yolk testosterone allocation and embryonic neural gene expression in tree swallows

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ABSTRACT

Offspring from females breeding in competitive social environments are often exposed to more testosterone (T) during embryonic development, which can affect traits from growth to behavior in potentially adaptive ways. Despite the important role of maternally derived steroids in shaping offspring development, the molecular mechanisms driving these processes are currently unclear. Here, we use tree swallows (*Tachycineta bicolor*) to explore the effects of the maternal social environment on yolk T concentrations and genome-wide patterns of neural gene expression in embryos. We measured aggressive interactions among females breeding at variable densities and collected their eggs at two timepoints, including the day laid to measure yolk T concentrations and on embryonic day 11 to measure gene expression in whole brain samples. We found that females breeding in high-density sites experienced elevated rates of physical aggression and their eggs had higher yolk T concentrations. A differential gene expression and weighted gene co-expression network analysis indicated that embryos from high-density sites experienced an upregulation of genes involved in hormone, circulatory, and immune processes, and these gene expression patterns were correlated with yolk T levels and aggression. Genes implicated in neural development were additionally downregulated in embryos from high-density sites. These data highlight how early neurogenomic processes may be affected by the maternal social environment, giving rise to phenotypic plasticity in offspring.

1. Introduction

A female's behavioral and physiological response to her environment can have long-lasting effects on the phenotype of her developing offspring, potentially providing a way to prepare them for the current environment (Mousseau, 1998; Uller, 2008). For example, hormone-mediated maternal effects, in which maternal hormones prenatally influence offspring phenotype, are seen across taxa, including fish (McCormick, 1999), reptiles (Uller et al., 2007), mammals (Dantzer et al., 2011; Dloniak et al., 2006), and insects (Crocker and Hunter, 2018). However, birds tend to be the best studied due to the relative ease of manipulating and measuring hormones in their externally developing embryos (Groothuis et al., 2005). One well-studied hormone-mediated maternal effect occurs in response to the social environment. In many species, females breeding in more competitive environments lay eggs with higher concentrations of yolk testosterone (T) (Bentz et al., 2013; Bentz et al., 2016a; Hargitai et al., 2009; Mazuc et al., 2003; Navara et al., 2006a; Pilz and Smith, 2004; Schwabl, 1997; Whittingham and Schwabl, 2002). Offspring that are exposed to elevated yolk T experience numerous phenotypic changes, including faster juvenile growth (Bentz et al., 2013; Eising et al., 2001; Navara et al., 2006b; Pilz et al., 2004; Schwabl, 1996), modified immune function (Navara et al., 2005; Navara et al., 2006b), and increased aggressive behaviors that persist into adulthood (Bentz et al., 2021a; Eising et al., 2006; Partecke and Schwabl, 2008; Strasser and Schwabl, 2004). Despite the wide-ranging and potentially adaptive effects of maternally derived steroids on offspring, the mechanisms causing these phenotypic changes remain unclear (Groothuis and Schwabl, 2008).

Few studies have examined the molecular mechanisms that underlie T-mediated maternal effects (Groothuis et al., 2019). Past work has primarily focused on neural sex steroid receptor genes, like estrogen (ER; Bentz et al., 2016b) and androgen receptors (AR; Pfannkuche et al.,

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2011), as candidates that could be sensitive to T and mediate the pleiotropic effects observed in offspring, including aggressive behaviors (Nelson and Trainor, 2007) and growth (Chang et al., 1995; Nilsson and Gustafsson, 2002). Yet, evidence from early embryonic stages suggests that sex steroid receptor expression in the head is not responsive to experimentally elevated yolk T (Kumar et al., 2019a). Steroid receptors likely play a more nuanced role, e.g., through receptor-mediated effects on downstream genes (Brinkmann et al., 1999) or non-genomic actions (Foradori et al., 2008). However, we need more information on how these processes interact with maternal hormones during development, because it is increasingly evident that maternally derived T acts through more diverse mechanisms than previously explored. For example, one of the best-studied mechanisms linking early life exposure to sex steroids and later adult traits involves immune signaling. Sexual differentiation of the brain causes lasting behavioral changes through interactions of sex steroids, inflammatory signals, and the resident immune cells of the brain (microglia; Arambula and McCarthy, 2020; Delage and Cornil, 2020; Nelson and Lenz, 2017). Microglia can shape neural development and synaptic connectivity, leading to lasting changes in neural function (VanRyzin et al., 2020), and are associated with other early life experiences that impact later-life cognition and behavior (e.g., maternal infection, Bilbo and Schwarz, 2009; prenatal stress, Gómez-González and Escobar, 2010). Additionally, T metabolites may also play a role. Maternally derived T is rapidly metabolized early in incubation to etiocholanolone (ETIO; Campbell et al., 2020; Kumar et al., 2019b), which does not bind to AR (Fang et al., 2003) but can still affect embryo development (Irving et al., 1976; Levere et al., 1967; Wang et al., 2023a; Wang et al., 2023b). Thus, narrowly focusing on transcriptional changes in classical nuclear receptors leaves uncertainty about the diverse processes potentially underlying hormone-mediated maternal effects. A transcriptome-wide approach during the critical window of exposure would help to clarify these mechanisms.

Here, we explore the effects of the maternal social environment on yolk T concentrations and genome-wide patterns of neural gene expression in tree swallow (*Tachycineta bicolor*) embryos. We predicted that if the maternal environment elevates yolk T, then genes involved in steroid synthesis, signaling, or metabolism, as well as neuroimmune processes, are likely candidates to be affected. Tree swallows are a good model for social maternal effects as females are highly territorial (Rosvall, 2008) and show transcriptomic (Bentz et al., 2021b; Bentz et al., 2022) and hormonal changes (George et al., 2022) in response to competition. Furthermore, elevated yolk T is found in response to competition, including elevated breeding density (Bentz et al., 2013) and natural territorial intrusions (Whittingham and Schwabl, 2002), and offspring exposed to elevated yolk T show faster growth and enhanced competitive ability as juveniles (Bentz et al., 2013). In this study, we observed a population of free-living tree swallows breeding in nest boxes at sites with variable breeding densities. We collected eggs at two timepoints, including the day laid to measure yolk T concentrations and eggs incubated to embryonic day (ED) 11 to measure gene expression in whole brains with RNA-seq. We assessed patterns of yolk T allocation across breeding density and determined the degree to which the maternal social environment relates to patterns of gene expression in the embryonic brain. Embryos included both males and females because yolk T can have sex-specific effects on growth, begging rate, and survival (Ruuskanen and Laaksonen, 2010; Sockman et al., 2008). Our data highlight several key genes associated with variation in breeding density and yolk T that point to potential mechanisms by which the maternal social environment could impact phenotypic plasticity in offspring.

2. Methods

2.1. Study system

We monitored breeding tree swallows across eight sites near Bloomington, IN (39°9 N, 86°31 W) between March and July 2020. Nests were checked every 3–5 days for breeding activity. 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. Breeding density

Field sites had 7–34 nest boxes (average: 20 boxes ±3.7 SE) spaced approximately 20 m apart, because previous work done on female tree swallows found density-dependent patterns with conspecific interactions and yolk T levels at this range (Bentz et al., 2013). All field sites were > 300 m apart, which is beyond the tree swallow home range (100-300 m) (McCarty and Winkler, 1999). Breeding density was calculated for each tree swallow pair as the number of boxes occupied by conspecifics within a 100 m radius of the focal box during the 5 days preceding the date of the first laid egg (i.e., the period of rapid yolk deposition; Ardia et al., 2006). Final breeding densities following settlement ranged from 2 to 23 pairs within a 100 m radius (average 12.8 pairs ±2.0 SE).

2.3. Behavioral observations

To quantify rates of physical aggression, we performed 20 min observations at field sites from 0730 to 1230 during territory establishment (April 2 to May 2), resulting in $n = 29$ observations (mean = 2.6 observations per field site). Observations were strategically randomized so that higher- and lower-density sites were counterbalanced, and repeat observations within a site were separated by 4–15 days. During each observation, the observer scanned across visible nest boxes to record the number of aggressive actions that resulted in physical contact between conspecifics, including hits, pecks, and grappling fights. Rates of physical aggression were calculated using a modified protocol from Bentz et al. (2021b). The number of minutes in which physical aggression occurred was divided by the total minutes of the observation. Observations in which only one box was occupied were removed from the analysis (n = 2 observations). A box was considered occupied if an individual was present at the box for ≥10 % of the observation. A linear mixed-effects model was used to test the main effects of average site breeding density, time of day, and proportion of boxes occupied during the observation on physical aggression rates, with field site as the random effect.

2.4. Sample collections and dissections

One egg per nest (3rd egg laid) was collected either the morning it was laid (ED0; $n = 19$ eggs) or after 11 days of incubation (ED11; $n = 14$ eggs). To facilitate collection of the 3rd egg, the first egg date for each nest was recorded and eggs were marked daily with a non-toxic sharpie as they were laid to indicate order. Incubation was considered to start on the day prior to clutch completion, but nests were checked daily to confirm the start of incubation (i.e., warm eggs and/or female on the nest). All egg collections occurred in the morning from 0800 to 1200 and they were stored at -80 °C until further analyses. Egg masses at collection ranged from 1.4 to 1.8 g (average 1.6 g ± 0.03 SE) and all eggs were laid within a ~ 2wk period from May 6 to May 22.

Eggs collected at ED0 were thawed and yolk was collected and stored at -80 °C until hormone analyses. For ED11 eggs, extra-embryonic tissues were washed from the embryo with phosphate buffered saline (PBS) and stored at -80 °C to be used for molecular sexing. Using a stereoscope, embryos were staged according to Murray et al. (2013) (average = stage 39.4 ± 1.0 SE). Developmental stage was not significantly related to breeding density (linear mixed-effects model: $p = 0.92$). Whole brains were collected and stored at -80 °C to be used for RNA-seq.

2.5. Yolk testosterone analysis

Eggs collected on ED0 were used to measure the concentration of T prior to embryonic metabolism and endogenous steroid production. Yolk T was extracted from homogenized yolk samples with an ethanol extraction according to Kozlowski et al. (2009). Briefly, 50 mg of yolk was homogenized with 200 μl of distilled water and incubated for 1 h at 37 °C. Then, 500 μl of 100 % ethanol was added to each sample, homogenized for 1 min, and incubated for 5 min on a plate shaker at 500 rpm at room temperature. Samples were centrifuged at 15,800*g* for 10 min and the supernatant was collected and dried under nitrogen. Dried samples were treated with 50 μl of 100 % ethanol and 300 μl of assay buffer and stored overnight at 4 °C. We quantified T with a commercial enzyme immunoassay (EIA) kit from Enzo Life Sciences (ADI-900-065). We performed several validations, including a spike-and-recovery and testing parallelism with a serial-diluted pooled tree swallow sample. For parallelism, we fit a linear model with sample type (pooled tree swallow sample vs standard curve), log dilution factor, and the interaction between the two with percent binding as the response variable. The interaction term was not significant ($F = 1.04$; $p = 0.348$) while log dilution was ($F = 270.20$; $p < 0.001$), suggesting there were no matrix effects. Yolk T concentrations averaged 1.33 pg/mg ±0.04 SE (range: 0.99–1.66 pg/mg) and recoveries averaged 81.8 % (±10.7 SE). Average intra-assay variation was 2.97 %. We used a linear mixed-effects model to test the main effects of focal nest breeding density and time of day the egg was collected on yolk T concentrations, with field site as the random effect.

2.6. DNA/RNA extractions and RNA sequencing

DNA and total RNA were extracted from ED11 brain and extra-embryonic tissues using the Quick-DNA/RNA Microprep Plus Kit (Zymo Research), following the manufacturer's protocol. Genomic DNA was resuspended in water, and quality and quantity were analyzed with spectrophotometry (Epoch Take3; BioTek Instruments, Inc.). Total RNA was resuspended in water and concentration of RNA was ascertained via fluorometric analysis on a Qubit fluorometer (ThermoFisher Scientific, USA). Overall quality of RNA was verified using an Agilent Tapestation instrument (Agilent Technologies, Santa Clara, CA). All RIN values were > 7.6. Total RNA from ED11 brain tissue was submitted to University of Oklahoma's Consolidated Core Lab for cDNA library construction using the xGen RNA Library kit (Integrated DNA Technologies, USA) according to the manufacturer's protocol. Sequencing was performed at the Oklahoma Medical Research Foundation Clinical Genomics Center using an Illumina NovaSeq 6000 with PE150 reads.

2.7. Molecular sexing

Extracted genomic DNA from ED11 extra-embryonic tissues was used for molecular sexing following the Bento Bioworks Ltd. protocol (https://bento.bio/). Briefly, we used polymerase chain reaction (PCR) to amplify a region of the CHD1-Z and CHD1-W genes on the Z and W sex chromosomes using the CHD1F/CHD1R primer set. PCR products were visualized on 3 % agarose gels stained with GelGreen DNA Stain (Bento Bioworks, Ltd.). Samples with two distinct bands were considered female and those with one band were male. Our dataset included 7 female and 7 male day 11 embryos.

2.8. RNA-seq mapping and differential gene expression

Reads were trimmed using fastp (version 0.20.1) (Chen et al., 2018). The resulting reads were mapped to the reference tree swallow transcriptome obtained previously by Bentz et al. (2019) using Bowtie2 version 2.4.4 (Langmead and Salzberg, 2012). Results were sorted and indexed using Samtools version 1.16.1 (Li et al., 2009). Custom scripts utilizing Samtools version 1.16.1 were used to include reads mapped in proper pairs and to count mapped reads to transcripts. Approx. 16.1 million read pairs per sample were mapped to the entire transcriptome accounting for ~73 % (range 65–78 %) of the total trimmed read pairs. When mapped against a high-quality protein-only subset of the transcriptome, approx. 5.3 million read pairs per sample were mapped, which accounts for ~25 % 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 (GSE246544).

We performed a differential gene expression analysis between ED11 brain tissues collected from high- and low-breeding densities, which we categorized using a median split (a density of 12 breeding pairs) resulting in 7 high- and 7 low-density samples. Transcripts with <10 counts in >10 % of the samples were filtered out ($n = 9816$ transcripts retained). Using the DESeq2 package (version 1.16.1) in R/Bioconductor (R version 3.4.1), we fit a negative binomial generalized linear model with a fixed effect for breeding density for each transcript and calculated per-transcript Wald test statistics to identify significant differences (Love et al., 2014). Sex was excluded from the model as a principal component analysis indicated genes did not cluster by this variable. *P*-values were corrected using Benjamini-Hochberg corrections and genes with an FDR ≤ 0.05 and log fold change ≥ |0.5| were considered statistically differentially expressed. Up- and down-regulated differentially expressed genes (DEGs) were separately assessed for enrichment of biological process Gene Ontology (GO) terms using PANTHER (Mi et al., 2021) 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).

2.9. Construction of weighted gene co-expression networks

We also 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 variation in the maternal social environment (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 (25 % least variable genes) ($n = 7310$ genes retained). We generated a signed hybrid network by selecting a soft threshold power (β) = 7 in accordance with scale-free topology (Fig. S1). We used a biweight midcorrelation (bicor) function and modules were calculated using a minimum module size of 30. A threshold of 0.25 was used to merge modules in Dynamic Tree Cut (Fig. S2). Genes not assigned to a module were classified to the colour gray.

2.10. 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., focal nest breeding density, embryo sex, and site averages for aggression and yolk T concentrations) using the cor function. We additionally included field site and developmental stage to account for these potentially confounding variables. We assessed each trait-associated module for enrichment of biological process GO terms using PANTHER (Mi et al., 2021). We only assessed genes with a significant ($p < 0.05$) module membership (MM; the correlation between the gene expression profile and module eigengene) and 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) (Horvath and Dong, 2008), and visualized these genes 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 interactions 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., 2023), thus networks depict relationships supported by likely interactions among gene products. To further identify densely connected regions within the larger network, we used the Cytoscape tool clusterMaker (Morris et al., 2011) to perform a community cluster (GLay) algorithm (Su et al., 2010).

3. Results

3.1. Breeding density, aggression, and yolk testosterone

Rates of physical aggression significantly increased with average site density ($\beta = 0.01 \pm 0.003$, $F = 15.71$, $p = 0.003$; Fig. 1A), but were not significantly related to time of day or proportion of boxes occupied during the observation (all $p > 0.29$). Yolk T also significantly increased with breeding density ($\beta = 0.02 \pm 0.01$, $F = 6.57$, $p = 0.031$; Fig. 1B) and time of day, such that eggs collected later in the day had lower yolk T concentrations ($\beta < 0.001 \pm 0.000$, $F = 6.99$, $p = 0.027$).

3.2. Differentially expressed genes

Whole brains from ED11 had $n = 1223$ DEGs between high- and low-density breeding sites ($n = 326$ genes downregulated and $n = 897$ upregulated; Fig. 2A; Table S2). The most significant DEG was glutamine-fructose-6-phosphate transaminase 2 (*GFPT2*) (log2 fold change = 1.66; Table S2), which is an enzyme involved in glutamate metabolism.

Upregulated DEGs were enriched for GO biological processes related to the growth of new blood vessels ('vasculogenesis', $n = 13$ genes, FDR = 0.001; 'stimulation of angiogenesis', $n = 27$, FDR < 0.001), immune response ('inflammatory response', $n = 45$, FDR = 0.002; 'activation of apoptosis', $n = 43$, FDR = 0.001; 'regulation of leukocyte migration', $n = 22$, FDR = 0.024; 'myeloid leukocyte differentiation', $n = 15$, FDR = 0.026), and endocrine function ('endocrine system development', n = 15, FDR = 0.021; 'response to estrogen', n = 12, FDR = 0.005; 'response to progesterone', n = 8, FDR = 0.014; 'regulation of steroid hormone biosynthetic process', $n = 5$, FDR = 0.030; 'response to peptide hormone', $n = 29$, FDR = 0.039) (Fig. 2B; Table S3). Notable genes among the DEGs involved in endocrine processes were growth hormone receptor (GHR) and glucocorticoid receptor (NR3C1).

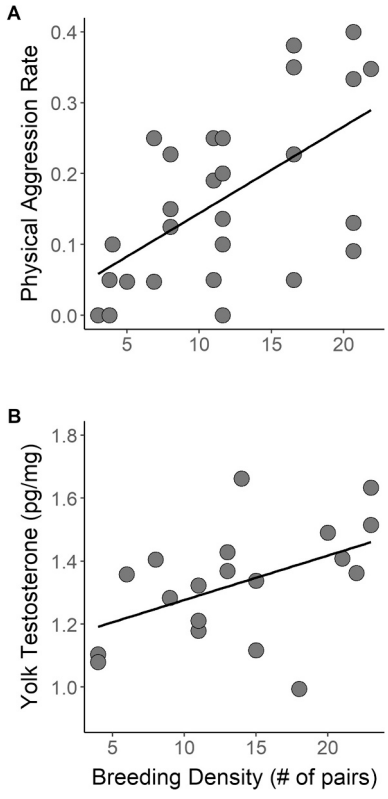

Fig. 1. Relationship between (A) rate of physical aggression (number of minutes with physical aggression / minutes observed) and (B) yolk testosterone (pg/mg) with nest site density.

Downregulated DEGs were enriched for GO biological processes related to neuron function and structure ('chemical synaptic transmission', $n = 21$, FDR = 0.001; 'regulation of axon extension", n=10, FDR=0.001; 'dendrite morphogenesis', n=7; FDR=0.016), with possible implications for learning and memory ('hippocampus development', $n = 9$, FDR = 0.003; 'learning or memory', $n = 14$, FDR = 0.025) (Fig. 2C; Table S3).

Given the number of immune-related processes, we explored whether these DEGs were specifically enriched in genes associated with the resident immune cells of the brain (i.e., microglia), which are implicated in the perinatal programming of later-life behaviors (Arambula and McCarthy, 2020; Bilbo and Schwarz, 2009; Delage and Cornil, 2020; Nelson and Lenz, 2017). The DEGs were compared against a list of core conserved microglia-specific genes identified in a cross-species single-cell analysis ($n = 822$ genes in cluster 1 from Geirsdottir et al., 2019) of which $n = 642$ were identified in our dataset. These core conserved microglia-related genes were more likely to be found among upregulated DEG (9.88 %, 77 of 856 genes) vs all other genes (7.24 %, 565 of 8374 genes) (chi-square test of independence; $X^2 = 6.07$, $p = 0.014$). Conversely, microglia-related genes were less likely to be found among downregulated DEG (3.27 %, 10 of 316) vs all other genes (7.63 %, 632 of 8914) ($X^2 = 7.27$, $p = 0.007$).

3.3. Trait-associated modules in embryonic brain gene networks

The WGCNA constructed 18 modules, not including the gray module of unassigned genes (Fig. 3; Table S4). The maroon module was significantly positively correlated with focal nest breeding density, site average yolk T, and site average rates of aggression (Fig. 3). The navy module was also positively correlated with breeding density and the cyan module was negatively correlated with breeding density (Fig. 3). Additionally, the lime and pink modules were negatively correlated with embryo sex (i.e., higher in females), while beige was positively correlated (i.e., higher in males; Fig. 3).

The maroon module had $n = 631$ genes that increased with breeding density (Fig. 4A) and these were largely enriched for processes related to blood vessel development ('angiogenesis', $n = 36$, FDR < 0.001; 'blood circulation', $n = 30$, FDR < 0.001), immune responses ('inflammatory response', $n = 40$, FDR < 0.001; 'regulation of leukocyte migration', $n = 20$, FDR = 0.002; 'phagocytosis', $n = 16$, FDR = 0.003; 'cytokine-mediated signaling pathway', $n = 25$, FDR = 0.01; 'apoptotic process', $n = 54$, FDR = 0.006), and extracellular matrix functions ('collagen fibril organization', n = 14, FDR < 0.001; 'cell-matrix adhesion', n = 16, FDR < 0.001; 'basement membrane organization', n = 6, FDR = 0.01; 'maintenance of blood-brain barrier', n = 8, FDR = 0.002) (Fig. 4B; Table S5). A number of differentially expressed intramodular hub genes were also found to be differentially expressed or methylated in the brains of adult songbirds exposed to prenatal T injections in past work (Bentz et al., 2021a), including CAV1, FGFR2, CD74, HMOX1, and SLK (Fig. 4B). Genes in the maroon module with high MM and GS for breeding density were visualized as a network (Fig. S3). The navy module also had $n = 12$ genes that significantly increased with density, but these had no significant functional enrichment.

The cyan module had $n = 59$ genes that jointly decreased with

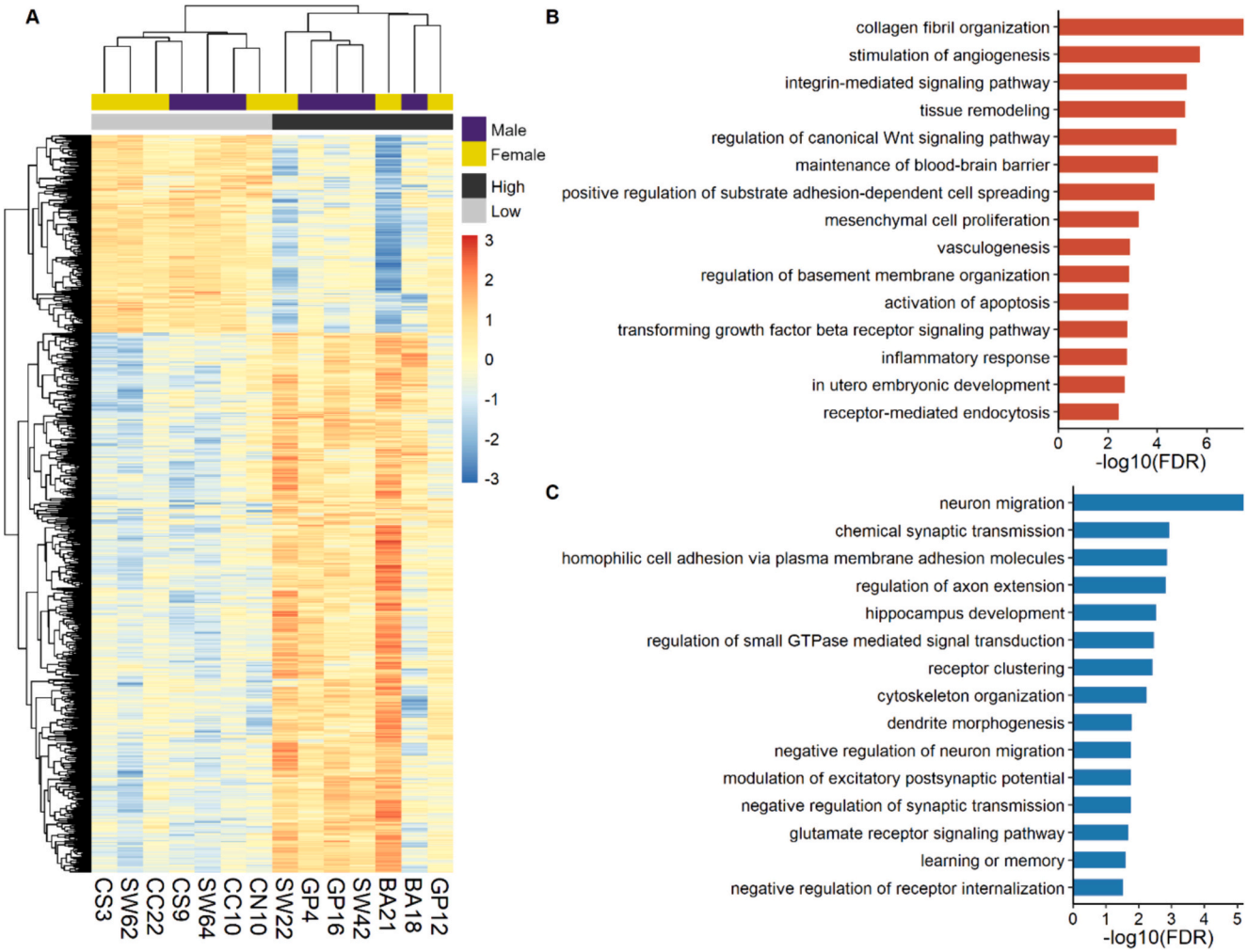

Fig. 2. (A) Heatmap depicting differentially expressed genes (DEG) between embryonic day 11 whole brains from high- (black) and low- (gray) density. Each column is an individual (males are purple and females are yellow) and each row is a gene. Rows are scaled to allow for comparisons of gene expression across individuals. Colour indicates log(norm count+1); with lower (blue) and higher (red) relative expression. Barplot of the top 15 most significant Gene Ontology (GO) Biological Processes enriched in (B) upregulated and (C) downregulated DEGs.

breeding density (Fig. 5A), and these were enriched for processes related to neural development ('neuron differentiation', $n = 13$, FDR = 0.006; 'cerebral cortex development', n = 5, FDR = 0.01; 'neural precursor cell proliferation', n = 5, FDR = 0.006) and gene regulation ('chromatin organization', $n = 10$, FDR = 0.004; 'regulation of transcription', $n = 23$, FDR < 0.001; 'histone H3-K14 acetylation', n = 3, FDR = 0.001) (Fig. 5B; Table S5). Genes in the cyan module with high MM and GS for breeding density were visualized as a network (Fig. S4).

The lime module had $n = 37$ genes with a significant negative correlation with sex (males tended to have lower expression) and high MM. These were enriched for 'lysosomal transport' (n = 5, FDR = 0.045) (Table S5). The pink module also had $n = 104$ genes with a significant negative correlation with sex and high MM. These were enriched for 'rRNA metabolic process' (n = 10, FDR = 0.001) and 'ncRNA processing' ($n = 11$, FDR = 0.010) (Table S5). The beige module had n = 23 genes with a significant positive correlation with sex (males tended to have higher expression) and high MM, but these had no significant enrichment.

4. Discussion

Maternal breeding density was related to patterns of yolk T and neural gene expression in embryonic whole brains. Female tree swallows breeding in higher density sites experienced elevated rates of physical aggression and their eggs had higher concentrations of yolk T compared to those at lower density sites, consistent with prior research (Bentz et al., 2013; Bentz et al., 2016a; Hargitai et al., 2009; Mazuc et al., 2003; Pilz and Smith, 2004; Schwabl, 1997; Whittingham and Schwabl, 2002). A gene network analysis indicated that co-expressed modules of genes linked to circulatory and immune processes were positively correlated with breeding density, yolk T, and aggression, while genes involved in neural development were negatively correlated with breeding density. Though we cannot rule out other egg components as the drivers of the patterns shown here (e.g., Love et al., 2008; Schmaltz et al., 2016; Verboven et al., 2005), several hub genes overlapped with genes that were found to be differentially regulated in prior work examining neural changes in adult songbirds exposed to experimentally elevated yolk T (Bentz et al., 2021a), suggesting these genes could be part of a lasting response to prenatal T exposure. Our findings apply to both male and female embryos, in conjunction with additional sex-related variation in neural gene expression. Below, we highlight key genes and processes related to breeding density and/or yolk T that could contribute to phenotypic plasticity in offspring.

Prior work has understandably emphasized changes in sex steroid receptor expression, yet AR and ER had low expression in the embryonic brain in our study and it was not related to density or yolk T, consistent with another study on yolk T in chicken embryos (Kumar et al., 2019a). Although this differs from past work in juveniles (Pfannkuche et al.,

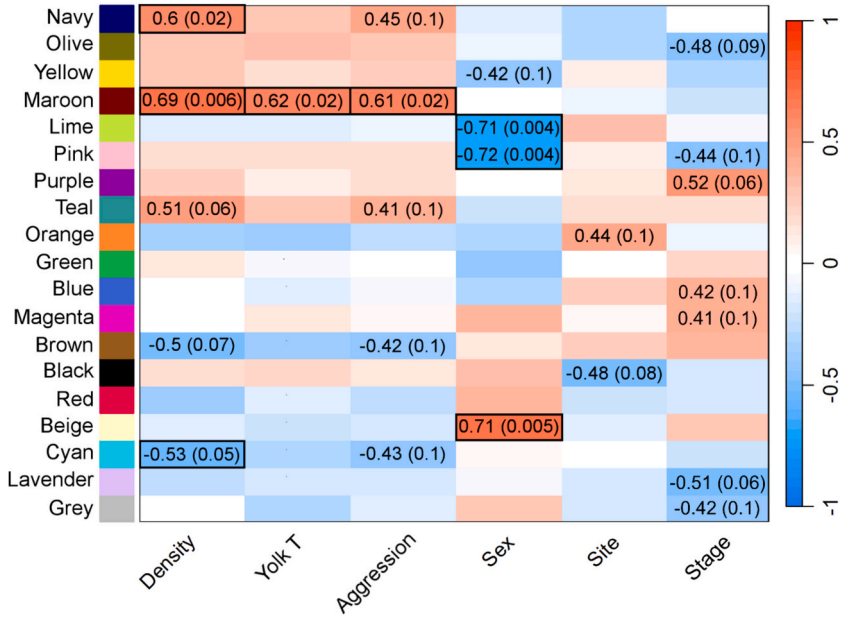

Fig. 3. Module-trait relationships determined by Pearson correlation tests in a WGCNA. Statistical associations are presented for focal nest breeding density, site average yolk testosterone (T), site average rate of aggression, embryo sex, field site, and embryo developmental stage. Correlation coefficients and associated *p*-values in parentheses are shown for all p-values ≤0.10. *P*-values ≤0.05 are denoted with a black box.

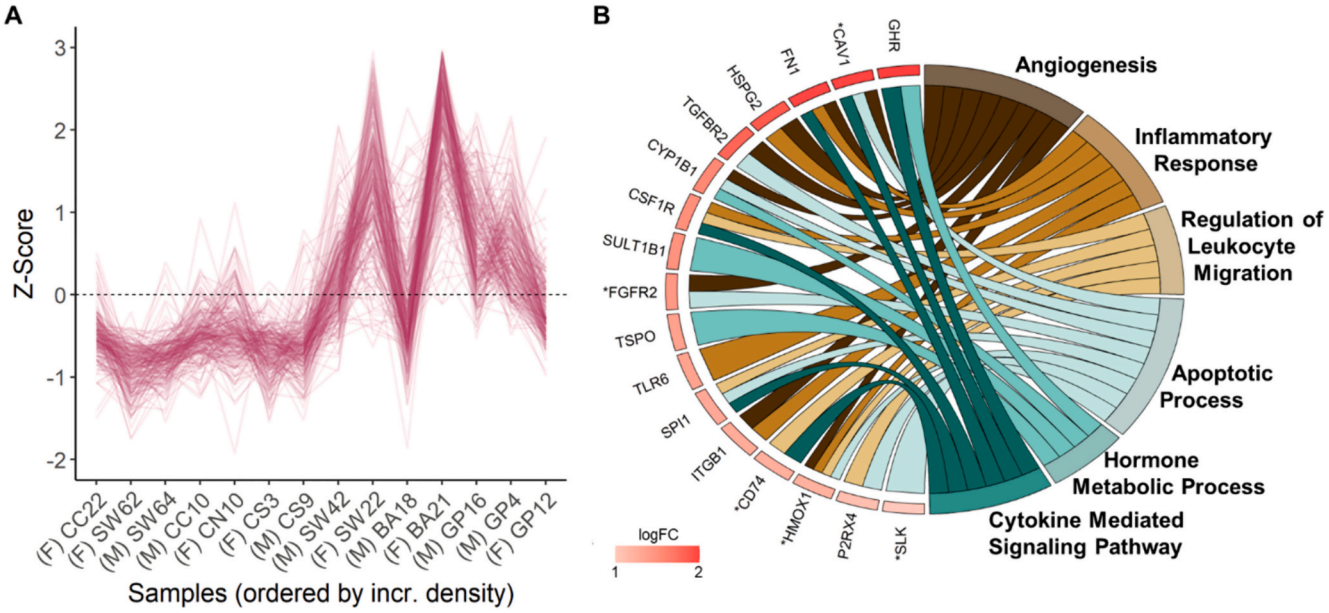

Fig. 4. (A) Expression profile of intramodular hub genes in the maroon module. For each gene, *Z*-score = (x-μ)/σ, where μ and σ are the average expression and standard deviation of a gene across all samples, respectively, and x is the expression of the gene in a specific sample; the y axis therefore represents relative expression levels. Samples are arranged in increasing order of focal nest breeding density and embryo sex is indicated in parentheses (M = male; F = female). (B) GOChord plot of maroon module hub genes linked to their assigned Gene Ontology Biological Process via colored ribbons. Genes are ordered according to the observed $\log_2$ fold change (logFC) across breeding density, which is displayed in descending intensity next to each gene. All of the genes were DEG in the current study; asterisks denote those that were also DEG or differentially methylated in an earlier study on adult songbirds treated with prenatal T (Bentz et al., 2021a).

2011), suggesting that transcriptomic changes in sex steroid receptor expression could arise later in life. Regardless, a lack of differential gene expression does not preclude steroid receptors from mediating important effects via transcriptional activation of other genes (Brinkmann et al., 1999) or non-genomic mechanisms (Foradori et al., 2008). Indeed, a gene that regulates androgen responsiveness via ligand-dependent AR activation (CAV1; Lu et al., 2001) was a differentially expressed hub gene in the maroon module and was also differentially regulated in adult songbirds subjected to yolk T injections (Bentz et al., 2021a), hinting at a lasting mechanism by which androgen sensitivity could be influenced by the early hormone environment. We additionally found metabolic enzymes, like CYP1B1 (metabolizes T to 6β-hydroxytestosterone) and

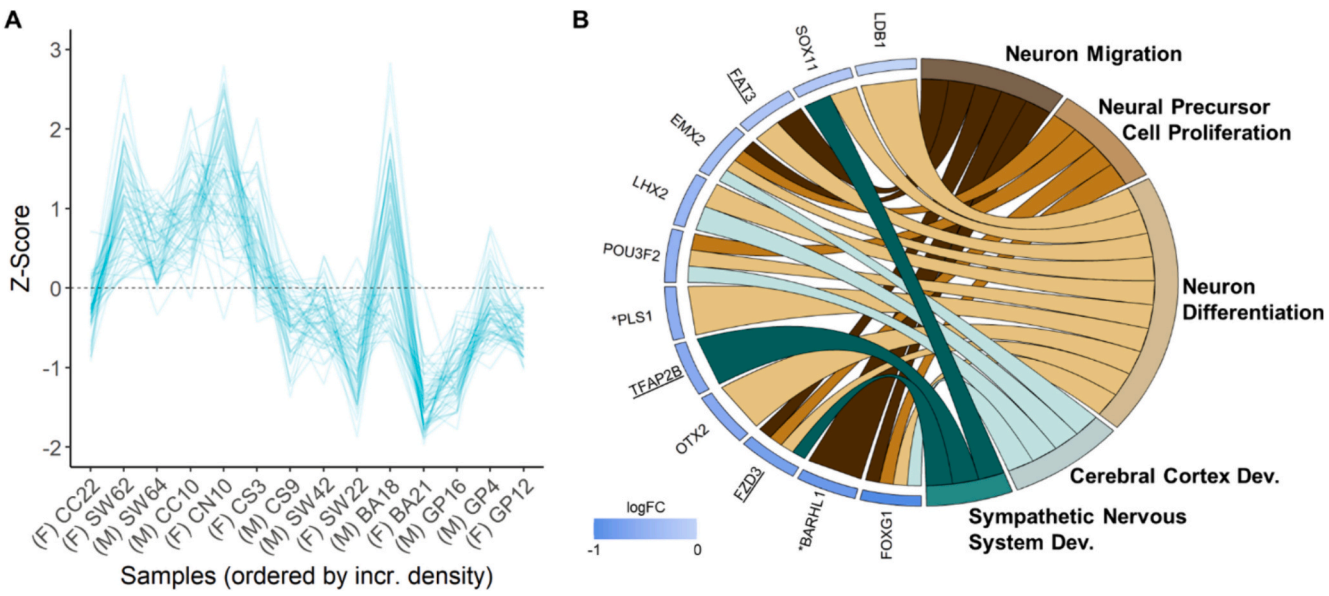

Fig. 5. (A) Expression profile of intramodular hub genes in the cyan module. For each gene, Z-score = (x-μ)/σ, where μ and σ are the average expression and standard deviation of a gene across all samples, respectively, and x is the expression of the gene in a specific sample; the y axis therefore represents relative expression levels. Samples are arranged in increasing order of focal nest breeding density and embryo sex is indicated in parentheses (M = male; F = female). (B) GOChord plot of cyan hub genes linked to their assigned Gene Ontology Biological Process via colored ribbons. Genes are ordered according to the observed $\log_2$ fold change (logFC) across breeding density, which is displayed in descending intensity next to each gene. Underlining denotes DEG in the current study; asterisks denote genes that were also DEG or differentially methylated in an earlier study on adult songbirds treated with prenatal T (Bentz et al., 2021a).

TSPO (associated with cholesterol transport in steroidogenic tissues), upregulated in embryos from high-density sites. Beyond sex steroids, the glucocorticoid receptor (NR3C1) and growth hormone receptor (GHR) were also upregulated in offspring from high-density sites, with the latter being a hub gene in the maroon module. NR3C1 plays an important role in mediating responses to environmental stressors, it is sensitive to early-life effects (Champagne, 2013; Oberlander et al., 2008; Ridout et al., 2020), and its expression can be regulated by the genomic actions of ligand-activated AR (Xie et al., 2015). Past work suggests yolk T may alter stress-related glucocorticoid responses in offspring (Schwabl and Partecke, 2020), and our data highlight a potential mechanism underlying this change. GHR is critical in mediating growth rate early in development (Schwartzbauer and Menon, 1998) and yolk T is associated with rapid early juvenile growth (Bentz et al., 2013; Eising et al., 2001; Navara et al., 2006b; Pilz et al., 2004; Schwabl, 1996). Thus, our data show that the maternal environment can upregulate key genes involved in the hormonal regulation of growth and stress responsiveness, but they also underscore the need to look beyond simple changes in hormone synthesis, signaling, or metabolism, to instead consider the diverse processes by which early T exposure may act to shape transcription in developing young.

Many of the genes with higher expression in embryos from high-density sites are involved in immune processes, including inflammatory response, cytokine-mediated signaling, and various leukocyte and apoptotic processes. Approximately 10 % of the upregulated DEGs, many of which were found in the maroon module, are considered to be core microglia-related genes identified in a cross-species single-cell analysis (Geirsdottir et al., 2019). Notably, CSF1R, a microglia-specific marker that regulates the self-renewal process, and SPI1, a marker specific to developing microglia, were differentially expressed maroon hub genes (Garcia-Morales et al., 2014; Satoh et al., 2014), and thus potentially affected by the maternal environment and/or yolk T levels. Microglia are the resident immune cells of the brain that respond to inflammation, but they also play a critical evolutionarily conserved role in shaping synaptic plasticity during development (Mosser et al., 2017; VanRyzin et al., 2020). Microglia are derived from the yolk sac during hematopoiesis and migrate to the brain during early development, where they remain as a self-renewing population (Ajami et al., 2007; Ginhoux et al., 2010), suggesting that effects occurring during early development can continue to persist throughout life. Microglia-mediated neuroinflammation is thought to play a critical role in sexual differentiation of the brain and behavior (Arambula and McCarthy, 2020; Delage and Cornil, 2020; Lenz et al., 2013; Nelson and Lenz, 2017), as well as lasting cognitive and behavioral effects arising from maternal infection (Bilbo and Schwarz, 2009) and prenatal stress (Gómez-González and Escobar, 2010). There is strong evidence that exposure to yolk T can also cause lasting changes to behavior, like enhanced aggression (Bentz et al., 2021a; Eising et al., 2006; Partecke and Schwabl, 2008; Strasser and Schwabl, 2004). Coupled with observations that transcriptional patterns are likely to shed light on later translation (Li and Biggin, 2015), our data suggest early inflammation and microglia could likewise play a role in T-mediated maternal effects. Indeed, several of the genes associated with steroidogenic processes that were mentioned above have strong relationships with neuroinflammation. For example, upregulation of neural TSPO is linked to neuroinflammation and activation of microglia (Cheung et al., 2023) and conversion of T to 6β-hydroxytestosterone via CYP1B1 is associated with neural hypertension and inflammation (Singh et al., 2020), thereby underscoring the need to better understand the role neuroimmune processes play in T-mediated maternal effects.

Circulatory processes were also significantly enriched in the maroon module and upregulated DEGs. This finding could be linked to past work showing yolk T and its primary metabolite ETIO cause increased embryonic metabolism and heart rate (Wang et al., 2023a; Wang et al., 2023b). ETIO, the effects of which cannot be separated from T in the current study, has also been shown to promote red blood cell production by increasing heme (Irving et al., 1976; Levere et al., 1967). While we did not measure heme, HMOX1 was a differentially expressed maroon module hub gene and it is upregulated in response to elevated heme to promote its degradation (Wu and Hsieh, 2022) because heme is an inflammatory molecule in excess (Figueiredo et al., 2007; Lin et al., 2012; Wagener et al., 2001). Thus, our findings may be indicative of a possible response to elevated heme in embryos from high density and one potential mechanism underlying the upregulation of genes associated with

inflammation and immune processes. If elevated yolk T can cause inflammation via its metabolite's actions on early blood cells, then this offers an intriguing connection to microglial activation and the later behavioral effects that frequently arise as a result of yolk T exposure in competitive maternal environments.

Downregulated DEGs and those found in the cyan module were largely involved in processes related to neural development, including learning and memory and hippocampal development. Prior studies show that prenatal T can have a positive effect on learning and memory (Bertin et al., 2009; Gurzu et al., 2008), although the cyan module was not significantly correlated with yolk T. While we did not measure yolk corticosterone, there is some evidence that it is also sensitive to the maternal environment (Love et al., 2008; Schmaltz et al., 2016; but see Bentz et al., 2013) and we did find that the glucocorticoid receptor was upregulated in embryos from high density. The hippocampus is particularly vulnerable to glucocorticoids (McEwen et al., 1992) and prenatal stress can cause learning deficits through impaired neurogenesis in the hippocampus (Lemaire et al., 2000). Among the downregulated DEGs were TMEM108 and DCLK2, which are associated with neurogenesis (Boseret et al., 2007; Yu et al., 2019). Future work should explore hippocampal-dependent learning in the context of hormone-mediated maternal effects, although yolk T may not mediate these effects.

We found few neurogenomic processes that differed by sex. No modules related to breeding density were also related to sex, but two modules containing genes related to transport and metabolic processes were downregulated in males. It is important to note that our data were acquired using whole brain samples, suggesting there are few sex-related differences across the entire brain. This does not exclude specific brain regions from being affected in a sex-specific manner. Additionally, steroid-induced sex differentiation of the brain in birds does not occur until the first week post-hatching (Adkins-Regan et al., 1994), thus, it is possible that ED11 is too early to detect sex-specific effects.

5. Conclusion

Using free-living tree swallows, we identified genes and processes within the embryonic brain that exhibit connections to the maternal social environment and yolk T. Specifically, our data show an upregulation of genes involved in circulatory and immune processes in embryos from high-density sites, characterized by elevated yolk T, while genes related to neurodevelopmental processes were downregulated. These observations offer potential mechanisms driving intergenerational phenotypic plasticity although future work should explore relationships within specific brain regions. Future experimental work should also clarify the relationships between maternally derived T and the key genes identified here, many of which are modulators of neuroinflammation and have exciting potential connections with previously established effects of yolk T.

CRediT authorship contribution statement

M. Leigh Bailey: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Cameron Nixon:** Writing – original draft, Investigation, Formal analysis. **Douglas B. Rusch:** Formal analysis, Data curation. **Aaron Buechlein:** Formal analysis, Data curation. **Kimberly A. Rosvall:** Writing – review & editing, Writing – original draft, Resources, Methodology, Funding acquisition, Conceptualization. **Alexandra B. Bentz:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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 (GSE246544).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2024.105561.

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