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THE NEUROGENOMICS OF COMPETITION AND PARENTAL CARE IN A SOCIALLY

POLYANDROUS SHOREBIRD

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

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ABSTRACT

Despite sharing largely similar genomes, females and males of the same species exhibit morphological, physiological, and behavioral differences. Sex-role reversal stems from a unique case of sexual selection in which females compete for mates and males conduct the majority of parental duties. We examined the extent to which aggression and parenting may be mediated by different neurogenomic mechanisms by comparing females versus males in the northern jacana (*Jacana spinosa*), a sex-role reversed shorebird. We hypothesized that sex differences in neural transcriptomic profiles explain sexual dimorphism in behavior. We conducted RNA-Seq in two brain regions within the social behavior network: the nucleus taeniae and the preoptic area of the hypothalamus. Several hundred genes were differentially expressed between females and males in both brain regions. These include androgen receptor (AR), which had higher expression in females, and prolactin receptor (PRLR), which had higher expression in parenting males. Additionally, we identified multiple gene networks associated with competitive traits including aggression, weaponry, and gonad size. These networks were enriched for biological processes such as central nervous system myelination, morphogenesis, and spermatogenesis. These comparative transcriptomic analyses suggest that molecular mechanisms associated with competition and parental care may be universal, regardless of which sex performs them.

CHAPTER ONE

INTRODUCTION

Sex differences

Sexual dimorphism in reproductive behaviors such as territorial aggression and parental care is widespread across the animal kingdom (Lande 1980; West-Eberhard 1983; McPherson and Chenoweth 2012; Zilkha et al. 2017). These complex behavioral traits have important fitness consequences, including resource defense and mate acquisition, as well as offspring survival. Differential selection on female and male reproductive behaviors may also shape underlying proximate mechanisms, including genomic, neural, and endocrine phenotypes that give rise to these traits (Ketterson et al. 2005; McCarthy 2016; Rosvall et al. 2020). Despite sharing vastly similar genomes, males and females of the same species often differ remarkably in morphology, physiology, and behavior. Thus, sexually dimorphic gene expression is an important mechanism for sex-specific adaptation (Rinn and Snyder 2005; Mank et al. 2007).

Sexually dimorphic behaviors are often termed "sex roles," which can include competition for mates, territorial aggression, mate choice, and parental care (Barlow 2005). Traditional hypotheses of sex role development suggest that males compete for access to choosy females and have higher variance in mating success than females (Andersson 1994, Trivers 1972). Indeed, male competition and female parental care constitute stereotypical sex roles in most animals. However, a comprehensive understanding of reproductive behaviors requires consideration of systems that contradict those norms.

Breeding season dynamics

The degree of sexual dimorphism may vary temporally (Robinson et al. 2008; Bukhari et al. 2019) as females and males shift their behavior across reproductive cycles. For instance, the transition from territorial establishment to parental care involves substantial shifts in behavior, facilitated by dynamic changes in hormone secretion, gene expression, and neural plasticity (Bentz et al. 2019; Bukhari et al. 2019; Feldman et al. 2019). One conserved physiological example is the decline in levels of testosterone in circulation from territorial establishment to parental care in both males (Wingfield et al. 1990) and females (Rosvall et al. 2020). An open question is how the degree of sexual dimorphism in neurogenomic mechanisms shifts across different reproductive stages of competition and parental care. Studies using brain transcriptomic analyses have identified neurogenomic changes associated with different breeding stages in both females and males (Bentz et al. 2019, Bukhari et al. 2019), but past works often focus on only one sex, potentially conflating sex and sex roles.

Neurogenomic mechanisms of behavior

In the vertebrate brain, a group of reciprocally connected regions known as the social behavior network regulates reproductive and other social behaviors, including competition and parental care, and these functions are conserved across species (Newman 1999; Goodson 2005; O'Connell and Hofmann 2012). In these brain regions, aggression is modulated by various neurotransmitters and neuroendocrine mechanisms, including sex steroid hormones and neuropeptide hormones (Maney and Goodson 2011; Ball and Balthazart 2008; Saudou et al. 1994; Soma 2006; Nelson and Chiavegatto 2001). Key regulators of parental care include the

signaling and reception of prolactin, oxytocin, arginine vasopressin, dopamine, and galanin (Fischer et al. 2019; Zilkha et al. 2017).

Although some mechanisms of parental care are shared between the sexes of species with biparental care (Feldman et al. 2019), other mechanisms of parental care may be specific to females, especially in species with maternal-only care (Zilkha et al. 2017). Less well understood, however, is what happens to the brains of females that have evolutionarily lost parental care, and to the brains of their care-giving male mates. Thus, progress towards understanding whether neurogenomic mechanisms of aggression and parental care are conserved between the sexes is currently impeded by the lack of studies in species with male uniparental care.

Social polyandry

Social polyandry, also known as sex-role reversal, is a mating system characterized by female competition for mates and male-biased parental care. As females compete to form sequential or simultaneous pair bonds with multiple males throughout the breeding season, sexual selection is stronger in females than in males. This unique mating system is found in only 1-2% of bird species (Emlen and Oring 1977; Fritzche et al. 2021). The females of these species are often larger and more ornamented than males (Jenni and Collier 1972; Blizard and Pruett-Jones 2017; Emlen and Oring 1977). Socially polyandrous species provide an excellent test of whether the proximate mechanisms of territorial aggression and parental care are conserved, regardless of the sex performing such behaviors.

The hormonal basis of competitive behaviors is well-studied in males, and particularly the role of testosterone in shaping male competition for territories and mates (Wingfield et al. 1990). Consequently, testosterone has been a mechanism of interest for female competition in socially polyandrous species. Some studies have found positive correlations between testosterone

and competitive traits, for instance in the wing spurs of female northern jacanas (Lipshutz and Rosvall 2020a) and the throat patch of the barred buttonquail (Muck and Goymann 2011). However, sex differences in the testosterone levels of socially polyandrous birds do not explain competition for mates among females, as they are not higher in females compared with males (Fivizzani et al. 1986; Fivizzani and Oring 1986; Rissman and Wingfield 1984; Lipshutz and Rosvall 2020a). For sex-role reversed species, the degree of sexual dimorphism in testosterone shifts with breeding stage (Lipshutz and Rosvall 2020b). Male parental care is reflected by a decrease in plasma steroid hormone levels between breeding stages of courtship and parental care (Lipshutz and Rosvall 2020b; Rissman and Wingfield 1984; Buntin et al. 1998). Additionally, levels of prolactin, a regulator of parental care and investment, are higher in males than females (Fivizzani and Oring 1986; Oring et al. 1986; Buntin et al. 1998), departing from trends in species with stereotypical sex roles.

Gene expression may also modulate female aggression and male parental care. Candidate gene approaches in socially polyandrous species have found that expression of androgen receptor is higher in females than males in several brain regions (Voigt 2016; Voigt and Goymann 2007). Tissue-specific sensitivity to hormones in the brain is therefore a potential mechanism through which sex-role reversed behaviors are regulated. A comparative microarray study found shared patterns of gene expression in whole brains of dominant males and females in two cichlid species, indicating that a conserved set of genes regulates aggressive phenotypes (Schumer et al. 2011). However, transcriptome-wide evidence for sexually dimorphic gene expression in the brains of polyandrous fish is mixed (Beal et al. 2018; Pappert et al. 2023). Thus far, no studies have sequenced whole transcriptomes in specific regions of the social behavior network for both males and females of a socially polyandrous species across breeding stages.

Scope

Here we investigate whether sexual dimorphism in competition and parental care is reflected in the neurogenomic profiles of the northern jacana (Jacana spinosa), a species in which females compete for mates and males provide uniparental care for offspring. Previous work in northern jacanas found that levels of testosterone in circulation were similar between females and parenting males and higher in courting males, reflecting patterns from species with conventional sex roles (Lipshutz and Rosvall 2020b). Among-individual variation in territorial aggression was also unrelated to testosterone (Lipshutz and Rosvall 2020a). Furthermore, the mechanisms underlying the transition from competition to parental care are currently unknown in socially polyandrous species, as past work examines one or another breeding stage (Voigt and Goymann 2007; Schumer et al. 2011; Voigt 2016). To evaluate conservation vs. divergence in the mechanisms of aggression and parental care, we compare sex- and stage-specific patterns of 1) differential gene expression and 2) coexpressed genes and pathways in two brain regions of the social behavior network. Due to the behavioral shift in males across the breeding season, we hypothesize that females will have more similar neurogenomic profiles to courting males than to parenting males, indicating that sexual dimorphism in the transcriptome can help explain sex-specific divergence in behaviors across breeding stages.

CHAPTER TWO

METHODS

Study population

We conducted field work in La Barqueta, Chiriqui, Panama (8.207N, 82.579W) from 4 June to 9 July 2018. We observed individuals for several hours to confirm their territorial, paired, and reproductive status. We mapped territories relative to geographic landmarks along water canals, small ponds, and irrigated fields. In jacanas, female territories encompass the territories of multiple male mates (Betts and Jenni 1991). Therefore, we avoided sampling adjacent male territories for which we could not distinguish the territory holders. We observed whether females were paired (n = 12), whether males were courting based on copulatory behavior (n = 5), or incubating based on their attending to a nest with eggs (n = 7). We later confirmed reproductive status upon collection (see below).

Measuring competitive traits

We measured aggression using a short (5-minute) simulated territorial intrusion (STI), as in Lipshutz (2017) and Lipshutz and Rosvall (2020b). Briefly, we made rotating taxidermy mounts from females in an aggressive posture and played conspecific calls from a Bluetooth speaker. We placed the mount and speaker in the center of the focal male and/or female's territory. We then observed behavior of the territorial female and/or male over the 5-minute trial, narrating into a voice recorder. Aggressive behaviors included average distance from the mount as well as number of wingspreads, hoverflights, threats, pecks, flyovers, and vocalizations. All STIs were conducted between 9am and 6pm.

Measuring morphological traits

We measured several morphological traits involved in mating competition in jacanas: wing spur length, gonad mass, body mass, facial shield length, and tarsus length. Wing spurs, body mass, facial shields, and tarsi are larger in females than in males (Lipshutz 2017). In a congener, the Wattled jacana (Jacana jacana), both female and male territory holders had larger wing spurs, body mass, facial shields, and tarsi than floaters, indicating the role of these traits in mediating competition for territories (Emlen and Wrege 2004). Wing spurs are sharp keratinous sheaths over metacarpal bone growth that jacanas use as weapons and signals during aggressive posturing. A previous study in northern jacanas, from the same individuals in the present study, found that wing spur length is positively correlated with testosterone in females, and that gonad mass is larger for parenting males compared to courting males (Lipshutz and Rosvall 2020).

Sample collection

Scientific collection in Panama was conducted with permission from landowners and prior approval of MiAmbiente, Panama's environmental authority (permit number: SE/A-17-18), and the Institutional Animal Care and Use Committee of the Smithsonian Tropical Research Institute (IACUC permit: 2018-0116-2021) and the University of Tennessee (IACUC permit: 2573).

Our goal was to quantify gene activity in the brain in a naturalistic state. All birds were collected using an air rifle, followed by anesthetic overdose of isoflurane and decapitation. Some individuals (n = 11) were collected quickly after the short aggression assay (average time from assay start to euthanasia = 9 min 20s ±1 min 35s). Because transcription of socially responsive genes after introduction of a social stimulus takes at least 30 minutes to be detected in other systems (Maney and Goodson 2011, Bukhari et al. 2019), we assume that measures of gene

expression represent constitutive-like levels. For individuals that could not be collected immediately, we returned to territories 5-8 days later for collection (n = 12).

We collected trunk blood for testosterone analysis, published in (Lipshutz and Rosvall 2020b). Using RNAse-free tools, we dissected whole brains from the skull, flash froze in powdered dry ice, and stored in dry ice and then a −80°C freezer until further processing (average time from euthanasia to brain tissue collection = 7 min 10s ± 20s). We also collected and flash froze gonadal tissue. All individuals were in breeding condition, as evidenced by yolky, developed follicles for females, enlarged testes for courting males, and brood patches for incubating males.

Brain microdissection and RNA isolation

To isolate RNA from focal brain regions, we used a CM1850 cryostat (Leica Biosystems, Buffalo Grove, IL, USA), and sectioned along the coronal plane (as defined by Puelles 2007). Because this is the first brain study for any species in the family Jacanidae, we first created a series of 40uM, undissected brain sections stained with cresyl violet and scanned using a Motic EasyScan (Motic, Richmond, British Colombia, CA) to aid in identifying anatomical landmarks and focal regions from brain atlases (see below). For the remaining RNA samples, we sectioned brain tissue at 200 uM. Cryostat temperature ranged from 14-16°C. We thaw-mounted sections for <10 sec onto glass slides (Fisher superfrost 12-544-2, uncharged) and then immediately refroze them at -43°C using the quick-freeze shelf within the cryostat chamber. We stored slides for up to a week at -80°C before microdissection.

Our microdissection methods followed Horton et al. (2020). We set up a Styrofoam cooler with a bed of dry ice and placed a chilled marble slab and coring mat on top. We used an LED magnifying lamp to locate brain regions using anatomical landmarks (e.g. ventricles, fiber

tracts). We collected neural tissue with rapid-core reusable biopsy punches (World Precision Instruments, Sarasota, FL, USA). Our punch tool size varied based on focal region (see below). We gave each tissue core a confidence score from 1-5, and only included cores with a score of 3 or higher. We expelled tissue cores into microcentrifuge tubes on dry ice and kept them frozen until all samples were dissected for that subject. We then added TRIzol (Invitrogen, Carlsbad, CA, USA) directly to the tubes, briefly vortexed, and stored at -80°C until RNA extraction (up to 5 months).

We focus on two conserved regions of the brain associated with agonistic behavior and parental care in other taxa: the nucleus taeniae (TnA), the avian homologue of the medial amygdala (Goodson 2005; Voigt 2016), and the preoptic area of the hypothalamus (POA). For TnA, we used neuroanatomical landmarks following the chick atlas (AHi subnuclei and ATn, Puelles 2007) and the ruff atlas (Loveland unpublished). Our POA samples contain anterior and medial preoptic area (AMPO and MPO according to Puelles 2007) and likely some or all of the anterior hypothalamus (Kuenzel and Masson 1988) and the periventricular hypothalamic nucleus (PHN, Kuenzel and Golden 2006). For POA, we used a 1.2 mm punch. For TnA, we used a 0.75 mm punch on either hemisphere, followed by 0.5 mm for the last two anterior/posterior sections (Figure 1).
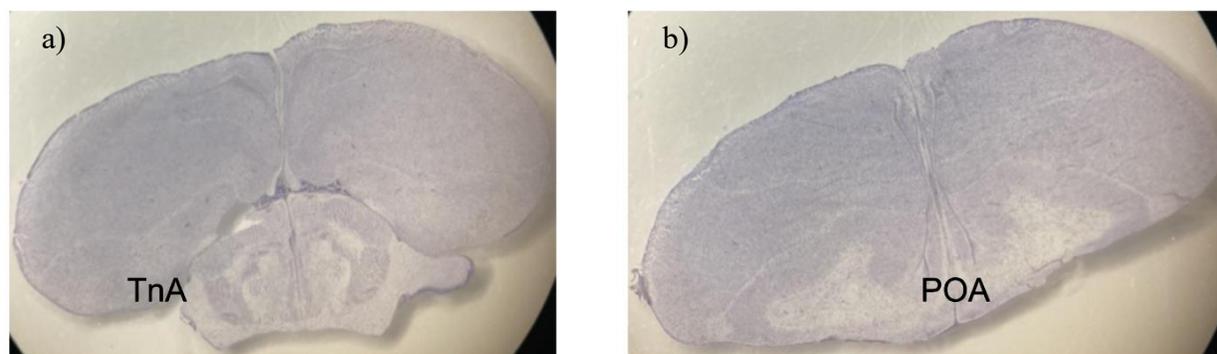
Figure 1. Brain regions. Stained cresyl violet sections of a jacana brain with orange squares around the a) nucleus taeniae (TnA) and b) preoptic area of the hypothalamus (POA).

Whole brains express a wide range of genes in the genome, compared to specific brain regions. To assess the specificity of expression in the POA and TNA relative to other tissues, we combined whole brains from one representative individual per group (female, incubating male, copulating male).

We extracted total RNA from tissues following the phenol-chloroform-based TRIzol manufacturer protocol, adjusted for nano-scale tissue volumes, and with the addition of phase-lock gel tubes (QuantaBio) and GlycoBlue (Invitrogen, Carlsbad, CA, USA) to facilitate pellet precipitation. We resuspended RNA in nuclease-free, Ultrapure water (Invitrogen). We analyzed RNA quantity and quality using a TapeStation 4150 and high-sensitivity RNA screentape (Agilent Technologies). Mean sample RIN for POA was 8.85 ± 0.18 and for TnA was 8.74 ± 0.12.

Genome sequencing and annotation

For one female northern jacana (NOF2), we conducted a high molecular weight gDNA extraction from flash frozen spleen tissue for a reference genome. The library was prepared for PacBio HiFI by Maryland Genomics, which generated two PacBio Sequel II 8M SMRT Cell

runs. We assembled the de novo genome using Hifiasm, a haplotype-resolved assembler for PacBio HiFi reads (Cheng et al. 2021).

We then lifted over gene annotations from the GalGal7 genome assembly (GRC7b, GCA_016699485.1) available on ensembl.org. We used Liftoff v1.6.3 (Schumate and Salzberg 2021) to align all GalGal7 genes to the jacana scaffolds. Liftoff identifies high confidence alignments for all exons using minimap (Li 2018), while taking into account transcript and gene structure. This method does not detect novel genes or novel splice events or exons but is suitable for RNAseq mapping. We successfully lifted over 14,588 out of 17,007 genes from the chicken genome.

RNAseq and mapping

We submitted total RNA to Indiana University's Center for Genomics and Bioinformatics for sequencing. We constructed complementary DNA libraries using a TruSeq Stranded mRNA HT Sample Prep Kit following the manufacturer's protocol. Sequencing was performed using an Illumina NextSeq 500 platform with a 75-cycle sequencing module generating 38-bp paired-end reads. We generated an average of ~28 million reads for the barcoded RNA samples (Table 1). We demultiplexed sequencing reads with bcl2fastq (v2.20.0.422). We cleaned reads with Trimmomatic v0.39 (Bolger et al. 2014) and mapped reads to the jacana genome using Hisat2 v2.2.1 (Kim et al. 2019). We only included reads mapped in proper pairs, sorted and indexed using Samtools v1.19.2 (Li et al. 2009). For TnA samples, ~21 million read pairs per sample were mapped, which account for ~82% of the total trimmed read pairs (Table 1). For POA samples, ~22 million read pairs were mapped, which account for ~85% of the total trimmed read pairs (Table 1). Reads were quantified into genes using featureCounts from the SUBREAD package v2.0.0 (Liao et al. 2019).

Table 1. Read count table. Paired read counts for each sample initially generated (red), paired reads trimmed with Trimmomatic (orange), paired reads aligned to the reference genome with Hisat2 (yellow), and alignments successfully assigned to genes using featureCounts (green).

Sample	NOF1_POA	NOF10_POA	NOF11_POA	NOF12_POA	NOF2_POA	NOF3_POA	NOF4_POA	NOF5_POA	NOF6_POA	NOF7_POA	NOF8_POA
Paired reads	31,464,747	22,989,447	30,057,786	30,336,027	29,775,473	22,223,037	23,397,099	26,740,913	29,852,122	30,683,730	31,513,809
% Reads paired	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Trimmed paired reads	29,405,011	21,418,129	28,104,490	28,261,037	27,773,585	20,668,544	21,883,902	24,924,540	27,952,259	28,297,608	29,098,979
% Paired reads surviving	93.45%	93.17%	93.50%	93.16%	93.28%	93.01%	93.53%	93.21%	93.64%	92.22%	92.34%
Aligned paired reads	25,130,123	18,149,120	23,956,316	24,035,778	23,571,268	17,384,277	18,806,026	21,246,547	23,770,194	23,988,063	24,584,136
% Aligned paired reads	85.46%	84.74%	85.24%	85.05%	84.87%	84.11%	85.94%	85.24%	85.04%	84.77%	84.48%
Assigned alignments	15,790,851	11,516,044	14,988,222	15,019,932	14,573,536	11,259,878	11,941,839	13,236,952	15,056,552	15,329,530	15,554,942
% Assigned alignments	49.10%	48.60%	48.80%	48.60%	47.40%	49.30%	49.50%	47.80%	49.00%	49.00%	47.90%

Sample	NOM1_POA	NOM10_POA	NOM11_POA	NOM12_POA	NOM2_POA	NOM3_POA	NOM4_POA	NOM5_POA	NOM6_POA	NOM7_POA	NOM8_POA
Paired reads	27,407,319	26,352,100	30,895,038	23,561,689	29,213,667	29,273,515	21,503,845	30,173,053	30,779,635	22,922,674	23,754,066
% Reads paired	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Trimmed paired reads	25,659,766	24,780,106	28,954,355	21,680,210	27,186,290	27,328,839	19,614,140	27,955,838	28,654,815	21,288,631	22,032,727
% Paired reads surviving	93.62%	94.03%	93.72%	92.01%	93.06%	93.36%	91.21%	92.65%	93.10%	92.87%	92.75%
Aligned paired reads	21,801,591	21,326,498	24,510,563	18,354,986	23,159,169	23,191,164	16,592,903	23,658,634	24,481,852	18,272,892	18,807,697
% Aligned paired reads	84.96%	86.06%	84.65%	84.66%	85.19%	84.86%	84.60%	84.63%	85.44%	85.83%	85.36%
Assigned alignments	13,949,420	13,258,111	15,444,592	11,730,159	14,787,352	14,955,133	10,598,459	14,584,793	15,241,948	11,823,793	12,185,473
% Assigned alignments	49.60%	49.10%	47.30%	48.80%	48.10%	50.00%	48.70%	47.10%	48.50%	50.60%	50.40%

Sample	NOF1_TnA	NOF10_TnA	NOF11_TnA	NOF12_TnA	NOF2_TnA	NOF3_TnA	NOF4_TnA	NOF5_TnA	NOF6_TnA	NOF7_TnA	NOF8_TnA
Paired reads	22,406,842	27,209,752	30,503,300	31,885,088	26,802,745	29,943,890	27,561,284	27,089,353	30,957,716	26,586,540	30,296,760
% Reads paired	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Trimmed paired reads	20,984,842	25,099,708	28,666,545	29,875,405	25,125,808	25,736,150	25,272,347	24,430,111	28,871,785	24,649,457	28,433,186
% Paired reads surviving	93.65%	92.25%	93.98%	93.70%	93.74%	85.95%	91.70%	90.18%	93.26%	92.71%	93.85%
Aligned paired reads	17,380,510	20,709,160	23,759,517	24,403,811	21,177,626	20,478,383	20,906,494	20,275,169	23,870,667	20,407,030	23,641,270
% Aligned paired reads	82.82%	82.51%	82.88%	81.69%	84.29%	79.57%	82.72%	82.99%	82.68%	82.79%	83.15%
Assigned alignments	11,343,652	13,469,163	15,113,209	15,999,423	13,565,601	13,904,771	13,686,000	13,174,941	14,945,361	13,362,387	15,457,598
% Assigned alignments	49.00%	48.80%	47.70%	48.90%	49.50%	48.50%	48.30%	48.60%	47.10%	49.60%	50.10%

Sample	NOM1_TnA	NOM10_TnA	NOM11_TnA	NOM12_TnA	NOM2_TnA	NOM3_TnA	NOM4_TnA	NOM5_TnA	NOM6_TnA	NOM7_TnA	NOM8_TnA
Paired reads	22,204,191	23,515,613	23,768,980	31,292,145	30,856,995	28,098,173	23,266,950	30,177,538	31,181,180	27,891,404	30,095,161
% Reads paired	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Trimmed paired reads	20,454,923	21,436,263	21,248,824	28,924,573	28,406,699	26,271,286	21,471,529	28,476,085	29,069,169	25,584,075	27,937,884
% Paired reads surviving	92.12%	91.16%	89.40%	92.43%	92.06%	93.50%	92.28%	94.36%	93.23%	91.73%	92.83%
Aligned paired reads	16,863,216	17,788,590	17,574,087	23,935,903	23,731,641	21,736,285	17,652,133	23,361,435	24,347,120	21,101,426	23,265,324
% Aligned paired reads	82.44%	82.98%	82.71%	82.75%	83.54%	82.74%	82.21%	82.04%	83.76%	82.48%	83.28%
Assigned alignments	11,057,585	11,393,999	11,613,221	15,465,148	15,308,553	14,185,820	11,514,631	14,895,434	15,668,169	13,758,714	15,179,660
% Assigned alignments	49.60%	46.80%	49.60%	48.30%	49.10%	49.90%	48.60%	47.30%	49.40%	47.90%	49.50%

Sample	NOF12_Blood	NOF12_Brain	NOF12_Gonad	NOM2_Blood	NOM2_Brain	NOM2_Gonad	NOM3_Blood	NOM3_Brain	NOM3_Gonad
Paired reads	28,956,897	30,501,062	31,837,046	27,172,580	30,538,409	26,560,242	26,098,034	28,539,050	29,303,535
% Reads paired	100%	100%	100%	100%	100%	100%	100%	100%	100%
Trimmed paired reads	27,266,634	28,567,795	29,347,444	25,349,725	28,081,290	24,033,478	23,690,429	26,713,306	27,442,330
% Paired reads surviving	94.16%	93.66%	92.18%	93.29%	91.95%	90.49%	90.77%	93.60%	93.65%
Aligned paired reads	23,965,795	23,526,171	25,490,185	22,110,150	23,237,453	20,096,973	20,589,808	22,224,258	23,353,581
% Aligned paired reads	87.89%	82.35%	86.86%	87.22%	82.75%	83.62%	86.91%	83.20%	85.10%
Assigned alignments	15,926,770	14,935,552	17,746,545	15,100,836	14,390,711	12,110,939	13,577,295	14,194,633	14,183,462
% Assigned alignments	50.90%	48.00%	54.90%	51.70%	46.50%	39.50%	48.80%	49.00%	40.90%

Differential expression analyses

We used DESeq2 v 1.38.3 (Love et al. 2014) to normalize read counts, which we visualized with a PCA (Figure 3). For each brain region, we compared logfoldchange patterns of differential gene expression between three dyads: females and courting males, females and parenting males, and courting and parenting males. Briefly, the data are fitted to a negative binomial generalized linear model with a fixed effect for sex and breeding stage for each transcript and filtered based on a per-transcript Wald test statistic to identify valid significantly differentially expressed transcripts. The p-values were corrected for multiple-testing (Benjamini–

Hochberg; adjusted p ≤ .05). Scripts and input files for DESeq2 are available in the Github

Repository: https://github.com/tpatton4/jacananeurogenomics.

For one individual from each group (female, courting male, parenting male), we also

compare brain-specific expression to two other available tissues - blood and gonad, to assess the

percentage of genes expressed in each tissue transcriptome.

Weighted Gene Co-expression Network Analyses

We conducted weighted gene co-expression network analyses (WGCNA, Langfelder and

Horvath 2008) for each brain region. Sample clustering to detect outliers identified one outlier in

the TnA analyses, a female, and one outlier in the POA analysis, a courting male, which we then

excluded from each dataset. We normalized and filtered out genes with <10 counts in 90% of

samples and used the 75% most variable genes (8455 in TnA, 9120 in POA). We generated a

signed hybrid network using a soft threshold power (β) = 12 for TnA and (β) = 14 for POA

(Figure 2). We used a biweight midcorrelation function and constructed modules with a

minimum size of 30. We then merged modules with Dynamic Tree Cut using a threshold size of

0.25, as these genes are highly co-expressed. We correlated expression levels for each module's

first eigengene with traits of interest: sex, male breeding stage, log testosterone, distance to

decoy, vocalizations, testes mass, ovary mass, body mass, facial shield width, tarsus length, and

wing spur length. We also included Julian date, time from STI to collection and time from

collection to brain dissection as potential confounding variables. For each module significantly

associated with traits of interest, we examined the top 10 genes with the highest module

membership (hereafter 'hub genes').

Scripts and input files for WGCNA are available in the Github Repository:
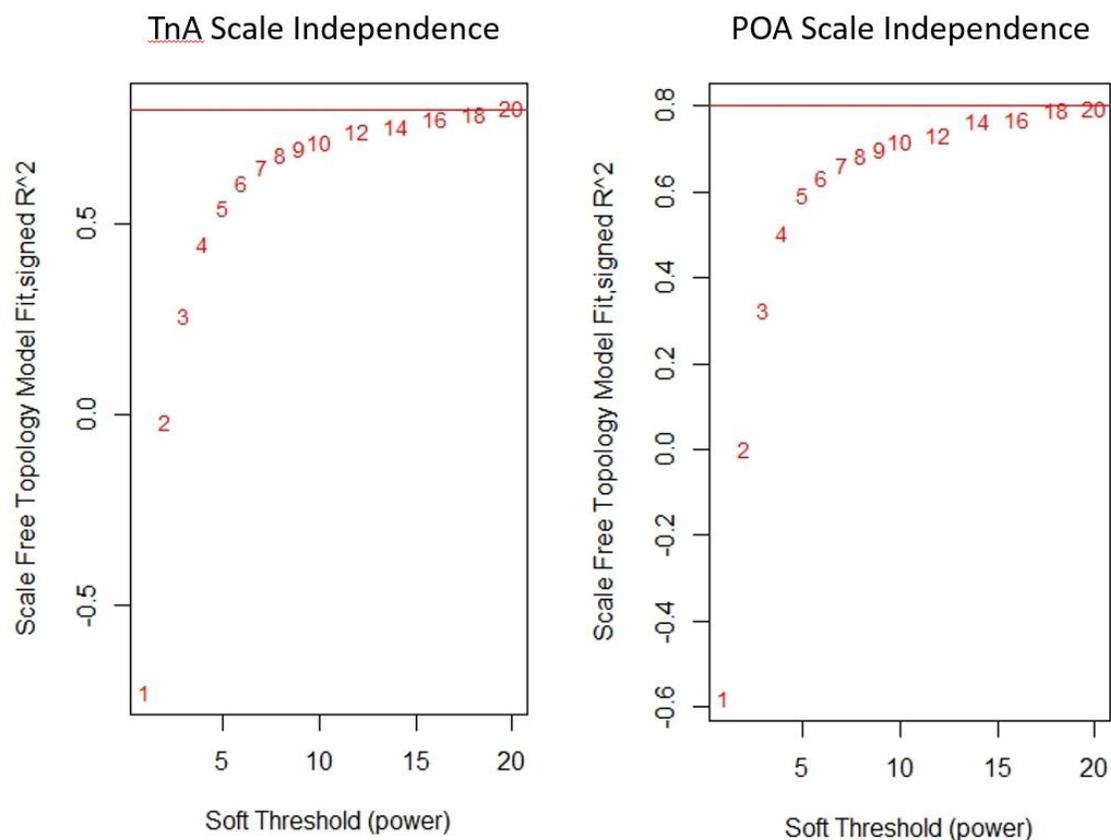
https://github.com/tpatton4/jacananeurogenomics.

Figure 2. Soft threshold power. Scale free topology fit for soft threshold power values in the a) TnA and b) POA.

Gene ontology

For each set of differentially expressed genes as well as genes from WGCNA modules associated with traits of interest (GS value $|>|$ 0.6) we inferred gene ontology (GO) of biological processes using PantherGO (Mi et al. 2019; Thomas et al. 2022). We evaluated gene enrichment using Homo sapiens as the reference because its ontologies are orthologous to, but more complete than avian references. To account for genes not present in our de-novo genome assembly and annotation, we limited our reference list to the 14,588 genes previously identified. We considered GO terms with an FDR < 0.05 as significantly enriched. We report the most specific subclass (first descendent) of each significant GO term

CAHPTER THREE

RESULTS

Differential gene expression

Between courting and parenting males, 19 genes were differentially expressed in the preoptic area of the hypothalamus (POA), and no genes were differentially expressed in the nucleus taeniae (TnA). Those DEGs in the POA were not significantly enriched for any biological processes. However, many of these DEGs are associated with the insulin-like growth factor (IGF) pathway, including insulin like growth factor binding protein 3 (IGFBP3), pappalysin 2 (PAPPA2), and ATP binding cassette subfamily C member 8 (ABCC8). Unexpectedly, genes encoding prolactin releasing hormone (PRLH) had higher expression in courting males than in parenting males in the POA, though expression was relatively low overall.

For both brain regions, comparison of DEGs between each dyad revealed that many more genes were differentially expressed between females and males than between male courting and parenting stages. Many of these DEGs are shared between the two dyads in each brain region (Figure 6). In the POA, 563 genes were differentially expressed between females and parenting males. These genes were not enriched for any biological processes. Of the top differentially expressed genes, many were involved in the hypothalamic pituitary gonadal (HPG) axis, including 17-beta-hydroxysteroid dehydrogenase 7 (HSD17B7), a gene that encodes an enzyme involved in the biosynthesis of steroid hormones including estradiol (Saloniemi et al. 2012), and 5'-AMP-activated protein kinase catalytic subunit alpha-1 (PRKAA1), a gene involved in the gonadotropin-releasing hormone pathway. Between females and courting males, 659 genes were

differentially expressed, and these DEGs were not enriched for any biological processes. Among the top differentially expressed genes between females and courting males, several were related to hormonal and neuropeptide signaling, including prolactin receptor (PRLR) and prostaglandin reductase 1 (PTGR1), which were higher in males than in females, and galanin receptor 1 (GALR1), estrogen receptor (ESR1), and adipolin (C1QTNF12), which were lower in males.

In the TnA, 520 genes were differentially expressed between females and parenting males. These genes were not enriched for any biological processes. Hydroxysteroid 17-beta hydroxysteroid dehydrogenase 4 (HSD17B4) was higher in parenting males than in females. Between females and courting males, 496 genes were differentially expressed. These genes were enriched for one biological process: cellular metabolic process. Among the top differentially expressed genes between females and males of both stages, several were related to cell cycle regulation and DNA repair and replication, including NSA2 ribosome biogenesis factor (NSA2) and valosin containing protein (VCP).
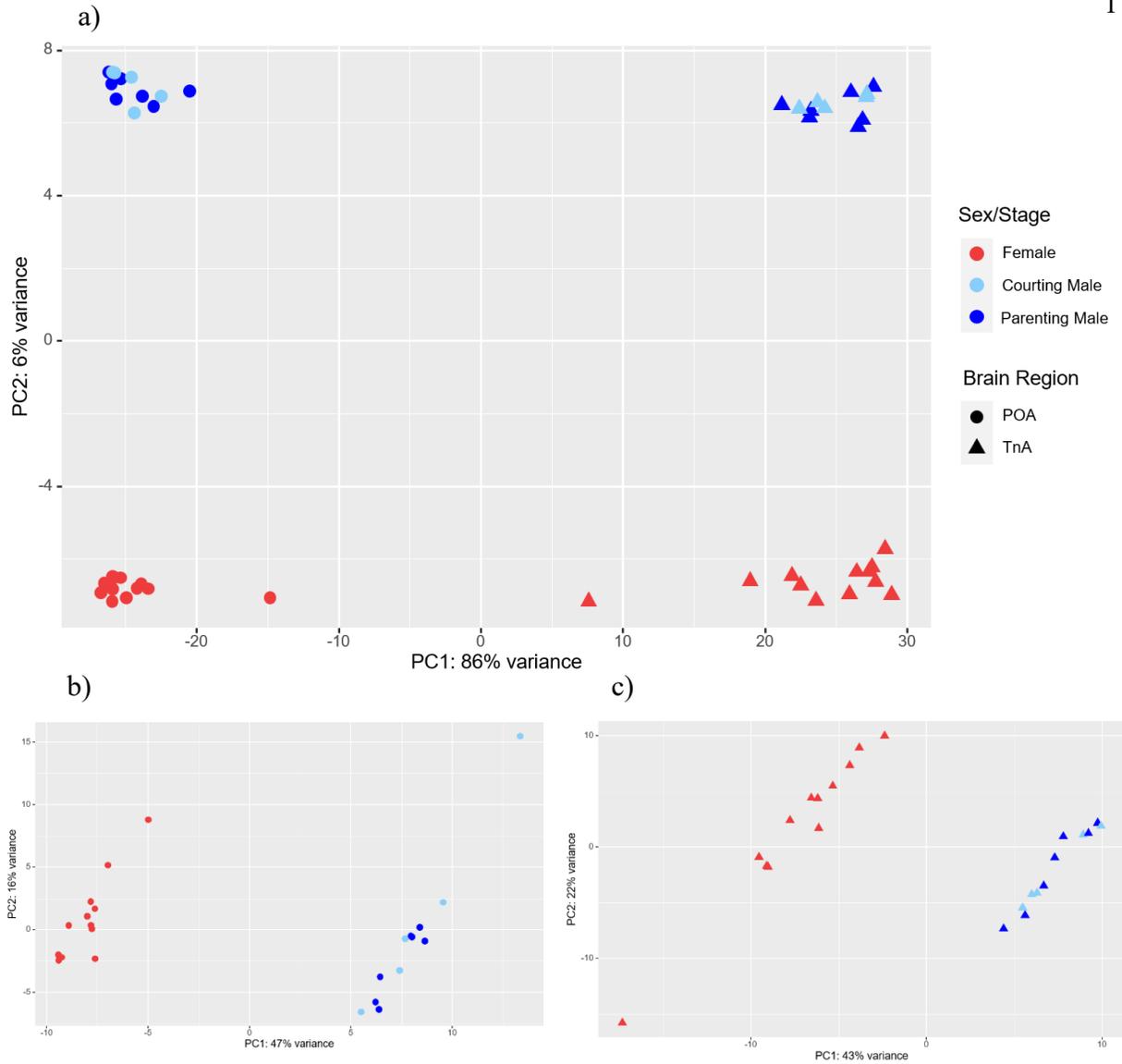
Figure 3. Principal component analysis (PCA) plots for differential expression data across a) all samples, b) the POA (circles), and c) the TnA (triangles). Each plot contains female (red), courting male (light blue), and parenting male (dark blue) individuals.
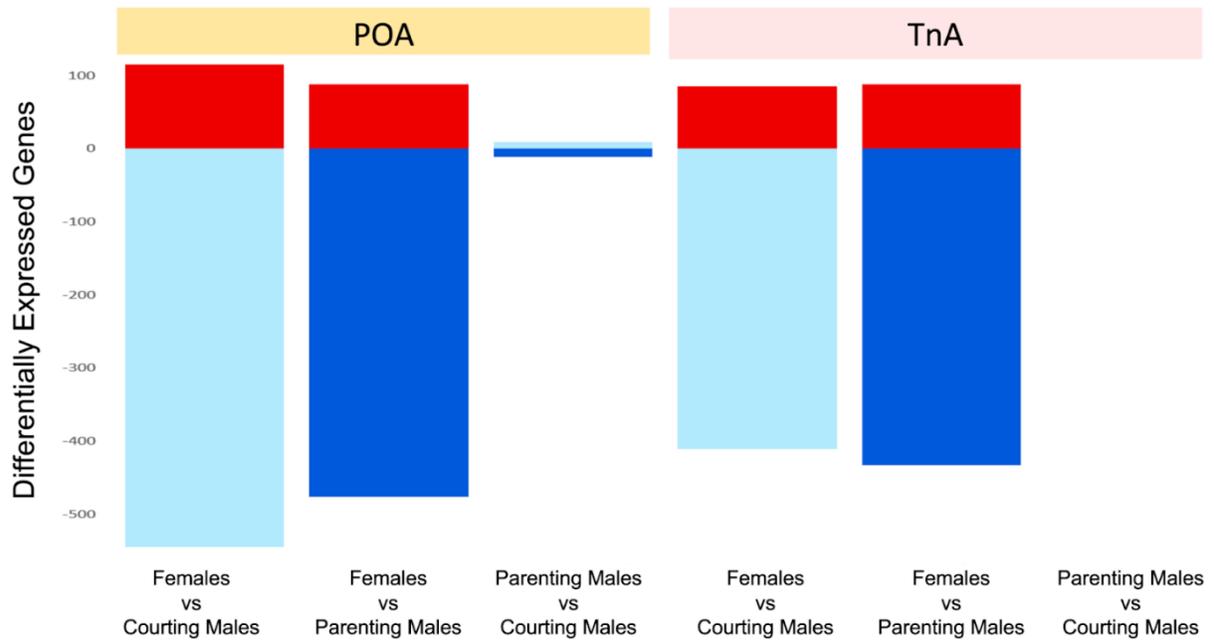
Figure 4. The number of significantly differentially expressed genes (adjusted p<0.05) for each sex-stage comparison in the POA and TnA. Genes more highly expressed in females are shown in red, genes more highly expressed in courting males are shown in light blue, and genes more highly expressed in parenting males are shown in dark blue.
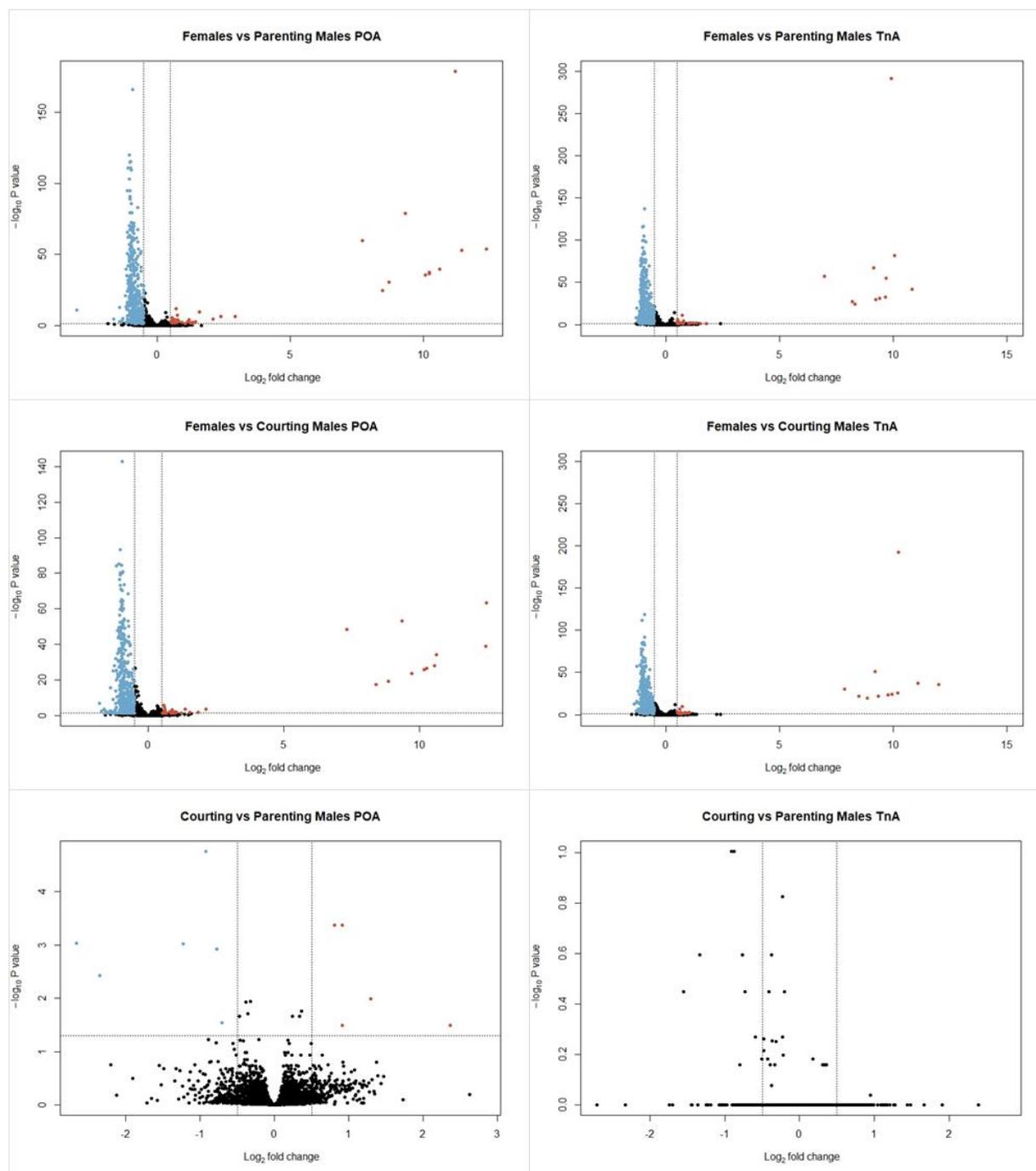
Figure 5. Volcano plots for each sex-stage comparison in each brain region. The -log10 Pvalue is plotted on the y-axis, with logfoldchange across the x axis. Each dot represents a gene. Black dots do not pass the Pvalue threshold (adjusted p<0.05), the logfoldchange threshold (-0.5>logfoldchange<0.5), or both. Colored dots pass these thresholds, and the colors represent which sex-stage group the gene was more highly a positive (red) or negative (blue) logfoldchange
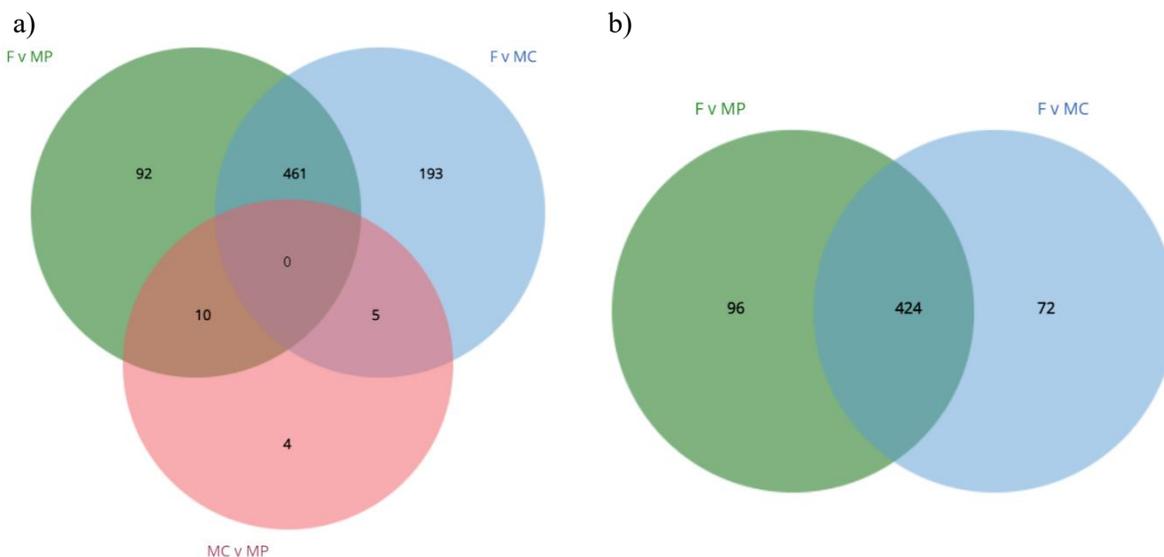
Figure 6. Venn Diagram of differentially expressed genes in the a) preoptic area of the hypothalamus (POA) and b) nucleus taeniae (TnA) between females vs courting males, females vs parenting males, and courting males vs parenting males.

Weighted Gene Coexpression Network Analysis

Weighted gene coexpression network analysis (WGCNA) created eight modules of coexpressed genes for each brain region (Figure 7). Multiple gene networks in both the POA and TnA were associated with traits of interest.

For the POA, four modules correlated with competitive behaviors and morphological traits, including tan, saddlebrown, brown, and cyan (Figure 7a).

The tan module correlated significantly with testosterone (r=-0.42, p=0.05). Hub genes with the top 5 module membership included WBP1, MSL1, and MAP7D1. The tan module was not significantly enriched for any GO terms.

The saddlebrown module correlated significantly with facial shield width (r=-0.49, p=0.02). Hub genes in the saddlebrown module included SERPINF1, OGN, COL3A1, CPXM1, and OSR1. The saddlebrown module was significantly enriched for smooth muscle tissue development, collagen fibril organization, endothelial cell migration, chondrocyte differentiation,

regulation of mononuclear cell migration, cell chemotaxis, regulation of vasculature development, blood vessel morphogenesis, anatomical structure formation involved in morphogenesis, animal organ morphogenesis, and negative regulation of multicellular organismal process.

The brown module correlated significantly with sex (r=0.88, p=3e-08) and many sex-related morphological traits including mass (r=-0.86, p=1e-07), facial shield width (r=-0.54, p=0.007), tarsus length (r=-0.83, p=8e-07), and wing spur length (r=-0.72, p=1e-04). Hub genes in the brown module included PIK3C3, HSD17B4, HOOK3, PHAX, and WDR70.

The cyan module correlated significantly with competitive traits, including distance to respond to a decoy competitor, a metric of aggression, (r=-0.41, p=0.05) and wing spur length (r=-0.46, p=0.03). Hub genes in the cyan module include RRBP1, GAB1, and BCAS1. The cyan module was significantly enriched for central nervous system myelination, cell-cell adhesion, cell migration, and nucleic acid metabolic process.

For the TnA, three modules correlated with competitive behaviors and morphological traits, including yellow, skyblue, and skyblue3. (Figure 7b).

The yellow module correlated significantly with sex (r=0.89, p=1e-08) and many sexually dimorphic morphological traits including mass (r=-0.86, p=2e-07), facial shield width (r=-0.44, p=0.04), tarsus length (r=-0.77, p=1e-05), and spur length (r=-0.7, p=2e-04). Hub genes in the yellow module included UBE2R2, GNAQ, ZCCHC6, and TARS. The yellow module was not significantly enriched for any GO terms, but did have significant overlap with the brown module in the POA (Fisher's exact test, p=2.2e-16).

The skyblue module correlated significantly with distance to respond to a decoy competitor (r=-0.58, p=0.003), vocalizations (r=0.45, p=0.03), and testis mass (r=-0.44, p=0.04).

Hub genes in the skyblue module included TEKT1, RSPH4A, RGS22, and CCDC78. The skyblue module was significantly enriched for inner dynein arm assembly, flagellated sperm motility, extracellular matrix organization, and germ cell development.

The skyblue3 module correlated significantly with sex ($r=-0.5$, $p=0.02$) and many sex-related morphological traits including ovary mass ($r=0.49$, $p=0.02$), mass ($r=0.54$, $p=0.007$), facial shield width ($r=0.45$, $p=0.03$), tarsus length ($r=0.47$, $p=0.02$), and spur length ($r=0.53$, $p=0.009$). Hub genes in the skyblue3 module include RFTN1, CBLB, TRPV2, MCTP2, and EPHA5.

Tissue specificity

For the three individuals for which we have expression data from the POA, TnA, whole brain, blood, and gonadal tissue, we compared expression in a presence/absence binary using UpSet plots (Figure 8). For all samples, most of the 14,588 genes were expressed in all tissues (female $n=11,716$, 80.8%; courting male $n=11,187$, 76.7%; parenting male $n=11,572$, 79.3%). The next largest set for all samples included every sample type except blood.

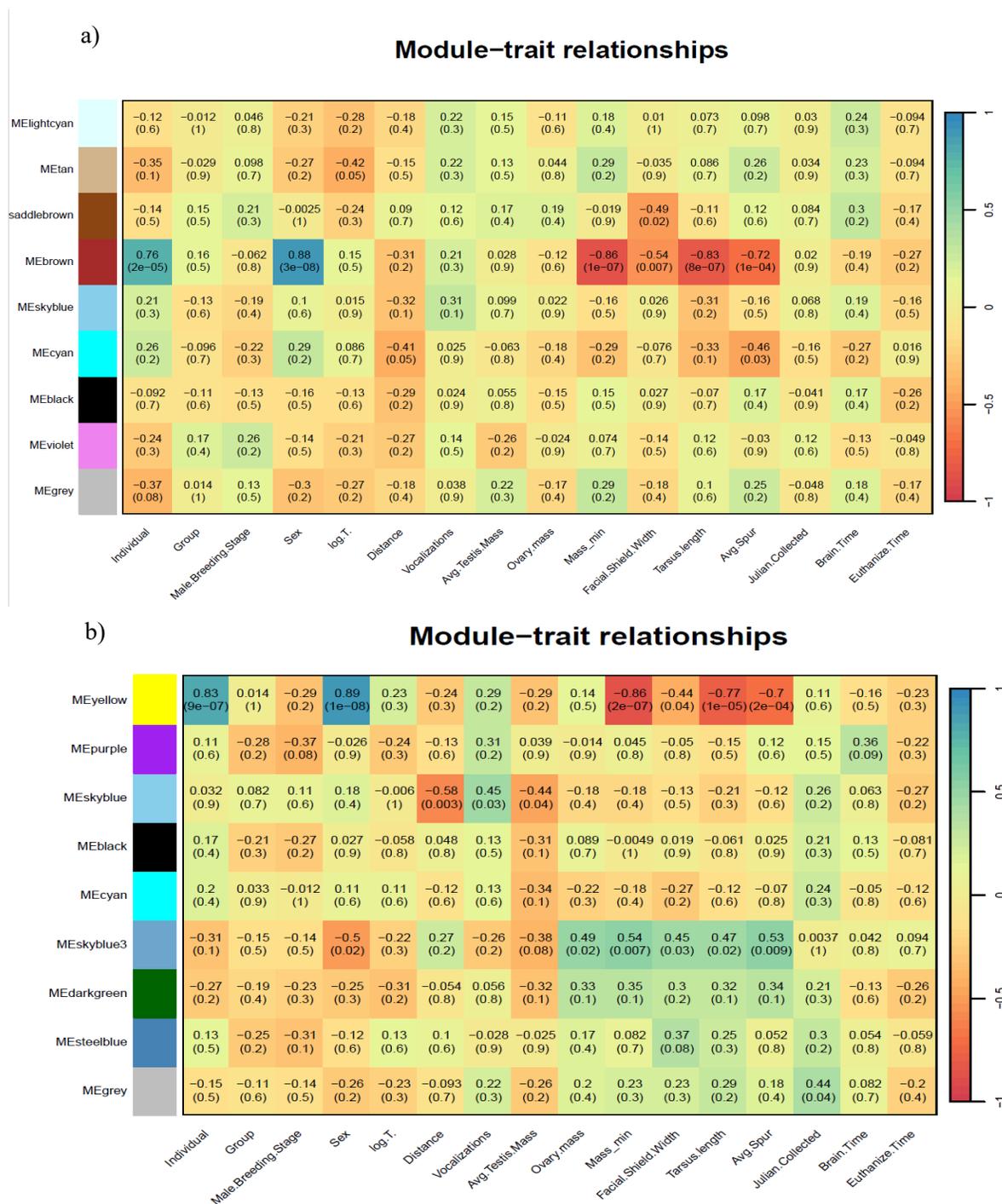Figure 7. WGCNA. Weighted gene coexpression network analyses heatmaps for a) the preoptic area of the hypothalamus and b) the nucleus taeniae. Modules on the y axis are assigned a random color. Competitive traits including aggression metrics and morphological measurements are on the x axis. Each module-trait has an r correlation coefficient and a p-value. The colors on the heatmap are determined by the r correlation coefficient.
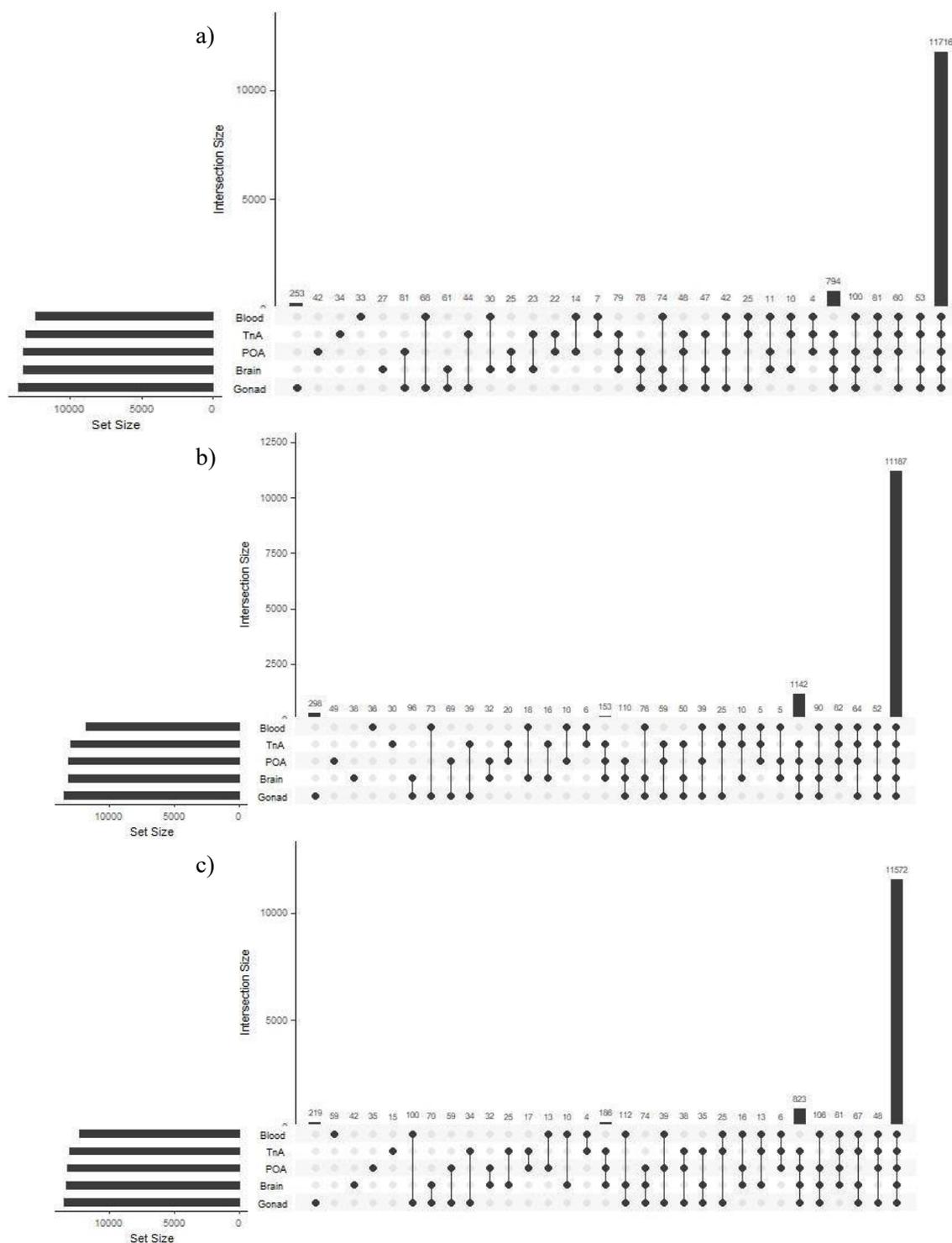
Figure 8. UpSet plot showing genes expressed in blood, TnA, whole brain, POA, and gonadal tissue and all intersections of these tissues in one individual of each sex-stage group: a) female, b) courting male, and c) parenting male. The intersection size on the y axis is the number of genes with a read count ≥ 1 in designated tissues or groups. For each row on the x axis, a black dot represents that the tissue is a part of that intersection.

CHAPTER FOUR

DISCUSSION

Here, we demonstrate that the neurogenomic profiles of northern jacanas, a sex-role reversed shorebird, are sexually dimorphic and reflect behavioral differences in parental care and competition between males and females, as well as between males in two breeding stages.

Gene expression reflects sex differences in behavior

The expression of hundreds of genes in both the preoptic area of the hypothalamus and the nucleus taeniae differ between female and male of northern jacanas, indicating sexually dimorphic neurogenomic profiles. These contrasts shed light on the gene regulatory mechanisms that may underlie sex-role reversal and associated sexual dimorphism in competition and parental care. Many of these genes are associated with mechanisms for competition and parental care, including hormone receptors. For instance, the expression of prolactin hormone receptor (PRLR) was higher in males compared to females, in both the POA and TnA. Prolactin is a hormone involved in mediation of parental care behaviors, stimulating the expression of parental investment (Angelier and Chastel 2009). However, in a species that exhibits bi-parental care, the rock dove, males had higher expression of PRLR than females in several tissues, including the hypothalamus (Farrar et al. 2022). Higher expression of PRLR may act as a primary mechanism for paternal care, as prolactin signaling has a demonstrated role in promoting parental behavior in both sexes (Smiley et al. 2022).

In another example, female jacanas had higher expression of androgen receptor (AR) in the POA compared with parenting male jacanas. Given that female jacanas have lower levels of

circulating testosterone than parenting males (Lipshutz and Rosvall 2020b), higher androgen sensitivity through receptor expression could be a potential mechanism promoting female competition. Our finding that AR is more highly expressed in the female POA, is supported by other studies that used candidate gene approaches for female aggression in sex-role reversed species black coucals and barred buttonquails (Voigt 2016; Voigt and Goymann 2007). The expression of these candidate genes reflects behavioral phenotypes more so than sex-specific trends among species with traditional mating systems, providing insight into conserved mechanisms for competition and parental care.

Breeding stage differences are reflected in candidate gene expression

We found relatively few differences in gene expression as males shift from breeding stages of courtship to parenting. No genes were significantly differentially expressed between male breeding stages in the TnA, and only 19 in the POA. Those DEGs in the POA include Prolactin Releasing Hormone (PRLH), Insulin Like Growth Factor Binding Protein 3 (IGFBP3), and Prodynorphin (PDYN). Interestingly, PRLH was more highly expressed in courting males than parenting males in the POA. PRLH encodes a prolactin-releasing peptide, which is one mechanism by which prolactin levels can increase (Spuch et al. 2007). This finding is somewhat surprising, as prolactin has been found to be higher in parenting males than females in sex-role reversed species (Fivizzani and Oring 1986; Oring et al. 1986; Buntin et al. 1998). Further work to explore these results in jacanas could include measuring prolactin from plasma, inspection of other genes in the prolactin biosynthesis pathway, and additional tissue analysis, for instance in the pituitary. IGFBP3 was more highly expressed in parenting males than in courting males. The insulin-like growth factor pathway contributes to growth and glucose homeostasis (Kim 2013), and insulin-like growth factor is essential for the regulation of body size in birds (Lodjak et al.

2017). As males shift between breeding stages, the differential expression of IGFBP3 indicates shifting energy demands associated with the trade-offs between courtship and parenting. Only a small number of differentially expressed genes reflect changes in male breeding stage in northern jacanas, in contrast to the shifting neurogenomic profiles of male stickleback during some stages of parental care (Bukhari et al. 2019).

One possible explanation for why there would be so few differentially expressed genes between male breeding stages could be that regulation of parental behavior occurs primarily through changes in circulating hormone levels, requiring few neurogenomic changes between breeding stages. In jacanas and other sex-role reversed birds, a decrease in steroid hormones occurs between stages of courtship and parental care (Lipshutz and Rosvall 2020b; Rissman and Wingfield 1984; Buntin et al. 1998), and studies of spotted sandpipers and wilson's phalaropes found that prolactin levels are higher in males than females (Fivizzani and Oring 1986; Oring et al. 1986; Buntin et al. 1998). Alternatively, neurogenomic differentiation in male northern jacanas may be more pronounced between periods of breeding and non-breeding, with the majority of neurogenomic changes occurring at the onset of the breeding season. Additional work in species with male uniparental care should include individuals at additional breeding stages to parse the function of gene expression on paternal behavior.

Competitive traits are correlated with gene networks

Competitive traits were associated with multiple gene networks in each region of the brain, indicating that subtle, coordinated changes in gene expression are reflective of competition in northern jacanas.

Wing spur length, a trait associated with territorial resident status in wattled jacanas, was correlated with multiple modules in each brain region. One such module with a negative

correlation with wing spur and positive correlation with aggression was enriched for central nervous system myelination, a biological process that can improve synaptic transmission (Hughes and Appel 2016). This perhaps hints at tradeoffs between neuronal energetics and the competitive behaviors associated with being a territory holder.

Gonad mass is associated with mating competition, and in male jacanas testes are larger during courtship than during parental care (Lipshutz and Rosvall 2020). Male jacanas compete to fertilize the eggs of female jacanas, and females increase their fitness by producing eggs for available males (Emlen et al. 1989). Testis and ovary mass correlated with several gene networks in both brain regions. One module associated with testis mass was enriched for flagellated sperm motility and germ cell development, suggesting that processes in the brain reflect some reproductive processes likely happening in the gonads.

Sex differences were apparent in two modules: the POA brown and TnA yellow networks. Though neither network was enriched for biological processes, these modules show us that coordinated changes in gene expression are associated with sexually dimorphic phenotypes. These gene networks overlapped significantly in the makeup of genes, suggesting a key set of genes that reflect sexual dimorphism in multiple regions of the brain. In both networks, HSD17B4 had high module membership. HSD17B4 encodes an enzyme involved in an oxidation pathway for fatty acids. Hydroxysteroid dehydrogenase 17b genes are involved in the biosynthesis and metabolism of sex steroid hormones (Kemilainen et al. 2016; Loveland et al 2022).

Most genes are expressed in all tissues

For three individuals, we compared the presence/absence of expression data for each gene in 5 tissues with an UpSet plot. Most genes (76.7-80.8%) were expressed in all tissues. The

next largest set included all tissues except blood. This is supported by other tissue-specific

analyses that utilize de novo transcriptome assembly (Bentz et al. 2019). Blood transcriptomics

offers the advantage of non-destructive sampling, but may not offer data as robust as those that

come from tissues. The presence/absence approach from few samples does not capture any other

information about gene count data, for example, dataset variability and low counts. These results

suggest that blood transcriptomics is a viable method for some studies. It is important to consider

the biological question before selecting a tissue for RNAseq to ensure meaningful data

collection.

Some mechanisms of behavior are conserved

Sex-role reversed species provide a unique opportunity to determine which mechanisms

of competition and parental care are conserved between sexes, and which are sex-specific. In

species with traditional sex roles, competition is often viewed as a "masculine" trait and parental

care is viewed as a "feminine" one. We acknowledge that this is an oversimplification that

overlooks behavioral diversity across the animal kingdom (Gowaty 2004, Ah-King and Ahnesjo

2013). However, sex-role reversed species allow us to explore an extreme case in which females

experience mating competition while they breed with multiple males, and males conduct all

parental care (Emlen and Wrege 2004). The expression of androgen receptor in female jacanas

indicates that the androgen pathway may be an important mechanism of competitive behavior,

similar to males in species with traditional sex roles. Likewise, prolactin may promote paternal

care in jacanas, inducing similar behaviors as in females of other species. Though gene

expression in northern jacanas is sexually dimorphic, differential gene expression does not

explain the shift from male breeding stages of courtship to parental care. Gene networks provide

an additional lens through which to view neurogenomic associations with behavior and

morphology. Gene networks were associated with competitive traits in jacanas, including male sperm competition and female mating competition. Our analysis of jacana neurogenomics brings us closer to understanding the molecular mechanisms underlying competition and parental care across sexes.

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VITA

Tessa Patton was born in St. Louis, Missouri and grew up in Knoxville, Tennessee. Before attending Loyola University Chicago, she earned her Bachelor of Science in Biological Sciences, Ecology and Evolutionary Biology with a Minor in Climate Change from The University of Tennessee, Knoxville. At UTK, Tessa was an undergraduate teaching assistant for Engineering Physics I and an undergraduate research assistant in a composite materials lab. She also joined the rowing team for two seasons and later started rock climbing.

Tessa began working with birds as an intern, then biotechnician, for the National Park Service, where she monitored piping plovers at Sleeping Bear Dunes National Lakeshore and Assateague Island National Seashore.

As a master's student, Tessa had the privilege of collaborating with amazing teams during two field seasons in Mono Lake, California where she studied spotted sandpipers. While at Loyola, Tessa received the Research Assistant Fellowship for both years of her schooling and a Grant In Aid of Research from the Society for Integrative and Comparative Biology.

Currently, Tessa is preparing to move to Duke University for her PhD. She is so excited for this opportunity!