Received: 28 August 2023 | Revised: 4 January 2024 | Accepted: 7 March 2024

DOI: 10.1111/mec.17327

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Neurogenomic landscape associated with status-dependent cooperative behaviour

Peri E. Bolton[1,2] | T. Brandt Ryder[3,4] | Roslyn Dakin[3,5] | Jennifer L. Houtz[6,7] | Ignacio T. Moore[8] | Christopher N. Balakrishnan[1] | Brent M. Horton[6]

[1]Department of Biology, East Carolina University, Greenville, North Carolina, USA

[2]Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, District of Columbia, USA

[3]Migratory Bird Center, Smithsonian National Zoological Park, Washington, District of Columbia, USA

[4]Bird Conservancy of the Rockies, Fort Collins, Colorado, USA

[5]Department of Biology, Carleton University, Ottawa, Ontario, Canada

[6]Department of Biology, Millersville University, Millersville, Pennsylvania, USA

[7]Department of Biology, Allegheny College, Meadville, Pennsylvania, USA

[8]Department of Biological Sciences, Virginia Tech, Blacksburg, Virginia, USA

Correspondence
Peri E. Bolton, Department of Biology, East Carolina University, Greenville, NC, USA.
Email: peri.bolton@gmail.com

Brent M. Horton, Department of Biology, Millersville University, Millersville, PA, USA.
Email: brent.horton@millersville.edu

Funding information
Directorate for Biological Sciences, Grant/Award Number: 1457541; Division of Integrative Organismal Systems, Grant/Award Number: 1353085

Handling Editor: Sarah Perry Flanagan

Abstract

The neurogenomic mechanisms mediating male–male reproductive cooperative behaviours remain unknown. We leveraged extensive transcriptomic and behavioural data on a neotropical bird species (*Pipra filicauda*) that performs cooperative courtship displays to understand these mechanisms. In this species, the cooperative display is modulated by testosterone, which promotes cooperation in non-territorial birds, but suppresses cooperation in territory holders. We sought to understand the neurogenomic underpinnings of three related traits: social status, cooperative display behaviour and testosterone phenotype. To do this, we profiled gene expression in 10 brain nuclei spanning the social decision-making network (SDMN), and two key endocrine tissues that regulate social behaviour. We associated gene expression with each bird's behavioural and endocrine profile derived from 3 years of repeated measures taken from free-living birds in the Ecuadorian Amazon. We found distinct landscapes of constitutive gene expression were associated with social status, testosterone phenotype and cooperation, reflecting the modular organization and engagement of neuroendocrine tissues. Sex-steroid and neuropeptide signalling appeared to be important in mediating status-specific relationships between testosterone and cooperation, suggesting shared regulatory mechanisms with male aggressive and sexual behaviours. We also identified differentially regulated genes involved in cellular activity and synaptic potentiation, suggesting multiple mechanisms underpin these genomic states. Finally, we identified SDMN-wide gene expression differences between territorial and floater males that could form the basis of 'status-specific' neurophysiological phenotypes, potentially mediated by testosterone and growth hormone. Overall, our findings provide new, systems-level insights into the mechanisms of cooperative behaviour and suggest that differences in neurogenomic state are the basis for individual differences in social behaviour.

KEYWORDS
behavior, birds, sexual selection, social evolution, transcriptomics

Christopher N. Balakrishnan and Brent M. Horton contributed equally to this work.

1 | INTRODUCTION

Cooperation is the synchronized behaviour(s) of individuals to produce a net fitness benefit for one or more individuals (Taborsky & Taborsky, 2015). An individual's decisions about cooperation are based on immediate social stimuli, past experiences, developmental effects and genotype. These effects contribute to repeatable, among-individual variation in behaviours—a behavioural phenotype—where some individuals are more cooperative than others. To understand the mechanisms of cooperation, it is important to understand not only the elements of neurophysiological signal processing but also the genomic and neurophysiological components that underpin behavioural phenotypes. Understanding these mechanisms enables us to link how genomic and neurophysiological architecture can constrain and facilitate the evolution of cooperation (Hofmann et al., 2014; Soares et al., 2010; Taborsky & Taborsky, 2015). However, the mechanisms underlying the diversity of behaviours related to cooperation remain unclear (Díaz-Muñoz et al., 2014; Kasper et al., 2017). In particular, our understanding of the neuroendocrine mechanisms underlying seemingly paradoxical male–male reproductive cooperative behaviours is only recently emerging (Díaz-Muñoz et al., 2014; DuVal & Goymann, 2011; Jones & DuVal, 2021; Loveland et al., 2021; Ryder et al., 2020; Vernasco et al., 2020).

Differences in cooperative behavioural phenotype are partly achieved through pleiotropic yet modular effects of hormone signalling systems (Cox, 2020; Hau, 2007; Ketterson et al., 2009). Hormonal pleiotropy mediates phenotypic integration, whereby multiple traits covary with a hormonal signal (Cox, 2020; Ketterson et al., 2009). Trait independence is facilitated by variation in hormone receptor expression within and across tissues and cell types, this modularity enables diverse and flexible responses to similar hormonal and social signals (Ketterson et al., 2009; Lipshutz et al., 2019). In particular, sex-steroid concentration and receptor distribution in neural tissues are important mediators of social behaviours, including cooperation (Kasper et al., 2017; Soares et al., 2010). The neural substrates modulating social behaviours are largely conserved across vertebrates, in the social decision making network (SDMN) (O'Connell & Hofmann, 2011). The SDMN comprises reciprocally interconnected brain nuclei rich in steroid receptors, steroidogenic enzymes and steroid-modulated neuropeptide systems (Goodson, 2005; Newman, 1999; O'Connell & Hofmann, 2011). This modular and dynamic network activates different combinations of brain regions in response to different social contexts (Goodson, 2005; Newman, 1999). Therefore, the neurological response in a single nucleus is not reflective of all systems engaged in modulating a given behaviour. Consistent variation among individuals in neuroendocrine gene expression across the SDMN has been shown to underlie repeatable individual differences in social behaviour and behavioural phenotype (Cardoso et al., 2015; Goodson & Thompson, 2010; Horton et al., 2014; Kingsbury & Wilson, 2016; Rosvall et al., 2012). For example, constitutive variation in the expression of genes involved in sex-steroid signalling pathways, such as those of the androgen receptor, across SDMN nuclei can modify the male brain's sensitivity to testosterone, resulting in distinct behavioural responses to a specific hormone signal (Fuxjager et al., 2010; Rosvall et al., 2012). Recent research has expanded this perspective into the transcriptomic realm: finding that neurogenomic states, characterized by variation in expression of suites of genes in behaviourally relevant brain regions, are associated with behavioural phenotypes (Antunes et al., 2021; Bell et al., 2016; Benowitz et al., 2017; Kabelik et al., 2021; Lattin et al., 2022).

For the first time, we characterize constitutive gene expression across the neuroendocrine system in relation to variation in a male–male cooperative behaviour. The wire-tailed manakin (*Pipra filicauda*) is a neotropical lek-breeding bird in which unrelated males perform cooperative displays to attract females (Figure 1a). Territorial and subordinate non-territorial (floater) males form long-term display partnerships which form complex social networks (Ryder et al., 2008; Ryder, Blake, et al., 2011). Status and cooperation are directly linked to fitness, whereby territory holders with more display partners sire more offspring, and floaters with more partners are more likely to ascend to territorial status (Ryder et al., 2008, 2009). Individuals have a repeatable 'testosterone phenotype' where territorial males tend to have higher testosterone than floaters (Figure 1c), and circulating testosterone has a status-specific effect on the cooperative display behaviour (Ryder et al., 2020; Ryder, Horton, & Moore, 2011). Experimental and observational evidence show that higher testosterone levels are antagonistic to cooperation in territorial males but promote cooperation in floater males (Ryder et al., 2020; Vernasco et al., 2020) (Figure 1b).

To explore the neurogenomic basis of the status-specific androgenic modulation of cooperative behaviour in these birds, we quantify transcriptome-level gene expression in the SDMN, pituitary and testes of free-living male wire-tailed manakins in the Ecuadorian Amazon. While useful for neuro-ethological experiments, captive studies can obscure ecologically and evolutionary relevant variation in environmental and behavioural responses (Griffith et al., 2017; Kasper et al., 2017), and we demonstrate the exciting feasibility of neuro-transcriptional methods in challenging rainforest conditions. We aim to characterize the genes and neuroendocrine pathways that correlate with individual variation in cooperative behaviour, social status and testosterone phenotype as a modifier of both social status and cooperative behaviour. Given the androgenic regulation of cooperative display (Ryder et al., 2020; Vernasco et al., 2020), we hypothesize that differences in gene regulation will manifest in SDNM nuclei with high density of sex-steroid receptors and endocrine tissues (Goodson, 2005; Newman, 1999), and will involve the expression of candidate genes relating to sex-steroid signalling, metabolism and neuropeptide systems (Goodson & Thompson, 2010; Kingsbury & Wilson, 2016). We anticipate brain region-specific gene expression patterns within the modular SDMN (Figure 1g), as well as systemic effects of testosterone on gene expression across the brain (Cox, 2020; Ketterson et al., 2009; Lipshutz et al., 2019). We discuss

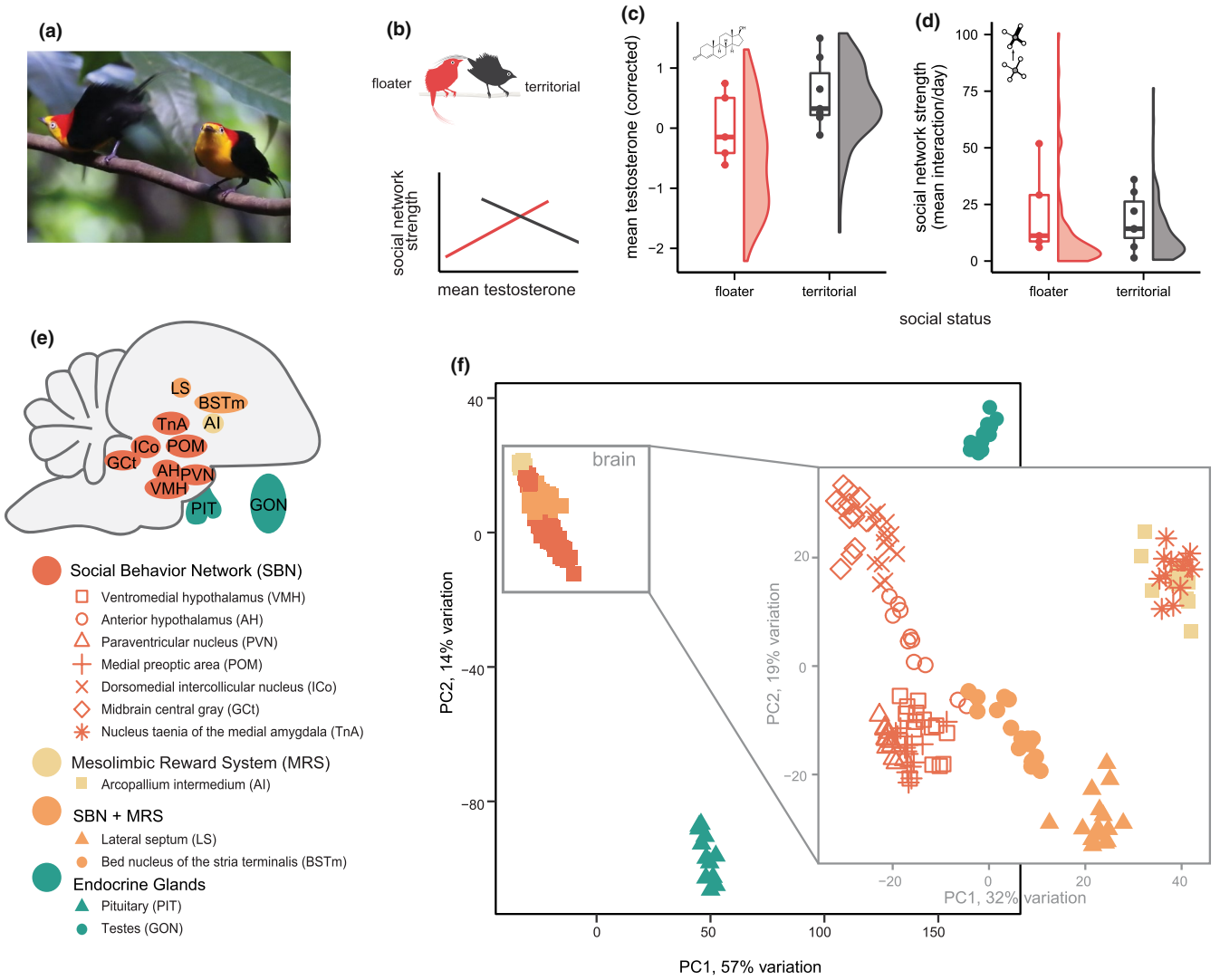


FIGURE 1 Testosterone and neuroendocrine tissues are involved in the cooperative behaviour of wire-tailed manakins. (a) Males engage in cooperative displays forming complex social networks. (b) Testosterone phenotype (mean testosterone [corrected]) influences cooperative tendency in territorial and floater males differently, where red colour is floater and black is territorial males. (c) Repeated measures of testosterone for 12 individuals (points) from a larger population (density). (d) Same as C but proximity-data logging measures of social network strength. (e) Brain and endocrine tissues sampled for RNAseq. Structures not to scale. Colours represent functional groups (O'Connell & Hofmann, 2011). (f, inset) Principal component analysis reveals distinct gene expression profiles among brain regions and endocrine tissues (colours and shapes as e). Photo: Alice Boyle. *Note*: Sample size $N = 16$ for social status, $N = 12$ for testosterone and network strength.

our results in the context of modes of phenotypic evolution and similarities with mechanisms of other cooperative and social behaviours.

2 | MATERIALS AND METHODS

All methods described here were approved by Smithsonian Animal Care and Use Committee (protocols 12-12,14-25,17-11), as well as the Ecuadorian Ministry of the Environment (MAE-DNB-CM-2015-0008), and samples were transported with export permit (006-016-EXP-1C-FAU-DNB/MA) and import permit (USDA APHIS 126133). Detailed methods can be found in Supporting Information, and full code from analyses in R v 4.0.2 is available on GitHub (https://peripheripatus.github.io/PIFI_brain_transcriptome/) and FigShare (https://doi.org/10.25573/data.22186516).

2.1 | Field methods

From a larger study on the role of testosterone on male cooperative lekking behaviour (Ryder et al., 2020), we sampled wild territorial and floater wire-tailed manakin males for RNA sequencing from Tiputini Biodiversity Station in the lowland Amazon rainforest of Ecuador (Ryder et al., 2020) (Table S1.1: Appendix S1). Males were categorized by social status: where the territorial birds ($n = 9$) were males in adult plumage that held a consistent display territory over

the study period, while floaters ($n=7$) had either adult or second-year plumage and did not occupy consistent territories within leks (Ryder et al., 2008). Twelve of the sampled males were part of the larger population study that used repeated testosterone sampling to characterize their testosterone phenotype (n(territorial)$=7$, n(floater)$=5$, Figure 1c, Appendix S1) (Ryder et al., 2020). A male's testosterone phenotype is derived from repeated testosterone sampling within and between field seasons. Specifically, mean corrected testosterone is represented by the mean of the residuals of a linear regression on the broader population of log-transformed testosterone accounting for capture conditions, that is, net time, Julian date, time of day and field season (see Table S1.3 for correspondence of these values to concentration) (Ryder et al., 2020). This measure of testosterone phenotype was previously shown to be repeatable within individuals (Appendix S1 for more detail, see also (Ryder et al., 2020)). Previous research has shown that territorial males have, on average, higher circulating testosterone than floaters (Ryder et al., 2020; Ryder, Horton, & Moore, 2011), a pattern also reflected in our 12 sampled males (Figure 1c).

Social network structure and individual variation in cooperative display were characterized using autonomous radio-telemetry and tag proximity detections within territories on a total population of 180 male manakins over multiple years (Dakin & Ryder, 2018; Ryder et al., 2012, 2020). We used this tagging data to calculate social network strength for the same 12 males with repeated testosterone measures that we sampled for RNA sequencing. Social network strength is a proxy for cooperative tendency and is the time spent interacting with other males averaged over the study period (Figure 1d) and was the most repeatable social network metric within individuals (Ryder et al., 2020). This particular proxy for cooperation showed the strongest status-specific relationship with an individual's testosterone phenotype (Ryder et al., 2020) (Figure 1b), but the overall frequency of cooperative interactions did not differ between territorial and floater males in the broader population or our sampled males (Figure 1d).

To collect brain and endocrine tissues, we captured birds with mist nets over three field seasons (2015–2018). In the field, birds were euthanized by decapitation immediately after blood sampling for testosterone, and brains were extracted within 4–6 min and immediately placed on dry ice (Appendix S1). Testes and pituitary tissues were extracted immediately after the brains, with the testes also placed on dry ice and the pituitaries were preserved in RNAlater™ (Invitrogen) at ambient temperatures until importation into the United States.

2.2 | Microdissection and RNAseq

Brains were cryosectioned and microdissected using the methods developed on the samples from the first field season (Horton, Michael et al., 2020), with additional detail in Appendix S1. We microdissected tissue from 10 different nuclei involved in social behaviour—nine of which are from the SDMN. The SDMN reflects the interconnectivity of two networks involved in regulating social behaviour (O'Connell & Hofmann, 2011): Social behaviour network (SBN) (Goodson, 2005; Newman, 1999) and the mesolimbic reward system (MRS) (O'Connell & Hofmann, 2011; Olds & Milner, 1954). Figure 1f indicates the nomenclature used and the relationships of these nuclei to the SBN and MRS. To clarify bird-specific nomenclature: the arcopallium intermedium (AI) is a region that may be partly homologous to the basolateral amygdala of mammals (Figure S2.6), which is hypothesized to play a role in androgen-dependent manakin display behaviour (Fusani et al., 2014). The nucleus taenia (TnA) is homologous to the mammalian medial amygdala (O'Connell & Hofmann, 2011), but see (Mello et al., 2019; Appendix S1). The paraventricular nucleus (PVN) is not yet formally recognized as part of the SDMN, despite its neuropeptide projections to nodes of the SDMN and established role as a major regulator of vertebrate social behaviour (Goodson & Kingsbury, 2013; Goodson & Thompson, 2010).

Microdissected tissues from both hemispheres were combined for RNA extraction and sequencing, using previously described methods (Horton, Michael et al., 2020). RNA libraries for the various tissues and last two field seasons were randomized among two sequencing batches and included replicates from the first field season to validate the inclusion of a third sequencing batch from an earlier season (Tables S1.1 and S1.2, Figure S1.3) (Horton, Michael et al., 2020). All samples analysed are associated with BioProject PRJNA437157 and SRR12660169-198, SRR19521260-271 and SRR19521432-575.

2.3 | Gene expression analyses

Reads were aligned to the annotated *Pipra filicauda* genome (GCA_003945595.1) using splice-aware aligner STAR v2.7.5 (Dobin et al., 2013), and gene-level counts were obtained from featureCounts v2.0.1 (Liao et al., 2014). A total of 170 libraries were retained after quality filtering in each tissue (Figures S1.2–S1.4, S3.1 and S3.2), but the number of individuals used in each tissue varies (Tables S1.1 and S1.2).

We conducted hierarchical analyses within and across tissues to address multiple aims (detailed descriptions of methods and results can be found in the corresponding section of the supplement). All analyses were conducted in R v4.0.2 (R Core Team, 2020). First, to further characterize tissue and brain nucleus function in this species (Horton, Michael et al., 2020), and to characterize modular and system-wide differences in gene expression, we characterized differences in gene expression among hypothalamic–pituitary-gonadal (HPG) tissues and brain nuclei using principal components analysis in PCAtools v2.8.0 and weighted gene co-expression analysis (WGCNA v1.71) (Blighe & Lun, 2020; Langfelder & Horvath, 2008) using signed adjacency matrices with scale-free topology (Appendix S2, eight analyses). Then, to describe the role of tissues and brain nuclei in relation to hormonal and behavioural phenotypes, we analysed differential expression using DESeq2 v1.36 (Love et al., 2014) (Appendix S3, 36 analyses) and co-expression using WGCNA

(Langfelder & Horvath, 2008) (Appendix S5, 12 analyses) in separate data subsets each tissue type. The differential expression analyses were then used to form the basis of further candidate gene- (Appendix S4) and additional system-wide analyses using sets of genes that overlap among analyses (Appendix S6).

To identify differentially expressed genes in each tissue or nucleus, gene counts were normalized and were modelled using a negative binomial generalized linear model framework (Love et al., 2014). For a subset of tissues, we had an extra four individuals characterized for social status from a previous study (Horton, Michael et al., 2020), for which behavioural and hormone sampling data were unavailable. Owing to this, and to avoid overparameterizing the models that account for batch effects, we identified differentially expressed genes (DEGs) associated with each of our traits of interest (social status, testosterone phenotype and social network strength) separately. These were run as models for each trait that accounted for batch effects where necessary using DESeq2 (Love et al., 2014) (Appendix S3: Figures S3.1 and S3.2). Testosterone and social network strength were treated as continuous variables. Significant DEGs were correlated with the predictor variable, and $\log_2$ fold change (LFC) value represents the LFC of gene expression per unit of the predictor variable. In all cases, significant DEGs were identified using the Wald Test and the default FDR corrected p-value ($q < 0.1$).

To identify tissues with similar patterns of gene expression in association with our traits of interest, we examined pairwise correlations of ranked gene lists among tissues (Appendix S6). The gene-lists from differential expression results were ranked using $-\log_{10}$ transformed p-values multiplied by the expression direction. Similarity between tissue pairs was measured using Pearson correlation and rank-rank hypergeometric overlap (RRHO) test (Cahill et al., 2018).

Due to the key role testosterone plays in modulating behaviour in this species (Ryder et al., 2020), we further analysed nine candidate genes involved in sex-hormone metabolism and signalling as well as nine steroid-sensitive neuropeptides and their receptors (Table S4.1). Candidate genes showing differential gene expression with an uncorrected $p < .05$ were considered 'significant' and examined further. In addition to analysing models of status, mean testosterone and social network strength separately, we also explored whether candidate genes were involved in mediating status-specific relationships between mean testosterone and cooperative behaviour (Ryder et al., 2020). Here we extracted 'significant' candidate genes for further inspection from DESeq2 interaction analyses (model: expression ~ status × mean testosterone) using the likelihood-ratio test. To minimize the effects of overfitting, we only selected candidate genes that had a significant interaction term in a reduced model without batch effects. We did not use this method to identify novel genes owing to power and overfitting concerns. All putative gene-trait relationships were plotted (Figures S4.1–S4.22), and those with highly influential observations or $R^2 \leq .2$ were excluded. For candidates derived from the interaction effect models or genes that were associated with multiple traits, the most convincing relationship was determined using a stepdown AIC procedure of multiple linear regression models (Appendix S4). Considering the smaller set of candidate genes, we report both uncorrected p-values as well as the FDR corrected values from DESeq2. We consider genes with relationships that passed the AIC process and/or had a raw p-value $< .05$ as worthy of discussion.

We reasoned that testosterone's role as a phenotypic integrator and the interplay between social status and basic biological processes such as immunity, metabolism and ageing (e.g. Anderson et al., 2021, 2022; Newhouse & Vernasco, 2020) would have systemic effects on gene expression, as well as flexible tissue specific responses investigated above (Cox, 2020; Ketterson et al., 2009). Systemic effects could be apparent in two ways: through shared patterns of gene expression (gene identity and gene functions), and through changes in the properties of gene co-expression networks. To explore systemic gene expression patterns, we calculated median p-values from the previous analysis and identified genes consistently differentially expressed across the entire brain (p-value $< .05$, transformed value $> |1.3|$). Here, we focused on the brain because we expected neural tissues would show more similarities than divergent endocrine tissues. Further, we correlated eigenvalues of each principal component (PC) from the PCA analyses (Figure 1f) with our variables of interest using pcatools::eigencorplot() (Blighe & Lun, 2020), (Appendix S2). System-wide WGCNA modules were also correlated with interest variables using standard WGCNA protocols (Appendix S2) (Langfelder & Horvath, 2008). Correlations with interest variables were validated using linear models and ANOVAs with a model form ~ [Batch] + Tissue + Trait (Appendix S2). Further, we explored status-specific differences in co-expression network connectivity and density by conducting module preservation analysis (Langfelder et al., 2011). We permuted gene labels 200 times to calculate new adjacency matrices under the null hypothesis of no difference in preservation. We further tested for differences in distribution of total gene connectivity (kTotal) in each module using a two-sided Mann–Whitney U-Test (Appendix S2). We focus on the Median Rank statistic, which summarizes and ranks network connectivity metrics among groups, because this metric is not sensitive to the number of genes in the module (Langfelder et al., 2011).

We used clusterProfiler v3.16.1 (Yu et al., 2012) to describe patterns of functionality in DEGs and WGCNA modules with gene ontology (GO) enrichment analyses using a custom set of GO annotations (Appendix S3). We used module membership and the differentially expressed genes with uncorrected p-value $< .05$ as foreground and the full gene list of the tissue as the background. To additionally describe the direction of expression of functional categories along the full p-value distribution, we used gene set enrichment analysis on the full gene list. This method leverages the rank order of p-values and direction of expression and prioritizes GO terms clustering at the ends of the list with lower p-values (Appendix S3). We used a threshold for FDR corrected p-value is $q < 0.05$ to determine significant enrichment. To summarize the GO in DEGs ($p < .05$) across all tissues, we used *rrvgo* v 1.0.2 to identify functional groups (based on semantic similarity clustering) commonly differentially expressed (Sayols, 2020).

3 | RESULTS

Gene expression analyses revealed substantial differences among tissues and brain regions. WGCNA analysis of the HPG axis revealed 11 modules of co-expression (Figure S2.12), and PCA analyses revealed substantial differences in the transcriptional composition of the endocrine versus brain tissues (Figure 1f). In the brain, these expression patterns were partitioned into 21 co-expression modules, and expression was more similar among adjacent nuclei, forming major brain-region groupings of the amygdala, hypothalamus and midbrain (Figure 1f, Figure S2.5). Additionally, our 18 candidate neuroendocrine genes (Table S4.1) displayed tissue and brain region-specific expression (Figure 3a, Tables S2.1 and S2.2).

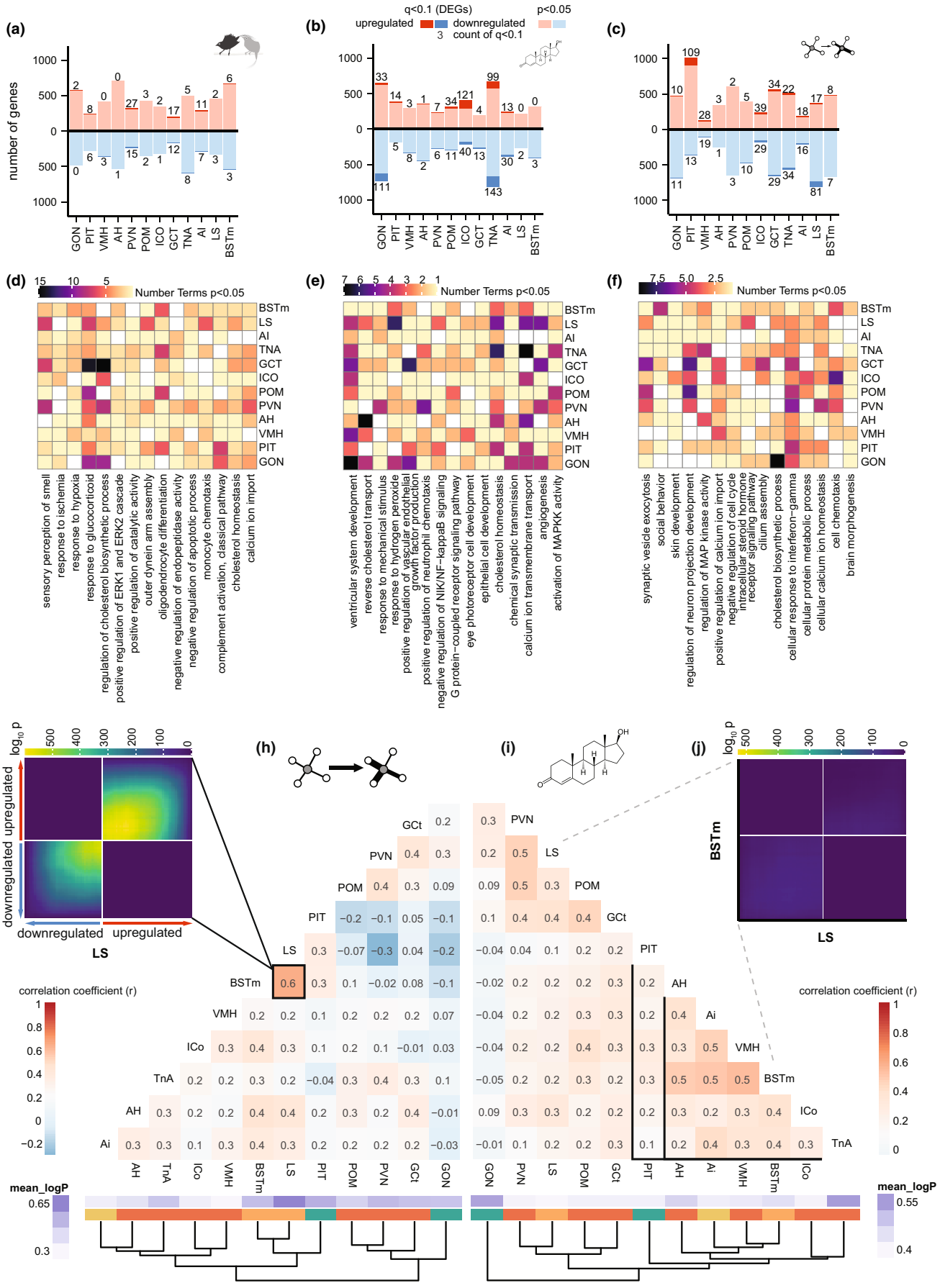
3.1 | Social status, testosterone phenotype and cooperative behaviour are associated with distinct landscapes of tissue and brain region-specific patterns of gene expression

We observed landscapes of gene expression in association with social status (Figure 2a), testosterone phenotype (Figure 2b) and cooperative behaviour (Figure 2c), with a varying number of differentially expressed genes (DEGs) or co-expression modules identified across different tissues. The number of DEGs in each tissue was more correlated between testosterone phenotype and status compared to number of DEGs associated with social network strength ($R=.6$, $p=.03$, Figure S3.3A). Although there was less correspondence in overall DEG identity (Figure S3.3B), many co-expression modules and candidate genes were shared between testosterone phenotype and status (Figure 3b,c, Figure S5.15, Tables S4.2 and S4.3). This similarity was expected due to the correlation between testosterone and status (Figure S1.1) (Ryder et al., 2020; Ryder, Horton, & Moore, 2011). Indeed, we observed a distinct gene expression landscape in relation to cooperative behaviour, which was also reflected in a different pattern of clustering of shared expression among tissues (Figure 2h,i, Figures S6.1–S6.4). Here, we primarily focus on the tissues with large numbers of DEGs and significant candidate genes for the main manuscript, but the co-expression results were similar to our differential expression results, and all results are shown in Figures S3.4–S3.39 and S5.1–S5.14. Among tissues, there was evidence for regulation of common functional groups, as summarized by number of GO terms clustered together by semantic similarity (Figure 2d–f). Notably, across all analyses there were common signatures for developmental processes, cholesterol regulation and biosynthesis, and steroid signalling, membrane potentiation and synaptic signalling.

The testes had over 1000 genes differentially expressed (at uncorrected p-value $<.05$) in association with both testosterone phenotype and social status (144 genes after FDR correction $q<0.1$ in association with mean testosterone; 2 after FDR correction in association with social status). DEGs identified in association with testosterone phenotype in the testes were enriched for genes involved with response to corticosterone (GO:0051412, $q=0.03$) and non-significant enrichment for others (Figure S3.16). Notable DEGs included steroidogenic acute regulatory protein (STAR, LFC $=-1.1$, $p=2\times10^{-7}$, $q=0.0003$, $R^2=.6$) and candidate gene vasopressin receptor 1A (AVPR1A, Figure 3g, LFC $=0.8$, $p=5\times10^{-5}$, $q=0.01$, $R^2=.7$). Log_2 Fold Change (LFC) represents a change in gene expression per unit of the mean testosterone variable (Ryder et al., 2020), see Table S1.3 for correspondence of this variable to serum testosterone concentrations. These transcriptional differences do not correspond to significant differences in testis size in relation to testosterone phenotype (Figure S3.40, Table S3.1), but territorial males have larger testes ($p=.04$, Table S3.1).

Among the 242 DEGs associated with testosterone phenotype in TnA (13 in social status), there was non-significant enrichment for long-term synaptic potentiation (GO:0060291, $p=4\times10^{-5}$, $q=0.08$), neural development (GO:0001755, $p=4\times10^{-4}$, GO:0048843, $p=4\times10^{-4}$, $q=0.16$) and memory (GO:0007613, $p=.001$, $q=0.26$) (Figure S3.24). Notably, the candidate gene oestrogen receptor 2 (ESR2; aka Erβ) was upregulated in TnA of males with higher testosterone (Figure 3h; LFC $=0.8$, $p=.001$, $q=0.08$, $R^2=.4$). Other notable DEGs in TnA included upregulation of immediate early gene JUN, also a 'coral1' hub gene (module membership (MM) $=0.93$ Figures S5.10 and S5.11; LFC $=0.61$, $p=.0004$, $q=0.05$) and corticotropin-releasing hormone receptor 2 (CRHR2, LFC $=1$, $q=0.006$, $R^2=.5$).

FIGURE 2 Neurogenomic landscape differential expression. (a) Landscape of differential gene expression in relation to social status, where red indicates genes upregulated in territorial males. Red bars indicate number genes upregulated in territorial males, and blue indicates genes downregulated in territorial males. Darker colours and text indicate number of differentially expressed genes after FDR correction. (b) Landscape of gene expression in male wire-tailed manakins with high testosterone phenotype (mean testosterone [corrected]) relative to lower testosterone phenotype, where red indicates upregulation in males with higher testosterone phenotype. (c) Gene expression landscape in male wire-tailed manakins with higher social network strength, where red indicates upregulation in males with higher social network strength. (d) Number of GO terms (unadjusted $p<.05$) in top 15 semantic clusters based on representation across tissues from the social status analysis, (e) mean testosterone and, (f) social network strength. (g) RRHO2 analysis shows similar gene expression profiles associated with social network strength in the LS and BSTm, (h) while network strength-related gene expression similarity is low among other tissues. (i) Gene expression similarity among tissues in relation to testosterone phenotype follows the ontogeny of brain regions, (j) No correlation between testosterone-related gene expression profiles in LS and BSTm. (h, i) Pearson correlation coefficients of gene expression between tissues, represented by cell colour and number. Gene expression p-values were $\log_{10}$ transformed and multiplied by direction of expression. Black boxes outline significant tissue correlation clusters based on 1000 bootstrap resamples. *Note*: Sample sizes vary in each tissue due to quality filtering. Maximum sample size for analysis of social status was $N=16$, while only 12 individuals were sampled for testosterone and behaviour.



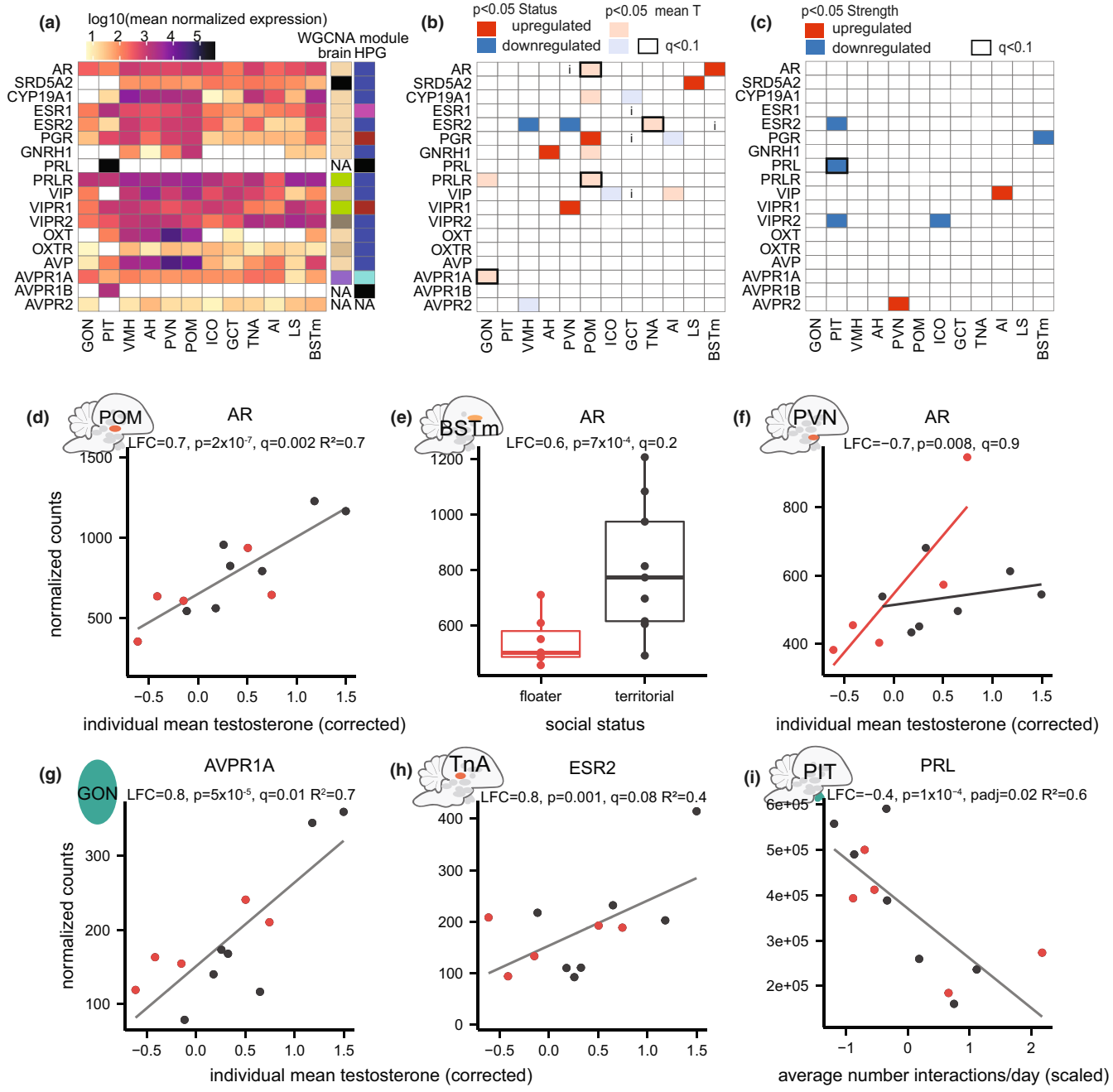


FIGURE 3 Landscape of candidate gene expression. (a) Candidate gene expression across tissues, where darker colour indicates higher mean expression. (b) Summary of candidate gene analysis and filtering for social status and mean testosterone, where colours indicate direction of expression, intensity indicates which trait is associated, and '*i*' indicates there was an interaction effect, and black boxes indicate the result was significant after FDR correction. (c) Summary of candidate gene analysis and filtering, where colours indicate direction of expression in in individuals with higher social network strength. (d) Upregulation of AR in POM of males with higher testosterone, where point colours indicate social status (red = floater; black = territorial). Fitted line from linear regression. Subheader includes results from DESeq2: $\log_2$ fold change (LFC), uncorrected *p*-value (*p*) and correction for multiple testing (*q*) and R^2 calculated separately. (e) Upregulation of AR in BSTm of territorial males. (f) Status-specific expression of AR in PVN in relation to testosterone phenotype. (g) Upregulation of AVPR1A in the testes of males with higher testosterone. (h) Upregulation of ESR2 in the TnA of males with higher testosterone. (c) Downregulation of prolactin (PRL) in the pituitary of males with higher social network strengths.

Intriguingly, although the POM did not reveal a high number of DEGs in association with testosterone phenotype (45), our analysis revealed differential expression for compelling gene ontology categories and candidate neuroendocrine genes (Figures S3.21 and S5.7, Figure 2b). Notably, males with higher testosterone exhibited upregulation of candidate gene androgen receptor (AR) in POM (Figure 3d). The 'floralwhite' module, which comprised many upregulated DEGs, was significantly associated with both testosterone phenotype and, to a lesser extent, social status (Figures S5.6 and S5.7). This module showed enrichment for biological

processes related to G-protein-coupled receptor signalling pathways (GO:0007186, $q=0.007$) and neuropeptide signalling pathway (GO:0007218, $q=0.03$), with non-significant enrichment for steroid receptor signalling (GO:0030518, $q=0.2$), learning or memory (GO:0007611, $q=0.2$) and nervous system development (GO:0007399, $q=0.2$, Figure S5.7), with similar patterns among DEGs (Figure S3.21D). Indeed, many of the shared hub-genes and DEGs interact with candidate sex-hormone pathways, such as prolactin-releasing hormone (PRLH, MM = 0.91, LFC = 2.4, $q=5\times10^{-5}$, $R^2=.6$, Figure S3.21B), growth-regulating oestrogen receptor binding 1 (GREB1, MM = 0.86, LFC = 0.6, $q=3\times10^{-5}$, $R^2=.7$, Figure S3.21B), prolactin receptor (PRLR, MM = 0.78, LFC = 0.25, $q=0.05$, Figure 3b) and gonadotropin releasing-hormone 1 (GNRH1, MM = 0.76, LFC = 0.4, $p=.006$, $q=0.2$, $R^2=.4$, Figure 3b). Other notable 'floralwhite' hub genes include the candidate gene progesterone receptor (PGR, MM = 0.81) which was associated with social status (Figure 3b; LFC = 0.4 $p=.006$, $q=0.7$) and serotonin receptor 5A (HTR5A, MM = 0.87, LFC = 0.4, $p=.04$, $q=0.6$, Figure S5.7). Candidate gene aromatase (CYP19A1) was also upregulated in males with higher testosterone phenotype (Figure 3b, LFC = 0.6, $p=.02$, $q=0.4$, $R^2=.5$), but was found in the 'lightcoral' co-expression module (MM = 0.86).

Certain brain regions exhibited stronger associations with status-specific expression (Figure 2b). That is, either an additive effect among social classes or a significant interaction between status and testosterone phenotype (Figure 2c). PVN of the hypothalamus showed the most DEGs associated with social status after FDR correction (564/42). DEGs before FDR correction were significantly enriched for cilia-related genes (e.g. GO:0003341, $q=9\times10^{-5}$), cholesterol biosynthesis (e.g. GO:0006695, $q=0.01$) and non-significant enrichment for response to progesterone (GO:0032570, $p=8\times10^{-4}$, $q=0.1$ Figure S3.8D). Candidate gene expression also revealed potential status-specific expression with a male's testosterone phenotype in the PVN (Figure 3b). In particular, AR expression was positively correlated with testosterone phenotype in floater males, but not in territorial males (Figure 3f; LFC = −0.7, $p=.008$, $q=0.9$). The PVN of territorial males also showed higher VIPR1 (LFC = 0.3, $p=.02$, $q=0.4$) and lower ESR2 expression (LFC = −0.6, $p=.006$, $q=0.2$, Figure 3b, Figure S4.6). In BSTm, a region that bridges the SDMN and MRS, there were more than 1000 DEGs, but only nine after FDR correction. Among these, territorial males showed higher AR expression compared to floater males (Figure 3e; LFC = 0.6, $p=7\times10^{-4}$, $q=0.2$). Further, testosterone metabolism gene 5-alpha reductase (SRD5A2) was upregulated in the LS of territorial males (Figure S4.19; LFC = 0.7, $p=5\times10^{-4}$, $q=0.4$).

GCt was a hotbed of genes associated with social status (29 DEGs after FDR correction) and revealed multiple candidate genes with potential status × testosterone phenotype interactions (Figure 3b, Figures S3.11, S4.13 and S4.14). Gene set enrichment analysis revealed significant downregulation of genes involved in response to peptide hormone (GO:0043434, $q=0.007$), as well as upregulation of genes related to RNA processing (e.g. GO:0000184, $q=6\times10^{-5}$) and translation (e.g. GO:0006412, $q=6\times10^{-6}$) (Figure S3.11C) in association with status. Candidate neuroendocrine genes showed status-specific expression in association with testosterone phenotype in this region including progesterone receptor (PGR, Figure S4.14; $p=.003$, $q=0.1$), oestrogen receptor 1 (ESR1; aka Erα; $p=.04$, $q=0.4$) and vasoactive intestinal peptide (VIP, $q=0.005$, $q=0.1$). Further, aromatase (CYP19A1) was downregulated with higher testosterone phenotype in the GCt (Figure 3b, LFC = −1.3, $p=.002$, $q=0.3$, $R^2=.4$).

The pituitary showed an abundance of differentially expressed genes associated with male's network strength (122 genes after FDR correction $q<0.1$), but there were no significantly enriched GO-terms among these (Figure 3.29D). Within the pituitary, candidate genes prolactin (PRL) (Figure 3c; LFC = −0.6, $p=5\times10^{-5}$ $q=0.03$, $R^2=.7$), ESR2 (Figure S4.3; LFC = −0.7, $p=.04$, $q=0.5$, $R^2=0.5$) and VIPR2 (Figure S4.3; LFC = −0.4, $p=.008$, $q=0.3$, $R^2=0.4$) were downregulated in highly cooperative males. Furthermore, although there was less representation of sex-steroid-related pathways in the candidate gene analysis (Figure 3c), PGR was downregulated in males with higher social network strength in BSTm (Figure S4.21; LFC = −0.3, $p=5\times10^{-4}$, $q=0.2$, $R^2=.5$).

Many genes in the LS were differentially expressed in association with an individual's social network strength (98 genes after FDR correction $q<0.1$). Among the top DEGs, lipoprotein receptor 4 (LRP4, LFC = −0.2, $q=0.01$) is involved in synaptic development and organization and there was non-significant enrichment for genes broadly involved in development and synaptic processes (e.g. GO:0045176, $p=6\times10^{-5}$, $q=0.1$; GO:0006904, $p=.001$, $q=0.62$; Figure S3.38D). No candidate genes were linked to social network strength in LS. Although few genes were differentially expressed in BSTm related to social network strength, RRHO analysis showed that LS and BSTm shared similar expression associations with strength (Cahill et al., 2018) (Figure 3g,h). This effect was primarily driven by a high-degree of similarity in the p-value rank and direction of genes with weak and non-significant effects (Figures S6.1–S6.3). In contrast, LS and BSTm showed limited similarity in gene expression related to testosterone phenotype (Figure 4i,j) or social status (Figures S6.1–S6.5), suggesting their unique role in modulating cooperative behaviour. The correlation between LS and BSTm in strength-related gene expression is unique as no other tissue pairs had such a high correlation coefficient (Figure 2g). This contrasts with the broader pattern of pairwise correlations of gene expression with testosterone phenotype, where neuroanatomically similar regions show are clustered (Figure 2i).

3.2 | Social status is associated with systemic gene expression

There was no evidence for global gene regulation across the HPG axis (Figure S2.12), as no co-expression modules were associated with our interest variables. There was a weak correlation with social status and PC7 across all 12 tissues in the PCA analysis (4% variation, Figures S2.1 and S2.2).

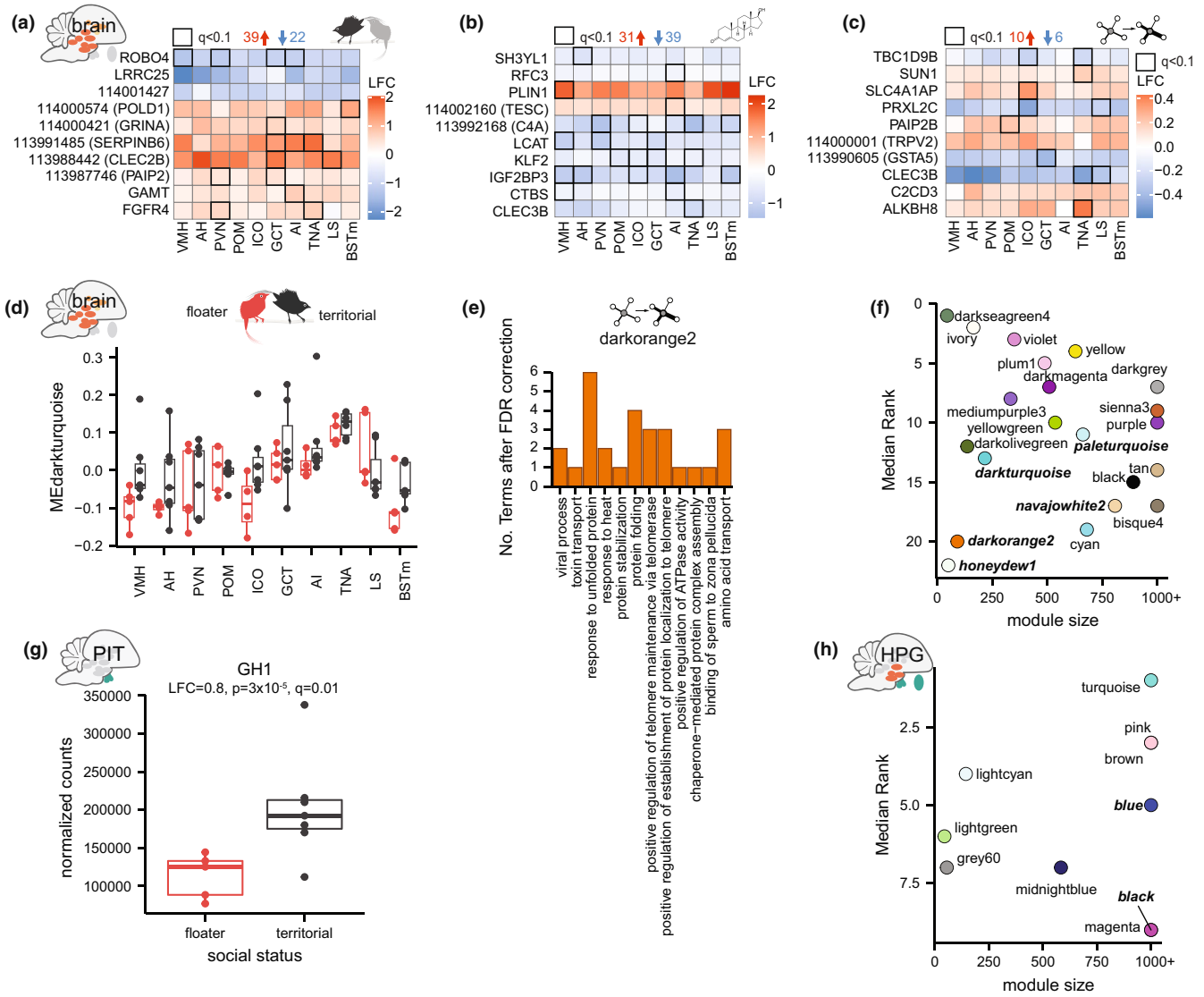


FIGURE 4 Global neural gene expression differences between social classes. (a) Top 10 consistently regulated genes (based on median raw *p*-value) across brain tissues according to social status, where colour and intensity indicate $\log_2$ fold change (LFC), black boxes indicate genes that were significant after FDR correction and graphic above summarizes the total number of genes with median $p < .05$. (b) Top 10 consistently regulated genes across brain tissues according to mean testosterone phenotype, and (c) social network strength. (d) Genes in the whole-brain WGCNA module 'darkturquoise' were consistently upregulated in most brain tissues of territorial males (Figure S2.6). (e) Genes in the whole brain WGCNA module 'darkorange2' were negatively associated with a higher cooperative tendency (Figure S2.9). This figure shows the number of significant terms after FDR correction ($q < 0.05$) as they were clustered by semantic similarity. (f) The whole-brain module 'darkorange2' shows low preservation (high medium rank) between territorial and floater males, and discussed modules are italicized and bolded (Figures S2.10 and S2.11). (g) Upregulation of growth hormone transcript in the pituitary of territorial males could explain global variation in gene expression between status classes. (h) The black module, in which GH1 is a hub, is poorly preserved (high medium rank) in territorial and floater birds (Figures S2.13 and S2.14).

There was evidence for brain-wide consistent differential expression of some genes. Social status and testosterone phenotype showed the highest number of consistently differentially expressed genes across tissues (61 and 70 genes with median uncorrected $p < .05$, respectively, Figure S4A,B), while social network strength had only 16 consistently differentially expressed genes (Figure S4C). There were no significant GO terms after FDR correction, but there were differences in the expression of an immune gene (e.g. CLEC2B-like) according to status and mean testosterone (Figure 4a,b, Table S6.2), and developmental gene functions (e.g.ROBO4, Figure 4a, Table S6.1) and of telomere maintenance genes (e.g.POLD1-like, Figure 4a) with social status. Further, there was evidence for global regulation of cholesterol biosynthesis (e.g. LCAT, Figure 2d–f, Figure 4b), and glutathione transferases (Figure 4c, Table S6.2), both of which are involved in steroid hormone biosynthesis. Further, PC5 of the whole brain analysis was significantly associated with social status, even after accounting for batch effects in an ANOVA, accounting for up to 10% of PC5 (Figure S2.4–S2.5). There was overlap between genes and GO categories among the consistently differentially regulated genes with

social status and testosterone phenotype (Tables S6.1 and S6.2), as well as with genes associated with PC5 (Figure S2.3).

Across the brain, the 'paleturquoise' (662 genes) and 'darkturquoise' (216 genes) modules were significantly associated with social status after accounting for batch variables in an ANOVA, and account for approximately 6% and 7% of module eigengene variation respectively (Figure 4d, Figures S2.6 and S2.7). These were also strongly associated with gene expression in the LS and TnA respectively (Figure S2.5). The 'darkturquoise' module showed strong signatures for enrichment in GO terms relating to immune, transcription and translational processes (Figure S2.6D), while 'paleturquoise' showed no enriched GO terms after FDR correction (Figure S2.7D). WGCNA identified the brain-wide 'honeydew1' module (50 genes) as correlated with cooperation (Figure S2.5), but this effect was discarded after linear modelling accounting for batch effects (Figure S2.7). Meanwhile, the 'darkorange2' (91 genes) module showed global downregulation in more cooperative males, accounting for between 5% and 17% of module eigengene variation (Figure S2.9). In this module, there was significant enrichment ($q<0.05$) for multiple GO terms relating to telomeric regulation and cellular stressors (Figure 4e). This module showed little evidence of preservation with increased connectivity in territorial males (Figure 4f, Figures S2.10 and S2.11). The 'navajowhite2' module containing multiple candidate genes and other important neuroendocrine genes (808 genes), was less connected in territorial males (Figure S2.11) and was among the least preserved modules (Figure 4f), though this was not a significant difference in *Z*-score-based statistics (Figure S2.10).

These findings of global expression differences among status classes could be partially explained by the upregulation of growth hormone (GH1) in the pituitary of territorial males (Figure 4g; LFC = 0.8, $p=3\times10^{-5}$, $q=0.01$). This was a hub gene in the 'black' module (MM = 0.97), which was among the least preserved modules in the HPG co-expression analysis (Figure 4h).

4 | DISCUSSION

Using the most comprehensive transcriptome-level sampling of the SDMN to-date (Antunes et al., 2021; Bentz, George, et al., 2021; Bentz, Niederhuth, et al., 2021; Kabelik et al., 2021; Lopes & König, 2020), our findings highlight both modular and integrative neurogenomic organization correlated with individual variation in cooperative behaviour. The relative roles of integration and modularity are central to the development and expression of any complex trait (Cox, 2020; Ketterson et al., 2009; Lipshutz et al., 2019).

4.1 | Different landscapes of gene expression with each trait reflect modular organization of the neuroendocrine system

Modularity is reflected in how different brain nuclei, endocrine tissues and genomic pathways are associated with the expression of different traits. These neurogenomic landscapes, characterized by the upregulation of neuroendocrine genes in some brain regions and downregulation in others, enabling differential sensitivity to testosterone and other signalling molecules among SDMN nuclei. These neurogenomic states can be likened to the activation of immediate early genes (IEGs) across the SDMN after a social stimulus (Goodson, 2005; Newman, 1999). In the 'landscape' model, it is the pattern of expression across multiple brain nuclei that correlates with behavioural responses, rather than the up or downregulation of particular genes within a single nucleus. In the landscape of constitutive gene expression in male wire-tailed manakins, most brain regions exhibit differences in gene expression but the brain regions with higher numbers of DEGs or candidate gene expression may be more important for mediating certain traits. The specific identities of these brain regions suggest derivation of cooperative display from aggressive behaviours (Díaz-Muñoz et al., 2014).

Some brain regions showed associations between gene expression and both social status and testosterone phenotype (e.g. POM) or only testosterone phenotype (e.g. TnA, testes). This may be because these regions are involved in testosterone-mediated traits irrespective of social status (O'Connell & Hofmann, 2012). Meanwhile, others appeared to be more related to social status and status-specific relationships with testosterone phenotype (e.g. PVN, GCt), or an individual's strength of cooperative behaviour (e.g. PIT, LS). Across brain regions, there were variations in the identity and function of genes, but certain functions consistently emerged, including neural development, synaptic potentiation or organization and genes related to cellular metabolism or transcription/translation regulation. These results suggest that individual variation is mediated by neurogenomic states that encode synaptic modifications and connectivity (mechanism of memory formation), larger scale shifts in cellular activity, and neural structure (Clayton et al., 2019; George et al., 2020). These mechanisms may interact with variation in elements of sex-steroid signalling that we investigated in detail (sensu 'neuroendocrine action potential'; Clayton et al., 2019). Moreover, the pairwise similarities in gene expression between different nuclei could reflect developmental similarities and/or specific circuitry involved in mediating particular traits (Kelly, 2022). For example, similarities in testosterone phenotype-associated gene expression may suggest synergistic hormone-mediated neural activity between forebrain regions (e.g. PVN & POM) and between forebrain and midbrain nuclei (e.g. POM & GCt; Figure 2i). Moreover, such gene expression similarity was also seen among SBN and MRS regions (e.g. VMH, BSTm, & AI; Figure 2i) that could serve as key functional links between the SBN and MRS to modulate cooperation. Finally, similarities in gene expression between PVN and LS, POM and GCt (Figure 2i) add to the growing evidence that PVN should be considered as an integral player in the SDMN and its regulation of social behaviour.

Our findings provide strong evidence of modularity in the expression of steroid-related genes, particularly in relation to social status, testosterone phenotype or their interaction. We found evidence that status-specific regulation of behaviour could be

modulated directly by testosterone through AR in multiple nuclei (e.g. POM, PVN, BSTm), which is consistent with previous findings of AR expression linked to display performance in golden-collared manakins (Schlinger et al., 2013). The increased expression of the enzyme 5-alpha-reductase (SRD5A2) in LS would lead to the conversion of testosterone to dihydrotestosterone and binding of the AR while preventing the conversion of testosterone to oestradiol and binding of oestrogen receptors (ERs) in this region. Because reductase gene expression is higher in the LS of territorial males (Figure 3b), LS is likely a key region for regulating status-specific responses to testosterone and, ultimately, mediating cooperation. Meanwhile, the conversion of testosterone to oestradiol by the enzyme aromatase and subsequent binding to ERs is a key pathway in the regulation of testosterone-dependent male behaviours (Schlinger & Balthazart, 2013), and we found evidence for the role of aromatase and oestrogen signalling in multiple nuclei. Moreover, differences in connectivity in the steroid and neuropeptide-rich 'navajowhite2' co-expression module among status classes suggest that some of these steroid DEGs are involved in or drivers of larger status-specific differences in regulation.

The co-upregulation of steroid-related genes in the POM of males with high testosterone phenotype (and territorial males) point to this region's role as a potent steroid regulator. The POM is involved in regulating male aggression, sexual behaviour and parental care (O'Connell & Hofmann, 2011), and stimulates gonadal testosterone production through the expression of gonadotropin-releasing hormone (*GnRH*). We observed an increase in GNRH1 transcription in males with high testosterone, which suggests enhanced steroid production in the testes. Indeed, we find a tendency for territorial males to have larger testes and an association between testosterone phenotype and expression in genes that influence steroidogenesis (e.g. AVPR1A and STAR). Further, the activation of POM and its steroid receptor concentrations have been associated with social status differences and social status ascension cichlid fish (Maruska et al., 2013), and mice (Lee et al., 2022). In the same cichlid species, different preoptic cell types, including homologues for PVN, showed higher activation in one of the two partners in cooperative territorial defence (Weitekamp & Hofmann, 2017). Testosterone stimulation of the POM links it to the hypothalamic–pituitary–adrenal (HPA) axis via the PVN (Williamson et al., 2010) – another important endocrine regulator of social status (DuVal & Goymann, 2011; Goymann & Wingfield, 2004; Jones & DuVal, 2021). In our study, the POM and PVN showed similar gene co-expression patterns, and HPA regulatory genes showed appropriate membership to hypothalamic and pituitary modules (Figures S2.5 and S2.12, Tables S2.1 and S2.2), and overall enrichment for genes involved in response to glucocorticoids. The status-specific expression of AR and other steroid-related genes, points to the PVN as a potential mediator of status-specific regulation of cooperative display. Thus, close linkages between the PVN and POM (Williamson et al., 2010), suggest these nuclei could be involved in multiple elements of manakin behaviour depending on cell types, social contexts and timescales investigated.

Our results also highlight the importance of neural VIP expression in modulating multiple aspects of social behaviour (Horton, Michael et al., 2020; Kingsbury & Wilson, 2016), as this neuropeptide system exhibited widespread differential expression patterns across all traits examined (Figure 3b,c). However, we measured VIP preprohormone transcript concentration not final neuropeptide, so follow-up immunohistological work is warranted to discern how transcript expression links with neuropeptide expression. A specifically intriguing finding in our study is the potential involvement of VIP and other steroid receptors in mediating status-specific responses to testosterone in GCt. Although tyrosine hydroxylase (TH) neurons in GCt have been associated with courtship behaviours (Ben-Tov et al., 2023; Goodson et al., 2009), our novel findings point to the association between the VIP system and courtship behaviours in the GCt (Kingsbury & Wilson, 2016). GCt coordinates social stimuli and downstream motor processes that control behaviour. For example, in songbirds, GCt links POM to the song control system and thereby mediates androgen-dependent singing behaviour (Haakenson et al., 2020). Indeed, we observed similar patterns of gene expression in the POM and GCt in association with testosterone phenotype. Given that cooperative display in wire-tailed manakins requires coordinated physical displays (and vocal displays in cooperative *Chiroxiphia* manakins; Trainer et al., 2002), the status-specific differential expression of genes for steroid receptors and steroid-sensitive neuropeptides in GCt suggest that this SDMN region may play a unique role in mediating status-specific relationships between testosterone and cooperative behaviour.

The differential gene expression observed in TnA suggests its role as a hub for regulating testosterone-mediated traits. TnA, the avian homologue of the medial amygdala (O'Connell & Hofmann, 2011), integrates sensory information and regulates diverse social behaviours (Raam & Hong, 2021). Our results may reflect persistent activation of this region, based on the upregulation of IEGs such as JUN and SYT7 in males with high testosterone phenotype, as well as differential regulation of genes involved in long-term synaptic potentiation (Clayton, 2000; Clayton et al., 2019; Marrone et al., 2008; O'Connell et al., 2012). Further, our results implicate potential estrogenic regulation in this region via upregulation of ESR2 in males with higher testosterone phenotype. Estrogenic pathways through ESR1 have been shown to regulate status-specific aggression in birds (Horton et al., 2014; Merritt et al., 2020) and prosocial behaviour in prairie voles (Cushing et al., 2008). Although ESR2 is not well studied in birds, it may function in the medial amygdala of mammals to regulate social recognition (Lymer et al., 2018), and sexual regulation (Nakata et al., 2016), and is associated with higher social rank in the forebrains of cichlid fish (Burmeister et al., 2007). Finally, the fish homologue of TnA appears to mediate aggression and cooperation in cooperative territory defence in an actor-specific manner (Weitekamp et al., 2017; Weitekamp & Hofmann, 2017), supporting a role for modulating cooperation in wire-tailed manakins.

LS showed many differentially expressed genes associated with cooperation, and highly cooperative individuals may show

differences in neural structure based on the enriched GO terms. Patterns of expression in BSTm were similar but weaker based on fewer statistically significant DEGs. BSTm has neural projections into LS, and vasopressin (AVP) and VIP neurons in the BSTm and receptors for these neuropeptides in the LS are associated with affiliative behaviour and gregariousness in birds (Kelly et al., 2011; Kelly & Goodson, 2013; Kingsbury & Wilson, 2016). This suggests cooperative display is regulated by some of the same conserved brain regions as other types of affiliative behaviour. These previous studies focused on the acute processing of social information in LS and BSTm. In contrast, our study suggests that constitutive differences in gene expression and neural structure in these regions may underlie individual variation in cooperative tendencies, potentially affecting their excitability in response to social information.

In sum, our results reveal a variety of mechanisms that could contribute to individual variation in cooperative behaviour, and its status-specific modulation by testosterone. We identified multiple forms of neuroplasticity that are involved, through steroid receptor variation, synaptic potentiation, neural development, as well as cellular activity (Clayton et al., 2019). Indeed, testosterone manipulations are known to induce changes in neuroanatomy in behaviourally relevant brain nuclei (Kabelik et al., 2008), suggesting a direct role for testosterone-mediated neural growth and flexibility in modulating male–male cooperation (Soares et al., 2010). Our results provide promising avenues for further study on the mechanisms underlying cooperative behaviour, such as examination of neuroanatomical features and the use of hormonal or other pharmaceutical manipulations and RNAi technologies to examine causal relationships between neural gene expression and behaviour (e.g. Merritt et al., 2020).

4.2 | Brain-wide expression patterns reflect the role of testosterone as a phenotypic integrator

The pleiotropic effects of testosterone and the interplay of social status with organism immune function and life history could lead to similarities in gene expression among multiple tissues (Anderson et al., 2021, 2022; Newhouse & Vernasco, 2020). The extent of gene expression similarities and differences among tissues may reflect the degree of integration and independence (or modularity), that can either constrain or facilitate adaptive evolution (Cox, 2020; Ketterson et al., 2009; Lipshutz et al., 2019). We identified consistent (systemic) differential expression of specific genes throughout the brain, as well as PC-axes and co-expression modules associated with social status and cooperation. However, our systemic results did not explain as much variation in overall neural gene expression compared with prior work on personality traits (Kabelik et al., 2021; Lattin et al., 2022), suggesting that the brain region-specific patterns may be more important in modulating male wire-tailed manakin behaviour.

The genes and co-expression modules with brain-wide gene expression patterns we described were linked with development, metabolic, immune and telomere gene ontology categories. The representation of these functions particularly among status-associated DEGs and in the 'darkturquoise' module likely reflects differences in allostatic load among status classes in male–male cooperative systems (Goymann & Wingfield, 2004; Jones & DuVal, 2021; Vernasco et al., 2021). The differential global regulation of telomeres by POLD-1 may explain the relative stability of telomere length in territorial wire-tailed manakin males (Vernasco et al., 2021), suggesting metabolic profiles associated with territoriality. The 'darkorange2' module, which showed strong enrichment for other telomere and cellular stress processes, was downregulated in the brains of more-cooperative males, and status-specific differences in gene connectivity. Further, cooperation itself may also have metabolic costs, as more cooperative male manakins have been shown to have shorter telomeres (Vernasco et al., 2021), and social bond strength has been associated with signatures of energy metabolism and stress (Anderson et al., 2022; Simons et al., 2022). Thus, this 'darkorange2' module may be related specifically to status-specific regulation of cooperation, but implication in causality remains an open question.

Our results highlight two additional hormones, prolactin (PRL) and growth hormone (GH1), that may have a system-wide role in the status-specific modulation of cooperative display behaviour. Transcripts for these hormones were differentially expressed in the pituitary and may reflect organism-wide metabolic profiles. The anterior pituitary is the production site for both hormones, and thus gene expression patterns are likely good predictors for circulating levels of those hormones and thus signal strength. PRL transcript was downregulated in more cooperative males. Prolactin is best known for its role in promoting parental care behaviours, including in cooperative breeders (Schoech et al., 1996), but emerging studies suggest other functions such as inhibiting aggression in cooperative breeders (Gilbert et al., 2022; Medger et al., 2019) and promoting nest defence behaviour (Mohamed et al., 2016). The negative relationship observed appears contradictory to the role of prolactin in promoting prosocial behaviour, and may reflect the origin of these cooperative displays from aggressive interactions (Díaz-Muñoz et al., 2014). In addition, growth hormone transcript (GH1) showed higher expression in territorial males, and along with PRL was a hub gene in a HPG co-expression module that showed evidence for status-specific differences in connectivity. This was surprising because growth hormone is typically highly expressed during juvenile growth, yet ascension in social rank in the cooperatively displaying manakins is associated with older age (DuVal, 2007; McDonald, 1989; Ryder et al., 2008). Status differences in GH1 expression may provide an explanation for the consistent patterns of neural development and protein synthesis observed in our results (Sonksen, 2006), and there is a growing appreciation of the role of growth hormone in adult metabolism, tissue repair and lifespan (Berryman et al., 2008). These expression differences could reflect the heightened metabolic demands placed on territorial males during cooperative displays, involving enhanced muscle performance and the regulation of oxidative stress (Hu et al., 2019). The neuroethological role of

growth hormone is poorly understood, but it may be linked to stress (Greenwood & Landon, 1966; McCormick et al., 1998), and has been shown to increase aggression (Johansson et al., 2004; Jönsson et al., 1998; Matte, 1981). The growth hormone inhibition system (via somatostatin) is an androgen-independent modulator of aggression and social status in cichlid fish (Hofmann & Fernald, 2000; Trainor & Hofmann, 2006). Moreover, the growth hormone axis is linked with the HPG axis through the facilitation of sex-steroidogenesis (Cox et al., 2017; Sirotkin, 2005), and natal androgen treatment increased somatostatin receptor expression in zebra finches (Bentz, Niederhuth, et al., 2021). Thus, in wire-tailed manakins growth hormone could be involved in testosterone production or act synergistically with androgens in territorial males potentially inhibit cooperation by promoting aggression. It may also be linked to status-specific regulation of neural development, metabolism and allostasis—functional processes that appeared consistently in our analyses. Future work on social behaviour should consider the interplay between growth hormone, somatostatin, sex-steroids and glucocorticoids to gain new insights into their roles in behavioural regulation.

5 | CONCLUSION

Our study reveals correlations between gene regulation, social status, cooperation and testosterone phenotype across the male wire-tailed manakin's SDMN and HPG axis, involving a plurality of gene functions, including sex-steroid and neuropeptide signalling. Our results suggest that neurogenomic states underpinning male–male cooperation may have origins in pathways for aggression and may involve differences in HPG regulation among status classes. These states could be encoded not only by differences in steroid signalling, but in synaptic potentiation, neural development, metabolism and pathways regulating allostasis. While our findings are correlative, and likely represent a combination of cause and effect, they provide valuable insights into the genomic, endocrine and neural pathways mediating understudied male–male cooperative display behaviours, and its status-specific regulation in this species. This landscape of gene expression speaks to the modular nature of behavioural mechanisms (Goodson, 2005; Lipshutz et al., 2019; Newman, 1999) and highlights the continuum between phenotypic integration and modularity (Ketterson et al., 2009). Unlike previous studies focusing on limited candidate genes or specific brain regions, our work is the most comprehensive examination of patterns across multiple nuclei in the SDMN. Although questions remain on the acute mediators of cooperative display behaviour in the wire-tailed manakin, we demonstrate the power and flexibility of a system-wide approach, and our findings generate hypotheses for candidate genes and pathways for further validation and experiments to implicate causality. We also contribute to the emerging concept that constitutive gene expression landscapes across the brain are associated with consistent behavioural phenotypes (Antunes et al., 2021; Horton, Ryder et al., 2020; Kabelik et al., 2021; Lattin et al., 2022). We propose that landscapes of constitutive gene expression, comprising multiple genes across many tissues and brain regions, form the foundation of consistent among-individual differences in behaviour, upon which flexible and acute responses to social stimuli are built.

AUTHOR CONTRIBUTIONS

TBR, BMH, ITM and CNB designed and funded the research. TBR, RD, BMH, ITM and JLH collected data and performed field and/or laboratory data analysis. PEB led the bioinformatics analysis and writing. CNB and BMH provided supervision. All authors contributed to review and editing of the manuscript.

ACKNOWLEDGEMENTS

We acknowledge long hours of fieldwork in Ecuador by Ben Vernasco and Camilo Alfonso-Cuta that made the behavioural foundation of this work. We thank them, and the staff at Tiputini Biodiversity Station. We thank Eric Schuppe and Matthew Fuxjager for use of laboratory resources. We also thank David Clayton, Julie George, Sarah London and Barney Schlinger for helpful discussions and manuscript reviewing, and Vanessa González for advice on data analysis.

FUNDING INFORMATION

Funding for the reference genome came from a private donor at Millersville University. Funding for the behavioural and RNAseq study came from the National Science Foundation (IOS 1353085; DBI 1457541), the Smithsonian Migratory Bird Center, the Global Change Center at Virginia Tech, Millersville University and the Tiputini Biodiversity Station of the Universidad San Francisco de Quito.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at FigShare (https://doi.org/10.25573/data.22186516) or (https://github.com/periperipatus/PIFI_brain_transcriptome).

DATA AVAILABILITY STATEMENT

Raw read data are available associated with BioProject PRJNA437157, and all code used to conduct analyses is available at FigShare (https://doi.org/10.25573/data.22186516) or (https://github.com/periperipatus/PIFI_brain_transcriptome).

ORCID

Peri E. Bolton https://orcid.org/0000-0002-2057-1973
T. Brandt Ryder https://orcid.org/0000-0002-5517-6607
Roslyn Dakin https://orcid.org/0000-0002-3140-3975
Jennifer L. Houtz https://orcid.org/0000-0002-1022-1398
Ignacio T. Moore https://orcid.org/0000-0001-8875-8913

Christopher N. Balakrishnan https://orcid.org/0000-0002-0788-0659
Brent M. Horton https://orcid.org/0000-0002-3355-1613

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SUPPORTING INFORMATION

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How to cite this article: Bolton, P. E., Ryder, T. B., Dakin, R., Houtz, J. L., Moore, I. T., Balakrishnan, C. N., & Horton, B. M. (2025). Neurogenomic landscape associated with status-dependent cooperative behaviour. *Molecular Ecology*, *34*, e17327. https://doi.org/10.1111/mec.17327