# Neurogenomic landscape associated with status-dependent cooperative behavior

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**Abstract**

The neurogenomic mechanisms mediating male-male reproductive cooperative behaviors are unknown. We leveraged extensive transcriptomic and behavioral data on a neotropical bird (*Pipra filicauda*) that perform cooperative courtship displays to understand these mechanisms. Cooperative display is modulated by testosterone, it promotes cooperation in non-territorial birds, but suppresses cooperation in territory holders. We sought to understand the neurogenomic underpinnings of these three related traits: social status, cooperative behavior, 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 behavior. We associated gene expression with each bird's behavioral 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 signaling appeared to be important in mediating status-specific relationships between testosterone and cooperation, suggesting shared regulatory mechanisms with male aggressive and sexual behaviors. 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 behavior and suggest that differences in neurogenomic state are the basis for individual differences in social behavior.

## Introduction

Cooperation is the synchronized behavior(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 genetics. These effects contribute to repeatable, among-individual variation in behaviors – a behavioral 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 genetic and neurophysiological components that underpin behavioral phenotypes. Understanding these mechanisms enables us to link how genetic 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 behaviors 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 behaviors is only 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 behavioral phenotype are partly achieved through pleiotropic yet modular effects of hormone signaling systems (Cox, 2020; 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, where tissues across the body are modular in their response to hormone signals, enabling diverse and flexible responses to 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 behaviors, including cooperation (Kasper et al., 2017; Soares et al., 2010). The neural substrates modulating social behaviors are 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

72 reflective of all systems engaged in modulating a given behavior. Consistent variation among
73 individuals in neuroendocrine gene expression across the SDMN likely underlies repeatable
74 individual differences in social behavior and behavioral phenotype. For example, variation in the
75 expression of genes involved in sex-steroid signaling pathways across SDMN nuclei can modify
76 the male brain's sensitivity to testosterone, resulting in distinct behavioral responses to a specific
77 hormone signal. Indeed, recent research suggests that constitutive gene expression or
78 neurogenomic state, characterized by co-expressed gene suites in behaviorally relevant brain
79 regions, is associated with behavioral phenotypes (Antunes et al., 2021; Kabelik et al., 2021;
80 Lattin et al., 2021).

81 For the first time, we characterize constitutive gene expression across the neuroendocrine
82 system in relation to variation in a male-male cooperative behavior. The wire-tailed manakin
83 (*Pipra filicauda*) is a neotropical lek breeding bird in which unrelated males perform cooperative
84 displays to attract females (Figure 1A). Territorial and subordinate non-territorial (floater) males
85 form long-term display partnerships and form complex social networks (Figure 1A, B) (Ryder et
86 al., 2008; Ryder, Blake, et al., 2011). Status and cooperation are directly linked to fitness,
87 whereby territory holders with more display partners sire more offspring, and floaters with more
88 partners are more likely to ascend to territorial status (Ryder et al., 2008, 2009). Individuals have
89 a repeatable 'testosterone phenotype' where territorial males tend to have higher testosterone than
90 floaters (Figure 1D), and circulating testosterone has a status-specific effect on the cooperative
91 display behavior (Ryder et al., 2020; Ryder, Horton, et al., 2011). Experimental and
92 observational evidence show that higher testosterone levels are antagonistic to cooperation in
93 territorial males but promote cooperation in floater males (Ryder et al., 2020; Vernasco et al.,
94 2020) (Figure 1C).

95 To explore the neurogenomic basis of the status-specific androgenic modulation of
96 cooperative behavior in these birds, we quantify transcriptome-level gene expression in the
97 SDMN, pituitary and testes of free-living male wire-tailed manakins in the Ecuadorian Amazon.
98 Captive studies can obscure ecologically and evolutionary relevant variation in environmental
99 and behavioral responses (Griffith et al., 2017; Kasper et al., 2017), and we demonstrate the
100 exciting applicability of neuro-transcriptional methods in challenging rainforest conditions. We
101 aim to characterize the genes and neuroendocrine pathways that modulate individual variation in

102 cooperative behavior, social status, and testosterone phenotype as a modifier of both social status
103 and cooperative behavior. Given the androgenic regulation of cooperative display (Ryder et al.,
104 2020; Vernasco et al., 2020), we hypothesize that differences in gene regulation will manifest in
105 SDNM nuclei with high density of sex-steroid receptors and endocrine tissues (Goodson, 2005;
106 Newman, 1999), and will involve expression of candidate genes relating to sex-steroid signaling,
107 metabolism, and neuropeptide systems (Goodson & Thompson, 2010; Kingsbury & Wilson,
108 2016). We anticipate brain region-specific gene expression patterns within the modular SDMN
109 (Figure 1G), as well as systemic effects of testosterone on gene expression across the brain (Cox,
110 2020; Ketterson et al., 2009). We discuss our results in the context of modes of phenotypic
111 evolution and similarities with mechanisms of other cooperative and social behaviors.

## 112 Materials and Methods

113 All methods described here were approved by Smithsonian Animal Care and Use Committee
114 (protocols 12-12,14-25,17-11), as well as the Ecuadorian Ministry of the Environment (MAE-
115 DNB-CM-2015-0008), and samples were transported with export permit (006-016-EXP-1C-
116 FAU-DNB/MA) and import permit (USDA APHIS 126133). Detailed methods can be found in
117 the supplementary material, and full code from analyses in R v 4.0.2 are available on GitHub
118 (https://periperipatus.github.io/PIFI_brain_transcriptome/) and FigShare
119 (10.25573/data.22186516).

### 120 *Field Methods*

121 We sampled 16 wild territorial and floater wire-tailed manakin males for RNA sequencing from
122 Tiputini Biodiversity Station in the lowland Amazon rainforest of Ecuador (Table S1.1). Males
123 were categorized by social status: where the territorial birds (n=9) were males in definitive
124 plumage that held a consistent display territory over the study period, while floaters (n=7) had
125 either definitive or pre-definitive plumage and did not occupy consistent territories within leks
126 (Ryder et al., 2008). We used repeated testosterone sampling on 12 of these males to characterize
127 their testosterone phenotype (Figure 1D, Supplement 1). A male's testosterone phenotype is
128 represented by mean of the residuals of a linear regression from repeated sampling of
129 testosterone concentration, and this measure was previously shown to be repeatable within
130 individuals (Supplement 1 for more detail, see also (Ryder et al., 2020)). Previous research has

131 shown that territorial males have, on average, higher circulating testosterone than floaters (Ryder
132 et al., 2020; Ryder, Horton, et al., 2011), and is reflected in our sampled males (Figure 1D).

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

143 ***Microdissection and RNAseq***

144 Methods for brain extraction, preservation, cryosectioning, microdissection, and RNA
145 extraction followed those published previously (Horton, Ryder, et al., 2020). Pituitaries were
146 preserved in RNAlater™ (Invitrogen) at ambient temperatures until imported into the United
147 States. We excised tissue from 10 different nuclei involved in social behavior – nine of which are
148 from the SDMN. The SDMN reflects the interconnectivity of two networks involved in
149 regulating social behavior (O'Connell & Hofmann, 2011): Social Behavior Network (SBN)
150 (Goodson, 2005; Newman, 1999) and the Mesolimbic Reward System (MRS) (O'Connell &
151 Hofmann, 2011; Olds & Milner, 1954). Figure 1F indicates the nomenclature used and the
152 relationships of these nuclei to the SBN and MRS. To clarify bird specific nomenclature: the
153 arcopallium intermedium (AI) is a region that may be, in part, homologous the basolateral
154 amygdala of mammals, a node of the MRS (O'Connell & Hofmann, 2011), which is
155 hypothesized to play a role in androgen-dependent manakin display behavior (Fusani et al.,
156 2014); and the nucleus taenia (TnA) is homologous to the mammalian medial amygdala
157 (O'Connell & Hofmann, 2011). The paraventricular nucleus (PVN) is not yet formally
158 recognized as part of the SDMN, despite its neuropeptide projections to nodes of the SDMN and
159 established role as a major regulator of vertebrate social behavior (Goodson & Kingsbury, 2013;
160 Goodson & Thompson, 2010). We additionally sampled the pituitary gland and testes
161 immediately after the brains. Testes were handled the same as brains (Horton, Ryder, et al.,

162 2020) and pituitaries were preserved in RNAlater™ (Invitrogen) at ambient temperatures until
163 imported into the United States.

164 Microdissected tissues from both hemispheres were combined for sequencing. RNA was
165 extracted and sequenced according to previously described methods (Horton, Ryder, et al.,
166 2020). RNA libraries for the various tissues and field seasons were randomized amongst
167 sequencing batches (Supplement 1, Table S1.1 & S1.2). All samples analyzed are associated
168 with BioProject PRJNA437157, and SRR12660169-198, SRR19521260-271, SRR19521432-
169 575.

170 ***Gene expression analyses***

171 Reads were aligned to the annotated *Pipra filicauda* genome (GCA_003945595.1) using
172 splice-aware aligner STAR v2.7.5 (Dobin et al., 2013), and gene-level counts were obtained
173 from featureCounts v2.0.1 (Liao et al., 2014). A total of 170 libraries were retained after quality
174 filtering in each tissue (Supplement 1 & 3), but the number of individuals used in each tissue
175 varies (Table S1.1 & S1.2). To further characterize tissue and brain nucleus function in this
176 species (Horton, Ryder, et al., 2020), we characterized differences in gene expression among
177 tissues and brain nuclei using principal components analysis in PCAtools v2.8.0 and Weighted
178 Gene Co-Expression Analysis (WGCNA v1.71) (Blighe & Lun, 2020; Langfelder & Horvath,
179 2008) (Supplement 2). All analyses were conducted in R v 4.0.2 (R Core Team, 2020).

180 To describe the role of tissues and brain nuclei in relation to hormonal and behavioral
181 phenotypes we analyzed differential expression using DESeq2 v1.36 (Love et al., 2014) and co-
182 expression using WGCNA (Langfelder & Horvath, 2008) in each tissue and nucleus separately.
183 For a subset of tissues, we had an extra four individuals characterized for social status from a
184 previous study (Horton, Ryder, et al., 2020), for which behavioral and hormone sampling data
185 were unavailable. Owing to this, and to not overparameterize the models that account for batch
186 effects, we identified differentially expressed genes (DEGs) associated with each of our traits of
187 interest (social status, testosterone phenotype and social network strength) separately. These
188 were run as separate negative binomial generalized linear models for each trait that accounted for
189 batch effects where necessary using DESeq2 (Love et al., 2014) (For detailed description see
190 Supplement 3) Testosterone and social network strength were treated as continuous variables.
191 Significant DEGs were considered to be correlated with the predictor variable, and $Log_2$ Fold

192 Change (LFC) value represents the LFC of gene expression per unit of the predictor variable. In
193 all cases, significant DEGs were identified using the Wald Test and the default FDR corrected p-
194 value ($q<0.1$). The identity of many hub genes identified by WGCNA were similar to DEGs
195 identified byDESeq2, so for brevity and simplicity we focus mostly on the differential expression
196 results, but the full results are presented in Supplement 5 (WGCNA) and Supplement 3
197 (differential expression).

198 Due to the key role testosterone plays in modulating behavior in this species (Ryder et al.,
199 2020), we further analyzed nine candidate genes involved in sex-hormone metabolism and
200 signaling (Figure S4.1), as well as nine steroid sensitive neuropeptides and their receptors
201 (Figure S4.2; Table S1.3). Candidate genes showing differential gene expression with an
202 uncorrected $p < 0.05$ was considered "significant" and examined further. In addition to analyzing
203 models of status, mean testosterone and social network strength separately, we also explored
204 whether candidate genes were involved in mediating status-specific relationships between mean
205 testosterone and cooperative behavior (Ryder et al., 2020) by extracting "significant" candidate
206 genes from a final DESeq2 interaction analysis (model: expression ~ status x mean testosterone).
207 We primarily used this model to identify candidate genes with status-specific expression rather
208 than discovering new DEGs due to overfitting concerns. The resulting putative gene-trait
209 relationships were plotted (Supplement 4), and those with highly influential observations or $R2 \leq$
210 $0.2$ were excluded. For candidates derived from the interaction effect models or genes that were
211 associated with multiple traits, the most convincing relationship was determined using a
212 stepdown AIC procedure (Supplement 4). Considering the smaller set of candidate genes,
213 uncorrected p-values were deemed significant ($p<0.05$), but we also report the FDR corrected
214 values.

215 To assess neuroanatomical constraints on gene expression patterns, we examined overlap
216 among brain nuclei by comparing ranked gene-lists (Supplement 6). The gene-lists were ranked
217 using -log10 transformed p-values multiplied by the expression direction. Similarity between
218 tissue pairs was measured using Pearson correlation and rank-rank hypergeometric overlap
219 (RRHO) test (Cahill et al., 2018).

220 Testosterone has pleiotropic effects, influencing gene expression in similar ways in
221 multiple tissues (Cox, 2020; Ketterson et al., 2009). We reasoned that these effects would be

222 reflected in shared (systemic) signatures of differential expression across brain nuclei. To
223 explore systemic gene expression patterns, we calculated median p-values from the previous
224 analysis and identified genes consistently differentially expressed across the entire brain (p-value
225 $< 0.05$, transformed value $> |1.3|$). Further, we correlated eigenvalues of each principal
226 component (PC) from the whole-brain PCA analysis (Figure 1G inset) with our variables of
227 interest using pcatools::eigencorplot() (Blighe & Lun, 2020) (Supplement 1). Correlations with
228 social status were validated by a linear mixed model in the nlme package v3.1 (Pinheiro et al.,
229 2016), which included individual as a random effect and accounted for correlated batch variables
230 (Supplement 1).

231 We used clusterProfiler v3.16.1(Yu et al., 2012) to determine patterns of functionality in
232 DEGs and WGCNA modules with Gene Ontology enrichment analyses. If the total number of
233 FDR corrected DEGs was less than 40, we used gene set enrichment analysis instead which
234 leverages the rank order of p-values and direction of expression (Supplement 3).

235

236 **Results**

237 As expected, gene expression analyses revealed substantial differences among tissues and
238 brain regions (Figure 1G; Figure S2.7). These expression patterns were more similar among
239 adjacent nuclei, forming major brain-region groupings of the amygdala, hypothalamus, and
240 midbrain (Figure 1G). Additionally, our 18 candidate neuroendocrine genes (Table S1.3)
241 displayed tissue and brain region-specific expression (Table 2.2, Figures S4.1-4.2).

242 ***Social status and testosterone phenotype are associated with tissue and brain region-specific***
243 ***patterns of gene expression***

244 We observed a landscape of gene expression in association with testosterone phenotype
245 (Figure 2A) and social status (Figure 2B), with a varying number of differentially expressed
246 genes (DEGs) or co-expression modules identified across different tissues. Similarly, across
247 brain regions, we identified relationships between many of our 18 candidate genes and status,
248 testosterone phenotype, or their interaction (Figure 2C). Mean testosterone was a continuous
249 predictor in DESeq2, as such DEGs indicate up-or downregulation with a higher testosterone
250 phenotype (Figure 2A), while DEGs identified in social status indicated up or down regulation in
251 territorial males (Figure 2B). Here, we primarily focus on the tissues with large numbers of

252 DEGs and significant candidate genes for the main manuscript, but the co-expression results
253 were similar to our differential expression results, and all results are shown in Supplements 3 and
254 5. The number of DEGs in each tissue showed a similar pattern between testosterone phenotype
255 and status compared to other trait comparisons ($R = 0.5$, $p = 0.08$, Figure S3.7), which was
256 consistent across co-expression modules (Supplement 5) and candidate gene analysis (Figure 2C
257 & Figure 3B). This similarity was expected due to the correlation between testosterone and status
258 (Figure 1D) (Ryder et al., 2020; Ryder, Horton, et al., 2011).

259 The testes and TnA each had over 1,000 genes differentially expressed (at uncorrected p-
260 value $< 0.05$) in association with both testosterone phenotype and status (144 and 252 genes after
261 FDR correction $q<0.01$ in association with mean testosterone; 2 and 13 after FDR correction in
262 association with social status). DEGs identified in association with testosterone phenotype in the
263 testes were enriched for genes involved with response to corticosterone (GO:0051412) and
264 synaptic transmission and organization (e.g., GO:0007268, GO:0050808) among others (Figure
265 S3.20). Notable DEGs included steroidogenic acute regulatory protein (STAR, LFC = -1.1, p =
266 $2x10^{-7}$, $q = 0.0003$, $R^2 = 0.6$) and candidate gene AVPR1A (LFC = 0.8, $p = 5x10^{-5}$, $q = 0.01$, $R^2 =$
267 0.7; Figure S4.4). Note that $Log_2$ Fold Change (LFC) represents a change in gene expression per
268 unit of the predictor variable. These transcriptional differences do not correspond to significant
269 differences in testis size in relation to testosterone phenotype (Table S3.2), nor social status
270 (Table S3.1 & S3.2).

271 Among the 252 DEGs associated with testosterone phenotype in TnA, there was non-
272 significant enrichment for genes involved in neural crest migration (GO:0001755), learning and
273 memory (GO:0007613, GO:0007612), and long-term synaptic potentiation (e.g., GO:0060291)
274 (Figure S3.28). Notably, the candidate gene estrogen receptor 2 (ESR2; aka Erβ) was
275 upregulated in TnA of males with higher testosterone (Figure 2C; LFC = 0.8, $p = 0.001$, $q =$
276 $0.08$, $R^2 = 0.4$). Other notable DEGs in TnA included upregulation of immediate early gene JUN,
277 also a 'coral1' hub gene (module membership (MM) = 0.93; LFC = 0.61, $p = 0.0004$, $q = 0.05$)
278 and corticotropin releasing hormone receptor 2 (CRHR2, LFC = 1, $q = 0.006$, $R^2 = 0.5$).

279 Not all nuclei showed similar patterns between status and testosterone phenotype. For
280 example, ICo of the midbrain showed 161 DEGs associated with testosterone phenotype after
281 FDR correction but did not have substantial association with social status (3 DEGs). Enriched

282 GO terms in ICo were primarily related to motor proteins and cilia (e.g., GO:0003341, Figure
283 S3.26). Intriguingly, although the POM did not reveal a high number of DEGs in association
284 with testosterone phenotype (45), our analysis revealed differential expression for compelling
285 gene ontology categories and candidate neuroendocrine genes (Figure S3.25, Figure S4.10,
286 Figure S5.7). Notably, males with higher testosterone exhibited upregulation of candidate gene
287 androgen receptor (AR) in POM (Figure 2D). The 'floralwhite' module, which comprised many
288 upregulated DEGs, was significantly associated with both testosterone phenotype and, to a lesser
289 extent, social status (Figure S5.6). This module showed enrichment for biological processes
290 related to G protein-coupled receptor signaling pathways (GO:0007186) and neuropeptide
291 signaling pathway (GO:0007218), with statistically non-significant enrichment for steroid
292 receptor signaling (GO:0030518), learning and memory (GO:0007611), and nervous system
293 development (GO:0007399) (Figure S3.7). Indeed, many of the shared hub-genes and DEGs
294 interact with candidate sex hormone pathways, such as prolactin releasing hormone (PRLH, MM
295 = 0.91, LFC = 2.4, q = $5x10^{-5}$, $R^2$ = 0.6, Figure S3.25A), growth regulating estrogen receptor
296 binding 1 (GREB1, MM = 0.86, LFC = 0.6, q = $3x10^{-5}$, $R^2$ = 0.7, Figure S3.25A), and
297 gonadotropin releasing hormone 1 (GNRH1, MM = 0.76, LFC = 0.4, p = 0.006, q = 0.2, $R^2$ =
298 0.4). Other notable 'floralwhite' hub genes include the candidate gene progesterone receptor
299 (PGR, MM = 0.81) which was also associated with social status (Figure 2C; LFC = 0.4 p =
300 0.006, q = 0.7) and serotonin receptor 5A (HTR5A, MM = 0.87, LFC = 0.4, p = 0.04, q = 0.6).
301 Candidate gene aromatase (LOC113993669/CYP19A1) was also upregulated in males with
302 higher testosterone phenotype (Figure 2C, LFC = 0.6, p = 0.02, q = 0.4, $R^2$ = 0.5), but was found
303 in the 'lightcoral' co-expression module (MM= 0.86).

304 Despite overall similarity in gene expression associations with testosterone phenotype
305 and status, certain brain regions exhibited stronger associations with either status or the
306 interaction between status and testosterone phenotype (Figure 2B). Likewise, the 18 candidate
307 neuroendocrine genes also showed varying associations with status or the interaction across
308 different nuclei (Figure 2C). PVN of the hypothalamus showed the most DEGs associated with
309 social status after FDR correction (42). These DEGs were enriched for cytoskeletal movement
310 genes involved in intracellular transport and cilia (e.g., GO:0003341, GO:0061640), and non-
311 significant enrichment for cholesterol biosynthesis (GO:0006695, GO:0045540) (Figure
312 S3.12B). Candidate gene expression also revealed status-specific associations with testosterone

313 phenotype in the PVN, that is a statistical interaction between status and testosterone phenotype
314 (Figure 2C). In particular, AR expression was positively correlated with testosterone phenotype
315 in floater males, but not in territorial males (Figure 2F; LFC = -0.7, p = 0.008, q = 0.9). The PVN
316 of territorial males also showed higher VIPR1 (Figure S4.8; LFC = 0.3, p = 0.02, q = 0.4) and
317 lower ESR2 expression (LFC = -0.6, p = 0.006, q = 0.2). In BSTm, a region that bridges the
318 SDMN and MRS, there were more than 1000 DEGs, but only nine after FDR correction. Among
319 these, territorial males showed higher AR expression compared to floater males (Figure 2E; LFC
320 = 0.6, p = 0.0007, q =0.2). Further, testosterone metabolism gene 5-alpha reductase (SRD5A2)
321 was upregulated in the LS of territorial males (Figure S4.20; LFC = 0.7, p = 0.0005, q = 0.4). In
322 the TnA, the VIP system was implicated in status-specific responses to testosterone, with VIPR1
323 upregulated in association with testosterone in territorial males (Figure S4.17; LFC = 1, p = 0.04
324 q = 0.7).

325 GCt was a hotbed for gene expression associated with social status and status ×
326 testosterone phenotype interaction (Figure 1B, Figure S3.6). In relation to social status, gene set
327 enrichment analysis revealed a significant downregulation of genes involved in synapse
328 assembly (GO:0051965), response to estradiol (GO:0032355), as well as upregulation of genes
329 related to RNA processing (e.g., GO:0000184, GO:0006396), and translation (e.g., GO:0006413,
330 GO:0006412) (Figure S3.15). Candidate neuroendocrine genes showed status specific expression
331 in association with testosterone phenotype in this region including progesterone receptor (PGR,
332 Figure S4.15; LFC = 1.9, p= 0.003, q = 0.1), estrogen receptor 1 (ESR1; aka Erα; LFC = 1.1 p =
333 0.04, q = 0.4), prolactin receptor (PRLR; LFC = 0.7, p = 0.01, q = 0.2), and vasoactive intestinal
334 peptide (VIP; Figure S4.15; LFC = 1.6, q = 0.005, q = 0.1). Further, aromatase was
335 downregulated with higher testosterone phenotype in the GCt (LFC = -1.3, p = 0.002, q = 0.3, $R^2$
336 = 0.4).

337 ***Cooperative behavior is associated with a distinct gene expression landscape***

338 Social network strength (the number of cooperative interactions between individuals)
339 varies widely among territorial and floater males (Figure 1E), representing a key dimension of
340 social behavior in this species. We predicted that the associations between gene expression and
341 cooperative behavior would be different from those observed for social status and testosterone
342 phenotype. Indeed, across tissues we found a distinct landscape of constitutive gene expression

343 in relation to cooperative behavior (Figure 3A, Figure S3.5), as well as among candidate genes
344 (Figure 3B). AH had the highest number of DEGs associated with a male's network strength,
345 with non-significant enrichment for genes involved in RNA metabolism (e.g., GO:0043928) and
346 regulation of translation (e.g., GO:0017148), development (e.g., GO:0061061, GO:0042472) and
347 synaptic potentiation (e.g., GO:0007274) (Figure S3.35). The pituitary also showed an
348 abundance of differentially expressed genes associated with male's network strength (109 genes
349 after FDR correction $q < 0.1$), with non-significant enrichment for actin filament organization
350 (GO:0007015), neural differentiation (e.g., GO:0031103, GO:0045664) and the cell cycle (e.g.,
351 GO:0043065, GO:0071158.) (Figure S3.33B). Within the pituitary, candidate genes prolactin
352 (PRL) (Figure 3C; LFC = -0.6, $p=5x10^{-5}$ $q = 0.03$, $R^2 = 0.7$), ESR2 (Figure S4.5; LFC = -0.7, p =
353 0.04, $q = 0.5$, $R^2 = 0.5$) and VIPR2 (Figure S4.5; LFC = -0.4, $p = 0.008$, $q = 0.3$, $R^2 = 0.4$) were
354 downregulated in highly cooperative males. Intriguingly, in the PVN there was a weak negative
355 correlation between the expression of the candidate gene oxytocin (OXT) and network strength
356 (Figure 3D; LFC = -0.3, $p = 0.04$, $q = 0.6$, $R^2 = 0.2$). Furthermore, although there was less
357 representation of sex-steroid related pathways in the candidate gene analysis (Figure 2B), PGR
358 was downregulated in males with higher social network strength in BSTm (Figure 2E; LFC = -
359 0.3, $p = 5x10^{-4}$, $q = 0.2$, $R^2 = 0.5$).

360 Many genes in the LS were differentially expressed in association with an individual's
361 social network strength (98 genes after FDR correction $q < 0.1$). Among these, the top non-
362 significant terms were broadly involved in development (e.g., GO:0048538, GO:0048593), and
363 neural organization (e.g., GO:0099175, GO:0050771) (Figure S3.42). No candidate genes were
364 linked to social network strength in LS. Although few genes were differentially expressed in
365 BSTm related to social network strength, RRHO analysis showed that LS and BSTm shared
366 similar expression associations with strength (Cahill et al., 2018) (Figure 3G, F). In contrast, LS
367 and BSTm showed limited similarity in gene expression related to testosterone phenotype
368 (Figure 4H, I) or social status (Figure S6.1A), suggesting their unique role in modulating
369 cooperative behavior. Pairwise correlations of gene expression associated with testosterone
370 phenotype aligned with neuroanatomical similarity (Figure 3H) possibly reflecting similarities in
371 pathways related to sex-steroid signaling (Figure 2C), but LS/BSTm similarity was the only
372 neuroanatomical signal in pairwise gene expression associated with strength.

373 ***Testosterone phenotype and social status are associated with systemic gene expression***
374 We reasoned that testosterone, with its pleiotropic effects and role in social status in
375 manakins (Ryder et al., 2020) would have systemic impacts on gene expression, resulting in
376 similar expression patterns across multiple regions. Social status and testosterone phenotype
377 showed the highest number of consistently differentially expressed genes across tissues (60 and
378 66 genes with median uncorrected $p < 0.05$ respectively), while social network strength had only
379 10 consistently differentially expressed genes . Further, PC5 of the whole brain analysis was
380 significantly associated with social status in a linear mixed model, accounting for batch
381 confounds and repeated sampling of individuals (Figure S2.5). There was overlap between genes
382 and GO categories among the consistently differentially regulated genes with social status and
383 testosterone phenotype, as well as with genes associated with PC5. But hereon we focus on
384 genes related to social status for simplicity (see Supplement 6).

385 The top two consistently differentially regulated genes were upregulation of the immune
386 system gene CLEC2B in the brains of territorial males (Figure 4A) and downregulation of the
387 development gene ROBO4 (Figure 4B). Enrichment analysis reflected the functional categories
388 of these genes, although no significant terms were found after FDR correction (Figure 4C). There
389 was also evidence for global differential regulation of telomere maintenance genes (Figure 4C),
390 with downregulation of POLD-1-like (LOC114000574) and upregulation of RAD51 in territorial
391 males. Further, there was evidence for global regulation of cholesterol biosynthesis (Figure 4C),
392 and glutathione transferases (Figure S2.6, Table S6.2), both of which are involved in steroid
393 hormone biosynthesis. These findings of global expression differences among status classes
394 could be partially explained by the upregulation of growth hormone (GH1) in the pituitary of
395 territorial males (Figure 4D; LFC = 0.8, $p = 3x10^{-5}$, $q = 0.01$), which remained consistent in a
396 model accounting for the interaction between social status and mean T (LFC = 0.9, $p = 6x10^{-5}$, q
397 = 0.05).

398 **Discussion**
399 Using the most comprehensive transcriptome-level sampling of the SDMN to-date
400 (Antunes et al., 2021; Bentz, George, et al., 2021; Bentz, Niederhuth, et al., 2021; Kabelik et al.,
401 2021; Lopes & König, 2020), our findings highlight both modular and integrative neurogenomic
402 organization underlying individual variation in cooperative behavior. The relative roles of each

are central to the development and expression of any complex trait (Cox, 2020; Ketterson et al., 2009; Lipshutz et al., 2019).

**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 genetic 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 signaling 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 that 'landscape' model, it is the pattern of IEG expression across multiple brain nuclei that correlates with behavioral responses, rather than the up or downregulation of particular genes within a single nucleus. This original model considered this landscape of IEG expression (and later neuropeptides) in the context of proximate responses to social stimuli. Our findings extend this model in two ways: by considering transcriptomic variation and how long-term neurogenomic states underlie individual variation in social behavior.

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. 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 behavior (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.,

433 2019; George et al., 2020). These mechanisms may interact with variation in elements of sex-
434 steroid signaling that we investigated in detail (*sensu* "neuroendocrine action potential" (Clayton
435 et al., 2019)).

436 Our findings provide strong evidence of modularity in the expression of steroid-related
437 genes, particularly in relation to social status, testosterone phenotype, or their interaction. We
438 found evidence that status-specific regulation of behavior could be modulated directly by
439 testosterone through AR in multiple nuclei (e.g., POM, PVN, BSTm), which is consistent with
440 previous findings of AR expression linked to display performance in golden-collared manakins
441 (B. A. Schlinger et al., 2013). Further, the conversion of testosterone to estradiol by the enzyme
442 aromatase and subsequent binding to estrogen receptors is a key pathway in the regulation of
443 testosterone-dependent male behaviors (B. Schlinger & Balthazart, 2013), and we found
444 evidence for the role of aromatase and estrogen signaling role in multiple nuclei.

445 The co-upregulation of steroid related genes in the POM of males with high testosterone
446 phenotype (and territorial males) point to this region's role as a potent steroid regulator. The
447 POM is involved in regulating male aggression, sexual behavior and parental care (O'Connell &
448 Hofmann, 2011), and stimulates gonadal testosterone production through gonadotropin releasing
449 hormone (*GnRH*). Interestingly, we observed increase in GNRH1 transcription, which suggest
450 enhanced steroid production in the testes. Our finding of co-upregulation of AR and GNRH1
451 may seem contradictory due to the negative regulatory effects of androgens and AR on *GnRH*
452 (Brayman et al., 2012). However, these may not interact at the cell population level or may show
453 different temporal patterns of translation. The activation of POM and its steroid receptor
454 concentrations have been associated with social status differences and social status ascension
455 cichlid fish (Maruska et al., 2013), and mice (Lee et al., 2022). In the same cichlid species,
456 different preoptic cell types, including homologs for PVN, showed higher activation in one of the
457 two partners in cooperative territorial defense (Weitekamp & Hofmann, 2017). Testosterone can
458 link the POM to the Hypothalamic Pituitary Adrenal (HPA) axis via the PVN (Williamson et al.,
459 2010) – another important endocrine regulator of social status (DuVal & Goymann, 2011;
460 Goymann & Wingfield, 2004; Jones & DuVal, 2021). In our study, the PVN showed status-
461 specific gene expression many genes, including AR, and could be an important mediator of
462 status-specific regulation of cooperative display. Thus, close linkages between the PVN and

463 POM (Williamson et al., 2010), suggest these nuclei could be involved in multiple elements of
464 manakin behavior depending on cell types, social contexts and timescales investigated.

465 Our results also highlight the importance of neural VIP expression in modulating multiple
466 aspects of social behavior (Horton, Michael, et al., 2020; Kingsbury & Wilson, 2016), as this
467 neuropeptide system exhibited widespread differential expression patterns across all traits
468 examined. An intriguing finding in our study is the potential involvement of VIP and other
469 steroid receptors in mediating status-specific responses to testosterone in GCt. Although tyrosine
470 hydroxylase (TH) neurons in GCt have been associated with courtship behaviors (Ben-Tov et al.,
471 2021; Goodson et al., 2009), our novel findings point to the association between VIP and
472 courtship behaviors in the GCt (Kingsbury & Wilson, 2016). GCt coordinates social stimuli and
473 downstream motor processes that control behavior. For example, in songbirds, GCt links POM to
474 the song control system and thereby mediates androgen-dependent singing behavior (Haakenson
475 et al., 2020). Given that cooperative display in wire-tailed manakins requires coordinated
476 physical (and vocal displays in cooperative *Chiroxiphia* manakins (Trainer et al., 2002)), the
477 status-specific differential expression of genes for steroid receptors and steroid-sensitive
478 neuropeptides in GCt suggest that this SDMN region may play a unique role in mediating status-
479 specific relationships between testosterone and cooperative behavior.

480 The differential gene expression observed in TnA suggests its role as a hub for regulating
481 testosterone-mediated traits. TnA, the avian homolog of the medial amygdala (O'Connell &
482 Hofmann, 2011), integrates sensory information and regulates diverse social behaviors (Raam &
483 Hong, 2021). Our results may reflect persistent activation of this region, based on the
484 upregulation of IEGs such as JUN and SYT7 in males with high testosterone phenotype, as well
485 as differential regulation of genes involved in long-term synaptic potentiation (Clayton, 2000;
486 Clayton et al., 2019; Marrone et al., 2008; O'Connell et al., 2012). Further, our results implicate
487 potential estrogenic regulation in this region via upregulation of ESR2 in males with higher
488 testosterone phenotype. Estrogenic pathways through ESR1 have been shown to regulate status-
489 specific aggression in birds (Horton et al., 2014; Merritt et al., 2020) and prosocial behavior in
490 prairie voles (Cushing et al., 2008). Although ESR2 is not well studied in birds, it may function
491 in the medial amygdala of mammals to regulate social recognition (Lymer et al., 2018), and
492 sexual regulation (Nakata et al., 2016), and is associated with higher social rank in the forebrains

493 of cichlid fish (Burmeister et al., 2007). Finally, the fish homolog of TnA appears to mediate
494 aggression and cooperation in cooperative territory defense in an actor-specific manner
495 (Weitekamp et al., 2017; Weitekamp & Hofmann, 2017), supporting a role for modulating
496 cooperation in wire-tailed manakins.

497 There were distinct patterns of gene expression across sampled tissues in association with
498 social network strength (time spent interacting with other males), and some of these signals
499 suggest derivation of cooperative display from aggressive behaviors (Díaz-Muñoz et al., 2014).
500 Many genes were differentially expressed in AH in association with strength, a region primarily
501 linked to regulation of aggression through AVP and VIP (Horton, Michael, et al., 2020;
502 O'Connell & Hofmann, 2011). Although these were not differentially expressed in AH, we
503 found a weak negative relationship between OXT expression and social network strength in
504 PVN. This negative relationship is contradictory to oxytocin's role in promoting sociality (as in
505 humans (Feldman, 2012). The relationship observed in the wire-tailed manakin suggests that
506 oxytocin inhibits cooperation, further supporting the hypothesis that cooperation is derived from
507 aggressive or dominance displays in this species. However, nonapeptides (like OXT and AVP)
508 have complex and sometimes species-specific relationships with affiliative behavior and social
509 status (Goodson & Thompson, 2010; Lee et al., 2022; Maruska et al., 2022). Thus, further
510 research is needed to clarify the role of nonapeptides in this species.

511 LS showed many differentially expressed genes associated with cooperation, and highly
512 cooperative individuals may show differences in neural structure based on the enriched GO
513 terms. Patterns of expression in BSTm were similar, but weaker based on fewer statistically
514 significant DEGs. BSTm has neural projections into LS, and vasopressin (AVP) and VIP
515 neurons in the BSTm and receptors for these neuropeptides in the LS are associated with
516 affiliative behavior and gregariousness in birds (Kelly et al., 2011; Kelly & Goodson, 2013;
517 Kingsbury & Wilson, 2016). This suggests cooperative display is regulated by some of the same
518 conserved brain regions as other types of affiliative behavior. Those studies focused on the acute
519 processing of social information in these nuclei. In contrast, our study suggests that constitutive
520 differences in gene expression and neural structure in these regions may underlie individual
521 variation in cooperative tendencies, potentially affecting their excitability in response to social
522 information.

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

534 **Brain-wide expression patterns reflect the role of testosterone as a phenotypic**
535 **integrator.**

536 The pleiotropic effects of testosterone are predicted to lead to similarities in gene
537 expression among tissues, supporting its role as a mediator of phenotypic integration. The extent
538 of gene expression similarities and differences among tissues may reflect the degree of
539 integration and independence (or modularity), that can either constrain or facilitate adaptive
540 evolution (Ketterson et al., 2009; Lipshutz et al., 2019). We identified similarities in testosterone
541 associated gene expression between brain regions that broadly recapitulated tissue ontology.
542 Additionally, we identified consistent (systemic) gene expression differences throughout the
543 brain, predominantly associated with social status and testosterone phenotype. However,
544 systemic results did not explain as much variation in overall neural gene expression compared
545 with certain personality traits (Kabelik et al., 2021; Lattin et al., 2021), suggesting that the brain
546 region-specific patterns may be more important in modulating male wire-tailed manakin
547 behavior. The association between social status and metabolic, immune, and telomere gene
548 ontology categories reflect differences in allostatic load among status classes in male-male
549 cooperative systems (Goymann & Wingfield, 2004; Jones & DuVal, 2021; Vernasco et al.,
550 2021). The differential global regulation of telomeres may explain the relative stability of
551 telomere length in territorial wire-tailed manakin males (Vernasco et al., 2021), suggesting
552 metabolic profiles associated with territoriality. Cooperation itself may also have metabolic
553 costs, as more cooperative male manakins have been shown to have shorter telomeres (Vernasco

554 et al., 2021), and social bond strength has been associated with signatures of energy metabolism
555 and stress (Anderson et al., 2022; Simons et al., 2022).

556 Our results highlight two additional hormones, prolactin and growth hormone, that may
557 have a system-wide role in the status-specific modulation of cooperative display behavior.
558 Transcripts for these hormones were differentially expressed in the pituitary, and may reflect
559 organism wide metabolic profiles. The anterior pituitary is the production site for both hormones,
560 and thus gene expression patterns are likely good predictors for circulating levels of those
561 hormones and thus signal strength. PRL transcript was downregulated in more cooperative
562 males. Prolactin is best known for its role in promoting parental care behaviors, including in
563 cooperative breeders (Schoech et al., 1996), but emerging studies suggest other functions such as
564 inhibiting aggression in cooperative breeders (Gilbert et al., 2022; Medger et al., 2019) and
565 promoting nest defense behavior (Mohamed et al., 2016). The negative relationship observed
566 appears contradictory to the role of prolactin in promoting prosocial behavior, and again, may
567 reflect the origin of these cooperative displays from aggressive interactions (Díaz-Muñoz et al.,
568 2014). In addition, growth hormone transcript (GH1) showed higher expression in territorial
569 males. This was surprising because ascension in social rank in the cooperatively displaying
570 manakins is associated with age (DuVal, 2007; McDonald, 1989; Ryder et al., 2008) and GH1
571 expression is higher in older territorial males. However, in *Anolis* lizards, stimulation of the
572 growth hormone axis is associated with increased testosterone that enables phenotypic
573 divergence between males and females (Cox et al., 2017). In manakins growth-hormone could
574 thus be involved in testosterone production and have subtle effects on multiple systems enabling
575 the status-specific regulation of behaviors. Status differences in GH1 expression may provide an
576 explanation for the consistent patterns of neural development and protein synthesis observed in
577 our results (Sonksen, 2006). These expression differences could reflect the heightened metabolic
578 demands placed on territorial males during cooperative displays, involving enhanced muscle
579 performance and the regulation of oxidative stress (Hu et al., 2019). The neuroethological role of
580 growth hormone is poorly understood, but it may be linked to stress (Greenwood & Landon,
581 1966; McCormick et al., 1998), and has been shown to increase aggression (Johansson et al.,
582 2004; Jönsson et al., 1998; Matte, 1981). While the growth hormone inhibition system (via
583 somatostatin) is an androgen-independent modulator of aggression and social status in cichlid
584 fish (Hofmann & Fernald, 2000; Trainor & Hofmann, 2006), and natal androgen treatment

585 increased somatostatin receptor expression in zebra finches (Bentz, Niederhuth, et al., 2021).
586 Thus, our results suggest that the growth hormone axis may play a role in the status-specific
587 regulation of wire-tailed manakin cooperative displays, where increased GH and androgens in
588 territorial males potentially inhibit cooperation by promoting aggression. Future work on social
589 behavior should consider the interplay between growth hormone, somatostatin, sex steroids, and
590 glucocorticoids to gain new insights into their roles in behavioral regulation.

591 **Conclusion**

592 Our study reveals correlations between gene regulation, social status, cooperation, and
593 testosterone phenotype across the male wire-tailed manakin's SDMN and HPG axis, involving a
594 plurality of gene functions, including sex-steroid and neuropeptide signaling. This landscape of
595 gene expression speaks to the modular nature of behavioral mechanisms (Goodson, 2005;
596 Lipshutz et al., 2019; Newman, 1999) and highlights the continuum between phenotypic
597 integration and modularity (Ketterson et al., 2009). Unlike previous studies focusing on limited
598 candidate genes or specific brain regions, our work is the most comprehensive examination of
599 patterns across multiple nuclei in the SDMN. While our findings are correlative, they provide
600 valuable insights into the nuclei, genomic pathways and endocrine mechanisms underlying
601 understudied male-male cooperative behaviors, and its status-specific regulation in this species.
602 Although questions remain on the acute mediators of cooperative display behavior in the wire-
603 tailed manakin, we demonstrate the power and flexibility of a system-wide approach, and our
604 findings generate hypotheses for further experiments to implicate causality. We also contribute
605 to the emerging concept that constitutive gene expression landscapes across the brain are
606 associated with consistent behavioral phenotypes (Antunes et al., 2021; Kabelik et al., 2021;
607 Lattin et al., 2021). We propose that landscapes of constitutive gene expression, comprising
608 multiple genes across many tissues and brain regions, form the foundation of consistent inter-
609 individual differences in behavior, upon which flexible and acute responses to social stimuli are
610 built.

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

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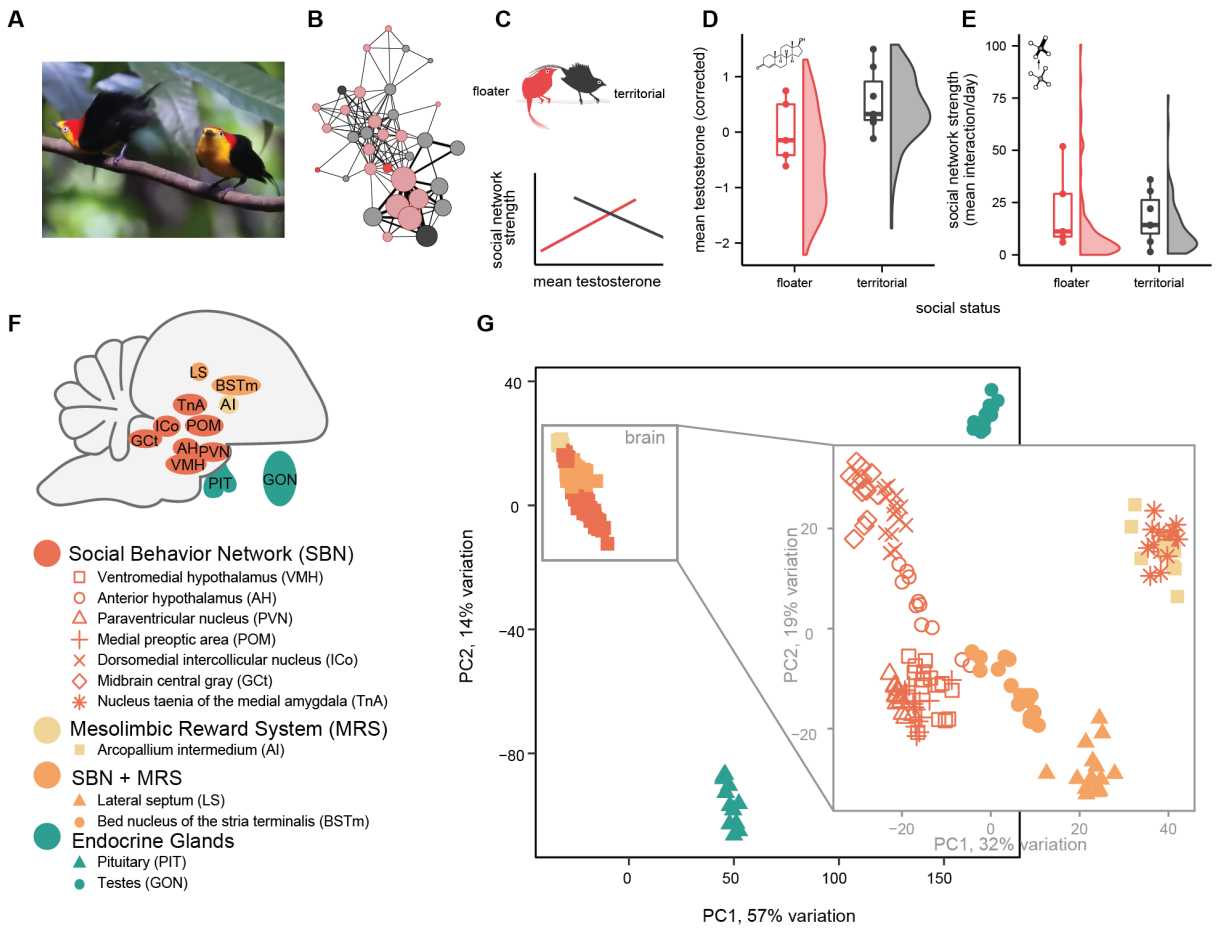


Fig. 1. Testosterone and neuroendocrine tissues are involved in the cooperative behavior of wire-tailed manakins.

A) Males engage in cooperative displays forming complex social networks. B) Example network shows single lek over one week, where node size indicates a male's average social network strength (measure of overall time spent interacting), and edge width represents interaction frequency. Node color denotes floater (red) and territorial (gray)status, and darker colors were included in the study. C) Testosterone phenotype (mean testosterone (corrected)) influences cooperative tendency in territorial and floater males differently. D) Repeated measures of testosterone for 12 individuals (points) from a larger population (density). E) as D but proximity-data logging measures of social network strength.. F) Brain and endocrine tissues sampled for RNAseq. Structures not to scale. Colors represent functional groups (O'Connell & Hofmann, 2011). G & inset) Principal component analysis reveals distinct gene expression profiles among brain regions and endocrine tissues (colors and shapes as F). (Photo: Alice Boyle). Note: sample size N=16 for social status, N=12 for testosterone and network strength.

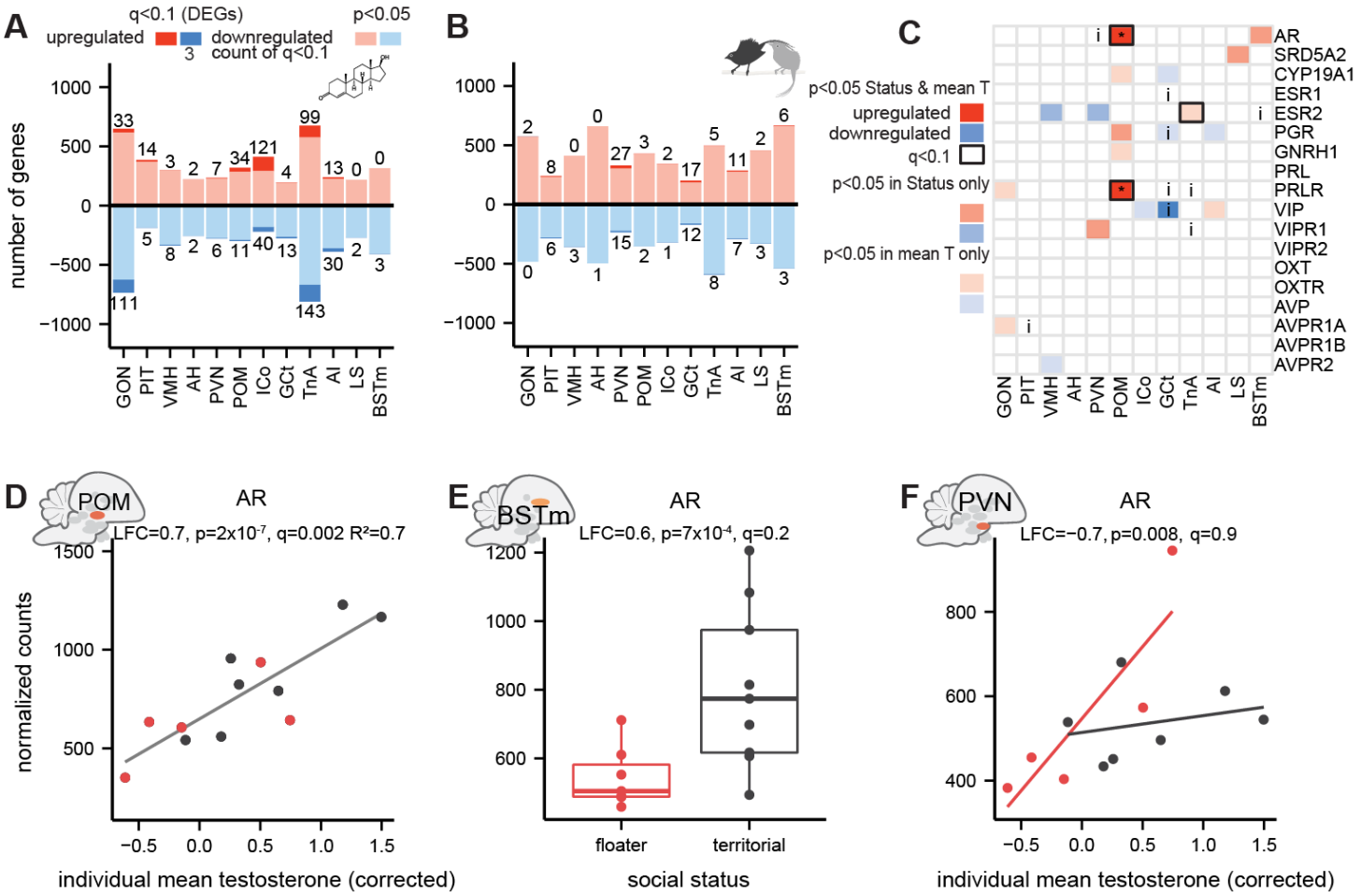


Fig. 2. Neurogenomic landscape of social status and hormone phenotype.
A) Landscape of gene expression in male wire-tailed manakins with high testosterone phenotype (mean testosterone (corrected)) relative to lower testosterone phenotype. Red bars indicate number genes upregulated in males with higher testosterone phenotype, blue indicates genes downregulated in males with higher testosterone. Darker colors and text indicate number of differentially expressed genes after FDR correction. B) Landscape of gene expression in relation to social status, where red indicates genes upregulated in territorial males. C) Summary of candidate gene analysis and filtering, where colors indicate direction of expression, intensity indicates which trait is associated, and "i" indicates there was an interaction effect, and black boxes indicate result was significant after FDR correction. D) Upregulation of AR in POM of males with higher testosterone, where point colors 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. 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 behavior.

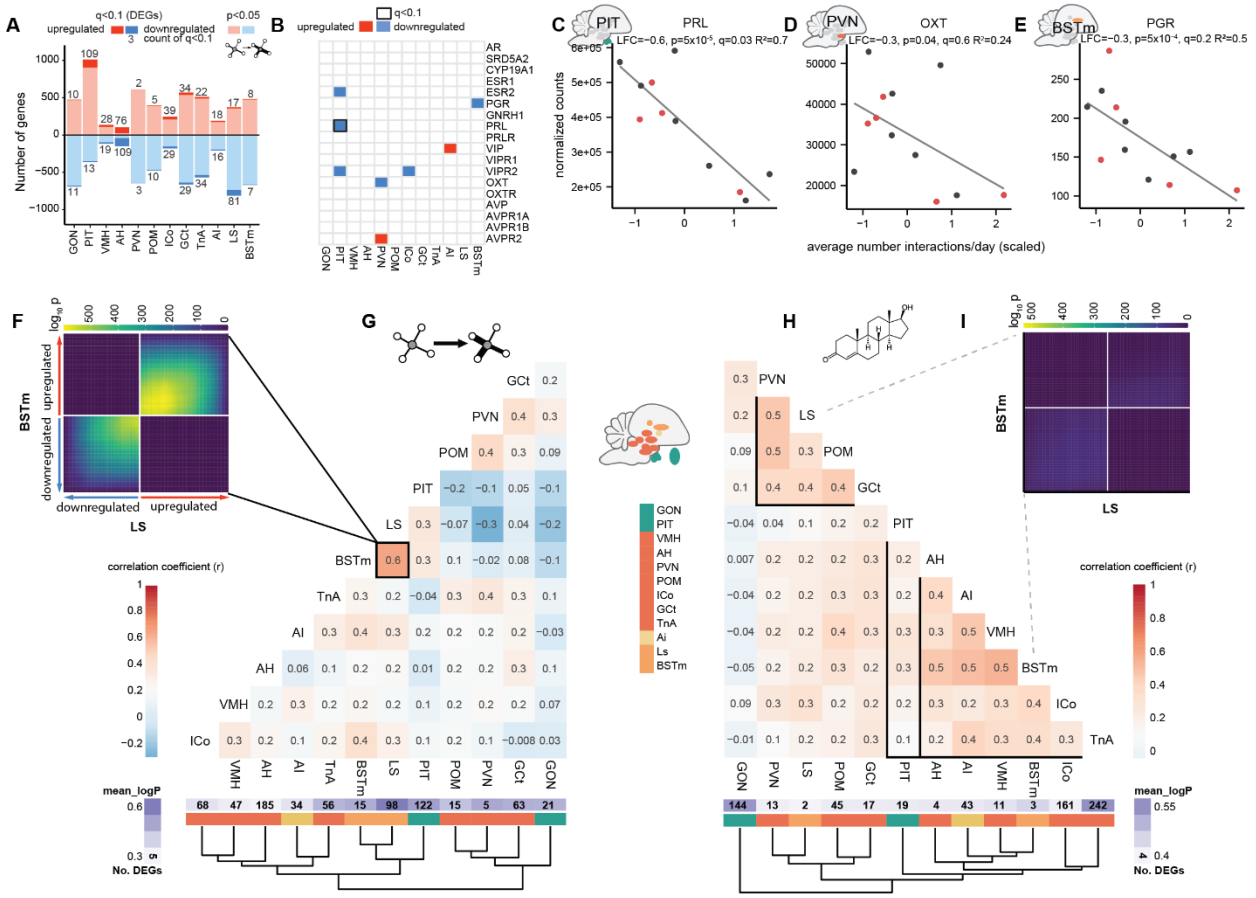


Fig. 3. A distinct landscape of gene expression in relation to cooperative behavior.
A) Gene expression landscape in male wire-tailed manakins with higher social network strength. Legend as fig. 2A. B) Summary of candidate gene analysis and filtering, where colors indicate direction of expression in in individuals with higher social network strength. Black boxes indicates significant results after FDR correction. C) Downregulation of prolactin (PRL) in the pituitary of males with higher social network strengths D) Downregulation of oxytocin (OXT) in PVN of males with higher social network strength E) Downregulation of progesterone receptor (PGR) in BSTm of males with higher social network strength. F) RRHO2 analysis shows similar gene expression profiles associated with social network strength in the LS and BSTm, G) while network strength related gene expression similarity is low among other tissues. H) Gene expression similarity among tissues in relation to testosterone phenotype follows the ontogeny of brain regions, I) No correlation between testosterone-related gene expression profiles in LS and BSTm. G,H) Pearson correlation coefficients of gene expression between tissues, represented by cell color 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.

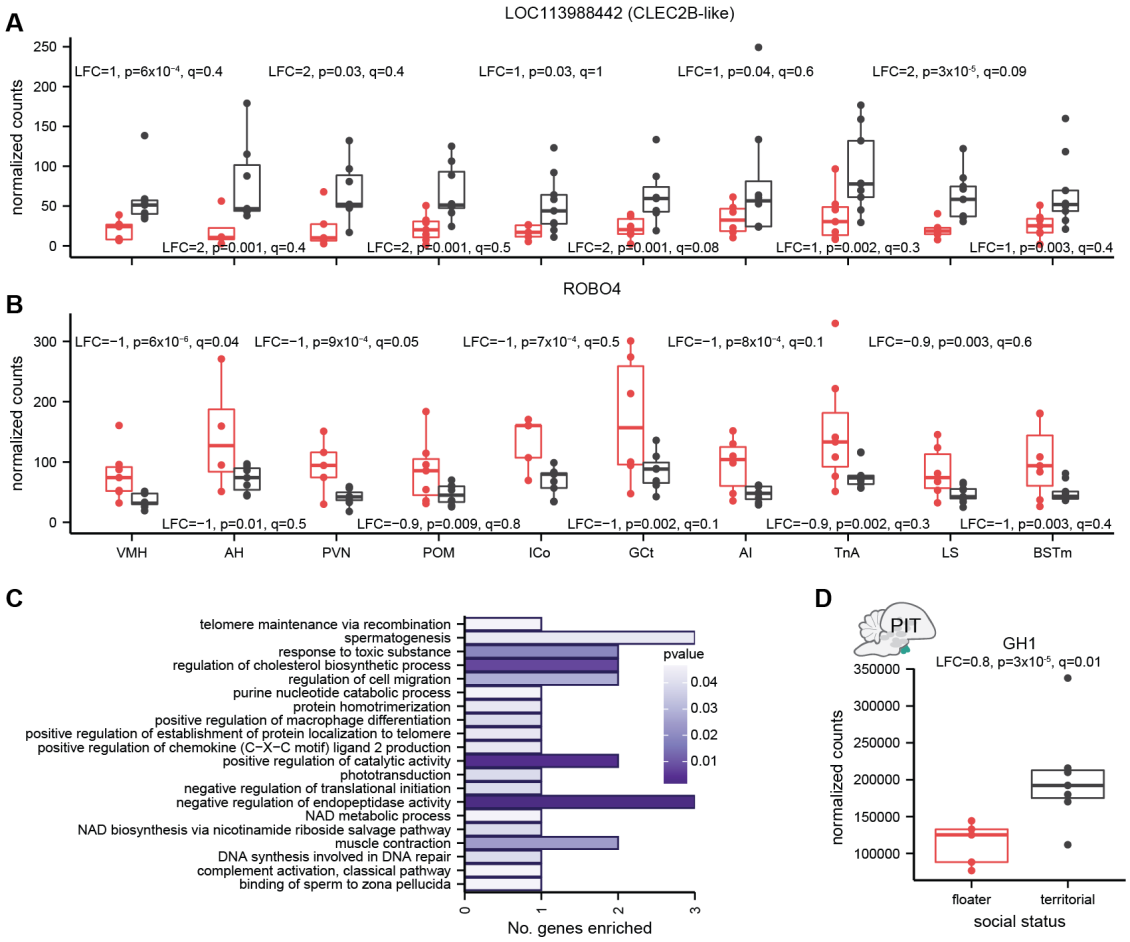


**Fig. 4: Global neural gene expression differences between social classes**
A) Immune gene CLEC2B-like was consistently upregulated in the brains of territorial individuals. B) Developmental gene ROBO4 was consistently downregulated in the brains of territorial males. C) Enriched GO categories among genes that were consistently up- or downregulated between territorial and floater males, where bar color indicates uncorrected p-value. D) Upregulation of growth hormone transcript in the pituitary of territorial males could explain differences between status classes.