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Repeated behavioural evolution is associated with convergence of gene expression in cavity-nesting songbirds

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Uncovering the genomic bases of phenotypic adaptation is a major goal in biology, but this has been hard to achieve for complex behavioural traits. Here we leverage the repeated, independent evolution of obligate cavity nesting in birds to test the hypothesis that pressure to compete for a limited breeding resource has facilitated convergent evolution in behaviour, hormones and gene expression. We used an integrative approach, combining aggression assays in the field, testosterone measures and transcriptome-wide analyses of the brain in wild-captured females and males. Our experimental design compared species pairs across five avian families, each including one obligate cavity-nesting species and a related species with a more flexible nest strategy. We find behavioural convergence, with higher levels of territorial aggression in obligate cavity nesters, particularly among females. Across species, levels of testosterone in circulation were not associated with nest strategy nor aggression. Phylogenetic analyses of individual genes and co-regulated gene networks revealed more shared patterns of brain gene expression than expected by drift, although the scope of convergent gene expression evolution was limited to a small percentage of the genome. When comparing our results to other studies that did not use phylogenetic methods, we suggest that accounting for shared evolutionary history may reduce the number of genes inferred as convergently evolving. Altogether, we find that behavioural convergence in response to shared ecological pressures is associated with largely independent evolution of gene expression across different avian families, punctuated by a narrow set of convergently evolving genes.

Biologists have long been fascinated by phenotypic convergence as a window into the predictability of evolution[1–3]. However, our understanding of the molecular mechanisms of behavioural convergence lags behind that of morphological or physiological traits[4–6]. Some studies of behavioural convergence find that evolution repeatedly uses the same (or similar) changes in particular genes or pathways[7–9]. Alternatively, the building blocks of behavioural convergence may be independent, with lineage-specific mechanisms across replicated evolutionary events[10,11]. Understanding the relative contributions of these two processes requires a comparative approach that embraces the likely polygenic nature of complex behaviours[12–16]. However, efforts to connect behavioural evolution to transcriptomics rarely use phylogenetic methods[6], despite the potential for changes in gene expression to shape phenotypic diversification. Likewise, genetic drift may shape gene expression independently of adaptation, and phylogenetic methods are necessary to disentangle these processes.

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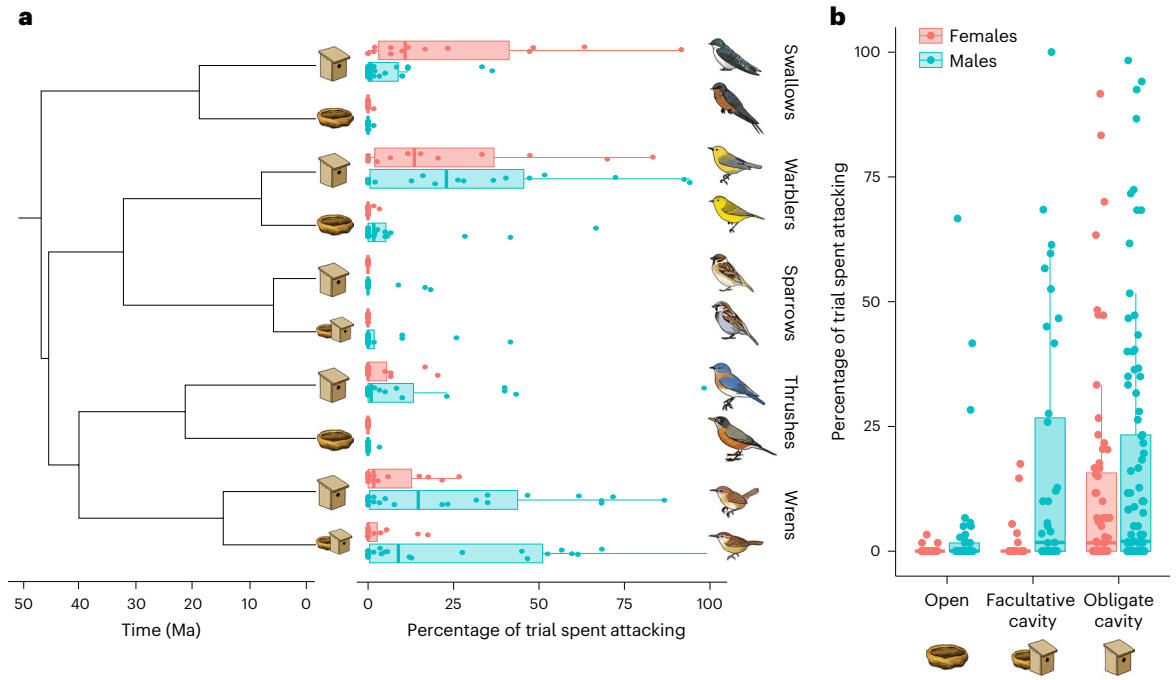

Fig. 1 | Obligate cavity nesters displayed more overt territorial aggression. **a**, Left: consensus phylogeny of 10 species from five families, which diverged from a common ancestor ~44 Ma (36–50 Ma). Species pairs diverged ~9–20 Ma (ref. 88). Nestboxes indicate obligate cavity nesters, nests represent open nesters and both symbols together represent facultative cavity nesters. Right: sex- and species-level aggression towards a conspecific decoy, measured by the proportion of 5 s intervals that contained physical contact during a 5 min aggression assay. Obligate cavity nesters spent more time attacking the decoy compared to species with more flexible nest strategies (PGLMM, 2 levels: post.mean = −4.29, lower 95% CI = −6.69, upper 95% CI = −1.92, pMCMC = 0.00105). Post.mean is the mean of the posterior distribution of the model coefficient, that is, the best model estimate for the relationship. *P* value is not adjusted. Species listed in descending order: swallows—tree swallows (*Tachycineta bicolor*), barn swallows (*Hirundo rustica*); woodwarblers—prothonotary warblers (*Protonotaria citrea*), yellow warblers (*Setophaga petechia*); sparrows—Eurasian tree sparrows (*Passer montanus*), house sparrows (*Passer domesticus*); thrushes—eastern bluebirds (*Sialia sialis*), American robins (*Turdus migratorius*); and wrens—house wrens (*Troglodytes aedon*), Carolina wrens (*Thryothorus ludovicianus*). **b**, Aggression, grouped by nest strategy and sex. Obligate cavity-nesting females were significantly more aggressive than females with more flexible nest strategies (PGLMM, 2-levels: post.mean = −2.55, lower 95% CI = −4.46, upper 95% CI = −0.7, pMCMC = 0.00637). *P* value is not adjusted. Each point is one assay on a unique free-living individual. Box plots convey the interquartile range (25th, 50th, 75th percentiles), with whiskers indicating 1.5× the interquartile range. Sample sizes for biological replicates of aggression per species are provided in Table 1. Illustrations: Tessa Patton.

Territorial aggression is a widespread behavioural trait that is well suited for evaluating these processes. In vertebrates, aggression is mediated by diverse neuroendocrine mechanisms[17], including the hormone testosterone[18] and its metabolite 17β-estradiol, both of which can act on sex steroid receptors in the brain to promote aggression[19,20]. Aggression is also linked to brain metabolic pathways[7] and G-protein coupled receptor signalling of dopamine, serotonin and glutamate[21,22]. Many of these candidates are shared across distantly related species[7,21], demonstrating the potential for conserved or repeatedly evolved mechanisms in the evolution of territorial aggression. However, there is also interspecific variation in the abundance and distribution of these endocrine-molecular building blocks[23,24], indicating that mechanisms of aggression can diverge over time.

Mechanisms of aggression can also differ within a species, including between males and females[25], both of whom exhibit territorial aggression in many species[26,27]. This provides a unique opportunity to include both sexes in analyses of behavioural evolution. After all, females and males share the majority of their genome, yet sex-specific selective pressures can alter gene expression[28] and hormone secretion[29], which together may shape aggression or the mechanisms that promote the expression of aggression, the latter of which is still remarkably understudied in females[30,31]. Critically, in both sexes, aggression shapes access to resources and mates, and the degree of such competition varies among species[26,27].

Competition for breeding territories is thought to be especially intense for obligate secondary cavity-nesting birds, which must secure an excavated space inside a tree or other substrate. They need a cavity to breed; they cannot excavate one themselves, and physical conflicts over cavities can lead to serious injury or death[32–34]. For many cavity-nesting species, both females and males compete to acquire and defend nesting territories[35–39], and our earlier case study showed elevated aggression in two species of obligate cavity nesters, although without quantitative phylogenetic methods[40]. There have been multiple evolutionary transitions in and out of secondary cavity nesting across passerines[41,42], providing a strong foundation to evaluate the degree of mechanistic convergence in behavioural evolution.

In this Article, we test the hypothesis of evolutionary convergence using a phylogenetic approach that integrates behavioural, hormonal and neurogenomic data. We compare species pairs from five avian families—Hirundinidae (swallows), Parulidae (woodwarblers), Passeridae (sparrows), Turdidae (thrushes) and Troglodytidae (wrens)—which each represent independent origins of obligate secondary cavity nesting (Fig. 1a; further details in Supplementary Section 1)[42]. Each pair includes one obligate secondary cavity-nesting species, which regularly nests in artificial nest boxes, and one species with a more flexible nest strategy, either open cup nesting or facultative cavity nesting. Each species pair is similar in other aspects of its ecology and life history (for example, foraging ecology, degree of biparental care). Across species, we compared territorial aggression, testosterone in circulation and brain gene expression. By applying quantitative phylogenetic approaches across these datasets, we evaluate convergence against a null hypothesis of shared evolutionary history and genetic drift.

Table 1 | Sample sizes for individual measurements of behaviour, testosterone and gene expression in the VmT

Family	Species	Nest strategy	Aggression		Testosterone		VmT RNA-seq	
			F	M	F	M	F	M
Swallows (Hirundinidae)	Tree swallow	Obligate cavity	14	24	14	8	7	6
	Barn swallow	Open cup	10	11	13	14	6	6
Woodwarblers (Parulidae)	Prothonotary warbler	Obligate cavity	12	19	9	9	6	6
	Yellow warbler	Open cup	10	20	8	8	6	6
Sparrows (Passeridae)	Eurasian tree sparrow	Obligate cavity	8	13	7	8	6	6
	House sparrow	Facultative	14	17	8	10	6	6
Thrushes (Turdidae)	Eastern bluebird	Obligate cavity	17	20	15	15	6	6
	American robin	Open cup	9	11	7	12	6	6
Wrens (Troglodytidae)	House wren	Obligate cavity	16	22	6	11	6	6
	Carolina wren	Facultative	15	22	6	8	6	6

RNA-seq, RNA sequencing; F, females; M, males.

Results

Behavioural convergence among obligate cavity nesters

Our study occurred during territorial establishment at the beginning of the breeding season, when birds are defending territories and acquiring mates, but before egg laying. We assayed aggression in 304 free-living female and male birds in Indiana, Illinois or Kentucky, USA (Supplementary Section 1 and Table 1). Each aggression assay lasted 5 min and included a conspecific, taxidermic decoy of either female or male sex, coupled with playback of female or male vocalizations that occur during natural aggressive interactions (details in Supplementary Section 2). We measured a variety of aggressive behaviours and focused on physical attacks (that is, contact with the decoy). To evaluate the role of nest strategy as a driver of territorial aggression, we used phylogenetic generalized linear mixed models (PGLMMs). Briefly, these models correct for covariation in traits among species due to common ancestry by incorporating phylogenetic relatedness as a random effect. Along with sex of the territory holder and sex of the decoy, we included nest strategy as a fixed effect with two levels: obligate cavity nesting versus more flexible strategies (including both facultative cavity and open nesting). We also ran three-level models differentiating obligate cavity, facultative cavity and open-nest strategies, for which results were qualitatively similar to those of the two-level models (Supplementary Sections 10–12).

We found significant effects of nest strategy, sex of the territory holder, sex of the decoy and their interaction on aggression. Obligate cavity nesters spent more time attacking the decoy, compared to species with more flexible nest strategies, indicating that obligate cavity nesters displayed more overt territorial aggression (PGLMM, $P = 0.00105$; Fig. 1a and Supplementary Table 1). This difference in aggression was larger between obligate cavity nesters and open nesters relative to facultative cavity nesters (Fig. 1a). Males attacked marginally more than females (PGLMM, $P = 0.058$). It is worth noting that sex interacted with nest strategy (PGLMM, $P = 0.00637$), a pattern driven by elevated levels of territorial aggression in obligate cavity-nesting females (Fig. 1b). We also found a significant interaction between sex of the territory holder and sex of the decoy (PGLMM, $P = 0.01073$), such that male territory holders were more aggressive towards male decoys, whereas female territory holders attacked female and male decoys with similar levels of aggression (Supplementary Fig. 1 and Supplementary Table 1).

In addition, we measured the average distance from the focal individual to the decoy, to confirm each subject was present and engaged during a trial, regardless of whether they were aggressively attacking. Distance from the decoy was not related to nest strategy (PGLMM, $P = 0.22$), sex ($P = 0.70$) nor their interaction ($P = 0.39$) (Supplementary Table 2 and Supplementary Fig. 2). This result indicates that decoy placement was a salient stimulus, in that all species were in audio-visual proximity to the simulated intruder (average distance = 5.0 m), but obligate cavity nesters spent more time attacking the decoy compared to their close relatives with more flexible nesting strategies (Fig. 1 and Supplementary Table 1).

Testosterone levels are not associated with nest strategy

Next, we sought to measure levels of testosterone and gene expression as potential physiological drivers of behavioural convergence. We focused our analyses on constitutive hormonal and neurogenomic states that are representative of unprovoked, free-living animals ($n = 196$ hormone samples, $n = 121$ brain gene expression samples; Table 1). We followed the assumption that social elevation of testosterone peaks around 30–45 min after hypothalamic–pituitary–gonadal axis activation[43,44] and that socially responsive genes likewise show peak transcriptional responses 30–60 min after a stimulus[45]. To maximize our number of aggression assays while meeting these assumptions, our sample collection took three approaches: (a) passive collection, in which we set up a mist net or trap in the target territory and waited to capture the focal individual without any stimulation (testosterone, $n = 108$; brain, $n = 51$), (b) immediate collection, in which we sampled individuals immediately after a short aggression assay (testosterone, $n = 55$; brain, $n = 43$) and (c) delayed collection, in which we sampled individuals several days after a short aggression assay (testosterone, $n = 33$; brain, $n = 27$). We found no differences in testosterone among immediate, passive or delayed sample collection approaches (analysis of variance, $F_{2,184} = 0.011$, $P = 0.90$; Supplementary Fig. 3a) and therefore combined these samples.

We previously identified a positive correlation between territorial aggression and testosterone within female tree swallows ($S = 2$, $P = 0.0028$, rho = 0.96)[40], which are included in this study, but we did not find this relationship in male tree swallows. To explore whether similar patterns apply to other species sampled here, we conducted Pearson's correlations across species for each sex and Spearman's correlations within each family for each sex, given the reduced sample size. We did not find any relationship between testosterone and aggression in Eastern bluebirds, house wrens, Carolina wrens, prothonotary warblers nor yellow warblers ($P > 0.13$), and we did not have enough data on testosterone and aggression from the same individuals for the other species. Considered collectively for all samples, we did not find a significant relationship between territorial aggression and testosterone for females ($t = 1.87$, d.f. = 25, $r = 0.35$, $P = 0.073$) nor for males ($t = 1.17$, d.f. = 35, $r = 0.19$, $P = 0.25$). Testosterone was not related to nest strategy (PGLMM, $P = 0.80$) nor the interaction between nest strategy

and sex (PGLMM, $P = 0.85$; Supplementary Fig. 4 and Supplementary Table 3). As expected, we found that males had significantly higher levels of testosterone in circulation than females (PGLMM, $P < 0.0001$).

Neurogenomic mechanisms of behavioural convergence

Finally, we examined convergent evolution in brain gene expression. Using RNA sequencing, we measured messenger RNA abundance for 10,672 orthologous genes expressed in all 10 focal species in the ventromedial telencephalon (VmT) (Table 1, Supplementary Fig. 5 and Supplementary Fig. 6); this region contains core nodes of the vertebrate social behaviour network, which regulates behaviours including aggression[46]. The number of differentially expressed genes generally increased with divergence time between species pairs within each family ($P = 0.08$, $R^2 = 0.83$; Supplementary Section 6, Supplementary Fig. 8 and Supplementary Table 4), underscoring the need for phylogenetic methods.

Gene expression is highly concordant between pairs of families

We used the rank–rank hypergeometric overlap (RRHO) approach to compare the magnitude and direction of gene expression differences among species pairs. If the same genes are repeatedly targeted by selection for the obligate cavity-nesting strategy, these genes should be differentially expressed in the same direction for cavity nesters across multiple family comparisons. As a null hypothesis, we also generated permuted differential expression datasets by randomly sampling the $\log_2$(fold change) and associated P values from the observed values with replacement for each gene. This was done independently for each family. The permuted datasets were then given to the same RRHO pipeline used for the empirical datasets.

In contrast to the largely random distributions of overlap from the randomly permuted dataset (Supplementary Fig. 9), RRHO for our empirical dataset revealed significantly concordant patterns of gene expression (all $P < 0.01$, permutation test; Fig. 2 and Supplementary Table 5). Genes that were more highly expressed in the obligate cavity nester of one species pair were also more highly expressed in the obligate cavity nester of another species pair, and vice versa for genes with lower expression (Supplementary Data 4), evidenced by clustering in the upper right and lower left quadrants of the RRHO heat maps (Fig. 2c). This concordant pattern applied to an average of 1,390 genes per family comparison (Supplementary Fig. 12), amounting to ~13.0% of genes studied, suggesting a large number of genes evolving in a similar direction of expression within each family. In comparison, the randomly permuted dataset had an average of 562 concordant genes per family comparison (~5.3% of total genes). The empirical dataset had more than twice as many concordant genes as expected by random chance from the permuted dataset. Therefore, our empirical RRHO data for each family comparison are overwhelmingly concordant, beyond the null expectation.

This high level of concordance in gene expression did not persist across multiple family comparisons, indicating a lack of convergence in the identity of genes differentially expressed between species pairs across the phylogeny. The number of shared genes decreased rapidly with increasing inclusion of additional family comparisons (Fig. 2a). Specifically, about 1,000 genes (~10% of orthologues) displayed concordant expression patterns among two or three families, and 497 genes (~5%) were shared by at least 5 out of 10 family comparisons at a time (Fig. 2a). Relative to the empirical data, the randomly permuted data had more shared genes across 2 family comparisons and fewer shared genes across 5+ family comparisons (Supplementary Fig. 9). Gene Ontology (GO) analyses of the 497 shared genes (Supplementary Data 4 and 5) revealed no significant functional enrichment. This pattern indicates that—despite a substantial degree of concordance between pairs of species—concordantly expressed genes are not repeatedly recruited from any particular set of known biological processes. However, we must recognize that GO databases are biased towards model organisms, and a lack of GO enrichment does not necessarily indicate an absence of a shared biological function.

Across all 10 family comparisons, 11 genes (0.1% of orthologues) were shared, exhibiting complete concordance across five independent origins of obligate cavity nesting (Supplementary Table 6). Although small, this number was still larger than expected by chance, considering that the randomly permuted dataset had only 1 gene shared across families. Some of these 11 globally concordant genes have established connections with behaviour or brain function. For instance, *EMC3*, *NPTX1* and *RGS19* are involved in neurotransmitter reception, *ATP6V0E1* and *DDX56* have ATPase activity, and most of the completely concordant genes have established connections with Alzheimer's (*ARRDC4*, *CTNND1*, *NPTX1*, *WIPF2*), addiction (*RGS19*) or other neurological disorders (*AFAP1L1*, *PQBP1*)[47]. These 11 genes also share regulatory motifs for transcription factors E2F, which is important for neurogenesis[48], and ETS-1, which plays a role in glial cell maintenance[49]. Future work could functionally validate these candidate genes for their role in aggression, cavity nesting and other co-evolving traits or explore alternative explanations for why these 11 genes are associated with repeated behavioural evolution (for example, mutation bias[3]). Overall, our results from RRHO indicate a high degree of independent gene expression evolution, with a subset of concordance, in association with the convergent evolution of obligate cavity nesting.

We repeated these RRHO analyses with females and males separately, because sex-specific selection pressures could generate concordance that would be masked by analysing both sexes together. We found similarly high concordance in these sex-specific analyses, in that significant gene overlap was concentrated primarily in the upper right and lower left quadrants. However, the degree (indicated by the scale of the heat map) and direction (concentration of significant overlap in the upper right versus lower left) of concordance differed between sexes (Fig. 2a, Supplementary Fig. 10 and Supplementary Fig. 11). Males had more cases of higher expression in the obligate cavity nester of both families (upper right), whereas females had more cases of lower expression (lower left).

Nest strategy is associated with convergently evolving genes

Next, we used PGLMMs to model expression of all ~10,000 orthologues as a function of nest strategy, sex, aggression, sex-by-nest-strategy interactions and aggression-by-nest-strategy interactions. To identify convergent expression evolution, these phylogenetic models explicitly account for shared expression due to evolutionary history. We expect some degree of convergence in expression evolution due to random chance (that is, genetic drift along the phylogeny), so we used a false discovery rate (FDR) correction to test this null hypothesis. Many genes showed some relationship with obligate cavity nesting, aggression or a relevant interaction, supporting a hypothesis of convergent expression evolution. Specifically, there were 234 genes associated with obligate cavity nesting and 79 genes associated with aggression, and 5 genes overlapped among these. Of these five genes (*RNASEH2B*, *ERN1*, *UGGT2*, *TAF1B* and *HIGD1A*), the first four relate to protein or RNA processing, the latter four relate to stress responses, and all have connections with neurodegenerative disorders[47,50–53]. An additional 62 genes exhibited an interaction between nest strategy and aggression. Seventy-six genes exhibited an interaction between sex and obligate cavity nesting, with approximately equal numbers of genes that had a stronger association in females versus males (Supplementary Table 7 and Supplementary Data 6). Sex had the largest influence on expression variation and was associated with 510 genes (Supplementary Table 7), the majority of which had higher expression in males than in females. Genes with sex-biased expression were significantly enriched for cellular metabolic process.

Because some nest-strategy and aggression-associated patterns may be driven by expression in one or two species, we developed additional criteria to pinpoint a robust set of convergently evolving genes. We retained only those that were (1) significantly different in expression

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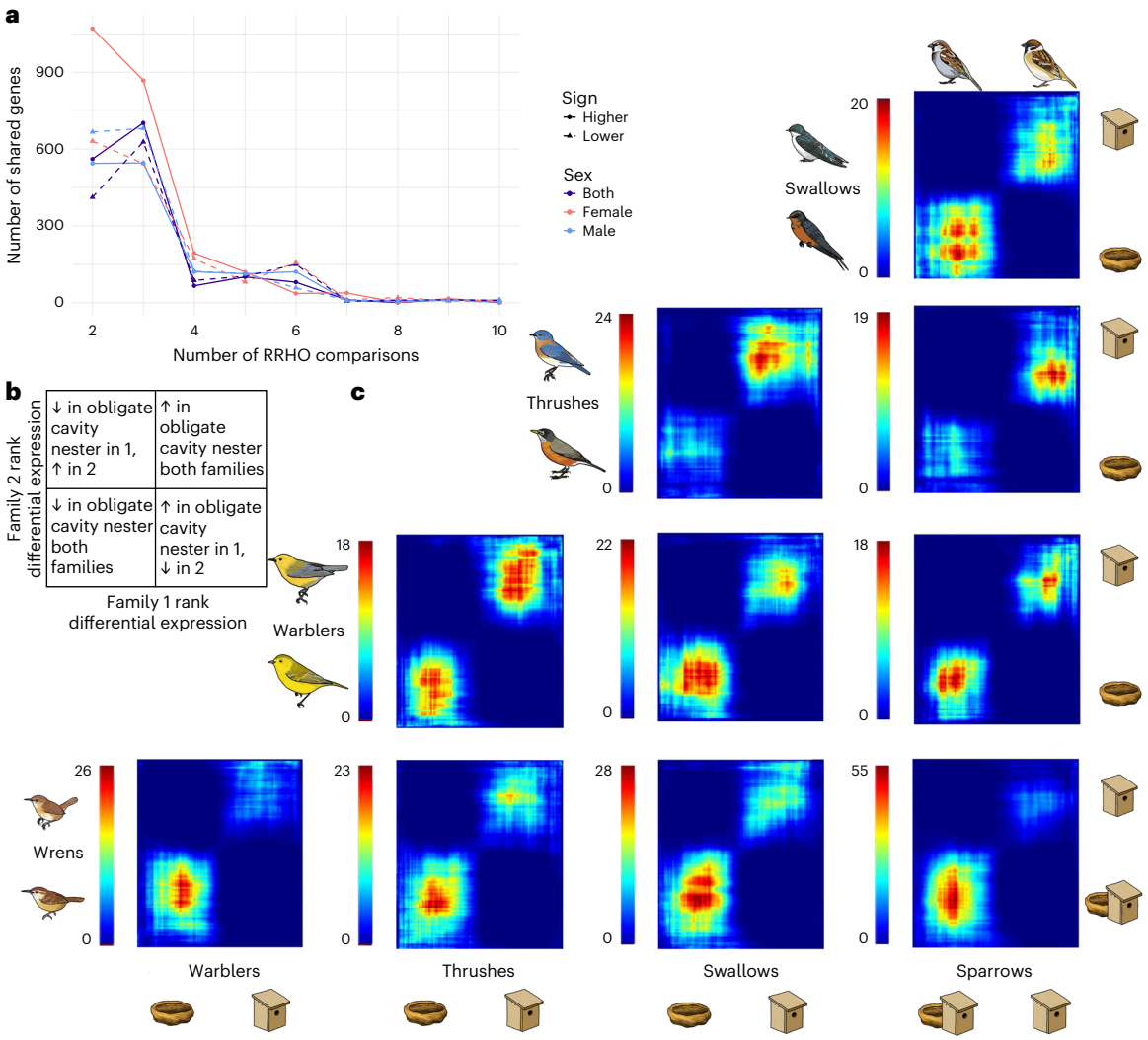

Fig. 2 | Concordance in differential expression based on RRHO. a, Number of concordantly expressed genes shared across family comparisons (that is, across heat maps) for males (blue), females (pink) and both sexes combined (purple). Solid lines indicate higher expression in obligate cavity nesters, dashed lines indicate lower expression. Across all 10 family comparisons, 11 genes were shared, exhibiting complete concordance. **b**, Key for interpreting individual heat maps. Each quadrant of the key corresponds to a quadrant of an individual heat map, shown in **c**. **c**, Each pixel within the heat maps contains two sets of approximately 100 genes being compared between the two families. Heat map colours reflect adjusted hypergeometric −log(*P* value), that is, the overlap between these gene sets. Larger values indicate more overlap in expression between families. Nestboxes indicate obligate cavity nesters, nests represent open nesters and both together represent facultative cavity nesters. Sample sizes for biological replicates of gene expression per species are provided in Table 1. Illustrations: Tessa Patton.

(by *t*-test) between species in at least 3 out of 5 family comparisons and (2) different in a consistent direction (that is, all higher or all lower expression) with respect to nest strategy in all families with a significant difference. Of 234 genes, 168 met these criteria of convergence for nest strategy (Supplementary Data 8). Further restricting our analyses to all 5 family comparisons, we identified 40 genes (0.4% of orthologues) associated with nest strategy. Several convergent genes associated with nest strategy have some connection to ATP and mitochondrial function (*ATP1B1*, *PITRM1*, *SLC25A24*), as well as behaviour or psychiatric risk (*TRMT1L*, *NT5C2*, *BCHE*)[47].

We also used PGLMMs to examine the expression of a subset of candidate genes related to testosterone and steroid hormone signalling in our transcriptome dataset, but we did not find a relationship with obligate cavity nesting (Supplementary Section 10).

Nest strategy is associated with convergent gene networks

Complex phenotypes are often regulated by subtle but coordinated changes in gene networks, which may also be targets of selection[54], and approaches that analyse genes individually may miss important emergent properties. Therefore, we used weighted gene co-expression network analyses (WGCNA) to estimate co-expression across all 10 species (Supplementary Section 9), yielding 93 networks of correlated genes (Supplementary Data 10). Using each network's eigengene (akin to a principal component of expression), we ran PGLMM with fixed effects of nest strategy, aggression, sex and their interactions. After accounting for phylogeny, and with FDR correction, two networks showed significant associations that are unlikely to occur by drift alone. Expression in the red network was higher in males than in females (PGLMM, coefficient = 0.169, adjusted $P = 0.0256$; Supplementary Fig. 14a), and 147 of 193 genes (76%) were on the zebra finch Z chromosome. Expression in the tan4 network was associated with nest strategy (PGLMM, coefficient = 0.166, adjusted $P = 0.0256$; Supplementary Data 10 and Supplementary Table 8), with lower eigenvalues and distinct expression values in each of the five independent origins of obligate cavity nesting (Fig. 3a,b and Supplementary Fig. 13). Although not significantly enriched for any GO terms (Supplementary Table 8),

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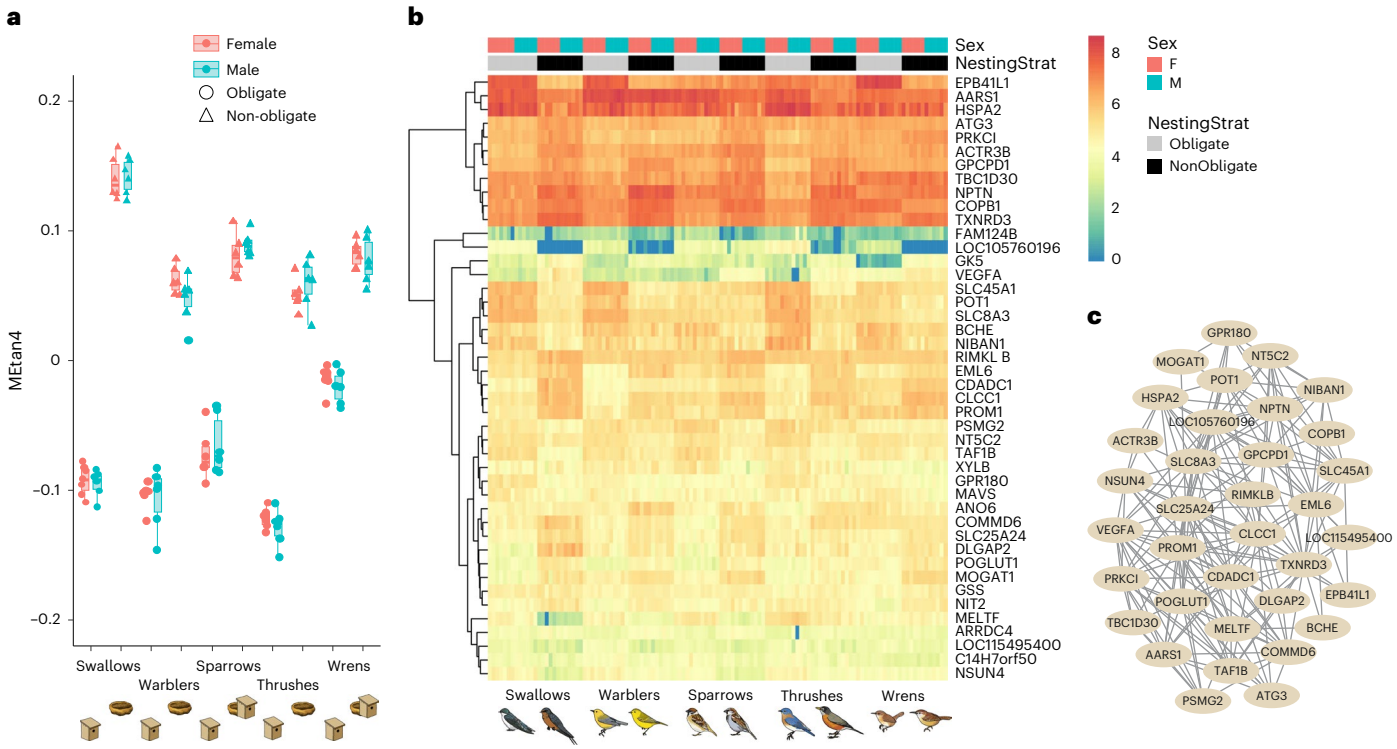

Fig. 3 | Tan4 gene network associated with obligate cavity nesting. a, WGCNA eigengene values for the tan4 network, which included 44 genes whose expression was significantly associated with obligate cavity nesting relative to non-obligate nesting strategies (PGLMM, coefficient = 0.166, adjusted $P = 0.0256$). Individual points represent the eigengene value for a sampled individual, coloured by sex. Nestboxes indicate obligate cavity nesters, nests represent open nesters and both together represent facultative cavity nesters. Box plots convey the interquartile range (25th, 50th, 75th percentiles), with whiskers indicating 1.5× the interquartile range. Sample sizes for biological replicates of gene expression per species are provided in Table 1. Module eigengene tan4 (MEtan4) is the first principal component of the tan4 gene network. **b**, Heat map of log-scaled genes in tan4 network. Columns represent individuals, grouped by sex and nest strategy. Colour scale indicates log of gene expression. **c**, Network depicts genes with network membership >|0.6|. Illustrations: Tessa Patton.

several genes in the tan4 network related to mitochondrial function and energy metabolism, cognition and stress response (Fig. 3c).

Because sexes have the potential to differ in mechanisms of behaviour, we repeated this analysis for each sex separately (Supplementary Section 11). In this sex-specific analysis, we identified two female-specific gene networks correlated with species-level aggression—pink4 and sienna4 (Pearson's correlation: $r > |0.78|$, $P < 0.0001$; Fig. 4, Supplementary Table 9 and Supplementary Data 10). These networks contained genes with established connections to aggression, including DRD3, a dopamine receptor in the pink4 network, and GRIA2, a glutamate receptor in the sienna4 network. Finding aggression-associated networks specific to females further suggests that there are multiple gene regulatory routes to aggression, including those that may differ between sexes.

Shared transcriptomic routes to behavioural convergence

To identify candidate genes that are robustly (that is, independently across multiple analyses) associated with nest strategy and/or aggression, we integrated results across differentially expressed genes, individual gene- and network-level PGLMMs, and concordant genes from at least 5 out of 10 RRHO comparisons (Supplementary Table 11). For nest strategy-associated candidates, 27 out of 44 genes (61%) in the tan4 network recapitulated convergent individual genes in the PGLMM analysis, a strong signal that this gene set is consistently associated with the convergent evolution of obligate cavity nesting. Out of the 221 differentially expressed genes shared among species pairs, 13 genes overlapped with nest-strategy-associated individual PGLMM analyses and the tan4 network. We also identified six aggression-associated genes that overlapped with nest-strategy-associated analyses, including three with concordant RRHO genes, two with individual PGLMM analyses and one gene, *TAF1B*, which overlapped with both the tan4 network and individual PGLMM analyses. Altogether, these results suggest that repeated use of a small core set of genes may have contributed to the evolution of obligate cavity nesting and associated changes in behaviour.

To place our results in a broader context, we compared our list of aggression-associated genes to previous studies (Supplementary Data 12), including those on the brain's response to an aggressive challenge[7,55] and those that are known to mediate aggression in diverse species, including humans[22,56]. Using a permutation analysis, we found significant overlap between our 79 aggression-associated genes and another study's network of 290 genes that were sensitive to experimental competition in the tree swallow brain (Supplementary Section 12, Supplementary Data 12, Supplementary Table 12 and Supplementary Fig. 20; ref. 55). Of the seven genes in common between these datasets (Supplementary Table 13), *DPF3*, *PLXND1* and *ZIC4* are involved in brain development, *CHN2* is associated with schizophrenia, *SPHKAP* is associated with neuroblastoma and apoptosis, and *GDF10* promotes neural repair after stroke[47], again linking aggression to key elements of brain function.

Discussion

Striking phenotypic similarities among distantly related organisms exposed to similar ecological pressures yields critical insights into the mechanisms of evolution. However, investigations of the proximate mechanisms by which natural selection generates evolutionary change are often limited to traits with a simple and well-understood genetic basis[2,3,57], and less is known about the underpinnings of convergence in complex behavioural traits. A particularly unresolved question is the relative contributions of repeated versus independent molecular

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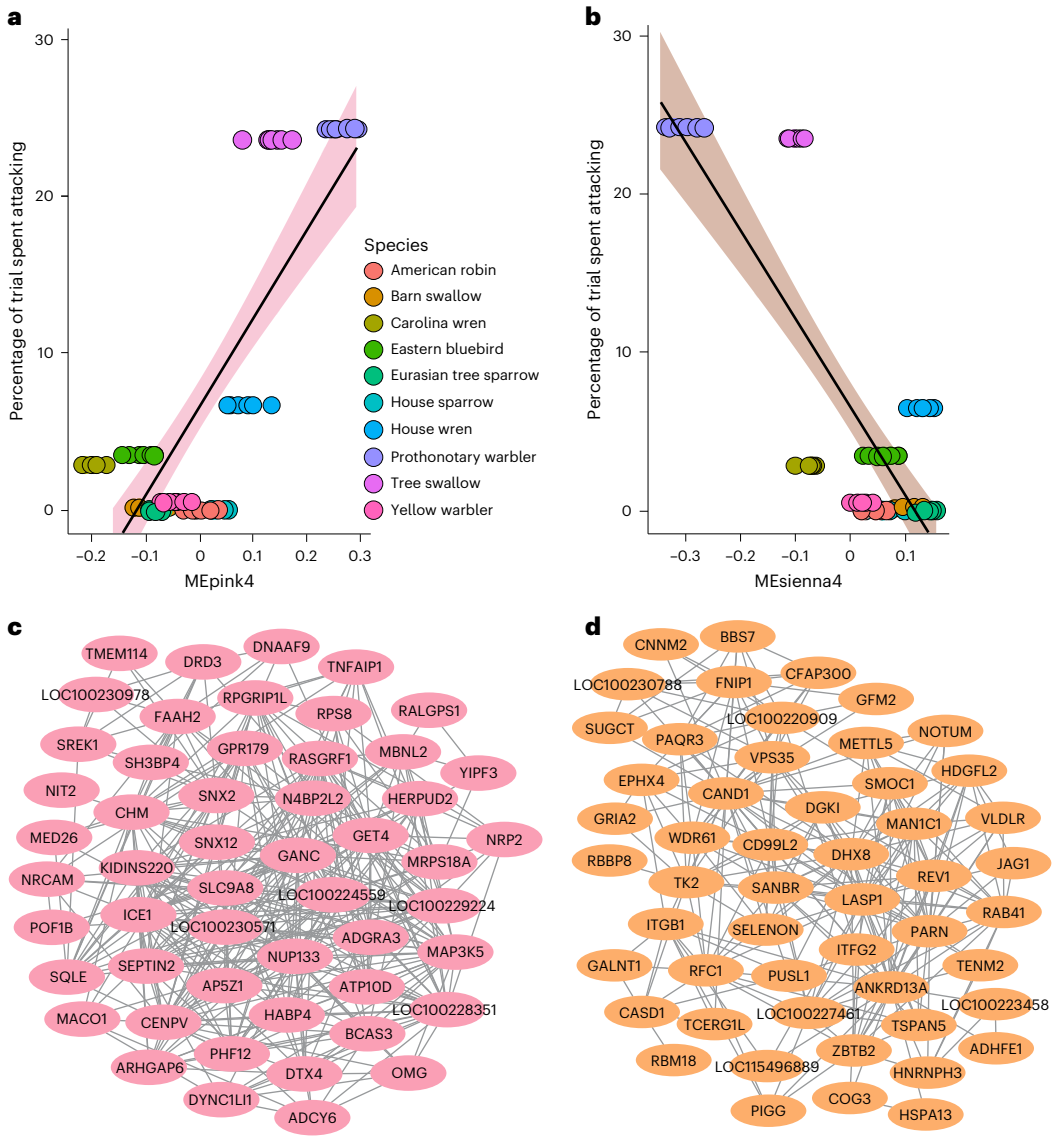

Fig. 4 | Gene networks associated with female aggression. a,**b**, WGCNA eigengenes for the female-specific pink4 network (PGLMM, coefficient = 1.39, adjusted $P = 0.0124$) (**a**) and sienna4 network (PGLMM, coefficient = −1.64, adjusted $P = 0.0124$) (**b**) were associated with average, species-level aggression towards a conspecific decoy, measured by the proportion of 5 s intervals that contained physical contact during a 5 min aggression assay. Error bands indicate the 95% confidence intervals. Module eigengene pink4 (MEpink4) is the first principal component of the pink4 gene network. Module eigengene sienna4 (MEsienna4) is the first principal component of the sienna 4 gene network. **c**,**d**, Networks for pink4 (**c**) and sienna4 (**d**) depict genes with network membership >|0.6|. Sample sizes for biological replicates of gene expression per species are provided in Table 1.

changes to convergent behavioural evolution. Across five avian families with independent origins of obligate secondary cavity nesting, we find behavioural convergence in territorial aggression, as well as transcriptomic convergence in the brain involving a small, core set of genes. These results highlight how complex behaviours can arise from largely independent evolution, punctuated by a few specific cases of repeated molecular changes in response to shared ecological pressures.

Across ten species, those with obligate cavity-nesting strategies exhibited greater physical aggression, although all species were present and responsive to the simulated intruder. Among females, aggression was particularly high for obligate cavity nesters, consistent with the hypothesis that female aggression is adaptive during competition for nesting territories[27,37,40,58]. Females were aggressive towards decoys of both sexes, whereas males were more aggressive towards their own sex. Conspecific aggression may serve multiple functions, most likely nest site defence or mate guarding[27,59], considering the timing of stimulated intrusions during the early spring period of territorial establishment before egg laying. Our results complement a macroevolutionary study finding that cavity nesters display more territorial behaviours against heterospecifics[60], underscoring multiple contexts linking territorial aggression with a cavity-nesting strategy.

Despite behavioural convergence, we did not observe higher levels of testosterone in obligate cavity nesters. Testosterone-focused hypotheses have dominated aggression-related research for decades[18], yet we find that testosterone does not explain species-level differences in female or male territorial aggression, at least when birds are sampled at a constitutive state, without a prolonged social challenge[40]. Recent phylogenetic analyses have found that breeding season length and mating system predict testosterone levels across species[61], but these life history traits are similar across our focal species. Interspecific variation in testosterone does not track interspecific variation in territorial aggression for either sex. This finding aligns with recent perspectives that variation in testosterone is context dependent[62,63] and that testosterone is but one of many potential mechanisms regulating aggression[64,65].

Using multiple phylogenetically informed approaches, we find that the convergent evolution of obligate cavity nesting is associated with a small set of convergently expressed genes in the brain, alongside a larger set of lineage-specific genes shared only by some families or species. With these quantitative phylogenetic approaches, we find changes that occur more than is expected due to shared evolutionary history and random chance. These patterns could be driven by expression evolution in either the obligate-cavity or open nesters, but we are currently unable to resolve this directionality, given that we did not explicitly construct ancestral states for expression values. RRHO analyses revealed striking patterns of expression concordance between family comparisons, with 0.1% of orthologues (11 genes) associated with obligate cavity nesting across all comparisons. Single-gene and network PGLMM analyses likewise revealed a small set of convergently evolving genes (0.4%; ~40 genes). These proportions are similar to two recent studies on parallel trait evolution[14,66]; however, they represent less convergence than most other studies of brain gene expression and behavioural evolution[9,13,67], which report that 4–6% of expressed orthologues are convergently evolving alongside behaviour. Although our study differs by the number of taxa and/or degree of evolutionary divergence, another key difference is our explicit phylogenetic approach to identify convergently evolving genes, which statistically eliminates shared patterns of gene expression that stem from common ancestry and more directly tests for convergence. Thus, ours and other phylogenetic approaches may reduce the number of genes inferred as convergently evolving. Altogether, our results suggest that the convergent evolution of complex behavioural phenotypes can be underlain by mostly independent, lineage-specific changes in gene expression, with some convergent expression in a small, core set of genes shared across species.

This constellation of changes in gene expression may be associated with obligate cavity nesting or aggression, and our PGLMMs disentangle these effects directly. Genes associated with nest strategy or aggression exhibited uncoupled regulatory evolution, with differing degrees of convergence and low overlap in gene identity. These patterns suggest that there are diverse neuromolecular correlates of aggression, including many that differ among species. It is worth noting that we also found significant gene overlap with our aggression-associated genes and a previously published socially sensitive gene network[55], indicating concordance between short-term functional responses to competition and long-term evolutionary change in competitive environments. Although our study focused on genes constitutively expressed during territorial establishment, future work should examine convergence in genes whose expression is activated or suppressed by acute social challenges, particularly as such environmentally sensitive genes may also play a key role in behavioural evolution[68].

By including both females and males, our study also assesses key elements of sex-specific evolution. Across species, we find sex-biased gene expression, for which males had higher expression than females. In birds, males carry two copies of the Z chromosome, and dosage compensation of the Z chromosome may be incomplete or absent[69], leading to higher expression of Z-linked genes in males than in females. This mechanism likely explains our results for the 'red' network of co-regulated genes, which had higher expression in males than in females. Higher expression in males could also stem from stronger sexual selection[70] or greater constraint on the evolution of reduced expression (that is, floor effects). Our sex-specific analyses further evaluate the potential for sex-specific behavioural and mechanistic convergence. Consistent with this hypothesis, we found that female obligate cavity nesters are more aggressive than females of both facultative and open-cup nesting species—a pattern that was not recapitulated in males. We also identified two female-specific gene networks that correlated with aggression. These sex-specific patterns suggest that female aggression has evolved differently from male aggression in obligate cavity-nesting species, again underscoring multiple regulatory routes to behavioural convergence.

In summary, convergent evolution provides a natural experiment to identify the genomic underpinnings of complex phenotypes, and our work highlights the power of comparative approaches to uncover these mechanistic bases of convergence[13,14,21]. Although future experiments are needed to functionally validate the nesting- and aggression-associated patterns we identify, our study is among the first to use phylogenetically explicit models of brain gene expression to understand the evolution of a complex and continuously varying behavioural trait. Our findings support the hypothesis that there are largely independent evolutionary routes to building an aggressive bird, layered atop a small set of convergently evolving genes.

Methods

This work was approved by Indiana University Bloomington Institutional Animal Care and Use Committee 18-004 and 21-003 and relevant federal (MB59069B) and state permits (Illinois, W20.6355; Kentucky, SC1911001; Indiana, 18-030, 19-272, 2545). Unless otherwise noted, analyses used R version 4 (R-Core-Team 2019).

Assay of aggression

For cavity-nesting species, we placed the conspecific decoy on the nestbox and hung a Bluetooth speaker nearby. For non-cavity nesters, we located nest sites or observed individuals for at least an hour to determine where they spent their time. For facultative cavity-nesting species, including Carolina wrens and house sparrows, some aggression assays were conducted on individuals for which the nest could not be located, and therefore the nest type could not be confirmed. We compared levels of physical aggression and distance from decoy between individuals with a known nestbox versus these other individuals and found no significant differences between groups for either species ($P > 0.19$). Additional details on audio stimuli and decoys can be found in Supplementary Section 2. We played a conspecific vocal lure to capture the attention of the focal individual and waited 30 s before beginning the 5 min aggression assay. We measured a suite of aggressive behaviours (Supplementary Section 2) and focused on the proportion of the trial spent physically attacking the decoy. We calculated a maximum attack score of 60, based on the number of 5 s intervals that contained any physical contact. To visualize this behaviour, we converted attack scores to a proportion of the trial spent attacking (number of intervals including attack/total number of intervals × 100). We also measured distance from the focal individual to the decoy to confirm that all focal territory holders were present and engaged with the simulated intruder. We evaluated the effects of nest strategy, sex, decoy sex and their interaction on physical attacks and distance from the decoy using PGLMMs (details below and in Supplementary Section 9). For downstream analyses, we include attack averages among species and sex categories as a fixed effect, referred to simply as 'aggression'.

Sample collection

For brain gene expression, we euthanized individuals with an anaesthetic overdose of isoflurane and rapid decapitation. We collected trunk blood into heparinized BD Microtainers (365965). Using tools cleaned with RNAse-away and 95% ethanol, we immediately dissected out whole brains and flash froze on powdered dry ice within 8 min 26 s ± 12 s of euthanasia followed by storage at −80 °C. Some additional hormonal samples were obtained from barn swallows via brachial venipuncture ($n = 4$ females, $n = 8$ males), and sex was confirmed from blood via PCR[71]. Blood was stored on ice packs in the field and later centrifuged for 10 min at 8,200 × g at 4 °C. Plasma was separated and stored at −20 °C. See Supplementary Section 3 for details on enzyme immunoassays.

Immediate collections occurred an average of 18 min and 25 s after the assay started. There was no relationship between latency to sampling and testosterone (Pearson's correlation: $r = 0.031$, $P = 0.84$; Supplementary Fig. 3b), or brain gene expression ($r < |0.32|$, $P > 0.13$).

For the facultative cavity nesters, we also found no difference in testosterone between individuals nesting in nestboxes compared with individuals whose nest type was unknown, for either species ($P > 0.51$), and no difference in brain gene expression for tan4 eigengene ($P > 0.88$).

Brain dissection and RNA isolation

For 6 to 7 individuals per sex per species, we dissected whole brains into functional regions following ref. 72. We removed the cerebellum, hindbrain, optic chiasm, optic tecta and hypothalamus to the depth of the anterior commissure. Gene expression analyses focused on the VmT, which contains nodes in the vertebrate social behaviour network[46] including the extended medial amygdala, bed nucleus of the stria terminalis, and lateral septum (Supplementary Fig. 5 and Supplementary Section 4). We collected this region by removing ~1 mm of the ventromedial portion of the caudal telencephalon. We extracted total RNA using Trizol (Invitrogen), and resuspended RNA in UltraPure water. Tape Station Bioanalyzer (Agilent) on 121 samples showed RNA integrity number = 8.56 ± 0.05 (mean ± s.e.).

Sequencing, alignment and mapping

We submitted total RNA to the Indiana University Center for Genomics and Bioinformatics, using paired end sequencing (Illumina NextSeq500, 75-cycle sequencing module) to generate an average of ~28.6 million reads per sample (Supplementary Data 1). We used Trinity version 2.13.2 (ref. 73) to assemble transcriptomes per species, which we aligned to the zebra finch (*Taeniopygia guttata*) proteome (National Center for Biotechnology Information assembly GCF_003957565.2_bTaeGut1.4.pri_protein.faa)[74] using tblastn v.2.2.9. See Supplementary Section 5 for additional bioinformatics details on alignment, mapping and downstream analyses. We identified 10,672 orthologous genes with high confidence in all 10 species. We normalized counts (Supplementary Data 2) and performed differential gene expression using DESeq2 (v.1.38.3)[75] (Supplementary Section 6). See Supplementary Section 7 for details on GO enrichment analyses.

RRHO

To identify shared transcriptomic patterns in obligate cavity-nesting species across pairs of families, we used the R package RRHO (v.1.46.0)[76] (details in Supplementary Section 8). RRHO uses normalized counts to rank genes based on the direction and magnitude of expression difference between groups, in our case each obligate cavity nester and its species pair within the same family. Hypergeometric tests evaluate overlap among these ranked lists, visualized as heat maps of *P* values from 100 permutations. The pipeline also identifies the set of genes with the highest degree of concordance for each comparison.

WGCNA

We constructed gene networks for both sexes combined using WGCNA (v.1.72.5)[77]. We used the normalized counts from DESeq2 and filtered out genes with <15 norm counts in 90% of the samples. We generated a signed hybrid network by selecting a soft threshold power (β) = 6, in accordance with scale-free topology. We calculated a minimum network size of 30 and used a biweight midcorrelation (bicor) function. We merged modules using the Dynamic Tree Cut method with a threshold of 0.25. Genes with an absolute network membership value of >0.6, which indicates high network connectivity, were assessed for enrichment of biological processes in PantherGO (v.19.0)[78], using our list of 10,672 orthologues as a reference set. Networks of interest were visualized in Cytoscape (v.3.10.1)[79]. See Supplementary Section 9 for gene network analyses run separately on females and males.

PGLMMs

PGLMMs used MCMCglmm (v.2.36)[80], with the phylogenetic covariance matrix from the consensus tree as a random effect (Supplementary Section 10). Our consensus tree (Fig. 1a) made use of the BirdTree.org[81] resource to obtain 1,000 ultrametric trees from Ericson All Species. We constructed a majority rule consensus topology from this tree set using the ape[82] function 'consensus' and inferred branch lengths using the phytools[83] function 'consensus.edges' with the least squares method. For each PGLMM, we evaluated Markov chain Monte Carlo convergence using Heidelberger and Welch's convergence diagnostic as implemented by the heidel.diag function in the R package coda (v.0.19.4.1).

To evaluate whether nest strategy predicted aggression, average distance from the decoy and testosterone, we fit models with nest type, sex and their interaction as fixed effects. Our aggression dataset contained many individuals that never attacked, and so we used a zero-inflated binomial distribution, with random effect and residual variance priors fixed at 1, the default burn-in period of 3,000, and 2,000,000 iterations. For this aggression analysis, technical limitations in using this specific model with a zero-inflated binomial distribution restricted our ability to specify obligate cavity nesting as the baseline factor level; therefore, we combined facultative nesting and open nesting into a single category to contrast with obligate cavity nesting. For all other PGLMMs, we fit models containing either two (obligate versus non-obligate) or three (obligate versus facultative versus open) factor levels (Supplementary Section 10). For these other models, we fit a standard Gaussian model with no priors, the default burn-in and 100,000 iterations.

To predict nest-strategy and aggression-associated genes from our set of ~10,000 orthologues, we included species/sex-level aggression sex, nest strategy, nest strategy by sex interaction and nest strategy by aggression interaction as fixed effects. After fitting a model for the log expression of each gene, we applied a Benjamini–Hochberg FDR correction in the statsmodels package[84] in Python version 3.7.11. As our PGLMMs could not estimate *P* values with precision lower than 1×10^{-4} (due to computational constraints), we set all raw *P* values reported as less than this value to exactly 1×10^{-4}. As the Benjamini–Hochberg procedure relies on the relative ranks of raw *P* values rather than their magnitudes, this should not affect our FDR correction.

To identify gene networks associated with nest strategy, sex and their interaction, we ran a similar phylogenetic analysis using the first eigengene value drawn from a WGCNA (Supplementary Section 11).

Our PGLMM-related scripts are available via GitHub at https://github.com/slipshut/CavityNesting/tree/main/PGLMM.

Functional interpretation

We inferred gene function based on genecards.org[47] and GO analyses. We inferred GO terms from *Homo sapiens* because its ontologies are orthologous to but more complete than avian references. We conducted an overrepresentation analysis of non-redundant biological process GO terms in PantherGO[78], using our list of orthologues as a reference set. To identify shared transcription factor motifs, we used g:Profiler (v.0.7.0) with chicken (*Gallus gallus*) as a reference 85.

We also conducted a custom permutation analysis to evaluate the degree of overlap between our aggression-related genes and published genomic studies of aggression[7,22,55,56] (Supplementary Data 12; Supplementary Section 12). Our enrichment scripts are available via GitHub at https://github.com/slipshut/CavityNesting/tree/main/Enrichment.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available in the Supplementary Information, on Github via Zenodo at https://doi.org/10.5281/zenodo.14715061 (ref. 86), and via Figshare repository at https://doi.org/10.6084/m9.figshare.26367061 (ref. 87). Raw sequence reads and count data can be obtained from the Gene Expression Omnibus database (GEO accession number GSE244480).

Code availability

Scripts for processing and analysing data are available on Github via Zenodo at https://doi.org/10.5281/zenodo.14715061 (ref. 86).

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S.E.L. and K.A.R. conceived of the study; S.E.L. led the data collection, supported by A.B.B., A.M.T., E.M.G, M.E.H., M.J.W., S.J.T, S.E.W., T.A.E. and W.M.S.; S.E.L., M.S.H., A.B.B., A.M.B., D.B.R., Q.K.T., M.W.H. and K.A.R. analysed and interpreted the data; S.E.L., M.S.H. and K.A.R. wrote the paper, and all authors contributed to revisions. The manuscript reflects the contributions and ideas of all authors.

Competing interests
The authors declare no competing interests.

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Policy information about availability of computer code

Data collection	No software was used to collect data for this study
Data analysis	Scripts for processing and analyzing data are available on Github via Zenodo: https://doi.org/10.5281/zenodo.14715061

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Ecological, evolutionary & environmental sciences study design

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Study description	We leverage the repeated, independent evolution of obligate cavity-nesting in birds to test the hypothesis that pressure to compete for a limited breeding resource has facilitated convergent evolution in behavior, hormones, and gene expression. Using aggression assays in the field, testosterone measures, and transcriptome-wide analyses of the brain in wild-captured females and males, we examined species pairs across 5 avian families, each including one obligate cavity-nesting species and a related species with a more flexible nest strategy.
Research sample	We focused on 10 North American songbird species (oscine Passeriformes) with well-characterized ecologies and life histories. In the Swallows (Hirundinidae), this included tree swallows (Tachycineta bicolor), which are obligate secondary cavity-nesters and barn swallows (Hirundo rustica), which build open-cup mud nests in human-made structures. In the Woodwarblers (Parulidae), this included prothonotary warblers (Protonotaria citrea), which are obligate secondary cavity-nesters, and yellow warblers (Setophaga petechia), which build open-cup nests in bushes. In the true Sparrows (Passeridae), this included two introduced species, Eurasian tree sparrows (Passer montanus), which are obligate secondary cavity-nesters, and house sparrows (Passer domesticus), which are facultative cavity-nesters that may nest in enclosed domed nests in dense foliage or human-made structures. In the Thrushes (Turdidae), this included Eastern bluebirds (Sialia sialis), which are obligate secondary cavity-nesters, and American robins (Turdus migratorius), which build open-cup nests in trees and shrubs. In the true Wrens (Troglodytidae), this included house wrens (Troglodytes aedon), which are obligate secondary cavity-nesters, and Carolina wrens (Thryothorus ludovicianus), which are facultative cavity-nesters that may build a cup or dome nest within loosely enclosed natural and artificial locations. For aggression assays, sample sizes ranged from 8-24 per sex and species (total = 304 individuals). For testosterone, sample sizes ranged from 6-15 per sex and species (total = 196 individuals). For brain gene expression, sample size ranged from 6-7 per sex and species (total = 121 individuals).
Sampling strategy	We did not perform sample-size calculations, but examined relevant literature to collect the appropriate number of samples for behavioral, hormonal, and neurogenomic studies typical of wild, free-living, nonmodel bird species.
Data collection	To assay territorial aggression, we simulated intrusion for 5-min using randomized combinations of 3-4 male or female taxidermic decoys and 3-5 conspecific vocalizations. We recorded behaviors from both the female and male in each territorial pair. Aggression assays were conducted by Sara Lipshutz, Alexandra Bentz, Tara Empson, Elizabeth George, Samuel Torneo, Abbigail Turner, Sarah Wolf, and Mary Woodruff. To sample testosterone and brain gene expression, we collected individuals using a mist-net or a nestbox trap, followed by an anaesthetic overdose of isoflurane and rapid decapitation. We collected trunk blood into heparinized BD Microtainers (#365965). Using tools cleaned with RNAse-away and 95% ethanol, we immediately dissected out whole brains and flash froze on powdered dry ice within 8 min 26 sec ± 12 sec of euthanasia followed by storage at -80°C. Some additional hormonal samples for were obtained from barn swallows via brachial venipuncture (n = 4 females, n = 8 males), and sex was confirmed from blood via PCR (22). Blood was stored on ice packs in the field and later centrifuged for 10 min at 10,000 rpm. Plasma was separated and stored at -20°C.
Timing and spatial scale	Our goal was to measure constitutive levels of testosterone and gene expression. We achieved this goal with three approaches: a)

Timing and spatial scale	passive collection, in which we set up a mist-net or trap in the target territory and waited to capture the focal individual without any stimulation (testosterone: n = 108, brain: n = 51), b) immediate collection, in which we sampled individuals immediately after a short aggression assay (testosterone: n = 55, brain: n = 43), and c) delayed collection in which we sampled individuals several days after a short aggression assay (testosterone: n = 33, brain: n = 27).
Data exclusions	We ran a principal component analysis and found that individuals clustered by family, species, and sex for the PCA of overall gene expression. PCA revealed clustering by sex, species, and family (Figure S5). PC1 explained 19% of the variance in gene expression, and PC2 explained 14%. One sample, a female house sparrow, had a divergent expression pattern from other samples, and upon further investigation also had lower overall expression relative to other individuals in same species and sex, and so was excluded.
Reproducibility	There were no attempts to reproduce these data
Randomization	We submitted total RNA to the Indiana University Center for Genomics and Bioinformatics in two rounds – once in 2019, for species pairs in the Thrush and Swallow families, and once in 2021 for species pairs in the Sparrow, Wren, and Warbler families. Because we sequenced species pairs together, we did not anticipate batch effects that would impact downstream gene expression analyses.
Blinding	Blinding was not relevant to this study, as the treatment groups were species and sexes whose identities needed to be observed in the field

Did the study involve field work? ☒ Yes ☐ No

Field work, collection and transport

Field conditions	Sampling dates ranged from mid-March to mid-May from 2018 – 2021, depending on the species and localities, and included COVID-19 impacted study seasons. We collected and processed data from species pairs within the same family during the same years. To ensure all species were in the territorial establishment and breeding stage, we monitored eBird records and observed our field sites directly and daily as pairs formed and began defending territories, evidenced by singing and/or displacing intruders. For both facultative and obligate cavity-nesting species, we also noted their nestbox contents because focal species claim their territory with a piece of nest material placed in the artificial cavity. Similarly, barn swallows reuse mud nests year after year, so we monitored nests for occupancy and signs of re-building with fresh mud. For other species, we observed pairs for multiple hours to determine territorial boundaries. Thus, some criteria used to predict breeding stage varied in a species-specific manner, but our observations suggest that all individuals were within the territorial establishment phase, which overlaps with the period of nest-building but occurs before any eggs were formed and laid. Indeed, when we confirmed breeding status by post-mortem inspection of the gonad(s), we noted that females had small white follicles and males had enlarged, white testes, consistent with this pre-breeding stage. No females had large yolky follicles that would have indicated fertility.
Location	We sampled barn swallows, yellow warblers, house sparrows, Eastern bluebirds, American robins, house wrens, and Carolina wrens at multiple sites around Bloomington, IN, USA (39142 N, 86.602 W). We sampled tree swallows near Lexington, KY (38.104 N, 84.489 W), prothonotary warblers near Belknap, IL (37.313 N, 88.984 W), and Eurasian tree sparrows near Havana, IL (40.353 N, 90.022 W) and Armington, IL (40.349 N, 89.349 W), USA. We studied additional barn swallows near Monticello, IL (40.028 N, 88.573 W) and additional house sparrows near Havana, IL.
Access & import/export	This work was conducted in compliance with federal (MB59069B) and state permits (IL: W20.6355, KY: SC1911001, IN: 18-030, 19-272, 2545).
Disturbance	Disturbance was minimized by collecting behavioral, hormonal, and gene expression data from as many of the same individuals as possible. None of these species are threatened or endangered. By establishing artificial nestboxes for five of our focal species, we created additional breeding habitat for 100s of birds.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

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☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	The study did not involve laboratory animals
Wild animals	Species included adult females and males of the following families: Swallows: tree swallows (Tachycineta bicolor), barn swallows (Hirundo rustica), Woodwarblers: prothonotary warblers (Protonotaria citrea), yellow warblers (Setophaga petechia), Sparrows: Eurasian tree sparrows (Passer montanus), house sparrows (Passer domesticus), Thrushes: Eastern bluebirds (Sialia sialis), American robins (Turdus migratorius), and Wrens: house wrens (Troglodytes aedon), Carolina wrens (Thryothorus ludovicianus). Our goal was to measure constitutive levels of testosterone and gene expression. We achieved this goal with three approaches: a) passive collection, in which we set up a mist-net or nest-box trap in the target territory and waited to capture the focal individual without any stimulation (testosterone: n = 108, brain: n = 51), b) immediate collection, in which we sampled individuals immediately after a short aggression assay (testosterone: n = 55, brain: n = 43), and c) delayed collection in which we sampled individuals several days after a short aggression assay (testosterone: n = 33, brain: n = 27).
Reporting on sex	We confirmed sex by the sex-specific vocalizations and plumage, PCR of the sex-specific CHD polymorphism, and by post-mortem inspection of the gonad(s). We report sample sizes for sex in Table 1 of the main manuscript.
Field-collected samples	To sample testosterone and brain gene expression, we collected individuals using a mist-net or a nestbox trap, followed by an anaesthetic overdose of isoflurane and rapid decapitation. We collected trunk blood into heparinized BD Microtainers (#365965). Using tools cleaned with RNAse-away and 95% ethanol, we immediately dissected out whole brains and flash froze on powdered dry ice within 8 min 26 sec ± 12 sec of euthanasia followed by storage at -80°C. Some additional hormonal samples for were obtained from barn swallows via brachial venipuncture (n = 4 females, n = 8 males). Blood was stored on ice packs in the field and later centrifuged for 10 min at 10,000 rpm. Plasma was separated and stored at -20°C.
Ethics oversight	This work was conducted in compliance with Indiana University Bloomington IACUC protocols #18-004 and #21-003.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	*Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.*
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