Sara E. Lipshutz, Mark S. Hibbins, Alexandra B. Bentz, Aaron M. Buechlein, Tara A. Empson, Elizabeth M. George, Mark E. Hauber, Douglas B. Rusch, Wendy M. Schelsky, Quinn K. Thomas, Samuel J. Torneo, Abbigail M. Turner, Sarah E. Wolf, Mary J. Woodruff, Matthew W. Hahn & Kimberly A. Rosvall. 2025. “Repeated behavioural evolution is associated with convergence of gene expression in cavity-nesting songbirds” Nature Ecology & Evolution. https://doi.org/10.1038/s41559-025-02675-x

Cavity nesters (with deep apologies to Charles Knight, James Audubon, and anyone who knows cardinal nesting natural history)

Class focus area: Behavior and genomics

EEB603: Brian O’Meara

All quotes and images from the above paper unless otherwise noted

https://www.adfg.alaska.gov/index.cfm?adfg=livingwithbirds.birdhousetips

https://www.birdsandblooms.com/birding/attracting-birds/bird-nesting/birds-nest-cavities/

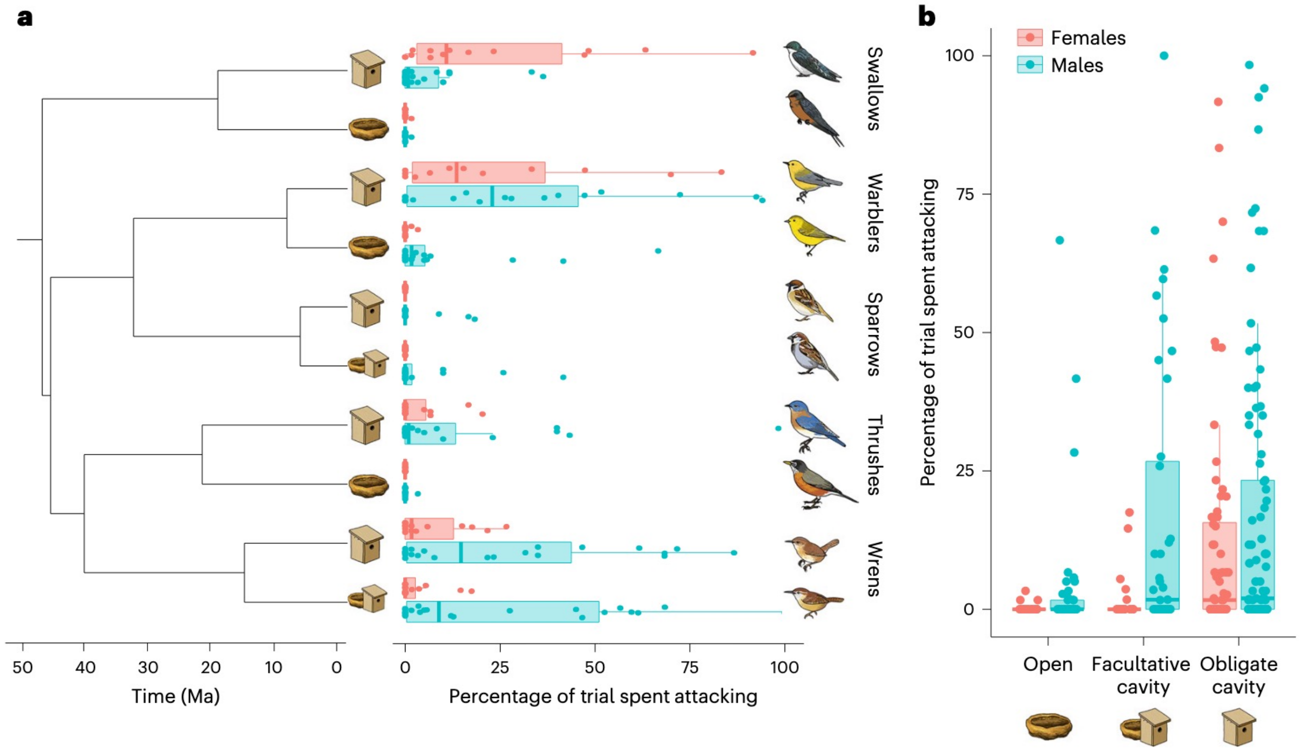


Fig. 1 | Obligate cavity nesters displayed more overt territorial aggression. **a**, Left: consensus phylogeny of 10 species from five families, which diverged from a common ancestor ~44 Ma (36–50 Ma). Species pairs diverged ~9–20 Ma (ref. 88). Nestboxes indicate obligate cavity nesters, nests represent 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.

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'.

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.

Table S1*: Model coefficient estimates from Phylogenetic Linear Mixed Models (PGLMMs) for physical attacks, as measured by physical contact with a decoy during the 5-min aggression assay. Zi is the zero-inflated class. For the 2-level models, the baseline is obligate for main effect terms, and non-obligate for interaction terms. For the 3-level models, the baseline is open for main effect terms and facultative for interaction terms. Post.mean = mean of the posterior distribution of the model coefficient, i.e. the best model estimate for the relationship. l = lower, u = upper confidence intervals, referring to the bounds of the 95% confidence internal. Eff.samp = effective samples, a measure of how effectively the Bayesian MCMC was able to search the space of possible solutions. A lower eff.sample relative to the total number of iterations (100k in this case) means a more poorly resolved model. P-values are not adjusted for the behavioral PGLMMs.*

coefficients	**post.mean**	**l -95% CI**	**u -95% CI**	**eff.samp**	**pMCMC**
2-Level Nest Strategies					
Physical Attacks	-4.28	-7.25	-1.34	7353.51	**0.01097**
Physical Attacks (zi)	-33.32	-60.55	-1.07	3.94	**0.00655**
Nest Strategy (Non-Obligate)	-4.29	-6.69	-1.92	3809.52	**0.00105**
Sex (Male)	1.85	-0.09	3.79	54657.15	0.05811
Decoy Sex (Male)	-0.34	-1.74	1.06	68149.38	0.63067
Nest Strategy (Obligate) x Sex (Male)	-2.55	-4.46	-0.7	53667.08	**0.00637**
Sex (Male) x Decoy Sex (Male)	2.26	0.52	4.03	88591.04	**0.01073**
3-Level Nest Strategies					
Physical Attacks	-9.514	-13.042	-6.067	7642.746	**0.00003**
Physical Attacks (zi)	-42.249	-65.167	-4.307	1.919	**0.00072**
Nest Strategy (Facultative)	2.163	-1.945	6.198	2586.864	0.281
Nest Strategy (Obligate)	5.282	2.221	8.42	4504.635	**0.00213**
Sex (Male)	2.394	0.00845	4.766	62315.019	**0.044**
Decoy Sex (Male)	-0.333	-1.738	1.075	91004.59	0.642
Nest Strategy (Obligate) x Sex (Male)	-3.024	-5.331	-0.799	74418.802	**0.00711**
Nest Strategy (Open) x Sex (Male)	-1.121	-4.29	2.026	59303.199	0.481
Sex (Male) x Decoy Sex (Male)	2.183	0.445	3.965	88715.232	**0.01414**

Table S2: *Model coefficient estimates from Phylogenetic Linear Mixed Models (PGLMMs) for distance from decoy during the 5-min aggression assay. Models were run with 2 nest strategies (obligate vs. non-obligate cavity-nesting) and with 3 nest strategies (obligate cavity, facultative cavity, and open cup nesting). For the 2-level models, the baseline is non-obligate cavity. For the 3-level models, the baseline is obligate cavity. Post.mean = mean of the posterior distribution of the model coefficient, i.e. the best model estimate for the relationship. l = lower, u = upper confidence intervals, referring to the bounds of the 95% confidence internal. Eff.samp = effective samples, a measure of how effectivily the Bayesian MCMC was able to search the space of possible solutions. A lower eff.sample relative to the total number of iterations (100k in this case) means a more poorly resolved model. P-values are not adjusted for the behavioral PGLMMs.*

Coefficients	**post.mean**	**l-95% CI**	**u-95% CI**	**eff.samp**	**pMCMC**
2-Level Nest Strategies					
Intercept	5.4643	2.9214	7.9813	9700	**0.00144**
Nest Strategy (Obligate)	-1.476	-3.8743	0.9726	9700	0.22392
Sex (Male)	0.3534	-1.4584	2.138	9700	0.70371
Nest Strategy (Obligate) x Sex	1.0811	-1.4082	3.4928	9366	0.3901
3-Level Nest Strategies					
Intercept	3.9925	1.2784	6.4632	9700	0.00701
Nest Strategy (Facultative)	1.1959	-2.2711	4.3114	9700	0.4532
Nest Strategy (Open)	1.6429	-1.4483	4.8598	9700	0.29814
Sex (Male)	1.4009	-0.2672	3.1057	9700	0.10412
Nest Strategy (Facultative) x Sex (Male)	-1.4911	-4.5875	1.6196	9700	0.33918
Nest Strategy (Open) x Sex (Male)	-0.6274	-3.6754	2.5242	9700	0.69423

Table S3: *Model coefficient estimates from PGLMM for testosterone. Models were run with 2 nest strategies (obligate vs. non-obligate cavity-nesting) and with 3 nest strategies (obligate cavity, facultative cavity, and open cup nesting). For the 2-level models, the baseline is non-obligate cavity. For the 3-level models, the baseline is obligate cavity. Post.mean = mean of the posterior distribution of the model coefficient, i.e. the best model estimate for the relationship. l = lower, u = upper confidence intervals, referring to the bounds of the 95% confidence internal. Eff.samp = effective samples, a measure of how effectivily the Bayesian MCMC was able to search the space of possible solutions. A lower eff.sample relative to the total number of iterations (100k in this case) means a more poorly resolved model. P-values are not adjusted for the behavioral PGLMMs.*

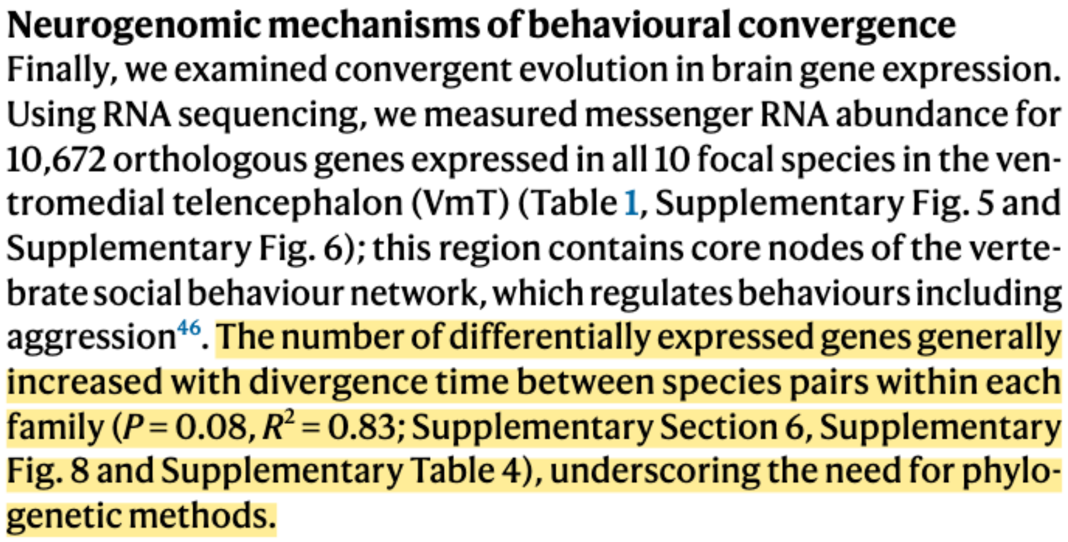
Coefficients	**post.mean**	**l-95% CI**	**u-95% CI**	**eff.samp**	**pMCMC**
2-Level Nest Strategies					
Intercept	-0.68207	-0.90066	-0.46649	10645	**<0.0001**
Nest Strategy (Obligate)	0.02489	-0.18095	0.21944	9700	0.799
Sex (Male)	1.11809	0.98293	1.25455	9700	**<0.0001**
Nest Strategy (Obligate) x Sex (Male)	-0.02052	-0.21309	0.16758	9700	0.846
3-Level Nest Strategies					
Intercept	-0.64969	-0.85172	-0.45655	9700	**<0.0001**
Nest Strategy (Facultative)	0.07451	-0.19652	0.33557	9700	0.572
Nest Strategy (Open)	-0.10393	-0.3311	0.11606	9700	0.352
Sex (Male)	1.10045	0.96776	1.23072	9700	**<0.0001**
Nest Strategy (Facultative) x Sex (Male)	0.07608	-0.18856	0.3479	9700	0.569
Nest Strategy (Open) x Sex (Male)	-0.01497	-0.22991	0.20409	9700	0.891

Table S7: *Phylogenetic Linear Mixed Models on individually expressed genes associated with nest strategy, aggression, and sex. Models run with 2 nest strategies (obligate vs. non-obligate cavity-nesting) had non-obligate as the baseline. Models run with 3 nest strategies (obligate cavity, facultative cavity, and open cup nesting) had obligate as the baseline. The total number of significantly associated genes for each model is listed, as well as genes that had higher or lower expression relative to the baseline. Gene Ontology terms listed were significantly associated with the total number of genes for each model.*

Model Term	**Total**	**Higher**	**Lower**	**Gene Ontology terms**
2-Level Nest Strategies				
Nest Strategy (Obligate)	234	127	107	None statistically significant
Nest Strategy (Obligate) x Aggression	62	19	43	None statistically significant
Nest Strategy (Obligate) x Sex (Male)	76	33	43	None statistically significant
Aggression	79	47	32	None statistically significant
Sex (Male)	510	372	138	cellular metabolic process (GO:0044237)
3-Level Nest Strategies				
Nest Strategy (Facultative)	278	85	193	None statistically significant
Nest Strategy (Open)	111	45	66	None statistically significant
Nest Strategy (Facultative) x Aggression	82	59	23	None statistically significant
Nest strategy (Open) x Aggression	92	39	53	None statistically significant
Nest Strategy (Facultative) x Sex (Male)	136	29	107	ATP metabolic process (GO:0046034)
Nest Strategy (Open) x Sex (Male)	99	53	46	None statistically significant
Sex (Male)	462	364	98	None statistically significant

Table S8. *Global Phylogenetic Linear Mixed Models (PGLMMs) on Weighted Gene Coexpression Network Analyses (WGCNA) that were significantly associated with nest strategy and/or sex. Models run with 2 nest strategies (obligate vs. non-obligate cavity-nesting) had non-obligate as the baseline. Models run with 3 nest strategies (obligate cavity, facultative cavity, and open cup nesting) had obligate cavity-nesting as the baseline. Networks with significant fixed effects are shown here. Gene Ontology terms listed were significantly associated with genes that had a network membership |>| 0.6.*

Network	**PGLMM Significant Terms**	**Number of Genes**	**Number of Genes < -0.6**	**Number of Genes > 0.6**	**High Network Membership Genes**	**Gene Ontology Terms**
2-Level Nest Strategies						
Red	Sex (Male)	193	9	114	123	None statistically significant
Tan4	Nest strategy (Obligate)	44	16	19	35	None statistically significant
3-Level Nest Strategies						
Brown	Nest strategy (Facultative), and all interactions with nest strategy (Facultative)	303	21	203	224	mtDNA translation (GO:0032543)
Dark Green	All interactions with nest strategy (Facultative)	102	68	18	86	None statistically significant
Red	Sex (Male)	193	9	114	123	None statistically significant
Tan4	Nest strategy (Open)	44	16	19	35	None statistically significant



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.

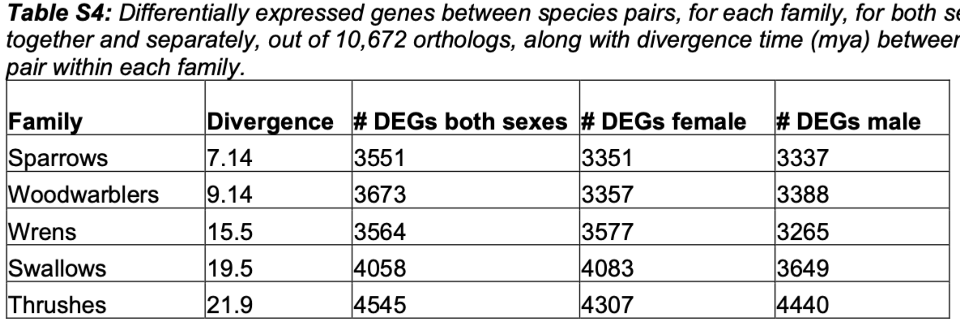


Table S4: *Differentially expressed genes between species pairs, for each family, for both sexes together and separately, out of 10,672 orthologs, along with divergence time (mya) between species pair within each family.*

Family	**Divergence**	**# DEGs both sexes**	**# DEGs female**	**# DEGs male**
Sparrows	7.14	3551	3351	3337
Woodwarblers	9.14	3673	3357	3388
Wrens	15.5	3564	3577	3265
Swallows	19.5	4058	4083	3649
Thrushes	21.9	4545	4307	4440

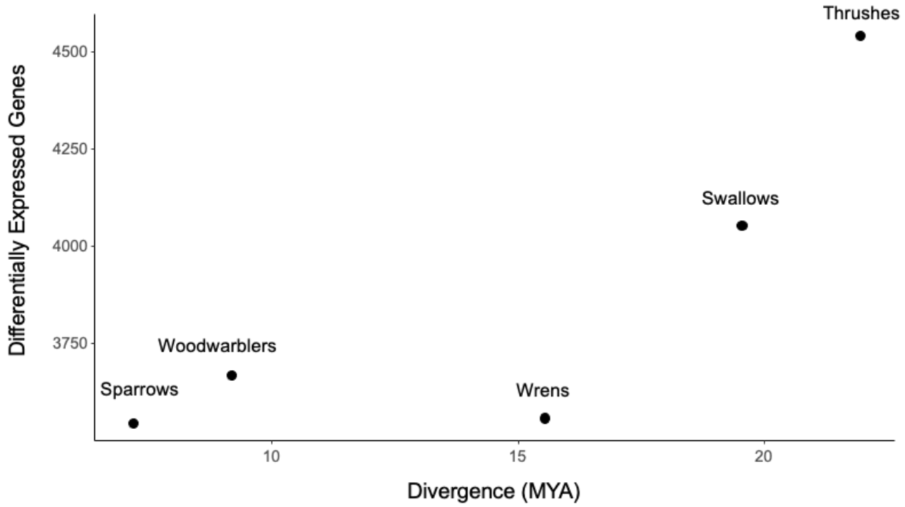


Figure S8: *Differential gene expression increased with divergence time. The number of differentially expressed genes generally increased with divergence time (in millions of years) between species pairs within each of five families ($p = 0.08$, $R^2 = 0.83$). Differentially expressed genes were classified using a log2foldchange > |0.5| and adjusted p-value < 0.05. Sample sizes for biological replicates of brain gene expression per species are provided in Table 1 in the main text.*

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.

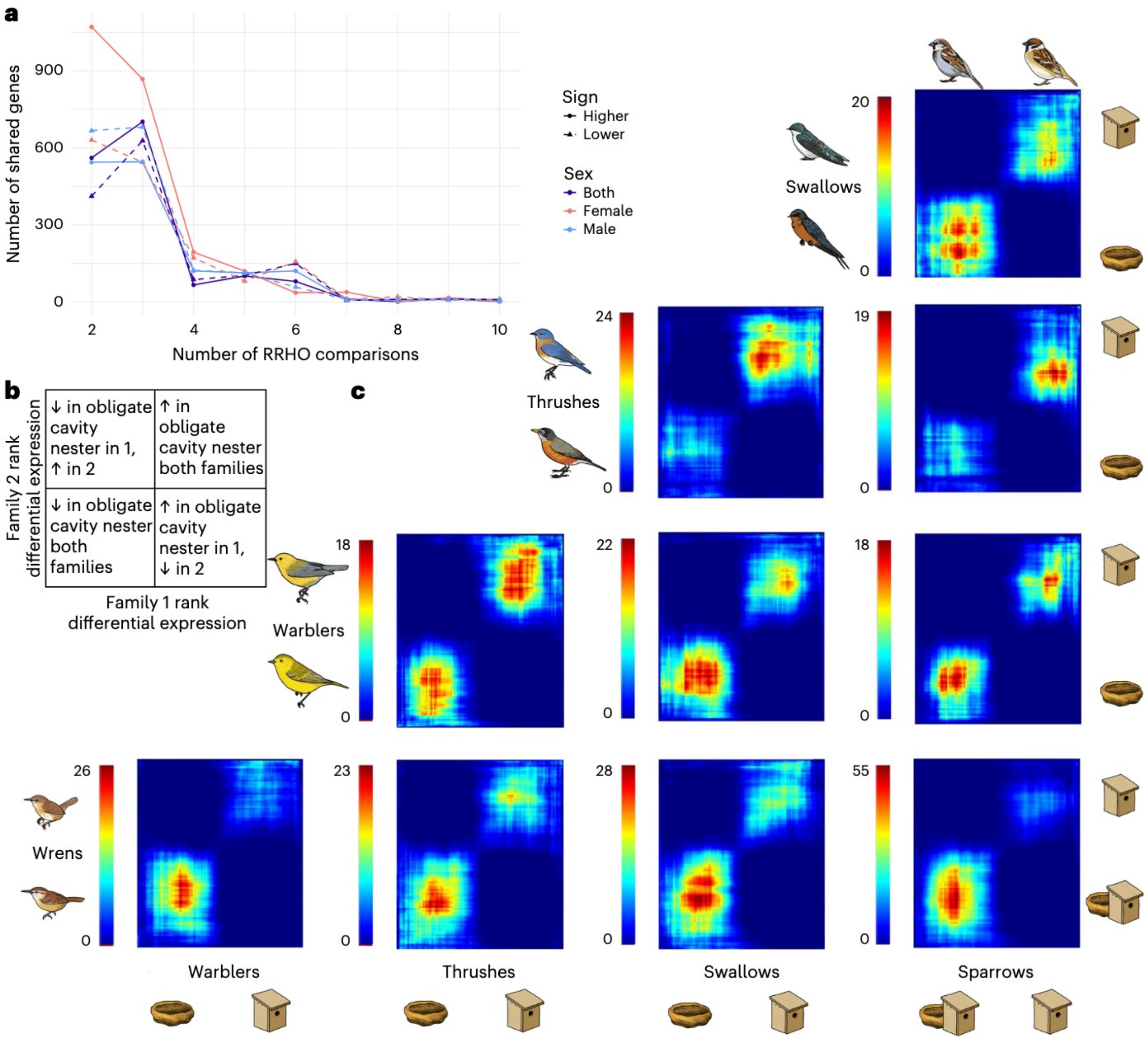


Fig. 2 | Concordance in differential expression based on RRHO. a, Number of concordantly expressed genes shared across family comparisons (that is, across heat maps) for males (blue), females (pink) and both sexes combined (purple). Solid lines indicate higher expression in obligate cavity nesters, dashed 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.

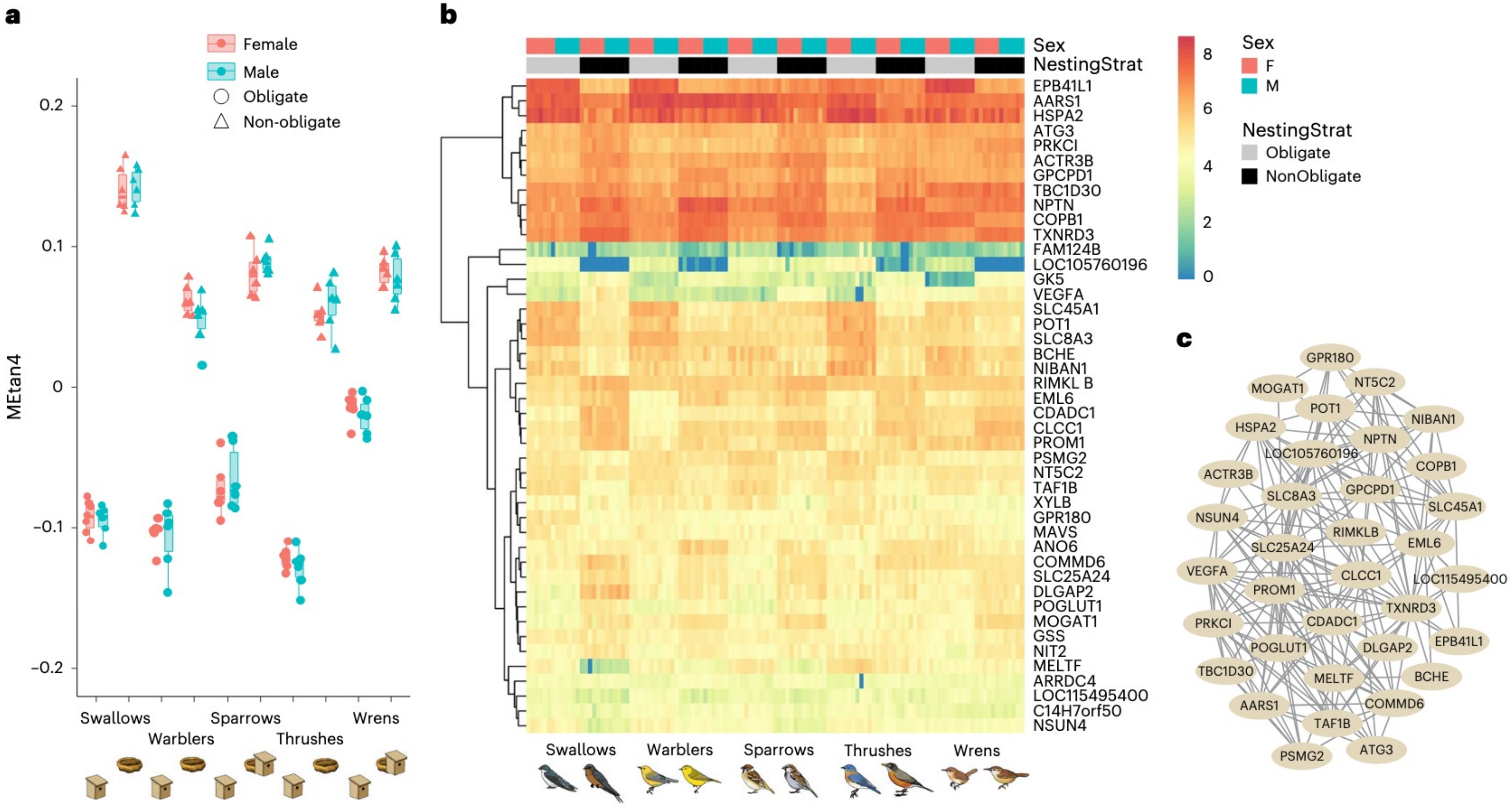


Fig. 3 | Tan4 gene network associated with obligate cavity nesting. a, WGCNA eigengene values for the tan4 network, which included 44 genes whose expression was significantly associated with obligate cavity nesting relative to non-obligate nesting strategies (PGLMM, coefficient = 0.166, adjusted $P = 0.0256$). 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.

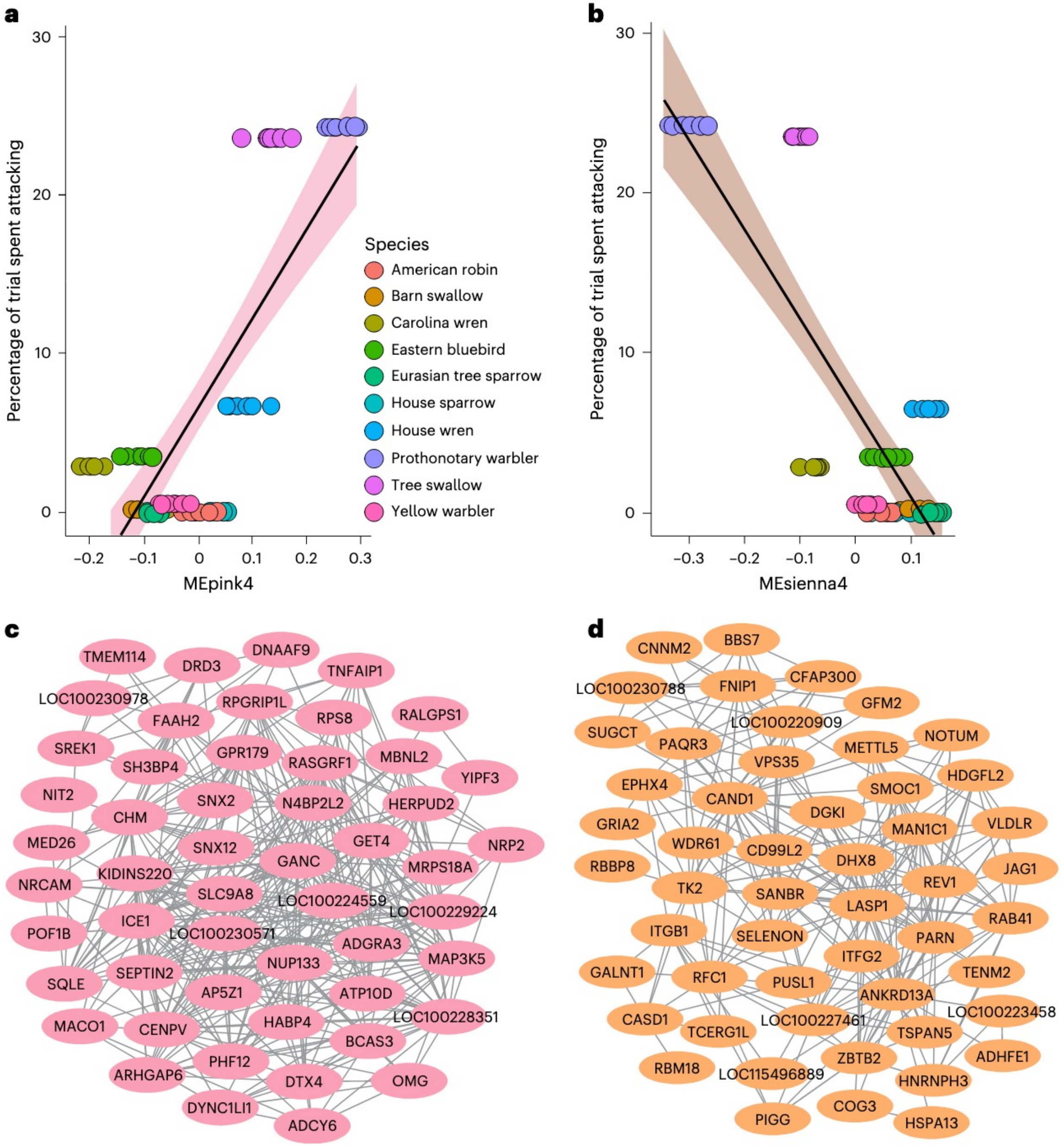


Fig. 4 | Gene networks associated with female aggression. a,**b**, WGCNA eigengenes for the female-specific pink4 network (PGLMM, coefficient = 1.39, adjusted $P = 0.0124$) (**a**) and sienna4 network (PGLMM, coefficient = −1.64, adjusted $P = 0.0124$) (**b**) were associated with average, species-level 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.

- How to convert glorious, messy behavior into something one can analyze?
- Why the use of pairs of species?
- PGLMM?
- Is this Bayesian?