Differential gene expression between urban and rural acorn ant populations

Sarah E. Diamond[1*], Lacy D. Chick[1†], Eddy Dowle[2], Catherine A. McManus[3], Gregory J. Ragland[2], Ryan A. Martin[1*]

[*] addresses for correspondence: sarah.diamond@case.edu, ryan.a.martin@case.edu
[†] present address: Hawken Upper School, Gates Mills, Ohio, USA

[1] Department of Biology, Case Western Reserve University, Cleveland, Ohio, USA
[2] Integrative Biology, University of Colorado, Denver, Denver, Colorado, USA
[3] Department of Biology, Temple University, Philadelphia, Pennsylvania, USA

ORCID:
SED 0000-0001-8518-773X
RAM 0000-0002-7448-5907
LDC 0000-0002-3059-4300
GJR 0000-0002-6501-3641
CAM 0000-0003-2437-5993

Credit:
SED - conceptualization, funding acquisition, formal analysis, visualization, writing - original draft, writing - review & editing
RAM - conceptualization, funding acquisition, formal analysis, visualization, writing - original draft, writing - review & editing
LDC - methodology, investigation, writing - review & editing
ED - methodology, investigation, writing - review & editing
GJR - conceptualization, methodology, writing - review & editing
CAM - formal analysis, writing - review & editing

Abstract

The acorn ant, *Temnothorax curvispinosus*, is a model system for rapid evolution of physiological traits to urban environments. Here, we performed a transcriptome-wide comparison of changes in gene expression between urban and rural populations of acorn ants in the southeastern United States. Our analyses revealed 287 differentially expressed genes. Overrepresentation in gene ontology terms was consistent with evolved differences in whole-organism traits such as metabolism and running speed. Transcriptome-wide comparisons also implicated an important role for cuticle development, which could directly aid in maintaining water balance in urban environments, with potential indirect effects on heat tolerance.

Description

Cityscapes can impose strong selection on populations, potentially leading to rapid, adaptive evolution (Diamond and Martin 2021). Shared selection pressures among species and among cities they inhabit can lead to shared, repeatable evolutionary outcomes (Losos 2011; Charmantier et al. 2024). For example, higher heat tolerance of ectothermic species has evolved repeatedly in association with urban heat island effects (Diamond et al. 2024). Yet, there is considerable variation in whether and how populations evolve in response to these novel urban environments (Santangelo et al. 2022). Part of this variation might be attributable to the multifarious changes in cities, set against the sensitivities of particular species to these environmental changes. Indeed, a diverse array of evolved changes in response to cityscapes have been documented. These changes include shifts in various aspects of physiology including thermal plasticity (Campbell-Staton et al. 2020) and immune defenses (Harris and Munshi-South 2017), life history including developmental rates (Tüzün et al. 2017) and pace-of-life syndromes (Brans et al. 2018), and morphology including body size (Winchell et al. 2023) and coloration (Fukano et al. 2023). Assessment of these changes has come via both whole-organism phenotypic approaches and molecular approaches. While molecular approaches that employ genome-wide or transcriptome-wide summaries of changes carry limitations on assessing causality between the environment, genotype or gene expression, and phenotype, they also carry many benefits (Hoban et al. 2016). Because these approaches are agnostic to initial choices of traits to study, they could point towards currently unrecognized attributes of cities and species sensitivities that underlie species responses to cityscapes.

Acorn-dwelling ants have become model systems for exploring rapid evolution in response to urbanization. Although most work in this area has focused on whole-organism phenotypes, molecular approaches have also been employed to understand transcriptome-wide changes in gene expression between urban and rural acorn ant populations (Smith et al. 2025). The term “acorn ant” is a broad descriptor of species in the ant genus *Temnothorax* that use hollowed-out acorns as their nest. This nesting habit allows for entire colonies to be collected and returned to the laboratory for rearing under common garden conditions to mitigate environmental effects on phenotypic and molecular differentiation. *Temnothorax longispinosus* and *T. curvispinosus* are two North American acorn ant species in which greater heat tolerance has been documented in urban populations compared with rural populations (Diamond et al. 2017; Harris et al. 2024). In

T. curvispinosus, these differences have been shown to have a genetic basis, with differences of over 1 °C in heat tolerance persisting into a second lab-reared generation (Martin et al. 2019). This species also shows divergence across urbanization gradients in metabolic rate, running speed, and heat tolerance plasticity in response to different rates of temperature change (Diamond et al. 2018a; Chick et al. 2021), though they have not diverged in other traits such as body size (Yilmaz et al. 2019). *Temnothorax nylanderi* is a European species of acorn ant, in which urban populations exhibit phenotypic changes in the form of improved heavy metal tolerance, greater foraging ability, and reduced aggression (Jacquier et al. 2021; Jacquier et al. 2023). Interestingly, this species shows fairly marginal divergence in transcriptome-wide change between urban and rural populations (Smith et al. 2025). Instead, the strongest differences were across environmental or social axes, i.e. warmer vs. colder developmental acclimation conditions and queen vs. worker castes.

In this study, we performed a transcriptome-wide comparison of worker ants from urban and rural populations of *T. curvispinosus* for which large, evolved differences in whole-organism traits have been documented. The specific urban and rural populations were sampled from Knoxville, Tennessee, USA. For this particular gradient, we previously found evolved differences in heat tolerance, cold tolerance, and potentially seasonal timing cues in the production of winged, reproductive ants (Diamond et al. 2018b; Chick et al. 2019). Although the acorn ants in our study were reared for two weeks under common garden conditions in the laboratory, mitigating immediate environmental effects of each environment, this was an insufficient amount of time to generate a new, laboratory-born cohort of workers.

Using gene-wise likelihood ratio tests on negative binomial generalized log-linear models of read counts for each gene as a function of environment (urban or rural), we found that 123 genes showed significantly increased expression in the urban population relative to the rural population and 164 genes showed significantly decreased expression in the urban population relative to the rural population (Figure 1A). In addition, 9,864 genes were not significantly differentially expressed between urban and rural populations. Of the subset of differentially expressed genes with annotations available in *Drosophila melanogaster*, we found that 7 genes had increased expression and 13 had decreased expression (Figure 1B). Gene ontology (GO) indicated trends in enrichment of metabolic processes (Figure 1C). These transcriptional changes match whole-organism phenotypic divergence between urban and rural populations of acorn ants. Urban ants exhibited higher whole-colony routine metabolic rates compared with rural ants when tested under non-stressful thermal conditions (Chick et al. 2021). In addition, the gene ontology analysis indicated important roles for tracheal development and control (Figure 1C), which could increase the aerobic scope of acorn ants and contribute to the observed pattern of improved running speed of urban ants relative to rural ants (Chick et al. 2021). Finally, genes involved in cuticle and chitin development and control were differentially expressed and significantly enriched (Figure 1C). Structural and molecular aspects of cuticle development have important functions in waterproofing and desiccation resistance (among other functions) in ants (Baumgart et al. 2022) and could play critical roles in coping with the often drier environments in cities driven by reduced groundwater infiltration and retention and hotter air and surface temperatures that lead to higher rates of evaporative water loss (Imhoff et

al. 2010). Waterproofing could likewise provide a mechanism underlying higher heat tolerance of urban acorn ants, as water balance can affect the ability of organisms to tolerate heat (Sinclair et al. 2024).

Evidence for differential gene expression between urban and rural populations of *T. curvispinosus* differs considerably from the observed lack of differential gene expression between urban and rural populations of *T. nylanderi* (Smith et al. 2025). As a caution, however, we note the potential for an inflated rate of false-positives with our analysis since the expression data are unreplicated (Robinson et al. 2010). In our study, individuals from multiple colonies in urban sites were pooled and compared with pooled individuals from multiple colonies in rural sites, whereas the *T. nylanderi* study had 4 replicates for each urban-rural comparison group. Thus, the disparity in differential gene expression between these two studies could reflect differences in experimental design and statistical power.

In addition, our study design relied on field-caught colonies that underwent a two-week laboratory acclimation at a common, non-stressful temperature, so we cannot determine whether the observed changes in gene expression were due to evolutionary divergence between the urban and rural populations or environmental effects from each location. It is possible that our study is detecting environmental temperature effects on gene expression in a similar manner to the *T. nylanderi* study that showed large environmental effects on gene expression but little evolutionary divergence. However, the relatively long laboratory acclimation period we employed here should act to minimize these environmental effects. The transcriptome changes we observed are bolstered by significant evolved differences in whole-organism physiological traits that match the transcriptional changes we observed. Furthermore, the fact that none of the gene ontology terms identified as exhibiting functional enrichment in *T. nylanderi* responses to warmer vs. colder rearing temperature were found among the enriched terms identified in our comparison of urban vs. rural *T. curvispinosus* (Figure 1C) lends additional support to the interpretation of evolved differences in gene expression between urban and rural *T. curvispinosus*.

Apart from study-level differences in the potential effects of the environment on the transcriptome, species-level differences in gene flow among populations used in the *T. nylanderi* study versus the *T. curvispinosus* study could also play a role. Specifically, *T. nylanderi* shows little evidence of population genetic structure between urban and rural sites and among more geographically distant sites distributed across Western Europe (Khimoun et al. 2020; Smith et al. 2025). By contrast, *T. curvispinosus* exhibits high population genetic structure in Eastern North America (Brandt et al. 2007; Pennings et al. 2011).

Methods

Colony collection and laboratory rearing

We collected queenright acorn ant colonies (queen was present plus her workers) from one urban and one rural site in the vicinity of Knoxville, Tennessee, USA. Collections occurred on 14

April 2017. The urban site was on the University of Tennessee campus and the rural site was at the Great Smoky Mountain National Park. These sites are separated by approximately 48 km. Four colonies were collected per site. Following collection, the colonies were brought to Cleveland, Ohio, USA for a two-week acclimation period during which colonies were reared at a constant 25 °C under a long-day summer light regime (14L:10D). Colonies were housed separately in containers and provided with unrestricted access to a water resource tube, sugar resource tube, and freeze-dried mealworms (see Diamond et al. 2018 for rearing protocol details).

RNA extraction and preparation of libraries

After the laboratory acclimation period, worker ants from each colony were frozen at -80 °C, ensuring that ants across the two source populations were frozen at the same time. Specimens were frozen on 28 April 2017. The ants were ground using the standard TRIzol method in batches of 25 individuals. There were two batches for each source location. Each batch contained an approximately equal number of workers across the colonies. RNA was extracted and purified from each of these four batches using Direct-zol Miniprep Plus. Following extraction, we pooled samples within each source location, resulting in 50 individuals from 4 colonies being represented in each pool.

Total RNA samples were submitted to the LRI Genomics Core Facility in 1.5ml tubes, normalized to 50ng/ul in 20ul. RNA quality was assessed using the Bioanalyzer. We prepared two libraries corresponding to each source location using the TruSeq Total Stranded RNA-RiboZero kit. Each library was quantified using Qubit and then pooled together. The library pool was quantified using qPCR, diluted to 4 nM, and loaded on the MiSeq v3 at 12 pM for paired-end sequencing (2 x 150 bp). The run was performed using a full flow cell (2 lanes); 5% PhiX was spiked in to check the run quality.

Differential gene expression analysis

All FASTQ files underwent quality control filtering and adapter trimming using Trim Galore! (Kreuger 2021). Following quality control, the FASTQ files were aligned to the *Temnothorax longispinosus* genome assembly (Jongepier et al. 2022) using the RNA STAR aligner (Dobin et al. 2013). We performed alignments separately for the urban population and rural population datasets. For the urban population, there were 7,551,370 uniquely mapped reads (73.3%). For the rural population, there were 4,992,186 uniquely mapped reads (67.1%).

Using the *featureCounts* function from the Rsubread package (Liao et al. 2019) in R (R Core Team 2025), we used the outputted BAM files from the STAR aligner and the gene annotation file (GTF format) from the *T. longispinosus* assembly to quantify the number of reads per gene. For the urban population, there were 2,417,036 successful assignments to features (with 2,100,447 unassigned due to not being mapped; 1,124,109 unassigned due to multi-mapping; 2,565,226 unassigned due to no features; and 12,706 unassigned due to ambiguity). For the rural population, there were 3,738,211 successful assignments to features (with 2,305,614 unassigned due to not being mapped; 1,312,227 unassigned due to multi-mapping; 3,794,574 unassigned due to no features; and 23,198 unassigned due to ambiguity). We summarized the

read counts for each source population group as *DGEList* objects using *edgeR* (Robinson et al. 2010), and then combined these into a single file for subsequent processing and analysis. We filtered out genes with low read counts using the *filterByExpr* function, using the default thresholds. We then normalized the library sizes using the *normLibSizes* function, again using the default settings. After filtering and normalization, we assessed differential expression among 10,151 genes.

Owing to the fact that we had one library per group of interest and thus no within-group replication, we used the *estimateGLMCommonDisp* function to estimate the dispersion of the normalized data. We then used the *glmFit* and *glmLRT* to model differences in expression and test their statistical significance, respectively. We used the topTags function to identify the differentially expressed genes at the $p < 0.05$ level, after adjusting for multiple comparisons using the Benjamini-Hochberg method.

Gene ontology

We used fuzzy matching to recode the NCBI RefSeq gene names to Entrez gene IDs for use in gene ontology analysis. We used *Drosophila melanogaster* as the reference species for annotations (org.Dm.eg.db; Carlson 2025). In total, our enrichment analysis was based on 20 genes with annotations available in *D. melanogaster* (Figure 1B). We used the *enrichGO* function from the clusterProfiler package (Yu et al. 2012) with the default settings except that we considered all three ontology categories (biological process, cellular component, and molecular function). We also modified the q-value and p-value cutoffs to be 0.2 and 0.1, respectively. We used a less stringent p-value cutoff owing to some borderline non-significant trends ($p \sim 0.06$) in our results and our fairly small number of differentially expressed genes that had corresponding annotation information. Multiple comparison corrections were done via the Benjamini-Hochberg method. We used the default cutoffs for gene set size, constrained to be between 10 and 500. For the pool of background genes, we used all genes from the differential gene expression analysis (both significantly and non-significantly differentially expressed) with available annotations from *D. melanogaster*. To plot the results from the gene ontology analysis, we used the *dotplot* function from the enrichplot package (Yu 2025).

References

Baumgart L et al. 2022. Why do ants differ in acclimatory ability? Biophysical mechanisms behind cuticular hydrocarbon acclimation across species. J Exp Biol. 225(16):jeb243847. https://doi.org/10.1242/jeb.243847

Brandt M, Fischer-Blass B, Heinze J, Foitzik S. 2007. Population structure and the co-evolution between social parasites and their hosts. Mol. Ecol. 16(10):2063-2078. https://doi.org/10.1111/j.1365-294X.2007.03300.x

Brans KI, Stoks R, De Meester L. 2018. Urbanization drives genetic differentiation in physiology and structures the evolution of pace-of-life syndromes in the water flea *Daphnia magna*. Proc R Soc B Biol Sci. 285(1883):20180169. https://doi.org/10.1098/rspb.2018.0169

254 Campbell-Staton SC et al. 2020. Parallel selection on thermal physiology facilitates repeated
255 adaptation of city lizards to urban heat islands. Nat Ecol Evol. 4(4):652–658.
256 https://doi.org/10.1038/s41559-020-1131-8

257 Carlson M. 2025. org.Dm.eg.db: Genome wide annotation for Fly. R package version 3.22.0.

258 Charmantier A et al. 2024. How does urbanization affect natural selection? Funct Ecol.
259 38(12):2522–2536. https://doi.org/10.1111/1365-2435.14667

260 Chick LD et al. 2019. Urban heat islands advance the timing of reproduction in a social insect. J
261 Therm Biol. 80:119–125. https://doi.org/10.1016/j.jtherbio.2019.01.004

262 Chick LD, Waters J, Diamond SE. 2021. Pedal to the metal: cities power evolutionary
263 divergence by accelerating metabolic rate and running speed. Evol Appl. 14:36–52.
264 https://doi.org/10.1111/eva.13083

265 Diamond SE et al. 2017. Rapid evolution of ant thermal tolerance across an urban-rural
266 temperature cline. Biol J Linn Soc. 121:248–257. https://doi.org/10.1093/biolinnean/blw047

267 Diamond SE, Chick LD, Perez A, Strickler SA, Zhao C. 2018a. Evolution of plasticity in the city:
268 urban acorn ants can better tolerate more rapid increases in environmental temperature.
269 Conserv Physiol. 6(1):coy030. https://doi.org/10.1093/conphys/coy030

270 Diamond SE, Chick LD, Perez A, Strickler SA, Martin RA. 2018b. Evolution of thermal tolerance
271 and its fitness consequences: parallel and non-parallel responses to urban heat islands across
272 three cities. Proc R Soc B. 285:20180036. https://doi.org/10.1098/rspb.2018.0036

273 Diamond SE, Kolaske LR, Martin RA. 2024. Physiology evolves convergently but lags behind
274 warming in cities. Integr Comp Biol. 64(2):402–413. https://doi.org/10.1093/icb/icae034

275 Diamond SE, Martin RA. 2021. Evolution in cities. Annu Rev Ecol Evol Syst. 52(1):519–540.
276 https://doi.org/10.1146/annurev-ecolsys-012021-021402

277 Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras
278 TR. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29(1):15-21.
279 https://doi.org/10.1093/bioinformatics/bts635

280 Fukano Y et al. 2023. From green to red: Urban heat stress drives leaf color evolution. Sci Adv.
281 9(42):eabq3542. https://doi.org/10.1126/sciadv.abq3542

282 Harris BA, Stevens II DR, Mathis KA. 2024. The effect of urbanization and temperature on
283 thermal tolerance, foraging performance, and competition in cavity-dwelling ants. Ecol Evol.
284 14(2):e10923. https://doi.org/10.1002/ece3.10923

285 Harris SE, Munshi-South J. 2017. Signatures of positive selection and local adaptation to
286 urbanization in white-footed mice (*Peromyscus leucopus*). Mol Ecol. 26(22):6336–6350.
287 https://doi.org/10.1111/mec.14369

288 Hoban S et al. 2016. Finding the Genomic Basis of Local Adaptation: Pitfalls, practical solutions,
289 and future directions. Am Nat. 188(4):379–397. https://doi.org/10.1086/688018

290 Imhoff ML, Zhang P, Wolfe RE, Bounoua L. 2010. Remote sensing of the urban heat island
291 effect across biomes in the continental USA. Remote Sens Environ. 114(3):504–513.
292 https://doi.org/10.1016/j.rse.2009.10.008

293 Jacquier L et al. 2021. Urban colonies are more resistant to a trace metal than their forest
294 counterparts in the ant *Temnothorax nylanderi*. Urban Ecosyst. 24(3):561–570.
295 https://doi.org/10.1007/s11252-020-01060-9

296 Jacquier L, Molet M, Doums C. 2023. Urban colonies are less aggressive but forage more than
297 their forest counterparts in the ant *Temnothorax nylanderi*. Anim Behav. 199:11–21.
298 https://doi.org/10.1016/j.anbehav.2023.02.004

299 Jongepier E et al. 2022. Convergent loss of chemoreceptors across independent origins of
300 slave-making in ants. Mol Biol Evol. 39(1):msab305. https://doi.org/10.1093/molbev/msab305

301 Khimoun A et al. 2020. Urbanization without isolation: the absence of genetic structure among
302 cities and forests in the tiny acorn ant *Temnothorax nylanderi*. Biol Lett. 16(1):20190741.
303 https://doi.org/10.1098/rsbl.2019.0741

304 Krueger F. 2021. Trim Galore. GitHub repository, https://github.com/FelixKrueger/TrimGalore

305 Liao Y, Smyth GK, Shi W. 2019. The R package Rsubread is easier, faster, cheaper and better
306 for alignment and quantification of RNA sequencing reads. Nucleic Acids Res. 47(8):e47.
307 https://doi.org/10.1093/nar/gkz114

308 Losos JB. 2011. Convergence, adaptation, and constraint. Evolution. 65(7):1827–1840.
309 https://doi.org/10.1111/j.1558-5646.2011.01289.x

310 Martin RA, Chick LD, Yilmaz AR, Diamond SE. 2019. Evolution, not transgenerational plasticity,
311 explains the divergence of acorn ant thermal tolerance across an urban-rural temperature cline.
312 Evol Appl. 12:1678–1687. https://doi.org/10.1111/eva.12826

313 Pennings PS, Achenbach A, Foitzik S. 2011. Similar evolutionary potentials in an obligate ant
314 parasite and its two host species. J. Evol. Biol. 24(4):871-886. https://doi.org/10.1111/j.1420-
315 9101.2010.02223.x

316 R Core Team. 2025. R: A Language and Environment for Statistical Computing. R Foundation
317 for Statistical Computing, Vienna, Austria. https://www.R-project.org/

318 Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential
319 expression analysis of digital gene expression data. Bioinformatics. 26(1):139–140.
320 https://doi.org/10.1093/bioinformatics/btp616

321 Santangelo JS et al. 2022. Global urban environmental change drives adaptation in white
322 clover. Science. 375(6586):1275–1281. https://doi.org/10.1126/science.abk0989

323 Sinclair BJ, Saruhashi S, Terblanche JS. 2024. Integrating water balance mechanisms into
324 predictions of insect responses to climate change. J Exp Biol. 227(10):jeb247167.
325 https://doi.org/10.1242/jeb.247167

Smith NMA et al. 2025. The transcriptome of the ant *Temnothorax nylanderi* is not affected by urbanisation but by rearing conditions. Insect Mol Biol. 34(6):889-899. https://doi.org/10.1111/imb.13011

Tüzün N et al. 2017. Microgeographic differentiation in thermal performance curves between rural and urban populations of an aquatic insect. Evol Appl. 10:1067–1075. https://doi.org/10.1111/eva.12512

Winchell KM et al. 2023. Genome-wide parallelism underlies contemporary adaptation in urban lizards. Proc Natl Acad Sci U S A. 120(3):e2216789120. https://doi.org/10.1073/pnas.2216789120

Yilmaz AR et al. 2019. Remarkable insensitivity of acorn ant morphology to temperature decouples the evolution of physiological tolerance from body size under urban heat islands. J Therm Biol. 85:102426. https://doi.org/10.1016/j.jtherbio.2019.102426

Yu G. 2025. enrichplot: Visualization of functional enrichment result. R package version 1.30.4. https://yulab-smu.top/contribution-knowledge-mining/

Yu G, Wang L-G, Han Y, He Q-Y. 2012. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS J Integr Biol. 16(5):284–287. https://doi.org/10.1089/omi.2011.0118

Acknowledgements

We thank the LRI Genomics Core Facility at the Cleveland Clinic Foundation.

Funding statement

Start-up funds to S.E.D. and R.A.M. supported this research.

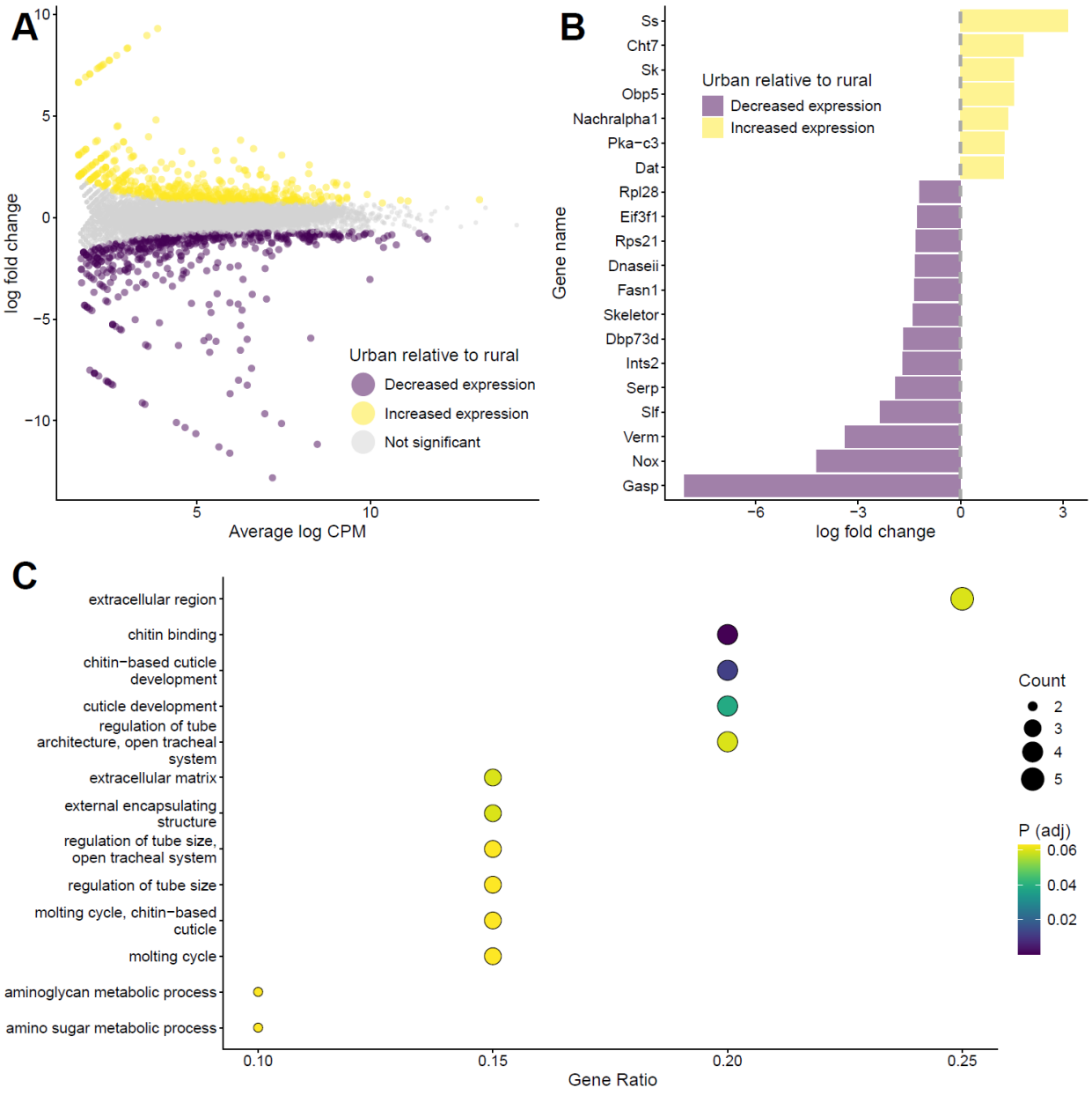

Figure 1. Differentiation between urban and rural acorn ants, *Temnothorax curvispinosus*. A) Differential gene expression summarized as the log2 fold change as a function of the average log counts per million (CPM). B) Significantly differentially expressed genes with annotation matches in *Drosophila melanogaster*. C) Gene ontology terms (y-axis) exhibiting trends ($p \sim 0.06$) or significant enrichment ($p < 0.05$). The x-axis indicates the ratio of genes enriched in a term. Point color corresponds with the associated p-value and point size corresponds with the number of genes showing significant enrichment in a particular term.