Trade-offs between cost of ingestion and rate of intake drive defensive toxin use

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Abstract

Animals that ingest toxins can become unpalatable and even toxic to predators and parasites through toxin sequestration. Because most animals rapidly eliminate toxins to survive their ingestion, it is unclear how populations transition from susceptibility and toxin elimination to tolerance and accumulation as chemical defense emerges. Studies of chemical defense have generally focused on species with active toxin sequestration and target-site insensitivity mutations or toxin-binding proteins that permit survival without necessitating toxin elimination. Here, we investigate whether animals that presumably rely on toxin elimination for survival can utilize ingested toxins for defense. We use the A4 and A3 *Drosophila melanogaster* fly strains from the Drosophila Synthetic Population Resource (DSPR), which respectively possess elevated and reduced metabolic nicotine resistance amongst DSPR fly lines. We find that ingesting nicotine increased A4 but not A3 fly survival against *Leptopilina heterotoma* wasp parasitism. Further, we find that despite possessing genetic variants that enhance toxin elimination, A4 flies accrued more nicotine than A3 individuals likely by consuming more media. Our results suggest that enhanced toxin metabolism can allow for greater toxin intake by offsetting the cost of toxin ingestion. Passive toxin accumulation that accompanies increased toxin intake may underlie the early origins of chemical defense.

Introduction

Most animals survive toxin ingestion by eliminating toxins through metabolic detoxification (1–3). Some chemically defended animals subvert this paradigm by sequestering dietary toxins to deter predators or parasites (4). Because metabolic detoxification serves to prevent toxin accumulation, toxin-sequestering taxa often employ resistance mechanisms that do not degrade toxins (5). For example, target-site insensitivity (TSI), which results from mutations in a protein that prevent toxins from binding, is common in toxin-sequestering insects (6, 7). TSI sometimes co-occurs with toxin-binding proteins that scavenge toxins and prevent them from binding to targets (8–11). Such non-metabolic resistance mechanisms may facilitate the transition from toxin elimination to sequestration by decreasing reliance on toxin breakdown for survival (12).

Although metabolic detoxification degrades toxins, it also permits toxin consumption and may ultimately lead to toxin sequestration so long as consumption outpaces degradation. To test this idea, we obtained two isofemale, homozygous strains of *Drosophila melanogaster* from the Drosophila Synthetic Population Resource (DSPR (13)) that possess high and low nicotine resistance (A3 and A4, Bloomington stocks 3852 and 3844), as well as the endoparasitoid figitid wasp, *Leptopilina heterotoma*. Compared to A3, A4 possess duplicate copies of cytochrome p450s *Cyp28d1* and *Cyp28d2* that are constitutively expressed at higher levels. A4 flies also harbor a mutation in the UDP-glucuronosyltransferase *Ugt86Dd*. *Ugt86Dd* is located in a Quantitative Trait Locus (QTL) that contributes 50.3% of the broad-sense heritability in nicotine resistance of DSPR lines, while a QTL containing *Cyp28d1* and *Cyp28d2* accounts for 5% (14, 15). The contributions of these three genes to nicotine resistance has been confirmed via gene knockout (16). Previous QTL and expression-QTL studies did not report evidence for TSI or

toxin-binding proteins in A3 or A4 lines. While these mechanisms could exist, variation in metabolic enzymes appears to underlie the major difference between A3 and A4 nicotine resistance.

We found that nicotine consumption by both flies killed many developing wasps, increasing A4 (but not A3) survival. A4 flies, which should detoxify nicotine more rapidly, accumulated higher levels of nicotine over a 24-hr period than A3 flies. Feeding assays suggested that this pattern was a result of reduced food consumption by A3 flies when on nicotine media. Thus, our findings indicate that increased toxin metabolism may facilitate the evolution of chemical defense by reducing the cost of toxin consumption and allowing for transitive accumulation of higher toxin loads.

Results and Discussion

We quantified A3 and A4 nicotine resistance by estimating the median lethal concentration (LC50) of nicotine (Fig. 1). Because A4 flies had low viability in general, to compare LC50 between strains for this assay we normalized percent survival by the maximum survival of each line on control food (see supporting data for non-normalized values). The A4 LC50 was nearly twice that of A3 ($LC50_{A4}$ = 1.9 ± 0.3 mM [mean ± SD], $LC50_{A3}$ = 1.1 ± 0.2 mM; Fig. 1). While A3 survival decreased significantly at 0.5 mM nicotine, A4 survival was not significantly impacted until 1.75 mM. We proceeded to use an intermediate level of 1.25-mM nicotine for subsequent experiments.

We next assessed whether ingesting 1.25-mM nicotine after parasitism by *Leptopilina heterotoma* increased *D. melanogaster* survival. *Leptopilina heterotoma* oviposits into the hemocoel of developing fly larvae, and actively suppresses the drosophilid defensive immune response against endoparasites (17). Thus, developing parasites are exposed to host hemolymph and, presumably, to circulating toxins consumed by fly larvae. In the no-nicotine + wasp treatment, 2.8% ± 2.7% of A4 larvae survived to adulthood, while in the nicotine + wasp treatment, A4 survival increased significantly to 6.8 ± 4.4% ($p = 0.03$, $Z = -2.2$; Fig. 2A). Correspondingly, *L. heterotoma* survival decreased five-fold from 37% ± 20% to 6.4% ± 6.8% ($p < 0.0001$, $Z = 7.0$; Fig. 2B). Thus, nicotine consumption provided A4 flies with a toxin-mediated fitness advantage.

In contrast, the survival of parasitized, nicotine-fed A3 larvae (15 ± 4.4%) was the same as parasitized, control-fed A3 larvae (19 ± 10%; $p = 0.36$, $Z = 0.92$; Fig. 2A). However, wasp survival on A3 flies halved from 41 ± 15% to 21 ± 9.3% when A3 flies consumed nicotine ($p = 0.0001$, $Z = 4$; Fig. 2B). This suggests nicotine consumption partially alleviated A3 parasitism-induced mortality. Nicotine consumption decreased unparasitized A3 fly survival by 44% ($p < 0.0001$, $Z = 7.6$), while nicotine consumption decreased parasitized A3 survival by only a tenth as much – 3.5%. The comparatively insignificant effect of nicotine consumption on parasitized A3 flies paired with a ~50% decrease in wasp survival suggests that nicotine may have offset parasitism-induced mortality for A3 flies, although to a lesser degree compared to A4 flies.

Next, we quantified nicotine accumulation in whole bodies of nicotine-fed larvae and adult flies. After ~24hr on nicotine media, third-instar A4 larvae contained over three times as much nicotine as A3 larvae (9.3 ± 4.6 ng vs. 3.2 ± 1.4 ng nicotine, $p = 0.021$, $W = 1$; Fig. 2D). Nicotine continued to accumulate until pupation in both strains and persisted through metamorphosis (Fig. 2DE; also observed with ouabain; 7), suggesting that nicotine remained after the meconium was shed and may provide a defensive advantage into adulthood. The greater amount of nicotine in A4 (Fig. 2DE) could underlie the stronger effect of nicotine on parasite survival in A4 versus A3 individuals (Fig. 2B). Although nicotine-fed A3 adults are smaller than nicotine-fed A4 adults (Fig. 2C), the difference is not three-fold and thus body size differences cannot completely explain variation in nicotine accumulation between strains.

Our finding that A4 larvae accumulated more nicotine than A3 contrasted with expectations, as A4 flies have genetic variants that are expected to increase nicotine breakdown (16, 18). To better understand this pattern, we compared relative amounts of cotinine, a metabolic by-product of nicotine (Fig 2E) between strains using integrated areas from GCMS traces (no standard was available). A4 larvae and pupae contained significantly higher levels of cotinine compared to A3 individuals (Fig. 2E). Intriguingly, A3 flies have greater relative amounts of cotinine than nicotine in all stages, yet A4 flies had relatively higher amounts of cotinine than nicotine in only the larval stage, suggesting that A4 flies have a distinct metabolic detoxification pathway compared to A3 (Fig. 2E; 13). This result matches expectations based on genotype, as the largest QTL underlying resistance in A4 contains several UGTs, which convert nicotine to glucuronides instead of cotinine (15).

The higher nicotine levels in A4 flies suggested that A3 flies are unable to survive high toxin loads, and thus might consume less to avoid nicotine accumulation. To quantify differences in feeding, we compared A3 and A4 adult body mass when reared on control versus nicotine food. While nicotine consumption significantly reduced A3 adult body mass, A4 mass remained unaffected (Fig. 2C), indicating that nicotine sensitivity constrained A3 food intake. The tobacco hornworm *Manduca sexta* employs a more extreme version of this pattern: nicotine exposure activates xenobiotic enzymes, which further stimulates feeding (19). Thus, perhaps unexpectedly, increased metabolic detoxification may promote rather than preclude toxin accumulation.

To our knowledge, *D. melanogaster* does not possess active nicotine sequestration mechanisms. Some drosophilids, such as *D. sechellia,* are known to acquire chemical defenses from toxic food sources (20), and *D. melanogaster* self-medicates against parasitoids using ethanol (21). However, other drosophilids that consume toxins have not been evaluated for chemical defenses (22, 23). Our finding that flies can utilize nicotine for defense without active sequestration mechanisms suggests that other organisms that tolerate toxin consumption could receive a transient defensive advantage, too. The biochemical properties and metabolic context of each toxin should affect their propensity to bioaccumulate. For example, glucosinolates (GLS) rapidly breakdown into toxic mustard oils; thus, GLS-sequestration requires adaptations that interrupt this process (23). Many organisms sequester toxic steroids or alkaloids (4, 24, 25), perhaps because these more readily diffuse or are transported across tissues into the hemolymph.

Here we find that in addition to having increased nicotine metabolism, A4 *D. melanogaster* flies also likely consume much higher quantities of nicotine than A3 flies (Fig. 2C). We hypothesize that higher intake may allow relatively more nicotine to escape metabolism and permeate into the hemolymph, affecting *L. heterotoma* development to a greater degree than A3 flies. This pattern could be confirmed with future studies that image nicotine abundance in different tissues of A4 and A3 flies.

In conclusion, we find that elevated resistance increases passive toxin accumulation. Further, this accumulation produces a toxin-mediated fitness advantage against natural enemies, in animals without identified sequestration mechanisms. Reliance on metabolic detoxification is likely the ancestral character state for organisms with acquired chemical defenses, and variation in toxin metabolism is common (27). We therefore propose that one of the first steps in the evolution of chemical defense may paradoxically be natural selection for increased toxin metabolism.

Methods

Fly and wasp stocks

Flies were maintained at room temperature on molasses media from the Fly Food Facility at UCB; survival and parasitism experiments used Ward's Instant Drosophila media to facilitate toxin dosing.

Wasps were maintained at room temperature on W118 *D. melanogaster* and 70%-honey water. Experiments used wasps within two weeks of eclosion.

Generation of fly larvae

Approximately one thousand flies were allowed to lay eggs for three days in three replicate resealable plastic containers with a layer of molasses-agar smeared with yeast paste. Larvae were then pooled from each container, and second instar larvae (L2) were selected based on morphology under a dissection microscope. Flies were not sorted by sex.

Nicotine-resistance experiment

Twenty L2 were transferred one-by-one from egg-laying chambers into 5 replicate vials containing the following nicotine concentrations: 0 mM, 0.5 mM, 1.25 mM, 1.75 mM, 2.25 mM, 2.50 mM, 3.00 mM, 4.00 mM, 5.00 mM nicotine-treated media. Vials were checked daily for eclosed flies.

Parasitism experiment

Four hundred A4/A3 L2 were transferred from egg-laying chambers into five plastic containers containing molasses agar. Forty female and twenty male wasps were added to three containers ("wasp" treatment) while the other two along with a sixth container of eighty L2s were left unmanipulated ("no-wasp" treatment); all containers were left for 24hr. The L2s were then transferred one-by-one (to avoid batch bias) into forty vials containing either control or 1.25-mM

nicotine media. Vials were checked every 1-2 days for pupation and emergence. Parasitism was performed prior to nicotine treatment to avoid exposing *L. heterotoma* adults to nicotine. Therefore, changes in fly and wasp survival reflect the effects of nicotine consumption by *D. melanogaster* larvae and not any behavioral change by *L. heterotoma*. This experiment was run twice with A4 flies and data were pooled across runs.

Nicotine accumulation experiment

One thousand A4/A3 L2 were distributed one-by-one from egg-laying chambers into five 1.25-mM nicotine-treated vials. At five developmental stages (3rd-instar larvae, day-1 pupa, day-3 pupae [A4 only], day-1 adult, day-3 adult), we collected five individuals and washed them individually in glass dissection wells with DI H_2O. Pupae were removed from vials prior to eclosion to avoid contamination of the adult exoskeleton with nicotine. Individuals from each stage for each vial were pooled and frozen at -20°C.

Frozen flies were thawed and soaked with methanol (50 µL) at room temperature for 2-3 days to reach equilibrium. Crude methanolic extracts were transferred to limited volume autosampler vials and injected directly. Gas chromatographic-mass spectrometric conditions were as previously described (28) and full details are given int the Supplementary Material.

Body Mass Measurement

300 A3/A4 L2 were placed one-by-one from egg-laying chambers into twenty vials containing either control or 1.25-mM nicotine media. Upon pupation, individuals were removed and placed onto food-free vials. Adults were starved for 48 hours and then weighed on a Mettler-Toledo XP6 scale.

Statistical Analysis

Statistical analyses were conducted using Rv3.6.1 (29). LC50s were calculated using adapted version of the ‘dose.p’ function from the 'MASS' package (30) to a binomial regression model of normalized percent survival versus nicotine dose generated by the ‘glmer’ function from lme4. Fly and wasp survival were each compared by applying a least-squared-means test to a binomial regression model of survival as a function of nicotine and (for flies) parasite treatments using the ‘glm’ function from the 'lme4' package (31). Adult fly mass was compared by applying the least-squared-means method described above to a model of average mass per vial as a function of nicotine and sex. Mean nicotine content of flies and was compared across fly strains using a Wilcoxon signed-rank tests in base R.

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Figure 1. A) Nicotine concentration-survival curve for DSPR A3 and A4 *Drosophila melanogaster*. Data are normalized by maximum survival of each strain on control food. Vertical dashed lines represent estimated LC50 of each strain.

Figure 2. A) Nicotine consumption significantly decreases survival in unparasitized A3 and A4 *Drosophila melanogaster* flies. Nicotine consumption increases survival of parasitized A4 but not A3 flies. **B)** Nicotine consumption by A4 and A3 flies significantly decreases *Leptopilina heterotoma* survival. **C)** Nicotine consumption reduced A3 but not A4 adult body mass. **D)** Nicotine-fed A3 and A4 flies accumulate nicotine across developmental stages. **E)** A4 flies contained higher amounts of cotinine than A3 flies, but A3 flies contained higher quantities of cotinine relative to nicotine than A4. Plot points and bars indicate mean and standard deviation; asterisks indicate significant differences (at $p < 0.05$).

References

1. P. J. Daborn, *et al.*, A single p450 allele associated with insecticide resistance in *Drosophila*. *Science* **297**, 2253–2256 (2002).

2. X. Li, M. R. Berenbaum, M. A. Schuler, Plant allelochemicals differentially regulate *Helicoverpa zea* cytochrome P450 genes. *Insect Mol. Biol.* **11**, 343–351 (2002).

3. P. Kumar, S. S. Pandit, A. Steppuhn, I. T. Baldwin, Natural history-driven, plant-mediated RNAi-based study reveals CYP6B46's role in a nicotine-mediated antipredator herbivore defense. *Proc. Natl. Acad. Sci.* **111**, 1245–1252 (2014).

4. S. S. Duffey, Sequestration of plant natural products by insects. *Ann Rev. Entomol*. **25**, 447-77 (1980).

5. G. Petschenka, A. A. Agrawal, Milkweed butterfly resistance to plant toxins is linked to sequestration, not coping with a toxic diet. *Proc. R. Soc. B Biol. Sci.* **282** (2015).

6. M. Karageorgi, *et al.*, Genome editing retraces the evolution of toxin resistance in the monarch butterfly. *Nature* **574**, 409–412 (2019).

7. A. J. Crossthwaite, S. Rendine, M. Stenta, R. Slater, Target-site resistance to neonicotinoids. *J. Chem. Biol. 2014 74* **7**, 125–128 (2014).

8. S. N. Caty, *et al.*, Molecular physiology of chemical defenses in a poison frog. *J. Exp. Biol.* **222** (2019).

9. F. Abderemane-Ali, *et al.*, Evidence that toxin resistance in poison birds and frogs is not rooted in sodium channel mutations and may rely on "toxin sponge" proteins. *J. Gen. Physiol.* **153** (2021).

10. R. D. Tarvin, *et al.*, Interacting amino acid replacements allow poison frogs to evolve epibatidine resistance. **357**, 1261-1266 (2017).

11. B. L. Whitelaw, I. R. Cooke, J. Finn, K. Zenger, J. M. Strugnell, The evolution and origin of tetrodotoxin acquisition in the blue-ringed octopus (genus Hapalochlaena). *Aquat. Toxicol.* **206**, 114–122 (2019).

12. S. Dobler, S. Dalla, V. Wagschal, A. A. Agrawal, Community-wide convergent evolution in insect adaptation to toxic cardenolides by substitutions in the Na,K-ATPase. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 13040–13045 (2012).

13. E. G. King, *et al.*, Genetic dissection of a model complex trait using the Drosophila Synthetic Population Resource. *Genome Res.* **22**, 1558–1566 (2012).

14. M. Chakraborty, J. J. Emerson, S. J. Macdonald, A. D. Long, Structural variants exhibit widespread allelic heterogeneity and shape variation in complex traits. *Nat. Commun.* **10**, 1–11 (2019).

15. T. N. Marriage, E. G. King, A. D. Long, S. J. Macdonald, Fine-mapping nicotine resistance loci in *Drosophila* using a multiparent advanced generation inter-cross population. *Genetics* **198**, 45–57 (2014).

16. S. J. Macdonald, C. A. Highfill, A naturally-occurring 22-bp coding deletion in Ugt86Dd reduces nicotine resistance in *Drosophila melanogaster*. *BMC Res. Notes* **13**, 1–6 (2020).

17. R. M. Rizki, T. M. Rizki, Selective destruction of a host blood cell type by a parasitoid wasp. *Proc. Nati. Acad. Sci. USA* **81**, 6154-6158 (1984).

18. C. A. Highfill, *et al.*, Naturally segregating variation at *Ugt86Dd* contributes to nicotine resistance in *Drosophila melanogaster*. *Genetics* **207**, 311–325 (2017).

19. M. J. Snyder, J. I. Glendinning, Causal connection between detoxification enzyme activity and consumption of a toxic plant compound. *J. Comp. Physiol. A* **179**, 255-261 (1996).

20. L. Salazar-Jaramillo, B. Wertheim, Does Drosophila sechellia escape parasitoid attack by feeding on a toxic resource? *PeerJ* **9** (2021).

21. N. F. Milan, B. Z. Kacsoh, T. A. Schlenke, Alcohol consumption as self-medication against blood-borne parasites in the fruit fly. *Curr. Biol.* **22**, 488–493 (2012).

22. J. C. Fogleman, Response of Drosophila melanogaster to selection for P450-mediated resistance to isoquinoline alkaloids. *Chem. Biol. Interact.* **125**, 93–105 (2000).

23. P. T. Humphrey, *et al.*, Aversion and attraction to harmful plant secondary compounds jointly shape the foraging ecology of a specialist herbivore. *Ecol. Evol.* **6**, 3256–3268 (2016).

24. S. C. Groen, *et al.*, Multidrug transporters and organic anion transporting polypeptides protect insects against the toxic effects of cardenolides. *Insect Biochem. Mol. Biol.* **81**, 51–61 (2017).

25. C. T. Hanifin, The chemical and evolutionary ccology of tetrodotoxin (TTX) toxicity in terrestrial vertebrates. *Mar. Drugs* **8**, 577–593 (2010).

26. G. Smagghe, L. Tirry, Insect midgut as a site for insecticide detoxification and resistance. *Biochem. Sites Insectic. Action Resist.*, 293–321 (2001).

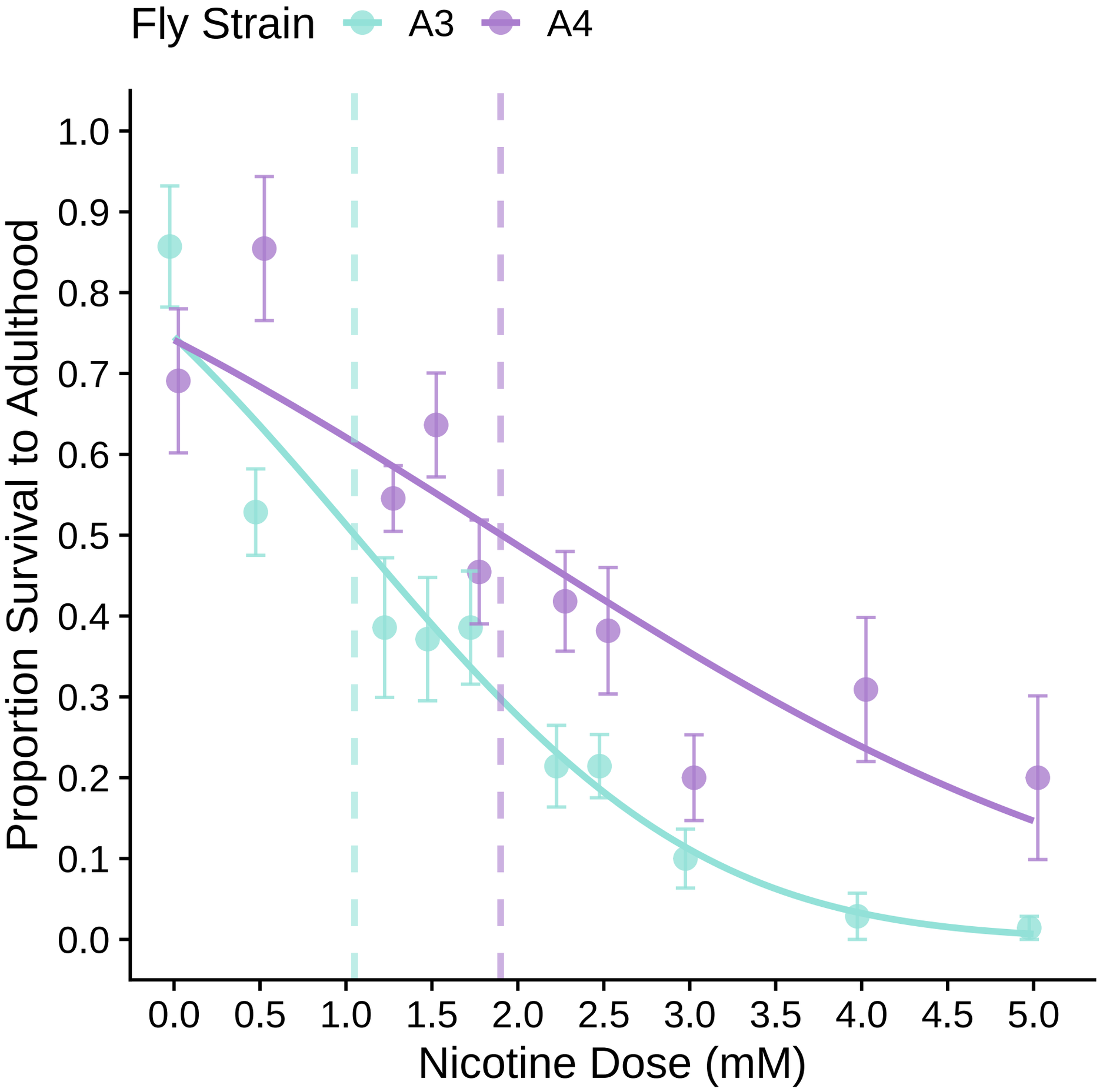
27. M. A. Najarro, *et al.*, Identifying loci contributing to natural variation in xenobiotic resistance in *Drosophila*. *PLOS Genet.* **11**, e1005663 (2015).

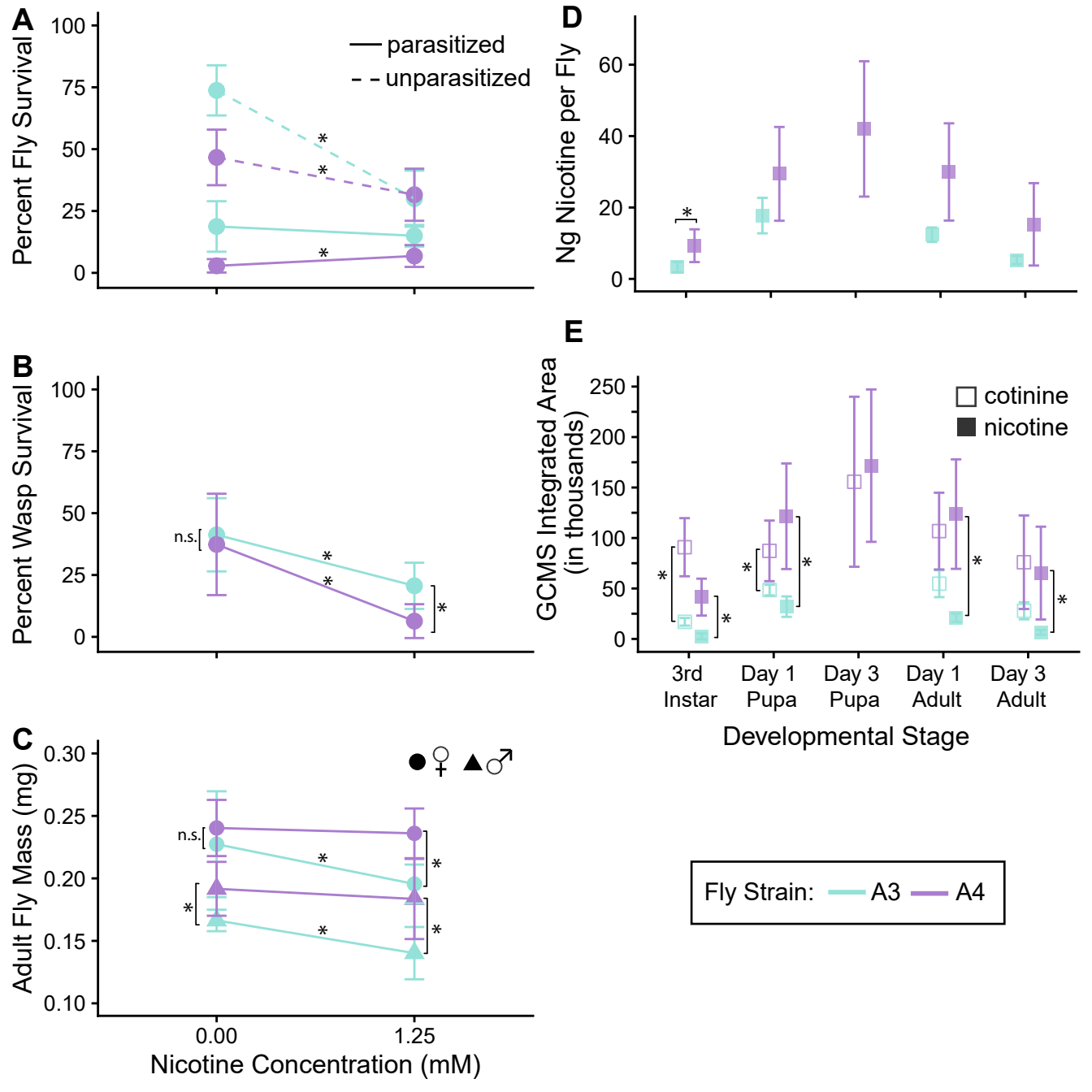
28. R. W. Fitch, T. F. Spande, H. M. Garraffo, H. J. C. Yeh, J. W. Daly, Phantasmidine: an Epibatidine congener from the Ecuadorian poison frog *Epipedobates anthonyi*. *J. Nat. Prod.* **73**, 331 (2010).

29. R Core Team. 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

30. W. N. Venables, B. D. Ripley, Introduction. 1–12 (2002).

31. D. Bates, M. Mächler, B. Bolker, S. Walker, Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1–48 (2015).


Fly Strain
A3
A4
Proportion Survival to Adulthood
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Nicotine Dose (mM)


A
Percent Fly Survival
parasitized
unparasitized
B
Percent Wasp Survival
n.s.
C
Adult Fly Mass (mg)
n.s.
Nicotine Concentration (mM)
0.00
1.25
D
Ng Nicotine per Fly
E
GCMS Integrated Area (in thousands)
cotinine
nicotine
3rd Instar
Day 1 Pupa
Day 3 Pupa
Day 1 Adult
Day 3 Adult
Developmental Stage
Fly Strain:
A3
A4