Toxin breakdown does not preclude the potential for defensive toxin use in a fruit fly

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Abstract

Animals that ingest toxins can themselves become toxic or unpalatable to predators and parasites. Because most animals rapidly eliminate toxins to survive toxin ingestion, it is unclear how species transition from susceptibility and toxin elimination to tolerance and accumulation as chemical defense emerges. Studies of chemical defense have generally focused on species that display active toxin sequestration and target-site insensitivity mutations that permit survival without necessitating toxin metabolism. Here we investigate whether animals that presumably rely on toxin elimination for survival can also 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 resistance to nicotine. We find that ingesting nicotine increased the survival of A4 but not of A3 flies against *Leptopilina heterotoma* wasp parasitism. Further, we find that despite possessing enhanced toxin clearance mechanisms, A4 flies accrued more nicotine than A3 individuals. Our results suggest that enhanced metabolic detoxification 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.

Keywords: xenobiotic metabolism, chemical defense, multi-trophic selection, bioaccumulation, enemy-free space

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Introduction

Most animals survive toxin ingestion by eliminating toxins through metabolic detoxification (1–3). Chemically defended animals subvert this paradigm by accumulating rather than eliminating toxins to deter predators or parasites (4). Given that pre-existing metabolic resistance mechanisms may preclude toxin accumulation, it is unclear how organisms transition from toxin elimination to accumulation. An important step appears to be evolving non-metabolic modes of resistance such as target-site insensitivity (TSI), which confer resistance without deactivating toxins (5). Animals with TSI often also evolve toxin-specific sequestration mechanisms that actively shuttle ingested toxins into storage tissues, driving further increases in TSI (6, 7). Because TSI acts on a narrow range of toxins, it may evolve through recurrent, long-term exposure to a single class of toxin (8, 9). Thus, if non-metabolic resistance is required to facilitate the switch from toxin elimination to accumulation, chemical defense evolution may be limited to species that specialize on particular toxic food sources. However, species lacking active sequestration or non-metabolic resistance may still acquire transient chemical defense through passive accumulation of dietary toxins so long as toxin consumption outpaces elimination. If so, then animals that consume toxins and survive may receive an advantage in the face of predation or parasitism, potentially leading to chemical defense.

Here we test whether an animal can passively accumulate a defensive level of toxin while simultaneously offsetting its cost through metabolic detoxification using Drosophila Synthetic Population Resource (DSPR) *Drosophila melanogaster* A4 and A3 fly strains (Bloomington stocks 3852 and 3844) and the parasitoid wasp *Leptopilina heterotoma*. The A3 and A4 strains are two of fifteen founder lines used to establish the DSPR, a panel of recombinant inbred lines that captures global *D. melanogaster* genetic diversity (10). The A4 fly strain harbors genetic variation in enzymes that increase its metabolic resistance to nicotine relative to A3; previous Quantitative Trait Locus (QTL) studies have not reported evidence for TSI in A3 or A4 (11, 12). Here, we tested whether nicotine consumption can increase fly survival against wasp parasitism. We found that nicotine ingestion increases A4 but not A3 survival, and that, surprisingly, nicotine-resistant A4 flies accumulate more toxin than nicotine-sensitive A3 flies. Thus, our findings indicate that, paradoxically, increased metabolic resistance may be a first step towards the evolution of chemical defense by reducing the cost of toxin consumption and thereby allowing for accumulation of a higher internal toxin load.

Results and Discussion

The A4 and A3 DSPR founder lines are respectively characterized by nicotine-resistant and nicotine-sensitive phenotypes (13). We quantified their resistance by measuring the proportion of second-instar larvae surviving to adulthood when reared on media containing 0 mM to 5.0 mM nicotine, then estimating the median lethal concentration (LC50) for each strain (Fig. 1). Because A4 flies had low viability in general (this was consistent between experiments), to compare LC50 between strains we normalized percent survival by the maximum survival of each line on control food (see supporting data for non-normalized values; reported values from subsequent experiments are uncorrected values). The A4 LC50 was nearly twice that of A3 ($LC50_{A4}$ = 1.9 ± 0.3 mM, $LC50_{A3}$ = 1.1 ± 0.2 mM; Fig. 1). While A3 survival decreased

significantly at the lowest nicotine dose administered (0.5 mM), 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* is a highly virulent parasitoid wasp that oviposits into the hemocoel of developing fly larvae, and successful wasp development is lethal to larval hosts. Although *L. heterotoma* actively suppresses the drosophilid defensive immune response against endoparasites (14), 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% (mean ± SD) of second-instar 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% ± 5% 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 not higher than parasitized larvae on control food (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 ($p = 0.0001$, $Z = 4$). This pattern suggests that nicotine consumption could have partially alleviated A3 parasitism-induced mortality in the nicotine + wasp treatment. Indeed, in non-parasitized treatments, nicotine consumption decreased A3 survival by 44% ($p < 0.0001$, $Z = 7.6$), while in the parasitized treatments, nicotine consumption decreased A3 survival by only 3.5%. The comparatively insignificant decrease in survival for parasitized A3 that were fed control versus nicotine media, as well as corresponding decrease in wasp survival when A3 flies were fed nicotine, suggests that nicotine may have partially offset parasitism-induced mortality but failed to increase net A3 survival.

To verify that flies contained nicotine, we quantified nicotine in nicotine-fed A4 and A3 larvae. After ~24hr on nicotine media, third-instar A4 larvae accumulated significantly more nicotine than A3 larvae (9.3 ± 4.6 ng vs. 3.3 ± 1.4 ng nicotine per individual, $p = 0.021$, $W = 1$; Fig. 2C). The greater amount of nicotine in A4 flies likely underlies the steeper decline in wasp survival observed on A4 nicotine-fed larvae relative to A3 (Fig. 2B). Nicotine levels increased throughout development in both fly strains and persisted through metamorphosis (also observed in fruit flies fed ouabain (15)), suggesting that nicotine persisted after the meconium was shed and may provide a survival advantage into adulthood. Our finding that A4 flies accumulated more nicotine than A3 contrasted with expectations, as A4 flies are known to exhibit elevated copy number and expression of multiple cytochrome p450s, as well as a structural mutation in a UDP-glucuronosyltransferase (UGT) gene, both of which should increase A4 nicotine metabolism relative to A3 (12, 16). We also found that A3 flies exhibit higher ratios of cotinine, a metabolic by-product of nicotine, across all stages compared to A4 flies (although non-significantly in larvae and pupae), which is unexpected given what is known about A4 metabolism (Fig. 2D). Although UGT enzymes may be converting nicotine into other metabolites, the overall higher levels of nicotine in A4 flies suggest that A4 flies simply consume

more nicotine-containing food. A similar dynamic has been observed in the tobacco hornworm *Manduca sexta*, wherein increased expression of cytochrome p450s stimulate an increase in nicotine consumption (17). Thus, enhanced survival in nicotine-fed A4 flies may also be due to higher overall nutrient intake.

Given that nicotine consumption conferred *D. melanogaster* with a survival advantage in a lab setting, we propose that gaining chemical defenses through passive toxin accumulation may be a general phenomenon in organisms that consume toxins. Several drosophilids consume toxins in the wild: *Scaptomyza nigrita* specializes on glucosinolate (GLS)-producing plants (18), while *Drosophila mojavensis* specializes on isoquinoline alkaloids-producing cacti (19), and *Drosophila sechellia* is known to acquire chemical defense through consumption of toxic fatty acids produced by its host *Morinda citrifolia* (20). We note that the biochemical properties and metabolic context of each toxin should affect their propensity to bioaccumulate. For example, GLS rapidly breakdown into toxic mustard oils, and sequestration may require adaptations that interrupt this process (18). Other small-molecule toxins may escape metabolic processes to bioaccumulate. Many organisms sequester toxic steroids or alkaloids, suggesting that these may more readily incorporate into the cuticle or hemolymph (21, 22).

In conclusion, we find that *D. melanogaster* flies with higher metabolic nicotine resistance passively accumulate more nicotine than nicotine-sensitive flies and can leverage accumulated nicotine for chemical defense against endoparasites. Thus, an increase in metabolic resistance may be a first step in the evolution of chemical defense, as increased resistance can facilitate passive toxin accumulation and generate a toxin-mediated fitness advantage against natural enemies without active toxin sequestration mechanisms. It is possible that any sufficiently resistant insect could consume a toxin and receive a toxin-mediated advantage against susceptible natural enemies through passive toxin accumulation. Our results support previous arguments that natural selection from predators and parasites on passive toxin accumulation might have been the starting point for many taxa that are currently recognized as chemically defended (15, 23).

Methods

Fly stocks

A4 and A3 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.

Wasp stocks

Leptopilina heterotoma were maintained at room temperature on W118 *D. melanogaster* and 70%-honey water. Experiments used wasps within two weeks of eclosion. *L. heterotoma* was chosen as our focal species based on its high virulence and inhibition of melanin encapsulation in *D. melanogaster* (14).

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 resealable 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 daily or every other day for pupation and adult fly and wasp emergence. The parasitism step occurred prior to nicotine treatment, in order to avoid exposing *L. heterotoma* adults to nicotine. Therefore, any changes in fly and wasp survival in this experiment 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 was 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. Our GC-MS data indicate that in A4 flies nicotine may have carried over between individuals during the wash step, as control samples show borderline detection of nicotine. However, these levels are low and suggest that cross contamination was insufficient to have influenced our conclusions. Moreover, because adult flies eclosed without physically contacting nicotine, we are confident that the signal detected in nicotine-fed flies represents genuine toxin accumulation.

GC-MS

Frozen flies were thawed and soaked with methanol (50 µL) at room temperature for 48 hours. The crude methanolic extracts were transferred to limited volume autosampler vials and injected into a Thermo Trace GC, equipped with a Restek RTX-5MS 30 m x 0.25 mm capillary column 0.5 µL with He carrier at 40 cm/s, temperature programmed at 100°C held 1 min, then a 10°C/min ramp to 280°C and held 10 min. Samples (1 µL) were injected splitless at 250°C with

a closed time of 1 min and surge pressure of 200 kPa. The GC was interfaced to a Thermo iTQ ion-trap mass spectrometer with a 250°C EI source, 70 eV ionization and autotuned with perfluorotributylamine, using automatic gain control (max ion time 25 ms). A solvent delay of 3 minutes was used. Nicotine di-dartrate (Sigma) standards were run before, midway through and at the end of test samples at log serial dilutions from 1.00 nM to 100 μM, eluting at 8.8 minutes and followed with blank methanol injections to avoid carryover. Integration analysis was done using extracted ion chromatograms at *m/z* 84 for selectivity (detection limit estimated at 0.2 ng (3 σ). The nicotine metabolite cotinine was identified by searching its mass spectrum and comparison with a known sample, eluting at 13.3 minutes. In the absence of quantitative standards for cotinine, we estimated relative rates of nicotine breakdown by ratioing the integrated area of cotinine at *m/z* 98 vs nicotine at *m/z* 84. Data were reviewed for but did not indicate presence of nicotine metabolites nornicotine and myosmine. Two other possible metabolites were found but remain unidentified. One nicotine-fed A3 pupal sample contained an order of magnitude more nicotine than all other measurements from both fly strains. Contamination by nicotine-treated media was likely the source of this inflated measurement, as the sample contained an extreme surplus of nicotine only and not its corresponding metabolic by-product, cotinine. We therefore excluded this outlier sample from our analysis.

Statistical Analysis

Statistical analyses were conducted using Rv3.6.1 (24). LC50s were calculated using adapted version of the ‘dose.p’ function from the 'MASS' package (25) 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. Mean nicotine content of flies was compared across fly strains using a Wilcoxon signed-rank tests in base R.

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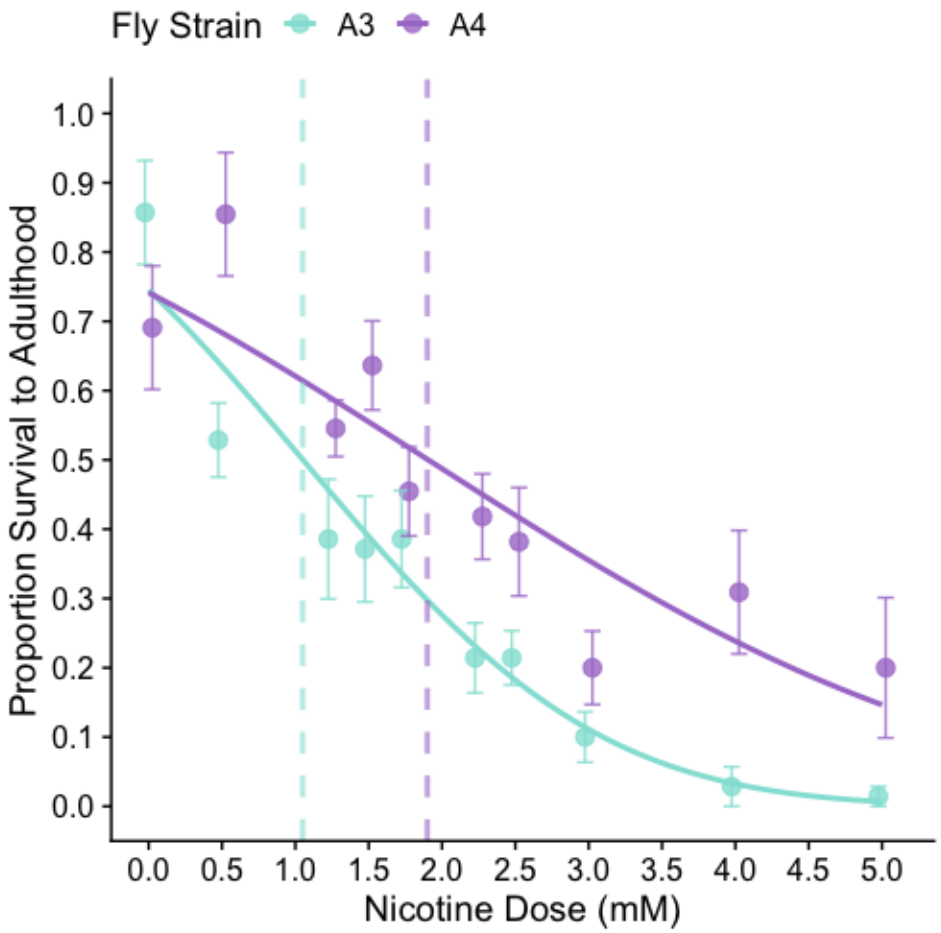

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290 **Figure 1.** Nicotine concentration-survival curve for DSPR A3 and A4 *Drosophila melanogaster*.
291 Vertical dashed lines represent estimated LC50 of each strain.

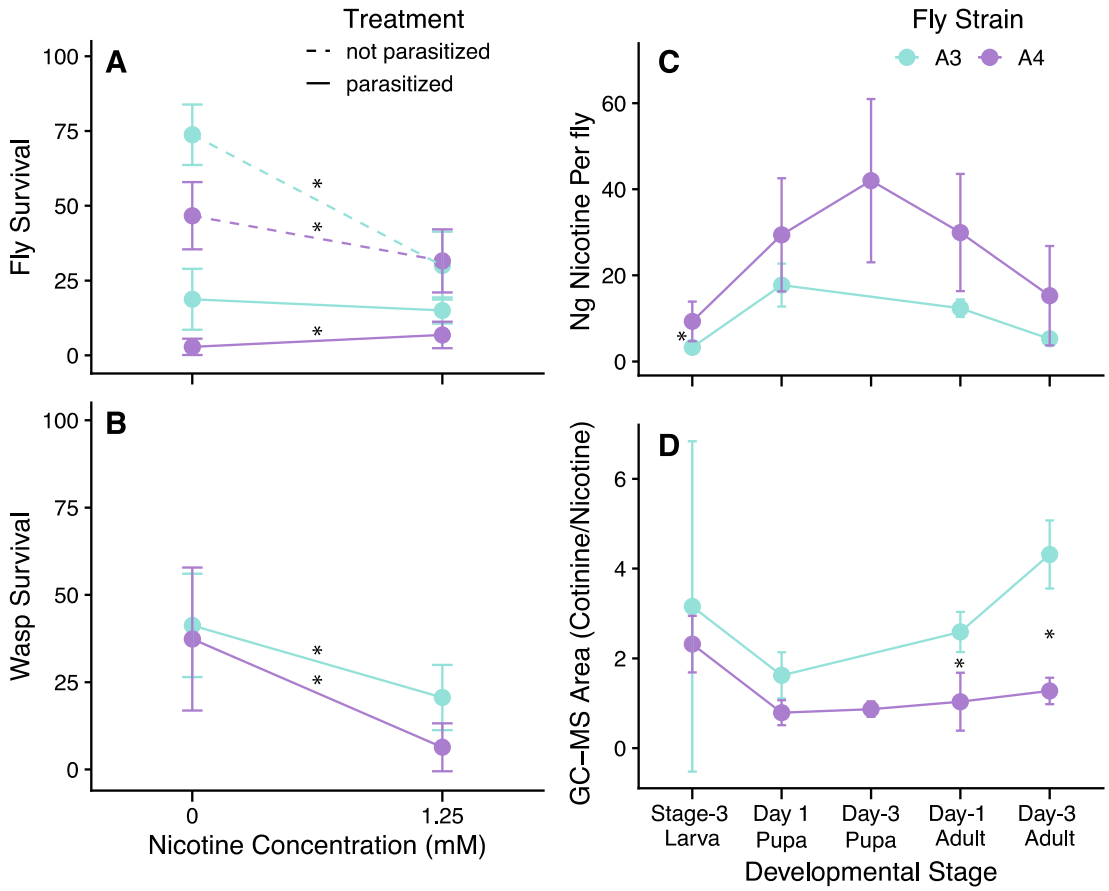

Figure 2. A) In unparasitized flies, nicotine consumption significantly decreases survival in A3 and A4 flies. When parasitized, nicotine consumption increases A4 but not A3 survival against parasitism. **B)** Nicotine consumption by A4 and A3 *Drosophila melanogaster* significantly decreases *Leptopilina heterotoma* survival. **C)** When fed nicotine-treated media, DSPR A3 and A4 *Drosophila melanogaster* accumulate nicotine across developmental stages. Larval nicotine values were significantly higher in A4 than in A3. **D)** A3 flies produced higher levels of the metabolite cotinine relative to accumulated nicotine as compared to A4 flies. Lines connect estimated means for each treatment; error bars indicate standard deviation; asterisks indicate significantly different survival (at $p < 0.05$) on nicotine versus control treatments for panels A and B and significantly different estimates of nicotine or cotinine-to-nicotine ratios between fly strains in panels C and D.