3-2020

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Tolerance of Novel Toxins through Generalized Mechanisms: Simulating Gradual Host Shifts of Butterflies

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Submitted May 8, 2019; Accepted September 12, 2019; Electronically published January 27, 2020

Online enhancements: supplemental PDF, Excel tables. *Dryad data:* https://doi.org/10.5061/dryad.pnvx0k6h3.

ABSTRACT: Organisms encounter a wide range of toxic compounds in their environments, from chemicals that serve anticonsumption or anticompetition functions to pollutants and pesticides. Although we understand many detoxification mechanisms that allow organisms to consume toxins typical of their diet, we know little about why organisms vary in their ability to tolerate entirely novel toxins. We tested whether variation in generalized stress responses, such as antioxidant pathways, may underlie variation in reactions to novel toxins and, if so, their associated costs. We used an artificial diet to present cabbage white butterfly caterpillars (*Pieris rapae*) with plant material containing toxins not experienced in their evolutionary history. Families that maintained high performance (e.g., high survival, fast development time, large body size) on diets containing one novel toxic plant also performed well when exposed to two other novel toxic plants, consistent with a generalized response. Variation in constitutive (but not induced) expression of genes involved in oxidative stress responses was positively related to performance on the novel diets. While we did not detect reproductive trade-offs of this generalized response, there was a tendency to have less melanin investment in the wings, consistent with the role of melanin in oxidative stress responses. Taken together, our results support the hypothesis that variation in generalized stress responses, such as genes involved in oxidative stress responses, may explain the variation in tolerance to entirely novel toxins and may facilitate colonization of novel hosts and environments.

Keywords: mutagens, plant defenses, oxidative stress, novel toxin, host shift.

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Am. Nat. 2020. Vol. 195, pp. 485–503.
DOI: 10.1086/707195

Introduction

Organisms have evolved an impressive range of mechanisms of toxin resistance (Hung et al. 1995; Danielson et al. 1998; Naumann et al. 2002), such as modification of sodium channels in snakes to resist tetrodotoxin (McGlothlin et al. 2016), or specific enzymes to detoxify plant defensive chemicals, such as the nitrile specifier protein in cabbage white butterflies (Wheat et al. 2007). Such evolved resistance mechanisms are specific to toxins that organisms experience regularly as part of coevolutionary relationships and may be less relevant toward novel toxins with entirely different chemical structures. Yet organisms may encounter novel toxins as their range or diet shifts, as predators or prey evolve new chemicals, or in anthropogenic environments (Ames 1983; Ames et al. 1987). How do organisms tolerate completely novel toxins? Understanding why organisms vary in their ability to cope with novel toxins has implications for understanding past evolutionary diet shifts (Ehrlich and Raven 1964) and the mechanisms by which organisms vary in their susceptibility to environmental carcinogens and pollutants (Aktipis et al. 2015). If some genotypes or populations are preadapted to tolerate novel toxins, they may be more likely to colonize new environments, whether in the context of a host shift or survival in a highly polluted site.

Organisms possess a range of "generalized" responses that help them resist or tolerate a variety of novel chemical challenges. Upregulation of these mechanisms in response to one stressor often confers resistance to additional stressors, some of which may be entirely novel. For instance, heat-shock proteins typically aid in protein folding, and their increased expression can confer resistance to temperature stress, oxidative stress, and some toxins (Feder and Hofmann 1999; Kregel 2002). Additionally, the enzymes involved in generalized physiological responses often have broad substrate reactivity. P-glycoproteins

can transport a broad range of toxins out of cells and have thus been implicated in tolerance to novel pollutants and toxins (Keppler and Ringwood 2001; Efferth and Volm 2017).

Some of the best-studied generalized mechanisms are pathways involved in combating oxidative stress (Valko et al. 2007), which can also affect tolerance of irradiation (Gerschman et al. 1954; Sun et al. 1998), carcinogens (Kensler et al. 2007), and pathogens (Deak et al. 1999). One group of such generalized enzymes are the glutathione S-transferases (GSTs), which conjugate glutathione to toxins prior to export and are responsive to a wide variety of harmful free radicals and diverse toxins (Schramm et al. 2012; Gloss et al. 2014; Halliwell and Gutteridge 2015). Antioxidant genes also combat oxidative stress associated with other detoxification mechanisms (Despres et al. 2007). For instance, cytochrome P450s (CYPs) recognize a broad range of toxins (Li et al. 2007; Schuler 2011). Both CYPs and GSTs play an important role in recently acquired pesticide resistance (Despres et al. 2007; Li et al. 2007), further implicating them in responses to novel toxins. Generalized stress responses, including genes responsive to oxidative stress, are upregulated in humans when plant defensive chemicals are consumed, reducing the risk of neural disorders, heart disease, and susceptibility to carcinogens (Mattson and Cheng 2006; Mattson 2008). Thus, it is possible that variation within and across species in constitutive or induced expression of these generalized stress responses could account for differences in susceptibility to novel toxins. In other words, genotypes with higher expression of generalized stress responses may be preadapted to colonizing polluted environments or consuming a new toxic diet (e.g., host shifts and pesticide tolerance in spider mites; Dermauw et al. 2013).

Despite the benefits of generalized stress responses, there is extensive variation in pathways and genes underlying such responses (Hackett et al. 2003; Fernandes et al. 2015; Yu and Huang 2015). The aging literature suggests that life-history trade-offs could maintain some of the variation in these pathways. Across both vertebrate and invertebrate model systems, the upregulation of generalized stress responses, such as antioxidant pathways and heat-shock proteins, has been linked to extended life span (Larsen 1993; Johnson et al. 2000; Harper et al. 2011). However, long-lived variants tend to suffer trade-offs in terms of fecundity (Johnson et al. 2000; Wang et al. 2004; Kim et al. 2010). We hypothesize that if such mechanisms also underlie responses to novel toxins, they will come with similar life-history trade-offs. It is also possible that pathways involved in generalized stress responses may trade off with their function in other contexts due to their pleiotropic nature. Melanin, for example, is important not only in oxidative stress responses (Sichel et al. 1991; Brenner and Hearing 2008) but also in immune function (Kanost and Gorman 2008) and wing structure (McGraw 2005).

This research focuses on the hypothesis that variation in generalized stress responses underlies variation in the ability to tolerate novel toxins, but that ability comes with associated trade-offs. We use butterflies as a study system because there have been hundreds of host shifts across plant families that vary drastically in the chemical structure of antiherbivory compounds (Ehrlich and Raven 1964; Fordyce 2010). Many of these defensive chemicals are poisonous and/or mutagenic to animals (Ehrlich and Raven 1964; Ames 1983). Studying the initial conditions that facilitated such host shifts is challenging, in part because rearing individuals on a novel toxic diet results in high mortality. We used an artificial diet to introduce small amounts of a novel toxic host into the diets of cabbage white butterflies (*Pieris rapae*). Such mixing of ancestral and novel hosts is similar to how some lepidopterans mix toxic and nontoxic hosts (Singer et al. 2002). This process may simulate how some host shifts occur, such as gradual transitions to spatially associated plant families in Pieridae (Braby and Trueman 2006) or through transient periods of polyphagy in Nymphalidae (e.g., Nylin et al. 2013). *Pieris rapae* utilizes host plants in the family Brassicaceae and thus primarily encounters glucosinolates as chemical defenses, for which they have specific, evolved detoxification responses (Wheat et al. 2007); Brassicaceae contain other plant defenses, such as cuticular waxes and trypsin inhibitors (Eigenbrode and Espelie 1995; Cipollini 2002; Halkier and Gershenzon 2006). Cabbage whites are an ideal species to test our hypothesis because they are easily reared on artificial diets (Snell-Rood and Papaj 2009) where different plant material can be incorporated. Furthermore, this species tends to harbor substantial variation within and between populations in behavioral and physiological traits, allowing comparisons across families in the ability to tolerate novel diets or toxins (e.g., Sikkink et al. 2017).

In this experiment, we introduced three plant species that represent plants to which host shifts have occurred in other lepidopteran lineages (Rothschild et al. 1979; Boppre 1990; Sime et al. 2000; Engler-Chaouat and Gilbert 2007) but which were entirely novel to cabbage whites. Each of these diets contains different suites of chemical defenses, including those that are both cytotoxic and mutagenic (e.g., aristolochic acids, pyrrolizidine alkaloids, or β-carbolines; Clark 1960; Picada et al. 1997; Frei et al. 1985; Boeira et al. 2001; Arlt et al. 2002; Fu et al. 2004). Such a diversity of toxins (both within and between the plant species considered) allows a test of generalized, rather than specific, detoxification mechanisms by examining genetic correlations in butterfly performance across diets. We focus on genes involved in the oxidative stress response as candidates for part of the

underlying generalized physiological response to a novel toxin. We test the predictions that (*a*) performance will be correlated across toxic diets, (*b*) variation in constitutive or induced expression of genes involved in oxidative stress responses will correlate with performance on novel toxic diets, and (*c*) variation in performance on novel toxic diets will come with life-history trade-offs.

Methods

Origin of Families and Egg Collection

Wild, gravid, female cabbage white butterflies were collected on and around the University of Minnesota, Saint Paul, campus in community gardens, weedy ditches, and agricultural areas. Approximately 40 females were captured over a 4-week period and placed in a greenhouse in individual mesh BugDorm cages (60 × 60 × 60 cm) with ad lib. access to 10% honey water (changed daily). Spermatophores were counted in wild-caught females to determine the number of times a female had mated (mean = 1.91), although *Pieris rapae* tends to have last-male precedence in the fathering of offspring (Wedell and Cook 1998). To increase egg collection, females were allowed to oviposit on three different host plants (green cabbage, *Brassica oleracea* var. COL Earliana; red cabbage, *Brassica oleracea* var. COL Red Express; and radish, *Raphanus sativus* var. Rabano Cherry Belle), increasing the probability of harvesting eggs from females with specific host preferences. There was no effect of oviposition host plant on patterns of gene expression (e.g., the lowest *P* value for the effect of original host plant in a model containing the predictors treatment, family, actin expression, and host plant was .32); additionally, there were no relationships between female plant preference and measures of performance on the novel hosts (e.g., survival on novel diets and Shannon index of hosts on which eggs were laid; $F_{1,11} = 1.13, P = .31$). Host plants were placed in 15-ounce plastic cups in a climate chamber at 23°C with 14-h day length until larvae were transferred to artificial diet 7 days after egg collection as early second instars. Of the original 40 captured females, 12 produced enough eggs for inclusion in the study.

Rearing on Novel Diets

Larvae were reared on artificial diet. The base diet, which also served as the control diet, is similar to that used in previous studies (Snell-Rood and Papaj 2009): 50 g of wheat germ, 10 g of cellulose, 15 g of cabbage flour, 27 g of casein, 24 g of sucrose, 9 g of Wesson salt mix, 12 g of Torula yeast, 3.6 g of cholesterol, 10.5 g of Vanderzant vitamin mix, 0.75 g of methyl paraben, 1.5 g of sorbic acid, 3 g of ascorbic acid, 0.175 g of streptomycin, 5 mL of flaxseed oil per 800 mL of water, and 15 g of fine-mesh agar. For each novel diet, we added 2 g of dried plant material from a nonhost plant (see below), comprising 1% dry mass of the diet. We chose to introduce novel plant material at a low dose to simulate plant "mixing" as may occur during host shifts (e.g., Braby and Trueman [2006] in Pieridae) and at low enough doses to permit study of both family and diet effects on survival in novel conditions rather than a large selective effect or complete refusal of the diet. To better place the 1% composition in context with respect to a host shift, we also reared a subset of butterflies on a range of concentrations for one diet (*Aristolochia*), as detailed below. We chose to introduce ground material from whole plants to simulate shifts to a novel host plant, although in the discussion section we further consider the complementary approach of introducing specific chemicals in more controlled doses.

We introduced novel plant material from three chemically distinct plant species fed on by other Lepidoptera but that are not a normal host plant for butterflies in the family Pieridae. First, we chose *Aristolochia macrophylla*, a member of the family Aristolochiaceae, consumed by troidine swallowtails. These plants contain nitrophenanthrene carboxylic acids, often called aristolochic acids (I and II), which have potent mutagenic and cytotoxic effects in bacteria, mammals, and *Drosophila* (Frei et al. 1985; Arlt et al. 2002); troidine swallowtails sequester these alkaloids as a chemical defense (Sime et al. 2000). *Aristolochia* were grown in the Fordyce laboratory (University of Tennessee), dried at 60°C for 2 days, and ground to a fine powder in an industrial strength blender (Waring Commercial Xtreme). Second, we chose *Tussilago farfara*, one of many species of Asteraceae that contains pyrrolizidine alkaloids, which are cytotoxic and mutagenic in both vertebrates and invertebrates (Clark 1960; Fu et al. 2004); this species in particular contains high concentrations of senkirkine, senecionine, and seneciphylline (Frei et al. 1992; Dreger et al. 2009). Moths of the genus *Euplexia* (Noctuidae) consume *T. farfara* as a host plant (Robinson et al. 2010), and pyrrolizidine alkaloids are readily consumed and sequestered by both arctiid moths and adult male danaine butterflies (Rothschild et al. 1979; Boppre 1990). Third, we chose *Passiflora incarnata*, a member of Passifloraceae, the host plant of *Heliconius* butterflies. *Passiflora* contain a range of toxins such as cyanogenic glycosides (Dhawan et al. 2004), which *Heliconius* synthesize and sequester as a defense (Engler-Chaouat and Gilbert 2007). However, they also contain the β-carboline alkaloid harman, which has toxic and mutagenic effects in bacteria, invertebrates, and vertebrates (Picada et al. 1997; Boeira et al. 2001). Both the *Passiflora* and the *Tussilago* were obtained as dried leaf material from Starwest Botanicals (items 209220-34 and 209483-34) and then ground further into a fine power with an industrial strength blender. While we did not measure the concentration or activity of these specific chemicals in our

experiments, we were careful to choose plants with chemically distinct profiles in order to measure correlated responses to novel diets. Our dose-response experiment (see fig. S1 and details below; figs. S1–S5 are available online) confirmed that, despite drying, toxins within the plants are still active and relevant to herbivores (indeed, most Ames test screens for mutagenicity of plant secondary compounds are done on dried plant material). As discussed in more detail below, frass production at all concentrations and with all host types confirmed that caterpillars were consuming each diet type.

One week after egg collection, early second-instar larvae were transferred onto a randomly assigned artificial diet (either the control diet or one of the three novel diets) with a paintbrush or feather forceps (three per cup except for <5% cups when fewer individuals were available for a given set of transfers). At this rearing density, each larva had ad lib. access to artificial diet throughout development (i.e., there was little to no larval competition; see also Jaumann and Snell-Rood 2017). Cups were stored at 24°C with a 14-h photoperiod in a walk-in climate chamber; larvae pupated within cups. At eclosion, individuals were numbered with a black, fine-tipped sharpie on their hindwing and placed into one of four mesh life span cages (35 × 35 × 61 cm) in the same walk-in climate chamber to measure adult longevity in controlled temperature conditions under T4 fluorescent bulbs (light brightness was considerably less than greenhouse levels, so general activity was reduced and no mating was observed). Butterflies had ad lib. access to 10% honey water. Cages were checked daily for dead individuals, which were immediately sealed in containers at −20°C.

To put our novel diet dosage in context, we additionally generated a dose-response curve for a broader range of toxin concentrations in the artificial diet, focusing on the *Aristolochia* diet. Wild-type *P. rapae* were obtained from Colorado State University in January 2019 and introduced to the control diet or one of five novel diets containing 0.5%, 1%, 2%, 4%, or 8% *Aristolochia* (by dry weight of diet). We manipulated the amount of cellulose in the diet (normally at 10 g per 800 mL of water) to ensure that total dry weight was comparable across each diet (however, the 8% diet, with 16 g of *Aristolochia*, had 6 g of extra material, so we added more water when mixing). Larvae ($N = 387$) were transferred onto the diets 7 days after egg collection on cabbage leaves, and performance was measured as survival to adulthood, development time, and adult dry mass (after drying for 24 h at 70°C). Our dose-response curve confirmed increasing stress (in a linear manner) with each doubling of novel host material (fig. S1; table S1; tables S1–S5 are available online), with 0% survival on the 8% *Aristolochia* diet. Increasing concentration of *Aristolochia* was associated with significant differences in survival, adult dry weight, and development time (table S1). The results confirmed that the 1% dose used in our study represents some degree of stress but is generally sublethal, thus potentially capturing responses that reflect both plastic and genetic variation. In addition, given that the diet is normally around 8% of cabbage flour (the normal host of *P. rapae*), we can roughly interpret our focal 1% toxic diet as "one part novel diet for every eight parts ancestral diet." Finally, although not significant (table S1b), the dose-response curve suggested there was a slight hormetic effect at the lowest concentrations of *Aristolochia* (fig. S1), where a low dose of toxins can have a slightly beneficial effect due to the induction of generalized defense mechanisms (e.g., Snell-Rood et al. 2018).

Performance and Phenotype Measurements

Survival was quantified as emergence as an adult with fully formed wings ($N = 581$). Development time was measured from the date of egg laying until the date of adult emergence ($N = 449$). Forewing length, defined as the distance from the forewing apex to the articulation of the forewing with the thorax, was used as a proxy for body size and was measured with digital calipers to the nearest tenth of a millimeter on individuals with intact forewings. We aimed to measure wing length for at least three males and three females from each treatment from each family ($N = 221$ total were possible given survival rates). Growth rate was calculated as body size divided by development time. Adult longevity was measured as the time from adult emergence until the date of adult death and ranged from 1 to 84 days (mean = 19 days, $N = 414$ individuals).

To determine whether differences in body size were due to direct effects of the novel toxins or that caterpillars were simply consuming less of the novel diets overall, we performed two additional comparisons. First, we reared a subset of larvae in individual cups to determine whether there were differences in consumption in the 24 h following transfer to diet from cabbage plants (at 7 days after egg collection). Neither mass- nor frass-based measures (see below) of food consumption differed across the four diets; in fact, there was a tendency toward greater initial consumption on the novel diets relative to the control (fig. S2; table S2). Given the starting mass of individuals at transfer and the average weight gain (fig. S2), most individuals doubled their initial mass during this 24-h period, suggesting significant exposure to the novel diets. Second, we measured total consumption over the entire larval period for a subset of individuals by scraping dried frass off of the artificial diet using a metal spatula and weighing to the nearest 0.0001 g; these measurements were taken only if three individuals in the cup survived to pupation ($N = 48$ diet cups). There were no differences in total consumption across the four diet types

($F_{3,44} = 0.63$, $P = .59$), although there were differences across families in the amount consumed regardless of diet type (in a model controlling for family; diet type: $F_{3,33} = 1.13$, $P = .35$; family: $F_{11,33} = 3.71$, $P = .002$). Despite family-level differences in the amount of diet consumed, this measure did not correlate with overall performance on the artificial diet (e.g., growth rate on the control diet; $F_{1,10} = 0.74$, $P = .41$) and thus is not considered further, although it is included in the online data set.

We measured egg size and the number of mature eggs in the ovary of females after death in the life span cage. Dissections were performed in 1× phosphate-buffered saline buffer at ×10–20 magnification using a Leica M165C microscope. To estimate egg size—defined as cross-sectional area of a mature egg—we captured images of up to five mature eggs, which were measured using ImageJ (NIH). We measured eggs from 75 females ($N = 360$ total eggs; on average, 4.75 females [range: 2–7] per family across all diet types). Egg size varied significantly between individuals (likelihood ratio of model fit, LR = 79.87, $P < .001$; models also accounted for effects of diet and family as described in "Statistical Analyses" below); however, neither body size nor age at death was significantly associated with egg size (analysis of deviance; size: $\chi^2_1 = 0.005$, $P = .945$; longevity: $\chi^2_1 = 0.66$, $P = .418$). To estimate fecundity, we counted the total number of mature eggs in individuals that were mature but not undergoing egg absorption (3–43 days after emergence, mean = 12.46 days old; $N = 57$ females). This represents a coarse estimate of reproductive potential given that female *P. rapae* emerge with immature ovaries and eggs develop with age but are further stimulated with mating and host plant exposure (Papaj 2000). Egg number did not vary with body size ($F_{1,15} = 0.02$, $P = .888$) or age at death ($F_{1,15} = 0.40$, $P = .539$) when diet and family were also accounted for.

Wing melanin investment was measured only on females, which have larger and darker black areas on their wings. We measured two to three females per diet type per family ($N = 68$ individuals), focusing on individuals <15 days old to minimize any effects of wing wear; age at sampling had no significant effect on our melanin measurements (area: $F_{1,52} = 0.484$, $P = .490$; darkness: $F_{1,50} = 0.161$, $P = .690$; for models containing additional predictors as described in "Statistical Analyses" below). Forewings were removed and imaged dorsally with a Canon Rebel T3 camera fitted with a 50-mm macro lens under controlled light conditions with a gray color standard. Photographs were analyzed via an image-processing algorithm in Matlab, which collected average red (R), green (G), blue (B), and gray (Gy) values from the center of upper dorsal black spot and the dorsal wing tip. To account for possible differences in light conditions, we used the reflectance value of the gray color standards to equalize the R, G, B, and Gy values. We defined the darkness of each wing region by subtracting the Gy value from 255 (the maximum RGB value, corresponding to white), so that higher values represent darker wing spots, that is, greater wing melanin investment. We also measured the total area of the black wing tip and the two dorsal wing spots using ImageJ. Measurements taken from the wing spot and wing tip were correlated for both area (Pearson's $R = 0.62$, $P < .001$) and darkness ($R = 0.72$, $P < .001$); thus, we selected the anterior wing spot as a proxy for melanin investment (the darkest area on the forewing).

Gene Selection

As discussed above, many genes and molecular pathways have been implicated in "generalized" physiological responses to various stressors. However, the number of diets and families included in this study made whole-transcriptome approaches cost prohibitive, and the importance of particular pathways allows for a candidate gene approach. Therefore, we chose to focus on pathways involved in oxidative stress responses because (1) many of the key genes are highly conserved across species, (2) they are important in general stress responses (Gerschman et al. 1954; Sun et al. 1998; Deak et al. 1999; Despres et al. 2007; Kensler et al. 2007; Li et al. 2007; Schramm et al. 2012; Gloss et al. 2014), and (3) other classes of candidate genes have large numbers of paralogs (e.g., approximately 85 putative CYPs have been identified in the monarch genome; Zhan and Reppert 2013). For primer development and analysis, we initially chose candidate genes involved in in vivo antioxidant defense based on conserved pathways across animals (see Halliwell and Gutteridge 2015, ch. 3). The selected genes (summarized in table 1) fall into four functional categories. First, we considered two genes involved in glutathione metabolism and glutathione-based detoxification, glutathione S-transferase D1 (*GstD1*) and pyrimidodiazepine synthase (*Se*), as these processes are important in combating oxidative stress and in toxin processing more generally (Schramm et al. 2012; Gloss et al. 2014; Halliwell and Gutteridge 2015). Second, we considered four highly conserved genes involved in antioxidant defense through removal of reactive species: superoxide dismutase [Cu-Zn] (*Sod1*), catalase (*Cat*), peroxiredoxin 4 (*Prx4*), and thioredoxin 2 (*Trx2*). These genes have been previously demonstrated to confer cross-tolerance to multiple stressors (Sagara et al. 1998; Sun et al. 1998). Third, ferritin (*Fer1HCH*) and transferrin (*Tsf1*) are genes that regulate and/or sequester metals involved in the generation of free radicals. These processes help minimize oxidative damage from a range of different stressors (Deak et al. 1999). Finally, tyrosine hydroxylase (*Th*) catalyzes a key step in the production of melanin pigments; melanin can act as a sink for free radicals and

Table 1: Summary of candidate genes

Gene	Protein	Putative function
GstD1	Glutathione S-transferase D1	Join glutathione to toxins as first step in detoxification
Se	Pyrimidodiazepine synthase (sepia)	Generates glutathione from glutathione disulfide
Sod1	Superoxide dismutase [Cu-Zn]	Processes superoxide radicals into oxygen or hydrogen peroxide; most common superoxide dismutase in eukaryotes
Cat	Catalase	Converts hydrogen peroxide into water and oxygen
Prx4	Peroxiredoxin 4	Converts hydrogen peroxide into water
Trx2	Thioredoxin 2	Reduction of disulfide bonds in the mitochondria
Fer1HCH	Ferritin	Storage and transport of iron in nontoxic form
Tsf1	Transferrin	Regulates levels of free iron
Th	Tyrosine hydroxylase	Converts tyrosine to L-DOPA using oxygen and iron

Note: L-DOPA = L-3,4-dihydroxyphenylalanine.

metals contributing to oxidative stress (Sichel et al. 1991; Hong and Simon 2007; Brenner and Hearing 2008).

Sequences for these candidate genes in monarchs (*Danaus plexippus*) or *Drosophila melanogaster* were retrieved from MonarchBase (Zhan and Reppert 2013) and FlyBase (Gramates et al. 2017), respectively. We used these sequences to perform a translated BLAST query (tblastx; Altschul et al. 1997; Camacho et al. 2009) against the *P. rapae* transcriptome (Sikkink et al. 2017), which identified significant matches for most of our initially queried genes. One gene involved in our initial list of candidates (metallothionein) did not have a significant match in either the *P. rapae* or the *D. plexippus* transcriptomes and was dropped from consideration. Actin was selected as a control gene on the basis of previous experiments in *P. rapae* and the validity of actin as a control gene for other lepidopterans (e.g., Lu et al. 2013; Zhu et al. 2014; Shu et al. 2018). Actin expression did not vary with treatment ($F_{3,76} = .23$, $P = .87$).

RNA Extraction and Measurement of Antioxidant Gene Expression

To measure gene expression, a subset of larvae ($N = 100$) were frozen in liquid nitrogen 24 h after transfer to artificial diet (either the control diet or one of the three novel diets). As detailed above, there was no difference in acceptance of the novel diets relative to the control diet during this time period—larvae fed on all diets during this 24-h period. Harvested tissue was homogenized by vortexing with 2.8-mm ceramic beads in PowerBead tubes (Qiagen; catalog no. 13114-50) in Buffer RLT (Qiagen) containing 2-mercaptoethanol. RNA was extracted from homogenized tissue using the Qiagen RNeasy Micro kit (catalog no./ID 74004) in accordance with the manufacturer's directions. Eighty of the highest-yield extracted samples (average concentration = 527 ng/μL RNA) from 10 families were chosen for subsequent quantitative polymerase chain reaction (PCR) analysis. We aimed for at least eight replicate individuals per family (with two individuals for each of the four diets); however, because of availability of larvae, our sample sizes ranged from four to 12 individuals per family (average = 8; median = 7.5) with an approximately even distribution across diets ($N = 23$ control; $N = 20$ *Aristolochia*; $N = 19$ *Tussilago*; $N = 18$ *Passiflora*).

Extracted RNA from each individual was sent to the University of Minnesota Genomics Center (UMGC) for complementary DNA synthesis (using Invitrogen SuperScript II reverse transcriptase) and quantitative real-time PCR using primers developed at UMGC (table S3) on an Applied Biosystems 7900HT real-time PCR instrument with two technical replicates per sample (cycle threshold [Ct] values were averaged for each sample).

Statistical Analyses

All raw data used in the analyses are available in the Dryad Digital Repository (Snell-Rood et al. 2019; https://doi.org/10.5061/dryad.pnvx0k6h3). Statistical analyses were performed using R version 3.4.0 (R Core Team 2017) unless otherwise stated. We fit linear models to test for factors affecting development time, wing length, or growth rate. For each of these traits, we included diet, family, sex, oviposition host, and the interaction between diet and family in our model. For wing length and growth rate measurements, we also included a factor to control for the identity of the person measuring wing length (ESR or RH). To test for factors affecting larval survival, we used a binomial logistic model including diet, family, the oviposition host, and the interaction between family and diet as fixed effects. Sex was not included as a factor for larval survival because it was not determined until after emergence as adults.

Throughout our analyses, we treat family as a fixed effect because we were interested in the specific direction and magnitude of the mean associated with each family line. As described below, our general approach is to treat family as a fixed effect in each initial model and use least square means as estimates for family characteristics in subsequent analyses. Because many models of family effects treat family as a random effect, we additionally reran analyses in this manner, using best linear unbiased predictors as estimates for each family. However, because these models generally failed to converge, we focus on results on analyses treating family as a fixed effect. Results are qualitatively the same regardless of approach (see the direct comparisons of two sets of analyses in tables S4 and S5).

To identify predictors with significant effects, ANOVA tables using type 3 sums of squares were generated using the car package (Fox and Weisberg 2011). Post hoc pairwise comparisons (Tukey honestly significant difference test) between all four diets were carried out using the pairs function of the lsmeans package (Lenth 2016). To test for effects of diet and family on adult longevity, we fit a Cox proportional hazards model using the coxph function in the R package survival (Therneau and Grambsch 2000). Diet, family, sex, oviposition host, and the interaction between diet and family were included as effects in the model. Analysis of deviance tables were generated using the car package to test for significant effects.

Traits associated with performance trade-offs—egg size, fecundity, and melanin—were also analyzed with linear models. To test for significant effects on egg size, we fit a linear mixed effects model using the package nlme (Pinheiro et al. 2017). This model considered individual females (for which we had multiple measures of egg size) as a random factor nested within family (which was treated as a fixed effect, as described above). Deviance tests were used to identify significant effects of diet or family on egg size; body size and life span had no significant effect on egg size and were excluded from the final model. To test for effects on fecundity, we fit a general linear model with the predictors diet and family. Wing length and life span were considered as covariates but were not significant, and therefore they were excluded from the final model. For estimates of wing spot size and darkness, we fit linear models including family and diet as predictors. For wing spot size, we also included wing size as a covariate. Wing size was not initially included as a predictor in models of wing darkness because we had no a priori reason to suspect spot darkness would scale with size. However, post hoc analyses indicated that there were significant relationships between wing size and spot darkness (smaller individuals had darker spots), so we repeated spot darkness analyses also controlling for body size. We used treatment contrasts to test for pairwise differences between the control and each novel diet for responses with significant effects of diet. Detailed statistical results for the final models are provided in the supplemental Excel tables (available online) for all performance metrics described above.

To compare responses of each family, we calculated the least square mean for each family on each diet from the above-described models using the lsmeans package. For development time, wing length, and growth rate, family means were averaged over the levels of sex, oviposition host, and the person performing size measurements (where applicable). For larval survival, the family means were averaged over the levels of oviposition host and were back transformed to the original scale of the response. In analyses of trade-offs, one must account for variation in condition, which can obscure underlying allocation trade-offs (Van Noordwijk and De Jong 1986; Reznick et al. 2000). For instance, large individuals with abundant larval reserves may be able to invest highly in all life-history traits, obscuring any underlying trade-offs in energy allocation. In this experiment, it is likely that the primary determination of "condition" across family lines is the ability to cope with artificial diet—families that perform better on diet should assimilate more nutrients, grow larger, and have more to allocate to a range of fitness-related traits. Indeed, previous studies have found differences between families and populations in performance on diet; for instance, more specialized populations do poorly on artificial diet (Espeset et al. 2019). In this study, families also varied in how well they perform on the normal cabbage-based artificial diet. Thus, in our analyses we defined a families' relative performance on a novel diet as the difference between each novel diet and the control diet for each performance metric, reasoning that this approach isolated responses to novel plant materials per se (rather than to artificial diet) and minimized variation in condition across families that mask underlying trade-offs. We used Spearman correlations to compare relative performance between the three novel diets in all pairwise comparisons. Because performance on novel diet types was correlated, we performed a principal component analysis (PCA) on the relative performance scores for each trait. For all traits analyzed, PC1 explained >50% of the variance in the trait across diets; thus, we used a family's PC1 score for each trait to summarize overall performance on the novel diet types within a family.

To test for effects on expression of the nine candidate genes, we fit a general linear model for each gene. Ct scores for each gene were multiplied by -1 so that higher values correspond to increased gene expression. Diet, family, and the $-$(Ct) value of the control gene, actin, were included as predictors in the model. We also tested for the effect of an interaction between diet and family in a subset of eight families that had observations in all four diets. Because the

interaction was not significant for any of the genes, we excluded it from the final model. Effect coefficients and additional statistics for the final models for each gene are provided in the supplemental PDF. Specific planned comparisons between the control diet and each of the novel diets were carried out using treatment contrasts with the control as the base level.

For comparisons of gene expression among families, we calculated the least square mean Ct value for each family, averaged over diet and actin expression. We used PCA to summarize overall transcriptional patterns for each family. Family scores on PC1 and PC2 were compared to the performance summary metrics (PC1 from each performance PCA described above) using Pearson correlations.

Results

Performance across Novel Diets Is Correlated across Families

Larvae ($N = 737$) from 12 wild-caught mothers were transferred to a control diet or one of three novel diets, which contained leaf material from the genera *Passiflora*, *Tussilago*, or *Aristolochia*, and allowed to develop to adulthood. Larval survival was significantly lower on the *Aristolochia* diet relative to the *Passiflora* or *Tussilago* diets, but none of the novel toxic diets differed significantly from the control diet (table 2; fig. 1). In the surviving larvae, performance on the *Aristolochia* and *Tussilago* diets was significantly poorer relative to the control diet: individuals reared on these diets had longer development times and slower growth rates, and emerging adults were smaller (measured as forewing length; table 2; fig. 1). Surprisingly, novel toxic diets did not significantly alter adult longevity, although there was substantial variation in life span among families (table 2; fig. 1).

Families varied in their performance across the four diet types, with significant family-by-diet interactions for body size (table 2; fig. 1). However, in many cases a family's performance on one novel toxic diet was also correlated with its performance on the other toxic diets (table 3) after correcting for performance on the control diet, since the ability to feed successfully on artificial diet varied among the families. For instance, families that developed relatively faster on *Aristolochia* developed faster on *Tussilago* as well; similarly, families with higher relative survival on *Aristolochia* also had higher relative survival on *Tussilago* (table 3; fig. 2). Thus, for each performance measure, we used PCA to create a composite measure across all three novel toxic diets, relative to the control (PC1 from a PCA for each performance trait; PC1 for larval survival, development time, adult body size, and growth rate explained 74%, 86%, 57%, and 74% of the variation across the three diet types, respectively).

Constitutive Antioxidant Gene Expression Is Higher in Families That Cope with Novel Toxic Diets

We measured whole-body expression of nine genes involved in antioxidant stress responses in a subset of 80 individuals from 10 families euthanized 24 h after transfer onto the artificial diets. Across most genes considered, variation in expression was primarily determined by family, not diet (table 4; fig. 3). However, contrasts comparing each novel diet to the control revealed significant upregulation of three genes for the *Passiflora* diet and two additional genes with a tendency to be upregulated for the *Passiflora* diet ($P = .052$ and .053; fig. S3). We found no evidence for family-by-diet interactions when considering only families with expression data for all four diets ($N = 70$, eight families; minimum $P = .49$).

To test the prediction that constitutive differences in expression contribute to performance differences on novel toxic diets, we summarized patterns of transcriptional variation in our nine genes (table 1) using a PCA (fig. 4). For each gene, we considered a family's expression level as the least square mean $-$(Ct) from a model controlling for diet and the control gene, actin. We identified two principal components, which together explained 74.5% of variation across families in gene expression. PC1 (47.5% of variation) had significant positive loadings (in decreasing order of magnitude) of catalase (*Cat*), transferrin (*Tsf1*), glutathione S-transferase D1 (*GstD1*), superoxide dismutase [Cu-Zn] (*Sod1*), tyrosine hydroxylase (*Th*), pyrimidodiazepine synthase (*Se*), and ferritin (*Fer1HCH*; fig. 4). PC2 (27.0% of variation) was positively associated with expression of thioredoxin 2 (*Trx2*), *Fer1HCH*, and *Th* and was negatively correlated with peroxiredoxin 4 (*Prx4*) expression. Families with higher expression of PC1 tended to have increased survival on novel toxic diets (Pearson's $R = 0.611$, $P = .06$; table 5; fig. 4). Families with higher scores on PC2 developed significantly faster (Pearson's $R = .735$, $P = .015$) and had faster growth rates (Pearson's $R = 0.829$, $P = .003$) across the three novel toxic diets (table 5; fig. 4).

Minimal Trade-Offs of Generalized Response to Novel Toxins

We tested for putative life-history trade-offs associated with the ability to survive on diets containing novel toxins. First, we considered egg size and egg number in the females reared on each diet. There were significant differences between families in egg size of these individuals

Table 2: Effects of family and diet on performance measures

Performance metric	Analysis method	Diet	Family	Family × diet	Oviposition host	Sex
Larval survival ($N = 581$)	Binomial logistic regression	$\chi^2_3 = 19.57$ (**<.001**)	$\chi^2_{11} = 118.12$ (**<.001**)	$\chi^2_{33} = 30.58$ (.588)	$\chi^2_2 = 7.53$ (**.023**)	NA
Development time ($N = 449$)	Linear regression	$F_{3, 398} = 16.47$ (**<.001**)	$F_{11, 398} = 17.26$ (**<.001**)	$F_{33, 398} = 1.32$ (.117)	$F_{2, 398} = 3.81$ (**.023**)	$F_{1, 398} = 1.70$ (.194)
Adult body size[a] ($N = 221$)	Linear regression	$F_{3, 169} = 11.94$ (**<.001**)	$F_{11, 169} = 10.75$ (**<.001**)	$F_{33, 169} = 1.53$ (**<.001**)	$F_{2, 169} = 3.98$ (**.021**)	$F_{1, 169} = 19.01$ (**<.001**)
Growth rate[a] ($N = 220$)	Linear regression	$F_{3, 168} = 16.67$ (**<.001**)	$F_{11, 168} = 8.58$ (**<.001**)	$F_{33, 168} = 1.40$ (.088)	$F_{2, 168} = 6.80$ (**<.001**)	$F_{1, 168} = 1.05$ (.306)
Adult longevity ($N = 414$)	Cox proportional hazards	$\chi^2_3 = 3.93$ (.367)	$\chi^2_{11} = 19.71$ (**<.001**)	$\chi^2_{33} = 38.31$ (.241)	$\chi^2_2 = 1.37$ (.382)	$\chi^2_1 = 11.64$ (**.001**)

Note: *P* values are shown in parentheses. Boldface indicates $P < .05$. NA = not applicable.

[a] Model included an additional term to control for two people measuring wing length; see the supplemental Excel tables for detailed statistics.

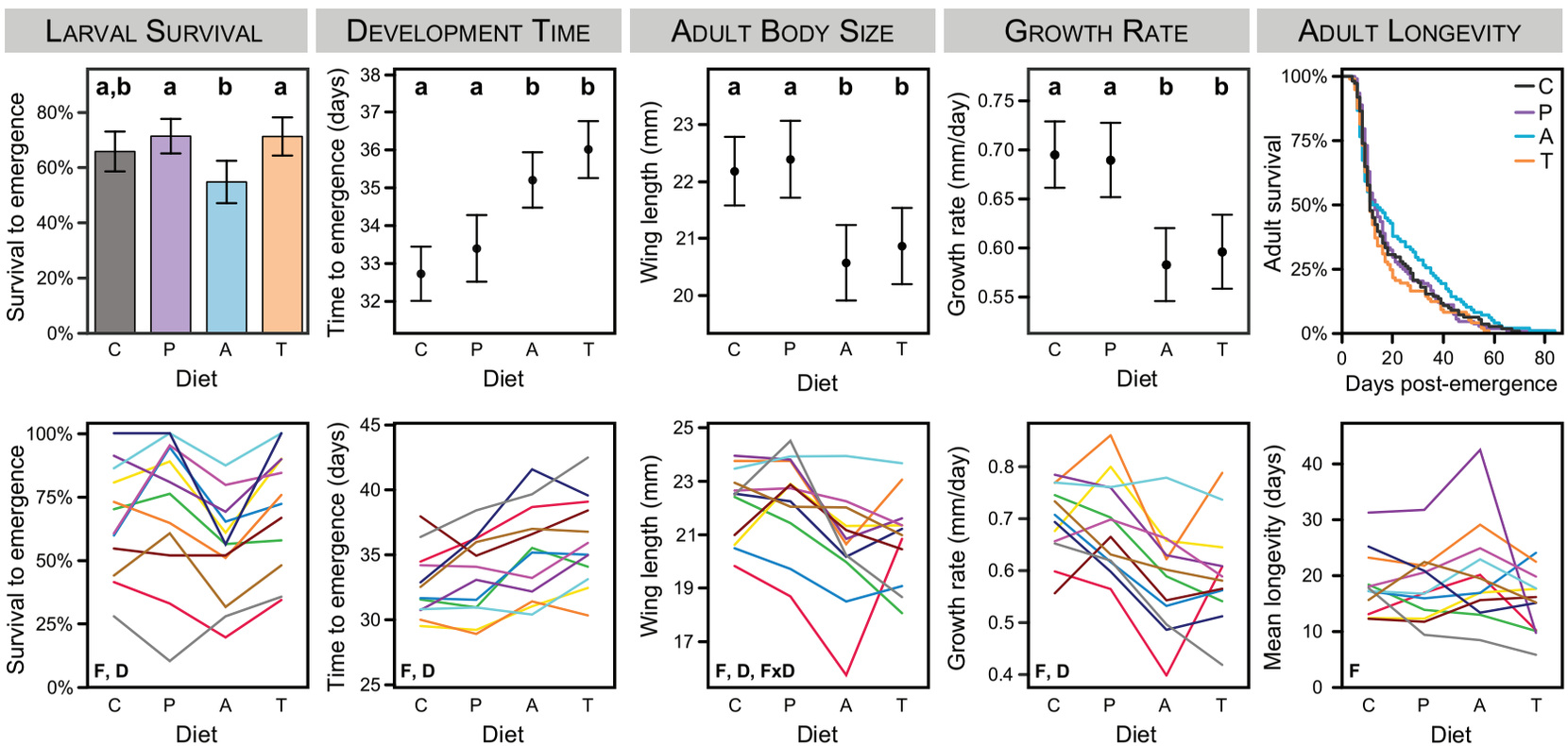

Figure 1: Effect of novel diets on butterfly performance. Least square means averaged over all families are shown in the top row (whiskers show 95% confidence intervals). Lowercase letters in each panel indicate differences between group means (Tukey honestly significant difference test, $P < .05$). In the bottom row, colored lines represent least square means for each family across the four artificial diets. The letters in each panel indicate significant effects of family (F), diet (D), or family-by-diet (FxD) interactions ($P < .05$; see table 2 for statistics). C = control diet; P = *Passiflora*; A = *Aristolochia*; T = *Tussilago*.

Table 3: Correlations in changes in family performance across diet types relative to control diets

Performance metric, diet comparison	Spearman's ρ	P
Larval survival:		
Passiflora vs. *Tussilago*	.580	.052
Passiflora vs. *Aristolochia*	.559	.063
Tussilago vs. *Aristolochia*	.776	**.005**
Development time:		
Passiflora vs. *Tussilago*	.832	**.001**
Passiflora vs. *Aristolochia*	.587	**.049**
Tussilago vs. *Aristolochia*	.797	**.003**
Adult body size:		
Passiflora vs. *Tussilago*	.231	.471
Passiflora vs. *Aristolochia*	.643	**.028**
Tussilago vs. *Aristolochia*	.266	.404
Growth rate:		
Passiflora vs. *Tussilago*	.587	**.049**
Passiflora vs. *Aristolochia*	.650	**.026**
Tussilago vs. *Aristolochia*	.357	.256

Note: Boldface indicates $P < .05$.

($\chi^2_{11} = 25.45$, $P = .008$) and a tendency for families to vary with respect to our measure of fecundity (egg counts of dissected individuals: $F_{11,42} = 1.94$, $P = .062$; fig. S4). Rearing diet had no effect on egg size ($\chi^2_3 = 6.14$, $P = .105$), but egg number was significantly lower in individuals reared on the *Aristolochia* diet ($t = -2.33$, $P = .0246$). There were no apparent trade-offs between reproductive investment and survival on novel diets for either egg size ($N = 12$ families; survival PC1: Spearman's $\rho = 0.02$, $P = .956$) or fecundity (survival PC1: Spearman's $\rho = 0.52$, $P = .089$). In fact, families with higher survival on novel toxic diets also tended to have higher fecundity.

We additionally considered possible trade-offs between melanin investment in antioxidant defense and melanin investment in wing structures. The size of the largest melanic wing spot varied with body size ($F_{1,53} = 104.74$, $P < .001$) and family ($F_{10,53} = 5.66$, $P < .001$) but not diet ($F_{3,53} = 1.56$, $P = .209$). Families with higher survival on novel toxic diets tended to have smaller melanic wing spots ($N = 11$; survival PC1: Spearman's $\rho = -0.55$, $P = .082$; fig. 5). The darkness of this wing spot varied with family and treatment (family: $F_{10,51} = 2.58$, $P = .013$; diet: $F_{3,51} = 3.30$, $P = .028$), being significantly lighter on the *Tussilago* diet relative to the control ($t = -3.10$, $P = .003$). Families with higher survival across the novel toxic diets had lighter wing spots, consistent with a trade-off ($N = 11$; survival PC1: Spearman's $\rho = -0.64$, $P = .040$; fig. 5). While we had no a priori reason to suspect wing spot darkness to be correlated with body size, there was a significant negative relationship across individuals, with smaller individuals having darker spots ($N = 65$, $F_{1,63} = 17.3$, $P < .001$; see also the supplemental Excel tables). When body size is included in estimates of family-level wing spot darkness, correlations between spot darkness and survival on novel diets are no longer significant, although the pattern is the same ($N = 11$, Spearman's $\rho = 0.56$, $P = .08$).

Discussion

Our results support the hypothesis that generalized responses play a role in tolerating novel toxins encountered during a diet shift. Performance of butterfly families was correlated across three novel and chemically distinct plants (fig. 2), consistent with the action of a generalized response. Furthermore, overall performance was correlated

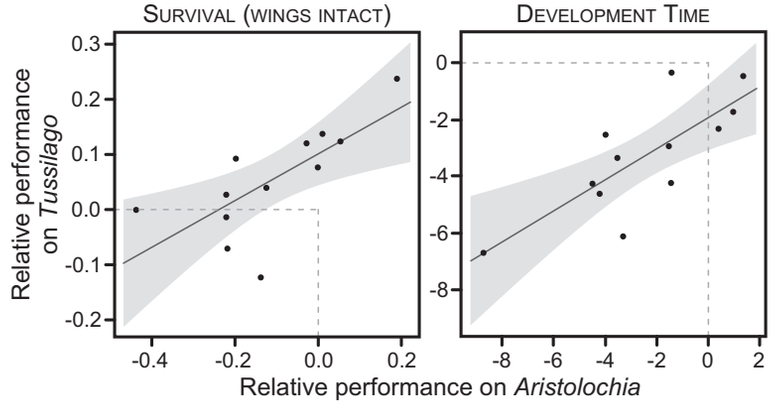

Figure 2: Correlated performance on novel diets across families. Points represent the difference between the least square mean on the novel diet and that on the control diet. The difference was calculated for each trait such that values greater than zero (dashed lines) represent higher performance—that is, higher survival and shorter development times—relative to the control. Solid lines and shading represent the linear fit and 95% confidence interval, respectively, for the relative performance metrics. See table 3 for correlation statistics. A color version of this figure is available online.

Table 4: Effects of family and diet on expression of antioxidant genes

Gene	Diet	Family	Actin (control gene)[a]
Sod1	$F_{3,66} = 2.29$ (.086)	$F_{9,66} = 3.56$ (**.001**)	$F_{1,66} = 67.56$ (**<.001**)
Cat	$F_{3,66} = 1.96$ (.129)	$F_{9,66} = 1.94$ (.061)	$F_{1,66} = 18.90$ (**<.001**)
Prx4	$F_{3,66} = 3.18$ (**.030**)	$F_{9,66} = 6.35$ (**<.001**)	$F_{1,66} = 130.87$ (**<.001**)
Tsf1	$F_{3,66} = 2.98$ (**.038**)	$F_{9,66} = 2.62$ (**.012**)	$F_{1,66} = 19.92$ (**<.001**)
Se	$F_{3,66} = .58$ (.627)	$F_{9,66} = 22.57$ (**<.001**)	$F_{1,66} = .93$ (.339)
GstD1	$F_{3,66} = 2.38$ (.077)	$F_{9,66} = 5.44$ (**<.001**)	$F_{1,66} = .06$ (.810)
Trx2	$F_{3,66} = .10$ (.958)	$F_{9,66} = 2.62$ (**.012**)	$F_{1,66} = 2.49$ (.119)
Fer1HCH	$F_{3,66} = 1.83$ (.150)	$F_{9,66} = 2.67$ (**.010**)	$F_{1,66} = 62.51$ (**<.001**)
Th	$F_{3,66} = .63$ (.595)	$F_{9,66} = 3.64$ (**.001**)	$F_{1,66} = 12.94$ (**.001**)

Note: P values are shown in parentheses. Boldface indicates $P < .05$.

[a] For some genes, there was no significant effect of the control gene, actin, suggesting that expression levels of these genes may not correlate to the total amount of tissue harvested.

with variation across families in expression of antioxidant stress response genes (fig. 4). Upregulation of these genes may be directly involved in detoxification through the actions of GST (Schramm et al. 2012; Gloss et al. 2014)—indeed, increased transcription of *GstD1* positively loaded with expression PC1, which tended to correlate with survival on the novel toxic diets. High antioxidant gene expression may also be important in minimizing the oxidative stress caused by CYP enzymes, which are often responsible for the first phase of metabolic detoxification for a broad range of toxins (Li et al. 2007; Schuler 2011). CYPs can process a wide range of chemicals, but studies contrasting specialists and generalists suggest that those with more permissive active sites are less efficient (Li et al. 2004, 2007), possibly resulting in greater oxidative stress (Gonzalez 2005). The link with CYPs underscores the fact that the generalized response to novel toxins likely involves other pathways, such as heat-shock proteins (Kregel 2002; Feder and Hofmann 1999), and upstream stress regulators, such as nuclear factor erythroid 2–related factor 2 (*Nrf2*; Kensler et al. 2007). In the future, whole-transcriptome approaches would allow an unbiased quantification of all genes that may be involved in a generalized response to a novel diet; however, observations that antioxidant pathways mitigate a range of novel and stressful environments (Gerschman et al. 1954; Sun et al. 1998; Deak et al. 1999; Despres et al. 2007; Kensler et al. 2007; Li et al. 2007; Schramm et al. 2012; Gloss et al. 2014) permitted a candidate gene approach.

Most variation in our candidate genes was constitutive rather than induced (table 4). This was unexpected given the large literature on induction of antioxidant

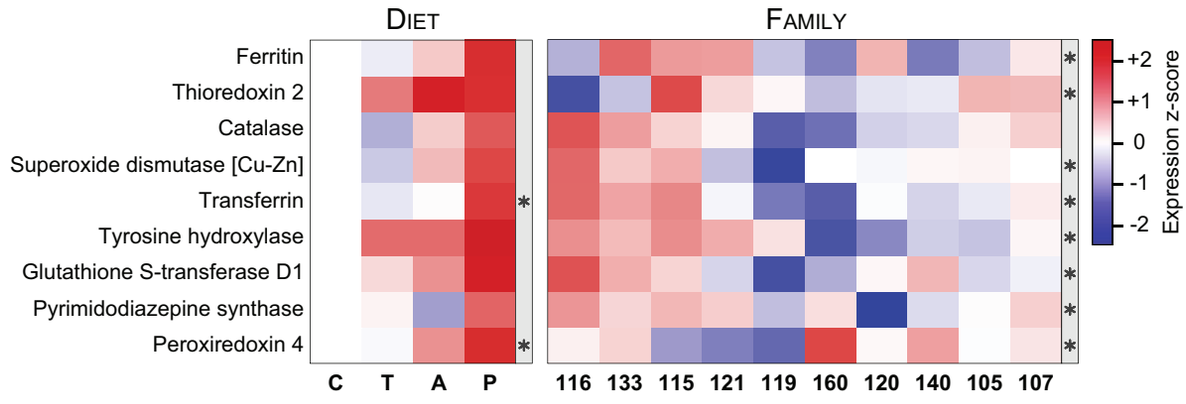

Figure 3: Effects of diet and family on expression of antioxidant genes. Expression levels were quantified as the least square means of expression after accounting for actin expression. Shown are expression levels for the nine candidate genes relative to either the control diet ("Diet"; *left*) or the overall mean ("Family"; *right*). Shading indicates upregulation (red) or downregulation (blue) of the gene, scaled to the standard deviation of expression for each gene. Genes that show significant effects of diet or family ($P < .05$; see table 4) are indicated with an asterisk to the right of the respective panel. C = control diet; T = *Tussilago*; A = *Aristolochia*; P = *Passiflora*.

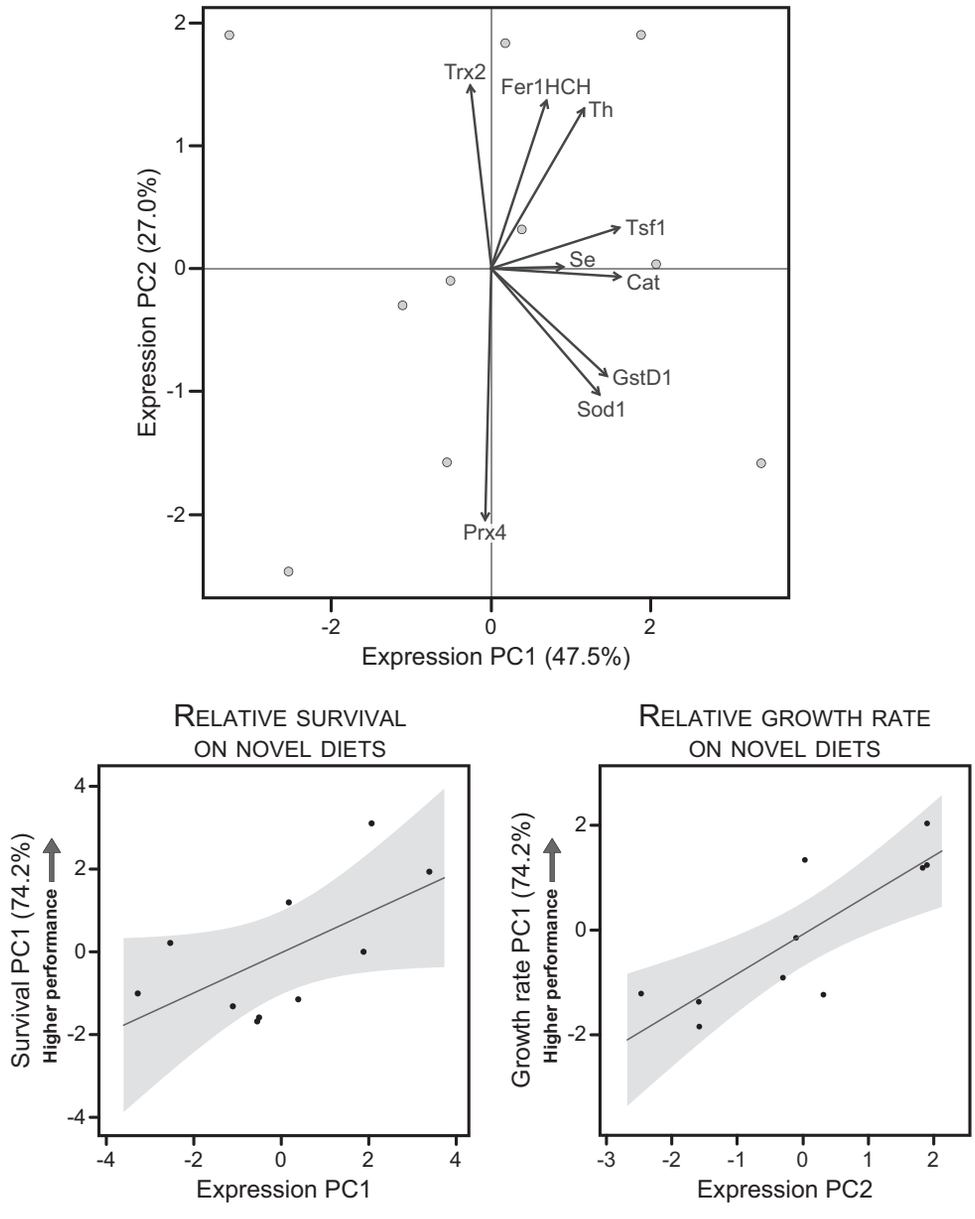

Figure 4: Families with higher constitutive antioxidant gene expression have higher performance on novel diets. Shown is a biplot from a principal components analysis (*top*) indicating the loadings of each candidate gene. For visualization, principal component values were transformed by multiplying by −1 (i.e., positive values on PC1 indicate higher expression levels of most genes). Points represent the mean scores for each family. Gene expression is correlated with composite measures of performance on the three novel diets (*bottom*). Shown are representative plots for larval survival (*bottom left*) and growth rate (*bottom right*). Solid lines and shading represent the linear fit and 95% confidence interval, respectively. Correlation statistics are reported in table 5. A color version of this figure is available online.

pathways by stressors, especially for hormetic responses, where a small amount of a stressor can have a beneficial effect through upregulation of generalized stress responses (Mattson and Cheng 2006; Mattson 2008). We did see significant induction of several genes on the *Passiflora* diet (fig. S3), which appeared to be the least stressful of the diets (fig. 1). While these changes in gene expression were no doubt modest—less than a twofold change in expression—such small changes can still be biologically relevant (St. Laurent et al. 2013). It is possible that the induction of antioxidant genes caused the higher performance on this diet, given that there were no overall differences in the willingness of caterpillars to feed on the *Passiflora* diet relative to the other novel diets during this time period (fig. S2).

Table 5: Correlations between gene expression and performance on novel diets across 10 lines

	Expression PC1[a]		Expression PC2	
	Pearson's *R*	*P*	Pearson's *R*	*P*
Larval survival PC1[b]	.611	.060	−.016	.965
Development time PC1	.210	.561	.735	**.015**
Adult body size PC1	.339	.339	.508	.134
Growth rate PC1	.106	.771	.829	**.003**

Note: Boldface indicates $P < .05$.

[a] Expression was quantified as the first or second principal component (PC) of expression of nine antioxidant genes after controlling for actin expression.

[b] Performance on novel diets was quantified as PC1 for a performance measure across all three diets (relative to the control diet) as performance across diets was correlated (see table 3).

The link between constitutive variation in expression and toxin tolerance recalls observations that evolutionary divergence in antioxidant defenses can occur through constitutive upregulation of such pathways (Arking et al. 2000; Carvalho et al. 2013). More generally, the variation in responses to the different diets shares similarities with examples in bacteria where resistance to some classes of antibiotics (e.g., penicillin) can be accomplished through transcriptional activation of existing defenses (AmpC β-lactamases), while resistance to newer classes of antibiotics (e.g., cephalosporins) is possible only through constitutive upregulation of these defenses (Paterson 2006).

We were unable to detect reproductive trade-offs with the ability to tolerate a range of novel toxins. In the aging literature, long-lived genetic variants in both vertebrates and invertebrates suggest that upregulation of antioxidant pathways and generalized stress responses come with fecundity trade-offs (Johnson et al. 2000; Wang et al. 2004; Kim et al. 2010). We found no correlations between survival on our novel diets and fecundity—indeed, the trend was in the opposite direction. It is possible that our measure of fecundity was insufficient. We dissected butterflies that died from natural causes in life span cages. However, the life span cages were in a low light climate chamber, meaning that no mating occurred, and they had no exposure to host plants; both mating and host plants stimulates further egg development (Papaj 2000). Our fecundity measures no doubt represent a lower estimate of egg production and may be insufficient to detect costs in a species that can produce hundreds of eggs. In addition, the relatively benign conditions of development on artificial diet, with ad lib. access to resources and low competition, may have further limited our ability to detect trade-offs with fecundity. Reproductive trade-offs are generally more pronounced when measured in stressful conditions (e.g., Armbruster and Reed 2005; Van Buskirk and Steiner 2009). However, some recent work suggests that the artificial diet we used is at least somewhat stressful to wild populations,

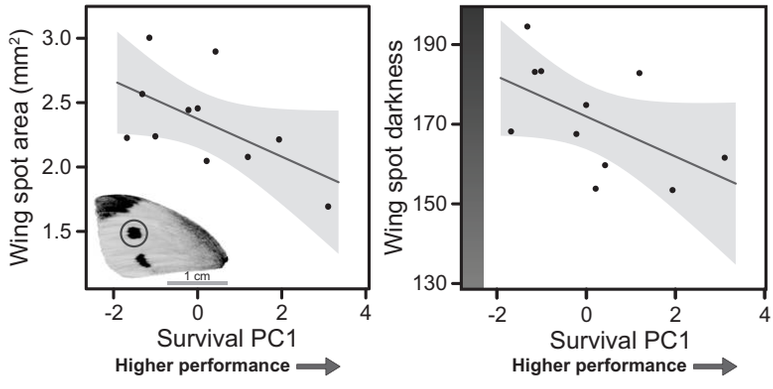

Figure 5: Families with higher performance across novel diets have smaller and lighter melanic wing spots. The size (*left*) and darkness (*right*) of the upper wing spot (circled in inset) of female butterflies was compared with the principal component of survival on the novel diets. Solid lines and shading represent the linear fit and 95% confidence interval, respectively. The dark gray bar in the right panel shows the gray value corresponding to the measured value for darkness. In this visualization, family means for spot size control for scaling with forewing area, but spot darkness does not as we had no a priori reason to suspect spot color would scale with size. A color version of this figure is available online.

as butterflies assimilate nitrogen more effectively from real host plants, even if absolute nitrogen content in the artificial diet can be elevated (Espeset et al. 2019). Regardless, before concluding there are no fitness trade-offs of the ability to tolerate novel toxins, future experiments should consider more complete measures of fitness in a range of rearing environments—for instance, egg laying by mated females over their lifetime after rearing on host plants with higher larval competition. It would be particularly interesting to simultaneously manipulate ascorbic acid, a typical component of lepidopteran artificial diets (at 1%–2%; e.g., Cappellozza et al. 2005), as it is a powerful antioxidant in some conditions (Griffiths and Lunec 2001), affecting redox reactions in lepidopteran guts (Johnson and Felton 1996).

Our data suggest that there may be trade-offs between a generalized response and investment in melanin, which plays structural and signaling roles in the wings (McGraw 2005) and is an important component of immune defense in insects (Kanost and Gorman 2008). We detected significant negative relationships between wing spot darkness and survival on novel diets (fig. 5), although this relationship was not significant when controlling for body size ($P = .08$). We saw a similar tendency for wing spot size to be negatively related to survival on novel diets ($P = .08$). Taken together these correlations are suggestive of a trade-off, although they are not conclusive. Melanin plays a prominent role in antioxidant function (Sichel et al. 1991; Brenner and Hearing 2008). Tyrosine hydroxylase (*Th*), which synthesizes melanin, was also a major component of our gene expression axis PC2. It is possible that allocation of melanin in antioxidant defense may trade off with melanin investment in other functions such as immunity (Kanost and Gorman 2008), but additional research here is needed. It is also possible that there are other costs of upregulated general stress responses that we did not detect with our measures. For instance, high GST expression may exhaust levels of glutathione, which can be costly to produce (Schramm et al. 2012). In another example, high heat-shock protein expression has been shown to be toxic in some contexts, which can explain why expression is often low early in development (Feder and Hofmann 1999).

It is likely that much of the variation observed across families in this experiment stems from standing genetic variation in the local population. However, we cannot eliminate the possibility of maternal effects causing some of the differences between families. Gravid females were collected from the same agricultural fields during the same 2–3-week period; however, it is possible that differences in larval nutrition or female experience could result in environmentally induced differences across families in egg size or composition that could affect the performance of a family's offspring (Rotem et al. 2003; Snell-Rood et al. 2013). Regardless of whether the variation across families stems from standing genetic variation or maternal effects, it is clear that there is substantial variation within a population in the ability of organisms to tolerate a novel toxic host plant in their diet. Future experiments that rear families through at least one generation in the laboratory could tease apart the relative contribution of genetic variation and maternal effects, which would clarify the evolutionary consequences of the present findings.

This experiment provides a way forward in studying adaptations to novel toxic diets. It is difficult to study the initial steps during such a host shift due to immediate or postingestion rejection of novel chemicals (e.g., Waldbauer and Fraenkel 1961; Glendinning 1996) or the necessity of feeding stimulants in normal host plants (e.g., Simmonds 2001; Müller et al. 2010), although species will often accept ancestral hosts (Pratt and Ballmer 1991; Janz et al. 2001; Braby 2012). In this work, we presented novel chemicals as ground plant material in an artificial diet, which overcomes limitations associated with rejection of novel toxic plants. For instance, while cabbage white butterflies would refuse to consume actual *Aristolochia* plants, mixture into artificial diet at quantities equal to their actual host (8%) resulted in consumption (as evidenced by observed frass production; see fig. S5) but not survival to adulthood (or advancement past second instar; see fig. S1). In addition, incorporating low doses of toxins into an artificial diet may be more representative of how host shifts occur in natural populations, that is, through mixing small amounts of novel plant material with the ancestral diet (Singer et al. 2002). For instance, our focal diets (1% novel toxic plants) represented about 1:8 novel to ancestral host material in the diet, which resulted in mostly sublethal stressful effects, whereas mixing at a ratio of 1:2 (a 4% diet) or 1:1 (an 8% diet) resulted in strong selection (80% mortality) or death (100%), respectively (see fig. S1).

Generalists often rely on enzymes with broad reactivity to deal with periodically encountered toxins (Li et al. 2004; Schramm et al. 2012). For example, species that only periodically encounter isothiocyanates rely on the general detoxification properties of GSTs (Wadleigh and Yu 1988), whereas more specialized species rely on the highly derived nitrile-specifier protein (Wheat et al. 2007). More specific mechanisms that are more efficient at detoxification (Li et al. 2004) no doubt take time to evolve, either through modifications of the genes involved in initial and general responses (Matzkin 2008; Gloss et al. 2014), via recruitment and diversification of entirely novel genes with novel detoxification functions (Naumann et al. 2002), or through alterations of the sites targeted by the toxin (Dobler et al. 2012; Carvalho et al. 2013). Periodic exposure to a novel toxin in the diet of a generalist may perhaps facilitate a host shift to a chemically distinct diet through gradual changes

in generalized mechanisms followed by longer-term changes in more specific mechanisms.

Future approaches with artificial diets could vary the specific chemicals introduced (e.g., Fordyce and Nice 2008) and the schedule of exposure to toxins or novel plant material to further simulate different types of diet shifts and colonization of novel toxic environments. For instance, phylogenetic analyses suggest that chemical defenses become increasingly more complex over time in arms races between plants and herbivores (Becerra et al. 2009; Edger et al. 2015). It may be more realistic to simulate a host shift by first introducing a single novel toxin that is relatively "simple." On the other hand, there are many examples of more recent host shifts to plants with established suites of "complex" chemical defenses: for instance, spatially assisted shifts from Brassicaceae to mistletoe parasites, then subsequently to pine and mangrove (presumably facilitated by mistletoe; Braby and Trueman 2006; Braby 2012). Regardless, using an artificial diet is a promising approach to introduce novel host plants and novel plant defensive chemicals to simulate host shifts, which is experimentally challenging due to rejection or death on toxic novel plant material. This approach may allow us to better understand how shifts to novel toxic host plants happened in the past and potentially how populations may respond to novel toxins in anthropogenic environments.

Acknowledgments

We are grateful to Kinsey Philips, Annie Stene, and Brandon Semke for help with animal care and Ryan Paul for sending *Pieris rapae* lines used to generate figures S1 and S2. Akshat Sakari provided assistance with wing melanin measurements. Jim Fordyce graciously provided *Aristolochia* host plant material for addition to artificial diets. The manuscript was greatly improved by insightful comments from two editors and four anonymous reviewers. This research was funded by a McKnight Land Grant through the University of Minnesota; the Snell-Rood laboratory was supported in part through National Science Foundation grant IOS-1354737.

Data and Code Availability

All data are available in the Dryad Digital Repository (Snell-Rood et al. 2019; https://doi.org/10.5061/dryad.pnvx0k6h3).

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Associate Editor: Sara Magalhães
Editor: Russell Bonduriansky

Cabbage white butterfly (*Pieris rapae*) and caterpillar. Photo credit: Emilie C. Snell-Rood.

Supplemental Material (not copyedited or formatted) for: Kristin L. Sikkink, Reilly Hostager, Megan E. Kobiela, Nathan Fremling, Katherine Johnston, Amod Zambre, Emilie C. Snell-Rood. 2020. "Tolerance of Novel Toxins through Generalized Mechanisms: Simulating Gradual Host Shifts of Butterflies." The American Naturalist 195(3). DOI: https://doi.org/10.1086/707195.

Online Supplement to

Tolerance of novel toxins through generalized mechanisms: simulating gradual host shifts of butterflies

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SUPPLEMENTARY FIGURES AND TABLES

Figure S1. ***Dose-response curve for performance on diets containing Aristolochia leaves.*** *Pieris rapae* larvae (N = 387) were transferred to artificial diet containing *Aristolochia* at 0, 0.5, 1, 2, 4, or 8% dry weight. Each diet additionally contained approximately 8% cabbage flour (their normal host) by dry weight. We measured survival to adulthood, adult dry mass, and development time from egg to adult emergence as measures of performance. Asterisks indicate whether a given diet concentration was significantly different from the control (0% *Aristolochia*) using post hoc individual t- or chi-square tests. The y-axis shows means and standard errors. Statistics are reported in Tables S1a and S1b.

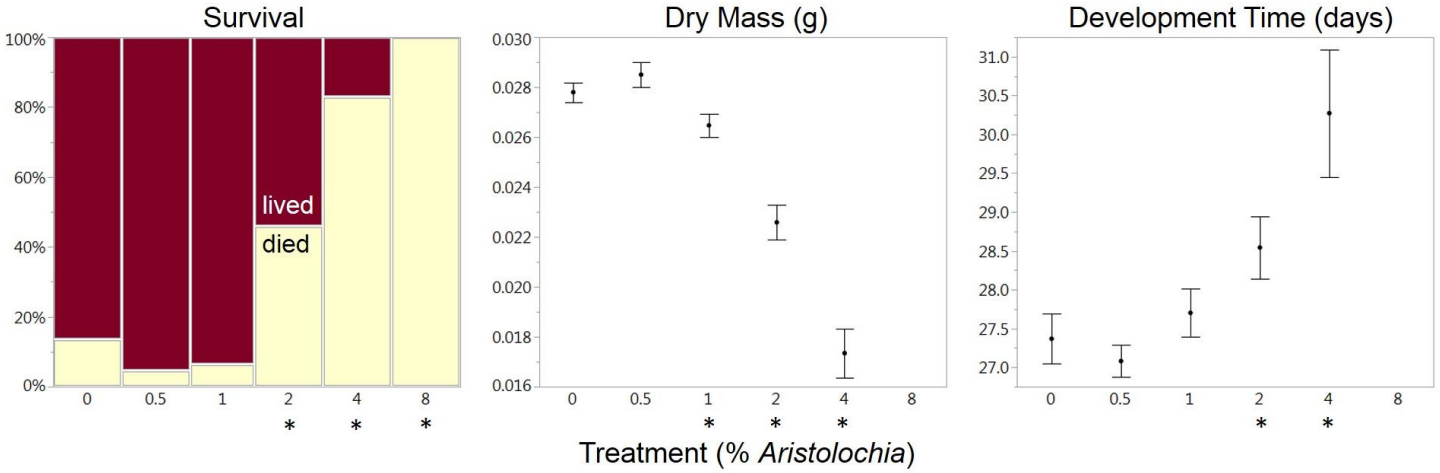

Figure S2. ***Individuals do not vary in consumption of novel diets 24 hours after transfer.***

A follow-up experiment was performed where individual caterpillars were transferred to small pieces of diet in 1-oz cups 7 days after egg collection. Individual larvae were weighed to the nearest 0.0001 g at the time of transfer and then 24 hours later. In addition, we counted the total number of individual frass produced during that time, using a 2X headband magnifier. The y-axis shows means and standard error. There were no significant differences across diet types using ANOVA -- statistics are presented in Table S2.

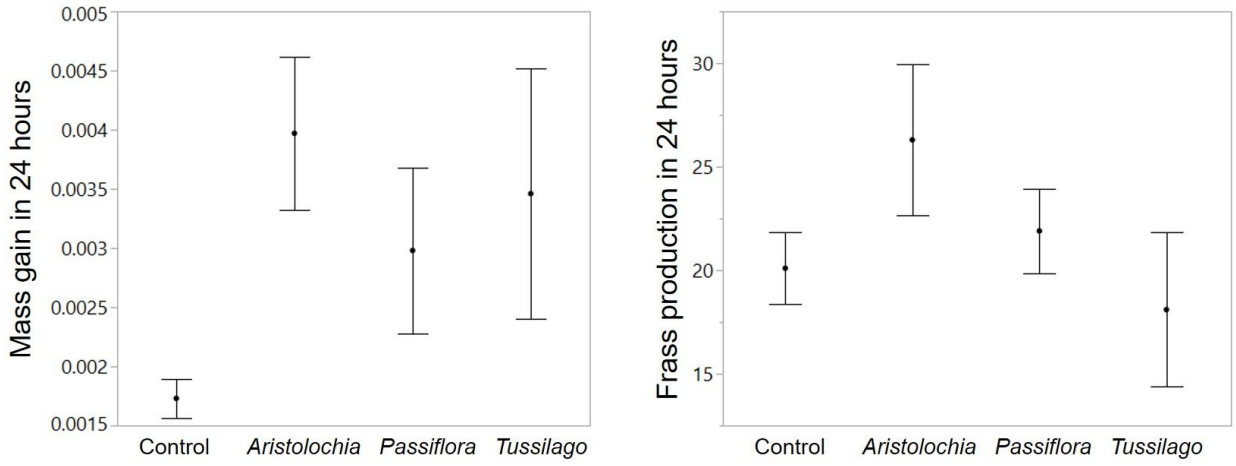

Figure S3. ***Change in antioxidant gene expression on novel diets.*** For each gene, change in expression is calculated as the difference between the least square mean Ct (± 95% CI) on a novel diet relative to the control diet. Positive values indicate upregulation on the novel diet. *P* values are given for significant or marginally significant contrasts from the linear model.

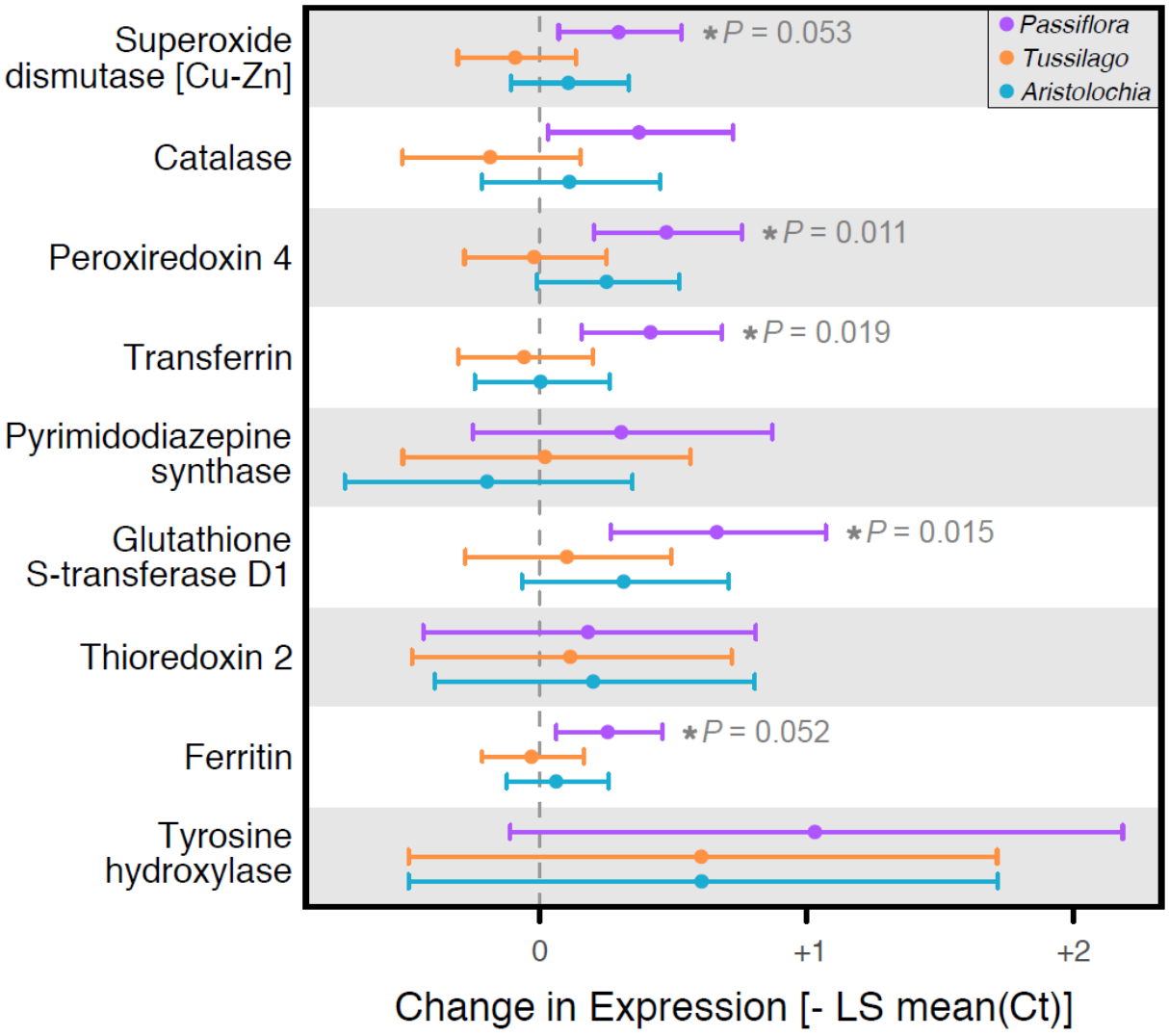

Figure S4. ***Differences in egg size and number among families.*** Least square means (±95% CI) are shown for each family.

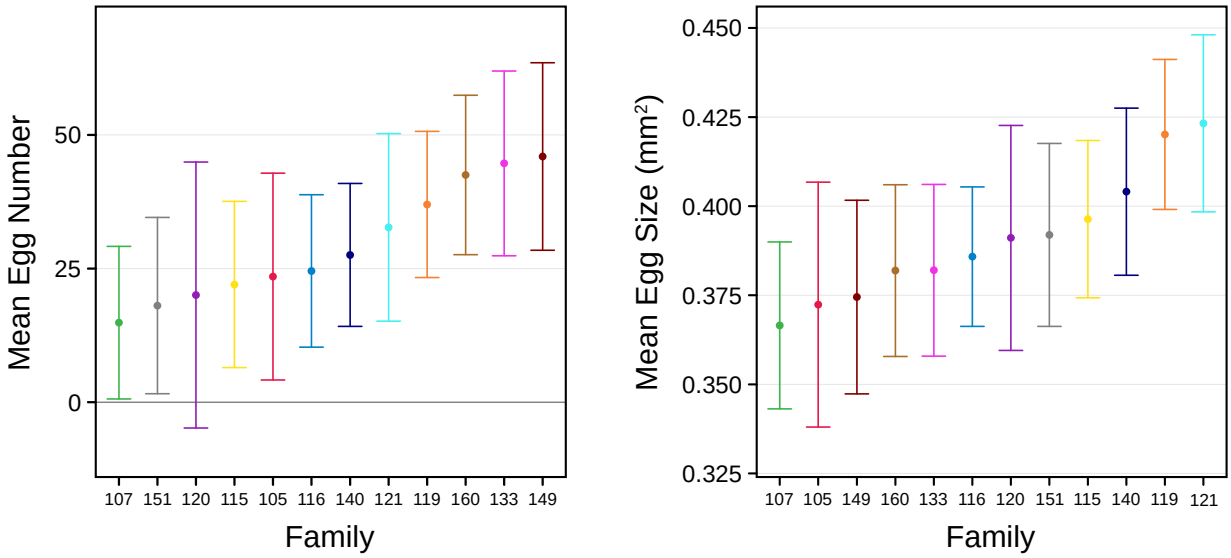

Figure S5. ***Image of frass production for dose-response curve***. Shown are representative cups 10 days after transfer to diets containing 0, 0.5, 1, 2, 4, or 8% dried *Aristolochia*. Note that the 8% treatment, which results in 0% survival (Figure S1) still contains one living 2nd instar larva (blue arrow) that is consuming the diet (as evidenced by individual frass, shown with red arrows, for which there are >30 in this cup). This suggests that even at the highest doses, larvae are consuming the diet, but fail to grow and survive.

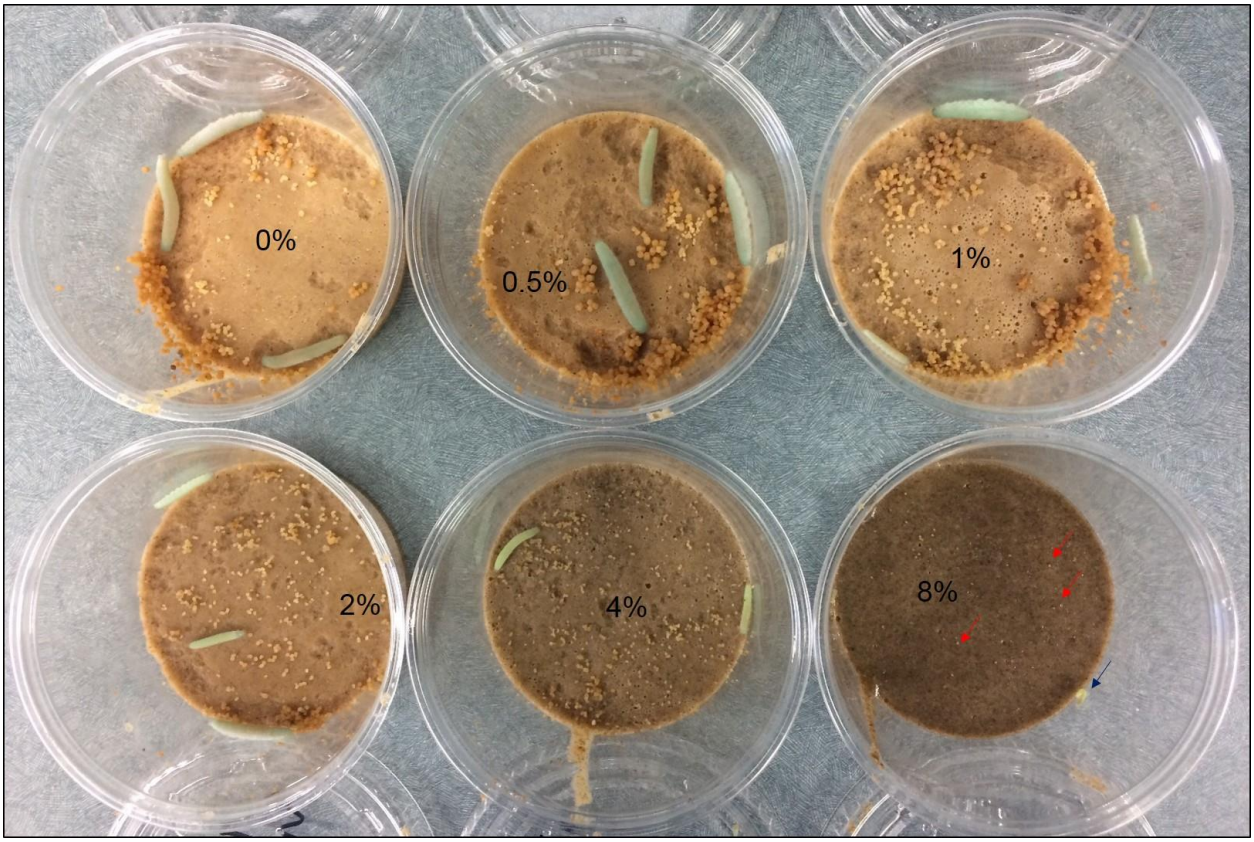

Table S1a. Results of models testing effects of increasing *Aristolochia* concentration on performance. Each treatment level was treated as a category (rather than a continuous amount) because we had no a priori expectation about the shape of the dose-response curve (e.g., linear versus hormetic effect at low levels). We used ANOVA (with *Aristolochia* concentration as the only predictor) to test for effects on adult dry mass and development time from egg to adult emergence and Pearson chi-squared test to test for effects on survival to adulthood (using JMP 14.0, SAS Institute). Post hoc, we additionally compared performance in each dosage level with the control group (Supplementary Table 1b). Results are shown graphically in Supplementary Figure 1.

Trait	**Test Statistic**	**P**	**Total N**
Survival	$X^2_{(5)} = 224.8$	<0.0001	387
Dry Weight	$F_{4,217} = 35.16$	<0.0001	222
Development Time	$F_{4,217} = 6.50$	<0.0001	222

Table S1b. Post-hoc comparisons of increasing *Aristolochia* dose with control treatment. Performance metrics for each treatment level were directly compared to the control diet using t tests or Pearson's chi-squared (using JMP 14.0, SAS Institute). No individuals survived in the highest dosage (8% *Aristolochia*) preventing analysis of dry mass and development time for those levels. Results are shown graphically in Supplementary Figure 1.

	Survival	**Dry Weight**	**Development Time**
0.5%	$X^2_{(1)} = 3.20$, $P = 0.07$	$t_{113} = 1.12$, $P = 0.26$	$t_{97.5} = -0.75$, $P = 0.46$
1%	$X^2_{(1)} = 1.73$, $P = 0.19$	$t_{109} = -2.19$, $P = 0.03$	$t_{111.7} = 0.75$, $P = 0.45$
2%	$X^2_{(1)} = 16.7$, $P < 0.0001$	$t_{55.3} = -6.54$, $P < 0.0001$	$t_{73.7} = 2.29$, $P = 0.025$
4%	$X^2_{(1)} = 63.3$, $P < 0.0001$	$t_{13.3} = -9.83$, $P < 0.0001$	$t_{13.3} = 3.29$, $P = 0.006$
8%	$X^2_{(1)} = 99.4$, $P < 0.0001$		

Table S2. Larval consumption of artificial diets for 24 hours after transfer. We used ANOVA (with diet as the only predictor) to test for effects of diet type (control, *Aristolochia, Passiflora, Tussilago*) on mass gain or frass production in the 24 hours after transfer to artificial diet (using JMP 14.0, SAS Institute). Results are shown graphically in Supplementary Figure 2.

Trait	**Test Statistic**	**P**	**Total N**
Change in mass	$F_{4,36} = 1.79$	0.17	40
Frass Production	$F_{3,36} = 1.42$	0.25	40

Table S3. ***Primer sequences for qPCR of candidate genes***

Gene	**Forward Primer Sequence**	**Reserve Primer Sequence**
Actin	tcaacacccccgctatgta	ccggagtcgagcacgata
Sod1	tgcagatccagatgacttgg	gtgcaccagcattacctgtg
Cat	acaatttgatggcacgctct	cccagaatatcgcaggaaac
Prx4	gctccatcagtttttggagactt	ccaggtcaagacactattataccaaa
Tsf1	agcaaagtggtatcccaagg	gcttgaatttgtcaccagca
Se	tctatcctgtgccttcacga	gagaaatacccgcggaatc
GstD1	tgcttatatctgcaatggtcatatt	tggcttggctcaacacatt
Trx2	acatcaaggattccgacgac	catgaagtcgatcaccactagc
Fer1HCH	cgacgagatggtagtcattgtc	aaagccatccgaactgtcat
Th	ccgctggtttactgactgct	gattggaagacacggaaagc

Table S4. Direct comparison of statistics for performance correlations when treating family as a fixed effect versus a random effect. When treating family as a fixed effect, family values are estimated as least square means; when treating family as a random effect, family values are estimated as best linear unbiased predictors. While the exact P values shift, the overall patterns and conclusions remain the same (there are correlations across diets in performance metrics).

		LSMeans		BLUPs	
Performance Metric	**Diet Comparison**	**Spearman's rho**	**P**	**Spearman's rho**	**P**
Larval survival	*Passiflora* vs. *Tussilago*	0.58	0.052	0.741	0.008
	Passiflora vs. *Aristolochia*	0.559	0.063	0.455	0.140
	Tussilago vs. *Aristolochia*	0.776	0.005	0.580	0.052
Development time	*Passiflora* vs. *Tussilago*	0.832	0.001	0.762	0.006
	Passiflora vs. *Aristolochia*	0.587	0.049	0.510	0.094
	Tussilago vs. *Aristolochia*	0.797	0.003	0.797	0.003
Adult body size	*Passiflora* vs. *Tussilago*	0.231	0.471	0.580	0.052
	Passiflora vs. *Aristolochia*	0.643	0.028	0.469	0.127
	Tussilago vs. *Aristolochia*	0.266	0.404	0.392	0.210
Growth rate	*Passiflora* vs. *Tussilago*	0.587	0.049	0.790	0.004
	Passiflora vs. *Aristolochia*	0.65	0.026	0.643	0.028
	Tussilago vs. *Aristolochia*	0.357	0.256	0.455	0.140

Table S5. Direct comparison of statistics for gene expression-performance correlations when treating family as a fixed effect versus a random effect. When treating family as a fixed effect, family values are estimated as least square means; when treating family as a random effect, family values are estimated as best linear unbiased predictors. While the exact P values shift, the overall patterns and conclusions remain the same (there are correlations between patterns of gene expression and performance on novel diets).

	Expression PC1		**Expression PC2**	
From LS Means	Pearson's R	*P*	Pearson's R	*P*
Larval survival PC1	0.611	0.06	-0.016	0.965
Development time PC1	0.21	0.561	0.735	0.015
Adult body size PC1	0.339	0.339	0.508	0.134
Growth rate PC1	0.106	0.771	0.829	0.003

	Expression PC1		**Expression PC2**	
From BLUPs	Pearson's R	*P*	Pearson's R	*P*
Larval survival PC1	0.690	0.027	0.095	0.795
Development time PC1	0.113	0.756	0.767	0.010
Adult body size PC1	0.187	0.605	0.706	0.022
Growth rate PC1	-0.107	0.768	0.866	0.001