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Rapid but narrow – Evolutionary adaptation and transcriptional response of *Drosophila melanogaster* to toxic mould

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Abstract

Insects have adapted to a multitude of environmental conditions, including the presence of xenobiotic noxious substances. Environmental microorganisms, particularly rich on ephemeral resources, employ these noxious chemicals in a chemical warfare against predators and competitors, driving co-evolutionary adaptations. In order to analyse how environmental microbes may be driving such evolutionary adaptations, we experimentally evolved *Drosophila melanogaster* populations by exposing larvae to the toxin-producing mould *Aspergillus nidulans* that infests the flies' breeding substrate. To disentangle the effects of the mycotoxin Sterigmatocystin from other substrate modifications inflicted by the mould, we used the following four selection regimes: (i) control without fungus, (ii) *A. nidulans* wild type, (iii) a mutant of *A. nidulans* Δ*laeA* with impaired toxin production, (iv) synthetic Sterigmatocystin. Experimental evolution was carried out in five independent *D. melanogaster* populations each, for a total of 11 generations. We further combined our evolution experiment with transcriptome analysis to identify evolutionary shifts in gene expression due to the selection regimes and mould confrontation. Populations that evolved in presence of the toxin-producing mould or the pure mycotoxin rapidly adapted to the respective conditions and showed higher viability in subsequent confrontations. Yet, mycotoxin-selected populations had no advantage in *A. nidulans* wild type confrontation. Moreover, distinctive changes in gene expression related to the selection-regime contrast were only associated with the toxin-producing-fungus regime and comprised a narrow set of genes. Thus, it needs the specific conditions of the selection agent to enable adaptation to the fungus.

KEYWORDS
chemical defence, experimental evolution, filamentous fungus, insects, transcriptome

1 | INTRODUCTION

The ubiquitous interactions between animals and microbes have led to adaptations through (co-) evolutionary processes in numerous ways. Special attention has been given to the often tightly linked adaptions in host-pathogen and host-microbiome systems. However, not only specialized parasites or obligatory symbionts are a selective force in animal-microbe interactions, but environmental microbes can also be evolutionary drivers; through utilization of same habitats, e.g., ephemeral rotting organic substrate,

where recurrent encounters of the same species can be expected (Dubinkina et al., 2019; Téfit et al., 2018). It is conceivable that these encounters may affect fitness positively or negatively. Although less tightly linked than, e.g., host-pathogen interactions, this can lead to long-standing associations between specific insects and microbes, and may provide the conditions to facilitate (co-)evolutionary adaptations (Sieber et al., 2021).

The saprotrophic insect *Drosophila melanogaster* is a classic example of an organism that evolved many traits related to interactions with diverse environmental microbes. It prefers fermenting fruits as egg-laying sites, has developed high resistance to alcohol, and is strongly attracted to the odours emitted by yeasts, which it perceives with distinct odorant receptors (Becher et al., 2010; McKenzie & Parsons, 1972; Semmelhack & Wang, 2009). The developing larvae feed not solely on fruit substrate but also on microorganisms that provide essential nutrients for which the larvae have deficiencies in their metabolism (Carvalho et al., 2010). This dependency on microorganisms as a food source may leads to hardwired preferences in their resource seeking behaviour. However, diverse microorganisms have the ability to produce highly toxic metabolites, which they deploy as a means to defend themselves and their substrate against microbial and other competitors. These biocidal substances, such as antibiotics and particularly mycotoxins, can have highly deleterious impacts on the development and survival of *D. melanogaster* larvae (Trienens & Rohlfs, 2011). Fungal-produced insect juvenile hormones may interfere with larval development (Nielsen et al., 2013). Nevertheless, larvae can be found foraging in close proximity to colonies of toxin-producing filamentous fungi, e.g., *Aspergillus* spp. (Trienens et al., 2010; Trienens & Rohlfs, 2011); they are attracted by fungal volatiles, seek out fungal colonies rather than avoiding them, and aggregate around their perimeter (Stötefeld et al., 2015; Trienens et al., 2017). Yet, *D. melanogaster* evolved a defensive repertoire as a counter-adaptation to cope with these noxious fungi, including behavioural adaptations like aggregated grazing (Trienens & Rohlfs, 2020), which can severely limit the growth of fungi, and an array of detoxification mechanisms (Trienens et al., 2010; Trienens & Rohlfs, 2011; Wertheim et al., 2002, 2006), including cytochrome P450 monooxygenases (CYPs), glutathione S-transferases (GSTs), the Keap1, cnc, AP-1 and AHR pathways (Luque & O'Reilly, 2002; Saisawang et al., 2012; Tijet et al., 2001; Trienens et al., 2017), and excretion of toxic substances through the Malpighian tubules (Chahine & O'Donnell, 2011).

We used our experimental system consisting of *D. melanogaster* populations and the saprotrophic filamentous fungus *Aspergillus nidulans* to study the potential of environmental microbes as evolutionary drivers. Both the fly and the fungus can utilize decaying organic resources, where they compete through interference. The effects of such competition are negative for both opponents as each can considerably decrease the fitness and survival of the other. While *D. melanogaster* larvae destroy fungal hyphae by feeding and moving activities at the perimeter of fungal colonies, the fungus produces and excretes toxic secondary metabolites that kill the larvae (Caballero Ortiz et al., 2018; Trienens et al., 2010; Trienens & Rohlfs, 2012). Previously, we showed that when larvae are exposed to *A. nidulans*, it can severely reduce larval survival (Trienens et al., 2010; Trienens & Rohlfs, 2011), but *D. melanogaster* can rapidly evolve higher tolerance (Trienens & Rohlfs, 2011; Wölfle et al., 2009). Further, exposure of larvae (from a base population) to the toxin-producing fungus had far more pervasive effects on gene expression than exposure to the isolated mycotoxin, Sterigmatocystin (Trienens et al., 2017). This indicates that the fungus introduces more complex changes to the environment – e.g., excretion of mycotoxins, antibiotics, and metabolic waste products, depletion of nutrients, change of pH-level –, which may also affect the counter-adaptations that *Drosophila* can evolve. Elucidating the genomic basis for the tolerance or resistance will provide novel insights into the full complexity of adapting to environmental microbes, and whether a defined feature, the toxic secondary metabolites, is the driving factor of the evolutionary counter-adaptations in *Drosophila*.

In the present study, we conducted an extended experimental evolution experiment to disentangle the insect's evolutionary response to the different substrate alterations inflicted by the toxic filamentous fungus and subsequently link them to changes in gene expression patterns induced by the different selection regimes. For this, *D. melanogaster* larvae of replicated populations developed over several generations repeatedly on breeding substrate infested with the toxin-producing *A. nidulans*. We compared viability, development time, and transcriptional responses of these evolved *D. melanogaster* populations with populations that were kept in parallel in three control selection treatments in order to account for distinct aspects of the fungal biology. Therefore, our selection treatments include: (i) absence of the fungus – no substrate alteration, (ii) exposure to *A. nidulans* wild type – substrate alterations including toxicity effect, (iii) exposure to *A. nidulans mutant* with impaired toxin production – substrate alterations with limited toxicity effects, (iv) exposure to a synthetic application of the purified mycotoxin – toxicity effect without further substrate alterations. Based on the severe detrimental impact that the wild type fungus and the mycotoxin can exert on *D. melanogaster*, we hypothesised that populations which encountered the toxin-producing wild type or the pure mycotoxin will evolve towards individuals that possess higher resistance or tolerance towards these agents, and potentially that cross-resistance can be found, considering the shared feature. However, the encountering of the toxin-production impaired mutant fungus was hypothesised to have limited selective force on *D. melanogaster* populations, as it is lacking the detrimental toxic feature. We found that the selection regimes with the toxin-producing fungus and the pure mycotoxin indeed rapidly changed the viability of those populations, after only a few generations, when exposed to their respective selection agents. However, populations that were selected on the isolated mycotoxin had no advantage when confronted with the toxin-producing fungus. We further tested whether changes in larval viability correlated with the extent to which the larvae suppress fungal growth, yet fungal growth was suppressed to similar extents by all populations of all selection regimes; which may indicate evolved tolerance rather than resistance.

Overall, in response to the toxin-producing fungus *D. melanogaster* larvae changed the expression of about 3000 genes; yet, with regard to the selection regimes, only populations that were selected on the toxin-producing *A. nidulans* showed considerable changes of a narrow set of genes.

2 | MATERIALS AND METHODS

2.1 | Organisms, substrate, and general conditions

We used a *Drosophila melanogaster* lab population of flies collected in Germany (surrounding of Kiel) that was established from 113 isofemale lines shortly after collection and kept at high numbers but low larval density in the lab for 123 generations prior to this study. The population was reared with non-overlapping generations on larvae-breeding substrate (see below) in breeding flasks, and eclosed adults were released into a population cage with supply of water and a sugar-inactivated-yeast mix. Rearing took place at 25°C and 12 h/12 h light/dark cycle. During the first selection-confrontation (see selection procedure below) larvae of this base population were randomly assigned to a given selection regime and population therein.

We used the toxin-producing *Aspergillus nidulans* RDIT2.3 as a wild type (WT) and the toxin-production impaired Δ*laeA*-mutant-stain RJW 46.4 (LA) – provided by Nancy P. Keller, Wisconsin, USA. The gene product of *laeA* is part of a regulatory complex (*velvet*) and considered the master regulator of secondary metabolite production in filamentous fungi (Calvo, 2008). In *A. nidulans* it affects the synthesis of, e.g., Sterigmatocystin, Penicillin, and Lovastatin (Bok & Keller, 2004), as well as pigmentation. Conidia were rinsed from colonies with saline solution (8.6 g NaCl, 300 mg KCl, 300 mg $CaCl_2$, 1 mL Tween80® per litre), counted with haemocytometry (Neubauer Improved), and titre adjusted to 1000 conidia/μL.

The mycotoxin Sterigmatocystin (ST) was purchased from Sigma Aldrich and dissolved in acetone. Sterigmatocystin was used as a single-agent selection pressure as it is present in the wild type fungus yet absent in the mutant strain. Further, as the metabolite of *A. nidulans* with the highest toxicity it has high potency as a selective force.

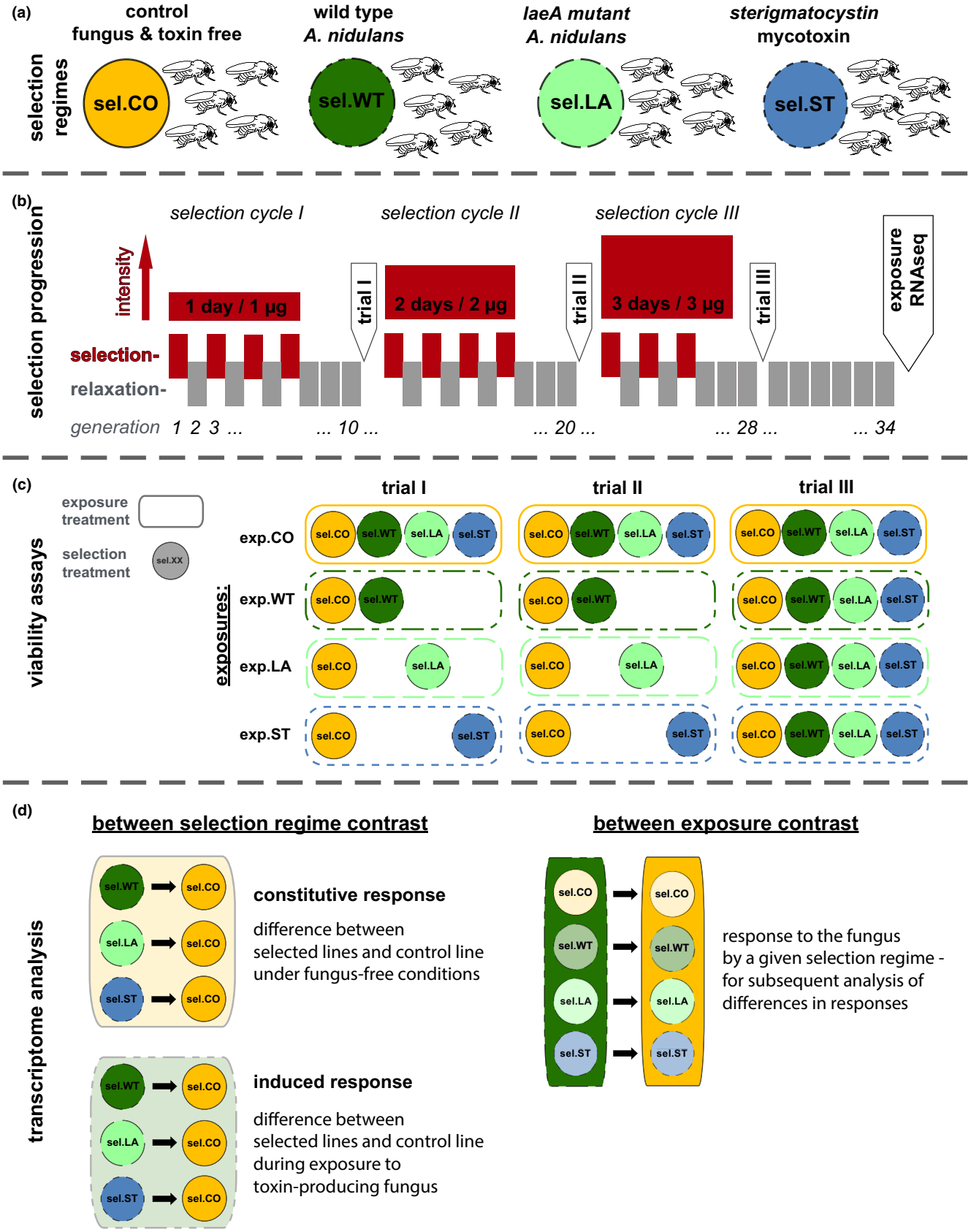
The larvae-breeding substrate consisted of 62.5 g of inactivated yeast (Leiber GmbH Bramsche), sugar and cornmeal, and 12.5 g of Agar, and 1 L purified water, and supplemented with 3 g Nipagin, 1 g Chloramphenicol, and 1 g Nystatin. This substrate was used in the selection- and exposure-experiments (see below), yet without the supplementation of anti-microbial substances. We used steam-sterilized solutions, media, and tools for all experiments. Although neither saline-solution (solvent for conidia) nor acetone (solvent for Sterigmatocystin) after evaporation of 15 min have an effect on larval survival or development, all substrates were pre-treated with the respectively lacking solvents prior to experimental treatment application.

For all assays, we transferred first-instar larvae that hatched from sodium hypochlorite treated eggs ('axenic larvae') to exposure- and confrontation-units to avoid the introduction of other microbes. Incubation took place at 25°C and 12/12 h light/dark cycle, if not stated otherwise.

2.2 | Selection procedure

We conducted four different selection regimes: (i) control without fungus (sel.CO), (ii) *A. nidulans* wild type (sel.WT), (iii) a mutant of *A. nidulans* Δ*laeA* with impaired toxin production (sel.LA), and (iv) synthetic Sterigmatocystin (sel.ST) (Figure 1a) in which we transferred *D. melanogaster* larvae for development to the respectively treated breeding substrate. Fungal conidia were applied by placing a 1 μL droplet of conidia solution in the centre of substrate surface, and Sterigmatocystin by applying 50 μL Sterigmatocystin-acetone solution, which spread over the entire substrate surface. To increase the selective pressure over the course of the evolution experiment, we first used fungal colonies of both strains with a development head-start of 1 day and 1 μg of Sterigmatocystin per confrontation vial (selection cycle I), then fungal colonies with 2-days head-start and 2 μg Sterigmatocystin (selection cycle II), and finally 3 days old fungal colonies and 3 μg Sterigmatocystin (selection cycle III). We repeated the selection cycle I and II four times each, and selection cycle III three times (Figure 1b). Each selection round included one selection- and one relaxation-generation. This was done to prevent population depression due to reduced fecundity of flies in populations with continually confronted generations (unpublished data). Further, the selection pressure alternation may reflect natural selection conditions in environmental microbe-insect interactions, as not each substrate patch available at times of different generations is inhabited to the

FIGURE 1 Scheme of selection procedure and exposure trials. (a) The four selection regimes applied to five independent *Drosophila melanogaster* populations per regime, with fungus-free and toxin-free control breeding substrate (sel.CO), breeding substrate inoculated with the toxin-producing *A. nidulans* wild type (sel.WT) or the toxin-production impaired mutant strain Δ*laeA* (sel.LA), or breeding substrate supplemented with the mycotoxin *sterigmatocystin* (sel.ST). (b) The selection progression was conducted with alternating selection- and relaxation-generations, where *D. melanogaster* larvae were confronted with the selection treatments and reared under standard breeding conditions, respectively. Selection intensity was gradually increased in three selection cycles, each of which included several selection confrontations. Prior to trials testing the viability of the selection lines three relaxation-generation were included to alleviate parental effects on offspring performance. (c) Setup of the exposure trials for viability assays: exposure treatment ('exp.xx') depicted as frames (solid: control, dash-dotted: wild type fungus, dashed: mutant fungus, dotted: mycotoxin) and selection treatment ('sel.xx') depicted as coloured circles. In trials I and II direct responses were assayed to the respective selection regime and control conditions, while trial III also included cross-responses to the other selection agents. (d) Overview of selection- and exposure-combinations used to define contrasts in the transcriptome data analysis.



exact same extent and with the exact same microbe composition, and thus selection pressure may not be uniform in each generation.

For each selection regime we conducted five independent fly populations of about 500 individuals each. For selection-confrontations, larvae were reared in respectively treated 50 mL vials (see above) with a density of 50 larvae per confrontation vial. As the selection treatment caused moderate mortality (up to approx. 25% in WT- and ST- selection regimes) 15 vials per population were prepared in order to ensure that 500 flies per population were gained. For relaxation-generations six larger breeding flasks (250 mL) with larvae-breeding substrate and 100–150 eggs per flask were used per population (Figure 1b). In each generation flies of a given population were merged in one population-cage shortly after eclosion for blending and mating, and population size adjusted to approx. 500 flies, each.

Prior to trials testing larval viability, populations were reared under relaxation conditions for three generations to eliminate the impact of epigenetic effects (Figure 1b).

2.3 | Viability of *D. melanogaster* larvae during exposure to *A. nidulans* and Sterigmatocystin

To test changes in the ability to develop in presence of the respective selection pressures, we exposed larvae in 2 mL tubes containing 1 mL breeding substrate that was inoculated with 1000 conidia of *A. nidulans* WT or LA (exp.WT, exp.LA), or Sterigmatocystin (exp.ST), or as fungi and toxin free controls (exp.CO). Acetone was allowed to evaporate for 15 min. Wild type colonies grew for 1 day and mutant strain colonies for 3 days prior to larval transfer. Sterigmatocystin was inoculated with 0.5, 1.0, and 1.25 μg per tube for trials after selection cycle I, II, and III, respectively. With these treatments we aimed to inflict a moderate mortality; where wild-type colonies with a longer development head start potentially induce a 100% mortality, whereas younger mutant-strain colonies have no measurable effect. We conducted 12 replicates per population and exposure condition, where larvae were exposed in groups of 10 per tube (120 larvae/population/condition). After first eclosion was observed we removed flies from tubes on a daily basis until no flies emerged across all conditions. Flies were counted and emergence day recorded to calculate viability and development time. Note, throughout the text we refer to the selection regime with 'sel.xx' and to exposure conditions for viability assessment as 'exp.xx', where xx represents the initials for the respective agents (CO, WT, LA and ST; Figures 1c and 2).

2.4 | Fungal growth suppression

We inoculated 1 μL conidia solution of *A. nidulans* WT on 4 mL fly breeding substrate in 35 mm Petri dishes and incubated them for 48 h. Then we transferred 10 *D. melanogaster* first-instar larvae to each dish and incubated them further. After 24- and 72-h confrontation with larvae we took pictures (~8 Mpix), analysed the proportion of substrate surface that was covered by fungal colonies using ImageJ (https://imagej.nih.gov/ij/), and calculated the fungal growth between the two time points. For larvae-free controls we conducted 20 replicates and for larvae-confronted colonies 10 replicates for each of the 20 populations. To reduce variation due to measuring error we measured each colony three times independently and used the mean for further analysis.

2.5 | Exposure and sampling for transcriptome analysis

We inoculated 4 mL breeding substrate in 35 mm Petri dishes with 1 μL *A. nidulans* WT conidia solution (exp.WT) or as fungi- and toxin-free controls (exp.CO) and incubated the Petri dishes at 25°C and 12 h/12 h light/dark cycle for 3.5 days. We transferred 20 axenic first-instar larvae to each dish and incubated them further for 24 h in darkness. Our earlier study showed that under these given conditions no significant differences in larval mortality or development time occurred (Trienens et al., 2017). We collected larvae after these 24 h of exposure, where we pooled 52 larvae for one biological replicate collected from four independent dishes (balanced design with 13 larvae per dish). Sampling order was randomized. Larvae were snap frozen in liquid nitrogen. The larvae used in this experiment were generated nine generations after the last selection generation. This was done to eliminate differences in gene expression patterns that are due to carry-over effects, such as epigenetics, and thus to emphasis gene expression changes due to evolutionary changes. It was further done to have the results from the viability assays available prior to sample generation for the transcriptome analysis.

For RNA extraction we combined phenol-chloroform and column-based methods (TRIzol® until ethanol addition followed by RNeasy mini, QIAGEN, according to manufacturer's instruction). Of the five populations per selection regime, we randomly selected three populations for library preparation for a total of 24 libraries (2 treatments × 4 selection regimes × 3 replicated populations). PolyA+ isolation, library preparation, and sequencing were performed by Eurofins Genomics (Ebersberg, Germany). We made use of Illumina HiSeq2500 technology and paired-end 125 base sequence chemistry. Samples were multiplexed in groups of 8; to efficaciously contrast the differences between selection-regime responses, a given population of each selection regime exposed to *A. nidulans* and to control conditions was blocked on one lane. Sequencing yielded on average 5.96 Giga base calls (Gbp) per library, with a minimum of 4.73 Gbp and a maximum of 7.18 Gbp.

2.6 | Transcriptome data analysis

We filtered the sequenced reads for a 99% probability of correctly identified bases (*FASTX 0.10.1*, *FASTQ Quality Filter*). Paired-end reads were mapped to the *Drosophila melanogaster*-reference-genome version 6.06 (flybase.org) allowing for 10% mismatches [*GMAP-GSNAP 2015-07-23* (Wu et al., 2016)]. Duplicates and non-unique reads were excluded from counting reads to

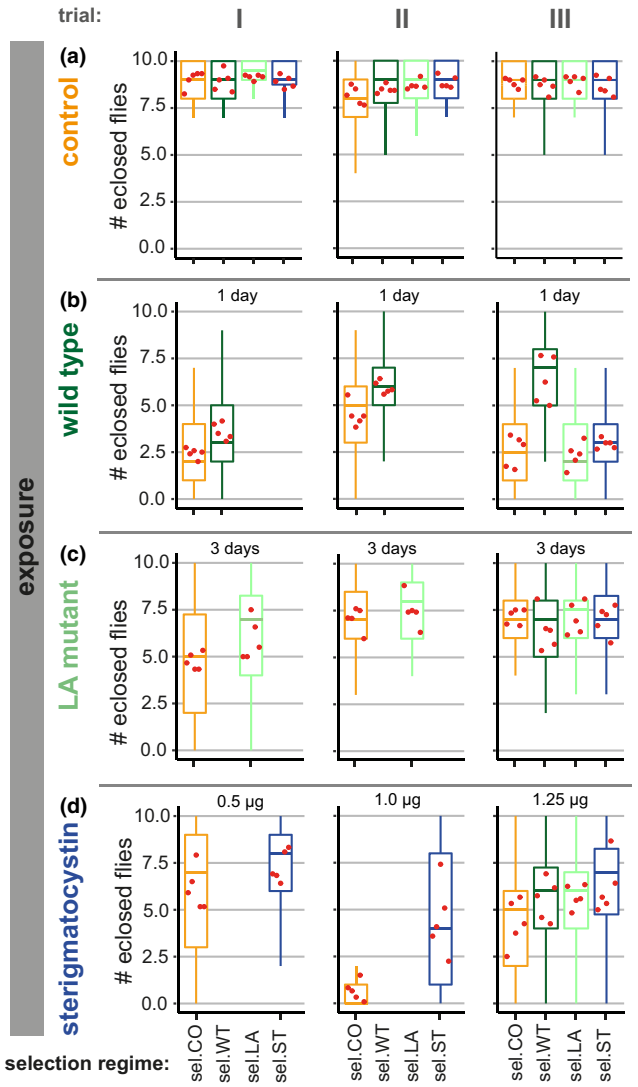


FIGURE 2 Adaptation through experimental evolution. Number of eclosed flies from larvae of the four different selection regimes (control: sel.CO, *A. nidulans* WT: sel.WT, *A. nidulans* LA mutant: sel.LA, and *sterigmatocystin*: sel.ST) developing on breeding substrate supplemented with (a) control solvents, (b) toxin-producing *A. nidulans* WT, (c) toxin-impaired *A. nidulans* LA mutant, or (d) the mycotoxin Sterigmatocystin after selection cycle I, II, and III (columns from left to right). The WT colonies were 1 day and LA colonies 3 days old at time of larval transfer. Sterigmatocystin was applied with 0.5, 1.0, and 1.25 µg per confrontation unit, respectively. Boxplots represent cumulative data of each selection regime with 600 larvae per boxplot – that is, five populations per selection regime and 12 replicates per population with 10 larvae per confrontation unit – and red dots within boxplots represent the means of the five replicated populations, numerically ordered from left to right. In trial I and II, direct responses to the respective selection regimes were assayed, while trial III was conducted in full-factorial design.

features (gene sequences; *HTSEQ 0.601*; Anders et al., 2014). Final feature count resulted on average in 9.74 M reads ±1.9 (SD) per library. For the analyses of differentially expressed genes (DEGs) we used *R* (version 3.3.2; R Core Team, 2019) and the package EDGER (Robinson et al., 2009; version 3.16.5, *limma* version 3.30.9). We used the subsample methods for normalization; that is, only libraries (read counts of samples) included in a given comparison were normalized together. Prior to analysis we removed all features that yielded less than 5 counts per million reads in less than 4 libraries in a given contrast. We then estimated tagwise dispersion and fitted the model with the grouping factor *selection:exposure*. For a first overview of the data set we generated a multi-dimensional-scaling (MDS) plot using EDGER standard setting.

We set contrasts to analyse *between-selection-regimes* differences with 'sel.WT vs sel.CO', 'sel.LA vs sel.CO', and 'sel.ST vs sel.CO' separately for control exposure (exp.CO, 3.3.1) and *A. nidulans* wild type exposure (exp.WT, 3.3.2; both Figure 5a). We further analysed *between-exposure* differences with the contrasts 'exp.WT vs exp.CO' within the different selection regimes (sel.CO, sel.WT, sel.LA, and sel.ST; 3.3.3, Figure 5b). These separate analyses provided us with insights in the constitutive differences in gene expression among selection regimes under control conditions, the induced differences in gene expression among selection regimes after exposure to the wild-type fungus, as well as the inducible differences in gene expression within each selection regime after exposure to the wild-type fungus. We considered genes to be differentially expressed when the false discovery rate (FDR) was <0.05 (BH adjustment method, EDGER).

We used *DAVID Bioinformatics Resources 6.8* (Huang et al., 2008) including functional categories, gene ontology (biological processes, cell component, and molecular functions), pathway involvement (KEGG) and protein domains (Interpro, Pfam) for functional annotation cluster analysis. We included genes that were marked as DEGs with an FDR <0.05.

2.7 | Statistic procedures

We analysed the viability of larvae with the glmer procedure [R package LME4 (Bates et al., 2015) version 1.1–12] as a binomial distribution and corrected for over-dispersion. We fitted the model to test the main effects of '*selection*', 'exposure', and 'trial' as fixed factor. By including interaction terms of the fixed factors we tested the effect of selection in a given exposure and trial. Initially '*population*' was included as random factor, yet removed during model reduction as applicable. For the analysis of the length of time until flies eclosed we used the R package COXME [(Therneau, 2012) version 2.2–7] with '*selection*' as fixed factor and fitted the Cox mixed-effects model by maximum likelihood on a survival object of type '*counting*', including '*population*' and '*tube-id*' as random factors.

We analysed the fungal fitness inferred by substrate surface covered by hyphae with the *glmer* procedure, REML fitted, with 'selection' as fixed factor and 'population' as random factor (LME4 version 1.1–12), and type III analysis of variance (R package CAR; Fox & Howlett, 2008; version 2.1–4).

3 | RESULTS

3.1 | Fitness-traits screening of evolved *D. melanogaster* populations after selection cycle I, II, and III

We conducted experimental evolution with four selection regimes: control without the fungus (sel.CO), *A. nidulans* wild type infested substrate (sel.WT), toxin-production impaired mutant *A. nidulans* Δ*laeA* infested substrate (sel.LA), and Sterigmatocystin supplemented substrate (sel.ST) on five independent *D. melanogaster* populations each, for in total 11 generations of selection (see Figure 1). At the end of each of the three selection cycles with increasing selective pressure (I, II, and III) the 20 *D. melanogaster* populations were exposed as larvae to the same four different treatments that were used as selection agents (see above; exposure labels: exp.CO, exp.WT, exp.LA, and exp.ST). The viability and development time of larvae were documented.

3.1.1 | Viability of *D. melanogaster* larvae during exposure to *A. nidulans* and Sterigmatocystin

Under control exposure conditions (exp.CO) all 20 populations showed high viability and little variation in the numbers of larvae that developed to adults. The lack of a global effect indicates that differences between selection regimes (sel.WT, sel.LA, and sel.ST) under control conditions were not significant (sel.WT: $p = .7577$, sel.LA: $p = .5299$, and sel.ST: $p = .6323$; Figure 2a and Table S1); yet in trial II two populations of sel.CO showed a slightly reduced number of eclosed flies. In contrast, all exposures (exp.WT, exp.LA, and exp.ST) had a negative effect on the viability of larvae from all selection regimes (exp.WT: $p < .0001$, exp.LA: $p < .0001$, and exp.ST: $p < .0001$; Figure 2a–d and Table S1) compared to control conditions (exp.CO).

Depending on the exposure the two most stringent selection-regimes had a positive effect on larval viability as a direct response. When exposed to wild type *A. nidulans* colonies (exp.WT) wild-type-fungus selected populations (sel.WT) had a higher viability compared to sel.CO populations (interaction term sel.WT*exp.WT: $p = .0409$; Figure 2b and Table S1) already after the first four selection generations. The difference in viability increased significantly from about 12% in trial I and II to 38% in trial III (sel.WT*exp.WT*trial.III: $p = .0100$; Figure 2b and Table S1). The mortality inflicted by the wild type fungus differed between the trials, with trial II having a lower reduction in the number of eclosed flies despite same age of the fungal colonies at time of confrontation. In this exposure two living organisms interact with each other, where external factors beyond control may affect this interaction, like, e.g., season, weather, and atmospheric pressure.

The mycotoxin selected populations (sel.ST) in exposure to the mycotoxin (exp.ST) had more variation in viability between the trials. However, compared to sel.CO populations, sel.ST populations also had a significantly higher viability from the first trial on (interaction term sel.ST*exp.ST: $p = .0357$, Figure 2d and Table S1), with a larger difference in viability in trial II (sel.ST*exp.ST*trial.II: $p = .0087$; Figure 2d and Table S1).

This increase of viability in subsequent exposures to the respective selection agents after only a few generations of selection shows that *D. melanogaster* populations can rapidly adapt to otherwise detrimental conditions created by toxic fungi or to the purified mycotoxin.

In contrast, although exposure to the toxin-production impaired mutant strain of *A. nidulans* (exp.LA) also led to a slight but significant reduction in viability compared to control exposure (Figure 2c), differences in viability between sel.CO and sel.LA in exposure to the mutant fungus (exp.LA) were not significant (interaction term sel.LA*exp.LA: $p = .2534$; Figure 2c and Table S1). With a 3 days developmental head start, the colony covers nearly the entire surface of the substrate. However, the fungus impaired in its toxic arsenal is incapable of eliminating the insect larvae.

In trial III populations of the different selection regimes were exposed to the respective other selection agents to test for changes in viability as an indication for cross-resistance. Whilst the selection with the single toxic agent (mycotoxin, sel.ST) did not elicit cross-resistance towards a multifaceted noxious agent (toxin producing WT fungus, interaction term sel.ST*exp.WT: $p = .2903$; Figure 2b and Table S1), selection on a non-toxic and on the toxic fungus resulted in reduced sensitivity to a mycotoxin (Sterigmatocystin, Figure 2d). However only for sel.WT-populations the difference was significant (interaction term sel.WT*exp.ST: $p = .0325$, sel.LA*exp.ST: $p = .1213$, Figure 2d and Table S1). No further cross-resistance was observed in any combination of selection regime and exposure condition (Figure 2 and Table S1).

3.1.2 | Development time of *D. melanogaster* larvae during exposure to *A. nidulans* and Sterigmatocystin

To characterize in more detail the potential effects of the different exposures on larval health and fitness, and the ameliorating effect of selection regimes on these exposures, we monitored development time of the larvae (from first instar larvae until eclosion of flies, Section 2.3). Across all exposure conditions, developmental time was on average fastest under control conditions and longest after exposure to Sterigmatocystin (Figure S1 and Table S3). Differences among larvae from different selection regimes in developmental time after the various exposures were overall marginal, except for the exposure with Sterigmatocystin after selection cycle II. Further, some of the selected populations also showed slight differences in developmental time compared with the control selected populations (sel.CO) under control conditions (exp.CO), as well as under the exposures they had been selected for, but these differences were only minor (for further details see Figure S1 and Table S3).

3.2 | Fungal growth suppression by selected *D. melanogaster* populations

The populations of all selection lines reduced the growth of *A. nidulans* WT colonies by about 34% compared to the growth of un-confronted colonies ($F_{1,218}=213.4$, $p<.001$). However, the populations of the different selection regimes did not differ in the degree of the inflicted growth reduction (Figure 3, glmer and type III analysis of variance, excluding data of un-confronted colonies: $F_{3,16}=0.4433$, $p=.725$).

3.3 | Gene expression analysis – Comparison within and between selection regimes

To screen for changes in gene expression in larvae of all four selection regimes, both under unexposed (control) conditions and after exposure to *A. nidulans* wild type, larvae were collected after 24 h of exposure duration. In the multidimensional scaling plot (MDS) the samples are predominantly divided into groups that had been exposed to *A. nidulans* opposing the fungal-free control condition (MDS, dimension 1; Figure 4 - solid versus open symbols). Further, the samples are distributed along dimension 2, which roughly groups the samples into sel.CO and sel.ST versus sel.WT and sel.LA.

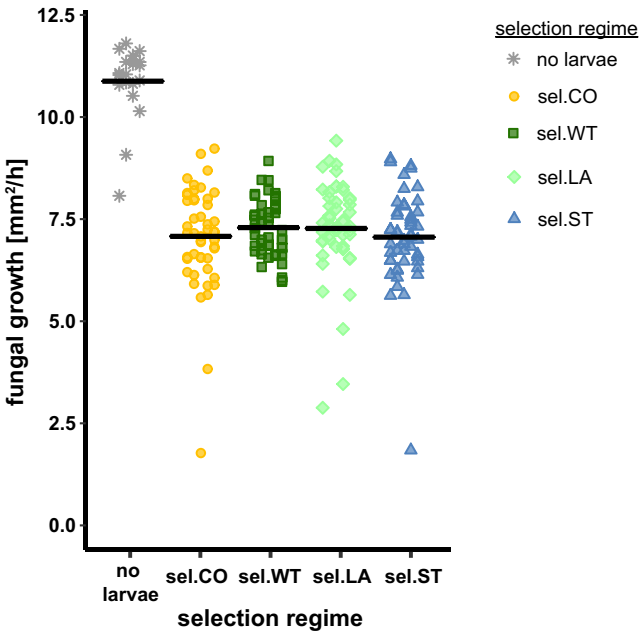


FIGURE 3 Fungal growth of WT colonies confronted with larvae of the selection lines. Fungal growth of *Aspergillus nidulans* WT colonies when confronted with larvae of the four different selection regimes (control: sel.CO, *A. nidulans* WT: sel.WT, *A. nidulans* LA mutant: sel.LA, and *sterigmatocystin*: sel.ST). The WT colonies were 48 h old at time of larval transfer (10 larvae per Petri dish), and growth was measured after 24 and 48 h as the increase in surface area of the colony. The graph represents data of the five populations for each selection regime, with 10 confrontations per selection regime per population.

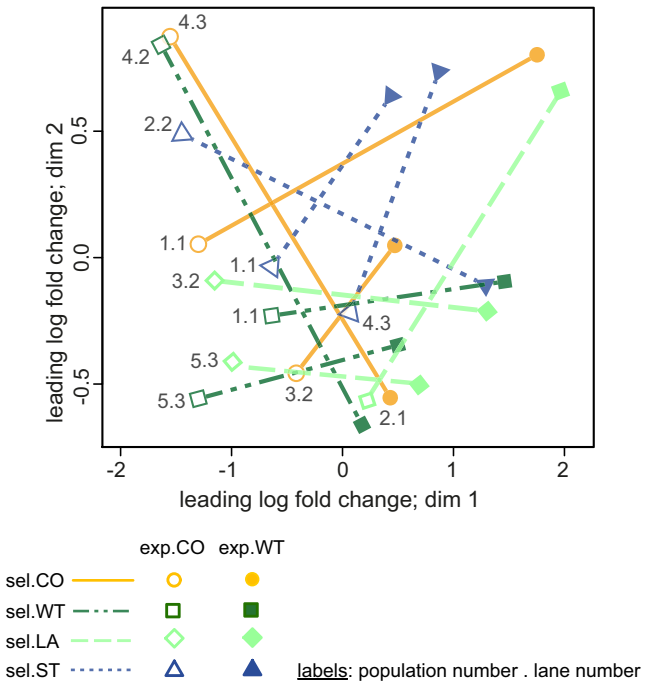


FIGURE 4 Multidimensional scaling plot (MDS plot). Multidimensional scaling analysis of the normalized mRNA sequence libraries (EDGER) from *D. melanogaster* larvae unexposed (control conditions, open symbols) and exposed to the toxic fungus *A. nidulans* (filled symbols). Three populations of each selection regime were randomly chosen for gene expression profiling. Symbol shapes represents populations from circle = sel.CO, square = sel.WT, diamond = sel.LA, and triangle = sel.ST. Sample names are composed of selection regime and population-number.

3.3.1 | Between selection regimes contrast – Constitutive response

We first analysed gene expression patterns of larvae on fungal- and toxin-free breeding substrate (exp.CO), comparing the selection regimes sel.WT, sel.LA, and sel.ST to the control selection regime sel.CO. The exp.CO-contrasts resulted in detection of a low number of differentially expressed genes (DEGs; Figure 5a and Table S4/DEGs) and thus, the constitutive gene expression of all populations under standard breeding conditions remained predominantly unchanged.

3.3.2 | Between selection regimes contrast – Induced response

When we compared the gene expression under wild type exposure (exp.WT) among selection regimes to the control regime, it resulted in 53 DEGs for 'sel.WT vs. sel.CO'-contrast, 10 DEGs for 'sel.LA vs. sel.CO'-contrast, and 2 DEGs for 'sel.ST vs. sel.CO'-contrast (FDR < 0.05, EDGER; Figure 5a and Table S4/DEGs), showing a more pronounced difference in the induced response of sel.WT populations than in the other selected populations. Of the 53 DEGs in the 'sel.WT vs.

sel.CO'-contrast 44 were upregulated only in this contrast. Despite the relatively low number of these genes for functional annotation cluster analysis, six clusters with significant over-represented annotation terms were detected (Benjamini correction < 0.05, *DAVID 6.8*). The 44 up-regulated genes clustered in terms like *Epidermal growth factor-like domain* (enrichment score 4.14), *Calcium ion binding*, *Laminin G domain*, *Insulin-like growth factor binding protein*, *Cadherin*, *receptor activity*, and *cell adhesion molecule binding* (enrichment score 3.40), *Plexin-like fold* (enrichment score 3.29), *membrane depolarization during action potential* and *Voltage-dependent potassium channel* (enrichment score 2.56), *Immunoglobulin-like fold* (enrichment 2.25), and *Glycoprotein* (enrichment score 2.23).

3.3.3 | Between exposure contrast – Response to fungus within selection regimes

We further compared the response of the larvae to the exposure to *A. nidulans* wild type fungus ('exp.WT vs exp.CO') within each selection regime. Comparisons resulted in 611 DEGs for sel.CO-populations, 493 DEGs for sel.WT-populations, 599 for sel.LA-populations, and 785 for sel.ST-populations, of which 80%–85% were down-regulated (Figure 5b). About 26.3% of the 904 down-regulated genes were shared between all contrasts and about 4.3% uniquely down-regulated in sel.WT and sel.LA, whereas 11.2% and 18.6% were uniquely down-regulated in sel.CO and sel.ST, respectively (Figure 5b). Of the 252 up-regulated genes throughout all contrasts about 12.3% were shared between all contrasts and about 12.3%–17.5% were uniquely up-regulated in only one given contrast. Cluster annotation analysis (using an 0.05 cut-off for clusters after Benjamini correction, *DAVID 6.8*) resulted in only few significant terms for the 31 shared up-regulated genes, namely *Glycosidase* and *mannose metabolic process* (enrichment score 3.48), and the 238 shared down-regulated genes in terms of *chitin-based cuticle development* (enrichment score 7.70), *DUF1676* (*'Osiris'*; enrichment score 5.61), *proteolysis*, *disulphide bond* (enrichment score 3.16), *apical constriction*, *zona pellucida domain*, *regulation of embryonic cell* (enrichment score 2.49), and *integral component of membrane* (enrichment score 2.19).

No significant clusters were detected for up-regulated DEGs of unique sets of sel.CO, sel.WT, sel.ST, whereas DEG cluster analysis of uniquely up-regulated genes in sel.LA clustered in terms like *proteolysis* and *serine protease* (enrichment score 3.58). Exclusively in sel.CO, down-regulated genes clustered in terms like *immunoglobulin-like domain*, *Fibronectin type III domain* (enrichment score 4.47), and *transmembrane helix* (enrichment score 3.37). DEGs down-regulated in sel.ST only clustered in terms like *Chitin binding domain* (enrichment score 4.79), *integral component of membrane* (enrichment score 2.94), and *Monooxygenase* (enrichment score 1.98).

No enrichment was found in genes involved in metabolic and detoxification processes, however, individual genes were consistently up-regulated (*cyp28d2*, *gstD2*) or down-regulated (*cyp18a*, *cyp301a1*, *cyp318a1*) in all selection regimes (Figure S3).

4 | DISCUSSION

Environmental microbes inhabiting natural resources can change the conditions for other organisms that rely on these resources in different ways. In such manner, the harmful filamentous fungus *Aspergillus nidulans* has a significant negative impact on the breeding environment of saprotroph *Drosophila melanogaster* larvae. Larvae that developed in the presence of the fungus or its isolated mycotoxin Sterigmatocystin had a reduced viability. To investigate how *D. melanogaster* larvae adapt to microbial-induced substrate changes by *A. nidulans* we used experimental evolution including four complementary selection regimes. We further analysed the transcriptome of the selected *D. melanogaster* populations to dissect the insect's traits that aid the larvae to cope with the detrimental impacts the fungus can exert.

4.1 | Rapid evolution of increased tolerance

The two most stringent selection regimes – toxin-producing fungus and pure mycotoxin – led the *D. melanogaster* populations evolve rapidly towards a higher ability to cope with the respective challenging condition, while the more lenient selection regime – toxin-impaired fungus – did not result in improved performance.

Cross-resistance between selection- and exposure-conditions was limited; populations that had evolved in the presence of the isolated mycotoxin did not achieve higher viability in the presence of the toxin-producing fungus. However, populations evolving on either of the two *A. nidulans* strains tended to a slightly improved viability towards the isolated mycotoxin compared to the control population. Note, *A. nidulans* does not infect *Drosophila melanogaster* as unlike some entomopathogenic fungi may infect insects (Lemaitre et al., 1996; Tinsley et al., 2006; Trienens et al., 2010).

Sterigmatocystin is one of the most potent mycotoxins of *A. nidulans*. As a single agent it severely hampers *D. melanogaster* development. Thus, the reduced susceptibility towards Sterigmatocystin of sel.ST-populations might be underlain by a specific mechanism that is directed at structural characteristics of the toxin. Which kind of mechanism leads to the reduced susceptibility to the isolated mycotoxin, e.g., more efficient degradation or faster emission via cellular drug efflux pumps, needs to be further investigated. However, these adaptations did not support further tolerance or resistance to other more versatile substrate alterations inflicted by *A. nidulans*. This implies that traits to defend against the isolated mycotoxin are not sufficient to provide cross-resistance towards related challenges, such as fungal exposure. In contrast, both in this study and a previously conducted experimental evolution experiment using *D. melanogaster* and *A. nidulans* as a model system (Trienens & Rohlfs, 2011), a selection regime that exposed fly populations to the toxin-producing *A. nidulans* showed an increased viability when larvae developed on Sterigmatocystin-supplemented breeding substrate, indicating an evolved cross-resistance.

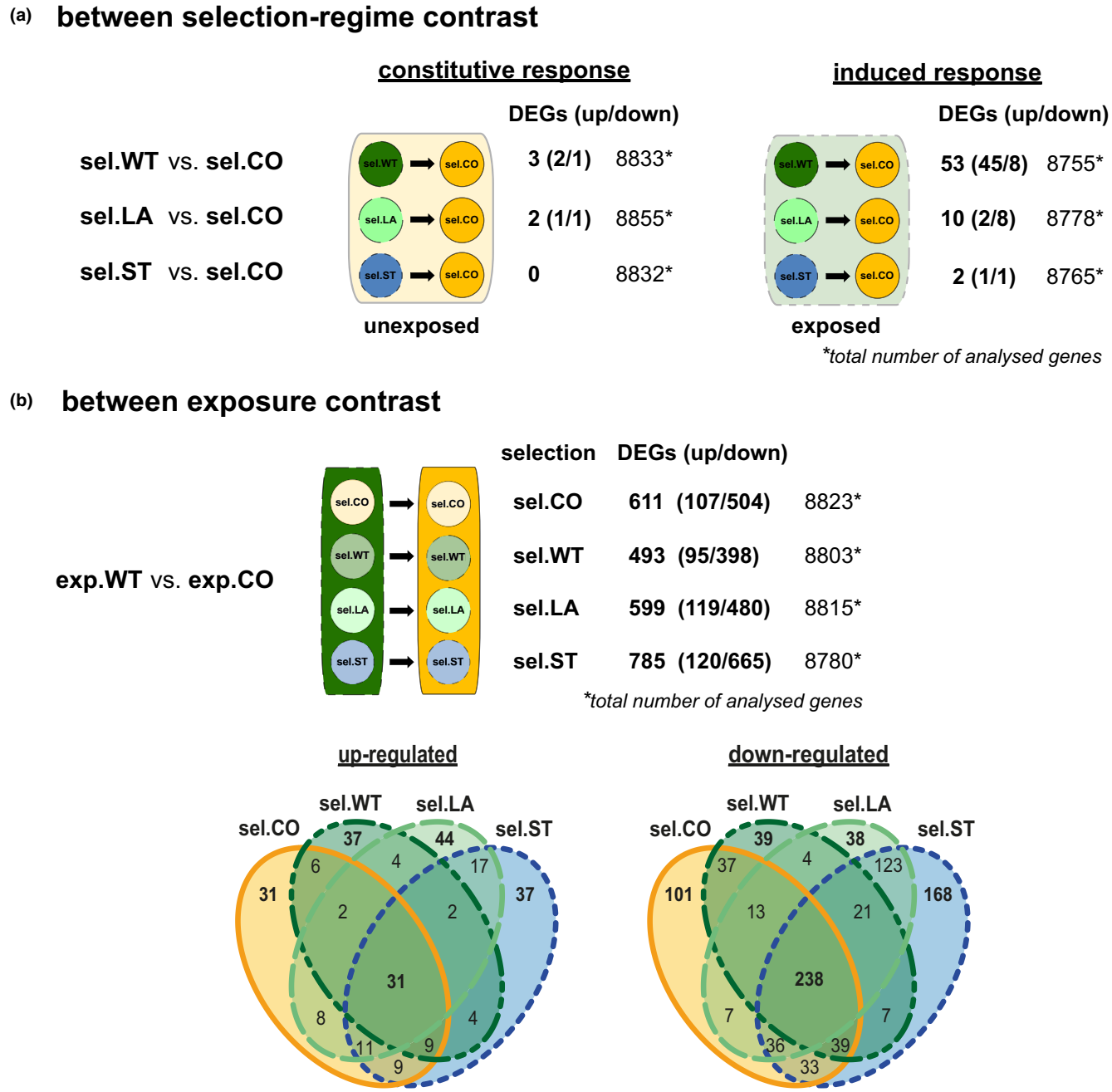


FIGURE 5 Gene expression of evolved lines upon exposure to control conditions or toxin-producing fungus. (a) 'Between-selection-regime' contrast within a given exposure condition; illustration of constitutive- (control condition) and induced- (fungus exposure condition) responses with numbers of differentially expressed genes (DEGs) and total number of analysed genes per contrast. (b) 'Between-exposure' contrast within a given selection regime; illustration of fungus exposure contrast with numbers of DEGs and total number of analysed genes per contrast. Venn diagrams of DEGs from 'between-exposure' contrast showing overlap of up- and down-regulated genes between the four selection regimes. The cut-off of DEG is FDR <0.05 (EDGER, GLMLRT).

Unexpectedly, in the present study we also found that populations selected on the toxin-production impaired fungus showed a tendency to a reduced susceptibility to Sterigmatocystin. In fungi, genes that are involved in the synthesis of secondary metabolites are clustered within the genome (Bok & Keller, 2004; Keller, 2019; Yu & Keller, 2005), where LaeA is considered to be a 'master switch' of positive expression regulation. In the toxin-production impaired strain Δ*laeA* the *laeA*-gene has been disrupted, leading to arrested synthesis of several secondary metabolites (Bok & Keller, 2004). Under the assumption that the populations selected on the impaired fungus had not been in contact with mycotoxins, this suggests that the repeated confrontation with the *A. nidulans* toxin-impaired strain during development induced a form of inherited non-specific stress response that simultaneously reduced susceptibility to Sterigmatocystin, leading to the cross-resistance. Alternatively, some toxic and non-toxic fungal metabolites have the

capacity to act in synergy leading to elevated or newly generated toxicity (Klarić et al., 2013). Thus, it is conceivable that toxic or non-toxic metabolites with synergistic effects that are not regulated by LaeA induced an evolutionary change in the respective populations. However, this cross-resistance did not extend towards the toxin-producing fungus, which holds the same toxic and non-toxic metabolites. Based on these findings it is thus premature to discriminate whether the underlying mechanism that led to increased larval survival in sel.WT-populations is of specific directed nature or of a general stress response.

With our selection procedure of selection- and relaxation-generations we generated a fluctuating environment. Potentially different traits were favourable in these two conditions that are of antagonistic nature. Then, the selection pressure imposed by the toxin-impaired fungus was not sufficient to shift the population towards increased viability, while the detrimental effects of the toxin producing wild type and the mycotoxin did.

The delay in development on fungal- and toxin-free breeding substrate of sel.WT- and sel.LA-populations (Figure S1a) might be caused by an investment in defence traits that require the allocation of resources that consequently are not available for other processes. Alternatively, rather than costs of defences inducing a shift in resource allocation, standing genetic variation in tolerance of *A. nidulans* within the base population for the selection regimes may be associated with slight variations in developmental time. Overall, however, the development time was much less effected than the viability of the larvae.

Reduced susceptibility of WT-selected populations was not related to a more efficient suppression of fungal growth by the larvae. Hence, we consider the evolutionary response as increased tolerance rather than resistance.

4.2 | Narrow evolution of divergent gene expression patterns

In the present study, multidimensional scaling analyses of gene expression patterns of *D. melanogaster* larvae showed pronounced separation into groups of larvae unexposed and exposed to *A. nidulans*. A further less pronounced separation was identified between sel.WT and sel.LA contrasting sel.CO and sel.ST (Figure 4). We previously assessed the gene expression patterns of the unselected base population after exposure to these same four different treatments used here as selection regimes (before the start of the experimental evolution), where we found similar patterns in the MDS-separation of the treatments, with larvae confronted with *A. nidulans* WT and Δ*laeA* strains separating from Sterigmatocystin and control conditions (Figure S6 in Trienens et al., 2017).

The similar separation in both analyses suggests that the fungi, irrespective of whether they are toxin-producing or toxin-impaired, induce changes that affects the flies, both through an immediate and an evolutionary response. Both fungal strains deplete the substrate for their own growth and may affect the metabolic or nutritional composition and the acidity. Thus, the impact of the fungus on a food resource goes well beyond the potential virulence factors (toxins) it may release. The separation of populations that evolved with and without fungus in this current study indicates that the flies adapted to the various facets of this fungal manipulation of the environment.

Within each selection regime we saw substantial variation among the three populations included in the RNAseq assay, as inferred from the divergent responses in the MDS-plot (Figure 4). Populations may be showing different molecular patterns underling similar phenotypic outcomes. Furthermore, while the selection regimes did not substantially vary in constitutive expression patterns in the absence of the toxin-producing fungus, exposure to the fungus resulted in a change in induced gene expression patterns that was fairly specific for each selection regime. We saw mostly that the induced response in the sel.WT-populations had changed (Figure 5a). This involved a narrow set of genes, but with several shared, yet eclectic combinations of functional annotations, including *epidermal and insulin-growth factor binding*, trans-membrane receptors (*Laminin*, *Cadherin*, *Plexin*) that may function in cell adhesion, *Voltage-dependent potassium channel*, *Immunoglobulin-like fold*, and *Glycoprotein*. Further, in sel.WT-populations the gene CG7900 was increased in its expression, both constitutively and through induction. CG7900 codes for a predicted Acylglycerol lipase (Flybase.org), yet how it may support larval viability in the WT selected populations is unclear.

The selection regimes were more concordant in the responses of down-regulated genes, but even there, less than half of the down-regulated genes upon confrontation were shared among all four regimes. This indicates that both within and among selection regimes the evolutionary responses had varied considerably, and that the different environmental changes that these fungi induce may select for different adaptive strategies.

4.2.1 | Comparison of two transcriptome studies

Comparison of our data of the evolved populations to an earlier transcriptome study on the unselected base population, in which larvae were confronted with *A. nidulans* wild-type using similar experimental conditions and analyses (24 h exposure in Trienens et al., 2017) showed large congruency in overall gene expression pattern, with similar expression changes for about 58% of the here detected DEGs. Genes up-regulated in both studies clustered in annotation terms of *Glycosidase* and *lysosome* (enrichment score 3.43). Currently, it remains speculative how and why these processes are consistently associated with confrontation with both toxic and toxin-impaired fungi. Perhaps it reflects the metabolism of ingested fungal hyphae. Genes that were down-regulated in both studies included the over-represented annotation terms of *Protein of unknown function DUF1676* ('Osiris'), *Zona pellucida domain*, *proteolysis*, *Transmembrane helix*, and *Chitin binding domain* (enrichment scores: 6.99, 3.54, 3.4, 2.91, and 2.63, respectively). In contrast to our study of 2017 (Trienens et al.), genes whose products are involved in metabolic- and detoxification-processes

(CYPs, GSTs, UTGs) were not upregulated to an extent that resulted in significant enrichment scores. However, individual genes showed significant up- or down-regulation. For example, cyp28d2 was up-regulated in exposure to *A. nidulans* in both studies. A complete list and comparison of expression of these genes between both studies can be found in Figure S3.

Thus, the transcriptional response to confrontation with the toxin-producing fungus in the populations that evolved with the toxin-producing fungus for several generations was largely similar to the response of the unselected base population and the control selection line, although it also included the up- and the down-regulation of several genes that were not responsive to such confrontation in the control selected or the unselected base population.

4.2.2 | Change in expression of *Osiris* genes

Both the present and the Trienens et al. (2017) study show a significant down-regulation of *Osiris* genes in *D. melanogaster* larvae at 24 h of exposure to *A. nidulans* wild type. The earlier study also included a sampling time point at 12 h of exposure; where, first instar larvae showed a peak expression of 4 Osiris members (*Osi6*, *Osi7*, *Osi15* and *Osi20*). This expression peak had the highest fold changes of expression of any of the differentially expressed genes (Trienens et al., 2017). It is known that *Osiris* genes in *D. melanogaster* have a peak-expression profile at mid-embryonal-, second instar-, and pupal-stage (Flyatlas: Chintapalli et al., 2007; Smith et al., 2018). Yet, the exact function of this gene family remains to be illuminated. Based on co-expression analysis, expression profile at distinct growth stages, and functional analysis it is hypothesised that *Osiris* genes may have a function in cuticle formation during development (Smith et al., 2018), including the formation of mouth hooks (Ando et al., 2019). However, the observed increase in expression of *Osi6*, *Osi7*, *Osi15* and *Osi20* at 12 h of exposure is outside of the typically expected expression peak in development, and the changed expression in response to exposure with toxic fungus could suggest that these genes may also have a function in coping with the effect that these fungi have on the breeding substrate.

Pathogenic fungi can destruct insect cuticle's integrity with proteinases to penetrate into the insect's cavity or cross the lumen barrier after oral intake (Ortiz-Urquiza & Keyhani, 2013). Further, toxic microbial substances can damage chitinous structures. Evidence is accumulating that *Osiris* genes might be involved in reinforcement or repair of protective barriers, such as the insect's integument and peritrophic membrane, which both contain chitin. As summarized in more detail in Trienens et al. (2017), *Osiris* genes have been found to show genomic modifications, changes in expression, and direct functions in several Drosophilidae in the context of evolutionary adaptation to a toxic fruit utilized as breeding habitat (*Morinda citrifolia*), in response to food derived glucosinolates, and octanoic acid (Andrade López et al., 2017; Huang & Erezyilmaz, 2015; Whiteman et al., 2012; Yassin et al., 2016). Also, oral infections of *Tribolium castaneum* larvae with the toxin-producing bacterium *Bacillus thuringiensis*, which induces pore formation in the gut of beetle larvae, modified gene expression of *Osiris* genes (Greenwood et al., 2017). Thus, *Osiris* genes are possibly involved in the repair or reinforcement of cuticular structures in response to fungal destruction of such features.

5 | CONCLUSION

Exploring evolutionary adaptation and life-history adaptation in *D. melanogaster*, we found that confrontations with a living and responding fungus (*A. nidulans*) comprises more versatile impacts than an isolated mycotoxic agent (Sterigmatocystin) and requires a more complex adjustment to environmental conditions by the insect. Naturally, whilst a single substance may affect only distinct (physiological) functions the fungus introduces more complex changes to the environment. These may include synergistic – toxic or otherwise detrimental – effects of metabolites and proteins, changes on abiotic factors (e.g., pH-level), and a response to the activities of the insect.

We showed that rapid evolutionary adaptation and transcriptomic changes in *D. melanogaster* can be induced by confrontations with toxin-producing filamentous fungi. Moreover, experimental settings often simplify the components and conditions, yet adaptation to natural conditions is likely to affect multiple traits simultaneously (Lazzaro & Little, 2009). Indeed, while we found a highly specific response to Sterigmatocystin selection, we also found that a general (stress) response in *A. nidulans* mutant-strain-selection populations led to cross-resistance to a toxin that was not experienced before. The transcriptomic changes in these evolved populations were rather dissimilar, both within and between selection regimes, suggesting the evolution of alternative strategies or molecular mechanisms. This signifies the versatile effects that environmental microbes may have on organisms, and their potential importance as evolutionary drivers.

AUTHOR CONTRIBUTIONS

MT designed the study, carried out the lab work, statistical analyses, sequencing data processing, and drafted the manuscript. JK gave suggestions on study design and analyses, and commented on the manuscript. BW participated in the design of the study, lab work, helped analysing the data and drafting the manuscript. All authors read and approved of the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

All authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The RNA-sequencing reads (Fastq files) for this study can be found in the BioStudies repository via https://www.ebi.ac.uk/biostudies/ under accession number: E-MTAB-9011. Raw data of phenotypic experiments and a list of identified DEGs can be found in the supplementary material data Table S4 in order of appearance.

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REFERENCES

Anders, S., Pyl, P. T., & Huber, W. (2014). HTSeq—A python framework to work with high-throughput sequencing data. *Bioinformatics*, *31*, 166–169. https://doi.org/10.1093/bioinformatics/btu638

Ando, T., Sekine, S., Inagaki, S., Misaki, K., Badel, L., Moriya, H., Sami, M. M., Itakura, Y., Chihara, T., Kazama, H., Yonemura, S., & Hayashi, S. (2019). Nanopore formation in the cuticle of an insect olfactory sensillum. *Current Biology*, *29*, 1512–1520.e6. https://doi.org/10.1016/J.CUB.2019.03.043

Andrade López, J. M., Lanno, S. M., Auerbach, J. M., Moskowitz, E. C., Sligar, L. A., Wittkopp, P. J., & Coolon, J. D. (2017). Genetic basis of octanoic acid resistance in *Drosophila sechellia*: Functional analysis of a fine-mapped region. *Molecular Ecology*, *26*, 1148–1160. https://doi.org/10.1111/mec.14001

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using LME4. *Journal of Statistical Software*, *67*, 1–48. https://doi.org/10.18637/jss.v067.i01

Becher, P. G., Bengtsson, M. S., Hansson, B., & Witzgall, P. (2010). Flying the fly: Long-range flight behavior of *Drosophila melanogaster* to attractive odors. *Journal of Chemical Ecology*, *36*, 599–607. https://doi.org/10.1007/s10886-010-9794-2

Bok, J. W., & Keller, N. P. (2004). LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryotic Cell*, *3*, 527–535. https://doi.org/10.1128/EC.3.2.527-535.2004

Caballero Ortiz, S., Trienens, M., Pfohl, K., Karlovsky, P., Holighaus, G., & Rohlfs, M. (2018). Phenotypic responses to microbial volatiles render a mold fungus more susceptible to insect damage. *Ecology and Evolution*, *8*, 4328–4339. https://doi.org/10.1002/ece3.3978

Calvo, A. M. (2008). The VeA regulatory system and its role in morphological and chemical development in fungi. *Fungal Genetics and Biology*, *45*, 1053–1061. https://doi.org/10.1016/j.fgb.2008.03.014

Carvalho, M., Schwudke, D., Sampaio, J. L., Palm, W., Riezman, I., Dey, G., Gupta, G. D., Mayor, S., Riezman, H., Shevchenko, A., Kurzchalia, T. V., & Eaton, S. (2010). Survival strategies of a sterol auxotroph. *Development*, *137*, 3675–3685. https://doi.org/10.1242/dev.044560

Chahine, S., & O'Donnell, M. J. (2011). Interactions between detoxification mechanisms and excretion in Malpighian tubules of *Drosophila melanogaster*. *The Journal of Experimental Biology*, *214*, 462–468. https://doi.org/10.1242/jeb.048884

Chintapalli, V. R., Wang, J., & Dow, J. A. T. (2007). Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nature Genetics*, *39*, 715–720. https://doi.org/10.1038/ng2049

Dubinkina, V., Fridman, Y., Pandey, P. P., & Maslov, S. (2019). Multistability and regime shifts in microbial communities explained by competition for essential nutrients. *eLife*, *8*, e49720. https://doi.org/10.7554/eLife.49720

Fox, E. M., & Howlett, B. J. (2008). Secondary metabolism: Regulation and role in fungal biology. *Current Opinion in Microbiology*, *11*, 481–487. https://doi.org/10.1016/j.mib.2008.10.007

Greenwood, J. M., Milutinović, B., Peuß, R., Behrens, S., Esser, D., Rosenstiel, P., Schulenburg, H., & Kurtz, J. (2017). Oral immune priming with *Bacillus thuringiensis* induces a shift in the gene expression of *Tribolium castaneum* larvae. *BMC Genomics*, *18*, 329. https://doi.org/10.1186/s12864-017-3705-7

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2008). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*, *4*, 44–57. https://doi.org/10.1038/nprot.2008.211

Huang, Y., & Erezyilmaz, D. (2015). The genetics of resistance to Morinda fruit toxin during the postembryonic stages in *Drosophila sechellia*. *G3 Genes|Genomes|Genetics*, *5*, 1973–1981. https://doi.org/10.1534/g3.114.015073

Keller, N. P. (2019). Fungal secondary metabolism: Regulation, function and drug discovery. *Nature Reviews. Microbiology*, *17*, 167–180. https://doi.org/10.1038/s41579-018-0121-1

Klarić, M. Š., Rašić, D., & Peraica, M. (2013). Deleterious effects of mycotoxin combinations involving ochratoxin A. *Toxins (Basel)*, *5*, 1965–1987. https://doi.org/10.3390/toxins5111965

Lazzaro, B. P., & Little, T. J. (2009). Immunity in a variable world. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*, 15–26. https://doi.org/10.1098/rstb.2008.0141

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., & Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette spatzle/toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell*, *86*, 973–983. https://doi.org/10.1016/S0092-8674(00)80172-5

Luque, T., & O'Reilly, D. R. (2002). Functional and phylogenetic analyses of a putative *Drosophila melanogaster* UDP-glycosyltransferase gene. *Insect Biochemistry and Molecular Biology*, *32*, 1597–1604. https://doi.org/10.1016/S0965-1748(02)00080-2

McKenzie, J. A., & Parsons, P. A. (1972). Alcohol tolerance: An ecological parameter in the relative success of *Drosophila melanogaster* and *Drosophila simulans*. *Oecologia*, *10*, 373–388. https://doi.org/10.1007/BF00345738

Nielsen, M. T., Klejnstrup, M. L., Rohlfs, M., Anyaogu, D. C., Nielsen, J. B., Gotfredsen, C. H., Andersen, M. R., Hansen, B. G., Mortensen, U. H., & Larsen, T. O. (2013). *Aspergillus nidulans* synthesize insect juvenile hormones upon expression of a heterologous regulatory protein and in response to grazing by *Drosophila melanogaster* larvae. *PLoS One*, *8*, e73369. https://doi.org/10.1371/journal.pone.0073369

Ortiz-Urquiza, A., & Keyhani, N. O. (2013). Action on the surface: Entomopathogenic fungi versus the insect cuticle. *Insects*, *4*, 357–374. https://doi.org/10.3390/insects4030357

R Core Team. (2019). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.r-project.org/

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2009). EDGER: A bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*, 139–140. https://doi.org/10.1093/bioinformatics/btp616

Saisawang, C., Wongsantichon, J., & Ketterman, A. J. (2012). A preliminary characterization of the cytosolic glutathione transferase proteome from *Drosophila melanogaster*. *The Biochemical Journal*, *442*, 181–190. https://doi.org/10.1042/BJ20111747

Semmelhack, J. L., & Wang, J. W. (2009). Select *Drosophila glomeruli* mediate innate olfactory attraction and aversion. *Nature*, *459*, 218–223. https://doi.org/10.1038/nature07983

Sieber, M., Traulsen, A., Schulenburg, H., & Douglas, A. E. (2021). On the evolutionary origins of host–microbe associations. *Proceedings of the National Academy of Sciences*, *118*, e2016487118. https://doi.org/10.1073/PNAS.2016487118

Smith, C. R., Morandin, C., Noureddine, M., & Pant, S. (2018). Conserved roles of *Osiris* genes in insect development, polymorphism and protection. *Journal of Evolutionary Biology*, *31*, 516–529. https://doi.org/10.1111/jeb.13238

Stötefeld, L., Holighaus, G., Schütz, S., & Rohlfs, M. (2015). Volatile-mediated location of mutualist host and toxic non-host microfungi by *Drosophila* larvae. *Chemoecology*, *25*, 271–283. https://doi.org/10.1007/s00049-015-0197-2

Téfit, M. A., Gillet, B., Joncour, P., Hughes, S., & Leulier, F. (2018). Stable association of a *Drosophila*-derived microbiota with its animal partner and the nutritional environment throughout a fly population's life cycle. *Journal of Insect Physiology*, *106*, 2–12. https://doi.org/10.1016/j.jinsphys.2017.09.003

Therneau, T. (2012). Coxme: Mixed effects cox models. R package version 2.2-3. *Oikos*, *123*, 589–598. https://doi.org/10.1111/oik.01149

Tijet, N., Helvig, C., & Feyereisen, R. (2001). The cytochrome P450 gene superfamily in *Drosophila melanogaster*: Annotation, intron-exon organization and phylogeny. *Gene*, *262*, 189–198. https://doi.org/10.1016/S0378-1119(00)00533-3

Tinsley, M. C., Blanford, S., & Jiggins, F. M. (2006). Genetic variation in *Drosophila melanogaster* pathogen susceptibility. *Parasitology*, *132*, 767–773. https://doi.org/10.1017/S0031182006009929

Trienens, M., Keller, N. P., & Rohlfs, M. (2010). Fruit, flies and filamentous fungi – Experimental analysis of animal-microbe competition using *Drosophila melanogaster* and *Aspergillus* as a model system. *Oikos*, *119*, 1765–1775. https://doi.org/10.1111/j.1600-0706.2010.18088.x

Trienens, M., Kraaijeveld, K., & Wertheim, B. (2017). Defensive repertoire of *Drosophila* larvae in response to toxic fungi. *Molecular Ecology*, *26*, 5043–5057. https://doi.org/10.1111/mec.14254

Trienens, M., & Rohlfs, M. (2011). Experimental evolution of defense against a competitive mold confers reduced sensitivity to fungal toxins but no increased resistance in *Drosophila* larvae. *BMC Evolutionary Biology*, *11*, 206. https://doi.org/10.1186/1471-2148-11-206

Trienens, M., & Rohlfs, M. (2012). Insect–fungus interference competition – The potential role of global secondary metabolite regulation, pathway-specific mycotoxin expression and formation of oxylipins. *Fungal Ecology*, *5*, 191–199. https://doi.org/10.1016/J.FUNECO.2011.07.009

Trienens, M., & Rohlfs, M. (2020). A potential collective defense of *Drosophila* larvae against the invasion of a harmful fungus. *Frontiers in Ecology and Evolution*, *8*, 79. https://doi.org/10.3389/fevo.2020.00079

Wertheim, B., Allemand, R., Vet, L. E. M., & Dicke, M. (2006). Effects of aggregation pheromone on individual behaviour and food web interactions: A field study on *Drosophila*. *Ecological Entomology*, *31*, 216–226. https://doi.org/10.1111/j.1365-2311.2006.00757.x

Wertheim, B., Marchais, J., Vet, L. E. M., & Dicke, M. (2002). Allee effect in larval resource exploitation in *Drosophila*: An interaction among density of adults, larvae, and micro-organisms. *Ecological Entomology*, *27*, 608–617. https://doi.org/10.1046/j.1365-2311.2002.00449.x

Whiteman, N. K., Gloss, A. D., Sackton, T. B., Groen, S. C., Humphrey, P. T., Lapoint, R. T., Sønderby, I. E., Halkier, B. A., Kocks, C., Ausubel, F. M., & Pierce, N. E. (2012). Genes involved in the evolution of herbivory by a leaf-mining, drosophilid fly. *Genome Biology and Evolution*, *4*, 900–916. https://doi.org/10.1093/gbe/evs063

Wölfle, S., Trienens, M., & Rohlfs, M. (2009). Experimental evolution of resistance against a competing fungus in *Drosophila melanogaster*. *Oecologia*, *161*, 781–790. https://doi.org/10.1007/s00442-009-1414-x

Wu, T. D., Reeder, J., Lawrence, M., Becker, G., & Brauer, M. J. (2016). GMAP and GSNAP for genomic sequence alignment: Enhancements to speed, accuracy, and functionality. In M. Ewy & S. Davis (Eds.), *Statistical genomics: Methods and protocols* (pp. 283–334). Springer. https://doi.org/10.1007/978-1-4939-3578-9_15

Yassin, A., Debat, V., Bastide, H., Gidaszewski, N., David, J. R., & Pool, J. E. (2016). Recurrent specialization on a toxic fruit in an Island *Drosophila* population. *Proceedings of the National Academy of Sciences*, *113*, 4771–4776. https://doi.org/10.1073/pnas.1522559113

Yu, J.-H., & Keller, N. (2005). Regulation of secondary metabolism in filamentous fungi. *Annual Review of Phytopathology*, *43*, 437–458. https://doi.org/10.1146/annurev.phyto.43.040204.140214

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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