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Published in:
Molecular Ecology

DOI:
[10.1111/mec.14254](https://doi.org/10.1111/mec.14254)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Trienens, M., Kraaijeveld, K., & Wertheim, B. (2017). Defensive repertoire of *Drosophila* larvae in response to toxic fungi. *Molecular Ecology*, 26(19), 5043-5057. <https://doi.org/10.1111/mec.14254>

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ORIGINAL ARTICLE

Defensive repertoire of *Drosophila* larvae in response to toxic fungi

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Funding information

Volkswagen Foundation, Grant/Award Number: AZ.:85982

Abstract

Chemical warfare including insecticidal secondary metabolites is a well-known strategy for environmental microbes to monopolize a food source. Insects in turn have evolved behavioural and physiological defences to eradicate or neutralize the harmful microorganisms. We studied the defensive repertoire of insects in this interference competition by combining behavioural and developmental assays with whole-transcriptome time-series analysis. Confrontation with the toxic filamentous fungus *Aspergillus nidulans* severely reduced the survival of *Drosophila melanogaster* larvae. Nonetheless, the larvae did not behaviourally avoid the fungus, but aggregated at it. Confrontation with fungi strongly affected larval gene expression, including many genes involved in detoxification (e.g., CYP, GST and UGT genes) and the formation of the insect cuticle (e.g., Tweedle genes). The most strongly upregulated genes were several members of the insect-specific gene family Osiris, and CHK-kinase-like domains were over-represented. Immune responses were not activated, reflecting the competitive rather than pathogenic nature of the antagonistic interaction. While internal microbes are widely acknowledged as important, our study emphasizes the underappreciated role of environmental microbes as fierce competitors.

KEYWORDS

chemical warfare, detoxification, *Drosophila*, filamentous fungi, interference competition, mycotoxins, transcriptome analysis

1 | INTRODUCTION

Severe limitation of resources leads to fierce competition. In the case of interference competition, the competitor may engage in offensive interactions, actively damaging the antagonist or reducing access to the resource. In lieu of physical confrontation deployed by mobile organisms, sessile organisms may make use of chemical substances to reduce physiological functions or induce mortality of the competitor. This mechanism of chemical warfare has been well established for plant–plant and plant–insect interaction (Mithöfer & Boland, 2012), yet applies to other organism groups as well (Rohlf & Churchill, 2011). In previous studies it has been shown that the saprotrophic insect *Drosophila melanogaster* can get engaged in interference competition with filamentous fungi that co-occur on

same substrates (Trienens, Keller, & Rohlf, 2010; Trienens & Rohlf, 2012). Here, we report on the defensive repertoire deployed by insects in this largely neglected interaction of interference competition between insects and their environmental microbes and its implications in evolutionary adaptations towards xenobiotics.

The fruit fly *D. melanogaster* frequents fermenting substrate as feeding and breeding sites. Their larvae do not exclusively feed on the fruit but also on the co-occurring microbes. Indeed, fruit tissue alone does not support larval development. The larvae depend on uptake of microbial biomass as they lack the ability to synthesize the cell-membrane component and steroid hormone precursor sterol (Carvalho et al., 2010). When searching for breeding sites, *D. melanogaster* is strongly attracted by yeasts, which support larval

development and can promote defence responses, for example, against parasitoids (Anagnostou, LeGrand, & Rohlf, 2010; Milan, Kacsoh, & Schlenke, 2012). Moreover, adults do not distribute their eggs across resources randomly or uniformly, but they tend to aggregate and share the same egg-laying resources, leading also to an aggregated distribution of larvae over resources (Wertheim, Sevenster, Eijs, & Van Alphen, 2000). Adults form these aggregations by responding to aggregation pheromones that males and mated females release, and that are attractive to both sexes. In these aggregations, adults mate, feed and oviposit. As a consequence of the multiple females laying (multiple) eggs on the same resources, the larvae also have an aggregated distribution (Wertheim, Allemand, Vet, & Dicke, 2006). Aggregative behaviour was shown to be advantageous for larval development, because adults inoculate the substrates with (beneficial) yeasts, and larvae in groups are better able to cope with (noxious) fungi that co-occur on the substrates (Rohlf & Hoffmeister, 2005; Wertheim, Marchais, Vet, & Dicke, 2002; Wertheim et al., 2006).

While the larvae feed on fermenting substrate, they encounter other saprophagous, including filamentous fungi, with whom they compete for food. Here, filamentous fungi and *Drosophila* larvae can interfere with each other's development and growth, where increasing abundance of one party leads to reduced fitness and abundance of the other (Rohlf & Hoffmeister, 2005; Trienens et al., 2010; Wertheim et al., 2002). For instance, *D. melanogaster* larvae can destroy and ingest young hyphae. Moreover, aggregations of larvae feeding on the hyphae benefit from "strength in numbers," as larger aggregations can effectively prevent filamentous fungi from establishing and spreading on fruit tissue. Within this process young filamentous colonies in particular can become severely damaged and destroyed (Figure 1a; Caballero Ortiz, Trienens, & Rohlf, 2013). Established fungal colonies also suffer from reduced growth and reproduction, but they can defend themselves with constitutive and induced production of toxic substances (Caballero Ortiz et al., 2013; Nielsen et al., 2013). These constitutive as well as induced defensive metabolite productions may constitute a major challenge for saprotrophic insect on rotting organic material, which are analogous to the inducible plant defences against herbivores (Mithöfer & Boland, 2012; Whiteman et al., 2012). The local outcome of a fly-filamentous-fungus encounter may range between entire loss of either of the antagonists, depending on fungal species—and thus composition of the toxic cocktail and further potential virulence factors; priority effects; and larval density (Trienens et al., 2010). We used *Aspergillus nidulans* as a fungal competitor that induces low to high mortality rates in *D. melanogaster* larvae, depending on substrate, developmental head start and larval density. It is important to note that *A. nidulans* may utilize larval carcasses as protein source, but that it does not grow in or on living insect hosts, and it does not invasively infect *D. melanogaster* larvae through the cuticle or gut like, for example, the pathogenic fungus *Beauveria bassiana*. Rather, it releases toxic substances into its surroundings, which interfere with insect growth and survival (Trienens & Rohlf, 2012; Trienens et al., 2010). Hence, based on the previous research, the antagonistic

interaction between *D. melanogaster* and *A. nidulans* does not comprise elements of an *infection mode-of-action*, and thus, can be defined as interference competition by destruction and chemical warfare.

In this study, we carefully dissected how *D. melanogaster* larvae cope with *A. nidulans* fungi and their toxic secondary metabolites in this interference competition. The responses of the fly larvae might be an accumulation of distinct responses to single facets of the fungi's manipulations, for example, alteration of pH level and nutrient availability, introduction of fungal tissue components and waste products, as well as the toxic secondary metabolites. We mimicked the natural scenario of hatching insect larvae to find their environment co-occupied with filamentous fungi. To assess the role of the different aspects of fungal interference competition, we compared the responses of larvae in control conditions (CO) to three treatments applied to the breeding-substrates: First, a toxin-producing wild-type (WT) fungal treatment; second, as a fungal-control we used a genetically manipulated strain $\Delta laeA$ (LA), which lacks a member of the regulatory complex for secondary metabolites, *velvet*, and is strongly impaired in the production of secondary metabolites including pigments and mycotoxins (Bayram et al., 2008; Bok & Keller, 2004); third, a highly toxic secondary metabolite synthesized by *A. nidulans*, sterigmatocystin (ST), was topically applied to the substrate. We also examined the behaviour and development of *D. melanogaster* larvae on fruit-substrates that are entirely microbe-free, which does not support larval development (MF). We examined whether larvae display escape or avoidance behaviours when confronted with a toxin-producing fungus, monitored larval development to estimate the consequences in nonchoice situations, and conducted a time series of whole-transcriptome sequencing to identify mechanisms and defensive processes allowing larvae to cope with the fungus and its toxic secondary metabolites. The possibility of our system to separate the different aspects between treatments and to apply them individually facilitates a comprehensive investigation of interference competition between insects and the environmental microbes.

2 | MATERIALS AND METHODS

2.1 | *Drosophila melanogaster* population and microbe strains

We used a *Drosophila melanogaster* population that was established from wild caught flies from Kiel in Germany and kept at high numbers in the laboratory for many generations. We used *Aspergillus nidulans* RDIT2.3 as a wild-type (WT) and the $\Delta laeA$ mutant strain RJW 46.4 (LA) (provided by Nancy P. Keller, Wisconsin, USA). Sterigmatocystin (ST) was purchased from Sigma-Aldrich (Germany). *Saccharomyces cerevisiae* DSM 70449 was obtained from DSMZ (German Resource Centre for Biological Materials, Braunschweig) and used for larval choice assays. For further details and media ingredients, see Appendix S1.

FIGURE 1 Larval escape and choice behaviour. (a) Larvae feeding at young colony of *A. nidulans* WT; dashed line indicates outermost expansion of explorative hyphae that are almost entirely disrupted, dotted line indicates rim of white hyphae forming the colony-body where later conidia/spores would be produced, arrows indicate some larvae on the fungal colony (contrast enhanced). (b) Larval escape behaviour: average proportion of larvae on a confrontation unit (breeding-substrate) sham-treated as control (CO) or inoculated with wild type (WT), $\Delta laeA$ (LA) or sterigmatocystin (ST) vs. alternative escape unit (breeding-substrate, equal to CO) at 24, 48, 72 and 96 hr after transfer to confrontation unit, n = number larvae observed. No significant differences between WT, LA and ST compared to CO (glm, quasi-binomial, units over time independent; all p -values $>.1$; for details, see Table S1). (c) Larval choice behaviour: average proportion of larvae on one of two alternatively inoculated fruit patches 24 hr after transfer to rim of choice-arena; yeast—YE; microbe-free—MF; WTvsMF: $p = .005$, LAvsMF: $p = .003$, YEvsMF: $p = .003$, YEvsLA: $p = .005$, YEvsWT: $p = .008$ and WTvsLA: $p = .105$; Wilcoxon signed-rank test with continuity correction using choice index data (p -values are approximate due to tie of values)

2.2 | Basic confrontation set-up: Escape, development progression and transcriptome analysis

To measure the behaviour, development progression and transcriptomic responses of *D. melanogaster* to fungi or mycotoxins, we incubated first-instar larvae in 35-mm petri dishes with sterile fly breeding-substrate (62.5 g of inactivated yeast (Leiber GmbH, Bramsche, Germany), sugar and cornmeal, 12.5 g of agar Kobe I and 1 L purified water) that had been inoculated with fungus, mycotoxin or control conditions. For all experiments, steam-sterilized solutions, media and tools were used. Although neither saline solution nor acetone after evaporation had an effect on larval survival or development, all confrontation units were inoculated with both the respective solvents prior to treatment application. For fungal confrontations, the 35-mm dishes were subsequently dot-inoculated with *A. nidulans* WT or LA conidia in saline solution (1,000 conidia/ μ l). Sterigmatocystin (ST) was solved in acetone and applied in 2 μ g/50 μ l concentration per dish and acetone was

allowed to evaporate for 15 min leaving a layer of ST on the substrate surface. Units for control conditions were sham-treated with sterile saline solution and acetone. Confrontation units were incubated at 25°C and darkness. Fifteen sterile first-instar larvae (hatched from sodium hypochlorite-treated eggs) were transferred to each dish 3 days postinoculation.

2.3 | Larval escape assay

To test whether *D. melanogaster* larvae exhibit escape behaviour when confronted with detrimental conditions generated by filamentous fungi, we transferred larvae to breeding-substrate patches, which had been inoculated with the toxin-producing *A. nidulans* wild-type strain (WT), the toxin-impaired strain (LA) or the mycotoxin sterigmatocystin (ST) or sham-treated as fungal and toxin-free controls (CO), providing them with an alternative “escape” patch, which is equal to CO (set-up sketch; see Figure 1b). Basic confrontation patches were prepared as described in *basic confrontation*

set-up. After 48 hr of incubation arenas were built by placing one treatment patch and one “escape” patch in a 90-mm dish, the space between patches was filled with a 5-mm layer of water-agar, and arenas were incubated for a further 24 hr. After 72 hr of total incubation time, 15 first-instar larvae were transferred to each confrontation patch and incubation continued with alternating orientations of arenas. Then, every 24 hr over a period of 96 hr, independent subset of units were sampled (i.e., each unit was only sampled once) to determine larval location (confrontation vs. escape patches). For each treatment and time point, 8–12 independently conducted arenas were sampled; as much as possible, all 15 larvae per dish were recaptured.

2.4 | Larval choice assay

To assess the preferences of *D. melanogaster* larvae towards fruit-substrate patches with different microbes (WT, LA or yeast [YE]) or microbe-free, “sterile,” patches (MF), we tested every possible combination in two-choice assays (see Figure 1c, *set-up* sketch). Choice arenas consisted of 90-mm dishes filled with 25 ml water-agar. Two 28-mm-diameter patches of water-agar were removed and refilled with 3 ml banana-agar (50/50 mashed banana/water + agar). Fibreglas filter discs of 12 mm diameter were dipped into malt-extract-agar, inoculated with 2 μ l conidia suspension (1,000 conidia) of WT or LA, 2 μ l cell suspension of *S. cerevisiae* (YE, 4,000 cells), or 2 μ l sterile Ringer solution (MF), and incubated at 25°C and darkness for 45 hr. Fibreglas discs with established fungal colonies and MFs were then transferred to fruit-agar patches in arenas, in the full factorial 2-choice combinations (WT-YE, WT-LA, WT-MF, LA-MF, YE-MF, LA-YE), and incubated for a further 15 hr. Twenty first-instar larvae were transferred onto water-agar at the rim of each arena. Arenas were 10 times replicated and incubated (25°C, darkness) with alternating orientations. Location of larvae was observed after 24 hr.

2.5 | Larval development assays

We tested the ability of single *D. melanogaster* larvae to develop on microbe-inhabited (WT, LA, YE) and microbe-free (MF) fruit-substrate. One millilitre of banana-agar (50/50 mashed banana/water + agar) in 2-ml tubes was inoculated with 1 μ l *A. nidulans* conidia suspension (1,000 conidia) of WT or LA at 3–0 days, or with 1 μ l *S. cerevisiae* cell suspension (YE, 1,000 cells) 1 day before a single first-instar larva was transferred. Microbe-free units (MF) were sham-treated with 1 μ l sterile saline solution. Incubation took place at 25°C and darkness. WT and LA treatments with 0 days development head start and YE treatment were replicated 12 times; MF and remaining WT and LA treatments were replicated 20 times.

Further, we assessed the ability of larvae to develop to adults in groups of 15 larvae on WT-, LA- and ST-treated breeding-substrate, using the *basic confrontation set-up* above with 10–12 replicates per treatment.

2.6 | Development-progression measurement

We analysed larval length and mouth-hook morphology to estimate delays in development. Confrontation was conducted as described in the *basic confrontation set-up* above. Larvae were collected at 3, 6, 12, 21, 24, 33, 36 and 48 hr after confrontation start from three independent replicated dishes for each treatment/time point combination. Order of sampling was randomized. Thirty-two to forty-one larvae were recaptured per treatment/time point combination.

2.7 | Confrontation and sampling for transcriptome analysis

Confrontation was conducted as described in *basic set-up* above. Sampling took place at 3, 6, 12 and 24 hr after confrontation start, where 50 larvae were pooled for one biological replicate collected from five independent dishes to account for “dish effects” (balanced design with 10 larvae per dish, and independents on replicate and time level). Four biological replicates were conducted resulting in 64 samples in total, and sampling order was randomized. Larvae were snap-frozen in liquid nitrogen. RNA was isolated using a combination of phenol–chloroform and column-based methods (TRIzol[®] until ethanol addition followed by RNeasy Mini; QIAGEN). PolyA⁺ isolation, library preparation and sequencing were performed by the Leiden Genome Technology Center (LGTC, Leiden University Medical Center, the Netherlands). We used Illumina HiSeq 2000 technology and paired-end 100 base sequencing. Samples were multiplexed in groups of 16, where a biological “replicate” of each condition/time combination was blocked on one lane. Each library was sequenced twice on two independent flow cells. Due to low read numbers in the first runs, three samples had to be sequenced for a third time.

2.8 | RNA-Seq—from raw data to counts

We filtered libraries for a 99% probability of correctly identified bases with FASTQC, where average quality score of libraries was well above the threshold. Paired-end reads were mapped to the *Drosophila melanogaster* reference GENOME version 5.54 acquired from Fly-Base (flybase.org) with GMAP-GSNAP (Wu & Nacu, 2010; Wu & Watanabe, 2005) allowing for 10% mismatches. Duplicates and non-unique reads were marked prior to counting of reads to features with HTSEQ (Anders, Pyl, & Huber, 2015). For further details, see Appendix S1.

2.9 | Statistical procedure for differential gene expression analysis

We analysed our multifactorial transcriptome data with the Bioconductor package EDGER (Chen, McCarthy, Robinson, & Smyth, 2015; Robinson, McCarthy, & Smyth, 2010) to estimate differential gene expression. As we observed a global shift of resulting DEGs by omitting single libraries, we prepared subsets of libraries, which we processed separately to minimize data distortion during the

normalizations. Subsets consisted exclusively of libraries that were included in a given contrast. Prior to analysis, we omitted features that did not reach at least five counts per million (CPM) in at least four libraries. Normalization factors were calculated to account for differences in library sequencing depths. Common and tagwise dispersion was estimated prior to fitting the model. Finally, the glmLRT function (likelihood ratio test) was applied to set contrasts. For each time point (3, 6, 12, 24 hr), we analysed contrasts as follows: WT-confrontation vs. LA-confrontation (WTvsLA) to contrast differences between toxin-producing vs. toxin-lacking fungus; ST-confrontation vs. CO-condition (STvsCO) to contrast responses to effects induced by the mycotoxin; finally, we compared the combined average of WT- and LA-confrontation to CO-condition ((WT+LA)/2 vs. CO = FUVsCO) to contrast responses of larvae to presence of filamentous fungi irrespectively of toxin production. We provide the detailed R script in Appendix S1.

2.10 | Functional annotation cluster analysis

We used DAVID Bioinformatics Resources 6.7 (Huang, Sherman, & Lempicki, 2008, 2009) including functional categories, gene ontology of biological processes, cell component and molecular functions, pathway involvement and protein domains for functional annotation cluster analysis. We included genes that were marked as DEGs with a FDR < 0.05 (BH method, EDGER).

2.11 | Inferring differential exon usage

We employed the *Bioconductor* package DEXSEQ (Anders, Reyes, & Huber, 2012) as described in the vignette (Reyes, Anders, & Huber, 2014). Briefly, uniquely mapped reads (see above) were counted using the implemented python script DEXSEQ_COUNT and the *D. melanogaster* annotation file obtained from ENSEMBLE release 5.78. We produced separate data sets for each time point including all four confrontation conditions. Then, libraries were normalized for sequencing depths and biological variation estimated. By fitting a generalized linear model with “~sample + exon + treatment:exon,” we estimated whether any of the three treatment conditions differed from control condition.

2.12 | Statistical analysis

Data of larval escape, choice, development and development progression were analysed with R version 2.2.5. Test statistics were adjusted for overdispersion where necessary. All sampling units over time were independent (i.e., units were only sampled once, with no repeated measurements on the same units). Escape: glm with family = quasi-binomial and logit link function; choice: Wilcoxon signed-rank test with continuity correction on choice index data; development: glm, family = quasi-binomial and logit link function; development-progression larvae length: ANOVA with log-transformed data (accounting for increased variance with increasing larval length over time), and adjusted *p*-values of Tukey's multiple comparisons 95%

family-wise confidence level; development-progression mouth-hook morphology: glm with family = quasi-binomial and logit link function.

We used R version 2.2.2 and the Bioconductor packages EDGER and DEXSEQ for statistical analysis of differential gene expression and differential exon usage (for details, see above and Appendix S1).

3 | RESULTS

3.1 | Larval escape assay

Shortly after transfer of larvae to confrontation patches, larvae were found in close proximity to fungal colonies (WT, LA), and explorative hyphae were affected by the larvae's activities (compare Figures 1a and S2). In all conditions and time points (CO, WT, LA, ST; 24, 48, 72, 96 hr) only a small proportion of larvae left the confrontation unit (max. proportion of 0.07 at 96 hr, LA strain) (Figure 1b). At none of the time points did fungus- and toxin confrontations differ significantly from control condition (for all combinations *p* > .10, glm, quasi-binomial distribution, logit link function; see Table S1). Thus, the larvae do not exhibit avoidance behaviour towards the fungus or its toxin on breeding-substrate patches.

3.2 | Larval choice assay

When larvae were offered microbe-infested fruit-substrate patches or sterile fruit-substrate patches, they showed a clear preference for microbe-infested food (Wilcoxon signed-rank test with continuity correction: WT-MF: *V* = 55, *p* = .005, LA-MF: *V* = 55, *p* = .004, YE-MF: *V* = 55, *p* = .004; Figure 1c). They favoured the yeast *Saccharomyces cerevisiae* over all other conditions (YE-WT: *V* = 0, *p* = .009, YE-LA: *V* = 0, *p* = .006; Figure 1b). However, larvae did not discriminate between the toxin-producing wild-type and toxin-production-impaired mutant strain of *Aspergillus nidulans* (WT-LA: *V* = 11, *p* = .106; Figure 1c). Thus, the larvae can perceive the microbial activity and showed a strong preference for patches with microbes over patches without microbes; among the microbe-infested substrates, they clearly preferred yeast- to fungal-inhabited substrates.

3.3 | Larval development assay

We tested the ability of single *Drosophila melanogaster* larvae to develop on microbe-inhabited (WT, LA, YE) and microbe-free (MF) fruit-substrate. No flies emerged from confrontation units with sterile banana substrate (MF). As expected, the sterile substrate did not support larval development. On banana-WT substrate, a maximum proportion of 0.1 flies emerged when the WT colonies had a 1-day head start (Figure 2a). No flies were observed to emerge from confrontation units where the WT colonies had a 3-day head start. Larvae developing on banana-LA substrate emerged as adults with a proportion of 0.66–0.95, dependent on the developmental head start of the fungus (Figure 2a). By contrast, all larvae transferred to *S. cerevisiae* inoculated units developed to adults. The probability of single larvae reaching the adult stage differed significantly between

FIGURE 2 Larval development and development progression. (a) Larval development to adulthood: accumulated proportion of eclosed flies from single larvae that were transferred to units with fruit-substrate, inoculated with wild type (WT) or $\Delta laeA$ (LA) 0 to 3 days before transfer, or as microbe-free control (MF). Note: *sterile fruit-substrate (MF) does not support larval development*. WT differed significantly from LA (glm, quasi-binomial, treatment: $t = -5.84$, $p = .001$, $df = 6$; data of MF (=0%) and YE (=100%) not included in test statistic). (b and c) Larval development progression: larval length (in pixel) of larvae transferred to units with breeding-substrate as sterile CO or with (b) 3-day-old WT or LA colonies and (c) 2 μ g sterigmatocystin (ST), mean $\pm$ SED. Note: *control breeding-substrate does support larval development*. Units of different time points were independent. Larvae of WT- and ST-confrontations were significantly shorter at time point 48 hr (log-transformed data, adjusted p-values of Tukey's multiple comparisons 95% family-wise confidence level, WT_48 hr: $p < .01$, ST_48 hr: $<.0001$; for all data, see Table S2)

WT- and LA-confrontation, $p = .001$ (glm, family quasi-binomial, for WT and LA data). The mortality of single larvae confronted with *A. nidulans* WT colonies on fruit-substrate was very high. While the toxin-impaired fungus supported larval development in otherwise microbe-free conditions, the toxins of the WT colonies did almost entirely obstruct larval development.

Previous research showed lower mortalities in larvae in the presence of the WT fungus, when alternative microbial sources of nutrients are available such as inactivated yeast and especially when the confrontation experiments are carried out with higher larval densities on *A. nidulans* WT-infested breeding-substrate (Trienens et al., 2010).

When groups of 15 larvae were confronted with WT colonies with a 3-day head start on breeding-substrate (see basic set-up), in average a proportion of 0.52 survived to adulthood (Fig. S4), which was not significantly different from the proportion of larvae that eclosed from fungal- and toxin-free control units (proportion CO = 0.66; glm family = quasi-binomial: WT: $t = -1.71$, $p = .095$; LA: $t = .96$, $p = .342$, ST: $t = -1.14$, $p = .263$; $df = 38$). Yet, also under these conditions the larvae do not avoid feeding on the fungal colonies. On the contrary, as Fig. S2 shows, aggregations of larvae are in close contact with the fungal colony, even though development-supporting breeding-substrate, which is not covered by fungal colony, is accessible.

3.4 | Development-progression measurement

We measured larval length as a proxy for the progression of larval growth on breeding-substrate in confrontation with WT and LA compared to CO conditions and separately ST compared to CO. During early larval development length increased equally between all

confrontation and control conditions. However, at 48 hr, surviving larvae from WT- and ST-confrontations had shorter body lengths than larvae from control conditions, indicating impaired developmental progress (anova: log-transformed data, Gaussian distribution, independent units, "treatment:time," WT-LA-CO: $F_{21,885} = 140.1$, $p < .0001$; ST-CO $F_{4,206} = 78.4$, $p < .0001$, Tukey's multiple comparisons, WT-CO_48 $p = .01$, LA-CO_48 $p = .82$; ST-CO_48 $p < .0001$, see Table S2 and Figure 2b,c).

We compared larval mouth-hook morphology, which shows distinct differences between the three larval developmental stages. The main factor "time" of sampling had a significant effect on mouth-hook morphology ("time": WT-LA-CO: $t = -8.38$, $p < .001$, $df = 67$; ST-CO: $t = -4.74$, $p = .0003$, $df = 15$), whereas "confrontation" and the interaction term "confrontation:time" were not significant (independent units over time, glm with quasi-binomial distribution, including model reduction; "confrontation": WT-LA-CO: $t = 0.14$, $p = .892$, $df = 67$; ST-CO: $t = 0.76$, $p = .457$, $df = 15$; "confrontation:time": WT-LA-CO: $t = 0.04$, $p = .966$, $df = 66$; ST-CO: $t = 0.005$, $p = .966$, $df = 14$) (Fig. S5). From the combination of assays, we conclude that the development of groups of larvae on *A. nidulans* WT- and LA-infested and ST-supplemented breeding-substrate appears to progress normally until approximately 36 hr postconfrontation, after which the growth of the confronted larvae slows, while the moulting rate is unaffected.

3.5 | Differential gene expression and functional annotation cluster analysis

To measure the transcriptional responses of larvae to confrontations with filamentous fungi and their toxic secondary metabolites, we characterized genomewide expression at 3, 6, 12 and 24 hr after

confrontation start. At these time points, larval growth (Figure 2b,c) and survival were not yet reduced by the presence of the fungus or toxin. Over the first 12 hr, the overall transcriptional profile of larvae from WT- and LA- confrontations was similar, but distinct from larvae from ST- and CO-confrontations, as judged from multidimensional scaling (MDS) plots (Fig. S6). At the 24-hr sampling time point, the transcriptional profiles also showed clear differences between WT and LA, and between ST and CO (Fig. S3, MDS plot). Of the 13,942 features to which reads were mapped, we omitted those with low counts. A total of 9,334 features passed our cut-off threshold of five counts per million (CPM) in at least four or more libraries per comparison and were included in further statistical analysis. We set contrasts separately for each of the four time points (3, 6, 12 and 24 hr) to address three questions: (i) What is the effect of toxic metabolites produced by the fungus (WTvsLA)? (ii) What is the effect of an isolated mycotoxin (STvsCO)? and (iii) What responses do larvae show towards the presence of filamentous fungi irrespectively of toxin production (FUvsCO; FU = WT + LA)?

Summarized across all contrasts and time points we marked 2762 genes as significantly differentially expressed (DEGs, *EDGER*, *glmLRT* FDR < 0.05, method = BH, full list of DEGs in Data S2). The number of DEGs were approximately evenly allocated over time points in the WTvsLA-contrasts, while it showed a late response in STvsCO-contrasts, and a higher and increasing number over time in FUvsCO-contrasts (number of DEGs for time points 3, 6, 12 and 24 hr, respectively: WTvsLA = 145, 105, 129, 172; STvsCO = 1, 8, 12, 487, FUvsCO = 356, 660, 627, 1,880) (Fig. S7). At each time point, and separated by up- and downregulation, the DEGs of the three contrasts showed only minimal overlaps, indicating confrontation-by-time specific transcriptional responses (Figure 3).

To assess which functional annotations were over-represented among the DEGs, we performed an annotation cluster analysis, using the Benjamini correction ≤ 0.05 (*DAVID*) as a cut-off to include annotation terms. We provide a graphical overview of the over-represented annotations for the complete set of DEGs at any time point across the

entire time course (Figure 4). In Figs S8–S11, we present these graphical overviews separately for each of the different time points, and a full list of annotation cluster terms with enrichment scores in Data S4.

Summarized over all time points (Figure 4), the mycotoxins, either in the presence of fungus (WTvsLA), or in the absence of fungus (STvsCO) induced classical detoxification mechanisms. The annotations cytochrome P450 monooxygenases (CYPs), glutathione S-transferases (GSTs) and uridine-diphospho-glucuronosyltransferases (UGTs) in conjunction with “metabolism of xenobiotics by cytochrome P450” and “drug metabolism” were among the most strongly over-represented terms of the 16 upregulated and overlapping genes of WTvsLA + STvsCO, and further among upregulated genes of the WTvsLA comparison and the overlapping genes of the WTvsLA+FUvsCO (Figure 4). In mycotoxin confrontations (STvsCO) we found differential expression in only a limited subset of the members of CYP, GST and UGT, which were almost exclusively upregulated at time point 24 hr (Figure 5, middle columns). In contrast, at the early time points many of the CYPs, GSTs and UGTs were first downregulated in fungal confrontations. For the CYPs, this was especially the case for members of the insect-specific *cyp6* family (right columns in Figure 5). Additionally, some of the CYP, GST and UGT genes that were downregulated in FUvsCO showed less downregulation in WT-confrontations than in LA-confrontations (Figure 5, right columns; and Fig. S13). The initial downregulation in fungal confrontations was followed by upregulation at 12 and 24 hr for some of the same and some other CYPs and UGTs (Figure 5).

Other transcriptional responses included “nonhomologous end-joining” among upregulated genes in the STvsCO comparison, genes with “CHK-like kinase (DUF227)” in upregulated genes in both the WTvsLA and the FUvsCO comparison, a strong upregulation in “insect cuticle protein” and an enrichment of “trypsin/serine proteases” in FUvsCO. However, we also found several of these same categories among the downregulated genes (e.g., “insect cuticle proteins” enriched in STvsCO, CYP, GST, UGT and CHK-like kinases in the FUvsCO), indicating a dynamic regulation of genes in these biological

FIGURE 3 Venn diagram of overlapping differentially expressed genes (DEGs, FDR < 0.05, BH methods *EDGER*) for contrast WTvsLA (dash-dotted), STvsCO (dotted) and FUvsCO (WT + LA) / 2 vs. CO (solid line) and time points 3, 6, 12 and 24 hr, separated by up- and downregulated DEGs. “tp any” combines all DEGs of a given contrast that at any time were up- and/or downregulated

FIGURE 4 Graphical summary of functional annotation terms separated by the three contrasts. Included are all genes that were differentially expressed (DEGs) at any of the four time points with a FDR <0.05 (EDGER, BH method). DEGs are assigned according to their up- and downregulation (up—left; down—right), to their respective contrasts, and overlaps between contrasts. Associated functional annotation cluster terms for gene groups were analysed using DAVID 6.7 and summarized in this graph; font sizes in word clouds are proportional to cluster enrichment score and ordered from small to higher scores (top to down), included terms pasted our cut-off threshold of 0.05 (DAVID, Benjamini correction). For each Venn diagram: upper left, WTvsLA-contrast—response to toxin produced by fungus; upper right, STvsCO-contrast—response to purified toxin; bottom, FUvsCO-contrast—general response to fungus; and their overlapping responses

processes, both among time points and between different types of confrontations. Among the downregulated genes in FUvsCO, several metabolic pathways were over-represented, including starch/sucrose metabolism, lipid biosynthesis, folate biosynthesis and limonene/pinene degradation. This exemplifies that the presence of the fungus on the food source may drastically affect the nutritional environment for the larvae. When the annotation cluster analyses were performed for separate time points, largely the same annotation categories also formed significant clusters (see Figs S8–S11). Additionally, downregulated genes of WTvsLA at time point 12 hr included an over-representation to annotation cluster terms as “lysozyme” and “bacteriolytic enzyme.”

We did not observe an activation of classical immune response related pathways. Instead, several of the *Toll*-pathway members—prevalingly regulating immune responses towards infections with

fungi and Gram-positive bacteria—were downregulated at several time points in fungal confrontations (see Data S2). Downregulated *Toll*-pathway members included the “Spätzle processing enzyme” SPE, Spätzle 3, 4 and 6, Toll, Myd88, *Drosomycin-like 2* (*Drsl2*) and *Drsl5*, Spirit, a serine protease of the trypsin family and positive regulator of Toll signalling pathway and Thor. The “peptidoglycan recognition proteins” (PGRP) SC1a and SC2, which maintained their enzymatic amidase function, were upregulated over all time points in FUvsCO-contrasts. However, PGRP-SC1a and PGRP-SC2 have been shown to stifle immune responses of the IMD pathway after activation (Bischoff et al., 2006).

A relatively small subset of genes were consistently changed in expression in fungal confrontations with comparable high log₂-fold changes (logFC) across time points (Data S2). “Niemann-Pick type C-2” genes c, d, g and h (*Npc2*) were each upregulated at 3–4 time

FIGURE 5 Heat map for a subset of DEGs that belong to the superfamilies of CYP P450, GSTs and UGTs. Colours indicate scaled log₂-fold change for the differential expression of the genes (left) in the contrasts at each time point (top; form left to right: 3, 6, 12, 24 hr); in white are genes without significant differential expression at that time point

points in FUvsCO. *Npc2* genes are involved in sterol homeostasis and sterol transport. The serine proteases *Ser6* and *Jon99Fii* were upregulated in FUvsCO at all time points, as well as *Jon99Fi* over time point 06–24 hr and *Jon99 Ci* at time point 24 hr. *Lysosomal α-mannosidase V* and *VI* were upregulated in FUvsCO over all time points with logFC of 1.1–3.8, and *lysosomal α-mannosidase I* in FUvsCO at time point 06–24 hr. *Shade (Shd)* involved in ecdysteroid biosynthetic process was upregulated in FUvsCO at time point 24 hr.

Among the DEGs with the highest upregulation in expression were four members of the “Osiris group” in FUvsCO at time point 12 hr (2.9–3.9 log₂FC, *Osi6*, *Osi7*, *Osi15* and *Osi20*; Table S3). However, 14 of the 15 differentially expressed Osiris genes were downregulated at time point 24 hr (note that we did not normalize for, for example, gene-length differences; thus, comparisons of fold changes between genes with different structures might be approximate).

3.6 | Differential exon usage

We tested for differential exon usage to assess whether genes may show splicing variation in response to confrontation with fungi or toxins. The majority of the 142 exons that were detected with differential exon usage (DEU)—any confrontation vs. CO, after accounting for differential gene expression—showed larger similarities in expression patterns for WT and LA, compared to the expression for ST and CO. Consistent differences were found for the first exon of the GSTs D1 and D9 at all four time points with LA and WT having fewer counts than CO and ST. Among others, *cyp4d1* and *Scheggia* (mitochondrial/transmembrane transport) were detected as having DEUs at three time points and *Multi drug resistance 50* (ABC transporter), at two time points, and *Vesicular monoamine transporter* (transmembrane/drug transporter), *CG6125* (transmembrane transport, sulphate transport), and GST Z2 showed DEU at one time point, respectively.

Although we found no over-representation of immunity genes among the DEGs, we did find a substantial number of immunity genes that showed differential exon usage (DEU). These included *Pipe* (Toll signalling pathway), *PNUTS* (phagocytosis), *PGRP-LB* (immune response), *Limpet* (defence response to fungus), *Serpin 42Dd* (negative regulation of proteolysis, defence response to fungus, regulation of antimicrobial peptide production), *Rm62* (antimicrobial humoral response, mRNA splicing), *Angiotensin converting enzyme* (response to symbiotic bacterium, proteolysis), *Rab14* (phagolysosome assembly, phagosome acidification; defence response to bacterium)

and lysosomal α -mannosidase II and VI. We provide a list with all DEUs in the supporting information (Data S3).

4 | DISCUSSION

We investigated interference competition between the saprophagous insect *Drosophila melanogaster* and the filamentous fungus *Aspergillus nidulans* by means of organismic and transcriptomic approaches. The fungus produces mycotoxins with insecticidal properties, which can strongly affect the development and survival of *D. melanogaster* larvae. Single larvae suffered high mortalities in confrontations with this fungus, while groups of larvae showed less reduced survival and reduced growth. Despite these negative impacts, we did not observe larval avoidance behaviour of filamentous fungal influences. Larvae were even attracted to toxin-producing fungi on fruit-substrate compared to sterile substrate (Figure 1), but preferred yeast when given the choice. However, filamentous fungi, irrespective of their ability to produce toxic secondary metabolites, induced early and considerable changes in genomewide gene expression patterns. After confrontations, genes that changed expression included established and putative detoxification genes and insect cuticle proteins, while the confrontation with the mycotoxin sterigmatocystin also upregulated nonhomologous end-joining genes.

4.1 | Larvae do not avoid but seek out development impairing filamentous fungi

The ability of fungi that co-occupy *D. melanogaster* breeding-substrate to produce toxic secondary metabolites can substantially determine the fate of the larvae. However, earlier studies showed that *D. melanogaster* larvae can adapt and actively feed on filamentous fungi and can thereby reduce, or even eradicate, the fungal growth (Trienens & Rohlf, 2011; Trienens et al., 2010; Wertheim et al., 2002). These larvae benefit from “strength in numbers,” where larger groups of larvae are more successful in containing the fungal growth—and by this conceivably hamper fungal-defence responses (toxin production)—and have higher survival rates. *Drosophila* larvae develop on ephemeral and distinct food sources and do not typically cross between separate food patches, potentially due to the risk of desiccation and predation. Thus, an adaptive explanation for why the larvae remain on, or seek out, the fungal colonies may be that they stand a better chance of surviving development by feeding on the fungus than fleeing from it, and generally, by remaining in a larger group and not spreading over potential food patches.

In the present study we conducted one larval choice and one development assay on a fruit-agar-substrate that by itself does not support larval development. The larvae depend on uptake of essential components for body-tissue construction and metabolism from fungal tissue or microbial activity, yet the toxin-producing fungus also can cause high mortality. Females have been shown to add favourable fruit-substrate supplements during egg-laying, such as yeasts, that facilitates larval development (Wertheim et al., 2002). In

the absence of these yeasts, however, and in secondary-metabolite-impaired mutant strains, the fungal tissue did support larval development. Dependence on microbial biomass uptake may also explain the lack of a strict fungal avoidance under certain substrate conditions, as sterile food sources caused an arrest of development and eventually death.

Adult females of *D. melanogaster* have been observed to be strongly attracted by odours emitted from substrates that are subject to alcoholic fermentation by yeasts and bacteria, mediated by distinct odorant receptors (Mansourian & Stensmyr, 2015). On the other hand, geosmin, a metabolite suggested to be a general identification cue for harmful microorganisms and synthesized by, among others, *Penicillium* fungi, induces pronounced avoidance behaviour (Stensmyr et al., 2012). In the larvae, we did not find expression of the *or56a* gene, the receptor to which geosmin is the exclusive ligand (Mansourian & Stensmyr, 2015), or *or33b* a receptor alternatively suggested to mediate avoidance of fungi in the larvae (Stensmyr et al., 2012). We did not specifically aim to measure gene expression of the nervous or sensory system, yet we did find gene expression for some other odorant receptors in our data set (e.g., *or45a*, *or45b*, *or63a*). The expression of odorant receptors in *D. melanogaster* larvae appears to be a reduced set of those expressed in adult flies (Kreher, Kwon, & Carlson, 2005). Again, this could be adaptive, because larvae are limited in their opportunities to behaviourally respond to unfavourable cues in their proximity, hence, predominantly depend on decisions made by females choosing suitable places for oviposition. Some of the volatile metabolites that induce attraction in larvae are synthesized by yeasts and filamentous fungi (Stötefeld, Holighaus, Schütz, & Rohlf, 2015). Yet, the larvae were able to perceive differences between food sources with and without microbial activity and showed a clear preference for substrates with yeasts over substrates with filamentous fungi. Cactophilic *Drosophila* larvae are even able to differentiate among various types of yeast, clearly preferring to feed on certain yeasts (Fogleman, Starmer, & Heed, 1981). When larvae had to choose between patches with and without fungi, they strongly preferred the food sources infested by fungi, indicating that their lack of avoidance is not due to a lack of ability to perceive the presence of the fungus.

Thus far it remains an open question what might be at the origin of the development of attraction to fungal volatiles: whether it is the need for food or for the benefit of tackling a foe, or its interplay. The larvae clearly aggregate in close contact with the fungus and feed on it, even when they do not need to for its nutrients, and while it is potentially (very) harmful causing increased mortality and reduced growth. It is still unresolved whether the larvae can recognize the difference between fungal food and a fungal foe; their lack of discrimination between toxin-producing and toxin-production-impaired fungal colonies might be due to an inability of larvae to detect the toxic metabolites. The relatively late transcriptional responses to the mycotoxin could also suggest that *D. melanogaster* larvae may not recognize the toxin as such, but rather suffer from its negative impact and respond to the potential damage. However, an inability to recognize toxin-producing fungal colonies would select

for larval avoidance of fungal growth when potentially harmful fungal colonies are frequently encountered under natural conditions, and feeding on these colonies incurs fitness costs. Alternatively, the larvae could be feeding on fungal colonies to reduce the access and impact of fungal competitors to the substrate, irrespective of whether the fungus is (perceived to be) harmful. The feeding behaviour on young hyphae is effective in reducing fungal cover, and this was shown to increase the survival probability of the larvae in the presence of fungal growth (Trienens et al., 2010). Moreover, selection for indiscriminate feeding behaviour is also expected when fungi can employ inducible defences, such that the currently perceived harmfulness is perhaps not a reliable indicator of the potential harmfulness. The fungus *A. nidulans* shows constitutive production of toxic secondary metabolites, but also increases the synthesis of these compounds after encountering grazing larvae and with increasing age of the colonies (Caballero Ortiz et al., 2013). While still speculative, this would suggest that the attraction towards fungal volatiles and the feeding on the fungal colonies may have evolved into a behavioural strategy in the defensive repertoire of the larvae against competing and potentially harmful environmental microbes.

4.2 | Antifungal immune pathway *Toll* is not activated

Studies using insects to investigate immune responses against pathogens often use septic injury/injection or oral application of infectious pathogens as a mean of transmission and find cellular and systemic humoral responses of the host within a few minutes to hours after infection (Shia et al., 2009; Vodovar et al., 2005). In the present study, and in contrast to these infections with entomopathogenic microbes, we did not find global activation of the *Toll*-mediated antifungal immune response within the first 24 hr of fungal confrontations. On the contrary, multiple members of the *Toll* pathway were downregulated, while the characteristic strong upregulation of antimicrobial peptides after an immune insult was absent. The underlying principles of oral infection with a pathogen and grazing at or in close proximity to a toxin-producing fungal competitor may differ substantially. Yet, we conclude that antagonistic confrontations with nonpathogenic environmental microbes do not lead to large transcriptional changes in the canonical immunity pathways. However, some of the genes that show differential exon usage (DEU) have annotation terms assigned that are related to antifungal responses, such as *Limpet* or the *Serpin protease inhibitor 42Dd*.

4.3 | Larval defence responses downregulated in fungal confrontation

Insects can become exposed to xenobiotics of various chemical structures and origin, such as natural metabolites of plants and microorganisms and synthetic pesticides. This has led to the evolution of sophisticated detoxification mechanisms that modify the chemical and physical properties of these compounds (e.g., water solubility), to render them less toxic, and/or to enhance their

excretion. These systems are not infallible, as depending on the specific mechanism, the substrate, the involved enzymes and their composition, even more harmful intermediates can be produced, a process called bioactivation (Guengerich, Johnson, Ueng, Yamazaki, & Shimada, 1996; Guengerich et al., 1998; Zeng, Niu, Wen, Schuler, & Berenbaum, 2006). Currently, we have only a limited understanding of the mechanisms, effectiveness or regulation of these detoxification genes, but gene overexpression, gene amplifications and mutations in protein-coding-gene regions of detoxification genes have frequently been implicated as factors for the evolution of insecticide resistance (Liu, 2015).

The degradation of aflatoxin, of which sterigmatocystin is a direct precursor, involves members of the conserved superfamilies of cytochrome P450 monooxygenases (CYPs) and glutathione S-transferases (GSTs) (Bbosa, Kitya, Odda, & Ogwal-Okeng, 2013; Lee & Campbell, 2000). Together with uridine-diphospho-glucuronosyltransferases (UGTs), CYPs and GSTs constitute one synergistic detoxification tool (Luque & O'Reilly, 2002; Saisawang, Wongsantichon, & Ketterman, 2012; Tijet, Helvig, & Feyereisen, 2001). While in our WTvsLA-contrast—addressing the effect of toxic metabolites produced by the fungus—several members of these gene families were upregulated in a faster response (6–12 hr) than in confrontations with the pure mycotoxin (24 hr, STvsCO), we found a remarkable number of CYPs, GSTs and UGTs to be downregulated in the overall response to the fungi (FUvsCO), especially at early time points and within the insect-specific *cyp6* family (Figure 5). A further pathway involved in detoxification includes *Keap1*, *cnc* (also *Nrf2*), *AP-1* and *AHR*. The gene *Keap1* is a negative regulator of *cnc* and was downregulated in fungal confrontations, but none of the other members showed differential expression in any of the contrasts at any time.

Pathogens, including those that are opportunistic, have been shown to manipulate immune responses of their hosts by different means (Rieber et al., 2015; Vallet-Gely, Lemaître, & Boccard, 2008), and secondary metabolites are suggested to be relevant agents (Chen et al., 2014; Vey, Matha, & Dumas, 2002; Vilcinskas, Matha, & Götz, 1997). While it remains speculative to invoke an active manipulation of larvae's responses by the fungus, these patterns might be indicative of an early but temporary impairment of insect classical detoxification countermeasures against the toxic secondary metabolites that fungi can produce. Perhaps in support of this manipulation hypothesis, larval responses to exposure with the pure mycotoxin sterigmatocystin comprised only a small set of CYP, GST and UGT genes, which were largely upregulated (Figure 5, middle columns).

The secondary metabolite sterigmatocystin that is produced by *Aspergilli* belongs to the substance group of xanthenes (aromatic carbons), is a potent hepatocarcinogen with the capability to build adducts with DNA (Essigmann et al., 1979) and leads to DNA strand breaks (Gao et al., 2015). In toxin confrontation (STvsCO) *nonhomologous end-joining* in association with *DNA-repair* was among the enriched annotation terms, while it was not over-represented in fungal confrontations.

A temporal restriction of insect countermeasures that reduce noxious effects of toxic secondary metabolites may not lead

immediately to an elevated mortality of the larvae, yet can lead to dysfunctional development with consequences on, for example, fecundity. Larvae of *D. melanogaster* are able to develop on breeding-substrate infested with young *A. nidulans* WT colonies without reduced eclosion rate, but emerging adults show a reduced fecundity (Wölflle, Trienens, & Rohlf, 2009).

4.4 | Osiris, Tweedle and CHK—putative toxin resistance genes activated

The Osiris gene family is insect-specific, originated at the early origin of insects and has maintained its sequences and synteny over time (Shah, Dorer, Moriyama, & Christensen, 2012). The members of this gene family, mostly located in a tightly linked cluster on chromosome 3R, have a secretion signal peptide and four domains that identify them as Osiris family members, one of those being a putative transmembrane domain and one a conserved domain of unknown function (DUF 1676). The gene NPFR1, which is unrelated but conserved within this gene cluster in several species, was not differentially expressed in any of the confrontations. Yet, we found four Osiris members significantly increased in expression in FUVsCO at time point 12 hr with a magnitude that was at the upper range of gene expression change in our data set (2.9–3.9 logFC, Osi6, Osi7, Osi15 and Osi20), and mostly expressed stronger in WT- than in LA-confrontations. However, at time point 24 hr, nearly all of the differentially expressed Osiris genes were significantly downregulated in FUVsCO. Thus far, the functions of its members remain predominantly unknown, but for Osi21 an involvement in regulation of endo-lysosomal membrane trafficking between late endosomes and lysosomes has been observed (Lee, Song, & Hong, 2013). Furthermore, a comparative genome study of a *Drosophila yakuba* island population that relatively recently (~29 kya) specialized on foraging on toxic noni fruit (*Morinda citrifolia*) showed differentiation compared to a mainland population at genomic regions that contain the Osiris gene cluster (Yassin et al., 2016). This was reported as recurrent evolution, as a QTL analysis of the genetic basis of specialization in *D. sechellia* identified a QTL that contained the same gene cluster (Huang & Erezyilmaz, 2015). Recently, Andrade López et al. (2017) found that knockdowns of Osi6, Osi7 and Osi8 led to reduced resistance towards octatonic acid, the primarily toxic compound of noni fruit, while tissue-specific downregulation via RNAi resulted in increased resistance. Furthermore, feeding by the herbivorous drosophilid *Scaptomyza flava* on leaves with high levels of glucosinolates also caused differential expression in eight genes in this same cluster (Whiteman et al., 2012). These studies, combined with our data, suggest that Osiris genes may be linked to the development of resistance against a range of different toxins.

Moreover, 10 Tweedle genes were significantly upregulated in the fungal confrontations; these genes are annotated as larval cuticular proteins. The specialization in both *D. yakuba* and *D. sechellia* on toxic noni also implicated the Tweedle gene cluster in the adaptation to the toxic food source (Huang & Erezyilmaz, 2015; Yassin et al., 2016). The gene TweedleO was increased in expression in both our

fungal and toxin confrontation. Additionally, the Tweedle gene cluster has also been implicated in the evolution of herbivory in drosophilids (Whiteman et al., 2012).

Further over-represented were genes with CHK-kinase-like/DUF227 domains. The biological processes in which choline-kinase-like proteins function are mostly unknown, yet they have been associated with pesticide resistance (Aminetzach, Macpherson, & Petrov, 2005). A study screening genetic variation in endoplasmic reticulum stress induced by tunicamycin, using the *D. melanogaster* lines of the *Drosophila Genetic Reference Panel* (DGRP), found CHK-kinase-like domain as the most enriched protein domain (Chow, Wolfner, & Clark, 2013). Endoplasmic reticulum stress is triggered by the accumulation of misfolded proteins in the endoplasmic reticulum.

4.5 | Early response of insect cuticle proteins

We found a strong enrichment of insect cuticle proteins among genes that were increased in expression in larvae that were confronted with fungi. For insects, the cuticle is the first line of defence against microbes. Pathogenic fungi have evolved proteinases that are used to penetrate the insect cuticle during infection and thus are considered as virulence factors (Vilcinskas, 2010). However, these can be reduced in their activity by some insect-derived inhibitors, such as *Serpin 42Dd*. This investment in cuticle proteins possibly reflects a counter measure of larvae to repair damaged tissue or to reinforce the protective capacity. We conducted a larval “Smurf assay”—in a nutshell: a food dye added to the breeding-substrate crosses the intestinal barrier if it is dysfunctional (Rera, Clark, & Walker, 2012)—to see whether the fungus affects the gut integrity, yet did not find indications that dye has crossed the gut membrane into the haemolymph. Moreover, the cuticle composition can impact the invasion success of entomopathogenic fungi (Greenfield, Lord, Dudley, & Butt, 2014), and cuticle thickening has been associated with pyrethroid (insecticide) resistance in *Anopheles funestus* (Wood, Hanrahan, Coetzee, Koekemoer, & Brooke, 2010).

Our transcriptomics experiment was conducted at four time points over the course of 24 hr. Surprisingly, the upregulation of a number of genes that belong to the classical detoxification gene families of CYP, GST and UGT, as well as members of the Osiris gene cluster, took considerable time (6–24 hr). On the other hand, some cuticular genes, including the Tweedle cluster were already upregulated at the 3-hr time point. The insect cuticle is often referred to as “the first line of defence” against the external milieu. Perhaps, this first line of defence is also involved in resistance to insecticidal substances, which might be of synthetic origin (pesticides) or produced by environmental microbes. The defence includes physical barrier, degradation and excretion functions performed by cuticular proteins, metabolic enzymes and transporters, of which all have been shown to be expressed in the integument in association with insecticide resistance (Zhu et al., 2013). Although in recent years attention has been paid to functions of the cuticle beyond being a boundary of the body to the environment, the involvement of cuticle proteins in insecticide resistance and defence responses seems to be underappreciated.

Multiple studies investigating the adaptation of *Drosophila* species to noxious food conditions, such as octanoic acid containing noni fruit, mustard oil producing plant (Andrade López et al., 2017; Whiteman et al., 2012; Yassin et al., 2016), and adaptation to toxic filamentous fungi (present study), show repeated occurrence of similar sets of genes in response to the xenobiotic compounds. In these studies above, as well as in an investigation of oral immune priming and subsequent oral infections in *Tribolium castaneum* larvae by the toxin-producing bacterium *Bacillus thuringiensis* (Greenwood et al., 2017), Osiris and Tweedle genes, “structural constituent of cuticle,” respectively, were significantly enriched in gene ontology analysis. Overlaps in *Drosophila* stress responses can also be found in comparison to other stressors (Whiteman et al., 2012). Perhaps, these two large, diversified and conserved multigene families may constitute a canonical defence mechanism against food-derived toxins in insects. However, further thorough analysis will be required to unveil the mechanism underlying this potential involvement.

5 | CONCLUSION

Multiple insect traits are likely to be affected by the interaction with noxious fungi (Lazzaro & Little, 2009). Therefore, we assessed a range of larval *D. melanogaster* phenotypic responses towards separate detrimental features of the filamentous fungi *Aspergillus nidulans*. We showed that larvae can detect the presence of a toxic fungus, but rather than trying to escape it, they actually feed on the fungus. This fungus is not known to be an (opportunistic) insect pathogen. Instead, using chemical warfare, *A. nidulans* is a serious competitor and has devastating consequences for *D. melanogaster* larvae. We found several potential insect countermeasures against *A. nidulans*, including changed gene expression of detoxification mechanisms, cuticular proteins and the induction of several gene families of largely unknown functions. The attraction to and feeding on these fungal colonies by the *Drosophila* larvae could also be part of a defensive strategy to stop the fungal competitor from overgrowing the substrate. As many insects co-occur and compete on food sources with environmental microbes, insects have evolved a repertoire of traits and strategies to cope with the xenobiotic substances in their environment.

ACKNOWLEDGEMENTS

We would like to thank Christiana Anagnostou for help during larvae collection; Laura Salazar Jaramillo for introducing RNA-Seq data analysis; Henk Buermans for handling our samples at LGTC, Leiden, NL; and Nancy P. Keller, Wisconsin, USA, for providing *A. nidulans* strains.

DATA ACCESSIBILITY

Raw count data and measurements of behaviour and development assays (Figures 1 and 2, Figs S4 and S5) are available in Data S1, in order of appearance.

Fastq files (fatsq.gz) of raw RNA-sequencing reads are deposited in EMBL-EBI via ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress>) and there available under Accession no. E-MTAB-5344, as well as in EBI Expression Atlas. List of used programs for count-data generation and complete R scripts for analysis of differential expressed genes (DEG) and differential exon usage (DEU) are provided in Appendix S1.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest. The project and MT were funded by the Volkswagen Foundation.

AUTHOR CONTRIBUTIONS

M.T. and B.W. designed the study; M.T. conducted experiments; K.K. acquired the sequencing data; M.T. and B.W. analysed and interpreted the data; M.T. and B.W. wrote the article; and K.K. commented on the article.

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SUPPORTING INFORMATION

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How to cite this article: Trienens M, Kraaijeveld K, Wertheim B. Defensive repertoire of *Drosophila* larvae in response to toxic fungi. *Mol Ecol*. 2017;26:5043–5057. <https://doi.org/10.1111/mec.14254>