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Myc Mediates Toxin Response in Female *Drosophila Melanogaster*.

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Abstract

Flies naturally contain microbes in their intestines after eating microbe-rich food like decaying fruits. When ingesting microbes, insects are also exposed to their toxins. The sensitivity of insects to ingested microbial toxins and their mechanism of response to toxins has not been thoroughly studied. Transcriptional regulator c-Myc has been shown to regulate the response to some but not all microbial toxins in mammals. We tested the sensitivity of wild-type and Myc mutant *Drosophila melanogaster* strains to two exotoxins, *Clostridium perfringens* α-toxin and *Vibrio cholerae* toxin, and two endotoxins, lipopolysaccharides (LPS) of *Salmonella minnesota* and *S. typhimurium*. We observed that both sexes of wild-type flies were insensitive to tested toxins. Similarly, Myc mutant males were insensitive to the four toxins. In contrast, female Myc mutants were significantly more sensitive to all tested toxins than wild-type females. The median survival of female Myc mutants was shortened by at least 54 hours in the presence of bacterial toxins. The component of LPS, lipid A, shortened the median survival of Myc females by 104 hours, indicating that the toxicity of LPS is caused by lipid A. This study demonstrates a sex-specific mechanism of the response of insects to toxins and describes that Myc protects female fruit flies from the tested microbial toxins.

Keywords: bacterial toxins; sensitivity; resistance; survival; immunity; *Drosophila melanogaster*

Abbreviations: Immune deficiency (Imd); antimicrobial peptides (AMP); mitogen-activated protein kinase (MAPK); Bloomington *Drosophila* stock center (BDSC); wild type (WT); lipopolysaccharide (LPS); adenosine diphosphate (ADP); Jun N-terminal Kinase (JNK); Nuclear Factor-κB (NF-κB); absorption, distribution, metabolism, and excretion (ADME); absorption, distribution, metabolism, excretion, and toxicity (ADMET); confidence interval (CI); deoxyribonucleic acid (DNA); intestinal stem cells (ISCs); Janus kinases (JAK); signal transducer and activator of transcription (STAT); epidermal growth factor EGF; and Wingless (Wg).

Introduction

Insects that eat decaying food, like rotting fruits, accumulate microbes and toxins in their intestines. Some of these microbes are mammalian pathogens, and insects can serve as their vectors (1). For example, the vectoring of toxin-producing mammalian pathogens by insects and other arthropods has been reported for *Vibrio cholerae* (2, 3), *Escherichia coli* (2, 4), *Clostridium perfringens* (5), and *Salmonella species* (6, 7).

Insects defend against microbes by targeting cell walls with antimicrobial peptides (AMP) and destroying pathogens with phagocytosing circulating hemocytes (8). To produce AMP, insects rely on Toll and the immune deficiency (Imd) signaling pathways, which are evolutionarily conserved to respond to invading pathogens. Lysine (lys)-type peptidoglycan found in the cell wall of most Gram-positive bacteria activates the Toll pathway (9). A lys derivative, diaminopimelate (dap)-type peptidoglycans found in the cell wall of all Gram-negative bacteria, Gram-positive *Bacilli* and most *Clostridia*, and components of fungal wall activate the Imd pathway (10-12).

The innate immune response to microbial toxins in insects is not well understood. In mammals, *Staphylococcus aureus* bacteria induce cells to secrete toxin receptor-bearing exosomes that scavenge pore-forming α-toxins as decoys (13). Moreover, once microbial toxins cause cellular damage, there exist numerous repair processes. Host cells repair toxin-induced damage to the plasma membrane by the lipogenic process and by clogging and removing toxin pores (14). The intracellular damage caused by microbial toxins is repaired by cytoskeleton remodeling and cell survival pathways such as Mitogen-Activated Protein Kinase (MAPK) pathways (14). Furthermore, host cells degrade organelles that contain microbial toxins by autophagy (14).

Ingested microbial toxins may cause stress and damage to cells of the intestinal lining, which would require a mechanism of enterocyte repair. *D. melanogaster* midgut contains intestinal

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stem cells (ISCs) located adjacent to the basement membrane of the midgut epithelium (15, 16). These ISCs undergo division by which each ISC produces a renewed ISC and an enteroblast, which in turn differentiates to the absorptive enterocyte (15, 16). Thus, *Drosophila* midguts regenerate in response to tissue damage and undergo a constant turnover (17). Midgut damage induced by feeding flies with chemicals or bacterial infection can stimulate ISC proliferation and mount tissue regeneration (18, 19). Several signaling pathways are involved in ISC regulation, such as Hippo, JAK/STAT, JNK, EGF, and Wg pathways. These pathways are activated during bacterial infection to stimulate ISC proliferation and converge to regulate the transcription factor Myc (17, 18).

Drosophila Myc is an evolutionally conserved transcription factor that controls cell growth and survival (20). Myc was identified as an essential regulator of ISC proliferation in *Drosophila*: tissue damage upregulates Myc expression in ISCs. Myc is required for elevated ISC proliferation and gut regeneration in response to tissue damage (21). Myc expression is also regulated by the stress-responsive Jun N-terminal Kinase (JNK), whose activation in progenitor cells leads to the over-proliferation of ISCs (22).

The human Myc family includes three members c-Myc, n-Myc, and l-Myc (23). Several previous studies have demonstrated that some but not all microbial toxins induce the expression of mammalian c-Myc. For example, it was shown that lipopolysaccharide (LPS), an endotoxin from Gram-negative bacteria, increased the expression of the human c-Myc (24-27). Additional toxins that have also been shown to induce the expression of mammalian c-Myc are cyanobacteria microcystin (28), *Bacteroides fragilis* toxin (29), *E. coli* enterotoxin B (30), and *Pasteurella haemolytica* leukotoxin (31). In contrast, cholera toxin does not activate the expression of the human c-Myc (32), and c-Myc does not contribute to the pathogenicity of the *C. perfringens* α-toxin(33).

Drosophila has only one Myc gene, which shares the closest homology to the human c-Myc (34). The gene encoding for Myc is located on the *D. melanogaster* X chromosome. Since females have two and males have one X chromosome, insects of different sexes employ a mechanism by which the transcription of X chromosomal genes is equalized between females and males. Nevertheless, it has been shown that the dosage compensation process in *Drosophila* is not absolute and that many X-linked genes are under- or over-expressed in males compared to females (35-37). It has been observed that the expression of the *D. melanogaster* Myc gene is higher in females than in males (38). In contrast to mammalian Myc, the role of Myc in *D. melanogaster's* response to ingested microbial toxins has not been studied.

We hypothesized that Myc contributes to the resistance to ingested microbial toxins in *D. melanogaster*, since Myc is involved in ISC proliferation, and ISC-derived intestinal regeneration is a likely response to toxin-induced midgut damage. To address this, we investigated the survival of wild-type and Myc mutant *D. melanogaster* intoxicated with various endo- and exotoxins. This study describes the role of Myc in the sex-specific response of insects to microbial toxins. Our experiments provide further

Methods

Chemicals and reagents

Toxins used for *Drosophila* feeding assays were purchased from List Biological Laboratories (Campbell, California). Toxins (product numbers) used for screening in *Drosophila* included: *C. perfringens* α-toxin (126A), *S. typhimurium* lipopolysaccharide (225), *S. minnesota* R595 lipopolysaccharide (304) and monophosphoryl lipid A (401), and *V. cholerae* cholera toxin (100B).

Drosophila rearing

Oregon-R *D. melanogaster* (Bloomington Drosophila Stock Center (BDSC) stock #2376) and MYC mutant (BDSC #64769) fly strains were used to conduct experiments. Fly strains were kept at 25°C with 12-hour light/dark cycles. All strains were fed a standard cornmeal-molasses-agar fly medium with yeast flakes.

Drosophila oral feeding survival assay

Oral toxin-feeding survival assays were conducted based on the *Drosophila* bacterial intestinal infection protocol by Alameh *et al*. (39). Flies were not starved before experiments. *Drosophila* vials were prepared by placing three 25 mm diameter circles of extra-thick Whatman blotting paper (Bio-Rad Laboratories, catalog #1703965) at the bottom of the vials and capping them with a cellulose plug. Bacterial toxins and toxin components were diluted in 50 mM sucrose solution at a concentration of 1 µg/mL, which falls within the range of the concentration of plasma-circulating toxins in infected mammals during the late stages of infections, and as previously tested (39). This concentration is also within the range of toxins found in water used to rinse food contaminated with bacteria (40). Whatman paper was not changed during the experiment.

Depending on the experimental condition, flies were exposed to 50 mM sucrose solution alone or sucrose solution containing 1 µg/mL of bacterial toxins. Flies were anesthetized via CO_2, separated by sex, and placed in vials containing 2.5 mL sucrose solutions. Each vial contained at least 20 males or 20 females per condition. All experiments were performed with male and female flies separately to determine a potential variability in survival and immunity due to sex differences. Because the immunity of flies changes with age (8, 41-43), the tested flies were of mixed ages. Experimental vials were incubated at 30°C and checked a minimum of 2 times per day to monitor the time of death. Insects that ingest toxins are also exposed to microbes. Other previous studies tested the immunity of *D. melanogaster* infected with various Gram-positive and -negative bacteria as well as fungi at 30°C. Thus, we designed our experimental intoxication model consistent with other studies (44-47). Although the optimal temperature for the flies ranges from 22 to 25°C, the toxin-treated flies were maintained at 30°C. The incubation temperature of 30°C after infection was also chosen because the optimal growth temperature for the pathogenic bacteria whose toxins we used in this study is 30°C or higher.

Data Analysis

We provided two types of quantitative analyses of our data: parametric and non-parametric. Parametric analysis was calculated as a P value, accounting for differences between the entire two Kaplan-Meier curves. We also provide non-parametric analysis, which does not make any assumptions, and the central tendency is measured as the differences in median survivals between two curves. Data analysis was conducted using GraphPad Prism software. All *P* values reported are products of the respective positive control to a single experimental condition using two statistical analyses: the Log-rank (Mantel-Cox) and the Gehan-Breslow-Wilcoxon tests. An alpha of 0.05 was deemed the threshold for significance. We report *P* values adjusted by the Bonferroni correction. A change in median survival was reported. Since the chance of dying in a small-time interval was different early in the study and late in the study, the values for the 95% CI of the ratio of median survivals were not meaningful and were not reported. Each insect experiment shown is representative of three independent experiments with 20 flies per condition. Figure 3 shows the average and standard errors of median survival of flies exposed to each toxin. Predictions of delivery, distribution, and physicochemical properties of peptides were calculated using ADMET Predictor v. 10.3 (SimulationsPlus, Lancaster, CA).

Results

The effect of exotoxins on the survival of wild-type and Myc mutant *Drosophila melanogaster*

We tested the response of *D. melanogaster* to four orally fed exotoxins and endotoxins produced by human bacterial pathogens. These toxins were chosen because they are made by various Gram-negative and Gram-positive bacteria and for their diversity in molecular structures and biochemical functions. The tested toxins are the pore-forming *C. perfringens* α-toxin ADP ribosylating *V. cholerae* toxin, and lipopolysaccharide (LPS) from *Salmonella typhimurium* and *S. minnesota*, as well as the lipid A component of *S. minnesota* LPS.

Since *D. melanogaster* Myc has been shown to play a role in the insect immunity (13), and its expression is higher in females than in males (38), we evaluated the sensitivity of Myc mutants to exo- and endotoxins in two sexes and compared it to that of the wild-type (WT) flies. WT fly survival varied when exposed to the various toxins of human bacterial pathogens. Pore-forming *C. perfringens* α-toxin significantly increased the median survival of both male and female flies by 84.5 and 20 hours, respectively (Fig. 1A-B). In contrast, *V. cholerae* (cholera) toxin did not affect the survival of male or female WT flies (Fig. 1C-D).

To determine if Myc is required for the response of *Drosophila* to microbial exotoxins, we measured the survival of MYC mutant males and females in the presence of toxins tested in the WT. This mutant strain is a null Myc expression mutant because the transcription starts and the first two exons of the Myc gene were deleted (48). In contrast to the extended survival observed in male WT flies, the survival of male MYC flies treated with α-toxin was insignificantly reduced by 15 hours compared to untreated male MYC flies (Fig. 1A). This effect was more striking in female MYC flies, in which α-toxin treatment reduced survival by 54 hours (Fig. 1B).

As with male WT flies, we observed that male MYC flies were insensitive to cholera toxin, as their survival was unaffected by the toxin (Fig. 1C). In contrast, cholera toxin shortened the survival of female MYC flies by 54 hours (Fig. 1D). Collectively, these results suggest that Myc is necessary for the response of female flies to exotoxins of various human pathogens, and that in the absence of Myc, male flies remain insensitive or become insensitive to the tested exotoxins.

The effect of endotoxins on the survival of wild-type and Myc mutant *Drosophila melanogaster*

Bacterial endotoxins are members of a class of toxins inside bacterial cells, which are released when the cell disintegrates. Such toxins include LPS, found in the outer membrane of gram-negative bacteria and consisting of lipid A and a polysaccharide. We tested the effect of LPS from *S. typhimurium* and *S. minnesota* on the survival of males and females of WT and MYC mutant strains. Both toxins did not affect the survival of male WT and female WT flies, as the median survival of flies was not significantly changed by either LPS (Fig. 2A-D). Male MYC flies responded similarly to male WT flies to both LPS treatments (Fig. 2A, C), showing no reduction in survival relative to untreated flies. In contrast, the median survival of female MYC flies treated with S. *minnesota* LPS (Fig. 2B) or *S. typhimurium* LPS (Fig. 2D) decreased by 72.5 and 56 hours, respectively.

Additionally, the *S. minnesota* LPS component lipid A did not affect the survival of WT and male MYC flies relative to untreated flies (Fig. 2E), but lipid A reduced the median survival of female MYC flies by 104 hours relative to untreated MYC females (Fig. 2F). As with LPS, female WT flies were insensitive to lipid A. These results indicate that the toxicity of LPS endotoxins in female MYC flies is at least partly due to the lipid A component. As summarized in Fig. 3, this study suggests that Myc is necessary for the response of female *Drosophila* to many exo- and endotoxins produced by various human pathogens.

Discussions

This study investigates whether *D. melanogaster* Myc gene affects the sensitivity of fruit flies to ingested microbial toxins. We observed that both sexes of wild-type flies were insensitive to the tested microbial toxins. In contrast, Myc mutant females, but not males, were significantly more sensitive to all tested toxins than wild-type females. This study identifies the toxin-response function of the Myc gene in *D. melanogaster*.

Only a few studies tested the effect of microbial toxins on flies by a physiologically relevant feeding assay. This was accomplished by exposing flies to purified recombinant cholera and anthrax toxins (39, 49). In contrast to toxins' effect on mammals, orally ingested anthrax and cholera toxins did not affect the survival of flies, and anthrax toxin component activated *Drosophila* resistance to bacteria through immune pathway mechanisms. Additionally,

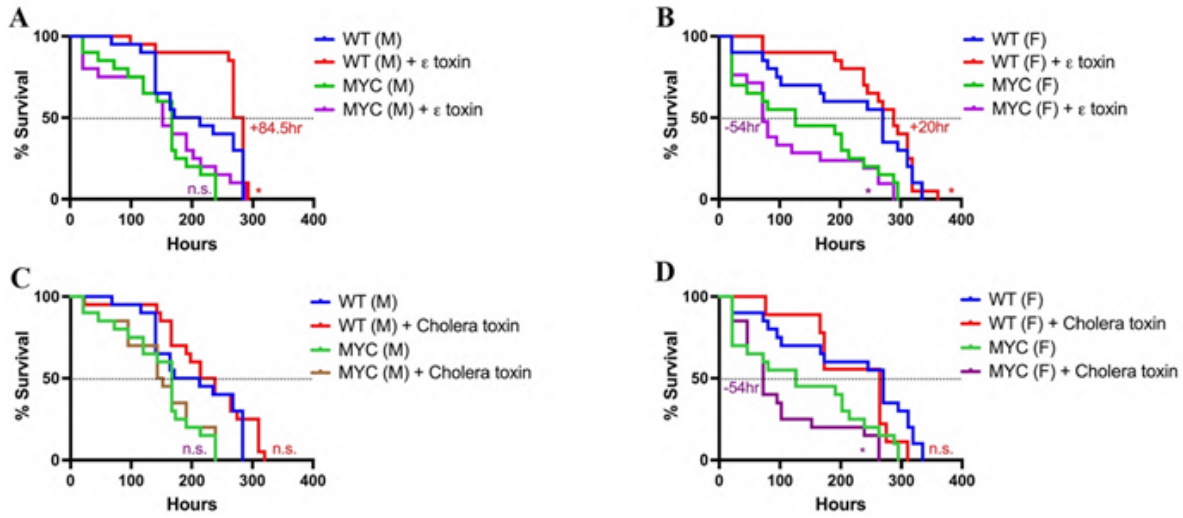


Figure 1: The effect of exotoxins of human pathogens on the survival of wild-type and Myc mutant *Drosophila melanogaster*. The survival of Oregon-R wild type (WT) and loss-of-function MYC flies was measured in the absence and presence of orally administered bacterial exotoxins: *C. perfringens* α-toxin in males (A) and females (B), *V. cholerae* (Cholera) toxin in males (C) and females (D). Toxins were tested orally at 1 μg/ml in a 50 mM sucrose solution. The effect of each toxin on fly survival was tested without toxins as a control for each sex. Positive and negative numbers indicate changes in the median survival, shown as a dotted horizontal line crossing Y-axis at 50%. Asterisks indicate statistically significant differences between treatment and control: *, 0.01 < *P* < 0.05; n.s., not significant.

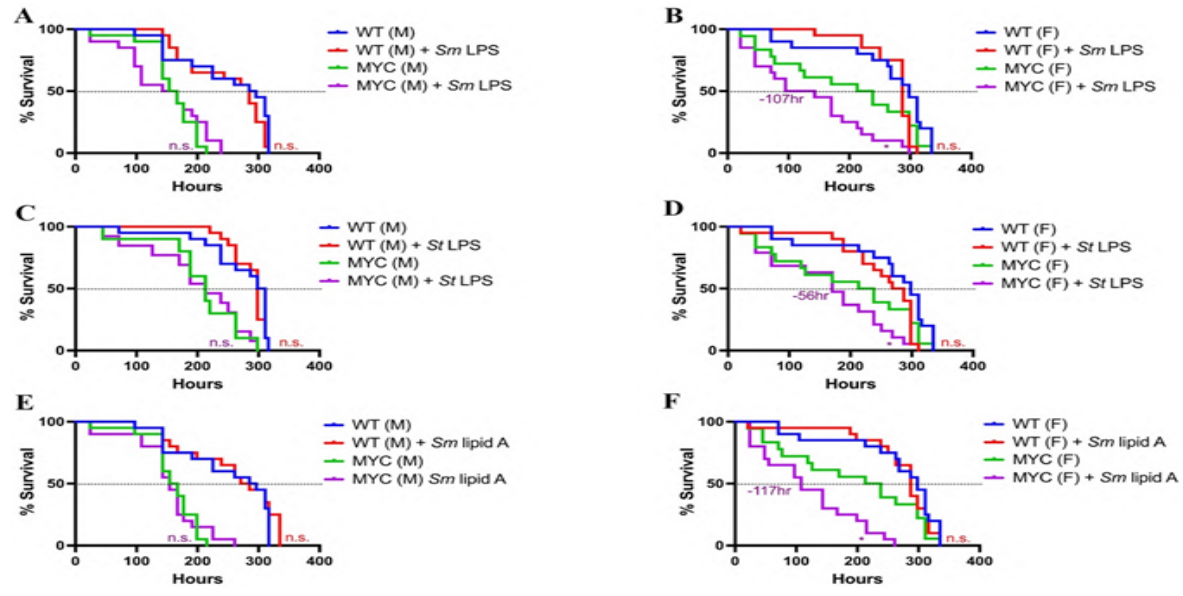


Figure 2: The effect of endotoxins of human pathogens on the survival of wild-type and Myc mutant *Drosophila melanogaster*. The survival of Oregon-R wild type (WT) and loss-of-function MYC flies was measured in the absence and presence of orally administered bacterial endotoxins: lipopolysaccharide (LPS) from *Salmonella typhimurium* in males (A) and females (B), and *S. minnesota* in males (C) and females (D). In addition to toxins, an LPS component, lipid A, from *S. minnesota* was tested in males (E) and females (F). Toxins were tested orally at 1 μg/ml in a 50 mM sucrose solution. The effect of each toxin on fly survival was tested without toxins as a control for each sex. Negative values indicate changes in the median survival, shown as a dotted horizontal line crossing Y-axis at 50%. *P* as in Figure 1.

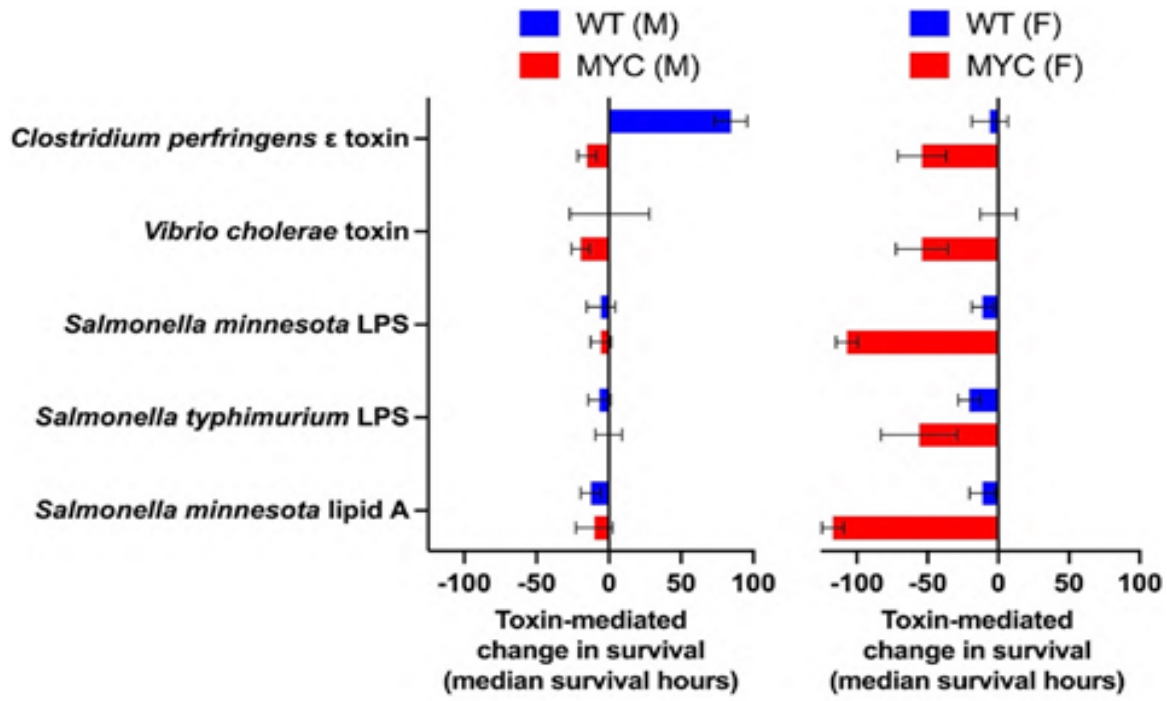


Figure 3: Summary of the sensitivities of WT and MYC mutant flies to toxins. Summary of toxin-mediated changes in the survival of WT and MYC mutant flies shown in Figures 1 and 2, expressed in changes in median survival hours: positive and negative values represent longer and shorter survivals, respectively. The left panel shows toxins response in males (M), while the right panel shows toxin response in female flies (F).

orally fed LPS from *E. coli* was tested in *D. melanogaster,* although the effect of LPS on flies' survival was not investigated (50).

This study tested the sensitivity of flies to four toxins of human bacterial pathogens by the feeding assay and investigated the mechanism of toxin resistance. We observed that while α-toxin prolonged the survival of male and female flies, other tested toxins did not affect the flies' survival at 1 μg/mL. This study shows that *D. melanogaster is* insensitive to many bacterial toxins, and that the toxin tolerance in females is mediated by the Myc protein. The role of Myc in male flies is toxin-specific: while the mutation in Myc negated α-toxin-induced survival in males, the response of Myc mutant males to other toxins was similar to that of WT males.

There are several confounding factors that limit the interpretations of our study. Only one toxin concertation was tested for each toxin, and future tests should study whether other concentrations of toxins affect flies' survival. In addition, the experiments were performed with flies of various ages, because the immunity of flies changes with age (8, 41-43). The age distribution of flies in control and experimental groups within an experiment should be similar. However, the age distribution may not be the same the next time the experiment is performed. Thus, although overall survival may have varied from experiment to experiment, the effect of microbial toxins on survival was reproducible, as seen by error bars for median survival in Figure 3. Additionally, since changing Whatman papers during the experiment was not practical, the evaporation of the sucrose solution limited the survival. Moreover, since the sucrose solution is not a well-balanced food, flies' survival is not expected to be as long as on the nutrient-rich fly food. Another factor that could potentially limit access to food is the deposition of feces and eggs on the surface of the Whatman paper, which could limit access to food. Moreover, the sex and the Myc mutation may affect the amount of food and microbial toxins consumed by flies.

Previously, the anthrax toxin component, Protective Antigen, was shown to protect insects against pathogenic microbes by activating the Toll pathway (39). However, in contrast to the non-pathogenic and non-toxic anthrax toxin component, other toxins, such as pore-forming α-toxin, ADP ribosylating cholera toxin, and pro-apoptotic LPS, can have harmful activity. Based on our results, we hypothesize that once ingested, microbial toxins trigger stress and immune responses in the gut. The stress to cells of the intestinal lining can cause damage or death of enterocytes in the midgut. In response to the loss of enterocytes, intestinal stem cells proliferate and differentiate to regenerate new enterocytes in the midgut. This step is mainly dependent on Myc in females. The expression of Myc itself is induced in mammalian systems by microbial toxins (24-31). The protein that transcriptionally regulates this process in male flies has not

been discovered yet, and future work should focus on identifying such proteins in males. The new enterocytes can induce immunity pathways to inhibit toxin-producing microbes. Based on our previous study (39), bacterial toxins may activate the Toll pathway to reduce the sensitivity of flies to pathogens (Fig. 4). Previous studies demonstrated that cholera toxin does not activate the expression of human c-Myc (32), and c-Myc does not contribute to the pathogenicity of the *C. perfringens* α-toxin (33). Nevertheless, our study showed that in Drosophila, the response to both toxins requires Myc (Fig. 1). Future studies should validate this model by gut-specific Myc knockdown by RNAi or mutagenesis by CRISPR/Cas9, as well as determine the gene that mediates toxin response in male *D. melanogaster*. Moreover, future studies should investigate specific tissues, in which Myc mediates microbial toxin response in females by tissue-specific gene inactivation. Additionally, as our results show that Myc mutation shortens flies' survival, more tests should determine the relationship between survival and toxin response.

While our study helps demonstrate how insects tolerate toxins of human microbial pathogens, resulting in their greater opportunity to transmit the disease-causing microbes, it further suggests that LPS in combination with Myc inhibitors could be used beneficially in agriculture as a new insecticidal combination. More generally, this study identifies insect Myc protein as a new target for future insecticidal compounds for controlling insect vectors of human pathogens. Although human c-Myc is identified as a critical player in tumor development, no specific c-Myc-targeting drugs are available clinically. Existing literature identifying potential compounds targeting mammalian c-Myc may be useful as a basis for optimizing their use as inhibitors of insect Myc. A short list of such possible Myc antagonists is proposed in Table 1. The list of Myc inhibitors in Table 1 was assembled based on the published potency information (51-54), coupled with in silico evaluation of permeability potential, distribution characteristics in biorelevant systems, and chemical or metabolic stability (i.e., ADME properties, using ADMET Predictor v. 10.3).

Myc is known to heterodimerize with another transcription factor, Max (23, 55). All four small molecules presented in Table 1 were discovered in drug screens to inhibit the cMyc-Max interaction. Most prior work has focused on developing cMyc-Max heterodimer inhibitors (55). While published drug candidates targeting mammalian c-Myc have historically tended to be small molecules, there is an effort to develop larger-sized inhibitors of c-Myc. For example, omomyc (56), a 90 amino acid-

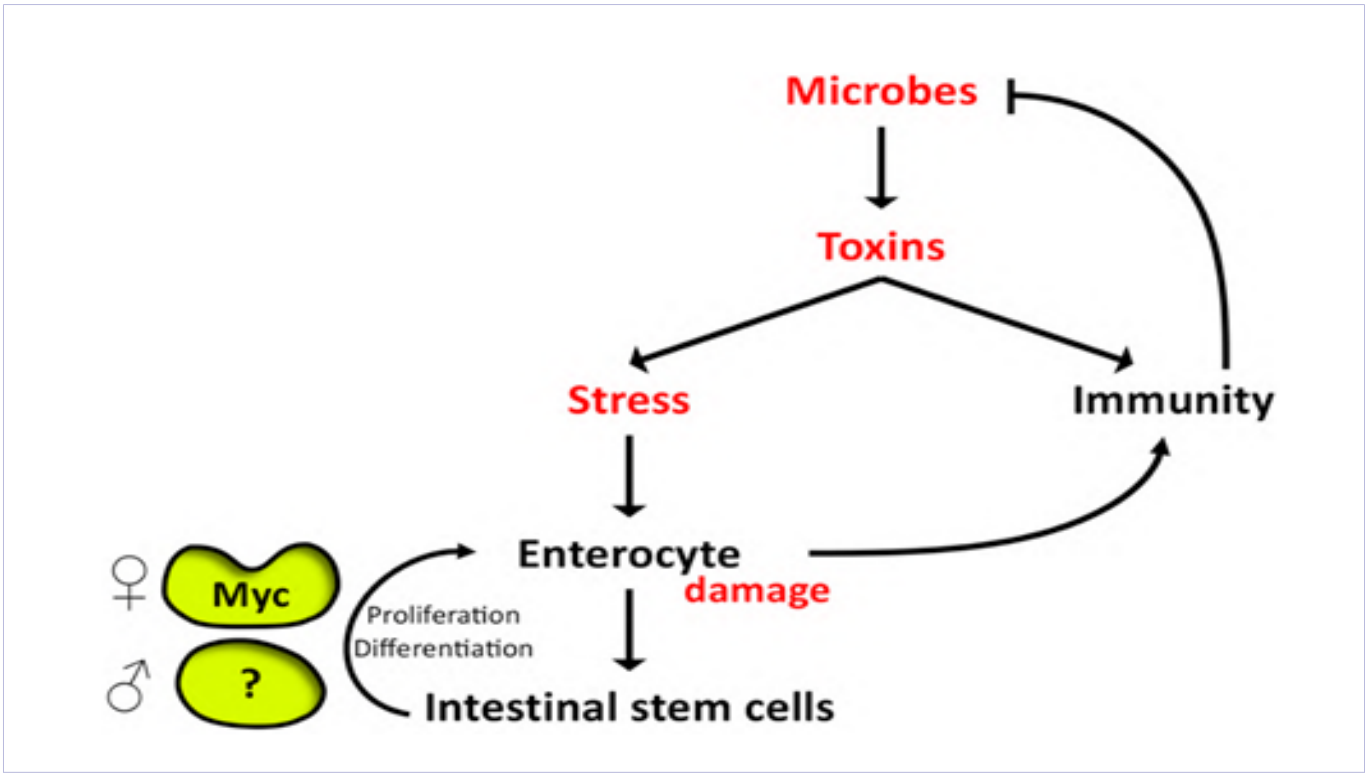
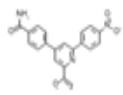
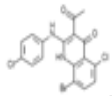
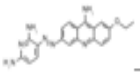
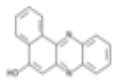


Figure 4: A model of Myc-mediated response to toxins in *D. melanogaster* midgut. During infections in mammals, microbial pathogens release toxins. Upon contact with infected animals or their carcasses, scavenging insects can be exposed to such microbes and their toxins. These toxins trigger stress and immune responses in the gut. The stress causes damage or death of enterocytes in the midgut. Enterocyte loss is compensated by the proliferation and differentiation of intestinal stem cells that regenerate new enterocytes in the midgut. This step largely depends on Myc in females and a yet-to-be-identified transcriptional regulator in males. The new enterocytes activate immunity pathways, which in turn control the pathogens. Once ingested by *Drosophila*, toxins themselves may trigger immunity by interacting with the Toll pathway to reduce the sensitivity of flies to microbial challenges.

Table 1: Predicted physicochemical and biopharmaceutical properties of putative Myc inhibitors.

Compound structure	Compound identifier	c-Myc %Inhibitory [conc] or Kd	Diffusion coefficient (cm^2/s x 10^5)	log P	Log (air-water partition coefficient) atm*m^3/mol	Permeability through human skin (cm/s x 10^7)	Intrinsic water solubility (mg/mL)	Volume of distribution (L/kg)	Cellular permeability classification	Absorption Risk (0-8 range). Exceeds 4 undersirable.
	KJ-Pyr-9	Kd (6.5nM)	0.682	4.65	-12.143	147.091	0.00001477	0.671	High (99%)	2
	KSI-3716	IC50 0.86 mM	0.709	3.81	-9.535	9.845	0.00006067	5.223	Low (77%)	1.488
	Mycmi-6	Kd of 1.6 ± 0.5 µM	0.674	3.6	-13.527	11.268	0.0003032	6.575	High (88%)	3.147
	sAJM589	IC50 of 1.8 ± 0.03 µM	0.885	3.41	-10.713	9.68	0.007	0.863	High (99%)	0.497

long peptide that comprises the DNA binding domain of c-Myc, acts as a dominant-negative c-Myc, by competing with the full-length c-Myc protein for DNA binding. However, while offering additional options for inhibiting insect Myc, there is a greater complexity for its delivery to target insect cells than small molecules (55). In that regard, small molecules targeting Myc-mediated insect response to microbial toxins, particularly those produced by vectored human pathogens, may prove useful in agricultural applications.

Declarations

a. Conflict of interest: NA

b. Ethical approval: NA

c. Clinical trial registration: NA

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