International Journal of Agriculture and Environmental Research

ISSN: 2455-6939

Volume: 12, Issue: 02 "March-April 2026"

SELECTION-DRIVEN ADAPTIVE RESPONSES IN *DROSOPHILA MELANOGASTER* EXPOSED TO *METARHIZIUM ANISOPLIAE* STRAIN ME1

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DOI: https://doi.org/10.51193/IJAER.2025.12221

Received: 17 Apr. 2026 / Accepted: 28 Apr. 2026 / Published: 30 Apr. 2026

ABSTRACT

Drosophila melanogaster was exposed to repeated applications of the entomopathogenic fungus *Metarhizium anisopliae* strain ME1 through both non-induced and induced exoskeleton contact. This study examines the development of transgenerational host resistance to *M. anisopliae*. After four generations, *D. melanogaster* expressed high levels of larval and adult resistance against *M. anisopliae* strain ME1. Without further selection a decline in resistance occurred over the next three generations. Resistance was re-established following two further selected generations. Resistant population adults were larger than parental adults at equivalent densities. Initially, all three fungal doses (10^6, 10^7, 10^8 sp/ml) significantly reduced larval emergence compared to controls. Some insects failed to pupate and/or emerge as adults in treated populations. Survived adult population expressed sensitivity to ether anesthesia and low levels of oviposition. Following the fourth selection round (inoculum: 10^9 sp/ml), where few insects failed to emerge and develop normally, selection was discontinued from generations 5-9. Generations 5-7 were subsequently used to assess *D. melanogaster* susceptibility to *M. anisopliae* at larval and adult stages in both selected and wild-type lines. Adults from both lines were weighed, and selected individuals regained resistance by the 11th generation. Resistance of *D. melanogaster* against repetitive applications of *M. anisopliae* induced bigger size adult individuals to overcome the microbial pressure. Overuse of microbial insecticides has the potential to lead into losses in microbial and biological pest management tactics, increasing chemical insecticide approach to suppress pest populations.

Keywords: Entomopathogenic fungi; insecticide resistance; insect body size.

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1. INTRODUCTION

The growing demand for innovative and selective pest control methods has driven increased interest in microbial control as an alternative to chemical pesticides, which face mounting challenges, including resistance development. Among microbial agents, entomopathogenic fungi represent a particularly promising group, with species distributed globally from Arctic Greenland to Antarctica, demonstrating varying host ranges and the capacity to initiate natural epizootics in local insect populations (Islam et al., 2021).

The cuticular exposome of *D. melanogaster* in the context of entomopathogenic fungi encompasses fungal spores and the associated biochemical and molecular factors that influence fungal adhesion, recognition, and invasion. The initial step involves fungal spore adhesion to the epicuticle, a critical interaction mediated by fungal surface molecules and insect cuticular cues. *Drosophila melanogaster*, a model organism, exhibits genetic variations that affect its susceptibility to virulent fungi. Variability in cuticular composition and immune defenses influences fungal infection efficacy, highlighting the dynamics between the fungal cuticular exposome and host defense mechanisms. This is exemplified by the differences in susceptibility observed among *D. melanogaster* genotypes from different ecological regions, possibly reflecting a co-evolutionary arms race at the cuticular interface (Tinsley et al., 2006).

Metarhizium anisopliae is an effective biocontrol agent against diverse insect pests (Schrank & Vainstein, 2010). Pathogenesis begins with spore adhesion to the host cuticle through hydrophobic and electrostatic interactions, followed by germination, which requires exogenous nutrients, including carbon, nitrogen, and amino acids, for appressorium development (Charnley, 1984; Charnley & St. Leger, 1991). Some isolates display extensive surface growth prior to appressorium formation, potentially enhancing the identification of vulnerable cuticular sites and improving invasiveness (Charnley, 1984).

The insect cuticle serves as the primary defense against entomopathogenic fungi, which must penetrate this multilayered barrier, making cuticular intrusion a critical determinant of infection success (Ortiz-Urquiza & Keyhani, 2013). The surface microbiome introduces a crucial biological dimension to cuticular defense mechanisms, as demonstrated by the capacity of *Lactiplantibacillus plantarum* and associated bacterial communities inhabiting *Drosophila* tarsi and body segments to effectively suppress the germination of *Beauveria bassiana* and *M. robertsii* spores (Hong et al., 2022).

Clavicipitaceae fungi deploy a coordinated enzymatic and mechanical arsenal to breach the host cuticle (Ortiz-Urquiza & Keyhani, 2013). Following hydrophobic adhesion of conidia, mediated by surface hydrophobins such as adhesin *MAD1* in *M. anisopliae*, germination occurs within 6–12 h (Wang & St Leger, 2007). Germ tubes differentiate into appressoria that generate turgor pressure

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through glycerol accumulation, while simultaneously secreting cuticle-degrading enzymes (Gao et al., 2013). A distinct melanization response is mounted at the sites of fungal penetration. The prophenoloxidase (PPO) cascade, activated by serine proteases, including *MP1, MP2* and *Hayan*, generates cytotoxic quinones and reactive oxygen species that are directly fungicidal (Dudzic et al., 2019; H. Tang et al., 2006).

After penetrating the cuticle, the fungus invades the hemolymph and releases toxins that compromise immunity and physiological functions, resulting in death (Charnley, 1984; Huxham et al., 1989). Insect defenses include melanin production, whereas *Drosophila* employs cellular (phagocytosis) and humoral (antimicrobial peptides) responses (Lu et al., 2015). Despite having reduced total hemocyte counts relative to Lepidoptera, Diptera achieve effective immunity through coordinated signaling pathways, exemplified by *D. melanogaster's* use of Toll, Imd, JNK, and JAK/STAT networks (Yu et al., 2022). Testing *Drosophila* immunity against five fungal species confirmed that prophenoloxidase-mediated melanization significantly contributes to blocking fungal entry, although the Toll pathway remains the dominant systemic defense (Liu et al., 2026).

Pesticide resistance is a global problem, with over 440 documented cases in insects and mites (Roush & Tabashnik, 1990). The *Drosophila melanogaster* genome has revealed resistance mechanisms involving gene mutations in conventional insecticide targets (Feyereisen, 1995; ffrench-Constant & Roush, 1990; Hall & Spierer, 1986; Loughney et al., 1989). While *M. anisopliae* provides an alternative for resistant populations (Zhang et al., 2022), some insects exhibit behavioral and physiological immunity through grooming and antibiotic production (Bonadies et al., 2019; Hughes et al., 2004; Yanagawa & Shimizu, 2007). The first entomopathogenic fungal resistance was documented in pea aphid *Acyrthosiphon pisum* clones resistant to 40 *Erynia neoaphidis* isolates (Milner, 1985).

Herein, the investigation of *D. melanogaster* against repetitive induced infections with Metarhizium anisopliae provides an opportunity to select for resistant wild-type subpopulations scaling the intensity of the phenomenon.

2. METHODS

Insects

Wild strain *D. melanogaster* stock was maintained in standard medium culture vials at 25°C under a 12:12h photoperiod. Larval medium included oat flakes (144 g), agar (10 g), black treacle (70 g), nipagin solution (12 ml) per approximately 1 liter. Agar was dissolved in 800 ml of distilled water by heating in a microwave at 93 °C for 3 min. Oat flakes were soaked in 400 ml of distilled water. Black treacle (70 g) was liquefied by microwaving for approximately 45 s and then stirred thoroughly into the oat flake suspension to achieve a homogeneous color. Nipagin solution was

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cautiously added to the dissolved agar. The agar was subsequently combined with the oat flake mixture and stirred well to ensure uniform dispersion. The mixture was then boiled at 93 °C for 10 min with continuous stirring. Sterile paper was added after filling the culture vials with the medium. Yeast was introduced into the vials before adding adult flies. For population expansion, breeding groups of 10 females and 4 males were allowed to oviposit for 2 days before sequential transfer to fresh vials. The wild-type strain was maintained under CO_2 anesthesia for all stock manipulations.

Entomopathogenic fungus

M. anisopliae strain ME1, originally isolated from pecan weevil, was subcultured from stock cultures on Sabouraud dextrose agar (SDA). Sealed plates were incubated in the dark at 28°C for 2 weeks to promote conidial sporulation and then stored at 4°C for 1 month before use. Spore suspensions were prepared by aseptically harvesting spores from SDA plates using a sterilized bacteriological loop and transferring them to 5 ml of sterile 0.05% Tween 80 solution in centrifuge tubes. Following thorough vortexing and centrifugation at 3,500 rpm for 10 min, the pellet was resuspended in fresh 0.05% Tween 80, and spore concentration was determined using a Neubauer hemocytometer. Spore viability was confirmed *in vitro* by assessing germination rates in bacteriological broth with lactophenol cotton blue staining, achieving initial germination within 4-6 hours and 99% germination within 12 h.

Fungal application to D. melanogaster adult stage

For the spray bioassays, fungal inoculum was applied using a Potter Tower at compressed nitrogen pressure of 5 lb/in ² (Roditakis, 1999). Tower calibration was standardized using *M. anisopliae* spores suspended in 0.05% Tween 80 and enriched with Uvitex® fluorescent dye, with spray deposits quantitatively assessed on coverslips under fluorescence microscopy (Olympus BH2; 350 nm excitation, 400V dichroic mirror, L-435 barrier filter). Spore enumeration was conducted using phase-contrast microscopy after air-drying.

The spore deposition density on coverslips ranged from 72 ± 12 to 571 ± 45 spores/mm² for inoculum concentrations of 8×10^5 to 2×10^8 spores/ml, respectively. The Potter Tower was decontaminated with 70% ethanol and sterile water between applications, with controls applied first followed by ascending spore concentrations. The treated flies were subsequently transferred to vials using a fine paintbrush.

Response of wild-type D. melanogaster to M. anisopliae

Adult flies from parental populations (10♀:4♂) were exposed to *M. anisopliae* spore suspensions (5×10^4 to 5×10^7 sp/ml) alongside untreated and Tween 80 (0.05%) controls. Each treatment

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comprised five replicates of 20 adults (1:1 sex ratio), and mortality assessment revealed dose-dependent responses.

Selection Bioassay

Adult flies (10♀:4♂) were maintained in oviposition vials for two days (25°C, 12L:12D) before removal. The resulting progeny were inoculated with 100 µL *of M. anisopliae* spore suspension, and after 3-5 minutes, sterile filter paper strips (1.5 × 3.5 cm) were inserted to enhance the pupation surface area and absorb excess solution (Figure 1).

The inoculated vials were incubated at 27-29°C (12L:12D photoperiod). Adults were collected 2 days post-emergence (10% control threshold), anesthetized with diethyl ether, and organized into breeding groups (10♀:4♂) in fresh oviposition vials (Figure 1).

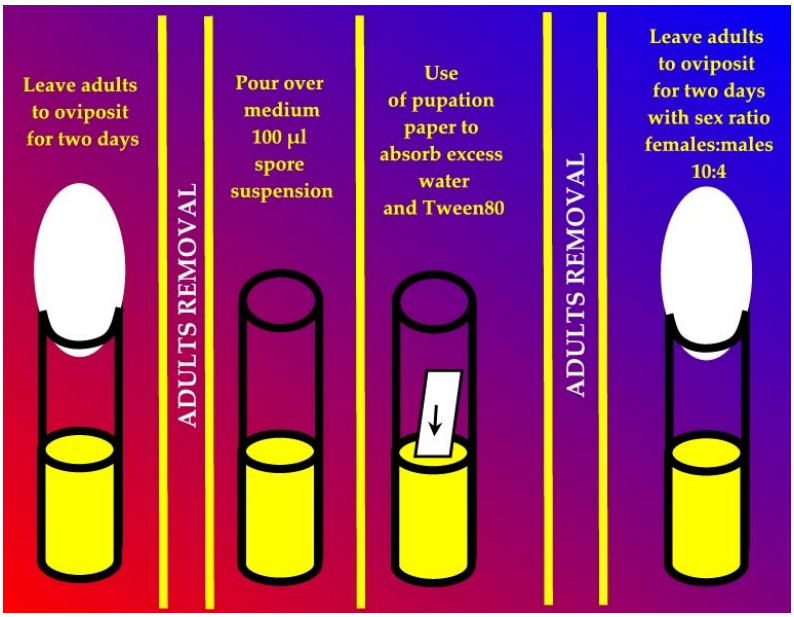

Figure 1: Selection bioassay of *D. melanogaster* larvae against *Metarhizium anisopliae* strain ME1

The adult lifespan was determined at 90% population mortality. Second-generation larvae received 100 µl spore suspension (10^8 spores/ml), with data stratified by parental treatment concentrations: (a) 10^6, (b) 10^7, and (c) 10^8 spores/ml.

Surviving second-generation individuals were pooled and treated with 10^8 spores/ml and maintained through the fourth generation. Wild-type populations underwent selection bioassays across generations 1-4, followed by the removal of selection pressure (generations 5-9) and resistance recovery assessment in generations 10-11.

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Preliminary assessment of the effects of water and 0.05% Tween 80 on *Drosophila* survival showed no detectable mortality when 100 µl of either solution was applied to oviposition vials following adult removal after the 2-day oviposition period.

Adult bioassay

Populations (10♀:4♂) were allowed 2-day oviposition periods at 25°C under 12h:12h photoperiod. Newly emerged adults (1-2 days old) were ether-anesthetized and organized into experimental units of 20 individuals (1:1 sex ratio). Flies were inoculated using a Potter spray tower, and the sprayer was decontaminated between treatments using 70% ethanol and sterile water. Treatments were applied in ascending order of concentration to prevent cross-contamination. Post-treatment, flies were transferred via a sterile paintbrush to glass vials (3×10 cm) equipped with water-moistened cork stoppers and a sucrose feeding apparatus. Inoculated insects were incubated at 27-29°C under 12h:12h photoperiod conditions.

Adult weight determination

Adults were separated by sex, killed by exposure to diethyl ether and weighed on a METTLER® precision microbalance.

Data analysis

A minimum of five replicates were used for each value unless otherwise stated. Data were analyzed using the 95% confidence limits overlap protocol (Sokal & Rohlf, 2012). LC_{50} values were determined by probit analysis using GENSTAT® (Hemel Hempstead, UK). Prism 8.0 (GraphPad, Boston, MA, USA) was used for data analysis and graphical presentation.

3. RESULTS

M. anisopliae spore deposits significantly reduced adult *D. melanogaster* lifespan (Fig. 1). The untreated control and Tween 80 solvent-only groups exhibited negligible mortality throughout the fungal application, thereby confirming that the observed mortality in the treated groups was attributable solely to *M. anisopliae* spores rather than the carrier solution. Mortality followed a sigmoidal pattern, shifting progressively as the spore concentration increased. At the lowest concentration (5×10^4 sp/ml), mortality onset was markedly delayed, with substantial die-off not occurring until approximately days 16–17, ultimately reaching full or near-full mortality by days 26–28. In contrast, the highest concentration (5×10^7 sp/ml) induced a considerably earlier and steeper mortality curve, with significant cumulative deaths recorded in 8–9th day, reflecting a shortened lethal time as the applicable dose increased. Probit analysis determined an LC_{50} of $1.4\times10^6 \pm 2.7\times10^5$ sp/ml for wild-type flies, with LT_{50} values of 13.07 ± 2.5, 9.83 ± 1.9, and 6.93 ± 1.0 days for 5×10^5, 5×10^6, and 5×10^7 sp/ml doses, respectively.

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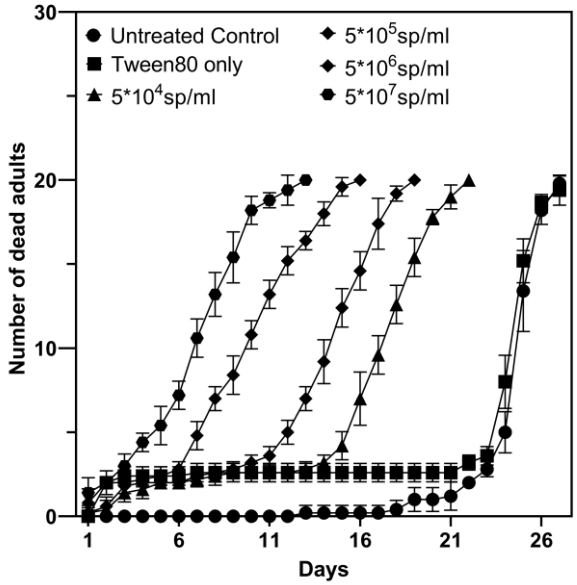

Figure 2: Dose response of *M. anisopliae* strain ME1 against *D. melanogaster* adults for different fungal inoculum sizes ($5 \times 10^4 – 5 \times 10^7$ sp/ml). Error bars represent the standard deviation (n=5).

Selection for *M. anisopliae* resistance in *D. melanogaster* involved treating the parental and three successive generations with fungal inocula. In the first generation, larval emergence (Figure 2A) revealed that treatment with ME1 substantially reduced the number of individuals completing larval development across all tested concentrations (10^6, 10^7, and 10^8 spores/ml), with approximately 50% or more of the individuals failing to survive within the medium, regardless of the dose. Notably, no clear dose–response relationship was observed among the three treatment groups, which clustered closely together and remained markedly below the non-treated control throughout the observation period, suggesting that even the lowest concentration was sufficient to exert near-maximal mortality in the larvae. The absence of dose-response relationships suggests a toxic rather than a pathogenic mode of action. Pupal emergence (Figure 2B) followed a broadly similar pattern, with all three fungal treatments producing considerably fewer pupae than the control group. The highest concentration (10^8 spores/ml) yielded the fewest emerged pupae, though the separation between doses remained modest, consistent with the plateau effect observed at the larval stage. Eclosion success (Figure 2C) was further compromised in the treated individuals, as evidenced by the reduced cumulative number of eclosed adults per vial. Even those individuals that successfully pupated under fungal challenge exhibited impaired or delayed eclosion, indicating that sublethal infection persisted through metamorphosis and continued to undermine the emergence of adults. The total number of emerged adults (Figure 2D) consolidated these cumulative effects, demonstrating a stepwise decline in adult yield with increasing spore

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concentrations. The untreated control produced approximately 63 adults per vial, whereas the 10^6, 10^7, and 10^8 spores/ml treatments yielded approximately 25, 19, and 8 adults per vial, respectively, representing reductions of approximately 60%, 70%, and 87%, respectively, relative to the controls. The adult lifespan (Figure 2E) was shortened with increasing fungal dose, with the mean survival declining from approximately 27 days in non-treated individuals to approximately 16 days at the highest concentration. Statistical differences confirmed that each treatment level produced a significantly different longevity outcome, indicating a clear dose-dependent acceleration of adult mortality. Oviposition (Figure 2F) was markedly suppressed in adults that had developed under fungal challenge. While untreated females maintained consistently high egg deposition (~80 eggs/vial) across the three-day observation window, females from all treated groups deposited significantly fewer eggs, with the most pronounced reduction observed at 10^8 spores/ml, which declined sharply to fewer than 20 eggs by day 2. This finding suggests that ME1 exerts lasting reproductive costs on surviving adults, likely through a combination of energetic depletion and sublethal pathophysiological damage sustained during the larval development.

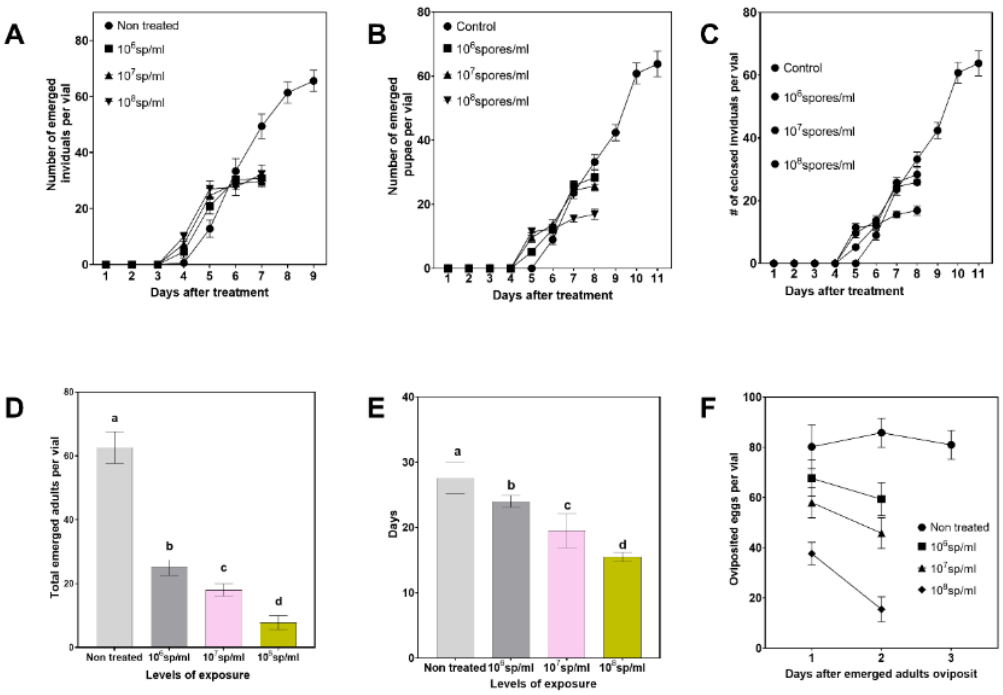

Figure 3: First generation of wild type *D. melanogaster* treated with *M. anisopliae* strain ME1: Larval emergence (A); Pupal emergence (B); Weakness in eclosion (C); Total emerged adults (D); Life span (E) and Oviposition of adults (F). Error bars represent the standard deviation (n=10). Different letters indicate statistically significant differences ($p < 0.05$).

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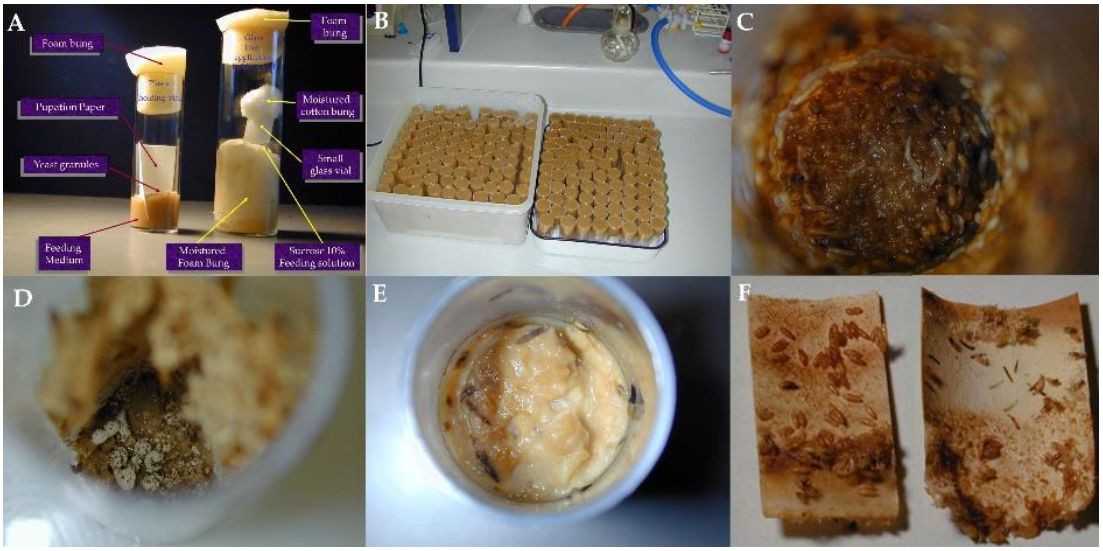

Figure 4: Feeding vials (left) and vials for adults post-treated with *M. anisopliae* (right) (A); feeding meals of *D. melanogaster* (B); healthy and productive colony of *D. melanogaster* on a feeding vial (C); fungal growth in vial after 16 days (D); dead larvae and pupae on pupation paper (E); unable to eclose individuals due to microbial stress (F).

In the second generation (Figure 4), larvae from adults previously inoculated with 10^6 sp/ml showed significantly reduced pupation success compared to the controls, whereas the other treatments accelerated larval emergence without affecting the numbers. Although the successful pupation rates were statistically similar among the treatments, larvae from adults inoculated with 10^7 and 10^8 sp/ml exhibited accelerated pupation. It was not possible to carry out in-depth statistics because there was no unified basis for comparisons among the treated populations (different sizes of oviposition). Although the number of pupae did not vary between treatments, the number of larvae that failed to pupate successfully appeared to differ significantly. The effect was greatest on larvae that originated from adults inoculated as larvae with 10^8 sp/ml.

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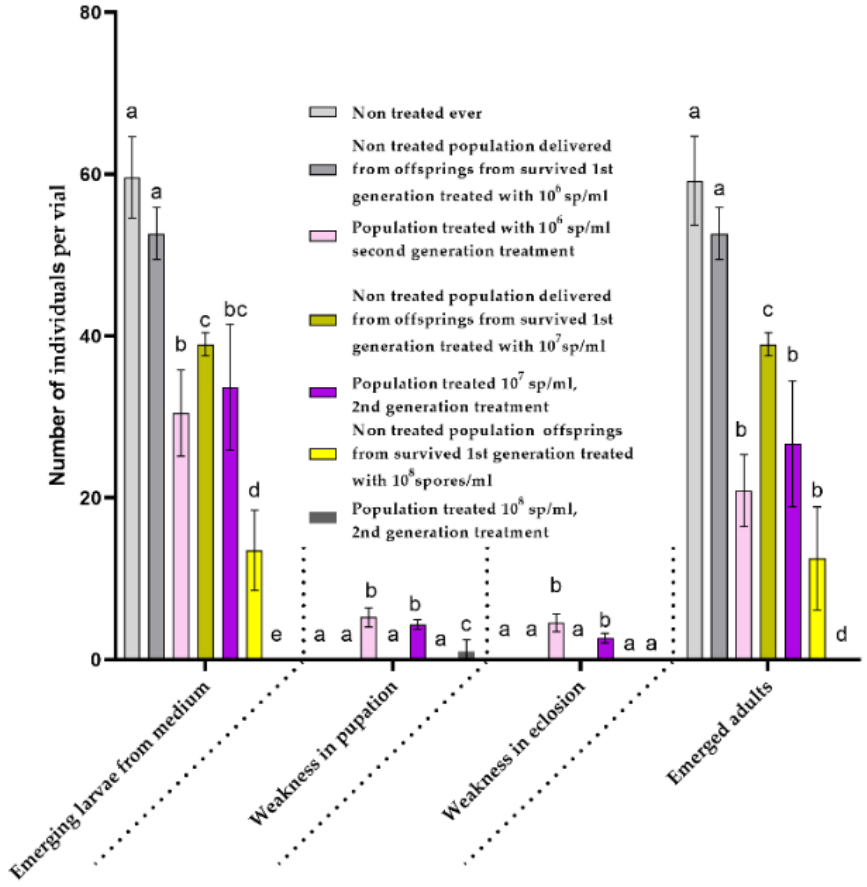

Figure 5: Second generation of wild-type *D. melanogaster* treated with *M. anisopliae* strain ME1. Data divided per treatment and previous microbial challenge at populational level with entomopathogenic fungi and life stage (emerging larvae from medium, weakness in pupation, weakness in eclosion, and emerged adults). Error bars represent standard deviation (n=10). Different letters indicate statistically significant differences ($p < 0.05$).

Larvae that survived fungal treatment exhibited reduced oviposition, creating variable starting populations between treatments. Flies treated with 10^8 sp/ml laid fewer eggs than those treated with 10^7 sp/ml, precluding direct comparisons with controls because of the absence of pupation or eclosion failures in untreated populations.

In the third generation, survivors from the previous selection were pooled into a single population (Fig. 5). The larvae received in situ treatment with 100 µl of 10^8 sp/ml spore suspension, resulting in reduced larval emergence from the media compared to the controls. Adult emergence from puparia was significantly lower in fungus-treated populations, with a small but significant proportion failing to complete pupation or eclosion, whereas control populations exhibited

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complete developmental success. Emerging adults from the treated populations exhibited increased body size relative to the controls, with this effect being particularly pronounced in females.

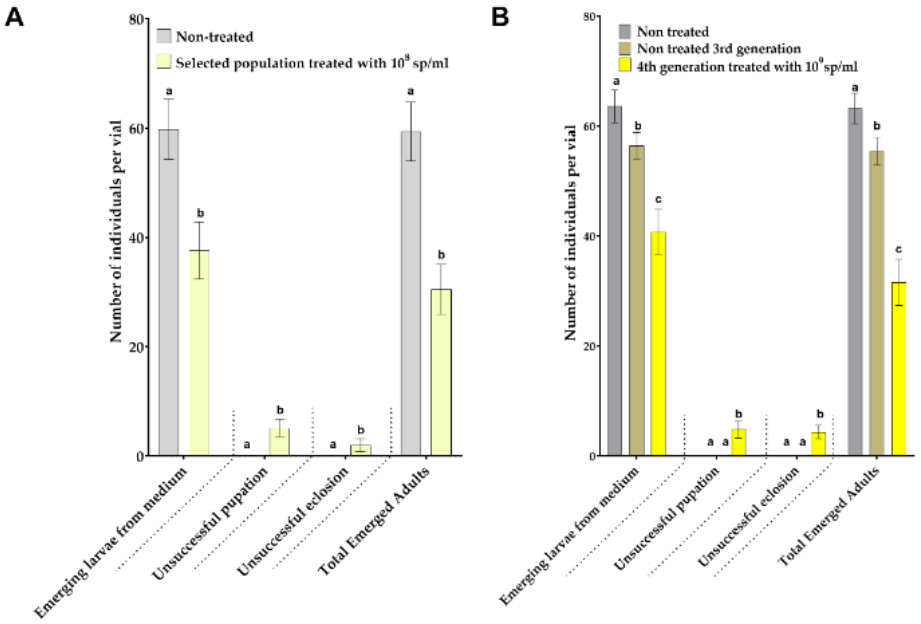

Figure 6: Third (A) and fourth (B) generations of wild-type *D. melanogaster* treated with *M. anisopliae* strain ME1. All previous groups were merged into one and challenged with one dose (10^8 sp/ml). Error bars represent standard deviation (n=10). Different letters indicate statistically significant differences ($p < 0.05$).

In the final phase of selection, fourth-generation larvae were subjected to fungal challenge using an inoculum concentration of 10^9 sp/ml. Fungus-treated populations exhibited significantly reduced pupation rates compared to the controls, with additional developmental abnormalities observed during pupation and adult eclosion in the treated groups.

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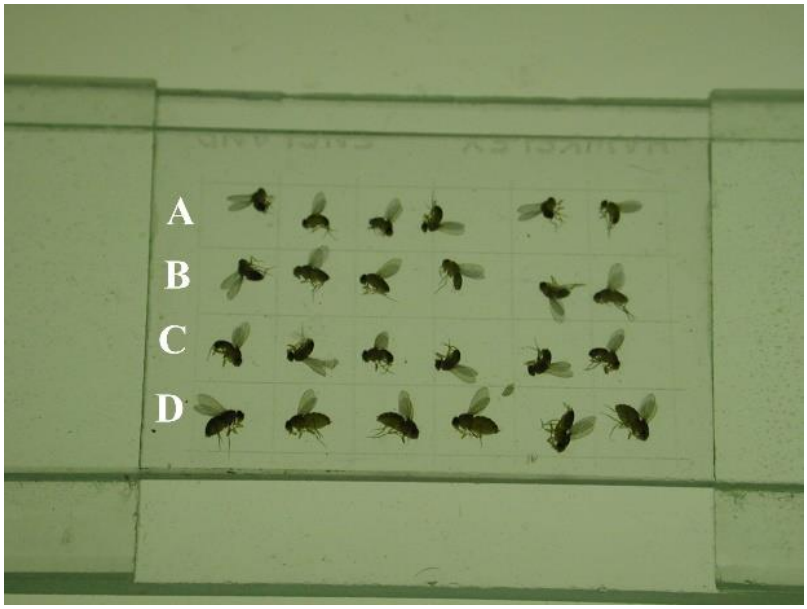

Figure 7: Adult size differences between control and selected individuals of oviposition at the 3rd generation: Untreated with *M. anisopliae* adult *D. melanogaster* males (A) and females (B); third-generation *D. melanogaster* adults from the population selected by *M. anisopliae* males (C) and females (D).

Following four rounds of selection, no further artificial selection pressure was applied between the 5th and 9th generations, providing an opportunity to assess whether resistance persisted in the absence of continued exposure to the fungus. Bioassays conducted in the 5th, 6th, and 7th generations consistently demonstrated that selected individuals retained high levels of successful pupation compared to non-selected individuals. When the pupal lines were subdivided into those proceeding to fast pupation (within 1–6 h) and those proceeding to normal pupation (beyond 24h), statistical differences between selected/non-selected lines were most pronounced among individuals undergoing normal pupation. The fast-pupating subgroup showed fewer significant differences between lines, suggesting that the resistance phenotype has diverse expression profiles throughout the developmental timeline.

The susceptibility of adult flies to *M. anisopliae* was evaluated in the 5th and 7th generations using median lethal time (LT_{50}) across three fungal doses (5×10^5, 5×10^6, and 5×10^7 conidia/ml). At the 5th generation, selected line adults exhibited a consistently and significantly longer LT_{50} than wild-type flies at each dose. At the lowest dose, the selected line survived a mean of 15.6 ± 6.4 days compared to 13.4 ± 2.6 days in the parental wild type, with analogous patterns observed at intermediate and high doses.

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However, by the 7th generation, the LT_{50} values of the selected line converged toward those of the wild type across all three doses, and the differences were no longer statistically significant. This suggests a partial erosion of adult resistance over just two unselected generations, in contrast to the more durable larval resistance pattern. Mortality confirmed this trend, with differences between wild type and selected lines being clearly apparent at generation 5 but narrowing considerably by generation 7, particularly at higher fungal doses.

Significant weight differences between selected and wild-type adults were observed in both generations examined (Table 1). At the 5th generation, selected line males were significantly heavier than controls at 6 and 10 days post-eclosion. The effect was more pronounced in females, where selected individuals were significantly heavier than the controls at all time points assessed, including immediately after eclosion.

At the 7th generation, the pattern was broadly maintained, although with some attenuation. Males showed no significant weight difference immediately after eclosion, but significant differences re-emerged on days 3, 6, and 10. Similarly, female selected individuals remained significantly heavier than controls from day 3 onward, although the magnitude of differences was somewhat reduced relative to generation 5. Cross-generational comparisons confirmed that females selected individuals were significantly heavier in the 5th than in the 7th generation, while male weights did not differ significantly between generations, suggesting a more dynamic weight trajectory in females over successive unselected generations.

Table 1: Average weight (± standard deviation) of 5th and 7th generation selected and non-selected adult *D. melanogaster* exposed to *M. anisopliae* strain ME1. First superscript letter defines statistically significant differences between individuals in the same developmental stage and generation; second superscript letter defines statistically significant differences between selected generations ($p<0.05$).

Male adults weight, 5th generation			Female adults weight, 5th generation	
Days from	Selected line	Wild type	Selected line	Wild type
0	539.05 ± 15.7 ^{a,a}	498.9 ± 14.0 ^{a}	881.8 ± 15.34 ^{a,a}	754.4 ± 18.1 ^{b}
3	708.6 ± 17.06 ^{a,a}	644.8 ± 14.68 ^{a}	1523.5 ± 25.9 ^{a,a}	1272 ± 23.7 ^{b}
6	763.12 ± 11.2 ^{a,a}	692.5 ± 11.0 ^{b}	1743.6 ± 22.8 ^{a,a}	1345 ± 17.23 ^{b}
10	782.9 ± 12.3 ^{a,a}	706.8 ± 10.8 ^{b}	1749.6 ± 23.0 ^{a,a}	1428.4 ± 22.6 ^{b}

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Male adults weight, 7th generation			Female adults weight, 7th generation	
Days from	Selected line	Wild type	Selected line	Wild type
0	533.5 ± 13.3 [a,a]	498.9 ± 14.0 [a]	803.1 ± 19.41 [a,b]	754.4 ± 18.1 [a]
3	691.7 ± 15.7 [a,a]	644.8 ± 14.68 [b]	1393.8 ± 25.8 [a,a]	1272 ± 23.7 [b]
6	745.7 ± 11.6 [a,a]	692.5 ± 11.0 [b]	1498.2 ± 18.5 [a,b]	1345 ± 17.23 [b]
10	754.8 ± 11.6 [a,a]	706.8 ± 10.8 [b]	1603.3 ± 25.11 [a,b]	1428.4 ± 22.6 [b]

To assess resistance recovery, a selection pressure was reapplied to the 10th and 11th generations using 10^8 sp/ml inoculation. Resistance was re-established more rapidly during the second selection cycle (11th generation). While the fungus significantly reduced larval emergence, pupation success, and adult eclosion in the 10th generation compared to the controls, no significant effects were observed at any life stage in the 11th generation. Notably, insects that successfully completed pupation in treated populations proceeded to normal eclosion without further fungal suppression, indicating that pupation represents a critical threshold for survival.

The reintroduction of *the M. anisopliae* strain ME1 (10^8 spores/ml) selection pressure in the 10th generation (Figure 8A) revealed significantly differential responses between treated and non-treated populations across multiple developmental parameters. During the oviposition phase, the reproductive outputs of both populations were comparable. However, a pronounced divergence emerged during subsequent developmental stages. At the emerged larvae stage, non-treated populations sustained high numbers, whereas treated populations experienced substantial reduction. This pattern intensified after successful pupation. The most severe impact was observed during normal eclosion, where the treated populations exhibited critically low emergence rates. By the 11th generation (Figure 8B), the response dynamics had shifted markedly. Reapplication of the same fungal concentration revealed a convergence in survival patterns between the treated and non-treated populations across all developmental stages. The oviposition rates remained comparable between the groups. Crucially, the previously observed susceptibility during larval emergence, pupation, and eclosion largely diminished, suggesting a rapid reacquisition of tolerance mechanisms. These findings demonstrate that the five generations of no selection pressure (generations 5-9) resulted in the loss of previously acquired resistance. However, the population's capacity to rapidly redevelop tolerance upon renewed exposure indicates the

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persistence of underlying genetic or physiological mechanisms that facilitate adaptation to fungal challenges.

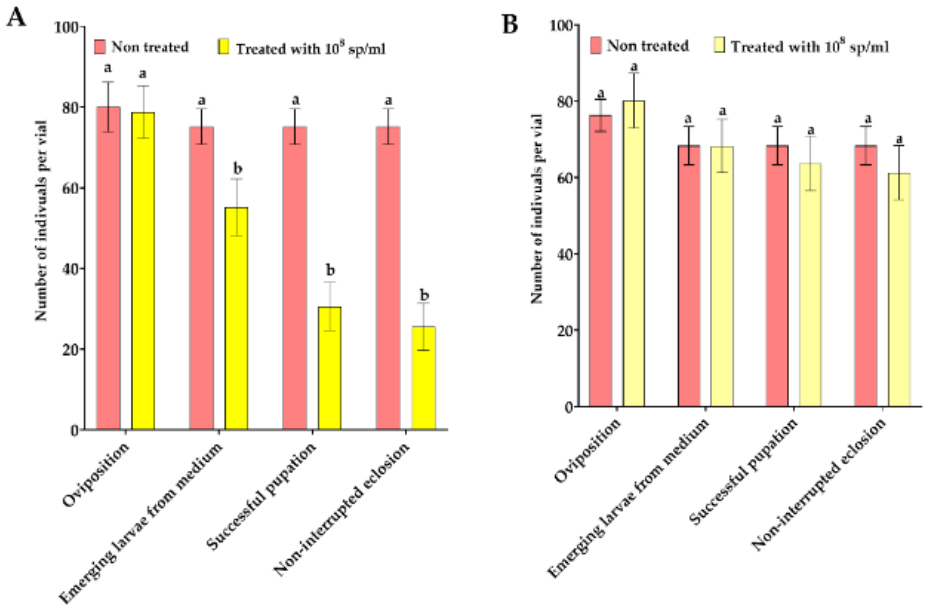

Figure 8: Response of *D. melanogaster* to the presence of *M. anisopliae* strain ME1 (10^8sp/ml) in the 10^{th} (A) and 11^{th} (B) generations, following a period of non-selection (5th to 9th generations). Error bars represent standard deviation (n=10). Different letters define differences for the specific parameter.

4. DISCUSSION

The emergence of insect resistance to *Metarhizium anisopliae*, a widely used entomopathogenic fungus, presents significant challenges to the global biopesticide industry by altering market dynamics and undermining its role as a sustainable pest management alternative (Rajula et al., 2021). This resistance development has profound implications for market stability, product development, and broader adoption of biological control technologies. Herein, the applicable selection bioassays did not permit flexible grooming behaviors, agonistic responses to fungal conidia, and modified habitat selection patterns, which could reduce exposure probability (Hughes et al., 2002).

Adaptation strategies within the industry have focused on several key approaches to mitigate resistance impacts and restore market confidence. Combination products incorporating multiple *Metarhizium* species or strains with different infection mechanisms have shown promise in delaying resistance development while maintaining efficacy. Additionally, the integration of *M. anisopliae* with other biological control agents, including competing entomopathogenic fungi,

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bacteria, and parasitoids, has created synergistic effects that can overcome individual resistance mechanisms while providing multiple modes of action (Vega et al., 2012).

D. melanogaster serves as an excellent model for host-pathogen studies, given its sequenced genome and extensive publicly available mutant collections. Mutations affecting body size, hemocytes, circulation, cuticle, melanization and signaling pathways offer valuable tools for elucidating resistance mechanisms (Lu et al., 2015; Mpamhanga & Kounatidis, 2024). The bioassay design facilitated the assessment of lethal and sublethal effects across all life stages while minimizing host handling, enabling multiple pathogen exposure routes. Selection for resistance in the first generation resulted in 50% larval mortality from mycosis and an additional 20-25% mortality from delayed infection effects during pupation and eclosion. Subsequent generations exhibited progressively reduced fungal susceptibility, with the fourth generation showing complete tolerance to 10^9 sp/ml applications.

Sublethal effects in first-generation survivors persisted in the second generation. Reduced fecundity characterizes microsporidian *Nosema locustae* infections in locusts (L. Zhang & Lecoq, 2021) and parasitoid-resistant *D. melanogaster*, which produce fewer and smaller eggs (Kraaijeveld & Godfray, 1997). However, second-generation adults in this study showed no oviposition differences between the treated and untreated groups.

Selection for fungal resistance coincided with increased insect size, visually apparent by the third generation but quantified only in the fifth. Significant weight differences emerged between treated and untreated flies of both sexes, developing during early adult life in males but were evident from eclosion in females.

Accurate weight measurements of larvae and adults by sex and developmental stage were not feasible; however, visual assessment suggested that larvae and pupae in the treated vials were larger than those in the untreated controls. This observation aligns with established patterns in other insects, where overcrowded populations typically produce smaller and darker individuals than non-crowded populations (Barnes & Siva-Jothy, 2000).

One possible explanation for the size difference is that the fungus reduces competition for food by killing a proportion of the population, so the survivors are bigger. This does not appear possible in the present case because the assessment of the status of treated and untreated lines was done on generations 5-9 which were set up with equal numbers of larvae and were not subjected to selection pressure.

The increased size of insects in the selected lines is the opposite of that found during experimental selection for parasitoid resistance, where treated *D. melanogaster* adults were smaller (Kraaijeveld et al., 1998; Kraaijeveld & Godfray, 1997). A direct comparison cannot be made because the

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mechanism of parasitoid virulence on the larval host is quite different than filamentous fungi due to the fact that many Drosophila parasitoids exert viral particles to downregulate host immune responses (Varaldi et al., 2006). However, host response comparisons between entomopathogenic fungi and parasitoids can be made on an immune-challenging basis.

In contrast to the present study, an inverse correlation between size and resistance to fungal infection was observed in Lepidoptera (Tang et al., 1999). The size difference in the latter study was expressed between instars so that physiology, as well as size per se, may influence susceptibility to fungus, although the authors suggested that bigger caterpillars acquire more spores. For bacterial lepidopteran pathogens, increased feeding rates reduce susceptibility to *Bacillus thuringiensis* toxins (Dhammi et al., 2022). Furthermore, members of other insect orders, such as Coleoptera, have optimum growth temperature and oxygen availability conditions that correspond to larger-sized insects with better melanization capacity after being immunologically challenged (González-Tokman et al., 2019).

The reduced susceptibility of larger flies to fungus may be due to a decrease in the ratio of surface area to volume, which could affect the invasive capability of a topically active pathogen. The larger the surface area to volume ratio, the more spores proportionately may attach to the cuticle per unit volume, thus increasing the chances of overwhelming the host immune system. However, it is also possible that larger flies have a thicker cuticle, a larger hemopoetic tissue and a more active fat body which may also contribute to humoral and cellular host defense (Dionne, 2014; Lu & St. Leger, 2016). The mechanisms of resistance to synthetic chemical insecticides usually result from altered target sites or increased metabolism of the toxin. In the case of fungal resistance in *Drosophila*, the immune response is likely to involve some aspect of the natural barriers to infection (cuticle) or hemolymph defense. Alternatively, some minor chemical changes could interfere with the signal exchange between the host and pathogen, leading to reduced fungal virulence. Russo et al. (2001) found that flies resistant to parasitoids produced more hemocytes. These participate in the encapsulation response, which is the major defense against parasitoid attacks in flies.

Interestingly, the selected adults were darker, especially on the abdomen and scutellum, possibly due to enhanced melanin production. *D. melanogaster* larvae become infected with *M. anisopliae* much quicker than adults, possibly because of the thinner unsclerotized cuticle of the larvae. The level of resistance differs when insects are exposed to fungi, parasitoids, or chemical pesticides (Fellowes et al., 1999a, 1999b). It is to be expected that selection against a single molecule, such as bt toxin, may result in high levels of resistance, as has been found for synthetic chemical insecticides e.g. 150-250 folds resistance of *Plodia interpunctella* to successive feeding with *Bt* toxin (McGaughey & Johnson, 1992).

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In the absence of selection, there was a fairly rapid loss of resistance to *Metarhizium*. In the absence of fungal pressure, the selected lines lost most of their resistance in three generations. This suggests that there is a cost to being large or whatever resistance mechanism associated with large body size that has been selected for. In *Drosophila* lines resistant to parasitoids the costs have been quantified (e.g. low fecundity in adults, selection in mating and feeding rates) (Fellowes et al., 1999a). Resistance to synthetic chemical insecticides also decreases in the absence of selection pressure. The rates of decline in chemical pesticides are especially dependent on the population structure and gene flow (Roush & Daly, 1990). Similar to these other systems, in the present study, resistance was acquired much more quickly in the second treatment round following reapplication of the selection pressure.

This study highlights the importance of further studying insect host resistance to *M. anisopliae*, which is a natural factory of toxins, antibiotics and useful for medicine metabolites (Dong et al., 2013; Ren et al., 2017; Sun et al., 2022). Since *D. melanogaster* is also an animal model organism for diseases, resistant larger flies to lethal fungus must be comparatively examined for potential long-term trade-off immunity costs in metabolic diseases. Host cross-resistance to different *M. anisopliae* isolates coupled with resistance management tactics should also be prioritized for investigation. The susceptibility of other publicly available *D. melanogaster* mutants will be helpful for simulating and studying alternative resistance management tactics. Exploring further the relationship between bigger size fruit flies and immunological resistance, potentially available *Gigas* mutant stocks with bigger cell size could contribute towards that direction due to the fact that regulation of the cell size of this mutant pass through tyrosine, a compound that is involved cuticle sclerotization (Ito & Rubin, 1999; Stocker & Hafen, 2000). Decreasing *D. melanogaster* body size at the population level, either by reducing space or food availability, could provide valuable insights. Insect body size is a complex metabolic and developmental issue regulated by numerous signaling pathways, including the *Target of Rapamycin* (TOR) pathway, the insulin pathway and *foxo* transcripts (Biglou et al., 2021; Shrivastava et al., 2023)

Farmers, professional groups, industry and the scientific community look for potential solutions to this problem as *M. anisopliae* is also a commercially emerging plant biostimulant. The fungus produces indole-3-acetic acid (IAA) at varying concentrations, achieving optimal production levels (Siqueira et al., 2020). The application of *Metarhizium* increased the root IAA content by 13.9% and 14.9% and the brassinolide content by 11.0% and 20.6% (Zhou et al., 2025). Stress alleviation protocols incorporating *M. anisopliae* applications may lead to increased fungal prevalence, potentially disrupting entomopathogenic activities while reinforcing plant stress memory pathways and consequently mediating ecosystem-level changes. *M. anisopliae* priming in rice and soybean improved shoot and root biomass, photosynthetic pigment content, leaf

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relative water content and osmoregulatory arrangements under salinity stress (Chowdhury et al., 2024; Khan et al., 2012).

D. melanogaster immune system research translates directly to agricultural bioinsecticide development, where model organism insights predict biocontrol failures, optimize *M. anisopliae* applications, and guide rotation strategies for controlling crop pests. This model organism framework accelerates sustainable and biologically informed pest management across diverse agricultural systems. From the other hand, future research in outdoor environments is likely to encounter specific challenges, including: a) the co-evolutionary characteristics of *M. anisopliae* with mycoviruses, which modify the fungal pathogenic profile (Camargo et al., 2025) and b) the heavy reliance on biomedical data from laboratory-bred *D. melanogaster*, which fails to account for agri-environmental variables (Overgaard et al., 2010).

5. CONCLUSION

This study demonstrated that *D. melanogaster* rapidly develops resistance to the entomopathogenic fungus *M. anisopliae* through multigenerational exposure, with successive generations exhibiting progressive reductions in mortality, culminating in complete tolerance by the fourth selected generation. This is accompanied by physiological adaptations, including increased body size and enhanced melanization, suggesting strengthened cuticular and immunological defenses. While resistance diminished upon removal of selection pressure, indicating fitness costs associated with the resistance phenotype, it was rapidly reestablished upon fungal rechallenge, revealing the persistence of the underlying adaptive mechanisms and underscoring the evolutionary plasticity of host-pathogen interactions. These findings illuminate the potential for the rapid evolution of fungal resistance in insect populations, which presents significant challenges for the sustainable deployment of *M. anisopliae* as a biocontrol agent. It also emphasizes the critical need for integrated resistance management strategies incorporating combination biopesticide formulations, cross-resistance assessments, and counter-resistance tactics to preserve the long-term efficacy of microbial pest control approaches in agricultural systems.

ACKNOWLEDGEMENTS

Sincere gratitude is expressed to A.K. Charnley from the Department of Biology and Biochemistry, Bath University, UK for providing valuable suggestions and creating a stimulating environment for this work.

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ISSN: 2455-6939

Volume: 12, Issue: 02 "March-April 2026"

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