Impacts of agricultural antibiotic exposure on the development and gene expression of *Musca domestica*

by

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Abstract

House flies (*Musca domestica L.*), common in livestock environments, often develop in substrates containing antibiotic residues from agricultural practices. This study assesses the effects of two widely used antibiotics, tetracycline and enrofloxacin, on larval physiology and gene expression. Developmental assays demonstrated dose-dependent impacts on survival, development time, and individual weights. To explore the molecular basis of these responses, we conducted RNA-seq to profile transcriptional changes in larvae exposed to environmentally relevant and elevated antibiotic concentrations. High concentrations of both antibiotics caused significant transcriptional stress. Tetracycline exposure led to altered mitochondrial and ribosomal pathways, while enrofloxacin broadly disrupted cellular transport and detoxification systems. Both treatments increased expression of detoxification-related genes, suggesting potential cross-resistance mechanisms. These findings reveal that antibiotic residues can significantly influence house fly biology through both physiological and molecular pathways. This study underscores the broader ecological implications of antibiotic use in livestock production, highlighting the need to consider non-target effects on pest species that play roles in disease transmission and resistance evolution.

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Chapter 1 - House fly presence in confined animal feeding operations

Introduction

Confined Animal Feeding Operations (CAFOs) are industrial-scale livestock facilities where animals are raised in dense populations to maximize production efficiency. These environments create ideal conditions for the transmission and persistence of infectious diseases due to high animal density, limited ventilation, and increased stress levels (Graham et al., 2008). The close confinement of animals facilitates the rapid spread of viral, bacterial, and parasitic pathogens, often requiring routine prophylactic treatments to prevent large-scale outbreaks (Silbergeld et al., 2008). House flies are opportunistic pests that thrive in the manure rich environments of CAFOs where swine, poultry, and cattle are raised. These flies breed prolifically in animal waste and decaying organic matter, making livestock facilities ideal habitats. Once established, house fly populations can grow rapidly, especially in warmer months, leading to swarms that harass animals. In swine and poultry houses, flies disturb resting and feeding behaviors, which can impair weight gain and productivity (Machtinger et al., 2021). In dairy operations, house flies cluster around the eyes, mouths, and wounds of cattle, promoting stress and the spread of pathogens (Machtinger et al., 2021). In livestock operations, heavy fly infestations lead to animal stress, reduced milk production, and increased risk of secondary infections, contributing to economic losses and higher pest control costs (Kaufman et al., 2001). This review aims to discuss CAFOs environments, house flies, their biology and current attempts at control methods as well as the research gaps within these topics.

Disease conditions in CAFOs

Commonly reported diseases include bovine respiratory disease complex (BRDC) in cattle (Ferraro 2021), swine influenza and dysentery in pigs, and avian colibacillosis and coccidiosis in poultry (Panth 2019). Another major concern in CAFOs is the accumulation of animal waste, which serves as a significant reservoir for enteric pathogens such as *Escherichia coli*, *Salmonella spp.*, *Campylobacter spp.*, and *Clostridium perfringens* (You et al., 2006). These pathogens not only infect the animals within the facility but can also contaminate local water sources and soils when manure is improperly stored or applied to fields. Waste lagoons, dust particles, and runoff can spread these microbes into the surrounding environment, increasing the risk of zoonotic infections in nearby human populations.

Antibiotic Use

In food animal production systems, antibiotics are heavily administered to livestock to prevent disease, enhance growth, and reduce mortality under intensive confinement. In the United States especially, this practice often involves the routine delivery of antibiotics to entire herds or flocks through feed or water, typically at weight-dependent dosages regardless of whether animals exhibit clinical signs of infection (Silbergeld et al., 2008). While this approach may reduce short-term economic losses from disease outbreaks, it contributes significantly to the accumulation of antibiotics in the environment.

A large proportion of administered antibiotics is excreted unmetabolized in animal waste, particularly for water-soluble compounds. This leads to elevated concentrations in urine and feces contaminated substrates, including manure pits and soil in and around CAFOs (Heuer et al., 2011). In northern China, across a range of livestock, water treatment plants, and organic

vegetable bases, soil and manure samples contained concentrations of tetracycline ranging from 0.09 µg/kg to 183.5 mg/kg with the highest concentrations found in livestock manure samples (Hu et al., 2010). A review across eight provinces in China took samples from poultry, swine, and cattle manure where antibiotic residues of enrofloxacin ranged from 0.03-1420 mg/kg and tetracycline from 0.015-59 mg/kg (Zhao et al. 2010). These residues not only persist in the environment but also create selective pressure on microbial communities, fostering the proliferation of antibiotic-resistant bacteria (ARB) and resistance genes. Accordingly, Zhang et al. 2013 found that antibiotic-laden waste harbors resistant strains of *Escherichia coli*, *Enterococcus*, and *Salmonella*, which can persist in manure, runoff, and contaminated dust particles. These ARB can spread through various pathways, including surface water, groundwater, insects, and aerosols posing risks to farm workers, surrounding communities, and downstream ecosystems (Zhang et al., 2013). Moreover, horizontal gene transfer among environmental bacteria can facilitate the dissemination of resistance traits, expanding the ecological and clinical footprint of antimicrobial resistance originating from animal agriculture (Marti et al., 2013).

When looking at the changes in antibiotic use within the US (Fig 1.1), from 2016-2020, overall use decreased 31.6% (Wallinga 2022). However, this decline is caused by poultry antibiotic use dropping 48% while other major animal production systems increased by 5-12% in the same time period (Wallinga 2022). The repercussions of this system are ecologically and medically far-reaching. The overuse of antibiotics in CAFOs undermines the effectiveness of critically important antimicrobials for human medicine, contributing to a growing global crisis in antibiotic resistance. The WHO has warned that the agricultural sector must play a more responsible role in mitigating resistance, especially as the link between food animal production

and the rise of multidrug-resistant bacteria becomes increasingly evident (Van Boeckel et al., 2015).

Regardless of the method, a significant portion of these drugs passes through the animals unmetabolized and is excreted in urine and manure (Masse et al., 2014). This antibiotic-laden waste accumulates in environments where house flies breed and feed, creating a direct pathway for flies to encounter and potentially disseminate both antibiotic residues and resistant bacteria. In this way, house flies serve as important mechanical vectors, bridging the gap between animal waste management practices and the wider environmental spread of antimicrobial resistance.

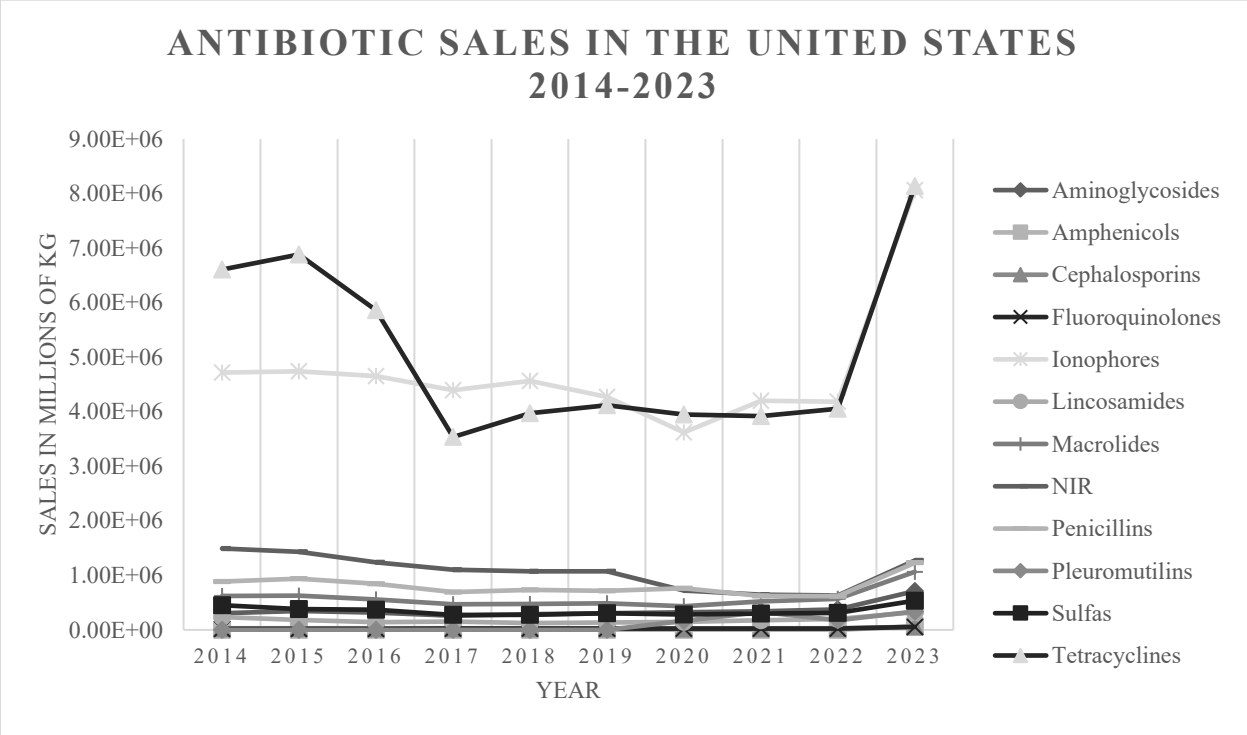


Figure 1.1. Antibiotic use within the United States from 2014-2023 Data collected from reports made by the FDA under the Animal Drug Use Fee Act.

House fly biology

House flies (*Musca domestica* L.) are present on every continent except Antarctica and documented in at least 190 countries worldwide (Bonnefoy et al., 2009). Their generalist diet,

rapid life cycle, and adaptability allow them to colonize urban, rural, and agricultural environments across diverse climatic zones. Their larvae play a role in decomposing organic matter, such as manure, carrion, and garbage, thereby accelerating nutrient recycling in ecosystems. This can be ecologically beneficial in waste reduction, vermicomposting, and soil health (Graczyk et al., 2001: Zhang et al., 2014).

House flies are holometabolous insects which have three distinct stages after hatching from their egg: larva, pupa, and adult (West 1951). Adult females (Fig 1.2A) lay clutches of eggs (Fig 1.2B) on almost any moist substrate that contains sufficient nutrients for larval growth. Females can produce 100-150 eggs, 3-6 days after eclosion under laboratory conditions (Howard 1911). If multiple matings occur, a single female can produce up to 900 eggs in her lifetime (West 1951). This high reproductive capacity results in house flies often reaching pestiferous levels. After hatching from their egg casing, larvae (Fig 1.2C) thrive in decomposing organic matter rich in microbes which they rely on for a food source (Zhao et al., 2017). This organic matter can range from decaying plant substrates to food and animal waste (Fig 1.2E and F) Larvae have three developmental instars which must be completed before pupation. During the larval stages the individual gains mass at a rate dependent on their environmental conditions such as temperature and food availability.

Microbes are an important part of the diet of larvae, which Schmidtmann and Martin explored in 1992 by examining the importance of microbe presence to larval development. When reared on blood agar plates without additional inoculation of microbes, no development past the first instar occurred (Schmidtmann and Martin 1992). Individuals raised on monoxenic cultures of *E. coli, Klebsiella* spp., *Staphylococcus* spp., and *Streptococcus* spp. were able to fully develop but showed less development than those in typical rearing media of animal feed and

wheat bran indicating that increased diversity in the microbial community or their metabolic excrement leads to improved larval development (Schmidtmann and Martin 1992).

Given the importance of microbes to the development of houseflies, any xenobiotics, like insecticides or antibiotics, that influence microbe populations can have significant impacts on the larvae within it. For example, a meta-analysis of manure samples in China found high residue levels of tetracyclines and quinolones in pig and chicken manure, indicating that larvae developing in that substrate are likely exposed to those compounds (Li et al., 2023). Exposure to antibiotic residues like oxytetracycline has been shown to suppress microbial decomposition of manure, altering nutrient cycling and likely changing the microbial food base that larvae depend upon (Fang et al., 2025). Neonicotinoids like imidacloprid can cause oxidative stress and disrupt overall larval development and fitness (Donahue et al., 2017). The insect growth regulator cryomazine, reduced survival, fecundity, and slowed development when exposed to third instar larvae (Khan 2023).

After reaching the third instar and gaining sufficient mass, larvae migrate to dryer ground to pupate (Fig 1.2D). Conversion to the adult may take little as 3 days under warm conditions but may take as long as several weeks under adverse conditions (Lysyk and Axtell, 1987). Both males and females will take sugar meals throughout their adult life to fulfill general energy requirements and occasionally operate as generalist pollinators when taking this meal from flowering plants (Cook et al. 2020). The adults are not sexually mature immediately after emergence from their puparium, females must acquire a meal containing substantial protein before reproducing and males will feed on other decaying organic matter in order to gain necessary nutrients.

Egg clutch size is dependent on the quality of the protein source for the females. Shipp and Osborne (1967) tested the effects of protein sources on egg production, finding that the highest fecundity was yielded by females fed on a yeast diet, slightly better than the typical powdered skim milk solids diet utilized in lab colony maintenance. While yeast may be the best on its own, the highest fecundity is achieved with a diet of sugar, milk, and yeast (Pastor et al.,2011). Conversely, protein sources in nature and around animal production facilities are unlikely to have edible amounts of these sources for access by flies. Rather females will diet on decomposing organic matter like carrion, feces, or even spilt animal feed and blood.

As adults, house flies are highly mobile, enabling them to traverse landscapes and serve as mechanical vectors moving pathogens between sanitary and unsanitary areas. House flies have shown the ability to mechanically vectors over 100 pathogens including *Salmonella*, *Escherichia coli*, *Shigella*, *Campylobacter*, and various parasitic protozoa (Gilchrist et al. 2007: Gioia et al. 2022). Several of these pathogens can be transmitted when flies come into contact with human or animal food, water sources, or wounds, making them serious public health threats (Graczyk et al., 2001; Scott & Lettig, 1962).

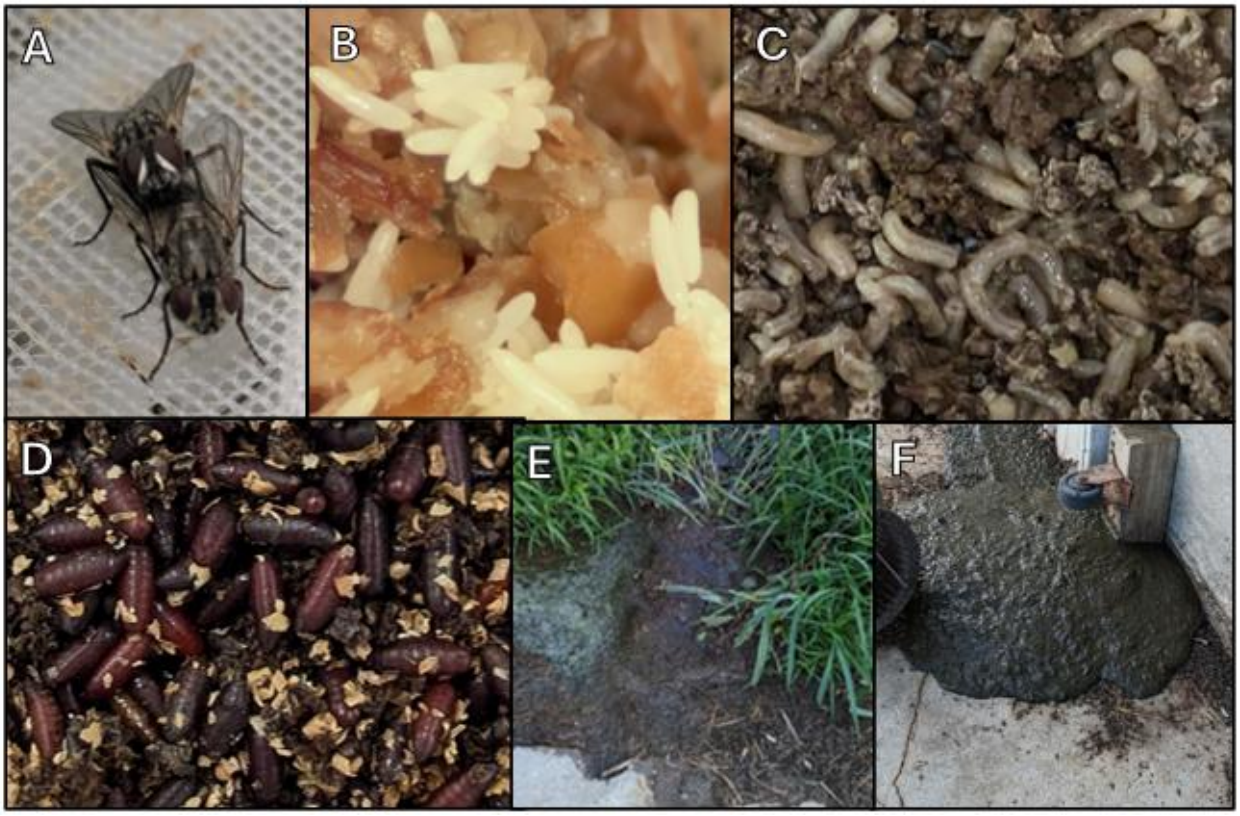


Figure 1.2. House fly biology and breeding sites A- Adult house flies mating; B- eggs deposited on a moist substrate; C- larvae within a moist decomposing substrate; D- house fly pupae; E&F- breeding and oviposition sites found within food animal production sites.

Mechanical pathogen transmission by house flies

House flies serve as mechanical vectors either through feeding or landing on contaminated surfaces, they can successfully transfer pathogens from surface to surface. The first and possibly most medically important instance of this transmission was an outbreak of typhoid in the late 19th century, where house flies were identified as the main driving force of spreading the disease. Even though sanitation in the US has evolved since then, the World Health Organization (WHO) still estimates 110,000 people die globally every year from typhoid as of 2019. In addition to typhoid, house flies have been implicated in the mechanical transmission of numerous other bacterial, viral, and parasitic pathogens affecting both humans and livestock.

Their sponging mouthparts, tarsal pads, and body hairs readily pick up microorganisms from fecal matter, garbage, and decaying organic material, which are then deposited on food, water, and animal wounds during subsequent visits (Graczyk et al., 2001). Pathogens such as *E. coli*, *Shigella spp.*, and *Campylobacter jejuni* have been isolated from flies collected near farms and markets, highlighting their role as bridge vectors between contaminated environments and susceptible hosts (Meerburg et al., 2016). In some cases, flies can regurgitate or defecate on food surfaces, introducing pathogens directly into potential transmission pathways (Greenberg, 1973). Studies have also shown that house flies can harbor infectious organisms internally for several days, allowing for dispersal over significant distances as adults are highly mobile. This ability to transport viable pathogens contributes to outbreaks of diarrheal diseases, salmonellosis, and cholera in regions with inadequate waste management and poor food hygiene practices (WHO, 2020).

Their role as mechanical vectors, therefore, makes house fly control a critical component of integrated strategies to reduce the burden of enteric diseases globally. In CAFOs flies have been implicated in the transmission of avian *E. coli* strains that cause colibacillosis, a leading cause of mortality in broilers. Similarly, in cattle operations, flies can exacerbate the spread of pinkeye (*Moraxella bovis*) and mastitis-associated bacteria that would be slower in the absence of flies. In swine production facilities, flies can carry and spread *Lawsonia intracellularis*, a pathogen associated with swine dysentery and proliferative enteropathy. The interactions between house flies and livestock go beyond simple nuisance, they are key players in the ecology of disease within animal production systems. The constant cycling of pathogens between animals and the environment via fly movement compromises biosecurity and contributes to antibiotic use, further accelerating antibiotic resistance issues.

Modern Control Methods

Due to their persistent nature, house flies are widespread and present at almost all livestock production facilities. One of the most common methods being chemical control in the form of insecticides. Producers are urged to use Integrated Pest Management (IPM) strategies to attack pest species at different life stages and locations. These IPM strategies include cultural/sanitation, mechanical habitat removal, biological control, and finally pesticide use. Historically, utilizing all four tiers of IPM is effective, but consistent efforts become expensive and laborious, leading to producers relying solely on chemical control. While each control method on its own is better than none, the conjunction of several methods yields the best insect control results.

Chemical Control

In many CAFOs, chemical control remains a cornerstone of house fly management, particularly in high-density livestock operations. Spot treatments, baits, and residual sprays are commonly deployed to target areas where adult flies rest and congregate, such as walls, fences, and manure storage structures. However, the ease of access and extensive use of insecticides in these CAFO settings has led to widespread insecticide resistance in house flies, particularly to organophosphates, pyrethroids, and neonicotinoids, complicating management (Kaufman et al., 2010).

Rotation of insecticide classes is critical to slow the development of resistance, which has been documented in *M. domestica* across multiple active ingredients (Kristensen & Jespersen, 2008). Fluralaner, a chemical that historically has not been registered for use as house fly

control, was used on a fly colony to select for 11,000-fold resistance compared to the susceptible strain in just 4 generations (Norris et al. 2023). Although EXZOLT, an oral treatment for mites in poultry, was approved for use in July 2025, Norris et al. were able to make this resistant selection in house flies in 2023. This highlights the adaptability of house flies to overcome new advances in insecticides.

While an important component of fly control, overreliance on insecticides without concurrent habitat management can result in rapid population rebound and diminished long-term control (Gerry, 2020). Resistance to a broad range of insecticides are often the result of a point mutation at the insecticide's point of action, or enzyme detoxification. Historically, resistance traits for pyrethroids such as *kdr, his-kdr,* and *skdr* are very well documented in house flies across the continental US (Mertz et al., 2023) after more than 35 years of consistent use. These point mutations change the active site of the voltage gated sodium channels within the house fly and allows their nerves to function normally in the presence of pyrethroids and organochlorines.

Similar point mutations have evolved to combat organophosphates and carbamates which bind to acetylcholinesterase (ACHE) an essential enzyme for proper nerve function (Liming et al., 2006). In resistant fly populations, the Esteratic binding site of ACHE is altered to combat the presence of these insecticides. Both glutathione-S transferases (GSTs) and cytochrome P450s (CYPs) are enzymes that play roles in the detoxification of xenobiotics within the house fly body. GSTs play a major role in the detoxification of both carbamates and organophosphates by conjugating the molecule with glutathione. CYPs are a large superfamily of genes, with 146 genes in the house fly genome. Specifically, CYP 4, 6, and 9 families are associated with environmental response and resistance to insecticides like permethrin and DDT (Li et al., 2023).

In addition to protecting the fly from insecticides, these enzymes can metabolize endogenous compounds, plant allele chemicals, and other xenobiotics they may encounter.

Larval Habitat Removal

Mechanical strategies are designed to physically disrupt breeding and resting sites of house flies, thereby reducing larval development and adult survival. These approaches include routine manure removal, aeration of bedding, and improved drainage to eliminate moist substrates conducive to larval growth (Imai, 1984). Physical exclusion techniques, such as installing screens on windows and vents, can limit adult entry into animal housing. Traps baited with attractants or sticky ribbons can help capture adult flies and reduce local populations when used in conjunction with other management tactics. Additional use of sticky traps could assist producers in estimating the fly population sizes within a certain area using the methods described in Anderson and Poorbaugh 1964. While physical control or habitat removal has been considered the most effective fly reduction method, results vary across treatment areas. These measures are most effective when integrated into a broader sanitation program and maintained consistently to prevent re-establishment of fly populations.

Biological Control

Biological control exploits the natural enemies of house flies to suppress populations below economic thresholds. Parasitoid wasps, particularly species in the genera *Muscidifurax* and *Spalangia*, are widely used to attack fly pupae, reducing adult emergence, and reducing stress on livestock (Rutz & Patterson, 1990). Predatory beetles and mites can also play a role in reducing larval numbers when manure is managed to favor their presence (Kaufman & Rutz, 2012). Augmentative releases of these beneficial organisms can enhance natural population

regulation, particularly during warm months when fly breeding rates are highest. Successful biological control programs rely on minimizing broad-spectrum insecticide applications that would otherwise harm beneficial insect populations and disrupt ecological balance (Geden & Hogsette, 2001).

Concluding Remarks

CAFOs concentrate large numbers of animals and generate substantial waste, creating conditions that promote the heavy use of antibiotics. These practices not only contribute to the accumulation of antibiotic residues in the environment but also expose house flies, prolific pests in manure-rich habitats, to these compounds. While adult flies are well recognized as mechanical vectors of pathogens and possess strong detoxification abilities, the effects of larval antibiotic exposure on their development, microbial associations, and detoxification potential remain poorly understood. This gap is particularly significant given the possibility that flies may both carry antibiotic residues and contribute to the spread of antimicrobial resistance beyond livestock operations. Accordingly, this thesis addresses larval development in this habitat, advancing our understanding of how antibiotic use in CAFOs intersects with house fly biology and informing broader efforts to manage pests, safeguard animal production, and mitigate public health risks.

Chapter 2 - Impacts of antibiotics on *Musca domestica* larval development

Introduction

House flies (*Musca domestica* L.) are synanthropic insects that thrive in livestock production systems, particularly confined animal feeding operations (CAFOs), where manure and organic waste provide abundant breeding sites. These facilities create favorable conditions for larval development by maintaining moist, nutrient-rich substrates and high microbial loads (Schmidtmann & Martin, 1992; Nayduch et al., 2023). While house fly larvae contribute to organic matter decomposition, they also facilitate pathogen persistence and amplification within these systems by supporting adult fly populations that act as mechanical vectors of numerous bacterial, viral, and parasitic pathogens (Hald et al. 2008). Adult flies readily acquire microbes on their mouthparts, tarsi, and body hairs when feeding or resting on contaminated surfaces, and can subsequently deposit these organisms onto feed, water, or animal wounds (Graczyk et al., 2001). Pathogens such as *Escherichia coli*, *Salmonella spp.*, and *Campylobacter jejuni* have been isolated from flies in livestock facilities, linking them to the transmission of enteric diseases in both animals and humans (Meerburg et al., 2016). Thus, house fly ecology in CAFOs intersects directly with animal production efficiency, biosecurity, and public health.

The larval environment in CAFOs is more than just manure; it is a chemically complex habitat shaped by routine agricultural practices that can affect house fly populations. Antibiotics administered to livestock can be excreted unmetabolized, accumulating in waste at sufficient concentrations to alter microbial communities (Hu et al., 2010; Heuer et al., 2011). Such residues can suppress decomposition, shift bacterial community composition, and change the quality of microbial food available to larval house flies (Schmidtmann & Martin, 1992; Fang et al., 2025;

Li et al., 2023). At the same time, insecticides and insect growth regulators (IGRs) are widely deployed for house fly management, and their residues can persist in larval substrates (Vakhidova and Ulmasov 2024). Sublethal exposures during larval development can generate oxidative stress, reduce survival, prolong development, and negatively affect the reproductive capacity of adults (Donahue et al., 2017; Khan, 2023). Together, antibiotics and insecticides represent major chemical stressors shaping the ecology of larval house flies in livestock facilities.

Insects and microbes are not the only organisms affected by consistent overuse of antibiotics in CAFOs. Persistence of antibiotics like enrofloxacin and tetracycline is well documented in both soils and freshwater sources surrounding these operations. Enrofloxacin was added to soil samples and found persistent at 55.8–107.1 ng/g of soil following 133 days of observation (Strando et al. 2022). When excreted, 25-70% of the treatment amount of tetracycline is excreted from animals and up to 75-90% according to monitoring data in the US and Denmark (Jjemba 2006; Halling-Sørensen 2000). As a consequence, tetracycline residues are found in wastewater, streams, and soils around the world even outside of CAFOs (Koplin et al., 2002; Miao et al., 2004; Deblonde et al., 2011; Arikan et al., 2008). The presence of enrofloxacin is more infrequent globally due to the differing regulations regarding its use in agricultural practices. Nonetheless, it is present at 2.4 μg/L in rivers and as high as 8.77 μg/L in animal wastewaters in China (Mei et al., 2012). Through widespread use over the last century, antibiotics are present all over the globe, as well as their detrimental effects on the organisms and mediums they occupy.

While adult house flies are well recognized for their ability to develop resistance to insecticides (Kaufman et al., 2010; Zhang et al., 2013), much less is known about how exposure

to other chemical stressors during the larval stage influences survival, development, and fitness. This gap is significant because larval exposure may not only determine population dynamics but also influence microbial associations and resistance mechanisms that persist into adulthood. Furthermore, environmental variation between production systems, such as beef versus dairy for example, may create distinct selective pressures on local fly populations, resulting in differences between field populations and laboratory colonies that have not been fully explored.

Accordingly, the purpose of this study was to evaluate how house fly larvae respond to chemical stressors, like antibiotics, that may be present in typical CAFO environments. Laboratory colony flies and wild populations collected from a university beef and dairy operation were exposed to two commonly used antibiotics during larval development. By comparing larval development and survival after exposure to antibiotics, this study aims to enhance our understanding of how environmental exposures influence house fly ecology and resilience, with implications for pest management strategies and the broader issue of antimicrobial resistance in livestock systems.

Materials and Methods

Colony History and Rearing

The Carolina 2024 (CR) house fly colony was raised from pupae purchased from Carolina Biological Supply, Burlington, NC, in 2024. These flies were considered our control strain since they have not been exposed to the selective pressures that are present in and around animal production facilities. Carolina colony of house flies were maintained at ~300 adults per cage (1 ft^3) at 28°C, 50 ± 5% RH, and a photoperiod of a photoperiod of 14:10h (L:D). Flies were provided with sugar, low-fat milk powder, and beef liver powder diet *ad libitum* with water

available via a cup with a cotton wick. Egg laying media (30g) containing wheat bran (11.4g), Nutrena SafeChoice pelleted horse food (4.3g), and water (14.3mL) was put into a weighing boat and added weekly to adult fly cages. Media was removed after 16 hours and transferred to larval media containing the same ratio of ingredients. Larvae were grown in aluminum rearing pans (9x9x3 inches), covered by cotton pillowcases, and stored at 28°C, 50 ± 5% RH, and a photoperiod of 14:10h (L:D). Pupae were removed from the larval media and placed in a clean cage with food and water. Three days after eclosion, egg laying media was offered to adults. For egg use in this study, egg laying substrate containing a moistened paper towel with yeast and milk powder was used to reduce possible egg destruction caused by transfer from egg laying media to experiments.

Field colonies were collected from a dairy and feedlot. The dairy (DR) line of adults was collected from the Kansas State University Dairy Teaching and Research Center, Manhattan, KS, in 2025. Flies (~200) were collected directly from the field prior to each assay. Adults were sweep-netted from feed bunks and immediately placed into cages with food and water *ad libitum*. The beef stocker (ST) line of flies was collected in the same fashion as the DR line from the Kansas State University Beef Stocker Unit in Manhattan, KS, in 2025. Wild caught flies were provided with an egg laying substrate containing a moistened paper towel with yeast and milk powder 3 days post-collection.

Larval Antibiotic Exposure

To assess the effects of antibiotics on larval development, three biological replicates were conducted for each treatment of antibiotics with each fly colony. The antibiotics used in this study were enrofloxacin and tetracycline, selected for their different antibacterial modes of

action. In bacteria, tetracycline targets the 30S subunit of the ribosome, and enrofloxacin targets DNA gyrase and topoisomerase IV. Antibiotic concentrations were selected based on global results of antibiotics found in manure within CAFOs. For tetracycline environmental residues of approximately 43.5 mg/kg have been observed (Hu et al., 2010; Daghrir and Drogui, 2013). Enrofloxacin environmental concentrations of approximately 10.5 mg/kg have been observed (Zhao et al. 2010). In addition to testing these concentrations, 10 times and 100 times increased concentration of the environmental concentration was tested, as well as a combination of both antibiotics together but at half concentration (Table 2.1).

Table 2.1. Antibiotic treatments and their respective concentrations, reported in mg/kg

	Control	*Environmental*	*10X Env*	*100X Env*
Tetracycline	0	43.6	436	4360
Enrofloxacin	0	10.5	105	1050
Tetracycline: Enrofloxacin	0	21.8:5.25	218:52.5	2180:525

Following successful egg collection from adult colony cages, the wheat bran and horse pellets were autoclaved to ensure sterility. Treatment media were prepared in sterile cups to a final weight of 30 g per cup and mixed thoroughly to ensure homogeneity and avoid compaction. Treatment volumes of the respective antibiotics were applied to each cup, using prepared tetracycline and enrofloxacin stock solutions. Approximately 30 eggs were applied to each experimental unit. Experimental units were covered with vented lids and wrapped in aluminum foil to prevent antibiotic degradation by light. All treatments were incubated at 28°C at a humidity of 50 ± 5% and monitored throughout the developmental period. Individual weights were measured at the second and third instars, as well as 1-day post-pupation. The timing of initial and final pupation was recorded. Once pupation was complete, at least 10 pupae per

experimental unit were weighed and transferred to emergence cages maintained in the insectary. Biological replicates were conducted in triplicate for each antibiotic treatment using eggs from a single egg-laying event within each respective fly strain. A two-way ANOVA was preformed to examine the effects of each treatment and the untreated control within each replicate across all life stages and colony strains. All data analyses were conducted using GraphPad Prism (version 10.6.1 for Windows, GraphPad Software, Boston, MA).

Bacterial Community Analysis

Media was collected during second and third instar development weight measurements. Media (0.1 g) was removed and added to sterile 1X phosphate buffered saline (PBS) rehydrated at 1 pellet per 100mL of water and vortexed. Ten-fold serial dilutions were performed until a dilution of 10^{-10} with PBS was achieved. Plates were incubated at 37ºC overnight before counting colonies. Colony morphotypes were further grown on blood agar plates and submitted to the Kansas State University Veterinary Diagnostic Laboratory (Manhattan, KS, USA) for bacterial identification via Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass spectrometry (MALDI-TOF MS).

Results

Antibiotic Exposure Bioassays

Second instar larvae treated with tetracycline for the CR strain showed significant reductions in weight at the highest antibiotic exposure for each. Untreated larvae had an average weight of 10.1 mg (SD = 0.9), while tetracycline-treated larvae weighed on average 6.1 mg (SD = 1.1) (Figure 2.1). Although weight reductions were observed at 43.6 mg/kg and 436 mg/kg treatments, these were less pronounced and not statistically significant. Similarly, enrofloxacin treatment at the highest concentration (1050 mg/kg) experienced a significant reduction in

weight. Control larvae weighed 10.6 mg (SD = 0.5), whereas enrofloxacin treated larvae weighed 5.6 mg (SD = 0.5) ($p<0.01$). Combinations of 2180 mg/kg tetracycline with 525 mg/kg enrofloxacin resulted in the greatest weight reduction, 2.8 mg (SD = 1.0) compared to control 10.6 mg (SD = 0.4) ($p<0.0001$). While the CR strain's larval growth was significantly impacted at the second instar, individuals of the DR and ST field-collected flies showed no significant reductions in weight, except in the DR strain at the highest concentration of enrofloxacin (1050mg/kg), where control larvae weighed 10.3 mg (SD = 0.5) and treated larvae weighed 8.9 mg (SD = 0.7) ($p<0.05$).

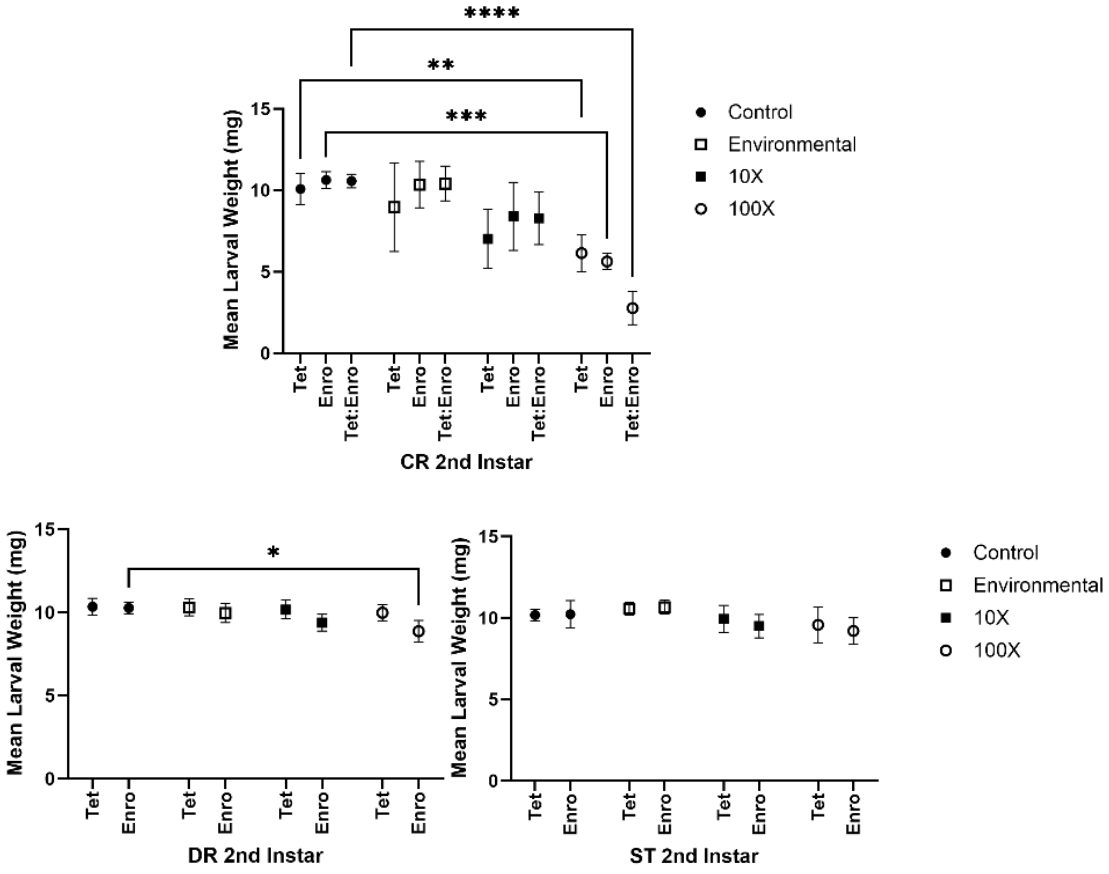


Figure 2.1. Mean second instar larval weight significantly reduced in laboratory strain with antibiotic exposure. Significance levels annotated as * = $p<0.05$, ** = $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$ (Two-way ANNOVA).

Responses in the third instar CR individuals showed a wider range of weight reductions across treatments (Figure 2.2). Tetracycline at the 436 and 4360 mg/kg treatments showed

significant decreases in larval weight, 13.8 mg (SD = 1.0) ($p < 0.001$) and 12.5 mg (SD = 1.8) ($p < 0.0001$) respectivley, compared to the control at 23.4 mg (SD = 3.0). Enrofloxacin treatment at 1050 mg/kg resulted in a weight reduction to 0.0 mg (SD=0.0) ($p < 0.0001$) compared to the untreated larvae at 21.6 mg (SD= 2.2). Tetracycline:enrofloxacin combination treated larvae at 218:52.5 and 2180:525 mg/kg weighed 15.2 mg (SD = 3.3) ($p < 0.05$) and 0.0 (SD = 0.0) ($p < 0.0001$) compared to the untreated at 22.0 mg (SD = 2.6). In a similar fashion observed in the second instar, DR and ST strains showed a lack of significant weight reductions across any antibiotic treatments.

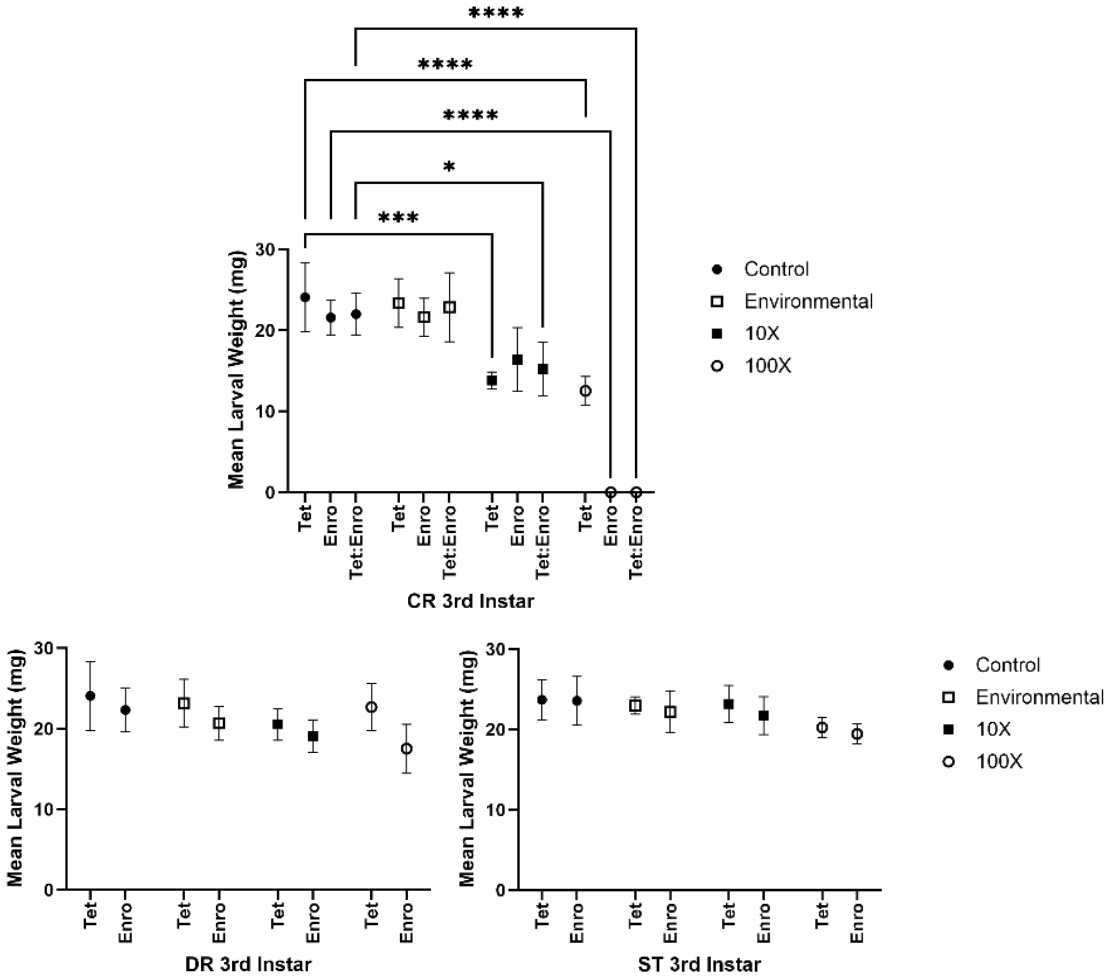


Figure 2.2. Mean third instar larval weight significantly reduced in laboratory strain with antibiotic exposure. Significance levels annotated as * = p<0.05, ** = p<0.01, ***= p<0.001, ****= p<0.0001 (Two-way ANOVA).

Pupal weights were significantly impacted at all 10x and 100x levels within the CR strain (Figure 3.3). Tetracycline treated individuals at 436 and 4360 mg/kg concentrations showed reduced weights of 12.8 mg (SD = 1.1) ($p < 0.01$) and 6.1 (SD = 5.3) ($p < 0.0001$), compared with control pupae averaging 21.3 mg (SD = 2.4). Pupae derived from enrofloxacin treated larvae withn 105 and 1050 mg/kg treatments weighed 14.6 (SD = 3.8) ($p < 0.05$)and 0.0 mg (SD = 0.0) ($p < 0.0001$), representing a significant weight reduction compared to untreated larvae at 20.6 mg (SD = 1.7). Tetracycline:enrofloxacin combination treated pupae at 218:52.5 and 2180:525 mg/kg resulted in reduced pupal weight, 13.5 mg (SD = 3.4) ($p < 0.01$) and 0.0 mg (SD

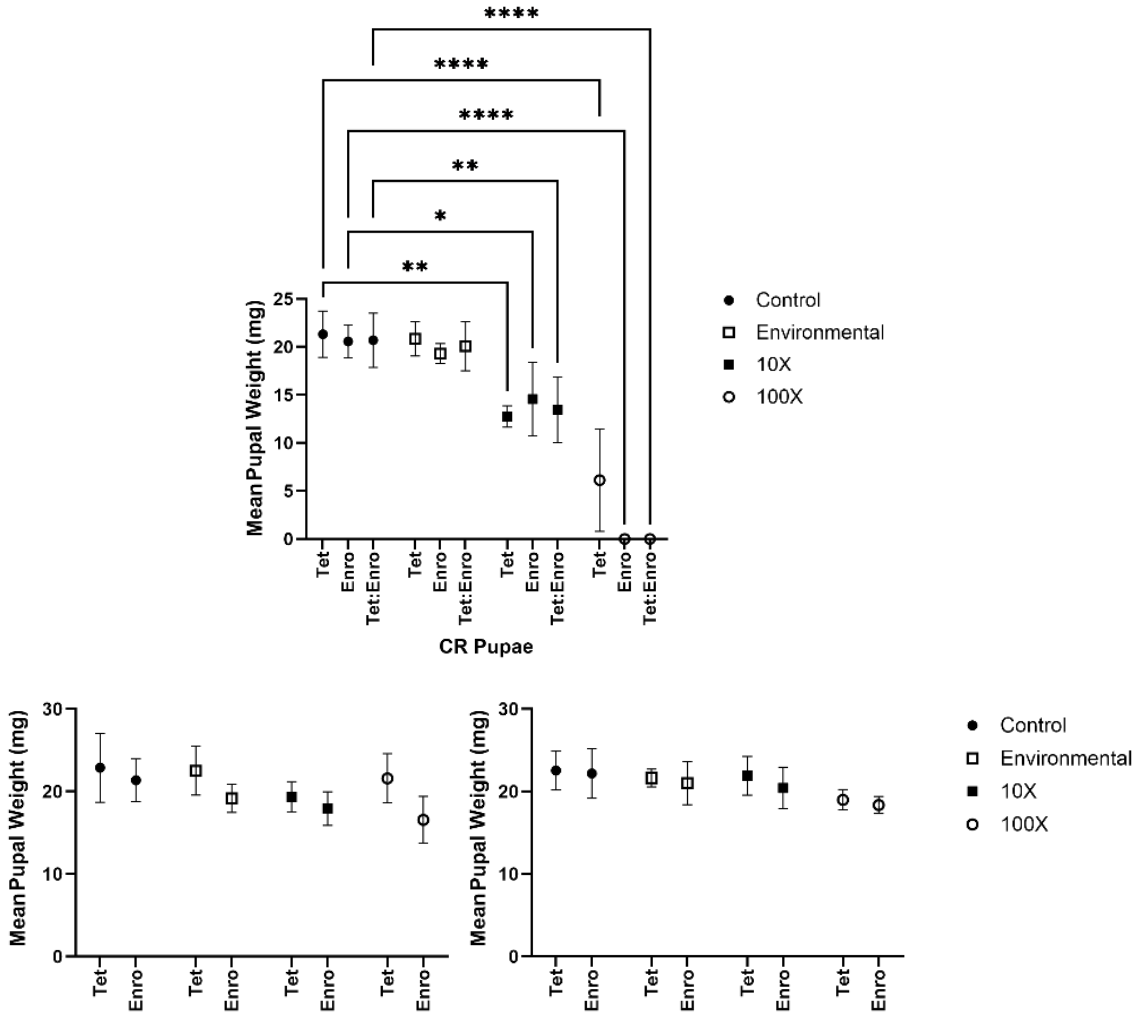


Figure 2.3. Pupal weight 1-day post-pupation significantly reduced in laboratory strain after antibiotic exposure. Significance levels annotated as * = $p < 0.05$, ** = $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$ (Two-Way ANOVA).

= 0.0) ($p < 0.0001$) compared to the controls at 20.7 mg (SD = 2.8). As with larval weights, DR and ST individuals showed no significant differences in pupal weight across treatments.

Although larval exposure to antibiotics carried forward into reduced pupal weights, eclosion rates were largely unaffected by previous antibiotic exposure (Figure 2.4). An exception occurred in the highest antibiotic treatments where tetracycline treatment (4360 mg/kg) resulted in 55.0 % (SD = 47.7) and enrofloxacin (1050 mg/kg) and combined tetracycline:enrofloxacin exposure (2180:525 mg/kg) resulted in no adult emergence. Control groups exhibited high eclosion success, at 96.7 (SD = 2.9) for tetracycline, 96.7 (SD = 2.9) in enrofloxacin replicates, and 98.3 (SD = 2.9) for the combined antibiotic repliciates. Successful eclosion occurred in all DR and ST treatments, with a total eclosion percentage of 93.2 (SD = 6.5) with no significant differences across antibiotic exposures.

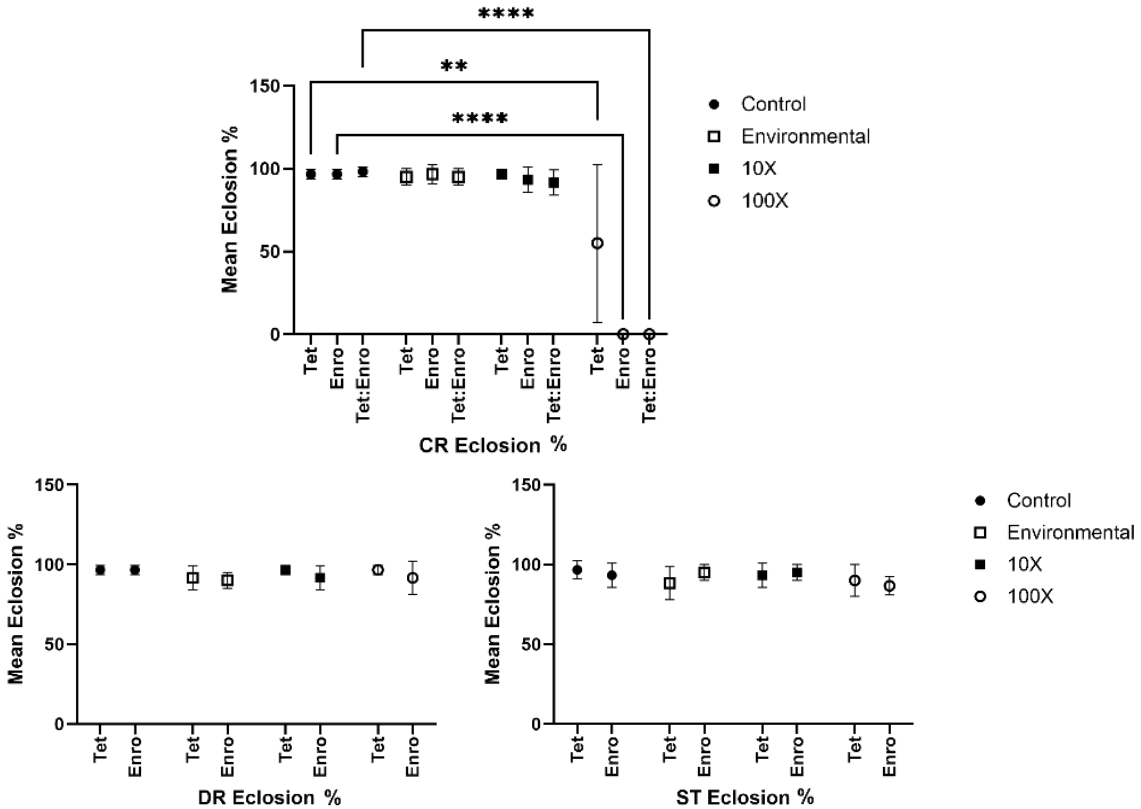


Figure 2.4. Laboratory strain eclosion rates unneffected by prior larval exposure to antibiotic except at the highest concentration. Significance levels annotated as * = p<0.05, ** = p<0.01, ***= p<0.001, ****= p<0.0001 (Two-Way ANOVA).

Bacterial Community Analysis

Neither enrofloxacin nor tetracycline exposure significantly altered the number of colony forming units (CFU) on plates (Table 2.2 and 2.3), nor were differences observed in overall bacterial species diversity across concentrations. To ensure microbial food source abundance was negatively impacted by antibiotic exposure, samples were taken from media and microbial communities were identified. Four selected morphotypes were identified through MALDI-TOF MS as *Providencia stuartii*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Staphylococcus gallinarum*. These taxa were consistently detected, after blood agar selection, in the bioassays regardless of antibiotic exposure.

Table 2.2. CFU counts and CFU/ml

	Control	*Env*	*10X Env*	*100X Env*	*Control CFU/mL*	*Env CFU/mL*	*10X CFU/mL*	*100X CFU/mL*
Tetracycline	836	696	601	672	8.36E+13	6.96E+13	6.01E+13	6.72E+13
Enrofloxacin	315	489	328	438	3.15E+13	4.89E+13	3.28E+13	4.38E+13
Tetracycline: Enrofloxacin	751	656	659	702	7.51E+13	6.56E+13	6.59E+13	7.02E+13

Discussion

Larval growth was negatively impacted by exposure to antibiotics in the Carolina laboratory colony. This response was seen to be dose dependent with larval weights being more impacted as antibiotic concentrations increased. Similarly, pupal weights were negatively impacted by antibiotic exposure, most likely a carryover from the reduced larval weights. This impact was not observed in the field caught flies from the ST or the DR fly strains indicating that sufficient food resources were available even under antibiotic treatment. Four bacterial species (*Providencia stuartii*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Staphylococcus gallinarum*) were consistently present, and colony abundances were not altered by antibiotic exposure. This

result suggests that the antibiotics are negatively impacting the CR strain, while strains ST and DR consisting of field collected flies may have a coping mechanism to nullify the toxic effects of the antibiotics. Overall, eclosion rates were unaffected. This may be because pupa were removed from the larval substrate and no longer exposed to antibiotics.

The differential responses of the CR, DR, and ST strains to antibiotic exposure may reflect their distinct ecological histories and levels of environmental adaptation. The CR strain, originating from a long-term laboratory colony unexposed to the chemical and antibiotic pressures typical of animal production systems, exhibited pronounced sensitivity to both tetracycline and enrofloxacin across all developmental stages. Significant weight reductions were observed in second and third instar CR larvae at higher tetracycline concentrations (notably 4360 and 436 mg/kg) and at elevated enrofloxacin levels (105 and 1050 mg/kg), with combination treatments producing the most pronounced effects. In some cases, no pupa were collected, most likely due to the larva dying. In contrast, the DR and ST strains showed considerable tolerance to antibiotics, showing no significant reductions in larval, pupal, or adult. The DR strain demonstrated a minor weight reduction in the second instar to enrofloxacin at the highest concentration. The lack of measurable effects in the DR and ST strains likely reflects prior adaptation to chronic, antibiotic exposure common in animal production environments, resulting in either physiological tolerance or the establishment of antibiotic-resistant gut microbiota. A diverse gut microbiome containing AMR bacteria would allow individual larvae to consume antibiotic laden food sources without the risk of those antibiotics damaging their microbiome. Collectively, these findings suggest that antibiotic exposure strongly influences larval development and may predispose adults to be resilient to other chemical stressors like insecticides.

These findings align with broader evidence from other immature insects inhabiting chemically stressful environments that show increased resilience as adults. Mosquitoes exposed to insecticides in polluted breeding habitats, cockroaches from pesticide-rich urban waste environments, and beetles associated with treated grain all exhibit reduced susceptibility to chemical stressors through both physiological resistance and microbiome-mediated tolerance (Zhu et al., 2016; Nyka et al. 2016; Siddiqui et al., 2022). In a similar vein, house flies being utilized for vermicomposting show an increase in gene expression of genes related to metabolic pathways and detoxification mechanisms (Li et al., 2021). House flies developing in livestock waste are exposed not only to antibiotics directly but also to resistant microbes that thrive under chronic antibiotic pressure. Beyond their implications for insect physiology, these results highlight the broader ecological and public health risks of agricultural antibiotic use.

Conclusions

Antibiotic exposure affected larval development in *Musca domestica*, with the laboratory colony showing the greatest sensitivity. Enrofloxacin prevented pupation entirely, while tetracycline reduced larval and pupal mass in a dose-dependent manner. Field-derived populations in contrast, maintained the ability to reach third instar, pupate, and emerge as adults, even under high antibiotic concentrations, indicating reduced susceptibility likely shaped by prior environmental exposure.

Bacterial analysis confirmed that larval growth suppression in the laboratory strain should not be attributed to reduced microbial nutrition availability. Differences in development are more likely driven by physiological tolerance mechanisms in wild caught flies which are undeveloped in the laboratory colony.

These results demonstrate that house flies from livestock environments can withstand antibiotic stress that impairs naïve laboratory colonies. This resilience has implications for pest management and highlights the potential for stable microbial communities to act as reservoirs of AMR genes, which mobile adult flies can disperse beyond CAFOs, bridging agricultural and human environments.

Chapter 3 - Transcriptomic analysis of *Musca domestica* larvae under antibiotic stress

Introduction

House flies (*Musca domestica* L.) are ubiquitous synanthropic insects that flourish in livestock production systems, especially in confined animal feeding operations (CAFOs), where animal waste and organic byproducts provide ample breeding substrates. Manure, bedding, and organic spoilage represent moist, nutrient-rich, microbially dense habitats, ideal for larval growth (Schmidtmann & Martin, 1992; Nayduch et al., 2023). While larvae play a role in decomposition and nutrient cycling, the adult flies carry considerable epidemiological significance: they act as mechanical vectors of bacteria, viruses, and parasites by acquiring microbes on mouthparts, tarsi, and integument, and depositing them onto feed, water, or animal wounds (Graczyk et al., 2001). In livestock systems, house flies have been implicated in the transmission of important enteric pathogens such as *Escherichia coli*, *Salmonella* spp., and *Campylobacter jejuni* to animals and humans (Meerburg et al., 2016).

The larval environment in such systems is not just biologically complex but contain chemicals associated with routine agricultural practices. Antibiotics administered to food animals are frequently excreted in unmetabolized or partially metabolized forms, accumulating in manure, lagoon effluent, and surrounding soils (Hu et al., 2010; Heuer et al., 2011). These residues can perturb microbial community composition, reduce decomposition rates, and alter the nutritional quality or microbial food available to larvae (Schmidtmann & Martin, 1992; Fang et al., 2025; Li et al., 2023). Simultaneously, residues of insecticides or insect growth regulators (IGRs) used against flies or other pests may persist in larval media (Vakhidova & Ulmasov, 2024). Sublethal exposures to these chemicals during development have been shown to generate

oxidative stress, extend developmental time, reduce survival, and impair adult reproductive potential in insects (Donahue et al., 2017; Khan, 2023). In this context, antibiotics and insecticidal residues act as co-occurring chemical stressors that may shape the developmental physiology, microbial associations, and evolutionary trajectories of larval flies.

Among antibiotic classes frequently detected in agricultural settings, tetracycline and enrofloxacin are of special interest due to their widespread use and distinct modes of action. Tetracycline's mechanism of action (MOA) involves binding to the 30S ribosomal subunit, blocking access of aminoacyl-tRNA to the ribosomal A-site and inhibiting protein chain elongation. In non-target eukaryotic species, tetracycline can similarly disrupt mitochondrial protein synthesis (Moullan et al., 2015), presumably since mitochondrial ribosomes are evolutionarily derived from bacterial ancestors. Inhibition of mitochondrial translation impairs oxidative phosphorylation and may provoke mitochondrial stress responses. Additionally, tetracycline exposure can perturb host gut microbiome composition, altering nutrient exchange, immune signaling, and metabolic homeostasis (Jaroszewski et al. 2023).

Enrofloxacin, a fluoroquinolone antibiotic, exerts its primary MOA by inhibiting DNA gyrase and DNA topoisomerase IV in bacteria, preventing the re-ligation of DNA strands halting DNA replication, transcription, and repair. In non-target organisms, enrofloxacin exposure has been associated with DNA damage, induction of oxidative stress, and mitochondrial dysfunction via reactive oxygen species (ROS) production (Liu et al., 2015). Such ROS generation can damage lipids, proteins, and mitochondrial membranes, potentially triggering apoptosis or necrosis in susceptible cells. Enrofloxacin has also been shown in some systems to cause mtDNA breaks or impair mitochondrial biogenesis under stress (Grabowski et al., 2022).

Despite the theoretical plausibility of these off-target impacts, surprisingly few studies have directly examined the molecular and transcriptomic consequences of antibiotic exposure in insects. A noteworthy exception is Li et al. (2021), who performed combined transcriptome and microbiome profiling of larval house flies treated with antibiotics (tetracycline, gentamicin, and streptomycin). That study revealed significant shifts in gut microbiota and identified differentially expressed genes associated with immunity, detoxification, and metabolic processes (Li et al. 2021). Beyond *M. domestica*, studies in other insects (e.g., *Bactrocera dorsalis*) have shown that antibiotic feeding can reduce gut symbiont diversity and modulate expression of immunity genes, including peptidoglycan recognition proteins and antimicrobial peptide pathways (Fu et al., 2021). Overall, the field remains largely underexplored: the frequency, amplitude, consistency, and functional consequences of antibiotic-induced transcriptomic changes across insect taxa are not well resolved (Archer et al. 2025).

Given this research gap, especially in the context of ecologically relevant exposures, our study aimed to characterize the transcriptomic response of *M. domestica* larvae to tetracycline and enrofloxacin at both environmentally relevant and elevated concentrations. In doing so, we hope to extend the finding of Li et al. 2021 and to provide a foundation for interpreting potential long-term evolutionary or ecological consequences of environmental antibiotic contamination in insect populations.

Materials and Methods

Fly history and rearing conditions

This study utilized the *M. domestica* lab colony (CR) maintained at Kansas State University in Manhattan KS, generated from the Carolina Biological Supply©, Burlington, NC *M. domestica* CR strain in 2023. Egg clutches were placed on larval media with one of four

antibiotic treatments or an untreated control. Five biological replicates were conducted with egg clutches from separate colonies of the CR line. Treatments of antibiotics were administered at either environmentally relevant concentrations or ten times that. Enrofloxacin was administered at 10.5 (EE) or 105 mg/kg (EX), and tetracycline at 43.6 (TE) or 436 mg/kg (TX) to the media prior to the addition of eggs. Larval media contained wheat bran, horse food, and water at a ratio of 10:3:6 ml, and approximately 30 eggs in 30 grams of media. Biological replicates were stored in an Isotemp Incubator (Fisher Scientific) at 28°C at a RH of 50 ± 5%. Development and conditions were monitored throughout the duration of the experiments to ensure identical treatment for all replicates.

RNA Extraction

Three individuals from each experimental unit were removed at the 2nd instar, pooled, and homogenized using 500 µL of TRIzol and RNase free pestles. Samples were centrifuged to separate body solids from solution, mixed with 0.2X 1-bromo-3-chloropropane, centrifuged at 13,000 RPM for 15 minutes, and supernatants were transferred to new nuclease free tubes. TRIzol suspended samples were treated according to manufactures protocol, including the optional on column DNase treatment to prevent DNA contamination during subsequent steps. Total RNA was extracted from each sample using the RNA Purification protocol from the Direct-zol™ RNA Miniprep Kit (Zymo Research, Irvine, CA). RNA was eluted in 100 µl of DNase/RNase-free water, then stored at −80 °C. RNA purity was evaluated using a NanoDrop 2000 Spectrometer (Thermo Fisher Scientific, Waltham, MA) and RNA concentrations were quantified using a Qubit Flex fluorometer (Thermo Fisher Scientific).

Library Preparation

mRNA-enriched libraries were generated with the Watchmaker mRNA library preparation kit (Watchmaker Genomics, Boulder, CO) according to the manufacturer's protocol. Samples from five replicates across five treatments—EE, EX, TE, TX, and control—were processed separately (N = 5). mRNA was isolated using mRNA capture beads, fragmented, and converted into cDNA through reverse transcription. Stubby adapters (Element Biosciences, San Diego, CA) were attached to the cDNA fragments and subsequently purified using Ampure XP beads (Beckman Coulter, Brea, CA). Library amplification incorporated indexes through 10 PCR cycles, with two extra cycles included to accommodate the selected insert size of 290–310 base pairs. The resulting libraries were assessed for fragment size using an Agilent 4150 TapeStation and a D5000 reagent kit (Agilent). Concentrations were determined via a Qubit Flex fluorometer and the 1×dsDNA High Sensitivity kit (Thermo Fisher Scientific). Sequencing was carried out on an Element Aviti platform (Element Biosciences) using the AVITI 2×150 Cloudbreak sequencing kit (Element Biosciences).

Demultiplexing and Quality Control

Demultiplexing was carried out with bases2fastq (https://github.com/Elembio/bases2fastq-nf), which was implemented through Nextflow (Di Tommaso et al., 2017) and executed using Apptainer (Gregory M. Kurtzer et al., 2017). During this process, adapter trimming was activated and paired end reads of 150 bp were generated. To further remove Element adapters and low-quality regions from the raw data, the edwardbirdlab/element_cleanup pipeline (https://github.com/edwardbirdlab/element_cleanup) was employed. This workflow incorporated BBDuk v38.96

(https://sourceforge.net/projects/bbmap/) to identify and excise adapter sequences at any position within the reads using a k-mer–based detection method. Reads were then subjected to additional quality trimming and screened for residual adapter sequences using FastP v0.24.0 (Chen, 2023). For this dataset, trimming parameters required a minimum read length of 60 bp and a mean quality threshold of 20. Quality assessment summaries from FastQC v0.12.1 (Andrews, 2010), FastP, and BBDuk were aggregated and visualized through MultiQC v1.22.2 (Philip Ewels et al., 2016). Two outliers (one from each EE and EX) were removed from quality assessment summaries due to low quality libraries.

Differential Expression Analyses

RNA sequencing data were analyzed using the nf-core/rnaseq pipeline v3.18.0 (Harshil Patel et al., 2025), a standardized workflow designed for RNA-seq processing and quality control. The workflow was executed through Nextflow v24.04.2 in combination with Singularity v1.3.1. Initial quality inspection of raw reads was conducted with FastQC v0.12.1, followed by removal of low-quality bases using Trim Galore! v0.6.10 (Krueger et al., 2023). The filtered reads were then aligned to the *M. domestica* reference genome (M3 Strain) with eGAPx annotation employing STAR v2.7.11b (Dobin et al., 2013). Quantification of transcript abundance was performed using Salmon v1.10.3 (Patro et al., 2017), generating expression estimates. Comprehensive quality evaluations were carried out using multiple tools: RSeQC v5.0.2 (Wang et al., 2012) for read distribution and junction saturation, Qualimap v2.3 (García-Alcalde et al., 2012) for alignment quality and feature coverage, and dupRadar v1.32.0 (Sayols et al., 2016) to assess PCR duplication levels relative to expression. Differential expression analysis was conducted with DESeq2 v1.46.0 (Love et al., 2014) in R v4.2 (Core Team, 2021).

Transcript-level counts were aggregated to gene-level data using tximport v1.34.0 (Soneson et al., 2015) before being imported into DESeq2. Principal component analysis (PCA) was carried out using the prcomp function on rlog-transformed data from DESeq2. Pairwise Pearson correlation coefficients were computed using the cor function, and sample correlation heatmaps were visualized with pheatmap v1.0.12 (Kolde R, 2019). A Wald test was applied with the design formula ~ Treatment to assess differential expression between treated and control samples. Adjusted p-values were derived using the Benjamini–Hochberg correction to control for false discovery. Two significance cutoffs were used: a strict threshold, testing for expression changes equal to or exceeding an absolute $\log_2$ fold change (FC) of 0.58 (1.5-fold) under the null hypothesis $|\log_2 FC| < 0.58$ with adjusted $p < 0.05$; and a loose threshold, testing for any deviation from baseline expression ($|\log_2 FC| > 0$, adjusted $p < 0.05$). This dual approach resulted in two distinct categories of differentially expressed genes (DEGs).

Term Enrichment

To better understand the biological relevance of the DEGs, orthologs between *M. domestica* and *Drosophila melanogaster* were identified. Orthologs were identified by performing reciprocal blasts between *M. domestica* and *D. melanogaster* (GCF_000001215.4) roteins (longest isoform) using Diamond. Genes were called orthologous if they were determined to be reciprocal best hits between species, with a value $< x10^{-10}$. A genomic background set was constructed by including all genes tested in the RNA-seq experiments that had a corresponding *D. melanogaster* ortholog (8,621 out of 10,010 total tested genes). The eight gene sets, which included the significantly upregulated (adjp < 0.05 & $\log_2 FC > 0$) and downregulated (adjp < 0.05 & $\log_2 FC < 0$) genes from the four antibiotic experiments under the loose threshold, were

also converted to *D. melanogaster* orthologs. STRING v12.0 analysis (Szklarczyk et al., 2023) was attempted for all 40 gene sets using their *D. melanogaster* orthologs as input. Analyses were conducted through the STRING web interface (https://string-db.org) using default settings. Enrichment analysis was performed in STRING using the full set of 8,621 *D. melanogaster* orthologs as the background for statistical comparison.

Results

Demultiplexing and Quality Control

RNA sequence (RNA-seq) data were initially processed to remove sequencing artifacts specific to the Aviti platform and aligned to the *M. domestica* reference genome. Raw-reads FastQC alignment resulted in 14.60 million (Control rep 1) to 23.73 million (TE rep. 4) reads. Final Fast QC reads following FastP trimming resulted in 13.93 million (Control rep. 1) to 23.31 million (TE rep. 4) reads. This excluded two (EE rep. 5 and EX rep. 1) outliers due to poor quality RNA-seq data. dupRadar was utilized to detect duplication bias and found no evidence of bias related to duplication. QualiMap reported the trimmed data aligned to our reference genome with 89.4-83.7% to exonic regions, 9.0-6.2% to intronic regions, and 7.4-4.4% to intergenic regions. Transcriptional structure related to treatment was evaluated via principal component analysis (PCA) (Figure 3.1) to determine variability correlated to different principal components (PC). Tetracycline treatments (Figure 3.1A) were best described by PC1, which accounted for 42.78% of the variability, and PC2 (22.66%). TX treatments were well described by PC2 and not by PC1, while TE was poorly described by both PCs. Enrofloxacin treatments were best described by PC3 (11.17%) and PC2 (23.07%). EX treatments were well described by both PC2 and PC3 while EE treatments were poorly described by both PCs. A heatmap of pairwise Pearson

correlation coefficients was generated using regularized log-transformed (rlog) counts to further analyze sample variability and detect potential outliers (Figure 3.2).

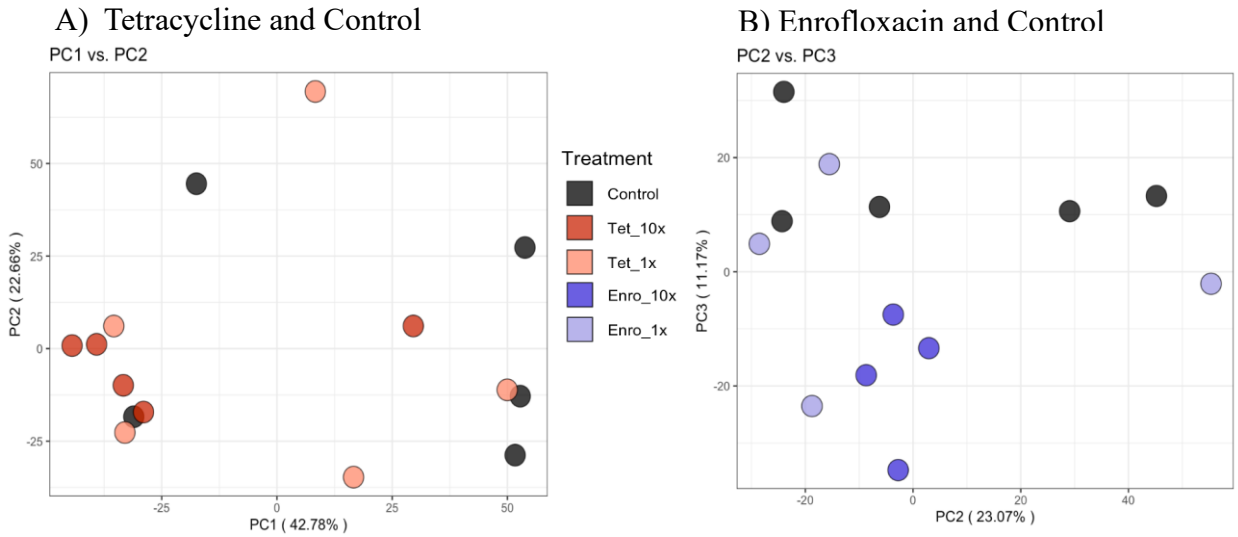


Figure 3.1. PCA of enrofloxacin and tetracycline treated *M. domestica* larvae samples. Graph A) Tetracycline treatments (TE and TX) graphed in conjunction with controls described by principal components PC1 and PC2. Graph B) Enrofloxacin treated samples (EE and EX) graphed in conjunction with controls described by principal components PC 2 and PC 3.

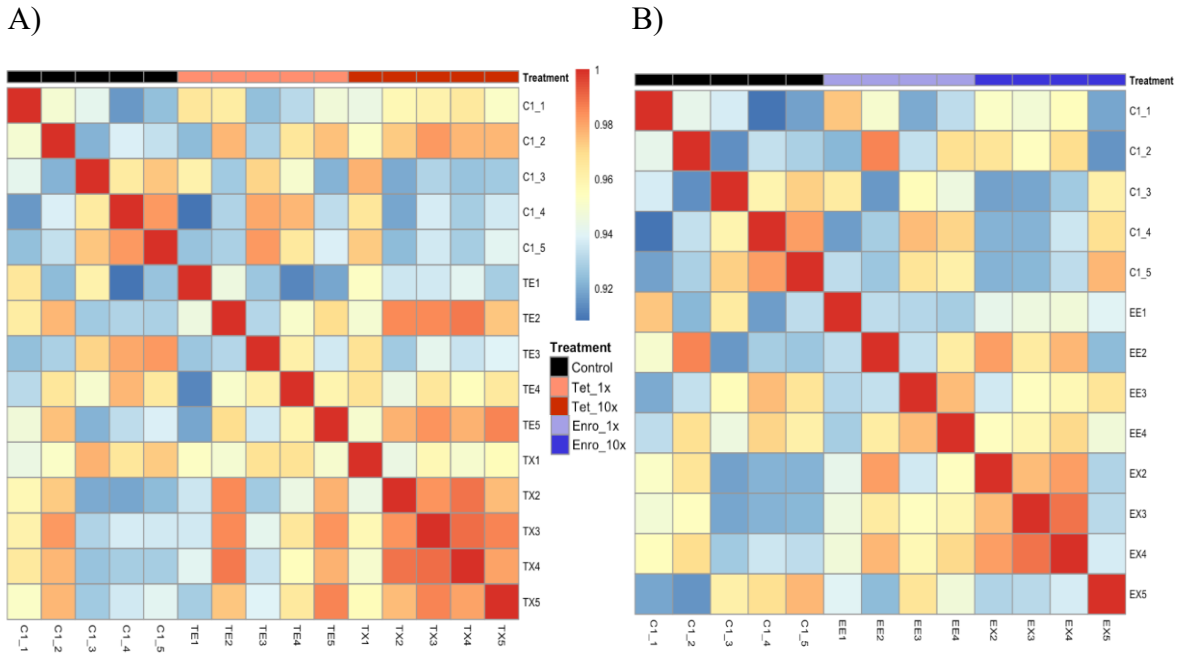


Figure 3.2. Pairwise correlation heatmap based on regularized log-transformed counts. Pairwise Pearson correlation coefficients were calculated from rlog-transformed counts using SummarizedExperiment (Martin Morgan, 2017) and visualized using the Phetmap (Kolde, 2019) packages in R. The color gradient indicates correlation strength between samples, with red equating to the highest correlation. A) Tetracycline treated vs. untreated samples. B) Enrofloxacin treated vs. control samples.

Differential Gene Expression

Under the strict threshold, only a limited number of genes were significantly differentially expressed in most treatments. EE resulted in two downregulated genes, while no upregulated genes were detected. In contrast, EX showed greater transcriptional modulation, with 11 upregulated and 13 downregulated genes. TE yielded no significant differential expression, whereas in the TX treatment, 17 genes were upregulated and three were downregulated.

Examining differential expression using the previously defined loose threshold, considerably more genes met the criteria than with the strict threshold. Under the loose threshold EE resulted in nine differentially expressed genes (six upregulated, three downregulated). EX exposure elicited the largest transcriptional response overall, with 773 genes differentially expressed (493 upregulated, 280 downregulated). TE treatments produced a modest transcriptional response, with five upregulated and 64 downregulated genes, while TX induced 567 DEGs (319 upregulated and 248 downregulated).

Several genes of potential biological relevance were identified among the differentially expressed gene sets. These included genes associated with ribosomes, detoxification, and cellular transport, such as *RpL10*, *probable CYP450 6a21*, and *Sec22* which were upregulated under both TX and EX. There were 22 instances of upregulated CYP450 genes across EX and TX gene sets, and 4 upregulated genes related to GSTs in TX. Among the most strongly upregulated genes under EX treatment were *GAT4* and *trypsin*, while *endocuticle structural protein SgAbd-6-like*

and *larval cuticle protein F1-like* represented two of the most strongly downregulated transcripts with changes of 6.66, 5.31, -23.9, and -25.1 respectively, under the loose threshold.

As observed across treatments, a substantial portion of DEGs were uncharacterized or lacked clear orthology to annotated genes, complicating interpretation of their biological significance. This trend was particularly evident in the higher concentration treatments, where the number of uncharacterized or non-coding transcripts was elevated relative to the low-dose groups.

Functional Characterization

Out of the 10,010 house fly genes analyzed in the differential expression analysis, 8,621 (86.1%) *D. melanogaster* orthologs were found with 8,486 (84.7%) orthologs having enrichment terms via String DB. Identifying orthologs of the well described *D. melanogaster* genome allows us to gain meaningful insight into possible biological pathways that are altered in response to the antibiotic treatments administered in this study. Major DEG trends seen in lower concentration treatments, EE and TE, were observed in EX and TX DEG sets, which also contained genes not seen at the lower antibiotic treatments.

Among these major trends, in upregulated DEG sets (FC > 0), 74 pathways (of 279 total enriched terms) directly related to ribosomal proteins were enriched in TE and TX treatments. Conversely, 31 (out of 146) pathways were enriched in the upregulated EX (FC > 0) (Figure 3.3), while none were enriched in EE gene sets. Specifically, cytosolic ribosome terms were enriched in both EX (FDR=2.63e-42) and TX (FDR=1.95e-56) under Cellular Component Gene Ontology (GO) terms. Within this cytosolic ribosome network (GO:0022626), 59 of 82 protein coding genes were observed in EX treated larvae, and 61 of 82 genes in TX treated larvae. Cellular Component GO terms showed stark differences in enrichment between tetracycline and

enrofloxacin treated groups. EX showed several enriched terms related to neurons (Supplementary Table 3.1), such as synapses (FDR=2.75e-07), neuron projection (FDR=2.12e-05), axon (FDR=5.6e-04), and synaptic vesicles (FDR=0.011). TX did not exhibit any neuron related genes within this gene set, rather there were several mitochondria related terms (Supplementary Table 3.2) that were not exhibited in enrofloxacin treatments. These included the inner mitochondrial membrane protein complex (FDR = 6.21e-07), mitochondrion (FDR = 0.0052), mitochondrial respiratory chain complex I (FDR = 0.0042), and the mitochondrial respirasome (FDR = 2.61e-05). Related to mitochondrial function, oxidative phosphorylation (FDR = 1.09e-05) under the KEGG Pathway term set (Figure 3.4), and Ubiquinone (FDR = 0.0013) under UniProt terms are both vital components to energy production at the cellular level.

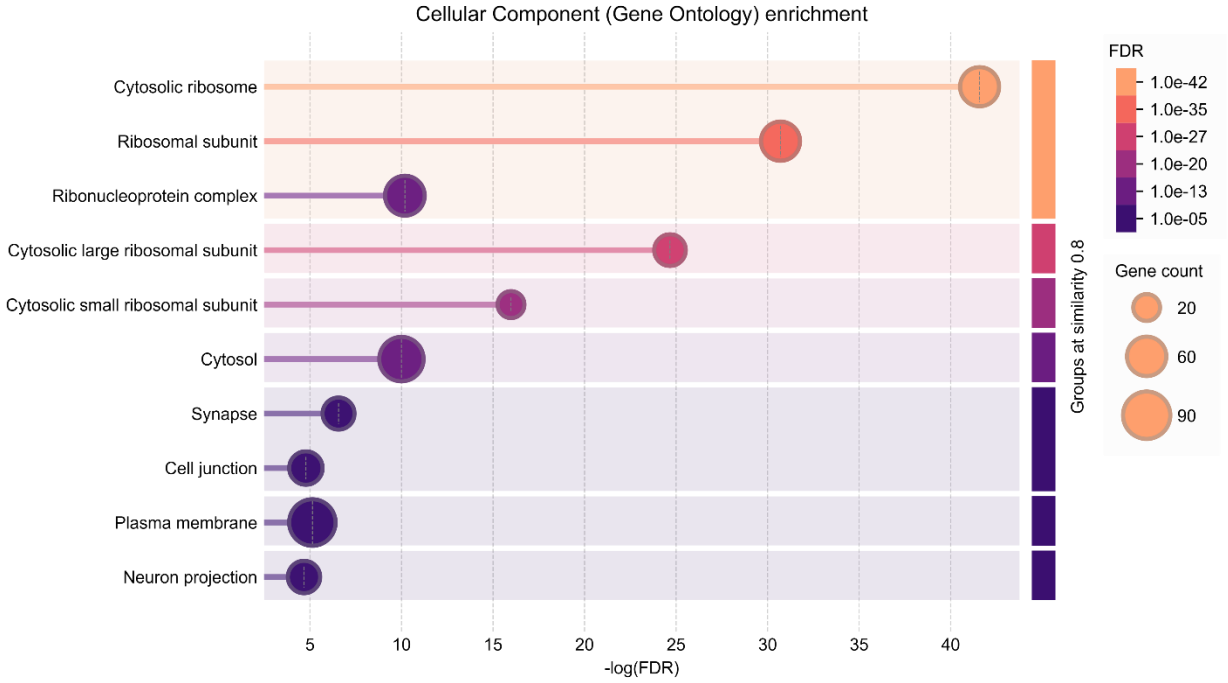


Figure 3.3. Cellular Component GO terms enrichment of upregulated genes within the EX treatment.

Upregulated genes (FC>0), belonging to the EX- vs Control DEG set, enriched following the loose threshold. FDR reported as shown by the color gradient, and gene count per GO term shown by circle size at the end of each respective bar graph.

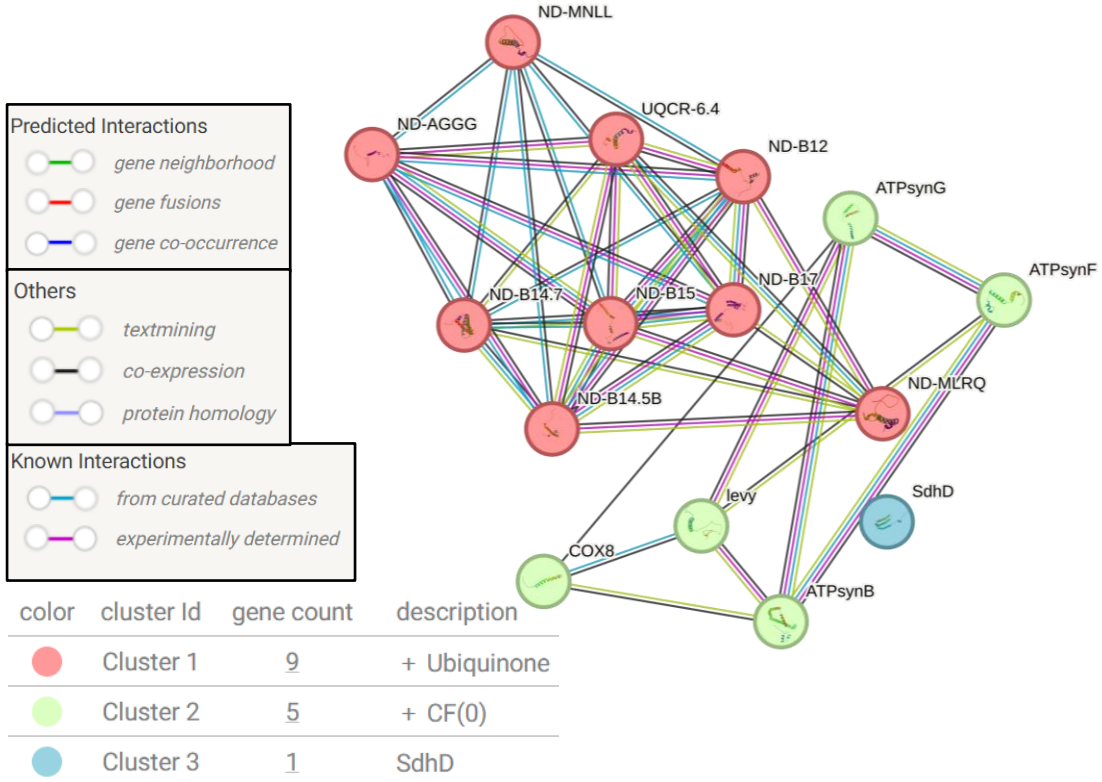


Figure 3.4. Differentially expressed *M. domestica* genes related to Oxidative Phosphorylation KEGG Pathway.

Network (DME00190) showing interaction between genes expressed by *M.* domestica within the TX vs Control DEG set. Clusters formed by k-means clustering (k = 3) based on centroids, interaction between genes shown by colored lines based on interactions. FDR=3.1e-06

DEG sets following the loose threshold exhibited far fewer instances of downregulated genes compared to the hundreds of upregulated genes seen above. Many of these differences were detected in EX (59) with 15 terms related to the Golgi body, and 6 terms related to the endoplasmic reticulum having reduced transcript levels. EE had no enriched GO terms while TE had one enrichment network related to the endoplasmic reticulum (FDR = 0.0089). TX had two enriched GO terms, the extracellular region network (FDR = 9.2e-04) and the Fertile *D. melanogaster* phenotype (MONARCH) with an FDR = 0.038. Under Cellular Component GO terms (Figure 3.5), EX larvae had downregulated genes related to the endoplasmic reticulum

(FDR = 3.1e-14), Golgi stack (FDR = 5.6e-04), organelle sub compartment (FDR = 9.96e-09), and SNARE complex (FDR = 0.0073). Terms related to vesicle transport, the Golgi body, SNARE complex, and endoplasmic reticulum were enriched in gene sets of biological process GO terms, Reactome Pathways, and Subcellular Localization (Figure 3.6) as well.

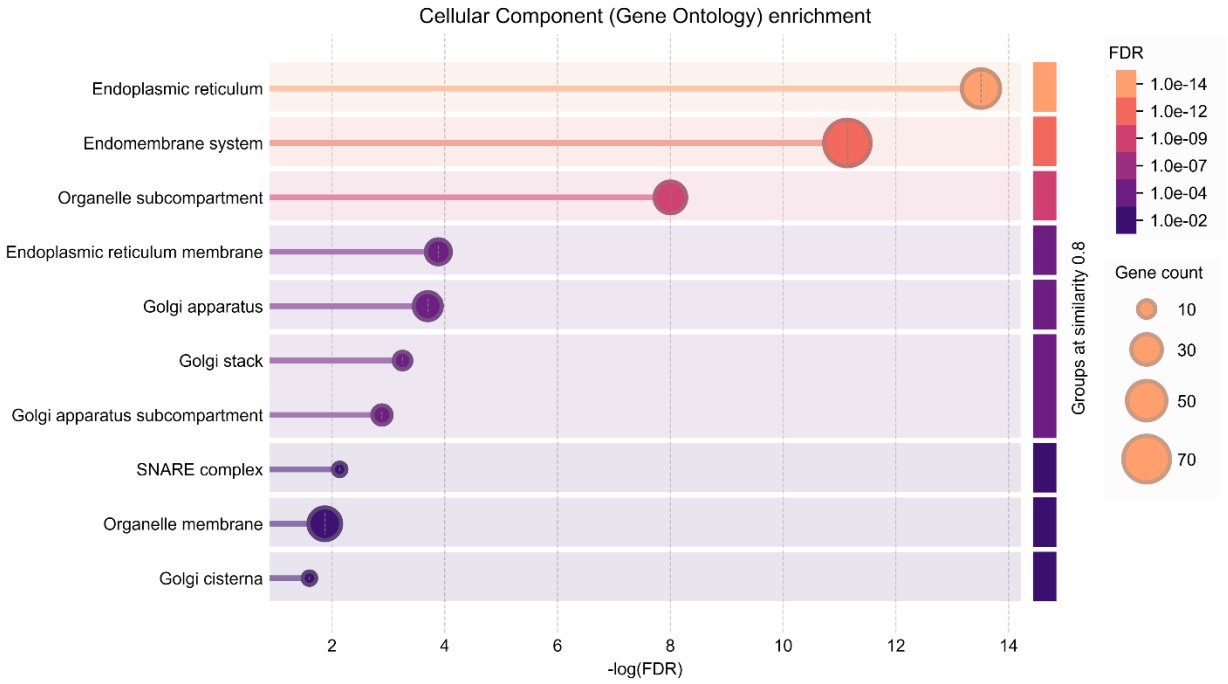
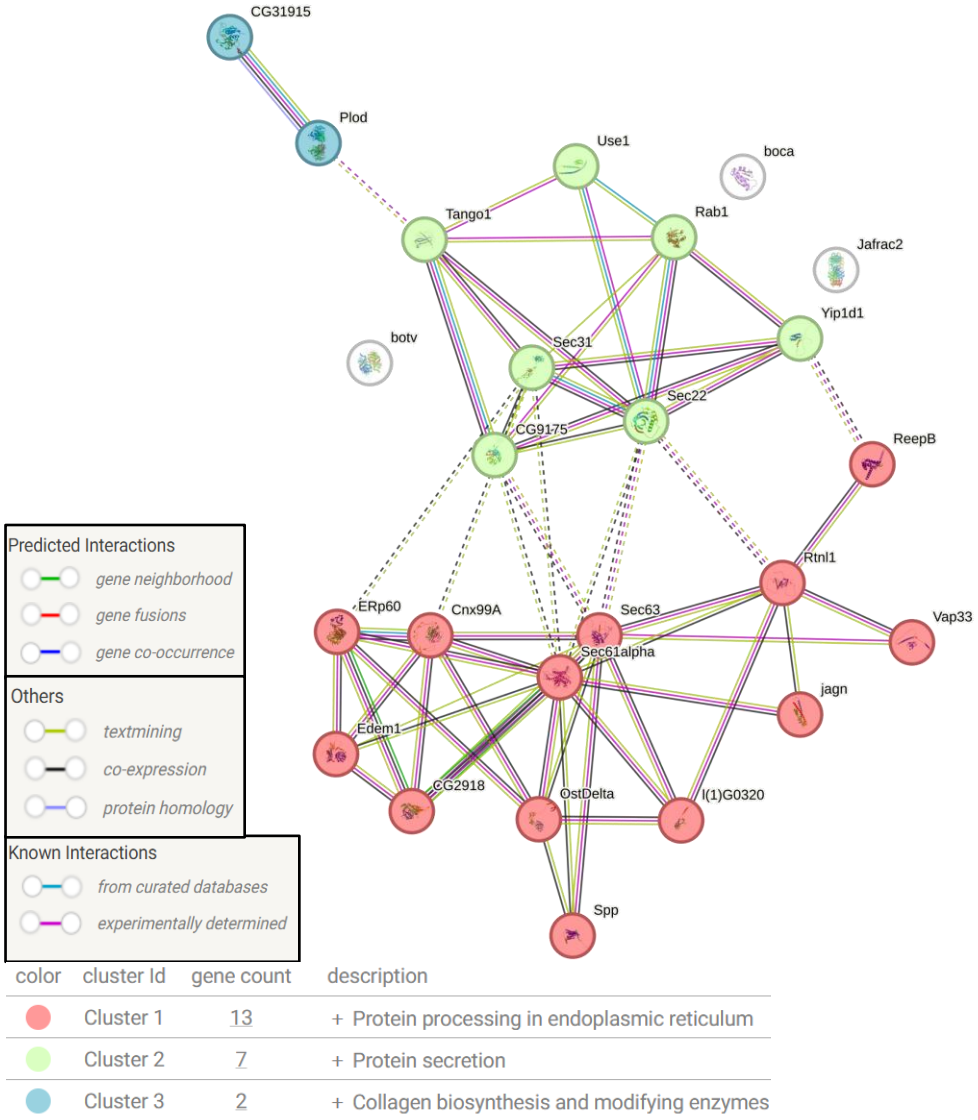


Figure 3.5. Cellular Component GO terms enrichment of downregulated genes within the EX treatment.

Enriched GO terms associated with the downregulated genes (FC < 0) identified in the EX vs Control DEG loose set. FDR reported as shown by the color gradient, and gene count per GO term shown by circle size at the end of each respective bar graph.



color	cluster Id	gene count	description
	Cluster 1	13	+ Protein processing in endoplasmic reticulum
	Cluster 2	7	+ Protein secretion
	Cluster 3	2	+ Collagen biosynthesis and modifying enzymes

Figure 3.6. Differentially expressed *M. domestica* genes related to the endoplasmic reticulum networks within the EX vs Control downregulated gene set.

Network interaction of endoplasmic reticulum associated genes (GO:0005783) that were significantly downregulated in response to EX treatment. Clusters formed by k-means clustering (k=3) based on centroids, interaction between genes shown by colored lines based on interactions, edge cluster interactions shown by dotted lines.

Discussion

Both antibiotics elicited a dose-dependent pattern of transcriptional change. High concentrations induced hundreds of DEGs whereas environmentally relevant doses produced relatively modest transcriptomic shifts. The greater number of DEGs in EX compared to TX suggests that enrofloxacin may impose a more globally disruptive cellular stress response, consistent with its DNA-damaging MOA and known capacity to generate reactive oxygen species (ROS) in eukaryotic cells (Grabowski et al., 2024). Conversely, tetracycline's primary non-target effects are expected to manifest through inhibition of mitochondrial ribosomes, leading to mitochondrial dysfunction (Moullan et al., 2015). These mechanistic differences are reflected in the distinct pathway enrichment patterns observed for each treatment.

Upregulation of mitochondrial and ribosomal genes in tetracycline exposed larvae, especially TX, suggests activation of compensatory mitochondrial biogenesis and increased protein synthesis machinery to counterbalance impaired translation within mitochondria. Mitochondrial stress often provokes nuclear transcriptional responses that enhance mitochondrial replication and oxidative phosphorylation (Martínez-Reyes & Chandel, 2020). The enrichment of GO terms related to the inner mitochondrial membrane, respiratory chain complexes, and oxidative phosphorylation in TX larvae supports this interpretation. This finding aligns with prior studies in mammalian and insect systems showing that tetracycline disrupts mitochondrial translation and stimulates the expression of genes involved in mitochondrial repair and energy metabolism (Chatzispyrou et al., 2015; Moullan et al., 2015).

With either antibiotic treatment, more so in TX and EX treatments, ribosomal protein gene families were significantly enriched and upregulated. This suggests that antibiotic-induced translational inhibition, whether by direct ribosomal binding or indirect stress signaling, prompts a compensatory increase in ribosomal biogenesis. Such responses are characteristic of cells

attempting to restore translational capacity following ribosomal or mitochondrial damage (Zhang et al., 2018). The strong representation of ribosomal GO terms further indicates a general translational stress phenotype shared between treatments. Following antibiotic exposure and ribosomal stress in wild fly populations, possible selections for stress-resistant genotypes may occur.

Enrofloxacin exposure, particularly in EX treatments, induced a unique set of transcriptional changes not observed in tetracycline treatments. Upregulated terms included neuronal and synaptic processes, suggesting that enrofloxacin exposure may interfere with neurodevelopmental signaling or synaptic maintenance. Similar neuroactive effects have been reported in vertebrate models, where fluoroquinolones disrupt GABAergic signaling and mitochondrial homeostasis in neural tissues (Hernandez et al., 2019). Continuous, non-lethal disruption of GABAergic signaling in wild fly populations could have future impacts on insecticidal development as insecticides that target GABA channels, like Fipronil or Fluralaner, may be developed for use in agricultural settings. Meaning that possible selection pressures enforced by fluroquinolones, like enrofloxacin, may predispose house fly larvae to be resistant to insecticides that target the same molecular sites, as adults.

Downregulated gene sets in EX larvae were enriched for endoplasmic reticulum (ER), Golgi apparatus, SNARE complex, and vesicle transport pathways. These results point toward a suppression of intracellular trafficking and secretory activity under enrofloxacin stress. In eukaryotic cells, fluoroquinolone-induced oxidative stress and mitochondrial dysfunction can trigger ER stress and reallocation of metabolic resources away from vesicle trafficking toward repair and survival pathways (Zhang et al.,2022). The simultaneous upregulation of ribosomal genes and downregulation of ER/Golgi-associated genes in EX may reflect a shift in cellular

priorities, favoring synthesis of stress response proteins while curtailing energetically expensive secretion processes. This resource reallocation hypothesis could be a short term strategy to keep the larvae alive in response to a possibly deadly chemical stressor.

Tetracycline and enrofloxacin both evoked transcriptional activation of detoxification pathways. Cytochrome P450 genes from families 4, 6, and 9 were upregulated in EX and TX treatments, suggesting xenobiotic metabolism similar to responses seen with insecticides and environmental pollutants (Li et al., 2021; Scott et al., 2019). These same P450 families are well known for metabolizing pyrethroids, organophosphates, and plant secondary compounds, indicating a generalized detoxification response. Glutathione S-transferase (GST) transcripts were specifically enriched in TX but not EX, implying that tetracycline exposure may engage phase II detoxification mechanisms more strongly than enrofloxacin. This may relate to tetracycline's amphipathic nature and its accumulation in mitochondria, where GSTs mitigate oxidative damage by conjugating reactive electrophiles (Lushchak, 2016).

Our transcriptomic findings reinforce insights from other eukaryotic models regarding the off-target cellular effects of antibiotics. Tetracycline's interference with mitochondrial translation leads to reduced ATP synthesis and increased ROS production, driving secondary transcriptional activation of antioxidant and biogenesis genes (Moullan et al., 2015; Chatzispyrou et al., 2015). The strong enrichment of mitochondrial and oxidative phosphorylation terms in tetracycline treated larvae aligns with this known off-target mechanism. In contrast, enrofloxacin's inhibition of DNA gyrase analogs, its capacity to intercalate with mitochondrial DNA, and its ROS-generating effects result in widespread oxidative and genotoxic stress (Kalghatgi et al., 2013). The extensive downregulation of vesicular trafficking and ER function in EX larvae may therefore reflect a cellular damage response to fluoroquinolone-

induced ROS and DNA fragmentation. Together, these results illustrate how structurally, and mechanistically distinct antibiotics can converge on shared stress pathways yet diverge in secondary cellular consequences. The greater breadth of DEGs and pathway diversity in EX relative to TX supports the hypothesis that fluoroquinolones exert broader cytotoxic effects on insect cells than tetracyclines.

The transcriptomic disruptions observed here likely extend beyond molecular changes to influence larval development, fitness, and ecological interactions. Alterations in mitochondrial and detoxification gene expression could translate to changes in energy metabolism, growth rate, and tolerance to other xenobiotics such as insecticides. Given that house fly larvae develop in manure often containing mixed residues of antibiotics and pesticides, this transcriptional plasticity could contribute to cross-resistance or altered susceptibility to chemical control agents.

Conclusions

Both tetracycline and enrofloxacin exposures produced clear, dose-dependent transcriptional responses in *Musca domestica* larvae, with higher concentrations eliciting broad cellular stress. The strong enrichment of ribosomal GO terms across treatments indicates a shared translational stress phenotype, reflecting disruption to protein synthesis and cellular homeostasis. Tetracycline primarily induced mitochondrial and ribosomal compensatory pathways, whereas enrofloxacin produced broader intracellular transportational effects, including suppression of ER and Golgi function.

These results suggest that antibiotic exposure can alter key metabolic and detoxification processes in house flies, potentially influencing their development, fitness, and response to other xenobiotics. Upregulation of detoxification-related genes, including cytochrome P450s and

GSTs, indicates that antibiotics may promote cross-resistance mechanisms like those involved in insecticide resistance. For producers, this highlights a potential unintended consequence of antibiotic use in livestock systems, as residues in manure may contribute to resistance evolution in pest fly populations. Overall, our findings emphasize that environmental antibiotic exposure acts as a stressor capable of reshaping house fly physiology and resistance potential, posing important implications for both pest management and agricultural sustainability.

Supplementary Tables

Supplementary Table 3.1. EX vs Control upregulated genes enriched in String DB under Cellular Component GO terms.

#term ID	*term description*	*observed gene count*	*background gene count*	*strength*	*signal*	*false discovery rate*
GO:0022626	Cytosolic ribosome	59	82	1.22	5.4	2.63E-42
GO:0044391	Ribosomal subunit	59	149	0.97	3.38	1.98E-31
GO:0022625	Cytosolic large ribosomal subunit	36	50	1.22	4.08	2.15E-25
GO:0022627	Cytosolic small ribosomal subunit	24	33	1.23	3.09	1.04E-16
GO:1990904	Ribonucleoprotein complex	61	489	0.46	1.09	6.55E-11
GO:0005829	Cytosol	78	747	0.39	0.96	1.01E-10
GO:0045202	Synapse	37	268	0.51	0.94	2.75E-07
GO:0005886	Plasma membrane	86	1112	0.26	0.62	7.40E-06
GO:0030054	Cell junction	41	379	0.4	0.71	1.67E-05
GO:0043005	Neuron projection	38	340	0.42	0.71	2.12E-05
GO:0005887	Integral component of plasma membrane	40	389	0.38	0.64	6.80E-05
GO:0071944	Cell periphery	94	1340	0.21	0.53	8.35E-05
GO:0098793	Presynapse	21	148	0.52	0.65	0.00035
GO:0030424	Axon	24	193	0.46	0.59	0.00056
GO:0099503	Secretory vesicle	14	77	0.63	0.62	0.0011
GO:0044306	Neuron projection terminus	13	70	0.64	0.59	0.0017
GO:0043679	Axon terminus	12	69	0.61	0.5	0.0056
GO:0070382	Exocytic vesicle	11	59	0.64	0.5	0.006

GO:0150034	Distal axon	14	95	0.54	0.46	0.0068
GO:0097060	Synaptic membrane	10	51	0.66	0.48	0.0078
GO:0043195	Terminal bouton	11	63	0.61	0.46	0.0087
GO:0099501	Exocytic vesicle membrane	6	16	0.94	0.51	0.0087
GO:0016021	Integral component of membrane	69	1044	0.19	0.35	0.0099
GO:0120025	Plasma membrane bounded cell projection	42	541	0.26	0.37	0.0099
GO:0008021	Synaptic vesicle	10	55	0.63	0.45	0.011
GO:0016020	Membrane	119	2137	0.11	0.27	0.0349
GO:0030672	Synaptic vesicle membrane	5	15	0.89	0.37	0.0356

Supplementary Table 3.2. EX vs Control upregulated genes enriched in String DB under Cellular Component GO terms.

#term ID	*term description*	*observed gene count*	*background gene count*	*strength*	*signal*	*false discovery rate*
GO:0022626	Cytosolic ribosome	61	82	1.43	7.68	1.95E-56
GO:0044391	Ribosomal subunit	62	149	1.17	5.07	5.98E-46
GO:0022625	Cytosolic large ribosomal subunit	38	50	1.44	5.88	1.29E-34
GO:1990904	Ribonucleoprotein complex	68	489	0.7	2	2.33E-25
GO:0005829	Cytosol	79	747	0.58	1.6	5.72E-23
GO:0022627	Cytosolic small ribosomal subunit	24	33	1.42	4.13	3.46E-21
GO:0032991	Protein-containing complex	131	2189	0.33	0.99	2.08E-19
GO:0015935	Small ribosomal subunit	25	60	1.18	3.04	1.04E-17
GO:0043232	Intracellular non-membrane-bounded organelle	81	1242	0.37	0.93	1.02E-11
GO:0005737	Cytoplasm	151	3515	0.19	0.66	4.11E-10
GO:0098800	Inner mitochondrial membrane protein complex	17	96	0.8	1.17	6.21E-07

GO:0005622	Intracellular anatomical structure	182	5080	0.11	0.5	1.73E-06
GO:0043229	Intracellular organelle	158	4253	0.13	0.49	1.27E-05
GO:0043226	Organelle	159	4330	0.12	0.47	2.48E-05
GO:0005746	Mitochondrial respirasome	12	60	0.86	0.97	2.61E-05
GO:0098803	Respiratory chain complex	12	61	0.85	0.96	2.88E-05
GO:0005743	Mitochondrial inner membrane	18	158	0.61	0.78	6.18E-05
GO:0031967	Organelle envelope	26	330	0.45	0.64	0.00017
GO:0098798	Mitochondrial protein-containing complex	19	191	0.55	0.69	0.00017
GO:0042575	DNA polymerase complex	6	13	1.22	0.89	0.00032
GO:0031966	Mitochondrial membrane	19	204	0.52	0.64	0.00037
GO:1990204	Oxidoreductase complex	10	62	0.76	0.68	0.00084
GO:0098796	Membrane protein complex	26	404	0.36	0.45	0.0035
GO:0005747	Mitochondrial respiratory chain complex I	7	35	0.86	0.57	0.0042
GO:0005739	Mitochondrion	36	673	0.28	0.4	0.0052
GO:0043596	Nuclear replication fork	5	17	1.02	0.54	0.0079
GO:1902495	Transmembrane transporter complex	10	104	0.54	0.35	0.0309
GO:0042788	Polysomal ribosome	3	6	1.25	0.37	0.0434
GO:0110165	Cellular anatomical entity	202	6595	0.04	0.23	0.049

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