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To Bee or Not to Bee Exposed:
Chemical Flux, Social Buffering, and Xenobiotic Defense in Honey Bee Colonies

By

ANGELA MARISOL ENCERRADO MANRIQUEZ
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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in

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in the

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of the

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DAVIS

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Julia Fine

Matt Hengel

Committee in Charge

2026

Dedication

To my family – thank you for being the foundation everything else was built on. I will never be able to repay all the unconditional love you have given me, all the kindness you have shown me, and all the opportunities you have provided me with.

To Isabel, Mariana, and Alan - I started this PhD journey very broken, and you all pieced me back together. I cannot see my life without any of you in it. Thank you for walking grad school with me, and above all, thank you for walking life with me. Let's get the next adventure started!

Dedicatoria

Para mi familia – gracias por ser el fundamento de todo lo que he construido. Nunca seré capaz de pagarles su amor incondicional, su amabilidad infinita, y todas las oportunidades que me han brindado.

Para Isabel, Mariana, and Alan – empecé este camino de Doctorado muy rota, y todos ustedes me ayudaron a volver a estar entera. No veo mi vida sin ustedes en ella. Gracias por caminar el doctorado conmigo y sobre todo gracias por caminar la vida conmigo. ¡Fuga a la siguiente aventura!

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To my committee members (Dr. Julia Fine and Dr. Matt Hengel) and my collaborators (Dr. David Baliu-Rodriguez, Dr. Sean Patrick Leonard, and Dr. Bruce A. Buchholz) – thank you for your constructive criticism and for the time you dedicated to advising me in writing proposals, manuscripts, experimental troubleshooting, and data analysis, I truly could have not manage without all of you. I would also like to thank the members of the USDA-ARS Pollinator Health Research Laboratory in Davis, CA, for their unwavering support in my research project. Special thanks to Eliza Litsey, with whom I have shared not only research questions but also life advice and a lot of laughter.

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Synopsis

The management of Honey bees (*Apis mellifera*) by humans has evolved over millennia to obtain commercial products such as honey, wax, and propolis. Around the world, pollination services increase crop production up to 50%, while also providing an annual income source for beekeepers (Porto et al., 2020). In the United States, specifically, honey bees pollinate over 100 commercially grown crops according to the 2022 USDA Annual Strategic Pollinator Priorities Report, increasing their total value by over ten billion dollars. In California alone, according to the National Agricultural Statistics Service (NASS) 2026 cost of pollination report, the almond industry required 1,630,000 colonies to pollinate 1,035,800 acres in 2025. From the 19 crops listed in the NASS report, almonds are the main solicitor of pollination services in the US.

The use of pesticides in agricultural crops is a common and necessary practice to keep pest populations low, preserve crop production, and improve global food security. The ongoing arms race to develop novel, more potent pesticides to combat pesticide resistance in agricultural pests is often a pyrrhic victory that can result in both acute and chronic toxicity in pollinators and other non-target insects (Serrão et al., 2022). In 2023, the California Department of Pesticide Regulation (“Pesticide Use Reporting - 2023 Summary Data,” 2023) reported that the almond industry registered the use of over 24 million pounds of 369 pesticides, including insecticides, herbicides, fungicides, insect growth disruptors (IGDs), and others. While integrated pest management practices aim to prevent harm to pollinators, pesticide residues can still be found across multiple hive matrices (Encerrado-Manriquez et al., 2024), including food sources consumed by all hive members (i.e., bee bread and nectar).

Additionally, assessment strategies for preventing honey bee exposure often focus on single-pesticide safety profiles based on adult worker bee lethality tests, ignoring caste-specific exposure risks and

multi-chemical exposures (US EPA, 2014). Given the social structure of the bee hive community and the intimate relationships between castes, chemical bioaccumulation, and transfer need to be investigated in the context of a colony “superorganism” that collectively absorbs, distributes, metabolizes, and eliminates pesticides. Two key biological mechanisms are central to understanding in-hive chemical fate: trophallaxis, the mouth-to-mouth food transfer that moves nutrients — and contaminants — between colony members, and maternal offloading, the process by which a reproducing queen may transfer accumulated xenobiotics to developing eggs. However, comprehensive evaluations that track the movement of chemicals between castes across diverse social contexts have been limited by the variability of chemical matrices in the hive and by the limitations of current analytical techniques (Charlton and Jones, 2007; Wu et al., 2021).

Moreover, commercial hives are often treated with miticides to prevent the dissemination of diseases across colonies and can also be exposed to other environmental contaminants, besides pesticides. It has been reported that chemical mixtures can cause toxic effects on the detoxification mechanisms in honey bees, thereby disturbing colony health and survival (Hawthorne and Dively, 2011; Johnson et al., 2009). Early studies on the effects of chemical mixtures on honey bee survival conducted by Hawthorne and coworkers introduced the idea that ATP-binding cassette (ABC) transporters, a family of membrane efflux transporters conserved across organisms, serve as a primary detoxification mechanism for worker bees (Hawthorne and Dively, 2011). Although ABC transporters have been widely studied in vertebrates and bacteria, their presence in insects is often overlooked, with attention mostly on pests rather than pollinators (Labbé et al., 2011; Srinivas et al., 2004). Furthermore, the few studies that investigate other parts of the honey bees' chemical defense (Ex, CYPs, GSTs) often fail to consider all hive members (Mao et al., 2013). ABC transporters in particular act as molecular gatekeepers in two key stages of cellular defense. First, as Phase 0 transporters, they prevent unaltered xenobiotic substances from entering cells, such as with ABCB1/P-glycoprotein. Later, as Phase III transporters, they remove compounds that have been metabolized by Phase I and II processing. However, there has not yet been a comprehensive study of

MDR-like ABC transporter expression in all castes and developmental stages of the honey bee superorganism. As such, it is still unclear if each caste has the same ability to protect against xenobiotic threats.

In this dissertation,

- I discuss the current routes of chemical exposure that commercial pollinators face. Furthermore, I discuss the lack of knowledge about the cumulative effects of multi-chemical and sublethal exposures in the field. I also raise the lack of consideration of peer-to-peer interactions in laboratory studies of chemical toxicity, especially across multiple castes (queen bees, drones, workers) and life stages. I identify that, to understand in-hive chemical flux, the use of higher-sensitivity analytical techniques is necessary to measure chemical pathways that account for peer interactions and realistic doses of xenobiotics. (**Chapter 1**)
- Using ¹⁴C isotopic tracing of a model pesticide and Biological Accelerated Mass Spectrometry (BioAMS), I found that at a sublethal dose, there are two key mechanisms that drive chemical movement within the honey bee colonies: trophallaxis and maternal offloading. Furthermore, I showed that worker bees protect queen bees under acute exposure to xenobiotics; however, if the exposure is chronic (as in the case of in-hive medicines or during the commercial pollination season), this protection eventually wears off, leaving the queen vulnerable to toxic outcomes. Once xenobiotics overcome this protective social buffering barrier, queen bees appear to protect themselves by sacrificing their offspring through maternal offloading. (**Chapter 2**)
- Given that xenobiotics can distribute across all hive matrices (**Chapter 2**), I wanted to determine whether xenobiotic defense would protect all hive members equally. To do so, I used qPCR to quantify the relative gene expression of 12 ABC transporter genes known to be involved in xenobiotic transport. Based on these findings, I proposed six genes in the honey bee genome that could have detoxification capacity (MDR-like transporters): Ame-ABCB1, Ame-ABCB6, Ame-

ABCC4a-c, and Ame-ABCG1. Finally, based on the expression of these genes across 13 caste-specific stages and tissues, I identified a vulnerability hierarchy (foragers > drones > queens) that suggests caste-specific trade-offs between reproduction and chemical defense. **(Chapter 3)**

- Finally, to promote interest in the study of environmental toxicology from an early age, the qPCR methodology central to Chapter 3 was translated into a science education outreach context: I developed a two-week, dual-module summer course that teaches high school students to use qPCR (quantitative polymerase chain reaction) to quantify gene expression of chemical defense proteins in response to environmental pollutants. The course is designed to be easily adaptable to other species. It includes a complementary laboratory manual that instructors can download, as well as a suggested questionnaire to evaluate students' satisfaction with the class and their understanding. **(Chapter 4)**

Based on my published findings and related thoughts while writing these dissertation chapters, future research should investigate how co-exposure to environmentally relevant chemical mixtures in the hive environment can alter chemical flux and accumulation patterns across hive matrices. Based on the results in Chapter 2, wax and eggs were the two sinks with the highest pesticide accumulation and should be prioritized in future mixture exposure studies. Additionally, given the queen bee's reduced MDR transporter expression (identified in Chapter 3) and the fact that she can accumulate pesticides in ovarian tissue (identified in Chapter 2), future studies should investigate the effects of chronic single compound exposures and mixture exposure on queen fecundity and overall health. Furthermore, given that maternal offloading can occur in the hive environment, even during acute exposures, it is important to assess offspring viability and the queen's health and reproductive organs. Future studies should specifically investigate markers of reproductive success, including egg cannibalism rates by workers, hatching rate, larval survival, and behavioral outcomes. Finally, further investigation of cell- and tissue-specific localization of ABC transporters in e.g., specific worker glands and in the queen ovaries using

immunohistochemistry (IHC) could help determine whether xenobiotics are actively pumped out of these tissues to protect the host and what other factors influence their final destination once inside the host. Specifically, IHC could help map potential transporter-mediated efflux into developing eggs as suggested in Chapter 2. Another critical next step based on my findings in Chapter 3 is the functional validation of the six proposed MDR-like transporters (Ame-ABCB1, Ame-ABCB6, Ame-ABCC4a–c, Ame-ABCG1) through heterologous expression in cell lines or microbial host systems combined with xenobiotic transport assays. Together, this could confirm their function in effluxing pesticides and identify caste-specific differences in substrate specificity. In addition, future studies should investigate the inducibility of these transporters in response to chemical exposures across castes. The results of such studies could reveal additional layers of caste-specific chemical defense adaptation. For instance, an inability of the queen to respond to chemical exposures by upregulating MDR transporters would significantly increase her vulnerability towards toxic chemical accumulation. Finally, the qPCR-based high schooler outreach course developed in Chapter 4 has been successfully run for four consecutive summers at UC Davis. Future efforts should focus on expanding this curriculum to upper-division high schools and other research institutions with a clear focus on early engagement with research. This means that students are getting introduced to hands-on laboratory training well before they enter college or university. By starting early in their academic journey, this program could help bridge the gap between high school and university-level lab work. As a result, it will cultivate the next generation of environmental toxicologists by giving these high schoolers direct experience with research challenges similar to those discussed in Chapters 2 and 3.

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Chapter 1. Environmental Exposures and Chemical Fate within the Honey Bee Superorganism

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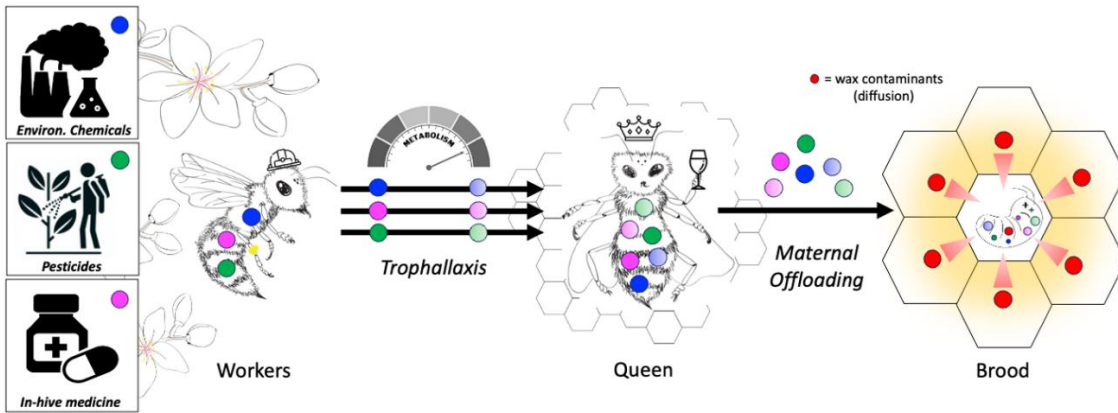
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Abstract

Honey bees are unintentionally exposed to a wide range of chemicals through various routes in their natural environment, yet research on the cumulative effects of multi-chemical and sublethal exposures on important caste members, including the queen bee and brood, is still in its infancy. The hive's social structure and food-sharing (trophallaxis) practices are important aspects to consider when identifying primary and secondary exposure pathways for residential hive members and possible chemical reservoirs within the colony. Secondary exposures may also occur through chemical transfer (maternal offloading) to the brood and by contact through possible chemical diffusion from wax cells to all hive members. The lack of research on peer-to-peer exposures to contaminants and their metabolites may be in part due to the limitations in sensitive analytical techniques for monitoring chemical fate and dispersion. Combined application of automated honey bee monitoring and modern chemical trace analysis techniques could offer rapid progress in quantifying chemical transfer and accumulation within the hive environment and developing effective mitigation strategies for toxic chemical co-exposures. To enhance the understanding of chemical fate and toxicity within the entire colony, it is crucial to consider both the intricate interactions among hive members and the potential synergistic effects arising from combinations of chemical and their metabolites.

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Graphical Abstract



Introduction

1.1 Economic importance of managed bees

Honey bees (*Apis mellifera*) are one of the best monitored and most widely used commercial crop pollinators worldwide. The practice of managing honey bees has been developing for millennia to obtain commercial products such as honey, wax, and propolis (Kritsky, 2017). It was not until 1851 that the invention of the movable frame hive gave rise to the existence of modern beekeeping practices and the extension of the use of honey bee colonies for commercial agriculture (Oxley and Oldroyd, 2010). Pollination services increase essential crop production by up to 90% while also providing an annual income source for beekeepers (Porto et al., 2020). According to the 2022 USDA Annual Strategic Pollinator Priorities Report, honey bees pollinate over 100 commercially grown crops in the U.S. and increase their total value by tens of billions of dollars annually (USDA, 2022a). While there are increasing efforts to identify additional wild insect species with the potential to be managed for crop production (Klein et al., 2018; Osterman et al., 2021), honey bees still remain the most economically valuable pollinators for global crop monocultures (Khalifa et al., 2021; Klein et al., 2018). However, the continuous expansion and geographic concentration of pollinator-dependent crop cultivation, such as the almond industry in California, has demanded an increase in the honey bee colony movements across states, ultimately exposing bees to multiple agrochemicals within a short period of time (Bond et al., 2021; USDA, 2022b).

1.2 Multi-chemical and chemical mixture exposure as emerging threats to colony health

Multiple categories of stressors, including biological (parasites, pathogens), dietary (poor nutrition), climate (warmer, dryer), and man-made factors (pesticides, miticides, biocides) (Daisley et al., 2020), can have negative effects on the populations of both commercial and wild bee species, compromising their productivity and survival. The issue of pesticide exposure is particularly complex in commercial beekeeping operations, as they are often migratory (Alger et al., 2018; Simone-Finstrom et al., 2016;

Traynor et al., 2016). Colonies are moved regionally and even nationally during massive monoculture pollination events in the U.S. (Bond et al., 2021). This constant annual migration exposes bee colonies to different types of crop pesticides, hive medications, and environmental chemicals within the span of only a few months (Sanchez-Bayo et al., 2016; Traynor et al., 2016). Managed honey bees live in a well-coordinated colony environment with their hives acting as superorganisms with intricate communication and food-sharing mechanisms. This makes chemical exposures a community problem, rather than an individual one, with hive-wide health implication. To begin to understand the dynamic nature and effects of multichemical co-exposures on colony health, it is important to first develop sensitive chemical analysis techniques to detect and monitor chemical residue levels and their possible metabolites within the hive environment.

1.3. Technical challenges to study chemical pathways and fate within the colony

Most studies on detection of trace chemicals in the hive environment have focused on quantifying pesticides in products of human use and consumption, such as wax, honey, and pollen (Calatayud-Vernich et al., 2018; Murcia-Morales et al., 2022). However, only a few studies have focused on identifying levels of pesticide residues as they are processed, transferred, and stored within different organismal and physical checkpoints of the honey bee colony (Murcia Morales et al., 2020). A major limitation when analyzing chemical residues in hive members and matrices is the fact that standard analytical methodologies, such as liquid chromatography (LC-) or gas chromatography (GC-) coupled with mass spectrometry (MS), are often not sensitive enough to detect trace levels of chemicals and their metabolites as they are moved and transformed within the hive environment (Charlton and Jones, 2007; Wu et al., 2021). Furthermore, chemical compounds need to be isolated through optimized extraction techniques, such as Quick Easy Cheap Effective Rugged Safe (QuEChERS) and solid-liquid extraction protocols (Barganska et al., 2018; Barganska et al., 2014; Calatayud-Vernich et al., 2016), which are in turn limited to sample size or amount. When evaluating small samples such as eggs (~0.0001g) or larvae (~0.002-0.2g) (Taber and Roberts, 1963;

Zoltowska et al., 2011), sample sizes can be prohibitively small. Yet bees are highly sensitive to pesticides even at sublethal concentrations and exposure to low-dose mixtures of these chemicals could promote synergistic effects on honey bee behavior and toxicity when acting the same molecular targets (Nicklisch et al., 2016; Nicklisch and Hamdoun, 2020; Taenzler et al., 2023; Wade et al., 2019). To develop a more holistic understanding of multichemical stressors in honey bees, it is important to apply modern tracking and quantification techniques to better elucidate the single and multi-chemical fate pathways, to identify chemical sinks within the honey bee hive environment, and to monitor and quantify how social interactions and caste structure can influence chemical exposures.

In this review, we synthesize current knowledge on sources and types of multi-chemical exposure experienced by commercial honey bees, the observed toxic effects on individual members and the impacts on colony health. We provide updates on the emerging information on possible chemical contaminant sinks and transfer from within-hive structures and food. We further shed light on how caste and developmental stage of the hive members can influence both chemical exposure and elimination pathways. We additionally highlight the importance of two unique but understudied features of the honey bee superorganism that can determine chemical fate within the colony, trophallaxis and maternal offloading. The overall goal of this review is to emphasize the need to consider social structure and colony interactions to advance our understanding of chemical fate in bee hives and for mitigating the impacts of multi-chemical stressor to honey bees.

Discussion

2.1 Routes of honey bee exposures to chemical mixtures.

2.1.1 Primary routes of exposure

Direct exposure of honey bees to pesticides and environmental chemicals can occur outside or within the hive (**Figure 1.1**). Forager bees, that collect food and material for hive maintenance and colony food supply, are regularly exposed to multiple chemicals in their environment (Johnson, 2015; Vannette et al., 2015). Thereby, exposure can typically occur via three different routes: i) orally through consumption of contaminated food (pollen, nectar) or water; ii) via direct contact (e.g., cuticle, antenna) with pesticides applied to plants or other surfaces or sequestered in wax comb; and iii) via air exposure when pesticides are applied as aerial sprays and drift onto bees or through active and passive air influx into colonies from nearby treated agricultural areas (Benuszak et al., 2017; Calatayud-Vernich et al., 2018; Johnson, 2015; Krupke et al., 2012; Murcia Morales et al., 2020; Peters et al., 2019; Southwick and Moritz, 1987; Zhu et al., 2015).

Within the hive, physical hotspots and reservoirs of chemical contaminants primarily include stored contaminated food and various hive matrices, such as propolis or wax. The systemic transfer of pesticides to pollen, nectar, beebread, and wax has been widely reported in the literature and poses a significant threat to honey bees (Alder et al., 2006; Benuszak et al., 2017; Calatayud-Vernich et al., 2018; Chauzat and Faucon, 2007; Dively and Kamel, 2012; Gunes et al., 2021; Mullin et al., 2010; Ostiguy et al., 2019; Sanchez-Bayo and Goka, 2014; Stoner and Eitzer, 2012; Stoner and Eitzer, 2013; Traynor et al., 2016; Traynor et al., 2021). This is particularly important for developing larvae, which, as one of the most vulnerable members in the hive, cannot relocate from contaminated wax cells and rely on the food produced by the nurse bees (Murcia Morales et al., 2020). The levels of pesticides detected in royal jelly is often relatively low, and there appears to be minimal transfer from worker bee diet to the glandular secretions they produce to feed to larvae. For instance, worker bees exposed in the field to a pollen-honey diet fortified

with a cocktail of 13 commonly used pesticides produced royal jelly with the majority of the detected concentrations of each active ingredient below 1 µg/kg (Böhme et al., 2017). However, in recent field studies, it was observed that pesticide residues are higher in royal jelly collected from natural comb compared to artificial queen cups, suggesting that there is some diffusion of pesticide residues from wax into the larval diet (Alkassab et al., 2022). Wu and coworkers showed that worker bees reared in pesticide-contaminated wax have shorter lifespans, which may be partly due to this diffusion and direct contact with the pesticide-laden wax (Wu et al., 2011). This highlights the importance of rotating comb to reduce pesticide contamination in wax and the need to assess long-term exposure and sublethal effects of pesticides on caste differentiation and queen bee development, which are still not fully understood (Böhme et al., 2017; Calatayud-Vernich et al., 2018; Payne et al., 2019; Schmuck et al., 2001).

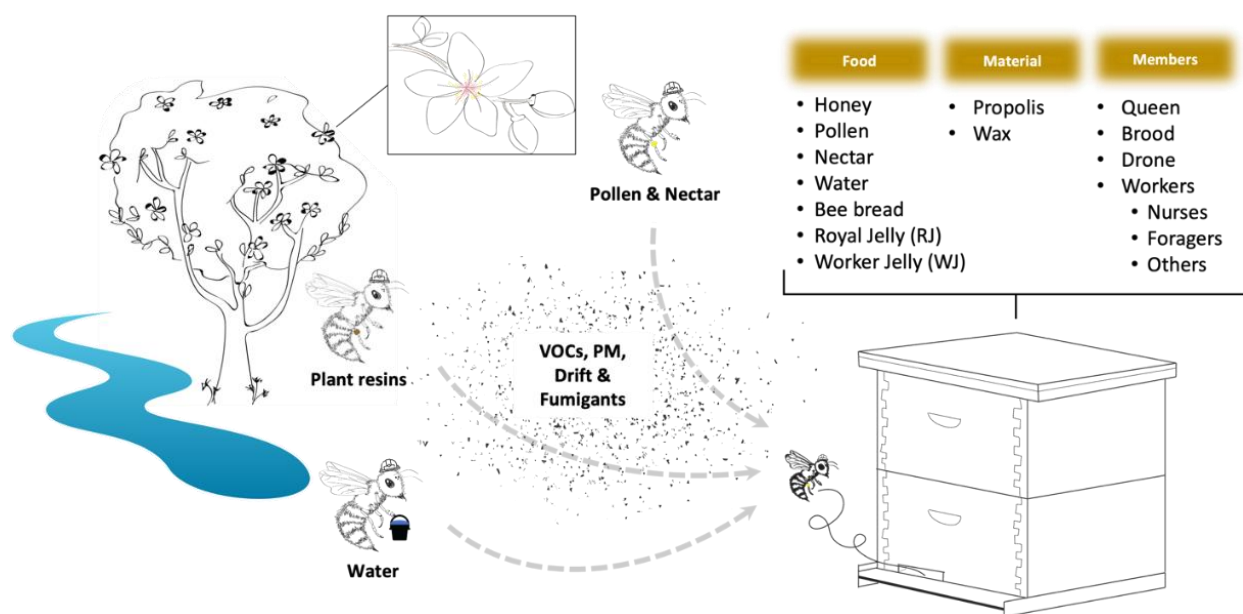


Figure 1.1. Routes of honey bee exposure to mixtures of pesticides and environmental chemicals.
VOCs = Volatile organic compounds; PM = particulate matter.

2.1.2 Secondary and tertiary routes of exposure

The complex dynamics within honey bee castes foster numerous biological intersections, creating various points where hive inhabitants engage in the processing and exchange of food. As such, food contaminant transfer and accumulation should be studied in the context of a colony “superorganism” that collectively absorbs, distributes, metabolizes, and eliminates (ADME) these chemicals. Indeed, pesticide residues have been detected in several hive members, such as foragers and larvae (Calatayud-Vernich et al., 2018; Kasiotis et al., 2014; Murcia Morales et al., 2020). These studies revealed that food sources consumed by all hive members (such as bee bread and nectar) were contaminated with a cocktail of pesticides (**Table S1.1**).

2.1..2.1 Trophallaxis: The unique process of food exchange through mouth-to-mouth regurgitation is a significant route of secondary exposure to pesticides for in-hive bees, including foragers, nurses, MABs, and drones. The queen bee and brood are likely to experience tertiary exposure since their food is first processed by nurse bees. As primary consumers of contaminated food, worker bees may dilute ingested pesticides across hive members involved in food handling (depending on the number of organisms in the food-sharing chain) (Böhme et al., 2017; Sponsler and Johnson, 2017). Moreover, the vectorial transport of contaminated food toward the queen bee creates a potential avenue for pesticide bioaccumulation in this vital individual.

While the impacts of peer-to-peer trophallaxis on viral transmission and spread have been well-documented (Chen et al., 2006; de Miranda et al., 2015; Dolezal et al., 2016; Posada-Florez et al., 2021; Ullah et al., 2021), studies on chemical residue levels across all hive members and on possible chemical transfer between bees are patchy and often do not consider continuous biomonitoring of chemical and/or metabolite pathways from the flower (via worker bees) to the reproductive hive members (i.e., queen, drone) and bee products for human consumption (i.e., honey). For instance, to our knowledge, almost no studies have been conducted to determine pesticide, hive medicine, and other chemical residues in queen

or drone bees (nor the manner of their transfer). Furthermore, the levels of ubiquitous environmental chemicals, such as the ICES-7 PCB compounds and other persistent, bioaccumulative, and toxic (PBT) chemicals, have so far only been studied in bee products of human consumption, including pollen, propolis and honey (**Table S1.1**) (Tang, 2013; Webster et al., 2013; Weisbrod et al., 2007).

Such a holistic approach toward sensitive multi-chemical tracing within a colony would allow for discriminating the extent to which interindividual trophallaxes of contaminated food and contact exposures could provide a biological pathway to mix and distribute toxic chemicals and their metabolites throughout the colony. Likewise, queen bee retinue behavior in worker bees, including grooming and trophallaxis, could facilitate toxic chemical and metabolite bioaccumulation in this longest-living hive member and have detrimental impacts on brood development and survival.

2.1..2.2 Maternal Offloading: Another aspect of superorganism ADME is the possibility of maternal offloading via the rapid elimination of accumulated contaminants from the queen bee. This process has been extensively characterized in marine fish and birds and involves the passive transfer of xenobiotic chemicals (including pesticides) through lipids from the fat storages of the reproductive female to nourish and support offspring development (Chynel et al., 2021; Jouanneau et al., 2022; Lyons and Adams, 2015; Lyons et al., 2013a; Lyons et al., 2013b; Mull et al., 2013). Honey bee larvae, consisting mostly of fat tissue, could serve as an ideal deposition site for hydrophobic chemicals originating from the queen bee's body (Bishop, 1961). Maternal environment influences traits in insects, including honey bees, such as the onset of diapause, egg production and size, and pupal and adult weight (Amiri et al., 2020; Fox, 1993; Mousseau and Dingle, 1991; Rossiter, 1991). However, the effects of maternal pesticide exposure on metrics such as disease susceptibility, behavior, and longevity of adult worker offspring are not known. Most evidence of maternal offloading in insects exists in the form of egg and immature insect toxicity data generated by exposing reproductive adults (Fine, 2020; Fine et al., 2017b; Hodgson et al., 2011; Joseph, 2019; Medina et al., 2010; Trostanetsky and Kostyukovsky, 2008). Information pertaining directly to the

amount of a pesticide that deposits in embryos is scarce, likely owing to the difficulty of performing quantitative assessments on small samples such as insect eggs. However, one study, using isotopically labeled pesticides, demonstrated that the insect growth disruptors pyriproxyfen and diflubenzuron are deposited into the eggs of the predatory lacewing bug (*Chrysoperla carnea*) following maternal exposure (Medina et al., 2002). This work generated precise quantitative data relating exposure scenarios to tissue-specific residue concentrations and physiological outcomes. Similar information about chemical residue levels in honey bees could be valuable, where the outcome and fate of an entire colony is dependent on a single reproductive queen.

2.2 Hive structure and interactions determine chemical exposure risk.

The colony structure of honey bees is characterized by a close-knit eusocial community where individuals share food, labor, and information (**Figure 1.2 and Table S1.2**). This complex and coordinated behavior evolved through a caste system, typically formed by a single queen bee, multiple worker bees (i.e., nurse, forager, and middle-aged bees (MAB)), drones, and brood (including egg, larvae, and pupa) (Johnson, 2010). Acknowledging the unique social structures and food-sharing practices within the bee hive is important to comprehensively track chemical exposure pathways and identify biological and physical reservoirs of chemical contaminants. This requires considering factors such as caste-specific routes of exposure, differences in metabolic enzymes among bees, physical contacts and feeding behavior within the hive, and the storage sites of contaminated food and bee products (Barascou et al., 2022; Medrzycki et al., 2015). These factors can significantly influence the movement, degradation, and potential accumulation of pesticides and other chemicals within the hive, ultimately impacting the overall health and well-being of the colony.

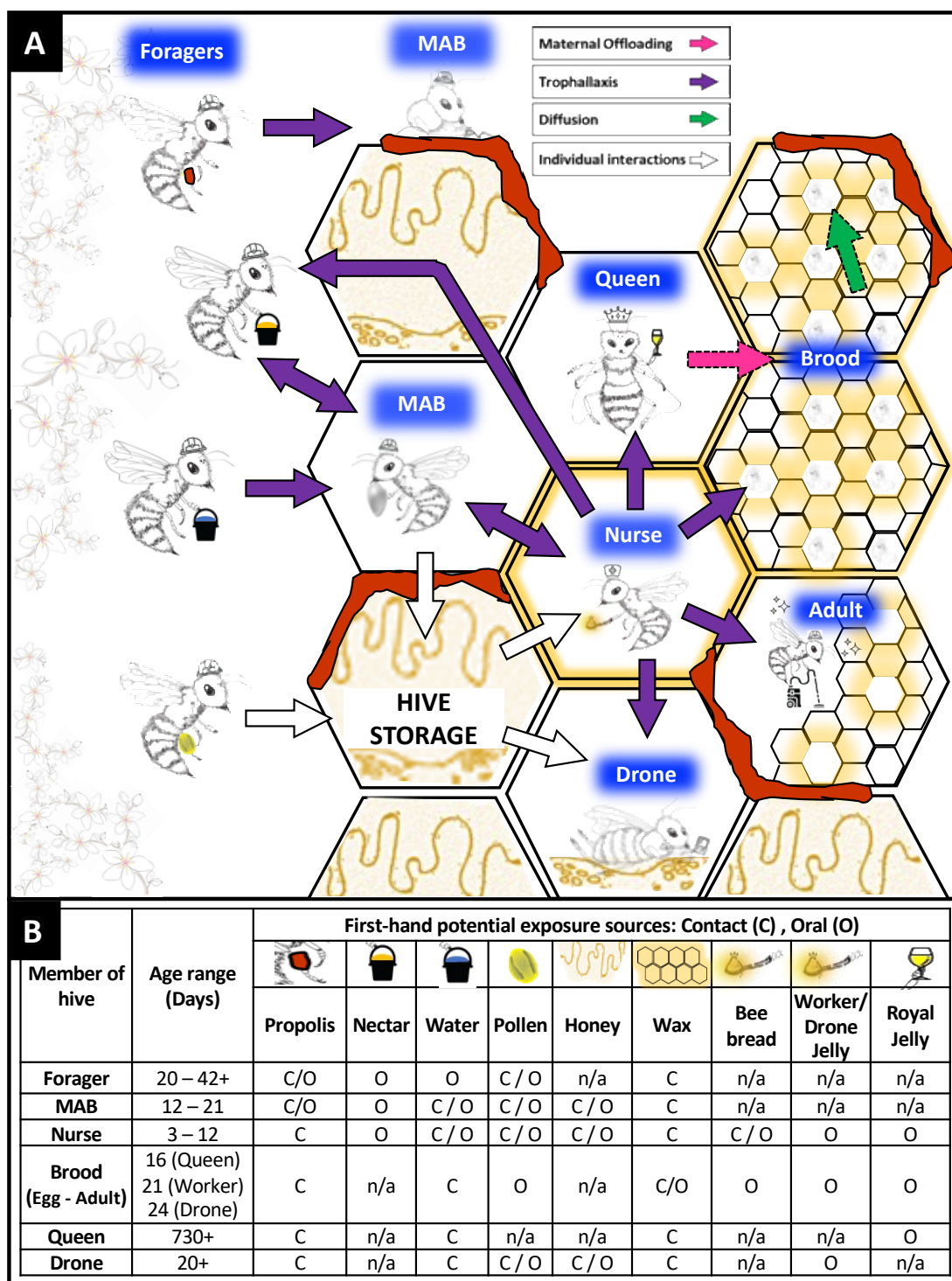


Figure 1.2. Hive member interactions determine chemical movement and fate within the honey bee superorganism. (A) Honey bee hive members and possible pathways of chemical contaminant deposition: Individual bee food deposition or consumption (white arrow), interindividual transfer across castes (purple arrows), passive absorption by the brood (green arrow), and active elimination by the queen (pink arrow). (B) List of possible first-hand oral (O), contact (C), and unknown (U) exposure routes for each hive member and bee product or food source. MAB = middle-aged bees.

2.2.1. Honey bee development and hive member interactions.

Forager honey bees are the oldest of all worker bees (**Figure 1.2 and Table S1.2**). Depending on the time of the year, foragers can live from 20 days (summer) to up to three months (winter) (Johnson, 2010). Forager honey bees venture outside of the hive and collect goods such as nectar, pollen, propolis, and water, which will serve the hive mainly as food sources. When forager bees return to the hive, they pass the collected goods to middle-aged bees (MABs), another type of worker bee, whose age ranges from 12 to 21 days old. At the entrance of the hive, MABs take nectar and pollen for storage, water for hive temperature control, and propolis for fixing gaps within the hive combs. MABs can also be in charge of handling comb building and hive defense. Deeper inside the hive at the brood comb region, nurse bees perform queen retinue behaviors and brood care. The life span of nurse bees ranges between 4 to 12 days, during which time they utilize gland secretions and hive-stored goods such as pollen, nectar, honey, and water to produce *brood* and *queen bee* food (Berenbaum and Liao, 2019) and share processed food via trophallaxis, the hive's main food-sharing mechanism. It is thought that queen bees are fed with royal jelly (though the full composition and extent of the queen bee's diet has not been well studied), while the worker brood are fed a mixture of primarily gland secretions, bee bread, and honey (though again, the specifics of these mixtures have not been established) (Berenbaum and Liao, 2019; Free, 1959; Haydak, 1943; von Planta, 1888; Wueppenhurst et al., 2022). The brood consist of eggs laid by the queen (~1500 per day), larvae (at different stages), and pupae (Remolina and Hughes, 2008). Brood are the youngest hive members and are taken care of by nurse bees until they become adult bees and replace them in their role. Finally, drones, or male bees are produced only to mate with virgin queens from other colonies to spread the colony's genes. During the summer, drones feed on stored goods and attempt to mate until they die. By winter any remaining drone will be removed from the colony to reduce the burden to the hive resources. Depending on the developmental stage and caste differentiation, each hive member can be exposed to multiple natural and synthetic chemicals in their foraging environment or within the hive.

2.3 Effects of multi-chemical exposure on colony health and survival

2.3.1. Synergistic toxicity of chemical mixtures

Managed and wild bees are continuously exposed to a wide-ranging mixture of natural compounds as well as man-made in-hive medicine, pesticides, and environmental chemicals. Once entering the organism, these complex mixtures of chemicals can interact with cells and biomolecules to directly exert harmful effects. These chemicals further interact with one another, creating a complex web of interactions that indirectly contribute to their overall impact on bee health. If the chemicals act on the same molecular target, such as receptors, transporters or enzymes, the interactions can be antagonistic (combined effect is less than the sum of its individual effects), additive (combined effect is the sum of its individual effects), or synergistic (combined effect is greater than the sum of its individual effect) (Nicklisch and Hamdoun, 2020). In a recent literature review on the effects of binary mixtures of pesticides on honey bee, wild bee, and solitary bee toxicity, it was noted that additive and synergistic toxicity were the most common interactions, representing 17% and 72% of reported data, respectively (Carnesecchi et al., 2019). One historical example of the synergistic toxicity observed in laboratory and field studies is the interaction between ergosterol biosynthesis inhibiting (EBI) fungicides and neonicotinoids or pyrethroids caused by the fungicide's inhibition of the cytochrome P450 monooxygenase system (Meled et al., 1998; Pilling et al., 1995; Pilling and Jepson, 1993; Tosi and Nieh, 2019). While enhanced toxicity of multi-pesticide combinations toward bees has been thoroughly described and tested in field and laboratory assays (Berenbaum and Johnson, 2015; Carnesecchi et al., 2019; Pal et al., 2022; Siviter et al., 2021; Wade et al., 2019; Wang et al., 2021b; Zhu et al., 2017a; Zhu et al., 2017b), intended and unintended co-exposures to mixtures of pesticide-adjuvant, pesticide-in-hive medicine, and pesticide-environmental chemicals have been less appreciated.

2.3.1.1 Intended chemical mixtures.

2.3.1.1.1 Tank mixing of pesticides and adjuvants: Pesticides are often applied in mixtures (known as tank mixing) to control multiple pests and diseases (e.g., an aphid infestation alongside a fungal infection) while reducing the number of trips over a field (Holloway and Western, 2003). Although intentional tank mixing of agrochemicals can be controlled and monitored by applicators, the possible synergistic toxicities of these chemical mixtures on non-target pollinators are not well understood. Toxicity to bees can also increase when pesticides are combined with adjuvants, a common agricultural practice of adding additional compounds like surfactants and oils to boost pesticide absorption and distribution with an eye toward improving their efficacy (Green, 2000; Hazen, 2000; Mullin et al., 2016). In a comparative study of over 1300 wax, pollen, and bee samples from hives across the US and Canada, Mullin and coworkers revealed a notable finding: only 70% of the targeted pesticide active ingredients were detected in the beehive samples, whereas 100% of the other sought-after formulation ingredients were present (Mullin et al., 2015). The ubiquitous and concurrent detection of pesticide contaminants and formulation ingredients in the hive environment could facilitate toxic pesticide bioaccumulation and toxicity in bees. Indeed, a 2015 field and laboratory study showed that the broad-spectrum insecticide Fipronil with the added spray surfactant and adjuvant, Sylgard, increased honey bee mortality up to 100% within 24 hours of application (Mayer and Lunden, 2015). Similar adjuvants typically considered biologically inert have also been shown in laboratory studies to promote viral pathogenicity in chronically exposed honey bee larvae (Fine et al., 2017a), enhance viral replication in adults, and impair queen oviposition rates (Fine et al., 2023). More recently, laboratory tests on 30 tank mixtures of different pesticide-adjuvant combinations showed that 50% of the combinations increased honey bee mortality (Wernecke et al., 2021). In another study, Walker et al. demonstrated that the adjuvant *Dyne-Acmin*, when combined with the fungicide tilt and the insecticide altacor, can result in elevated toxicity levels, attributed to increases in overall toxicity of the pesticide mixture (Walker et al., 2022).

Adjuvants can be added to the pesticide formulation as “inert” ingredients, in which case they are classified as formulation adjuvants, or they can be added separately on top of other formulated products in a tank mix, in which case they are classified as spray adjuvants. Each adjuvant can add a specific characteristic to the formulation or tank mix, therefore multiple adjuvants can be used in the same application to achieve several different functions (reduce drift, increase penetration, increase solubility, increase attachment, increase stability) (Hock, 2022; Wu et al., 2023). Adjuvants are thought to lack inherent pesticidal properties, and federal law exempts spray adjuvants from EPA pesticide registration, leaving them largely unregulated. However, this rule varies by state, and in California the CDPR does require spray adjuvants to be registered as pesticides prior to sale and use (Cox and Zeiss, 2022). Unlike in the EU, where toxicity testing of active ingredients and adjuvants is required to have a ‘representative formulation’, in the US toxicity testing of pesticide products does not involve inert ingredients such as adjuvants and mainly focuses on active ingredients (Straw et al., 2022). In addition, given the lack of scrutiny and toxicological testing of these “inert” formulation ingredients for lethal and sublethal effects on the reproductive Queen and vulnerable hive members, such as the bee brood, more research is needed to better predict the effects of various pesticide-adjuvant interactions on colony health and survival.

2.3.1.1.2 In-hive medicine: There is a growing body of evidence that in-hive medications applied to treat bee infections and hive infestations can be detrimental to bee health when bees are pre-exposed to other agrochemicals (Mullin et al., 2010; Rangel and Tarpy, 2016; Traver et al., 2018; Traynor et al., 2016). It has been suggested that such unintended co-exposures of agricultural pesticides affect bee colony health directly (by causing acute additive or synergistic toxicity in bees) or indirectly (by sensitizing bees to other xenobiotics). For instance, in a laboratory assay, bees pre-treated with oxytetracycline (OTC), an antibiotic treatment for foulbrood diseases, and sequentially exposed to the acaricide coumaphos, a therapy for mite infestation, experienced a 44% increase in mean mortality (Hawthorne and Dively, 2011). Likewise, bees fed with OTC and the acaricide tau-fluvalinate exhibited a 33% increase in mortality. In another laboratory study, researchers found that orally administering fumagillin (an antibiotic used to treat *Nosema* within

hives) together with tau-fluvalinate (an acaricide used to treat varroa mite infestations) can decrease the median LD₅₀ by 2-fold compared to sucrose syrup control treatment in exposed honey bees (Johnson et al., 2013).

As such, a precautionary approach accounting for potential synergies between in-hive medications and agrochemicals is prudent to ensure thriving honey bee colonies. Ongoing monitoring of pesticides exposures and a carefully timed application of in-hive medications to avoid simultaneous pesticide exposure may help mitigate interactive toxicity.

2.3.1.2 Unintended chemicals mixtures.

2.3.1.2.1 Foodborne toxicities: Arguably the primary route of honey bee exposure to toxic xenobiotics is through ingestion of contaminated pollen and nectar, typically a result of the widespread application, systemic distribution, and environmental persistence of modern insecticides such as neonicotinoids applied to growing crops (Bonmatin et al., 2015; Stoner and Eitzer, 2012; Tosi et al., 2018). Exposures to multiple pesticides at lethal and sublethal concentrations may cause synergistic toxicity across hive members and contribute to colony loss (Biddinger et al., 2013; Farooqui, 2013; Iwasa et al., 2004; Johnson et al., 2010; Levine and Borgert, 2018; Lu et al., 2014; Magal et al., 2019; Magal et al., 2020; Pilling et al., 1995; Pilling and Jepson, 1993; Spurgeon et al., 2016; Thompson et al., 2014; Williamson et al., 2013; Williamson and Wright, 2013). For instance, Zhu and coworkers showed in two separate laboratory studies that when honey bees were exposed to eight different insecticides (imidacloprid, acephate, λ -cyhalothrin, oxamyl, tetraconazole, glyphosate, sulfoxaflor, and clothianidin) in binary combinations, the mixtures showed synergistic toxicity that increased mortality between 15-26% (Zhu et al., 2017a; Zhu et al., 2017b). Notably, when bees were exposed to a mixture of all eight pesticides, the mortality increased to 100% (Zhu et al., 2017b). Johnson and coworkers performed a similar laboratory study using pairwise combinations of six different acaricides, eight fungicides, three antimicrobials, and three detoxification enzyme inhibitors

(Johnson et al., 2013). The group reported synergistic toxicity as median lethal dose (LD_{50}) and observed the highest toxicities for the acaricide tau-fluvalinate in combination with the fungicide prochloraz (LD_{50} = 0.01 mg/bee), the model CYP450 inhibitor piperonyl butoxide or PBO (LD_{50} = 0.01 mg/bee), and the acaricide coumaphos (LD_{50} = 0.78 mg/bee). Tau-fluvalinate also increased toxicity in honey bees pre-treated with different EBI fungicides, including myclobutanil (LD_{50} = 0.121 mg/bee) and metconazole (LD_{50} = 0.452 mg/bee).

2.3.1.2.2 Waterborne contaminants: Unintended co-exposures to water-soluble environmental chemicals, such as Per- and polyfluoroalkyl substances (PFAS), is another understudied research area and possible threat to honey bee health. PFAS are a group of persistent organic pollutants (POP) that are ubiquitously found in the environment and are recognized by the EPA as contaminant of emerging concern (CEC) in surface waters (Gluge et al., 2020; Miner et al., 2021; Podder et al., 2021). PFAS are regularly detected in aquatic environments, including wastewater, agricultural runoff waters, rainwater, groundwater, and receiving waters such as rivers, ponds, and lakes (Dauchy et al., 2019; Lin et al., 2014). These forever chemicals have also been shown to move from irrigated soil through roots into plants, including flowering crop plants known to be pollinated by commercial and solitary bees (Navarro et al., 2017; Xu et al., 2022). Honey bees need water as a dietary salt source, as a thinner to prevent honey crystallization, and for thermal homeostasis of the hive during the warm summer months (Butler, 1940; Lau and Nieh, 2016; Nicolson, 2009; Stabentheiner et al., 2021). As such, water consumption could be a significant route of PFAS exposure to honey bees. Indeed, PFAS compounds have been shown to bioaccumulate in different aquatic and terrestrial insects and can be transferred to their predators (Koch et al., 2021; Lan et al., 2020). Recently, Sonter and coworkers showed in a laboratory assay that the major water contaminant perfluorooctanesulfonic acid (PFOS) can accumulate in honey bees and increase mortality, reduce colony defense and maintenance behaviors, and even halt brood development (Sonter et al., 2021). Furthermore, Surma and coworkers showed the presence of perfluoroalkyl substance in honey from various European

countries, indicating that bees who undergo the intricate process of producing and storing this natural product within the hive, come into contact with PFAS (da Silva et al., 2016; Surma et al., 2016).

2.3.1.2.3 Airborne toxicities: Bees can also be unintentionally exposed to airborne pesticides, heavy metals, micro- and nano-plastics (also present in water) (Alma et al., 2023; Buteler et al., 2022; Edo et al., 2021), and air pollutant mixtures due to wind drift or when bees are foraging on neighboring crop fields with varying foliar spray applications (Botias et al., 2015; Davis and Williams, 1990; Krupke et al., 2012; Macri et al., 2021; Marzaro et al., 2011; Moffett et al., 1972; Thimmegowda et al., 2020; Ucar and Hall, 2001; Zaric et al., 2018; Zhu et al., 2015). It has been suggested that both airborne particles such as respirable suspended particulate matter (RSPM) and volatile agrochemicals interfere with pest insect and pollinator sensory physiology, chemical and pheromone signaling, and behavior (Gate et al., 1995; Jacobson, 1966; McFrederick et al., 2009). Smoke has been traditionally used by beekeepers to reversibly interfere with the bee's sense of smell and subdue the alarm response (Gage et al., 2018; Visscher et al., 1995). But chemical air pollutant mixtures, such as agriculture and horticulture pesticides, diesel exhaust, and other volatile organic compounds (VOCs) have been shown in field studies to sensitize pollinators to additional stressors and affect crucial pollinator foraging behavior and reproduction (Reitmayer et al., 2019; Samuelson et al., 2018).

2.3.1.2.4 Micro- and nanoplastics: Bees can also incorporate ubiquitous micro- and nanoplastics (MNPs) from a variety of media in the environment and deliver it to multiple hive matrices and members (Alma et al., 2023; Edo et al., 2021). MNP toxicity depends not only the particle size, but also on the type of polymer, and the presence of other chemicals (Al Naggar et al., 2021). For instance, in recent laboratory assays, micro particles of polystyrene have been shown to accumulate in honey bee tissues, and to promote adverse health effects (i.e., decrease gut microbiome diversity, changes in oxidative damage, impaired detoxification and immunity, and reduced lipid metabolism gene expression) when in combination with other pollutants or existing diseases (Alma et al., 2023; Buteler et al., 2022; Deng et al., 2021; Wang et al.,

2021a). Further research is needed to better characterize the effects of MNP exposure on a colony level and to elucidate possible synergistic effects with MNP-adsorbed or co-exposed chemicals in the hive environment.

2.4 Sublethal pesticide exposures

The effects of repeated exposures of commercial and wild bee species to low levels of pesticides have traditionally been underestimated. Sublethal exposure has been shown to interfere with the colony's survival, performance, winterization, behavior, development, and physiology (Barron, 2015; Bryden et al., 2013; Dively et al., 2015; El Hassani et al., 2008; Fine, 2020; Hatjina et al., 2013; Ingram et al., 2015; Lu et al., 2014; Negi et al., 2022; Williamson et al., 2014; Wu et al., 2011; Wu et al., 2022; Xavier et al., 2015). Specifically, delayed larval development and adult emergence, downregulation of detoxification enzymes, and shortened worker bee longevity have been identified as added stressors that impact a colony's fitness and survival (Fisher et al., 2021; Glass et al., 2021; Smodis Skerl et al., 2009; Tome et al., 2020; Wu et al., 2011).

2.4.1. Learning and memory impairment

Low-dose exposures have also been shown to cause direct neurological effects in bees, including decreased memory and learning performance. For instance, honey bee exposure to sublethal concentrations of the neonicotinoids thiamethoxam and thiacloprid in field studies caused an increase in mortality and affected the ability of forager bees to navigate home (Henry et al., 2012; Tison et al., 2016). Thiamethoxam was also shown in two other field studies to alter honey bee and bumble bee feeding rates, motor skills, and visual learning (Laycock et al., 2014; Ludicke and Nieh, 2020). As a result, sublethal studies triggered a re-evaluation of thiamethoxam which led to its eventual banning in Europe for application to flowering plants (Stokstad, 2018). Acute laboratory exposure of bees to sub-lethal doses of the organophosphate in-hive acaricide coumaphos impaired short-term (10-minute) and long-term (24-hour) memory in response to a

conditioned olfactory stimulus (Williamson et al., 2013), while environmentally-relevant subchronic treatment of bees over four days with either coumaphos or the neonicotinoid imidacloprid resulted in degraded olfactory learning and memory (Williamson et al., 2014). Using the proboscis extension response (PER) assay, Decourtye and coworkers found that worker bees exposed in the laboratory to sublethal concentrations of fipronil, deltamethrin, endosulfan, and prochloraz showed reduced learning performance (Decourtye et al., 2005). Since associative learning of odor, color, and shape of flowers with essential food sources such as pollen and nectar is an important behavior of foraging bees (Hammer and Menzel, 1995), the disruption of this vital sense through chronic exposure to sublethal concentrations of pesticides could both threaten colony survival and disturb the pollination services in the ecosystem.

2.4.2. Disruption of chemical communication

Laboratory assays examining low-level pesticide exposure have also demonstrated that chronic exposure can interfere with the production and quality of queen mandibular pheromone (QMP), thereby impacting communication between the hive and queen bee, affecting labor, queen-rearing suppression, and colony cohesion (Bortolotti and Costa, 2014; Milone and Tarpy, 2021; Rangel and Tarpy, 2016; Walsh et al., 2020). Laboratory pesticide exposures also affect chemically guided worker bee retinue behaviors toward the queen, which usually promotes attenuating, grooming, and trophallaxis (Litsey et al., 2021). Indirect effects of low-level pesticide exposures in the laboratory have also been shown to cause glandular degeneration and impact the nutritional quality and quantity of royal jelly produced by nurse bees (Milone et al., 2021), which can have dire consequences for adequate provisioning of queen bees and healthy colony maintenance.

2.4.3. Disturbance of gut microbiome

Exposure to low levels and mixtures of agricultural pesticides have also been shown to affect gut bacterial colonization of larval, newly emerged, and adult honey bees (Almasri et al., 2022; Motta and Moran, 2020; Motta et al., 2018; Vazquez et al., 2023). Furthermore, exposure to commonly used in-hive

medicine has been shown to affect honey bee microbiome structure and function (Kakumanu et al., 2016). Altering the bee gut microbiota, including core species like *Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus Firm-4*, *Lactobacillus Firm-5*, and *Bifidobacterium asteroides*, can have detrimental effects on bee development, metabolism, immunity, and behavior (Daisley et al., 2020; Kwong and Moran, 2016; Motta and Moran, 2023). However, to date only a few pesticides have been tested for their effects on honey bee and wild bee microbiomes at either sub-lethal or environmentally relevant concentrations, and more research is needed to better understand the impacts on the host's immune system and overall health (Hotchkiss et al., 2022).

2.4.4. Cumulative multi-stressor effects

Finally, sublethal pesticide exposure to larval and adult bees already battling pathogens may also have synergistic detrimental effects. For instance, Doublet and coworkers found in laboratory assays that honey bee larvae exposed to sublethal doses of the neonicotinoid thiacloprid in conjunction with Black Queen Cell Virus (BQCV) have a lower survival rate (65%) than the control (90%) or exposure to just thiacloprid (80%) or the virus (85%) (Doublet et al., 2015). In a similar way, Pettis and coworkers showed in field studies with three brood generations that sub-lethal exposure to the pesticide imidacloprid sensitized honey bee colonies towards increased infections with the *Nosema* gut pathogen (Pettis et al., 2012).

Chronic exposure to multiple agricultural chemicals, even at low sublethal doses, can negatively impact honey bee health. At the colony level, these sublethal effects can reduce productivity and increase susceptibility to disease and parasites, ultimately leading to higher mortality. While testing myriad potential mixtures of real-world residues in a laboratory setting poses challenges related to scope and analytical detection limits, the integration of innovative computer-aided approaches for individual bee monitoring, coupled with sensitive trace chemical analysis techniques will be essential for developing a holistic understanding of sublethal and toxic effects resulting from chemical mixtures within bee hives.

2.5 Toward improved understanding of chemical fate and toxicity in bee colonies

Pesticides in the U.S. are regulated by the Environmental Protection Agency (EPA) to ensure the safety of humans, pollinators, and other non-target organisms (EPA, 2014; EPA, 2016a; EPA, 2016b). A plethora of pesticides have been approved for U.S. agricultural crops, often applied in mixtures to combat several pests at once. Globally, according to the FAOSTAT statistics (<https://www.fao.org/faostat>) of the Food and Agriculture Organization (FAO) of the United Nations, between 1990-2021 herbicides were the largest applied pesticide class (by tons), followed by fungicides in second place, and insecticides in third. In contrast to Europe, where in 2021 fungicides were the most applied pesticide class, in the US herbicides continue to dominate the market for agricultural use. The pesticide classes most associated with honey bee and other pollinator toxicity are neonicotinoids, pyrethroids, carbamates, organophosphates, and the phenylpyrazole Fipronil (Cressey, 2017; Johnson et al., 2010; Pisa et al., 2015; Sanchez-Bayo, 2014; Sanchez-Bayo and Goka, 2014).

Historically, most evaluations of pesticide toxicity toward bees are based only on tests evaluating the effects of direct contact and oral exposures of adult bees or larvae to a single compound (Maus et al., 2022). Furthermore, assessments of pesticide hazards towards honey bee colonies by calculating a Hazard Quotient (HQ) or Risk Quotient (RQ) from pesticide application rate divided by the acute adult bee LD₅₀ values often produce poor correlation results between field application rates and actual pesticide residues reaching the individual colony members (Carlson et al., 2022; Drummond et al., 2018; Mu et al., 2022; Thompson, 2021). These methods were initially developed for foliar spray applications and assume that the combined total bee exposure from all routes (oral, contact, air) is directly related to the pesticide application rate. Due to a lack of residue-level validation studies, predicting effects of agrochemical exposure using these methods is hampered by the fact that dietary and contact exposure pathways during bee developmental stages are different (i.e., capped brood versus roaming adult bees).

The eusocial system within a honey bee hive forms another underexplored dimension of developmental and caste-specific exposure risks and interindividual chemical transfer and mixing that needs to be considered when evaluating effects of agrochemicals. Furthermore, within the hive, wax functions as the structural foundation for both present and future hive occupants, serving not only as their habitat but also as a repository for stored food. Its hydrophobic nature makes it an ideal sink for lipophilic environmental pollutants. However, there is a limited body of research exploring wax as a possible route of developmental exposure and the toxicity of contaminated wax across the diverse members of the hive. A major limitation to performing these studies is the fact that chemical concentrations in small samples such as eggs and larvae are typically below the detection limit of standard analytical methods. In addition, it is technically still challenging to determine the chemical-specific diffusion or transfer rates from the wax into larvae and other hive members to develop estimates of exposure (Alkassab et al., 2022; Kast and Kilchenmann, 2022; Murcia Morales et al., 2020; Wilmart et al., 2021).

Modern isotope tracing analysis techniques, such as accelerated mass spectrometry (AMS), that use stable radiolabeled chemicals to detect trace levels of multiple chemical compounds and their metabolites, can lower the detection capabilities to quintillion levels (10^{-18} mol) and sample size to milligrams (Enright et al., 2016; Kutschera, 2016; Nnane and Tao, 2005). This technique has shown its capability to do accurate quantification of parent pesticide and metabolite distribution in Argentinian ant colonies (Hooper-Bui et al., 2015). However, to our knowledge, to date no AMS studies have been performed on honey bees. An additional challenge to track chemical contaminant flow between hive members is the need to clearly identify and monitor individual bees and their interactions.

Artificial intelligence (AI)-based automated monitoring of insects using computer vision systems (Bjerger et al., 2021; Gernat et al., 2023; van Klink et al., 2022) is a promising, emerging technology in the field of animal behavior studies and could provide a new avenue to study parent compound and metabolite transfers within and across honey bee hive members. Such AI-guided systems could facilitate monitoring

the interactivity of individual bees, including trophallaxis and behavior, and – in combination with the sensitive AMS technology – could aid in the prediction of contaminated food flow, deposition, and the effects on colony survival.

Conclusions

Honey bees are exposed to complex chemical mixtures in their natural habitat that can significantly threaten colony health and survival. Although this article focuses on the honey bee hive superorganism and its interactions with chemicals, it is worth remembering that chemical exposure is not exclusive of commercial pollinators and more research needs to be done in unmanaged pollinator species, such as wild bumble bees and solitary bees, where the species survival can be entirely dependent on one individual. Levels and composition of these chemical mixtures may vary during the year and over time due to crop-specific pesticide application schedules and chemical degradation rates. Identifying levels and the metabolic fate of trace amounts of chemicals as they are processed, transferred, and stored within different organismal and physical checkpoints of the hive will be necessary to identify and apply appropriate mitigation strategies such as comb replacement (for physical sinks) or requeening (for biological sinks).

Based on the available literature, sublethal impacts on factors such as learning, immunity, and colony productivity can have substantial consequences for bee health at the colony level. Additionally, synergistic interactions between the multiple active and inert ingredients in commercial pesticide formulations and tank mixes may alter toxicity but are not fully captured in single active ingredient assays. To enhance protection of bees from pesticide-related harm, more research investigating sublethal endpoints across exposure scenarios at the individual and colony levels is needed. Continuing development of field-realistic chronic toxicity protocols, evaluation of pesticide mixtures, and increased use of semi-field and field studies can help ensure the intricate effects of toxicants are fully considered.

A fundamental understanding of the combined effects of the chemical mixtures on bees is crucial for addressing the complex and evolving challenges posed by multiple chemical exposures in their natural environment. This will require adopting modern tracing analysis techniques and artificial intelligence-based monitoring to better understand possible cumulative and synergistic effects of chemical mixtures, especially within the hive's social structure at field realistic concentrations. This comprehensive approach aims to achieve a fundamental understanding of chemical fate in hives for safeguarding honey bee health and colony survival in the face of diverse and changing chemical threats.

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Supplemental Data

Table S1.1. Summary of pesticide, in-hive medicine, and environmental chemical residue levels detected in honey bee hive members, food sources, and products. Listed are the top 20 agricultural pesticides applied in CA in 2021, the 13 EPA-approved in-hive medicines, and 20 environmental chemicals of concern (COC) according to the EPA. Residue levels were obtained from (Albero et al., 2022; Arceo-Gómez et al., 2018; Barganska et al., 2014; Barker et al., 1980; Bergero et al., 2021; Beyer et al., 2018; Bonmatin et al., 2005; Bonmatin et al., 2003; Botias et al., 2015; Byrne et al., 2014; Calatayud-Vernich et al., 2019; Calatayud-Vernich et al., 2018; Chauzat et al., 2011; David et al., 2015; David et al., 2016; de Souza et al., 2022; Dively and Kamel, 2012; El Agrebi et al., 2020; Erdoğrul, 2007; Gérez et al., 2017; Giroud et al., 2013; Hb Kanga, 2019; Henry et al., 2015; Issa et al., 2020; Jiang et al., 2018; Johnson et al., 2010; Kasiotis et al., 2014; Larson et al., 2015; Laurent and Rathahao, 2003; Lentola et al., 2017; Manning, 2018; Marti et al., 2022; Milone et al., 2021; Mititelu et al., 2022; Mullin et al., 2010; Murcia Morales et al., 2020; Nozal et al., 2021; Orantes-Bermejo et al., 2015; Pareja et al., 2011; Pohorecka et al., 2012; Pohorecka et al., 2017; Raimets et al., 2020; Roszko et al., 2016; Schaad et al., 2023; Schmuck et al., 2001; Simsek et al., 2021; Stoner and Eitzer, 2012; Tong et al., 2018; Traynor et al., 2021; Umsza-Guez et al., 2021; Waller and Barker, 1979; Wang et al., 2022; Wang et al., 2010; Wang et al., 2020; Wueppenhurst et al., 2022) and https://research.beeinformed.org/state_reports/pesticides/.

Chemical Class	Chemical Name	Pollen (ppb)	Nectar (ppb)	Propolis (ppb)	Brood (ppb)	Adult/Worker (ppb)	Beebread (ppb)	Honey (ppb)	Royal Jelly/Bee Jelly (ppb)	Bee wax (ppb)
Fungicides	Azoxystrobin	21 - 107 (USA) 0.44 - 40 (World)	-	-	-	n.d. (USA)	37.5 - 56.5 (USA) 0.29 - 201.81 (World)	4 (USA) 31 (World)	-	15.4 - 278 (USA) <LOQ - 447 (World)
	Propiconazole	110.3 - 361 (USA) nd (World)	-	-	-	n.d. (USA) n.d. (World)	51.7 - 69.9 (USA)	n.d. (USA) n.d. (World)	-	36.1 - 227 (USA) 2.06 - 378 (World)
	Pyraclostrobin	100 - 265 (USA) -0.24 - 19.6 (World)	<1.2 (World)	-	-	8.6 - 9 (USA)	61.9 - 105.7 (USA) 0.48 - 8.177 (World)	17 (USA)	-	22.8 - 438 (USA)
	Tebuconazole	19.5 - 34 (USA) -0.24 - 221 (World)	<0.36 (World)	-	412 (World)	1146 (USA) 18.2 (World)	67.5 - 103.8 (USA) 0.1 - 59.7 (World)	5 (USA) n.d. - 5 (World)	-	n.d. - 44.8 (USA) 3.5 - 32 (World)
Herbicides	Acetochlor	-	-	-	-	-	70.4 (USA)	-	-	241.3 (USA)
	Altrazine	13.6 - 49 (USA)	-	2753 (World)	-	15 (USA)	96 - 98.8 (USA) 1.8 - 293 (World)	81 (USA)	-	8.2 - 31 (USA) <LOQ - 192 (World)
	Bentazone	0.53 - 0.7 (USA)	-	-	-	-	0.52 - 6.43 (World)	-	-	-
	Bromoxynil	0.77 - 3.78 (USA)	-	-	-	-	1.30 - 2.09 (World)	-	-	-
	Dicamba	20 (USA)	-	-	-	-	-	-	-	-
	Dimethenamid	-	-	-	-	-	27.5 - 28 (USA)	-	-	5.7 (USA)
	Diuron	-	-	-	-	-	15 - 20.4 (USA)	-	-	17.5 (USA)
	Glyphosate	-	-	-	34 (World)	400 (World)	47 (World)	62 (World)	-	-
	Metolachlor	13.4 - 103 (USA) 3.84 - 51.6 (World)	-	-	-	n.d. (USA)	156.3 - 163.6 (USA)	n.d. (USA)	"traces" (USA)	n.d. - 74.1 (USA)
	Metsulfuron	6.3 - 44 (USA)	-	-	-	n.d. (USA)	5.8 - 59.4 (USA)	n.d. (USA)	-	Trace - 8 (USA)
Insecticides	Simazine	22.4 - 54 (USA)	-	-	-	n.d. (USA)	25.5 - 57.2 (USA)	17 (USA)	-	n.d. - 283 (USA) <LOQ - 55 (World)
	Thiodan	-	-	-	-	-	-	-	-	-
	Chlorpyrifos	2.85 - 830 (USA) 1.09 - 163 (World)	-	10 - 71 (World)	-	3.4 - 57 (USA)	22.6 - 25.2 (USA) 1.2 - 41.4 (World)	15 (USA) 30 - 80 (World)	-	19.6 - 890 (USA) <LOQ - 95 (World)
	Acetate	163 (USA)	-	-	-	n.d. (USA)	130.16 - 216.3 (USA)	52 (USA)	-	n.d. - 51 (USA)
In Hive Medicines	Dimethoate	100 - 22940 (USA) 1.5 - 144.5 (World)	741 - 21000 (World)	-	2 (World)	4864 (USA) 1.5 - 20.5 (World)	1.2 - 15 (World)	9 (USA) 5 (World)	-	26.6 (USA)
	Imidacloprid	<0.2 - 912 (USA) <0.16 - 73.9 (World)	<0.2 - 6588 (USA) <0.17 - 5.7 (World)	-	-	n.d. (USA) 0.3 - 5.74 (World)	19.5 - 20.6 (USA) 0.1 - 19.3 (World)	2 (USA) 0.7 (World)	-	8 - 14 (USA)
	Chlorfene ¹	-	-	-	-	25 (USA)	192 (USA)	-	-	1236.7 (USA) <LOQ - 151 (World)
	2,4-Dimethylamine ²	-	-	-	-	-	2245 (USA)	-	-	-
Environmental Chemicals	2,4-DMPF ²	147.9 (USA) 1.2 - 41 (World)	-	-	-	1249.1 (USA)	81.88 - 134.5 (USA) 1.4 (World)	2 (World)	5 (USA)	2199.8 - 3096.2 (USA)
	Amitraz	1117 (USA)	-	-	61.42 (World)	13780 (USA)	104.94 (World)	12.45 - 555 (USA)	-	17.6 - 446060 (USA) 46 - 2469.31 (World)
	Coumaphos	180.4 - 5828 (USA) 56.2 - 477.4 (World)	-	13 - 834 (World)	4.13	50.4 - 2777 (USA) n.d. - 1545.6 (World)	61.2 - 84.9 (USA) 25.3 - 78.2 (World)	2020 (USA) 37.9 - 60 (World)	0.011 - 210 (World)	215.3 - 94131 (USA) 2.85 - 3788 (World)
	Coumaphos oxon ¹	21.2 (USA)	-	-	-	4.5 (USA)	11.6 - 20.7 (USA)	-	-	19.5 - 102.7 (USA)
	DMA ²	-	-	-	-	2507.5 (USA)	-	-	-	742.1 (USA)
	DMP ²	17.6 (World)	-	-	-	-	71.2 (World)	-	-	<LOQ - 180 (World)
	Fluralinate	4.6 - 26770 (USA) 10.9 - 66.2 (World)	-	-	-	Trace - 5860 (USA)	60.6 - 66.2 (USA) 1.5 - 249.3 (World)	750 (USA)	-	54.85 - 204000 (USA) 742 (World)
	Permethrin	40.1 (USA) 2.20 - 46.81 (World)	-	-	-	2478.1 (USA)	175.6 - 364 (USA) 21 (World)	-	-	209.6 - 1598 (USA)
	Potanan ¹	1119.8 (USA)	-	-	-	-	-	-	-	-
	Tau-fluvalinate	14-334.1 (World)	-	0.587 - 0.012 (World)	2.48 - 15 (World)	7 - 65.5 (World)	10.24 - 563 (World)	8 - 44.7 (World)	0.005-0.028 (World)	<LOQ - 2384.37 (World)
	Thymol	-	-	-	-	-	1582.8 - 2183.8 (USA)	-	301 (USA)	10791.3 (USA)
	Diphenyl ether oxide (DPO)	-	-	-	-	-	-	0.03 (World)	-	-
	Diphenyl ether oxide (DPO)	-	-	-	-	-	-	2.26 (World)	-	-
	Phenanthrene	-	-	35 (World)	-	-	-	<LOQ (World)	-	-
	Pyrene	-	-	4.93 (World)	-	-	-	<LOQ (World)	-	-
	Benzo(a)anthracene	-	-	2.62 (World)	-	-	-	0.76 (World)	-	-
	Chrysene	-	-	2.175 (World)	-	-	-	<LOQ (World)	-	-
	PCB-28	61.3 (World)	-	9.12 (World)	-	-	-	<LOQ - 0.83 (World)	-	-
	PCB-52	15.9 (World)	-	8.87 (World)	-	-	-	<LOQ - 1.26 (World)	-	-
	PCB-101	36 (World)	-	-	-	-	-	-	-	-
	PCB-118	37.7 (World)	-	28.47 (World)	-	-	-	<LOQ - 0.1 (World)	-	-
	PCB-138	36.7 (World)	-	6.8 (World)	-	-	-	<LOQ - 0.1 (World)	-	-
	PCB-153	33.7 (World)	-	12.98 (World)	-	-	-	<LOQ - 0.21 (World)	-	-
	PCB-180	11 (World)	-	14.19 (World)	-	-	-	<LOQ - n.d. (World)	-	-
	BDE-3	0.2 (World)	-	-	-	-	-	20 - 260 (World)	-	-
	BDE-7	-	-	-	-	-	-	10 - 420 (World)	-	-
	BDE-15	-	-	-	-	-	-	25 - 95 (World)	-	-
	BDE-17	0.2 (World)	-	-	-	-	-	30 - 260 (World)	-	-
	BDE-28-33	0.9 (World)	-	-	-	-	-	20 - 220 (World)	-	-
	BDE-47	3 (World)	-	-	-	-	-	30 - 210 (World)	-	-
	BDE-49	0.5 (World)	-	-	-	-	-	30 - 210 (World)	-	-
	BDE-66	0.4 (World)	-	-	-	-	-	10 - 260 (World)	-	-
	BDE-71	0.2 (World)	-	-	-	-	-	10 - 50 (World)	-	-
	BDE-85	0.7 (World)	-	1.78 (World)	-	-	-	10 - 210 (World)	-	-
	BDE-99	2.7 (World)	-	-	-	-	-	10 - 180 (World)	-	-
	BDE-100	1.4 (World)	-	-	-	-	-	10 - 60 (World)	-	-
	BDE-133	0.6 (World)	-	-	-	-	-	140 - 1520 (World)	-	-
	BDE-154	0.9 (World)	-	-	-	-	-	20 - 260 (World)	-	-
	BDE-183	1.4 (World)	-	-	-	-	-	30 - 100 (World)	-	-

1 = metabolite of coumaphos

2 = metabolite of amitraz

Table S1.2. Honey bee colony structure and eusocial organization. The table lists the typical division of labor, food sources, products, potential chemical exposure pathways, and average life expectancy for each caste member according to (Page Jr, 2013; Page and Peng, 2001; Seeley, 2009). DSR = Developmental stage range, DPE = Days post emergence.

Hive Members & Developmental stages		Sex	Role(s)	Diet	Products	Active glands	Potential exposure pathways	Life expectancy	Location in hive	DSR (days)	DPE (days)
Developmental stage	Egg		Maintain colony population	Protein-rich egg yolk	n/a	n/a	Nectar, pollen	n/a	Central broodnest	3	n/a
	Larva (L1-L5)	F/M	Maintain colony population	Bee bread	n/a	n/a	Nectar, pollen	n/a	Central broodnest	4-13 (D) 4-12 (W) 4-11 (Q)	
	Pupa		Maintain colony population	Large larval cells	n/a	n/a	Nectar, pollen	n/a	Central broodnest	14-24 (D) 13-21 (W) 12-16 (Q)	
Worker (10,000-40,000)	NEB	F	Cell cleaner in the brood area, whole hive cleaning	Pollen stores	n/a	n/a	Nectar, pollen		Central broodnest		1-2
	Nurse		Brood food secretion for feeding the brood, tending the queen, capping brood, grooming and feeding nestmates. Young developing larvae are feed royal jelly while older larvae are feed bee bread (mix of pollen and diluted honey)	Bee bread	Brood food	Hypopharyngeal glands	Nectar, pollen		Central broodnest		3-12
	MAB		Wax production, storage of honey, conversion of nectar into honey, receiving nectar from foragers, storage of pollen, ventilation of hive, continues grooming and feeding hive mates. If additional comb is needed for honey storage will activate their wax glands and build comb.	Bee bread	Wax	Hypopharyngeal Glands, Poison gland, Wax glands, Salivary glands	Nectar, pollen, Water	21-42 days, up to 6 months in winter	Peripheral, food-storage region		12-21
		F	Orientation flights, guarding the hive entrance	Bee bread	n/a	n/a	Nectar, pollen, Water		Peripheral, food-storage region		
			Secretion of enzymes for converting nectar into honey, gathering of nectar, pollen, water, resin. Some forager bees concentrate on pollen foraging while others devote their entire foraging careers to the collection of water	Bee bread	Honey, pollen, resins, water	Hypopharyngeal glands	Nectar, pollen, water		Peripheral, food-storage region		
	Forager		Reproduce (lays about 1,500 eggs per day during spring and summer), maintains colony cohesion and regulates reproduction (using hormones)	Royal jelly	Brood	n/a	Nectar, pollen	2+ years	Center of hive		730+
Queen			Spread genes of their colony by mating with virgin queens, fly off to reproduce with other queens	Bee bread	n/a	n/a	Nectar, pollen	~1 month	Back of the hive		20+
Drone (~1/10 of worker population)		M									

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Chapter 2. Queen Bees Offload Pesticide Burden to Eggs When Social Buffering is Overwhelmed.

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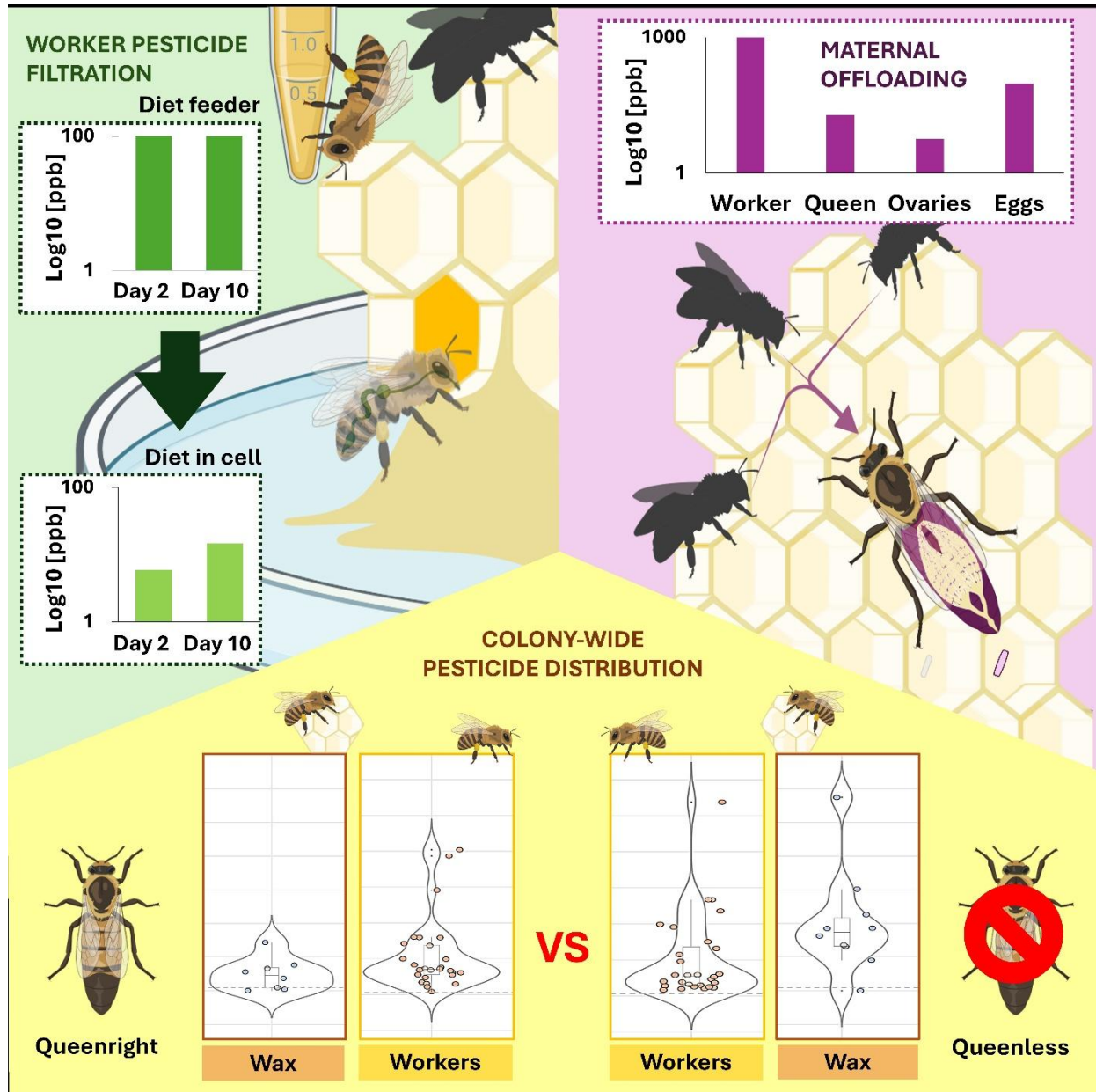
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Summary

Honey bee colonies pollinate about one-third of the world's food crops, and their rapid decline directly threatens agricultural productivity and ecosystem stability. Understanding how colony-level social defenses influence pesticide fate and the circumstances under which they fail is therefore a crucial question in pollinator biology. We used biological accelerator mass spectrometry (BioAMS), a sensitive radiotracer technique, to track the movement of a model pesticide through a small honey bee colony in laboratory conditions. We tested the hypothesis that social buffering protects honey bees from toxic accumulation and that this protection can be overcome, leading to maternal offloading of the pesticide to developing embryos. Consistent with these hypotheses, our results identified three key mechanisms governing chemical movement within a social insect colony: (1) worker bees initially decrease dietary pesticide levels by 95% through diet filtering and deposition in honeycombs, though this declines to 86% by day 10; (2) queen bees maintain markedly lower pesticide levels compared to workers but, over time, they accumulate the pesticide in their ovaries and transfer it into developing eggs, revealing a previously undocumented protective mechanism in reproductive individuals; and (3) the presence of a queen bee shifts colony-wide chemical distribution by concentrating worker exposure and increasing pesticide deposition in wax. Our findings show that honey bee colonies function as integrated detoxification networks, in which chemical fate depends on complex social behaviors and caste-specific physiology. When social buffering is overwhelmed, reproductive queens may survive by transferring their chemical burden to their offspring.

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Graphical Abstract



1 Introduction

2 The social buffering hypothesis states that the pathogenic effects of stress can be mitigated by social
3 interactions ¹. This phenomenon has been observed across diverse taxa and is often linked to hormonal
4 shifts that modulate an organism's response to stressors ². In social insects, sociality extends beyond
5 behavioral coordination to include physiological regulation mediated by pheromonal communication. For
6 example, the queen mandibular pheromone in honey bees chronically alters gene expression in the brains
7 of worker bees, activating genes associated with in-hive nursing tasks while repressing those linked to
8 foraging ³. These pheromone-mediated physiological changes suggest that social context may influence not
9 only behavior but also the hormonal and metabolic detoxification processes that govern how organisms
10 interact with their chemical environment. Despite the likelihood that physiological shifts influence the
11 uptake, distribution, and fate of chemicals within an organism, our understanding of how sociality shapes
12 the toxicodynamics of xenobiotics remains poorly understood ⁴. Honey bees, as social insects, provide an
13 ideal system for evaluating this, particularly given the established influence of social status and social cues
14 on their physiological characteristics. Walton and coworkers have recently conceptualized this idea as
15 "social stress protection", suggesting that collective defenses against environmental challenges, including
16 pesticides, are an evolutionary counterpart to the limited detoxification gene repertoires found in honey
17 bees ^{5,6}.

18
19 In a healthy honey bee colony, a single queen lays between 1,000 and 2,000 eggs per day ^{7,8}. In
20 contrast, non-reproductive female worker bees fulfill the colony's other needs. Younger worker bees
21 typically remain within the hive as nurses, tending and feeding the queen, whose diet consists almost
22 exclusively of royal jelly. They also provide larvae with glandular secretions and processed pollen and
23 nectar ⁹. In contrast, older workers undertake riskier tasks, such as foraging for resources ⁹. In agricultural
24 settings, forager bees are exposed to various agrochemicals, including pesticides ¹⁰⁻¹². These chemicals can
25 be transported back to the colony via contaminated pollen or nectar and shared among colony members

through trophallaxis, the mouth-to-mouth transfer of food, which serves as the primary vector for both nutrient redistribution and the potential spread of xenobiotics^{13,14}. Once inside the hive, pesticides persist in processed food sources, including honey, bee bread, and glandular secretions, as well as in forager-built matrices such as wax^{4,12,15,16}. Despite the potential for widespread in-hive contamination, the social organization of honey bee colonies may confer some protection against chemical accumulation, with recent field studies showing that chemical residues are progressively diluted as these contaminants move from foragers through stored hive products to nurse bees and larvae¹⁷.

Indeed, honey bee colonies have been proposed to exhibit greater resilience to pesticide exposure than solitary insects, thanks to their intricate sociobiology, which protects the most critical members of the hive, such as the queen and developing brood^{6,18}. Existing research primarily relies on residue studies assessing pesticide concentrations in hive matrices, including food, wax, and whole worker bee bodies. However, comprehensive evaluations that track the movement of chemicals between castes across diverse social contexts have been lacking⁴. In particular, the effect of queen presence on chemical distribution and accumulation remains unquantified. In addition, maternal offloading, the process by which pesticides accumulate in reproductive tissue and may be transferred to developing offspring, has not been established or refuted^{19,20}. This is partly due to the limited sensitivity of current analytical techniques for quantifying chemical disposition in small, heterogeneous samples, such as queen eggs, at environmentally relevant concentrations.

To overcome this analytical barrier, we used biological accelerator mass spectrometry (BioAMS) to trace radiolabeled compounds with attomole (10^{-18} mol) sensitivity in milligram-sized samples, which is several orders of magnitude beyond the parts-per-million limits of conventional analytical methods²¹ (Table S1). This sensitivity allows simultaneous tracing across multiple castes, tissues, and hive matrices, including low-biomass samples such as queen eggs and ovarian tissue that are inaccessible to conventional residue methods^{22,23}. This study used [^{14}C]-labeled methyl parathion (MP), a model lipophilic

organophosphate pesticide, to comprehensively map chemical distribution pathways in queen bees, worker bees, developing brood, and hive structures. MP is an ideal model compound because its organophosphate mode of toxic action has been extensively studied in honey bees (Table S2), and its moderate lipophilicity ($\log K_{ow} = 3.0$) and molecular weight (263.2 g/mol) align with those of many current organophosphates and neurotoxic pesticides^{24,25}. Furthermore, the commercial availability of high-purity [¹⁴C]-labeled standards enables precise quantification via AMS, and their well-documented environmental fate data facilitate comparisons with the existing literature^{19,20}.

We traced MP movement over 10 days in modified nano-colonies (see Methods for full description), tracking its progression from a dietary source through trophallactic interactions among worker bees, deposition into wax, transfer to the queen, and ultimately, maternal offloading to eggs. This approach allowed us to test the overarching hypothesis that honey bee colonies function as integrated social detoxification networks, in which sociality shapes pesticide fate, and that this protection has a finite capacity that determines downstream consequences for the queen and her embryos.

Results

Worker bees reduce pesticide concentrations in their diet before reaching queens

To quantify the extent of dietary filtration by workers, we compared MP concentrations in the source feeders with those in the processed diet within comb cells (**Figure 2.1A**). In [¹⁴C]-MP-treated nano-colonies, significant effects were observed for day ($\chi^2 = 7.89$, $df = 1$, $p = 0.005$), sample type ($\chi^2 = 778.43$, $df = 8$, $p < 2.2e-16$), and their interaction ($\chi^2 = 70.20$, $df = 8$, $p = 4.478e-12$). On day 2, the MP concentration in paraffin comb cells was 95% lower than in source feeders ($\Delta \approx -117.95$ ppb, $p < 0.0001$), indicating substantial filtration by worker bees. This difference persisted on day 10 ($\Delta \approx -90.2$ ppb, $p < 0.0001$), although filtration efficiency decreased significantly (86%). We refer to this drop in concentration from the source diet in the feeders to the processed diet stored in comb cells as the worker filtering function, which

results in a net reduction in MP mediated by workers through dietary handling, processing, and deposition. Importantly, MP concentration in comb cells increased significantly from day 2 to day 10 ($\Delta \approx 6.63$ ppb or 40%, $p = 0.0114$).

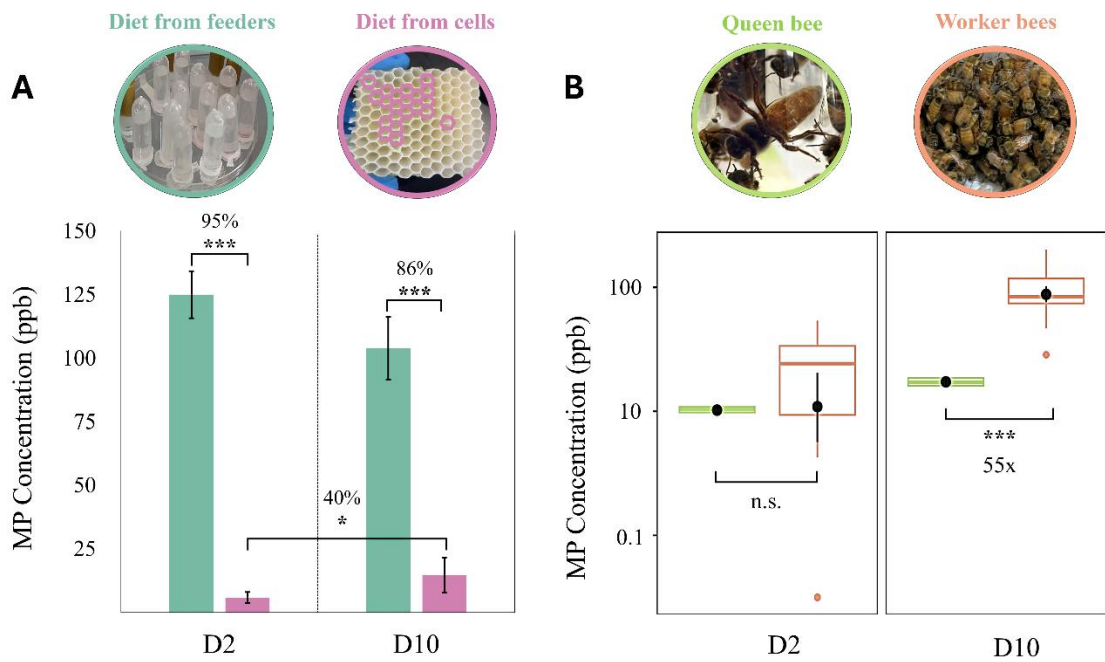


Figure 2.1. Diet processing and pesticide filtration by worker bees. In both graphs, the y-axis shows the MP concentration in parts per billion (ppb) of the sample, and the x-axis shows the sampling days. Statistical significance indicators: $p > 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). In both panels, the small circles arranged above each x-axis day label depict the individual samples measured for that day and group; circle frame colors are matched to the colors of the corresponding datasets in the graph below (panel A: feeders vs. paraffin-comb cells; panel B: queens vs. worker bees), so that frame color identifies the group and horizontal position identifies the sampling day. (A) Significant differences were observed between the average diet in the feeders $\pm$ SD ($n=8$) and the average diet in the paraffin-comb cells $\pm$ SD ($n=10$), at D2 ($\Delta \approx -117.95$ ppb, $p < 0.0001$) and D10 ($\Delta \approx -90.20$ ppb, $p < 0.0001$). MP concentration decreased when the diet was transferred to wax cells by 95 % at D2 and 86% by D10. (B) The concentration of [14 C]-MP in queen ($n=27$) and worker bees ($n=56$) is plotted on a logarithmic scale to facilitate comparison. Black points are group means and error bars represent 95% CI. Within individual days, differences between queens and workers were significant only at D10 ($\Delta \approx -1058.79$ ppb, $p < 0.0001$), but not at D2 ($\Delta \approx -63.66$ ppb, $p = 0.99$). Individual-sample distributions underlying these summary means are shown in Figure 2.2A (for the queen, ovary, and egg data) and in SI Appendix, Figure 2.4A–B (for the worker-bee distributions).

Body burden dynamics in worker bees differed markedly from those in queens (Figure 2.1B). On day 2, concentrations did not differ significantly between workers and queens ($\Delta \approx -63.656$ ppb, $p = 0.99$). However, by day 10, workers accumulated significantly more MP than queens ($\Delta \approx -1058.789$ ppb, $p <$

0.0001). On average, workers had accumulated 55-fold more MP than queens (1049 ppb vs. 19 ppb), indicating that caste-specific divergence in body burden emerges and accelerates over time. Individual worker concentrations varied widely (**Figure S2.1A**).

Maternal offloading in queen bees involves transferring a pesticide burden to developing eggs

As worker bees accumulate a much higher pesticide burden than queens do, we investigated whether and how queens might redistribute this chemical load to their reproductive tissues. To trace this chemical flux from dietary exposure through trophallactic networks to reproductive tissues, we measured [¹⁴C]-MP across multiple matrices in queenright colonies (**Figure 2.2**). Pairwise comparisons revealed significant concentration gradients along the exposure pathway. Worker bees accumulated significantly more MP than the source feeders ($\Delta \approx -461.55$ ppb, $p = 0.001$), whereas queens maintained significantly lower body burdens than workers ($\Delta \approx -561.22$ ppb, $p < 0.0001$) (**Figure 2.2A**).

Despite lower whole-body MP burdens than workers, queens exhibited preferential MP partitioning into reproductive tissues (**Figure 2.2, Table 2.1**). Statistical analysis showed significant enrichment of MP concentrations in eggs relative to queen ovaries and remaining somatic tissue (whole queen body excluding ovaries) ($\Delta \approx 73.45$ ppb, $p < 0.0001$), reaching 81–141 ppb by day 10, levels 5–10 times higher than queen body burdens (**Figure 2.2A, 2.2B**). Notably, ovarian concentrations, while measurable, were not significantly elevated compared with those in the remaining somatic tissues. This suggests preferential sequestration of pesticide specifically at the oocyte packaging stage, possibly via active transport at the follicular membrane or lipophilic partitioning into yolk lipids during oogenesis.

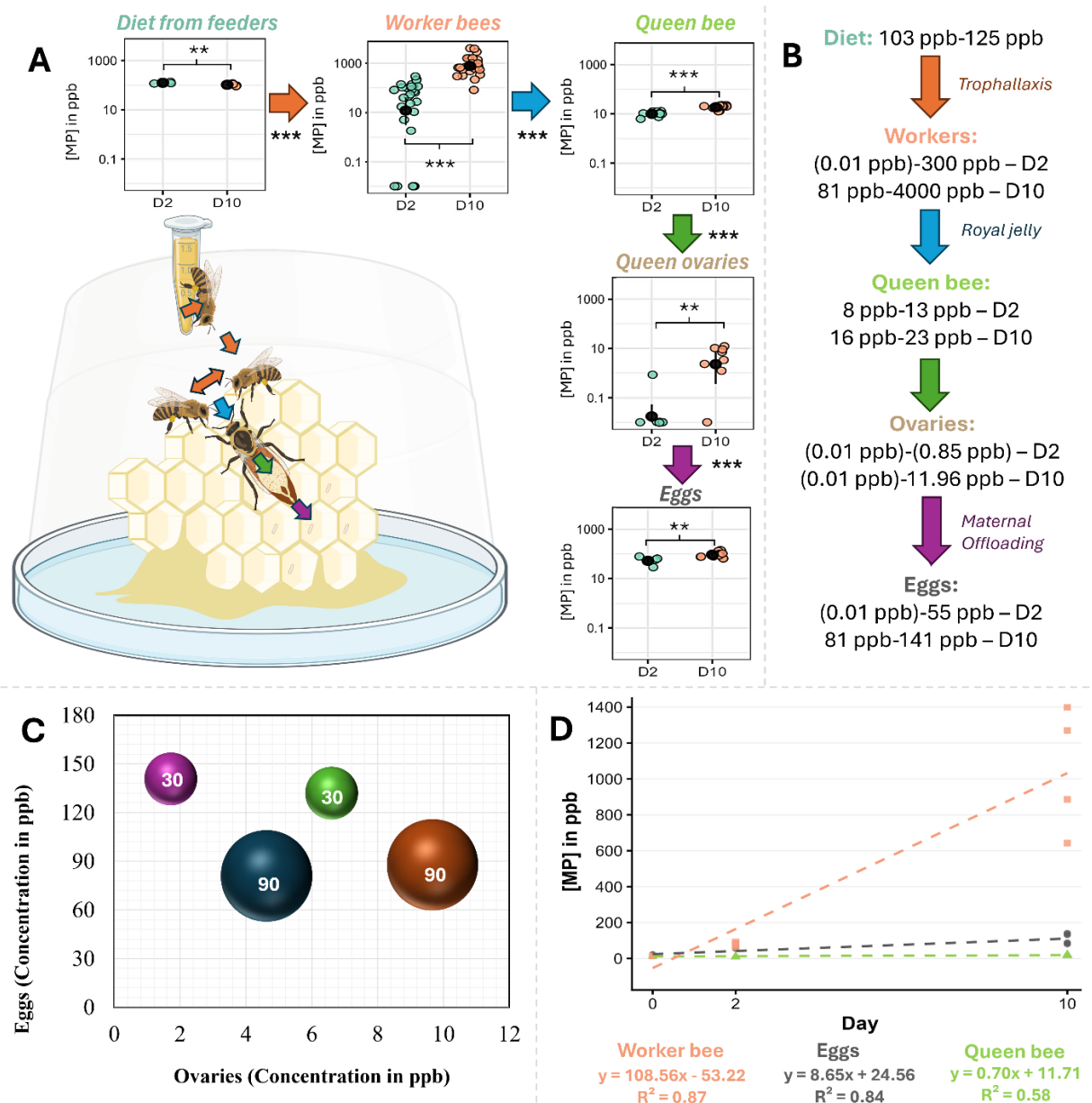


Figure 2.2. Maternal Offloading Pathways in Queen Bees. (A) Temporal progression of MP concentrations from source feeders through worker bees ($n=56$), queen bees ($n=27$), queen ovaries ($n=16$), and eggs ($n=18$) at D2 and D10. The LOQ for each tissue is shown in Table S3 (SI Appendix). The y-axis is on a log scale, showing the MP concentration in the sample (ppb), and the x-axis shows the sampling days. Significant differences are shown within tissues (D2-D10 comparisons) and across tissues (arrows) with $p > 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). (B) Major transfer pathways for MP, including trophallaxis, royal jelly provisioning, and maternal offloading. (C) Bubble chart shows the relationship between MP concentration in queen ovaries (x-axis) and eggs (y-axis) at D10. The size of the circle represents an approximate number of eggs for each MP-treated queen, with larger circles showing a larger egg production for that queen. Queens producing more eggs showed lower egg concentrations, consistent with dose dilution. (D) Transfer kinetics of MP dispersion along the worker–queen–egg axis. Accumulation rates were calculated by plotting the average concentrations at D2 and D10 (with D0 set to

the tissue-specific LOQ; SI Appendix, Table S3) for each of the four cages and each sample. Workers show the highest rate (108.56 ppb/day, $R^2 = 0.87$), eggs the intermediate rate (8.65 ppb/day, $R^2 = 0.84$), and queens the lowest (0.70 ppb/day, $R^2 = 0.58$), consistent with active maternal offloading rather than passive diffusion. Data are presented in orange for worker bees, dark gray for eggs, and green for queen bees. Slopes are indicated in the inset text boxes associated with each color. See also Table S3.

Individual queen data suggested a dose–dilution pattern, with queens that laid more eggs distributing their body burden across more offspring, resulting in lower per-egg concentrations (Figure 2.2C, Table 2.1). At day 10, Queen 1 laid approximately 30 eggs, with individual egg concentrations averaging 140.76 ppb (ovary concentrations: 3.42 and 0.01 ppb), while Queen 3 laid approximately 90 eggs, averaging 80.98 ppb per egg (ovary concentrations: 7.01 and 2.23 ppb). At day 10, whole-body queen MP concentrations ranged from 16–23 ppb ($n = 4$ queens; Table 2.1), while average per-queen ovary concentrations ranged from 1.71–9.67 ppb (mean of two ovaries per queen; Table 2.1; Figure 2.2C).

[¹⁴ C]-MP-treated queens			Whole Queen Body (ppb)	Average Ovaries (ppb)	Individual Ovaries (ppb)		Eggs (ppb)	Total Eggs measured (n)
Queen 1	Cage 1	Round 1	23 ± 0.5	1.71 ± 2.4	(3.42)	(0.01)	140.76 ± 3.7	30
Queen 2	Cage 2	Round 1	21 ± 0.3	6.60 ± 7.6	11.96	(1.23)	131.93 ± NA	30
Queen 3	Cage 1	Round 2	16 ± 2.1	4.62 ± 3.4	(7.01)	(2.23)	80.98 ± 16.4	90
Queen 4	Cage 2	Round 2	18 ± 4.7	9.67 ± 1.1	(10.44)	(8.89)	87.86 ± 10.7	90

Table 2.1. Concentration of [¹⁴C]-MP (ppb) in whole queen bodies, queen ovaries, and eggs at day 10 post-exposure. Each queen is identified by its cage of origin and the experimental round. Individual ovary measurements are shown (representing two ovaries per queen; values below the limit of quantification are indicated in parentheses). Egg concentration represents the average concentration across all measured eggs per queen.

All tissues exhibited significant increases in MP concentration from day 2 to day 10; however, these increases varied significantly among bee castes. Worker bees showed the greatest increase ($\Delta \approx 1003.82$ ppb, $p < 0.0001$), followed by eggs ($\Delta \approx 41.08$ ppb, $p = 0.003$), queen bees ($\Delta \approx 8.68$ ppb, $p < 0.0001$), and queen bee ovaries ($\Delta \approx 5.53$ ppb, $p = 0.005$) (Figure 2.2A). The only exception was feeders,

which showed a slight decrease over time ($\Delta \approx -21.12$ ppb, $p = 0.0052$), likely due to reduced [^{14}C]-MP stability in solution, volatilization, or adsorption to the glass walls of the storage container.

To determine whether pesticide enrichment in eggs reflects passive transfer or active concentration, we compared accumulation rates across castes. We quantified differential accumulation dynamics among colony members by calculating MP accumulation rates from average concentrations at D2 and D10, with D0 set to the limit of quantification (LOQ) for each sample (**Figure 2.2D**). Worker bees exhibited the highest accumulation rate at 108.56 ppb/day ($R^2 = 0.87$), consistent with their role as the primary contact point with contaminated food and their limited ability to offload contaminants. In contrast, queen bees showed the lowest accumulation rate at 0.70 ppb/day ($R^2 = 0.58$). Eggs displayed an intermediate accumulation rate of 8.65 ppb/day ($R^2 = 0.84$), exceeding the queen's rate by more than ten-fold and indicating active concentration of MP in reproductive tissue.

The presence of a queen alters pesticide distribution throughout the colony

To determine whether queen presence alters the total dietary pesticide load entering the colony, we first compared food consumption rates between queenright and queenless nano-colonies (**Fig. 2.3**). Estimated Marginal Means (EMMs) for diet consumption revealed two significant groups by day: D10 ($n=4$ cages) and D2 ($n=4$ cages) (Tukey-adjusted, $p < 0.05$) (**Fig. S2.1**). No significant differences were observed within the same days across rounds (R1 and R2) or treatments (control and [^{14}C]-MP). Queenright nano-colonies consistently showed higher consumption rates ($m=0.143$) than queenless colonies ($m=0.001$) (**Fig. S2.2**), indicating that queen presence stimulates worker feeding behavior at the source feeder.

To evaluate how this social context influences chemical distribution, we compared MP accumulation patterns between queenright and queenless nano-colonies (**Figure 2.3**). The total MP burden,

pooled across all comparable samples (workers, wax, and diet from cells), did not differ significantly between queenright and queenless colonies ($\chi^2 = 0.2062$, $df=1$, $p = 0.65$). Thus, despite the higher feeding rates observed in queenright colonies (**Fig.2.3; Fig. S2.2**), the queen's presence does not alter the total pesticide burden retained across measurable colony tissues by day 10, but fundamentally reshapes where the burden is deposited. Indeed, the queen's presence significantly influenced pesticide partitioning among tissues (**Fig. 2.4**). At day 2, both worker bee body burdens and wax samples had low frequencies of detection (FOD) and many measurements were at or near the LOQ in both conditions, preventing reliable statistical comparison at this early time point (**Fig. 2.4A and C**). However, by day 10, worker bees in queenright cages showed tighter concentration distributions (i.e., reduced interquartile range, IQR) and higher median body burdens than those in queenless cages (**Fig. 2.4B**). Furthermore, a higher frequency of detection (FOD) above quantification limits indicated greater [^{14}C]-MP deposition in wax from queenright cages on day 10 (**Fig. 2.4D**).

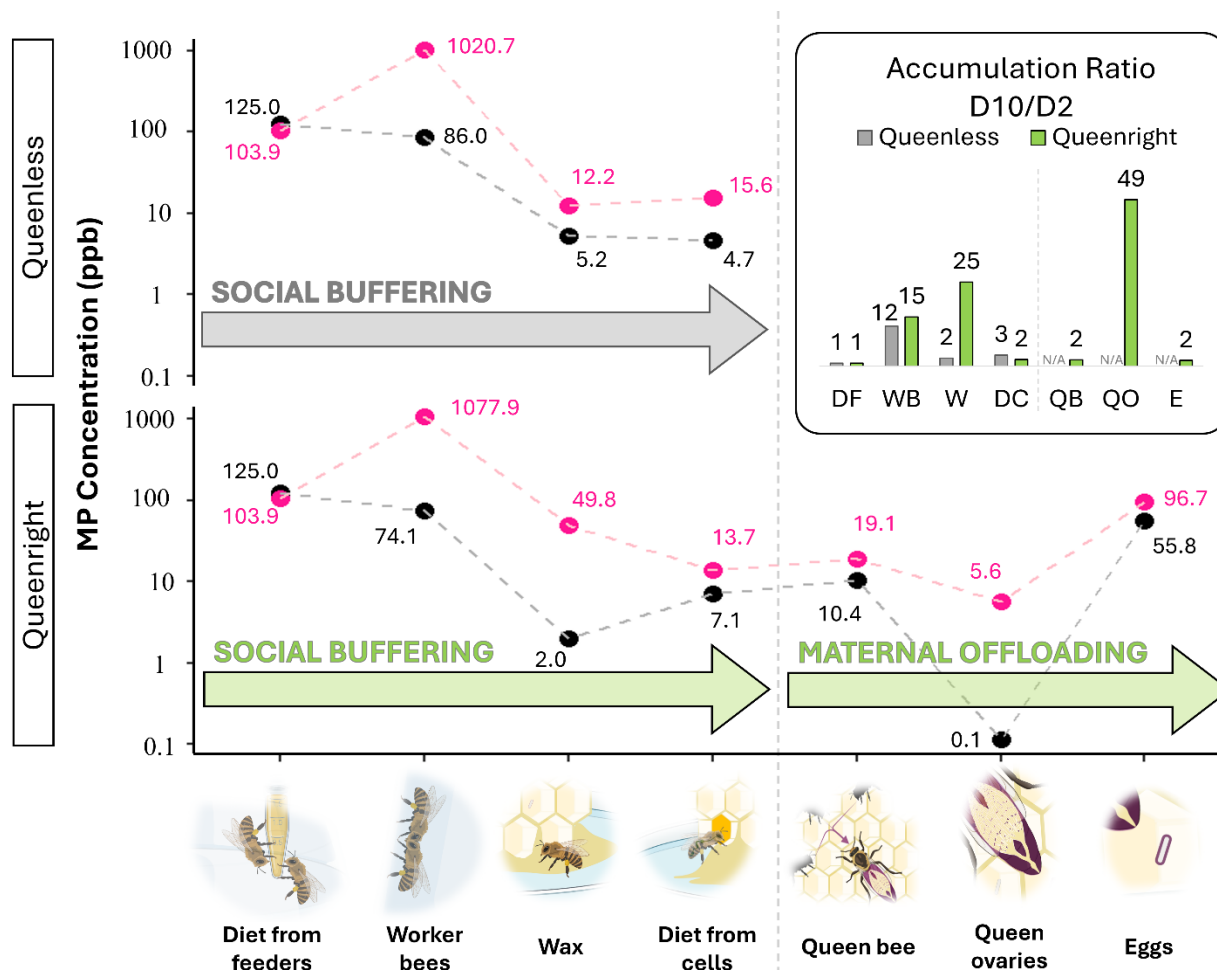


Figure 2.3. Pesticide transfer as mediated by social buffering and maternal offloading pathways. Concentrations of MP (ppb, log scale) across hive matrices in queenless (top panel, $n=8$ cages) and queenright (bottom panel, $n=8$ cages) nano-colonies are shown. Black dots indicate day 2 (D2, $n=4$ cages) mean, and pink dots indicate day 10 (D10, $n=4$ cages) mean. Dashed lines connect sequential matrices along each transfer pathway, with concentration values labeled in ppb. Gray arrows indicate the dominant transfer pathways in each colony type. The social buffering pathway, present in both colony types, demonstrates pesticide processing through worker bees to storage matrices, while the maternal offloading pathway (observed only in queenright colonies) diverts pesticides through queens to eggs. An inset displays accumulation ratios (D10/D2; values > 1 indicate net accumulation over the exposure period) for each matrix, comparing queenless (gray) and queenright (light green) colonies across diet sources: feeders (DF), worker bees (WB), wax (W), diet from cells (DC), queen bees (QB), queen ovaries (QO), and eggs (E). See also Figure S2.

The queenright and queenless colony comparisons (**Figure 2.3**) show that the presence of a queen redirects pesticide flux from purely social buffering pathways to an additional maternal offloading route. The calculated accumulation ratios (D10/D2) show the greatest relative accumulation in ovaries (49 $\times$) and

wax (25×), followed by eggs (2×). However, these values must be interpreted in the context of the underlying measurements: wax and egg concentrations were well above the LOQ at both time points, making their accumulation ratios direct comparisons of quantified values. In contrast, the high ovary accumulation ratio reflects a comparison between a well-quantified D10 mean and a D2 value at or below the LOQ. Therefore, the 49× ratio represents a lower bound rather than a precise fold-change. The lower egg accumulation ratio is further explained by baseline egg concentrations already being detectable at D2 in one queen (55.8 ppb in 90 eggs), which reduces the apparent fold-change relative to tissues where D2 signals were near the detection limit. We acknowledge that the queenright–queenless comparison is based on $n = 4$ cages per condition per time point at the biologically independent level. This setup includes multiple individual bees (56 workers across the cages), egg pools (18 in total), and tissue samples per cage, offering technical replication within each treatment. As a result, patterns within each treatment are well-defined, while differences between treatments should be considered descriptive for this group until further large-scale replication is performed.

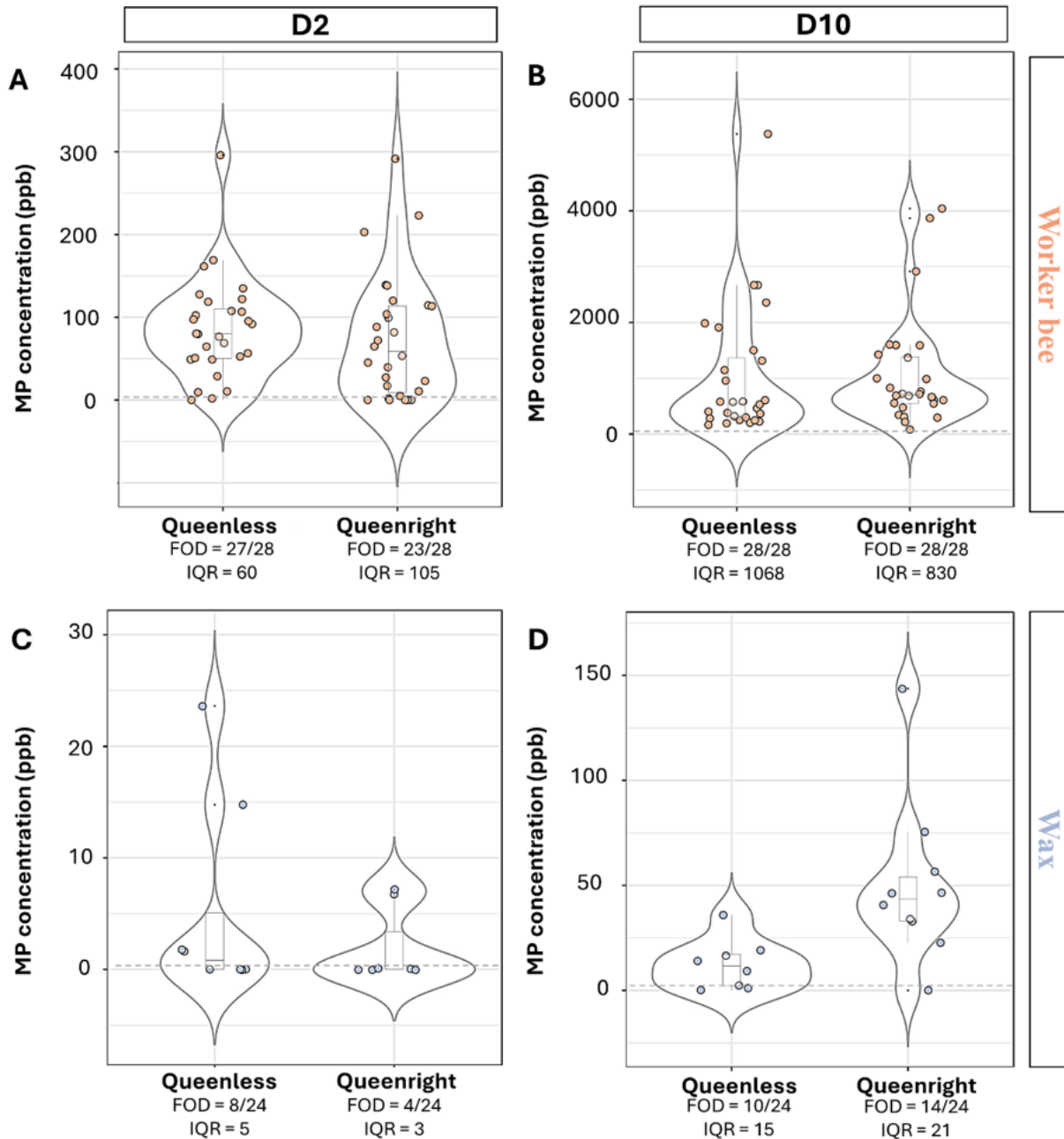


Figure 2.4. Queen presence influences pesticide distribution in worker bees and wax. The figure compares D2 and D10 samples of worker bees ($n=112$ total) (A, B) and wax ($n=96$ total) (C, D) from queenright and queenless nano-colonies. Each point represents an individual worker bee or wax sample; violin plots show the probability distribution. Gray dotted lines indicate the LOQ; FOD and IQR values are shown below each plot. A Type III ANOVA of a linear mixed effect model with Wald Chi-squared tests showed no significant difference across MP concentrations between queenright ($n = 8$) and queenless ($n = 8$) nano-colonies ($\chi^2 = 0.2062$, $df = 1$, $p = 0.65$). Day 2 data in both conditions show high variability in workers, and limited detection above the LOQ in wax. However, queenright cages at day 10 show reduced interquartile ranges in workers and higher FOD in wax, indicating queen-mediated effects on pesticide partitioning.

Discussion

Our study reveals three key dynamics governing pesticide flux in honey bee colonies: (1) worker bees actively reduce pesticide concentrations while processing and storing the diet in comb cells, a capacity that diminishes under chronic exposure; (2) queens maintain markedly lower body burdens than workers through workers' filtering function and queens' active offloading of accumulated pesticides to developing eggs; and (3) queen presence fundamentally reshapes colony-wide pesticide distribution by concentrating worker exposure and enhancing wax deposition.

The ability of worker bees to substantially reduce [^{14}C]-MP levels in a processed diet stored in wax cells, compared with source feeders, suggests active processing and selective deposition into wax matrices (**Figure 2.1A**). Passive diffusion of MP from the stored diet into the surrounding paraffin wax seems to play a minor role since our measured wax samples showed approximately 2 ppb of MP at D2 (**Figure 2.4C**), compared with ~13 ppb in the diet inside the comb cells and ~131 ppb in the source feeders. Therefore, passive diffusion into wax can explain only a small portion of the ~118 ppb (~95%) difference observed between feeders and stored diet. This filtration strategy aligns with known bee physiology: young workers contain high levels of detoxification enzymes, including cytochrome P450 monooxygenases, glutathione S-transferases, and carboxylesterases, which are used during nectar processing and royal jelly production²⁶. The conversion of nectar into honey through invertase, glucose oxidase, and amylase activities provides opportunities for simultaneous pesticide metabolism^{17,27,28}. Our data show that the processed diet on day 2 contained 95% less MP than the source feeders, suggesting that these enzymatic activities partially neutralize incoming resources before queens or developing broods can access them. However, by day 10, pesticide levels in the processed diet were significantly higher than at day 2 (**Figure 2.1A**), suggesting saturation of metabolic capacity, depletion of detoxification cofactors, or physiological stress-induced impairment of metabolic processes. The increase in comb-cell MP cannot be simply explained by an increase in upstream input. The feeder MP showed a slight decrease from D2 to D10 ($\Delta \approx -21.12$ ppb, $p =$

0.0052; **Figure 2.2A**), indicating that the pesticide pool entering the colony did not increase over the study. Additionally, the increase in comb-cell MP does not align with a passive parallel accumulation in both compartments, as the worker body burden increased approximately 55-fold over the same period, while comb-cell MP rose by only about 40%. This uneven increase suggests a gradual failure in the worker-mediated filtration process. The limited detoxification gene repertoire of *A. mellifera* compared to other sequenced insects⁵ would further limit its ability to maintain enzymatic processing under chronic exposure. Similar effects have been reported in predatory insects, where repeated insecticide exposure impairs detoxification²⁹, and in honey bees, where nutritional stress compromises pesticide metabolism³⁰. The progressive decline in worker filtration efficiency has far-reaching implications, suggesting that chronic, low-dose exposure may be more detrimental than acute, high-dose exposure once the colony's buffering capacity is saturated.

We observed a broad range of MP concentrations among individual workers (**Figure 2.4A and B**). This heterogeneity is likely consistent with the division of labor that is well-documented in honey bees^{9,31–34}. Honey bees exhibit plasticity in single-cohort cage assays and will adapt a division of labor independent of the age of the bees³². In colony settings, task specialization creates distinct exposure patterns, with foragers often carrying higher pesticide loads than nurses³⁵. Our data extend this observation to controlled environments, showing that toxicant fate varies among individuals and may be influenced by social role. However, direct assessment of task specialization in worker bees will require future behavioral testing, including whether a subset of workers preferentially absorbs the chemical burden through task-specific chemical processing.

Unlike the high and variable pesticide loads observed in workers, queen [¹⁴C]-MP body burdens remained consistently lower than those of workers despite continuous exposure (**Figure 2.1B, 2.2A**). By day 2, queen levels approached the LOQ, whereas worker levels had already reached high levels. By day 10, queen burdens increased but remained an order of magnitude below worker means. This difference is

likely attributable in part to dietary differences between workers and the queen bee. Workers ingest both nectar/syrup and pre-processed worker jelly, while the queen is almost exclusively fed royal jelly.^{36,37} This glandular secretion is derived from already processed dietary inputs in the nurse's head and contains a reduced pesticide load (consistent with our findings in **Figure 2.1A**). However, dietary filtration alone does not fully account for the more than 50-fold difference in body burden between workers and the queen by day 10 (**Figure 2.1B**)¹⁷. Instead, our data indicate that maternal offloading is a key detoxification pathway for reproductive females. For this study, eggs served as the most downstream MP repository we measured in the colony, with concentrations exceeding those in the whole queen body (**Figure 2.2A-C**). We did not track the subsequent fate of these eggs (hatching success, larval viability, or potential cannibalism). Determining whether contaminated eggs continue to circulate the chemical burden within the colony or are removed from it is an important next step. The increased concentration in reproductive tissue parallels findings in other insect systems, where lipophilic compounds selectively partition into developing eggs owing to their high lipid content^{38,39}. The negative correlation between egg number and egg concentration (**Figure 2.2C**) further indicates a dose-dilution effect: queens laying more eggs distribute their body burden among more offspring, thereby reducing per-egg contamination. However, with $n = 4$ queens, this remains a descriptive observation rather than a statistically tested correlation. Although maternal offloading has been documented in many aquatic and mammalian organisms^{20,40,41}, it has been largely ignored in social insects. Our findings indicate that this may be a critical yet underappreciated route for both queen detoxification and brood exposure.

Our analysis of pesticide accumulation kinetics revealed pronounced differences among workers, queens, and eggs (**Figure 2.2D**), further supporting the social buffering hypothesis within the toxicological framework. Workers showed the highest accumulation rate, queens the lowest, and eggs the intermediate rate. Notably, egg concentrations exceeded queen body burdens, suggesting that pesticides could be actively concentrated in the eggs rather than being passively transferred via lipophilic gradients. This concentration

gradient could indicate active transporter-mediated sequestration, for which further mechanistic studies are needed^{42,43}.

This protective effect of maternal offloading for queens comes at a cost: increased embryo exposure. By day 10, egg concentrations reached 81-141 ppb (**Figure 2.2A**), substantially exceeding queen burdens. Little is known about how pesticides may impact eggs⁴⁴, but larvae are generally more susceptible to pesticide toxicity than adults because they lack fully developed detoxification mechanisms during rapid development^{42,45,46}. Thus, maternal burden transfer could contribute to egg and brood mortality and developmental abnormalities in field colonies chronically exposed to pesticides⁴⁷. Egg and larval losses immediately reduce colony reproduction and future population size. Therefore, pesticide accumulation in eggs represents a potentially severe effect that is undetectable in standard adult toxicity studies. The majority of cage assays rely predominantly on queenless worker assays and, as our data show, do not capture embryonic or brood exposure or the colony-level consequences of chronic contamination⁴⁸⁻⁵⁰.

This demonstration of maternal offloading in honey bees extends beyond taxonomic novelty. Although this mechanism is well established in vertebrates, its discovery in social insects has fundamentally different implications given the eusocial colony structure. In a queenright colony, the queen is the only individual responsible for reproduction who can continuously produce a large number of eggs. This imbalance suggests that prioritizing the queen's survival, even at the cost of some eggs, could be an evolutionary advantage for social bees to ensure a colony's long-term health and survival. In solitary organisms, maternal offloading represents a trade-off between maternal protection and offspring exposure. By lacking the worker-mediated dietary filtration that buffers honey bee queens, solitary bees have no comparable upstream defense. In alfalfa leaf-cutting bees, for example, the phenomenon can be devastating to the individual female's fecundity, though a small percentage of eggs do survive and hatch⁵¹. In eusocial systems, however, this mechanism is embedded within a multilayered colony defense network, in which sterile workers serve as an initial chemical buffer and reproductive queens are the sole source of

replacement workers⁶. Failure of worker filtration under chronic exposure transforms maternal offloading from a potential adaptive mechanism into a colony-level vulnerability, as contaminated eggs cannot be replaced by alternative reproductive individuals. The intergenerational toxicological consequences of maternal transfer, including effects on larval survival, development, and fitness, have been demonstrated in fish⁵² and invertebrates⁵³ but remain uncharacterized in honey bees, representing a critical gap given the importance of brood replacement to colony recovery.

Beyond the queen–egg transfer axis, the queen's presence altered MP distribution across the hive. We acknowledge that the comparison between queenright–queenless conditions is based on four cages for each condition at each time point. Although the patterns within each treatment are well supported by repeated sampling of tissues and individual bees within each cage, the differences between treatments, as described below, should be viewed as descriptive of this cohort and should encourage validation in larger, replicated studies. Chronically exposed worker bees in queenright cages showed tighter concentration distributions and higher median body burdens than those in queenless cages (**Figure 2.4B**). These observations support the hypothesis that social buffering shapes chemical accumulation patterns and demonstrate the influence of social cues on this process. This suggests that queen pheromones or worker behavioral requirements related to queen care can alter task assignment and increase exposure. Queens produce pheromone bouquets, particularly queen mandibular pheromone (QMP), that regulate worker behavior, ovarian development, and foraging, nursing, and comb-building activities^{3,54}. Queenright colonies exhibit a more developed division of labor with clearer distinctions between nurses and foragers^{9,32,34}. While our data cannot directly establish whether the presence of the queen influences task-specific subpopulation exposure in nano-colonies, the higher and tighter worker bee body-burden distributions observed in the queenright cages (**Figure 2.4B**) suggest this possibility. By using artificial QMP exposure in queenless colonies, future studies could directly test whether queen pheromone signaling, directly or indirectly, modulates xenobiotic processing in workers, independent of task allocation^{3,54}.

367 Additionally, a higher frequency of detection (FOD) above the quantification limits indicated
368 greater [^{14}C]-MP deposition in the wax of queenright cages on day 10 (**Figure 2.4D**). Wax production
369 requires substantial honey consumption, and workers building comb to receive eggs inevitably ingest more
370 contaminated food while concentrating lipophilic compounds in secreted wax. This has important practical
371 consequences: wax contamination is often used as an integrative marker of colony exposure history ^{10,55}.
372 However, our results suggest that wax residues may underestimate colony exposure when queens are absent
373 or brood production is low. Conversely, wax serves as a long-term sink, potentially releasing accumulated
374 pesticides into the hive environment and extending exposure beyond initial contamination.

375
376 Together, our data reveal fundamentally different exposure dynamics, underscore the profound
377 influence of sociality on toxicodynamics, and highlight the strengths and limitations of colony-level
378 approaches. Initially, worker filtration protects the queens, suggesting that colony-level tolerance may
379 exceed individual-level predictions under acute exposure. However, chronic exposure saturates this
380 buffering capacity, concentrating pesticides in eggs to levels that approach or exceed larval LD₅₀ values for
381 organophosphates ^{46,56}. This temporal pattern, with an initial protective phase followed by a breakdown in
382 social protection, may explain field observations of delayed colony losses occurring months after initial
383 pesticide application ^{57,58}. In this context, maternal offloading is best understood as a last defense: a
384 mechanism that protects the queen when worker filtration is overwhelmed yet ultimately transfers the
385 colony's chemical burden to its most vulnerable and irreplaceable tissue. Our results emphasize that honey
386 bees must be studied as integrated superorganisms, rather than as aggregations of individuals, because both
387 the integration of their social defenses and the conditions under which those defenses fail determine colony
388 fate.

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Author contributions

A.M.E., S.C.T.N., J.D.F., S.P.L., and B.A.B. designed the study; A.M.E., J.D.F., and E.L. performed the study; D.B-R. contributed new reagents; A.M.E., D.B-R., S.P.L., and B.A.B. analyzed the data; and A.M.E., J.D.F, and S.C.T.N. wrote the manuscript with input from all authors.

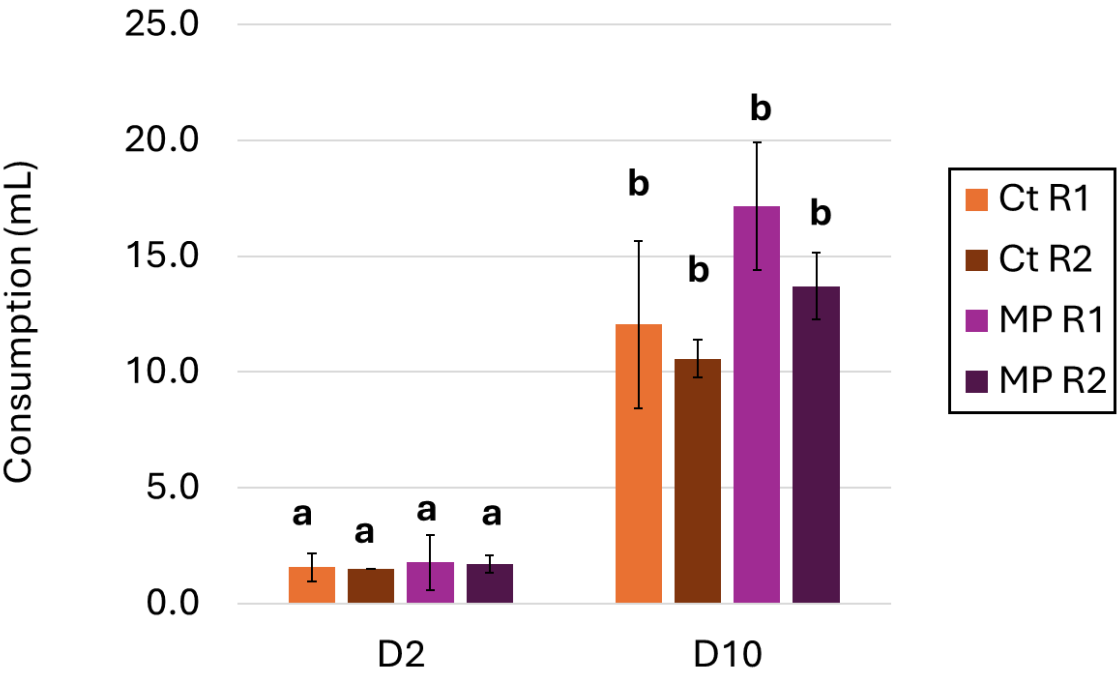


Fig. S2.1. Total diet consumption in control and [¹⁴C]-MP-treated nano-colonies. Related to STAR Methods. Bars show the average total diet consumption $\pm$ SD per round on sampling days (D2 and D10). A Type III ANOVA followed by a pairwise comparison of the estimated marginal means identified two significant groups. Letters (a, b) denote significantly different groups (Tukey-adjusted, $p < 0.05$, $n = 4$ cages per treatment); different letters indicate significant differences between sampling days, but not between treatments or rounds.

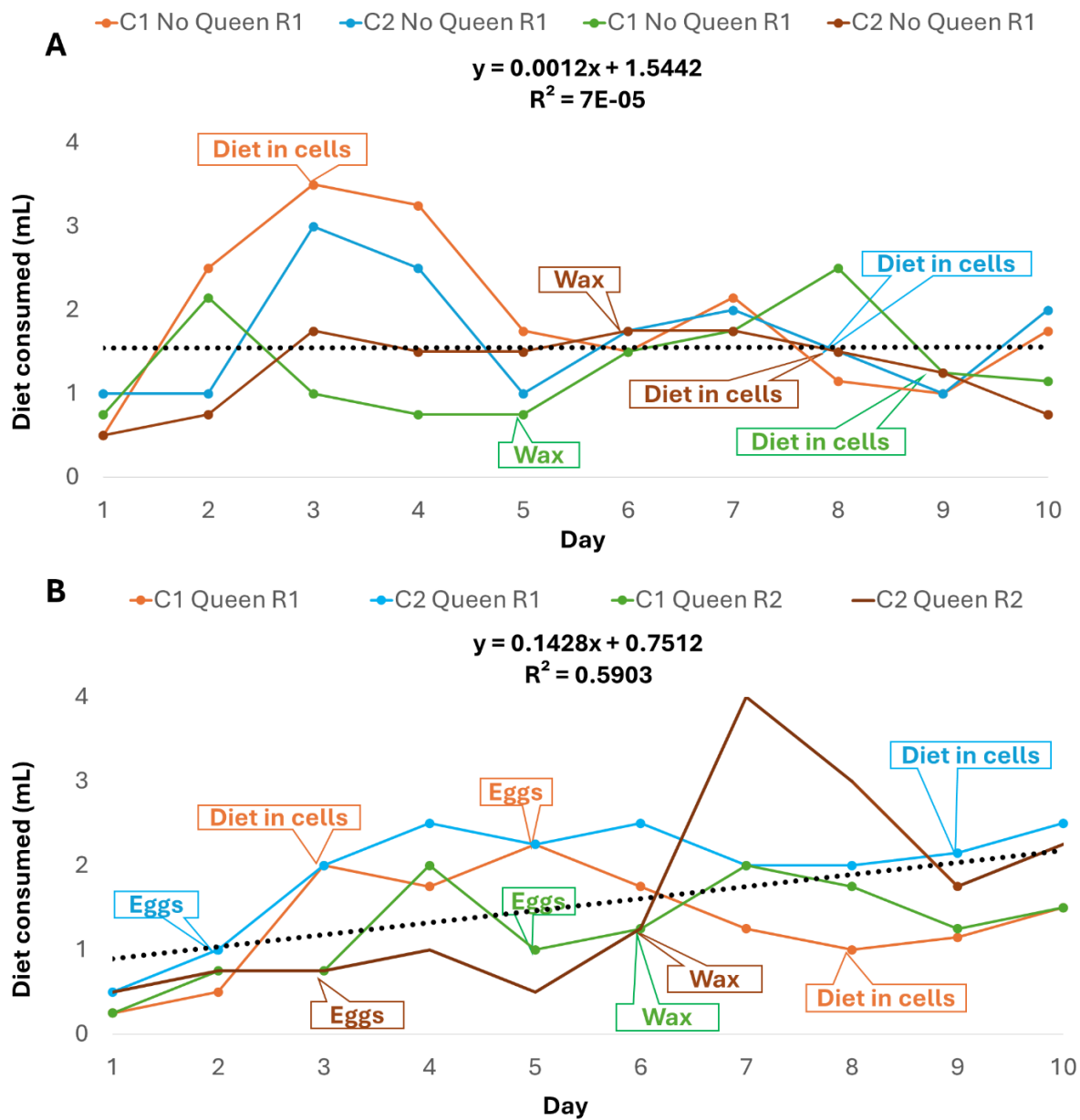


Fig. S2.2. Daily diet consumption (mL/cage) in queenless (A) and queenright (B) nano-colonies through day 10. Related to Figure 2.3. Callouts mark the first observed occurrence of eggs, wax, or diet in comb cells. Each cage's diet consumption is represented by a different color, and the overall consumption trend is shown with a black dotted line. Queenright cages showed increasing consumption over time ($m=0.143$, $R^2=0.59$), whereas queenless cages showed stable or decreasing consumption ($m=0.001$, $R^2=7E-05$). Consumption peaks often occurred before or after specific activities; for example, workers consumed more diet the day before egg laying but less on the day eggs were deposited.

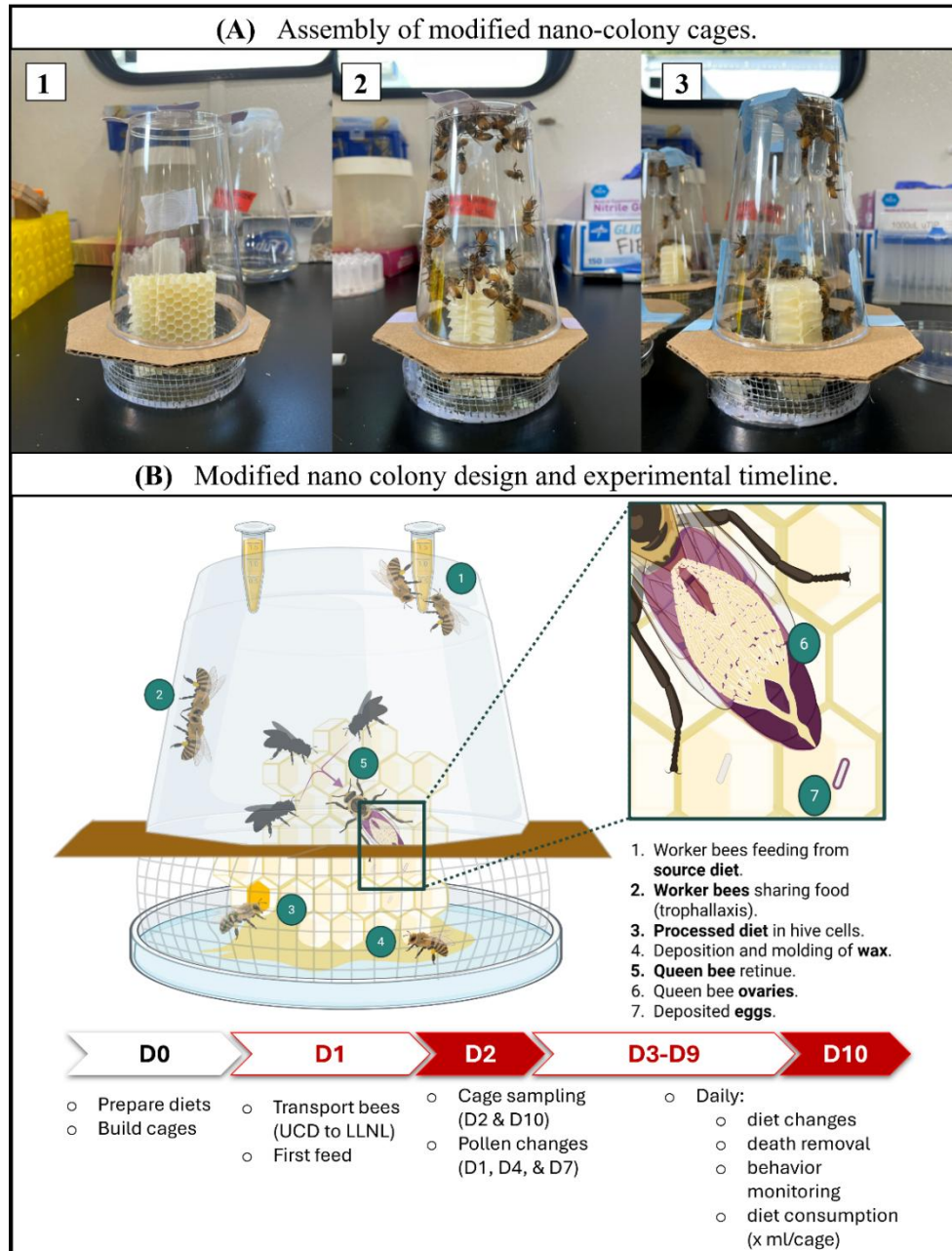


Figure S2.3. Modified nano-colony design, assembly, and experimental timelines. Related to STAR Methods. (A) Assembly of modified nano-colony cages. (1) Empty nano-cage with no feeders. (2) Side view of the nano-cage with worker bees added and no feeders. (3) Side view of the nano-cage with worker bees and feeders. (B) Schematic of the nano-colony setup and 10-day exposure timeline. The wax frame was placed vertically in the center of each cage, mimicking field-realistic hive frames. This design accommodated ~60 worker bees and one queen bee, enabling observation of: (1) worker bees feeding from the source diet, (2) trophallactic food sharing among workers, (3) processed diet stored in comb cells, (4) wax deposition and comb construction, (5) retinue behavior around the queen, (6) queen oviposition, and (7) egg deposition in cells. In the timeline, red shading indicates the 10-day exposure period; solid red boxes mark sampling days: day 2 (acute) and day 10 (chronic).

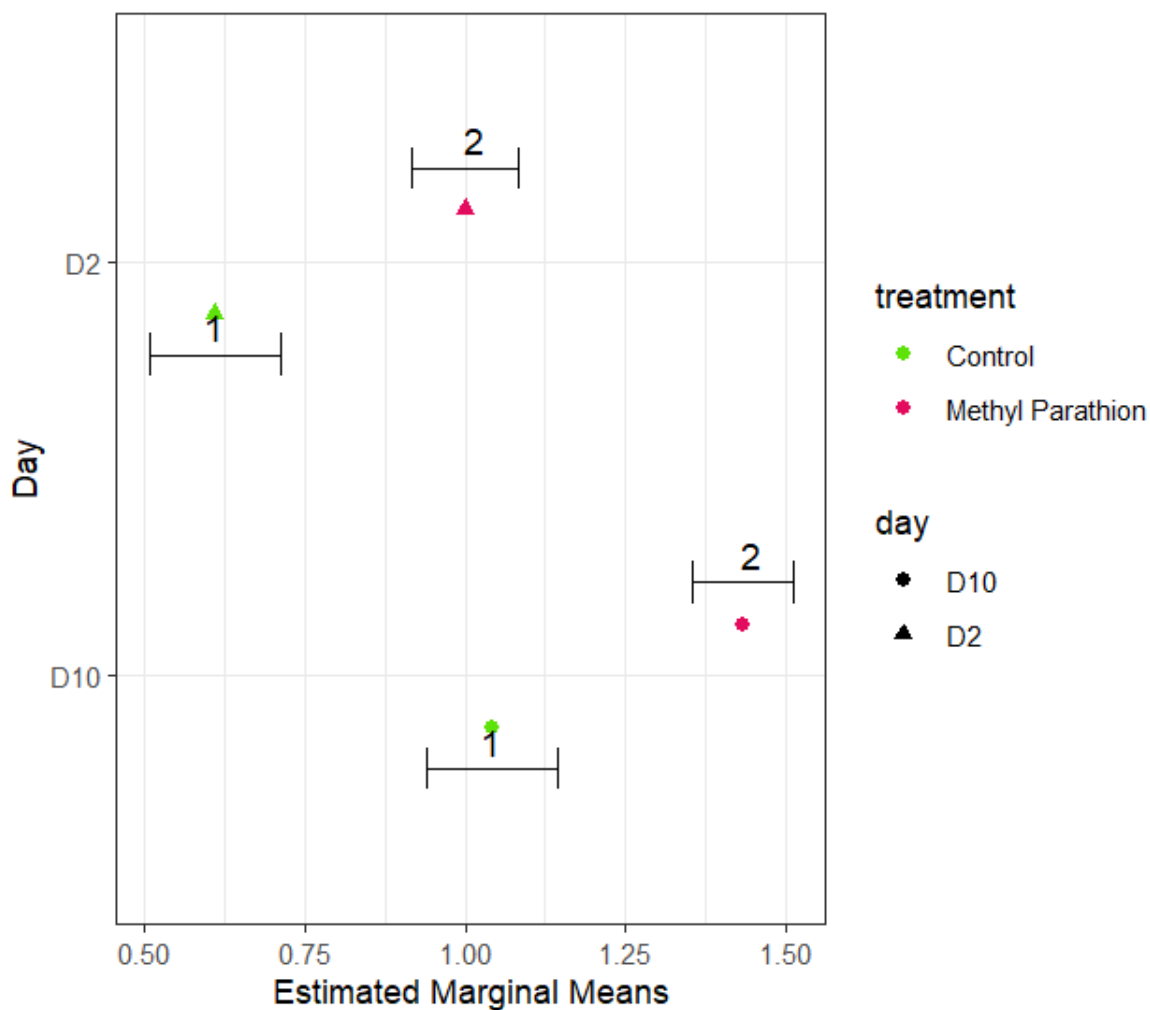


Fig. S2.4. Comparison of fraction modern values between control (green) and $[^{14}\text{C}]$ -MP-treated (pink) nano-colonies at D10 (day 10, •) and D2 (day 2, ▲). Related to STAR Methods. Values represent estimated marginal means $\pm$ SE across all samples for each treatment/day combination. $[^{14}\text{C}]$ -MP-treated ($n=297$; D2: $n=138$, D10: $n=159$) nano-colonies showed significantly higher fraction modern values than controls ($n=146$; D2: $n=75$, D10: $n=71$) at both days (Tukey-adjusted pairwise comparisons, $p = 0.0098$ for each), confirming successful tracer incorporation.

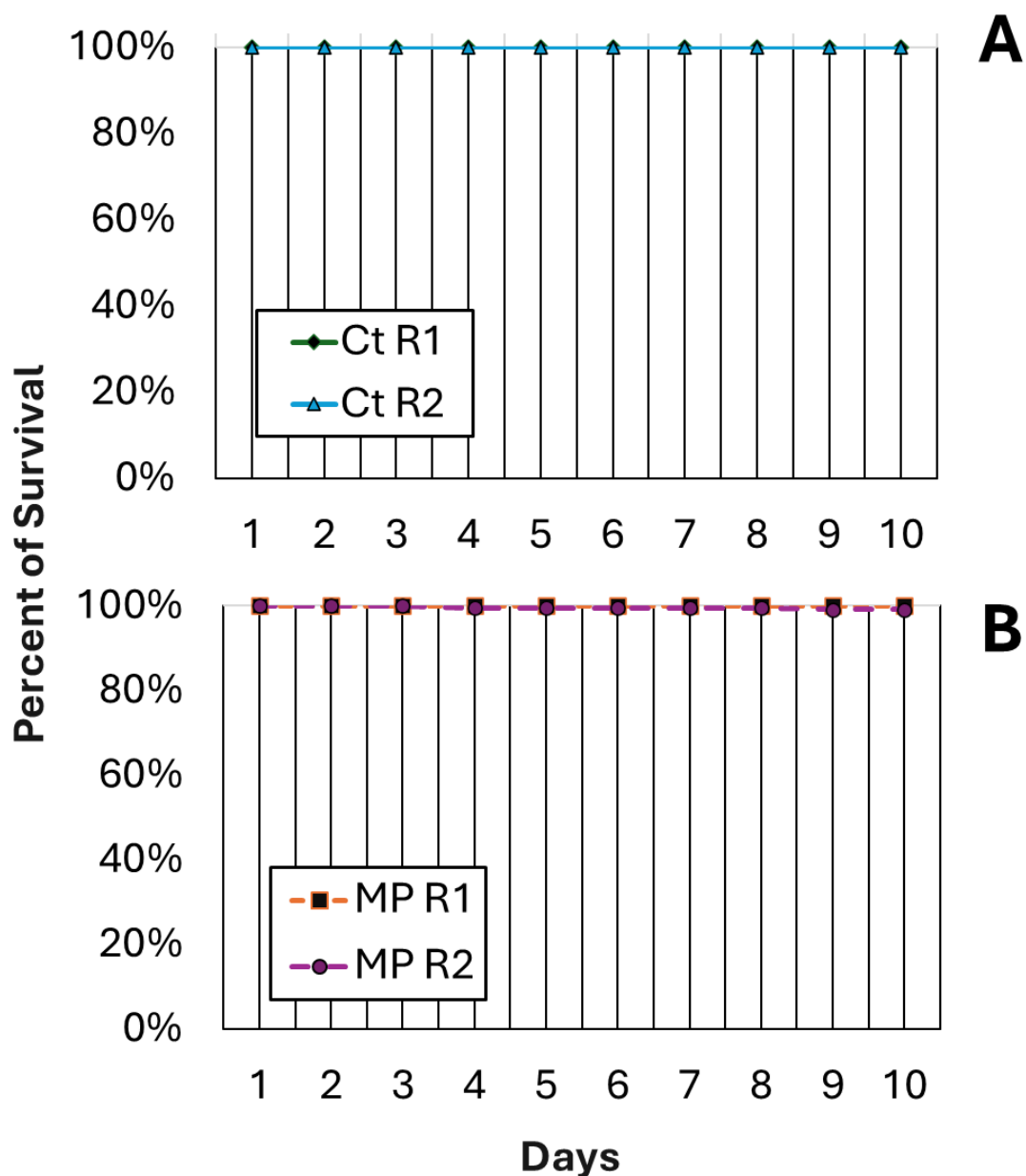


Fig. S2.5. Survival curves for control (Ct) and $[^{14}\text{C}]$ -MP-treated (MP) nano-colonies. Related to STAR Methods. Survival is shown for control cages ($n=8$) (R1: green; R2: blue) and $[^{14}\text{C}]$ -MP cages ($n=16$) (R1: orange; R2: red) over the 10-day exposure period. Lines represent the average percent survival $\pm$ SD across queenright and queenless cages combined. A Type III ANOVA revealed no significant difference in survival between treatments ($F\text{-value}=1.1053$, $df=1$, $p=0.3051$) or rounds ($F\text{-value}=2.2105$, $df=1$, $p=0.1519$). Tukey-adjusted pairwise comparisons and Sidak-adjusted mean-group summaries were consistent with this finding ($p > 0.05$), indicating that the 25 ppb daily exposure concentration did not induce acute mortality.

Table S2.1. Comparison of sample size requirements for common analytical techniques used to detect chemical residues in insects. Related to STAR Methods. Gas Chromatography (GC), Liquid Chromatography (LC), Mass Spectrometry (MS), Tandem Mass Spectrometry (MS/MS), Accelerator Mass Spectrometry (AMS), and Parts per Billion (PPB). Total carbon percentage per honey bee tissue was measured at the UC Davis Analytical Laboratory using SOP 522.1.

Instrument	Tissue Samples	Required amount of samples (n)	Sample weight	Required total sample weight	Total carbon (%)	References
GC-MS and LC-MS	Whole Worker bee	~50	100 mg	5 g	NA	s1
LC-MS/MS (1 ppb)	Whole Worker bee	~50	100 mg	5 g	NA	s2
BioAMS	Ants	~1	0.001 g	0.001 g	NA	s3
	Honey bees (all tissues)	~1	0.005 g	0.005 g	50	This study
	Eggs	30	0.0001 g	0.003 g	78	s4
	Wax	~1	0.002 g	0.002 g	80	This study
	Feeder diet (source)	1	0.010 mL	0.010 mL	21.06	This study
	Stored diet (comb)	1	0.006 mL	0.006 mL	33.69	This study

Table S2.2. Methyl parathion concentrations (ppb) reported in honey bee hive matrices from field surveys. Related to STAR Methods. These data demonstrate the environmental relevance of the 25-ppb daily exposure concentration used in this study.

Name	Class	Pollen ^{S5}	Honey ^{S5}	Bee bread ^{S6,S7}	Beeswax ^{S5,S8}	Adult honey bees ^{S5,S8}
Parathion methyl	Insecticide	26000.00	50	6.6	4.6-3085.00	1.8-3600.00

Table S2.3. Limits of quantification (LOQ; mean + 2σ, 95% CI) for AMS measurements by tissue type. Related to Figure 2.2 and STAR Methods. Fraction modern values are shown for each tissue/day/round combination; ppb equivalents are shown per tissue.

Tissue	Day & Round	LOQ (Fraction modern)	LOQ (ppb)
Worker bees	D2 R1	1.067	14.34
	D10 R1	1.058	
	D2 R2	1.031	
	D10 R2	1.033	
Queen body (no ovaries)	D2 R1	1.021	13.78
	D10 R1	1.022	
	D2 R2	1.021	
	D10 R2	1.023	
Queen Ovary	D2 R1	1.038	14.28
	D10 R1	1.034	
	D2 R2	1.039	
	D10 R2	1.035	
Wax	D2 R1	0.100	14.84
	D10 R1	0.146	
	D2 R2	0.103	
	D10 R2	0.110	
Diet in feeders	D2& D10. R1&R2	1.036	7.35
Diet in cells	D2& D10. R1&R2	1.037	
Eggs	D2& D10. R1&R2	0.951	21.78

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STAR★METHODS

KEY RESOURCES TABLE

Chemicals, peptides, and recombinant proteins		
Methyl parathion-ring-[¹⁴ C] (specific activity = 1.0 mCi/mL or 13.8 mCi/mmol)	Sigma Chemical Co (St. Louis, MO)	CAS: 179795-13-2
Dimethyl sulfoxide (DMSO)	Tokyo Chemical Industry Co., Ltd	CAS: 67-68-5
Pure cane granulated sugar	C&H, Crockett, CA	https://www.chsugar.com/
Honey bee feed protein-based powder	MegaBee, LLC, San Diego, CA	https://megabee.com/
Deposited data		
Figshare	Figshare LLP ("Figshare")	https://doi.org/10.6084/m9.figshare.32373060
Software and algorithms		
R-Studio (R v.4.5.1)	R Core Team	https://www.r-project.org/
Microsoft® Excel® for Microsoft 365 MSO (Version 2604 Build 16.0.19929.20136)	Microsoft	https://www.microsoft.com/en-us

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Acquisition of worker and queen bees

In 2024, two rounds of worker bee and queen bee acquisition were conducted (R1: August; R2: September), each lasting 10 days. Honey bees were sourced from three colonies maintained by the USDA-ARS Invasive Species and Pollinator Health Research Unit in Davis, CA. *Varroa destructor* mite levels were monitored monthly using alcohol washes and controlled with formic acid applications (Formic Pro™). Three weeks before the scheduled LLNL exposure, three Italian queens were placed on drawn frames in deep-frame

isolation cages with queen excluder material. After 48 h, the isolation cages were removed. Sixteen days later, frames with developing bees were transferred to USDA-ARS incubators ($34.5 \pm 1^\circ\text{C}$, 75% RH maintained with a saturated NaCl solution). Upon emergence, newly emerged bees (NEBs) from three colonies were pooled before placement in cup cages. Sixteen cup cages were assembled per round, provided with water and a 1:1 w/w aqueous sucrose solution, and incubated at $34.5 \pm 1^\circ\text{C}$ overnight (D0). Newly mated Italian queens obtained from a Northern California commercial queen breeder were labeled and introduced into eight prepared nano-colonies.

METHOD DETAILS

Preparation and administration of dietary treatments

Dietary treatments were prepared the day before use by adding 1 mL of 5 ppm [^{14}C]-MP in DMSO stock solution to 200 mL (246.2 g) of 50% aqueous sucrose solution (w/w) to achieve a final concentration of 85 nM (25 ppb, specific activity = 1.5 nCi/mol, **Table S2.2**), or 1 mL of DMSO to 200 mL of sucrose solution for controls. The diet was administered via 2 mL Falcon tubes with two 1/8" holes drilled at the tip. Tubes were changed daily to prevent [^{14}C] contamination or mold growth.

Cage and experimental design

Modified nanocages were assembled according to the design of Lamas et al.⁵⁹, using a plastic cup placed over a plastic Petri dish with mesh windows for ventilation. Modifications included using a paraffin-wax comb (3" H $\times$ 2.5" W) instead of natural wax to avoid additional contamination from pesticide residues and a metal mesh between the cardboard skirt and the Petri dish to facilitate handling and increase bee mobility (**Figure S2.3A**). Each round, 16 cages were made: half with ~60 NEBs (queenless) and half with ~60 NEBs + 1 queen (queenright). For each set, equal numbers of controls (n=4) and [^{14}C]-MP-treated samples (n=4)

were included. Control cages were divided equally between LLNL (n=2) and USDA-ARS (n=2) to evaluate contamination. On D1, 12 cages (4 control, 8 [^{14}C]-MP) were transported to LLNL (~45 min); the remaining control cages (n=4) remained at USDA-ARS. All cages received 4 mL of diet treatment, 2 mL of DI water, and one tube of pollen supplement daily. [^{14}C]-labeled diets were kept in separate mini-refrigerators from controls at LLNL. Diet and water tubes were replaced daily, and consumption was recorded as a change in volume. Fresh pollen supplement was provided on D1, D4, and D7. The experimental design is shown in **Figure S2.3B**.

Cage and tissue sampling

Approximately 2 mL of diet and water from each cage was sampled daily. Whole cages were sampled on day 2 (D2, acute, n=8) and day 10 (D10, chronic, n=8), with equal numbers per treatment. After feeder removal, cages were flash-frozen in dry ice (~3 min). Worker bees were placed in pre-chilled 50 mL tubes; queens in pre-chilled 15 mL tubes; and paraffin-comb samples wrapped in aluminum foil. USDA-ARS controls were sampled using a similar protocol and transported to LLNL. All tissues were stored separately at -80°C by treatment. Worker bees were randomly sampled (n=28 per day from each colony type [queenright and queenless]), ensuring intact specimens. Dedicated tools per treatment prevented contamination. Queen ovaries were dissected into individual 2 mL screw-cap tubes; the remaining queen somatic tissue (i.e., all non-reproductive tissues, including muscle, fat body, nervous system, and integument) was homogenized together. Paraffin comb samples (1–2 mg) were collected over dry ice. Owing to the small carbon mass of eggs, 30–35 eggs comprised the optimal sample size (determined via optimization with control eggs) and were run as duplicates. Control egg samples were obtained from USDA-ARS hives and were excluded from the exposure due to insufficient control cage egg production. On D2 in [^{14}C]-treated cages, only one queen laid sufficient eggs for analysis.

Tissue homogenization and AMS sample preparation

Honey bee tissues were homogenized in RNase/DNase-free 2-mL tubes using a Mini Beadbeater (Biospec Products) with 1.0-mm silica spheres (six cycles). For this, 100 mg of bee tissue was dissolved in 500 μ l of DI water. Bee tissue carbon content averaged 50%, and aliquots of 50 μ l containing 10 mg tissue (approximately 5 mg of carbon, **Table S2.1**) were pipetted into short quartz tubes (6 mm o.d., 3–4 cm long), vacuum-centrifuge-dried, loaded into combustion tubes with excess CuO, evacuated, torch-sealed, combusted to CO₂, and reduced to graphite using Ognibene's method ⁶⁰. Aliquots of wax, ovaries, and diets containing 0.5–2 mg of carbon were not homogenized prior to loading.

BioAMS measurement

Carbon isotope ratios were measured using a National Electrostatics Corporation 250 kV single-stage AMS spectrometer at LLNL Biosciences & Biotechnology Division ⁶¹. Typical measurement times were 5-10 min/sample with 0.5-3% counting precision (RSD). The standard deviation across three to ten measurements ranged from 1% to 3%. Measured [¹⁴C]/[¹³C] ratios were normalized to four identically prepared isotopic standards (IAEA C-6).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data processing and calculations

Data processing and calculations involved organizing the data in Microsoft Excel, subdivided by tissue, treatment (control versus [¹⁴C]-MP), and day (D2 versus D10). Instrument-measured [¹⁴C]/[¹³C] ratios were used to determine the fraction modern values. The control fraction and modern values outside the average error were removed as instrument error. Controls were averaged by day and tissue, and this average was

subtracted from individual [¹⁴C]-MP samples collected on each day; the resulting values were then multiplied by conversion factors and tissue %C (**Table S2.1**) content to yield (fmol ¹⁴C/mg tissue).

$$\text{Excess } 14\text{C in tissue (fraction modern)} \times 0.098 \text{ fmol } \frac{14\text{C}}{\text{mol}} \times (0.01 \times \text{Tissue \%C}) = \frac{\text{fmol } 14\text{C}}{\text{mg tissue}}$$

Samples were converted to parts per billion by multiplying by the molecular weight and fraction label.

$$\left(\frac{\text{fmol } 14\text{C}}{\text{mg tissue}} \right) \times \left(263.20 \frac{\text{g}}{\text{mol}} \right) \times (\text{fraction label}) = \frac{\left(\frac{\text{fg}}{\text{mg}} \right)}{1000} = \frac{\text{ng}}{\text{g}} = \text{ppb}$$

LOQs per tissue were calculated per tissue as 2× standard average error of controls (95% CI) (SI Appendix, Table S3). Frequency of detection (FOD) was defined as the ratio of positive detections to the total number of samples submitted.

Statistical analysis

All analyses were conducted using R v.4.5.1 (RStudio interface). For modern fraction comparisons between treatments, cage identity was defined as the interaction among round, treatment, day, cage number, and type (queenless/queenright). A linear mixed-effects model followed by a Type III analysis of variance (ANOVA) with Wald Chi-squared tests was used to analyze the fraction modern as the response variable, with treatment and day as fixed effects, and cage identity as a random intercept. Estimated marginal means (EMMs) were calculated for each tissue-day combination using the emmeans package. The estimated marginal mean difference between groups is expressed as Δ (delta), representing (EMM₁ – EMM₂). Significant differences between treatments (control and [¹⁴C]-MP) across days were evaluated using pairwise comparisons and Tukey-adjusted post hoc tests, with statistical significance assessed at α = 0.05 (**Figure S2.4**). Linear models followed by a Type III ANOVA, were used to evaluate survival (**Figure S2.5**) differences between treatments, with treatment and round as fixed effects. EMMs were compared using Tukey-adjusted pairwise comparisons, while Sidak adjustments were applied to mean-grouping

summaries. Diet consumption (**Figure S2.1**) was evaluated using a linear model with treatment, round, and day as fixed effects, followed by a Type III ANOVA. For diet consumption, EMMs of treatment, day, and round interactions were compared using Tukey-adjusted pairwise comparisons, while Sidak adjustments were applied to mean-grouping summaries (**Figure S2.1**). For tissue comparisons within [^{14}C]-MP queenright cages only, ppb-transformed values were used. Negative values were replaced with 0.01 for analysis. Cage identity was defined as the interaction of round, treatment, day, and cage number. A linear mixed-effects model was fitted using maximum likelihood (ML) estimation, allowing for heterogeneous variance structures across tissues, with day, tissue, and their interaction as fixed effects, and cage identity as a random intercept. Fixed-effect significance was evaluated using a Type III ANOVA with Wald Chi-squared tests. EMMs and Tukey-adjusted pairwise comparisons identified significance at $p > 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). All pairwise comparisons were two-tailed.

Chapter 3. Developmental and Caste-specific Expression Patterns of ATP-Binding Cassette (ABC) Transporters in Honey bees (*Apis mellifera*).

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Abstract

While honey bees play a vital role in global crop production, they face increasing exposure to xenobiotic chemicals during commercial pollination. Multidrug-resistance (MDR)-type ATP-binding cassette (ABC) transporters provide the first line of defense against xenobiotic chemicals. This study investigated the gene expression profiles of 12 ABC transporters involved in chemical detoxification across three honey bee castes and 13 life stages using quantitative real-time PCR. Six ABC genes showed increased expression during worker bee development and were identified as MDR-like transporters (Ame-ABCB1, Ame-ABCB6, Ame-ABCC4a-c, Ame-ABCG1). Four transporters showed pupal-specific expression during metamorphosis. Queens exhibited significantly reduced MDR transporter expression compared to workers between 1.7-fold lower (ABCB6) and 17.5-fold lower (ABCB1). Drones showed intermediate expression levels. Queen ovaries demonstrated tissue-specific upregulation of select transporters. These findings reveal a vulnerability hierarchy (foragers > drones > queens) and suggest caste-specific trade-offs between reproduction and chemical defense in honey bee superorganisms.

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Introduction

Honey bees (*Apis mellifera*) have been historically recognized as ideal pollinator for commercial pollination due to their gentle disposition, high honey production, and lack of floral specificity. The demand for commercial pollination, coupled with the use of large-scale agricultural chemicals, has led to agrochemicals infiltrating pollinator feeding habitats and hives [1]. Although worker bees are the caste that spends the longest periods outside the hive and are most frequently exposed to agrochemicals[2–4], interactions inside the hive facilitate chemical transport across multiple castes[5]. Consequently, workers potentially expose new generations during development as well as adults. Additionally, the use of in-hive medicines extends the presence of xenobiotic chemicals to the hive structure, with substantial residues often found in the wax (e.g., fluvalinate 204 ppm, Amitraz 446 ppm)[5].

1.1. Honey bee development and hive castes

The honey bee superorganism is structured into three genetically, physiologically, and behaviorally distinct reproductive castes: queens, workers, and drones. Within a hive, all three castes interact as a superorganism through intricate communication and food-sharing mechanisms[5]. Worker bees are diploids, containing chromosomes from both parents (drone and queen), while drones are haploids, containing only chromosomes from the queen. Importantly, drones only carry the genetic material of the queen, while workers inherit genetic material from both drones and queens. Chemical defense genes are therefore inherited from drones to worker bees, with their expression and effectiveness dependent on external factors [2]. Worker bees constitute most of the hive's population, fluctuating between 10,000 to 40,000 members per hive and are further subdivided based on age and colony needs: newly emerged bees, nurses, and foragers. As bees age, they transition from tasks inside the hive (cleaning cells, brood care, feeding) to tasks outside (foraging for water, pollen, nectar, and propolis) [6,7]. Worker bees perform a wide variety of behaviors related to hive maintenance and protection, caretaking, and resource acquisition, with physiological characteristics that assist with these tasks (such as functioning stingers for protection).

Drones spend their lifetime inside the colony until reaching sexual maturity for mating flights and represent only ten percent of the colony's population. A drone bee's primary function is to carry the genes of the hive; with a sole focus on reproduction, drones lack the physiological characteristics necessary to sustain and protect themselves from chemical exposure. Queen bee physiology is similarly designed to prioritize reproductive success; apart from mating flights and during swarming, they do not leave the colony[8]. In terms of protection, there is some evidence that queens may tolerate higher doses of certain topically applied miticides than workers[9], and the reclusive nature of queens may also protect them from environmental dangers. These differences across castes could affect their exposure and responses to xenobiotic chemicals.

1.2. The chemical defensome.

When exposed to potentially harmful foreign chemicals, known as xenobiotics, organisms can activate a sophisticated and evolutionarily conserved cellular defense system known as the chemical defensome [10,11]. A well-orchestrated defensome includes four metabolic stages: Phase 0 (modulation of membrane penetration by unmodified compound efflux via ABC transporters or uptake via SLC transporters), Phase I (functionalization), Phase II (conjugation), and Phase III (modified compound efflux) [12–18], which work in concert to protect cells from xenobiotic toxicity. Multidrug-resistance (MDR) ATP-binding cassette (ABC) transporter proteins can be categorized into Phase 0 or Phase III, depending on their substrate specificities for transporting unmodified or metabolically modified xenobiotics, respectively [16,17].

ABC transporters serve as molecular gatekeepers, with ABCB1 acting as the first line of defense by preventing initial cellular uptake of parent compounds, while other ABC transporters handle the final elimination of modified compounds after Phase I and II processing. The ABC transporter ABCB1/MDR1 is ubiquitously expressed in cellular membranes of tissue barriers and, unlike other ABC transporters, is categorized as a Phase 0 transporter since it mediates the immediate efflux of *unmodified xenobiotic* compounds, thereby reducing their cellular entry [16,17]. If chemical compounds evade ABCB1 detection,

they are typically modified via functionalization (Phase I) and/or conjugation (Phase II) enzymes to enhance their inactivation and/or are actively eliminated through Phase III efflux transporters, such as the ABCC or ABCG subfamilies [11]. Hence, cellular export of Phase I and Phase II modified compounds is intimately linked to subsequent recognition by Phase III efflux transporters.

Many studies on the insect chemical defense have focused on Phase I metabolic enzymes, such as those in the cytochrome P450 family (CYPs) [19–22]. While these enzymes are essential for xenobiotic metabolism, MDR-like ABC transporters have both the first and the last interaction with chemical compounds via Phase 0 (unmodified efflux) or Phase III (modified efflux). Additionally, multiple genes of the ABC superfamily are known to confer multi-drug resistance to cells [23]. Previous studies on arthropod MDR-like ABC transporters indicated them as the primary drivers of insecticide resistance in multiple insect species, including *Plutella xylostella* (L.), *Helicoverpa armigera*, and *Aedes aegypti* [24–26]. Hence, these efflux transporters represent versatile cellular defense mechanisms that influence how cells respond to chemical stress. Due to their evolutionarily conserved xenobiotic gatekeeping function and known involvement in pesticide resistance, MDR-like ABC transporters are key molecular targets for understanding an organism's chemical defensive capabilities and their cellular responses to chemical challenges.

Understanding the baseline expression patterns of MDR-like ABC transporters across different life stages and castes is a fundamental step toward characterizing the complexities of the honey bee superorganism defense and inherent chemical defense capacity [5,27]. This study examines the developmental and caste-specific expression of 12 different honey bee ABC transporter genes that are orthologs of ABC transporters known to be involved in chemical defense in other arthropod species [28–32] and **Table S3.1**]. Quantitative real-time PCR (qPCR) was used to establish baseline expression levels of these honey bee ABC transporter genes across 13 developmental stages across three different castes (workers, queens, drones). In addition, the transporter gene expression patterns in queen bee ovaries were

examined separately, as they represent a key reproductive organ. Our results suggest that queens and drones are more vulnerable to xenobiotic exposure than workers, highlighting caste-specific differences in chemical defense capacity within honey bee colonies

Materials and Methods

2.1. Sample Acquisition

Honey bee samples were collected from hives maintained by the USDA-ARS Pollinator Health Research Unit in Davis, CA. **Table S3.2** describes the sampling seasons, years, and locations. Quantitative monitoring of parasitic *Varroa destructor* mites was conducted using alcohol washes [33], and levels were found to be low throughout sample collection. Colonies were maintained according to standard best beekeeping practices in the United States [34], and levels of *Varroa destructor* mites were controlled using treatments with Formic ProTM (NOD Apiary Products Ltd., Trenton, Ontario).

Table 3.1. Honey bee samples collected for qPCR analysis of ABC transporter gene expression by caste, developmental stage, and tissue type. The sample abbreviations are used consistently throughout the text. Eggs represent those destined to develop into workers. For details on adult bee sample collection, please refer to the Supplemental Table S1.

Caste	Life Stage/Tissue	Abbreviation
<i>Worker bee</i>	Eggs	E
	Larvae (Instar 3)	WL3
	Larvae (Instar 5)	WL5
	Pupae	P
	Newly Emerged Bee	NEB
	Nurse	N
	Forager	F
<i>Drone bee</i>	Larvae (Instar 5)	DL5
	Adult	D
<i>Queen bee</i>	Ovaries	QO
	Larvae	QL
	Pupae	QP
	Adult	Q

Five samples of each life stage were collected for biological replication. Additional queens were collected for dissection of five ovaries. Eggs (E) were collected by taking a frame containing E (from a hive

or queen monitoring cage [35] used in the laboratory) and tapping them out onto dark-colored paper before pouring them into an RNase-free 1.5 mL tube. When E were sticking to the cells, they were removed using grafting tools. For each sample, 5-10 mg of E were collected. All larvae and pupae were collected by grafting directly from hive frames and were stored similarly.

Adult bees (NEBs, nurses, foragers, and drones) were identified on live frames, collected, and euthanized by flash-freezing in liquid nitrogen. During an annual queen replacement event in April 2022, mated queens were collected in the field and immediately put on dry ice. All bee samples were stored at -80°C until processing. A subset of five queen samples from these mated queens had their ovaries isolated as an additional treatment group (QO). All dissections were performed using a Leica S9i stereo microscope (Leica Microsystems, Deerfield, IL). Frozen queens were dissected in a petri dish containing chilled phosphate-buffered saline (PBS) (pH 7.4) over ice.

2.2. Tissue Homogenization and RNA Extraction

Honey bee tissues were homogenized using BeadBug™ 6 Six-Position Homogenizer (Benchmark Scientific, Sayreville, NJ). Steel beads of 2.8 mm (Omni International, Kennesaw, Georgia) were selected over ceramic beads to effectively break through insect exoskeletons and homogenize the samples. For each sample (except QO), the entire bee was used for total RNA extraction. All samples were homogenized in DNA/RNA Protection Reagent (New England Biolabs, Ipswich, MA) for three cycles of 30 seconds at 6 m/s with 45-second dwell periods. For samples with especially tough exoskeletons or large specimens (i.e., drones and queens) that impeded steel bead movement within the tube, up to three additional homogenization cycles were performed to ensure thorough soft tissue disruption. Samples were placed on ice between each cycle to prevent overheating that could potentially compromise RNA quality. RNA extraction was conducted using the Monarch® Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA). The quantities of reagents used in the initial steps varied based on sample weights. For example, each WL3 sample (weighing approximately 10 mg) required minimal buffer and Proteinase K, whereas Q and

D samples (exceeding 200 mg) necessitated maximum reagent usage. The optional on-column DNase I treatment was performed on every sample to ensure complete genomic DNA (gDNA) removal. RNA quality was validated using standard 2% w/v agarose gel electrophoresis and UV absorbance to determine the A_{260}/A_{230} and A_{260}/A_{280} ratios [36].

2.3. cDNA Conversion

Total RNA was converted to cDNA for qPCR analysis using the SuperScript IV First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific, Waltham, MA). The manufacturer's protocol was followed to prepare 1000 ng of cDNA per sample, except for E samples, which had particularly low RNA yields (200 ng). For qPCR analysis, all final cDNA concentrations were standardized to 20 ng/ μ l.

2.4. Primer Design and Quality Control

All qPCR primers were designed using KEGG, CLC Genomic Workbench v20.0.4, Primer-BLAST (NCBI), and Primer3Plus[36]. Gene sequences for all genes of interest, including their respective isoforms, were downloaded into CLC. Within each gene, isoforms were aligned, and gaps were recorded. Primers were subsequently designed within regions of the alignments where no gaps were present to capture all isoforms of each gene. Target amplicons were set between 80 and 200 base pairs (bp), with an optimal size of 120 bp. Potential for primer dimerization was assessed using Beacon Designer (Premier Biosoft International, Palo Alto, CA), and all primers were screened for potential non-specific binding using Nucleotide BLAST (NCBI). For this project, three reference genes were selected: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein S5 (RS5), and Ras-related protein Rab-1A (RAB1A). The first two are commonly used as reference genes for honey bees, and the latter was recently identified as the most stable reference gene for qPCR experiments across honey bee life stages [37,38]. The previously published primer pair was used for the housekeeping gene RAB1A [37]. High-purity salt-free (HPSF) primers (Eurofins Genomics, Louisville, Kentucky) were reconstituted and diluted using TE buffer (pH = 7.4). Working primer concentrations were 10 μ M.

The specificity of each primer pair was assessed using nurse bee cDNA via standard PCR and agarose gel electrophoresis [36]. Prior to qPCR analysis, primer efficiency was tested and optimized by creating a five-point cDNA serial dilution curve. The slope obtained from plotting concentrations against Ct values was used to calculate efficiency using the following equation [39]:

$$[10^{(-1/(\text{slope}))}-1]*100$$

Primer pairs with reaction efficiencies between 90-110% were considered acceptable. Calculated efficiencies of all validated qPCR primers can be found in Table S3.

2.5. Phylogeny

Phylogenetic analysis was performed to determine the evolutionary relationships of honey bee ABC transporters with their MDR-like orthologs in human and *Drosophila melanogaster* (**Figure 3.1 and Table S3.4**). Full-length protein sequences of honey bee ABC transporters were used to identify corresponding orthologs in human and *Drosophila* through reciprocal NCBI BLAST. The BLAST results were sorted by Max score, selecting only the top annotated sequence from each organism. Sequences for each organism were downloaded and organized in CLC Main Workbench v21.0.5 to perform multiple sequence alignments and construct a phylogenetic tree using the following parameters: Neighbor-Joining (algorithm), Jukes-Cantor (distance measure), and 1000 replicates (bootstrap). The tree was rooted with the human HRas GTPase (NP_001123914) as an outgroup.

2.6. Real-time PCR (qPCR)

All qPCR plates were designed with three technical replicates for each unique reaction. Each plate contained one biological replicate from three different life stages, with five genes of interest and three reference genes. A 20 µL reaction protocol using SsoAdvanced Universal SYBR® Green Supermix (Bio-

Rad, Hercules, CA) was applied to all samples and pipetted onto MicroAmp™ EnduraPlate™ optical 96-well fast clear reaction plates (Thermo Fisher Scientific, Waltham, MA). After loading the reactions, plates were sealed with MicroAmp™ optical adhesive film (Thermo Fisher Scientific, Waltham, MA) and centrifuged for one minute to thoroughly mix all reagents.

All plates were run on a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA) using the following protocol: a 30s hold at 98°C for polymerase activation and initial DNA denaturation (rate 4.3°C/s), followed by 40 cycles of 15s at 98°C for denaturation (rate 3°C/s), and 30s at 60°C for annealing/extension (rate 3°C/s). A melt-curve analysis was performed after 40 cycles: 15s at 95°C (rate 1.6°C/s), followed by 1min at 60°C (rate 1.6°C/s), followed by 15s at 95°C (rate 0.05°C/s).

Using biological replicates (n=5) ΔC_T values and technical plate replicates, an average ΔC_T was obtained for each of the eleven treatments (**Table 3.1**) and twelve genes of interest (**Table 3.2**). A geometric mean of all three housekeeping genes was used to calculate ΔC_T values. qPCR data were analyzed using the Livak method for fold change calculation using $\Delta\Delta C_T$ [36,40], comparing differences between treatments. Each tested caste member, developmental stage, and tissue type were considered a "treatment" except for E, which was designated as the "control" since all caste members pass through this stage. Gene expression was normalized to its corresponding expression in E (= value of 1). Fold change was calculated using the following equation:

$$2^{-\Delta\Delta C_T} = 2^{-(\Delta C_T ("treatment") - \Delta C_T ("control"))}$$

All qPCR results were assessed for quality criteria, including melting temperature, C_T values greater than 35, and technical replicate C_{TS} within 0.5 units of their mean. Quality parameters were applied using RStudio v.4.2.2 (R-Packages: dplyr, tidyverse, openxlsx) to ensure unbiased analysis. Filtered data were then used for statistical interpretation.

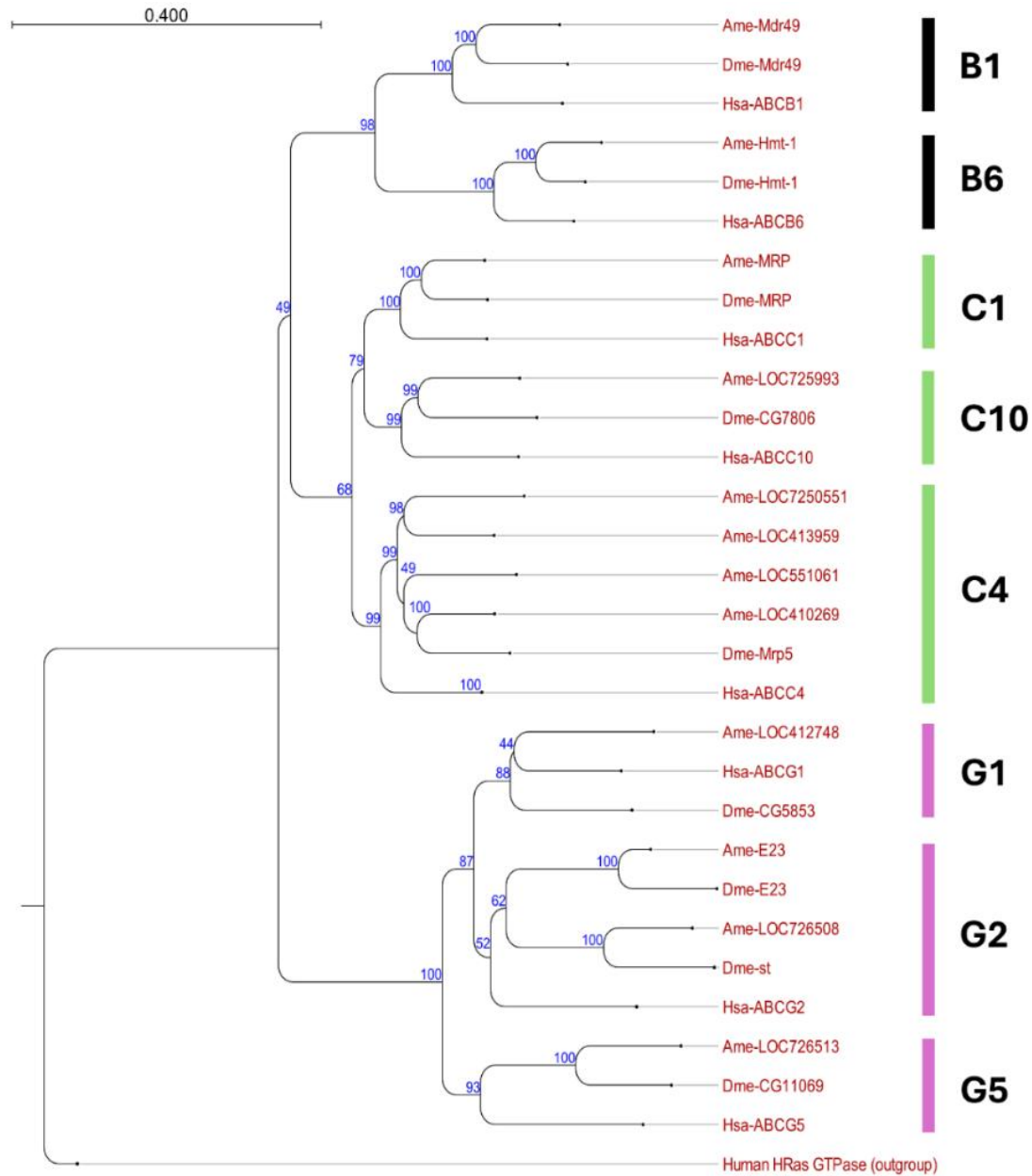


Figure 3.1. Phylogenetic analysis of full-length protein sequences of *Apis mellifera* MDR-like ABC transporters and their known homologs in humans and *Drosophila*. The percentage concordance based on 1000 bootstrap iterations is shown at the nodes (blue numbers). *Ame* = *Apis mellifera*; *Dme* = *Drosophila melanogaster*; *Hsa* = *Homo sapiens*. The human HRas GTPase gene was used as a reference point to determine evolutionary relationships between ABC transporter genes and to root the tree.

2.7. Statistical analysis

All statistical analysis was performed using RStudio v.4.2.2 (R-packages: dplyr, tibble, forcats, emmeans, multcomp, multcompView, ggplot2, openxlsx). Filtered data were first checked for normality

using histogram, QQ-plot, and Shapiro-Wilk normality test ($W=0.97$, $p\text{-value} < 2.2 \times 10^{-16}$). Normality testing and models created to analyze the data were applied to housekeeping gene-normalized C_T values (ΔC_T). A one-way ANOVA (“analysis of variance”) was conducted to determine whether significant differences were present across the twelve treatments (E, WL3, WL5, DL5, P, NEB, N, F, D, Q, QO) for each gene of interest. Following ANOVA, a Tukey Honest Significant Difference (HSD) test was applied to identify significant differences between treatments. The confidence interval for the Tukey model was set to 0.99. Comparisons were made across same genes and across same life stages, with values below 0.01 considered statistically significant.

Results

3.1. Identification of MDR-like ABC transporters orthologs in honey bees

NCBI BLAST searches and phylogenetic analyses were used to identify ABC transporter orthologs involved in multidrug resistance (MDR) and chemical defense across honey bees (*Ame*), humans (*Hsa*), and *Drosophila* (*Dme*) (**Figure 3.1**). Twelve honey bee ABC transporter genes from three different subfamilies (B, C, and G) with potential roles in chemical defense were identified and analyzed (**Figure 3.1, Table 3.2**). Phylogenetic reconstruction using the Neighbor-Joining method revealed clear orthologous relationships between honey bee ABC transporters and their known MDR transporter counterparts in human and *Drosophila* (**Figure 3.1, Tables S3.1 and S3.4**).

Within subfamily B, *Ame*-Mdr49 and *Ame*-Hmt-1 formed well-distinct clades with their respective *Drosophila* (*Dme*-Mdr49 and *Dme*-Hmt-1) and human (*Hsa*-ABCB1 and *Hsa*-ABCB6) orthologs. In each of these clades, the insect sequences group together in a subcluster of high bootstrap support, indicating a closer evolutionary relationship compared to the human orthologs.

Subfamily C showed more complex relationships: *Ame*-MRP and *Ame*-LOC725993 formed a clade, within which *Ame*-MRP grouped together in a subcluster with *Dme*-MRP1 and human ABCC1, opposing *Ame*-LOC725993 which formed a sub-clade related to *Dme*-CG7806 and human ABCC10. A larger subfamily C expansion was observed in honey bees for ABCC4, with *Ame*-LOC551061, *Ame*-LOC725051, *Ame*-LOC413959, and *Ame*-LOC410269 forming a distinct clade with *Dme*-Mrp5 that shares ancestry with human ABCC4. Within this clade, *Ame*-LOC551061 and *Ame*-LOC410269 form a sub-clade with *Dme*-Mrp5 that shows a close relationship to the other subclade formed by *Ame*-LOC725051 and *Ame*-LOC413959.

Table 3.2. Metadata summary of the 12 honey bee ABC transporters investigated in this study. Gene ID and naming conventions in NCBI and BeeBase/HGD for honey bee ABC transporters are shown. The four different genes for honey bee ABCC4 (a-d) are all paralogous to human ABCC4. Two ABCG (a-b) genes were paralogous to human ABCG2.

Protein name	BeeBase/ HGD ID	Gene ID	Gene symbol	Gene description	Length (aa)
<i>Ame</i> -ABCB1	GB55378	551167	Mdr49	Multidrug resistance protein homolog 49	1343
<i>Ame</i> -ABCB6	GB54214	726867	Hmt-1	ABC transporter ATP-binding protein/permease Hmt-1	840
<i>Ame</i> -ABCC1	GB53134	412217	MRP	Multidrug-Resistance like Protein 1	1536
<i>Ame</i> -ABCC10	GB44193	725993	LOC725993	Multidrug resistance-associated protein 7	1625
<i>Ame</i> -ABCC4a	GB50195	413959	LOC413959	Multidrug resistance-associated protein 4	1356
<i>Ame</i> -ABCC4b	GB45277	551061	LOC551061	Multidrug resistance-associated protein 4	1392
<i>Ame</i> -ABCC4c	GB45278	725051	LOC725051	Multidrug resistance-associated protein 4	1418
<i>Ame</i> -ABCC4d	GB17987; GB43189	410269	LOC410269	Probable multidrug resistance-associated protein lethal (2) 03659	1333
<i>Ame</i> -ABCG1	GB44770	412748	LOC412748	ATP-binding cassette sub-family G member 1	631
<i>Ame</i> -ABCG2a	GB49098	410967	E23	Early gene at 23	657
<i>Ame</i> -ABCG2b	GB43076	726508	LOC726508	Protein scarlet	623
<i>Ame</i> -ABCG5	GB41827	726513	LOC726513	ATP-binding cassette sub-family G member 5	625

Within subfamily G, *Ame*-LOC412748 showed close relationship to *Dme*-CG5853 and human ABCG1. *Ame*-LOC726508 and *Ame*-E23 both demonstrated relationships to human ABCG2, with *Ame*-LOC726508 being more closely related to *Dme*-st and *Ame*-E23 to *Dme*-E23. *Ame*-LOC726513 clustered with *Dme*-CG11069, both orthologous to human ABCG5.

Based on the identified phylogenetic relationships (**Figure 3.1**), protein sequence similarity (NCBI BLAST), and orthology database (OrthoDB) comparisons, standardized gene symbols were assigned to each honey bee ABC transporter for clarity and consistency throughout this study (**Table 3.2**).

3.2. Developmental expression patterns of MDR-like ABC transporters in worker bees

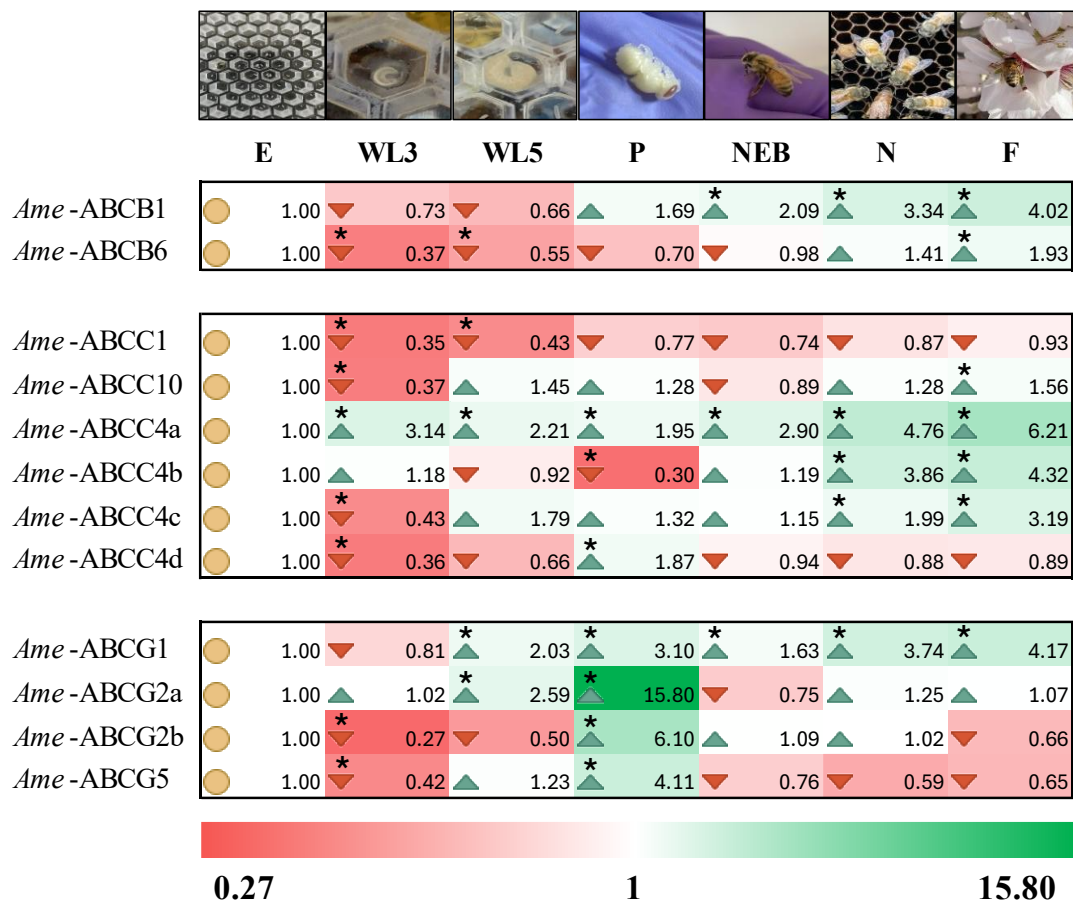


Figure 3.2. Heat map of relative gene expression (fold change) of twelve MDR-like ABC-type transporters during worker bee development. Color intensity reflects the expression of each gene, with green representing relative upregulation (>1) and red representing relative downregulation (<1). Significant differences between samples were validated by one-way ANOVA and are denoted by * ($p < 0.01$). The color scale reflects comparisons across all genes and all life stages: E (eggs), WL3 (Worker Larvae 3), WL5 (Worker Larvae 5), P (Pupae), NEB (Newly Emerged Bees), N (Nurses), and F (Foragers). The top images show corresponding tissues and caste members at different developmental stages (photographs taken by A. Encerrado).

Figure 3.2 shows the relative gene expression of twelve MDR-like ABC transporters, identified through phylogenetic analysis, across seven worker bee life stages, expressed as fold change relative to worker eggs (E). It is well established that foraging behavior specifically drives xenobiotic defense gene upregulation during worker bee development due to environmental xenobiotic exposure during nectar and pollen collection [41]. Here, we used seven worker bee developmental stages, from pristine eggs to environmentally exposed forager bees, to correlate environmental exposure patterns with detoxification transporter gene expression.

Both ABCB subfamily members, ABCB1 and ABCB6, showed initially decreased expression in early larval stages WL3 followed by increased expression in later developmental stages. The expression of ABCB1 was significantly upregulated between NEB (2.09-fold) to F (4.02-fold) and ABCB6 expression was significantly downregulated in WL3 (0.35-fold) and WL5 (0.55-fold) but increased while workers mature with significant upregulation in F (1.93-fold), showing an overall developmental increase in expression as worker bees mature.

The ABCC subfamily showed the most diverse expression patterns. ABCC1 gene expression was relatively low and stable throughout all developmental stages, indicating housekeeping or maintenance function during worker bee development. While ABCC1 expression moderately increased from WL3 (0.35-fold) to its highest expression peak in foragers (0.93-fold), only WL3 (0.35-fold) and WL5 (0.43-fold) showed statistically significant different expression compared to E and later developmental stages. ABCC10 showed variable expression with peak expressions in WL5 (1.45-fold) and moderate elevation in F (1.56-fold). ABCC4a was significantly upregulated in all life stages starting with WL3 (3.14-fold) and reaching a maximum expression (6.21-fold) in F. ABCC4b expression shows a significant decrease in expression during P (0.30-fold) and a late-stage activation in N (3.86-fold) with peak expression (4.32-fold) in F. ABCC4c displays an initial decrease in expression during WL3 (0.43-fold), and significant late-stage activation in N (1.99-fold) and peak expression (3.19-fold) in F. ABCC4d presents a unique pattern, with

significantly decreased expression in WL3 (0.36-fold), peak expression in P (1.87), followed by decline in later developmental stages. Overall, the expression of ABCC4a-c increased linearly as bees aged, while the expression of ABCC4d reached its maximum in P and decreased as bees aged.

Members of the ABCG subfamily recapitulated some of the distinct expression patterns identified in the B and C subfamilies. The expression of ABCG1 showed a steady and significant increase in WL5 (2.03-fold) with peak expression in F (4.17-fold). ABCG2a had a very unusual expression pattern with a dramatic pupal-specific expression (15.80-fold), followed by a return to baseline expression. This fold change was higher than in any other gene we measured. A similar, pupal-specific spike in gene expression was observed for both ABCG2b (6.10-fold) and ABCG5 (4.11-fold), with declined expression during all other developmental stages. Interestingly, the significant upregulation of ABCG2a, ABCG2b, and ABCG5 transporter genes was also conserved in queen pupae, indicating similar function during metamorphosis (Figure S1).

Based on the identified gene expression patterns and known gene expression patterns during worker bee development from literature (**Figure 3.2**), the 12 ABC transporters were grouped into three different functional categories:

- 1. MDR-like transporters with increasing expression toward foragers:** An overall increased gene expression from larval to forager stage, with significant peak expression in F, was observed for six distinct ABC transporters: *Ame-ABCB1*, *Ame-ABCB6*, *Ame-ABCC4a*, *Ame-ABCC4b*, *Ame-ABCC4c*, and *Ame-ABCG1*.
- 2. Developmental or metamorphosis transporters with pupal-specific expression:** We identified four transporter genes with expression peaks during the pupal stage (major metamorphic event) and a rapid

decline after this developmental transition: *Ame*-ABCC4d, *Ame*-ABCG2a, *Ame*-ABCG2b, and *Ame*-ABCG5.

3. Housekeeping or maintenance transporters with stable, low expression throughout development:

Both genes, *Ame*-ABCC1 and *Ame*-ABCC10, showed stable expression across the developmental time points with fold-changes remaining within 2-fold variation, indicating a housekeeping gene role during worker bee development.

Gene expression of these functionally categorized ABC transporters in worker bees was subsequently compared to the expression in queen and drone bees to assess caste-specific differences in transporter gene expression among worker and reproductive hive members.

3.3. Comparative analysis of caste-specific ABC transporter expression in foragers, drones, and queens

Figure 3.3 displays the fold-change gene expression for the 12 functionally categorized ABC transporters in forager bees and in the two reproductive caste members (drones and queens), as well as the fold-change ratios for forager:drone (F/D), forager:queen (F/Q), and drone:queen (D/Q) comparisons to highlight caste-specific specialization.

Overall, forager bees show higher expression of the suspected MDR-type transporters compared to all reproductive members (**Figure 3.3A**), consistent with the idea that foraging behavior can upregulate xenobiotic defense gene expression due to increased toxin and toxicant exposures during pollen and nectar collection. In contrast to this, queen bees show consistently low expression across most transporters, particularly of all MDR-type transporters, indicating that they have a reduced need for xenobiotic detoxification. Adult drones show intermediate expression levels of the MDR-type and most developmental

transporters between workers and queens, indicating that they are less intensively exposed to xenobiotics compared to foragers.

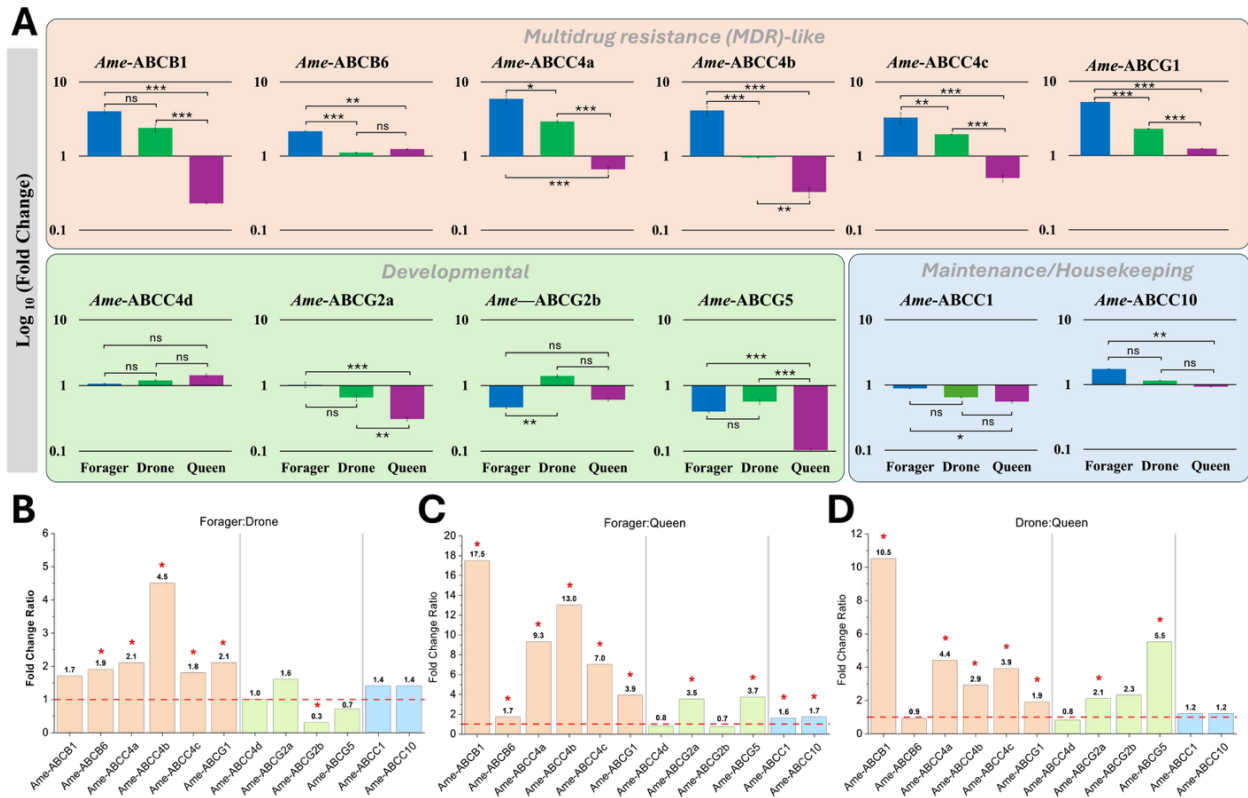


Figure 3.3. Adult stage comparison of functionally categorized ABC transporter gene expression. (A) Shown are fold changes of all 12 ABC transporter genes between foragers (blue), adult drones (green), and queens (purple) relative to worker eggs. Fold-change ratios of transporter gene expression for Forager:Drone (B), Forager:Queen (C), and Drone:Queen (D). Ratio values >1 indicate numerator-enriched, values <1 indicate denominator-enriched gene expression. Significant differences between samples were validated by one-way ANOVA and are denoted by * ($p < 0.01$). Boxes are color-coded by functional classification: light peach family = MDR Function transporters showing forager-specific upregulation for xenobiotic detoxification; light mint green family = Developmental Specialization transporters with pupal-specific expression peaks and caste-specific metamorphosis patterns; light blue family = Housekeeping/Maintenance transporters with stable expression across stages.

When comparing transporter gene expression in **foragers versus drones** (Figure 3.3B), foragers show five of the six suspected MDR transporters significantly upregulated, including ABCB6, ABCC4a-c, and ABCG1. Among these, ABCC4b showed the highest fold change ratio (4.5), indicating preferential or selective upregulation of this particular MDR-like transporter gene in foragers. Interestingly, drones show significant enrichment of the suspected developmental transporter gene ABCG2b and a non-significant

enrichment of ABCG5 relative to foragers, indicating that these two transporters might be involved in reproductive functions.

The gene expression comparison in **foragers versus queens (Figure 3.3C)** shows that ten or the 12 investigated transporters are significantly enriched in foragers, including all six suspected MDR transporters. A moderate, but non-significant, enrichment in queens relative to foragers can be observed for the two suspected developmental transporters ABCC4d and ABCG2b, indicating that the ABCG2b transporters are moderately enriched in reproductive caste members (queens and drones) versus foragers.

The comparison of gene expression fold-change ratios in **drones versus queens (Figure 3.3D)** reveals that drones have five of the six MDR transporter genes (ABCB1, ABCC4a-c, and ABCG1) and two of the developmental transporter genes (ABCG2a and ABCG5) significantly upregulated. Since drones also show a moderate enrichment of ABCG5 transporters relative to foragers (**Figure 3.3B**), these transporter genes could have drone-specific functions. Notably, for the suspected MDR transporter ABCB6 and the developmental transporter ABCC4d, queen bees show a non-significant enrichment relative to drones. Together, this shows that the expression of ABCC4d transporters is moderately upregulated in queens versus both drones and foragers, indicating a queen-specific function.

3.4. Developmental and tissue-specific ABC transporter expression in reproductive castes

Figure 3.4 illustrates the developmental regulation of all 12 ABC transporters during reproductive caste maturation and their tissue-specific expression in queen reproductive organs.

During drone development (**Figure 3.4A**), gene expression of three suspected MDR transporter genes (ABCB1, ABCB6, and ABCC4a), three suspected developmental transporters (ABCC4d, ABCG2b, and ABCG5), and the housekeeping gene ABCC1 is significantly upregulated. Thereby, the most dramatic

increase in gene expression can be observed for ABCB1 (5.14-fold) and ABCG2b (6.59-fold). Interestingly, the suspected MDR transporter ABCG1 shows similar gene expression in drone larvae (2.03-fold) and adult drones (1.99-fold) that is significantly higher compared to worker eggs (1-fold) (**Figures 3.2, S3.1, S3.2, and 3.4**). An almost two-fold upregulation of ABCG5 and transporter gene expression in adult drones compared to larvae further supports the hypothesis of a drone-specific function of this gene.

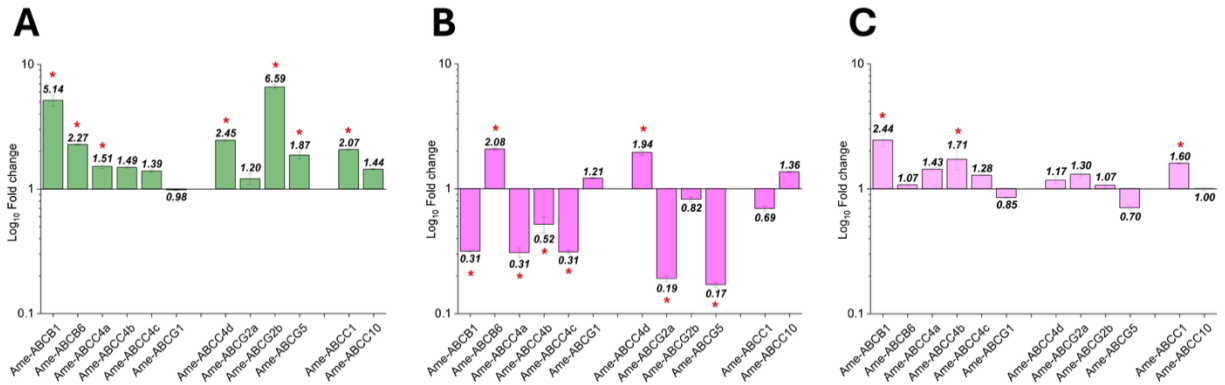


Figure 3.4. ABC transporter expression patterns during reproductive caste development and in specialized reproductive tissues. (A) Fold-change expression of ABC transporters comparing adult drones to drone larvae, showing developmental regulation during drone maturation. (B) Fold-change expression of ABC transporters comparing adult queens to queen larvae, highlighting developmental changes during queen maturation. (C) Tissue-specific expression of ABC transporters in queen ovaries compared to whole queen bodies, identifying ovary-enriched transporters associated with reproductive function. Significant differences between the two samples were validated by one-way ANOVA and are denoted by * ($p < 0.01$).

During queen bee development, adult queens significantly upregulate the gene expression of one MDR transporter (ABCB6) and one developmental transporter (ABCC4d) (**Figure 3.4B**). Notably, these two transporters are also moderately upregulated in queen ovaries compared to worker eggs (**Figure S3.1 and S3.2**), indicating female reproductive tissue-specific functions. In agreement with the consistently lower baseline expression levels of most transporters in the cross-caste normalization approach (**Figure S3.1 and S3.2**), adult queen bees show significant downregulation of four MDR transporter genes (ABCB1, ABCC4a-c) and two developmental transporters (ABCG2a and ABCG5) when normalized to queen larvae.

To identify reproductive tissue-specific expression patterns, we normalized transporter gene expression from queen ovaries to queen bees. In this normalization approach, most transporters show higher expression in queen ovaries than in the whole queen body (**Figure 3.4C**). In addition, several genes showed significant upregulation, including two suspected MDR transporters (ABCB1 and ABCC4b) and the suspected housekeeping/maintenance gene ABCC1. Notably, the ABCC4d transporter gene shows moderate but consistent ovary specialization, with relatively high expression in both queen tissues (1.09-fold in queens and 1.17-fold in ovaries) compared to foragers (0.89-fold) and drones (0.91-fold) (**Figure S3.1 and S3.2**).

Discussion

Honey bee colonies face unprecedented chemical challenges in modern agricultural landscapes, but our understanding of how different colony members respond to xenobiotic threats remains limited. Previous research has characterized Phase I detoxification enzymes like cytochrome P450s and carboxylesterases in worker bees and demonstrated that ABC transporter expression varies across worker developmental stages [2,41,28]. For instance, the comprehensive review of Dermauw and Van Leeuwen (2014) focused on ABC transporters across arthropod species for pesticide resistance mechanisms, while a more recent study by Maiwald et al. (2023) identified developmental patterns across the entire detoxification gene inventory in worker bees.

However, a comprehensive analysis of multidrug resistance (MDR)-like ABC transporters—which provide both the first and last lines of cellular defense—across all castes of the honey bee superorganism, with reproductive caste and tissue-specific resolution, has been lacking. This knowledge gap is particularly critical given that colony survival depends on the coordinated health of specialized castes with potentially different chemical defense capacities.

Here, we focused on twelve distinct MDR-like ABC transporter orthologs (**Figure 3.1**) from three subfamilies (B, C, G) and expanded the scope to cover the full hive superorganism, including reproductive castes (queen bees and drones) and specialized tissues (queen bee ovaries) in addition to worker bees.

4.1. Phylogenetic Identification of Honey Bee MDR-like ABC Transporters

Our phylogenetic analysis successfully identified twelve honey bee ABC transporter orthologs with established roles in xenobiotic defense in humans and *Drosophila melanogaster* (**Figure 3.1, Table S3.1**). We used these homology comparisons in conjunction with gene expression data (**Figure 3.2**) to infer the specific functionality of the identified honey bee orthologs (**Table 3.1**) and identify those involved in chemical detoxification.

Within subfamily B, the clustering of *Ame*-ABCB1 with human ABCB1/P-glycoprotein and *Dme*-Mdr49 confirms its likely role as a Phase 0 transporter mediating immediate xenobiotic efflux [23,29,43]. Similarly, *Ame*-ABCB6's phylogenetic relationship to human ABCB6 – a porphyrin transporter located in the outer membrane of mitochondria - suggests involvement in mitochondrial detoxification and protection against oxidative stress [44]. In arthropods, ABCB6 can also confer resistance against heavy metals [45]. Notably, both genes, ABCB1 and ABCB6, exhibited clear increase in expression during worker bee development (**Figure 3.2**), consistent with previous studies indicating that forager bees have a higher expression of detoxification genes compared to younger nurse bees [4].

The expansion of ABCC4 paralogs in honey bees (ABCC4a-d) compared to single orthologs in humans and *Drosophila* represents a particularly interesting evolutionary adaptation. In humans, *Hsa*-ABCC4 transports nucleosides, endogenous signaling molecules, and a wide range of xenobiotic drugs [46]. In lice (*Pediculus humanus humanus*), ABCC4 has been shown to protect against the antiparasitic drug ivermectin [47]. In honey bees, ABCC4(b) was reported to be highly expressed in the Malpighian tubules, an organ recognized for its central role in detoxification [42]. Gene duplications often provide raw

material for functional diversification, and the distinct expression patterns we observed for each paralog (**Figure 3.2**) suggest sub-functionalization has occurred. For instance, while ABCC4a-c gene followed the MDR-like expression pattern, ABCC4d expression was highest in queens and their ovaries, indicating a functional role in reproductive tissues. The expansion into at least three ABCC-type paralogs with MDR-like expression patterns may reflect the specialized detoxification demands faced by a eusocial species exposed to diverse environmental chemicals through complex foraging behaviors. Despite their identified gene orthology to important chemical detoxification transporters in humans and *Drosophila*, *Ame-ABCC1* and *Ame-ABCC10* gene expression patterns were remarkably stable across all worker and reproductive caste member life stages, indicating a maintenance or housekeeping role rather than a xenobiotic defense system. A potential explanation for this expression profile is that the gene is primarily upregulated in response to acute exposures and/or to specific environmental chemicals that our specimen did not encounter. Further research on these honey bee transporter genes is necessary to determine whether their expression can be triggered in response to xenobiotic exposures.

In insects, the ABCG subfamily of transporters typically comprises between 10 and 25 members, with *Drosophila* and honey bees having approximately 15 annotated G transporters [48]. However, humans have only five members of the ABCG subfamily, with four being mainly involved in lipid and sterol transport and ABCG2 (aka BCRP) being a key MDR efflux transporter [30,49]. The sheer number of different G transporters in insects, along with their presence in multiple detoxification tissues, has previously raised the question of whether these transporters are used for detoxification, rather than exclusively for sterol transport [28,50]. Indeed, in our study the expression pattern of *Ame-ABCG1* in worker bee development follows the trend of the other MDR-like genes with peak expression in foragers (**Figure 3.2**). This suggests that *Ame-ABCG1* may have additional functions besides cholesterol transport that relate to environmental exposure (such as transport of environmental chemicals with similar properties to sterols). Interestingly, the expression profiles of *Ame-ABCG2a* and *Ame-ABCG2b*, which showed close phylogenetic relationship to the major human drug efflux pump ABCG2, did not follow a typical MDR-

like pattern and were low in most life stages and caste members with a spike only in worker and queen bee pupae (**Figure 3.2, Figure S3.1**). This suggests a developmental role during major metamorphosis for these two transporters. In a similar way, *Ame-ABCG5* showed a pupal spike expression, indicating a role in lipid transport during major cellular reorganization events.

4.2. Developmental Expression Patterns and Functional Categorization

Our analysis revealed three distinct functional categories of ABC transporters based on developmental expression patterns: (1) MDR-like transporters with increasing expression toward foragers (*Ame-ABCB1*, *Ame-ABCB6*, *Ame-ABCC4a-c*, *Ame-ABCG1*), (2) developmental transporters with pupal-specific expression (*Ame-ABCC4d*, *Ame-ABCG2a-b*, *Ame-ABCG5*), and (3) housekeeping/maintenance transporters with stable expression (*Ame-ABCC1*, *Ame-ABCC10*).

The progressive upregulation of MDR-like transporters toward forager stages aligns with established patterns of detoxification gene expression in honey bees [41,42]. This developmental trajectory reflects the increasing environmental exposure risks associated with foraging behavior, where bees encounter pesticide-contaminated nectar, pollen, and water sources. The low expression in early larval stages likely reflects their protected environment within the hive and their diet of filtered secretions from worker bees, which may act as a biochemical barrier to direct xenobiotic exposure [2]. The pupal-specific expression of ABCG2 paralogs and ABCG5 suggests specialized roles during metamorphosis, possibly related to the efflux of developmental hormones or metabolic waste products generated during tissue reorganization. This pattern is consistent with the known function of ABCG2 orthologs in hormone transport in other organisms [51].

4.3. Caste-Specific Differences in Chemical Defense Capacity

Our findings reveal significant differences in ABC transporter expression among honey bee castes, with queens consistently showing the lowest expression of MDR-like transporters (**Figure 3.3**). This pattern suggests a physiological trade-off, as queens prioritize reproduction over chemical defense, allocating their energy primarily to egg-laying to maintain the hive's population. However, this reduced chemical defense capacity raises concerns given that toxins can accumulate within hives. For example, propiconazole residues have been found at levels up to 227 ppb in wax and 361 ppb in pollen in the United States, demonstrating that even secluded hive members may face chronic exposure [5]. Recent advances in social insect transcriptomics have revealed that caste-specific gene expression patterns often reflect specialized physiological demands and behavioral roles [52].

The reduced chemical defense capacity in queens may be partially compensated by behavioral and social factors. Queens rarely leave the hive except during mating flights and swarming events, limiting their direct exposure to environmental xenobiotics. Additionally, the food-processing behavior of worker bees may serve as a form of social immunity, pre-filtering potentially harmful compounds before they reach reproductive castes [5]. Worker bees likely act as a buffer system, protecting vulnerable colony members through food processing via trophallaxis. However, this protection is not absolute—the expression of suspected MDR transporter ABCC4a in workers is only 2 times higher than in larvae, suggesting that some xenobiotic exposure reaches all colony members.

Drones showed intermediate expression levels between workers and queens, which may reflect their unique life history. Unlike workers, drones do not engage in foraging or hive maintenance activities that would expose them to high levels of environmental chemicals. However, their higher expression relative to queens may be necessary for survival during mating flights and competition with other males. The vulnerability of drones to environmental stressors has particular implications for colony reproduction. Previous studies have shown that exposure to pesticides like thiamethoxam can prevent drones from

reaching sexual maturity, with mortality rates reaching 100% in some exposed colonies [53]. Early-stage pesticide exposure also negatively affects drone sperm viability, which could reduce colony reproductive success [54].

4.4. Implications for Colony Vulnerability and Chemical Risk Assessment

The differential expression of MDR-like transporters across castes has important implications for understanding colony-level responses to chemical stressors. Multi-drug resistance transporters play crucial roles in determining honey bee responses to pesticide combinations, and our findings suggest that this capacity varies dramatically within colonies.

The vulnerability hierarchy we identified (foragers > drones > queens, based on MDR transporter expression) may help explain observed patterns of pesticide sensitivity in field studies. However, colony survival depends critically on queen health and reproduction, making the low chemical defense capacity of queens particularly concerning for colony-level risk assessment. Even sublethal effects on queens could have disproportionate impacts on colony fitness through reduced egg-laying or altered pheromone production.

The genetic diversity of worker chemical defenses may partially offset the vulnerability hierarchy identified in our study. Queen mating behavior significantly enhances this genetic diversity, as queens can mate with multiple drones during mating flights, incorporating genetic material from different colonies into their spermatheca [55]. This polyandry enriches the genetic pool of worker offspring, potentially providing diverse chemical defense capabilities across the worker population that could compensate for the inherently lower expression levels we observed in reproductive castes [56].

The tissue-specific upregulation of certain transporters in queen ovaries (ABCB1, ABCC4b, ABCC1) suggests some compensation for the generally low whole-body expression in queens. This ovary-

specific expression may protect developing eggs from maternal transfer of xenobiotic compounds, representing an important mechanism for preventing intergenerational chemical effects.

4.5. Evolutionary and Ecological Perspectives

The caste-specific expression patterns observed here reflect broader evolutionary principles governing social insect biology. Gene regulatory mechanisms underlying social organization often involve trade-offs between different physiological functions [57], and our results suggest that chemical defense represents one such trade-off with reproductive specialization.

The social division of labor in honey bees extends beyond behavior to include molecular-level specialization in chemical defense systems [58]. This division may represent an adaptive response to the complex chemical landscapes encountered by foraging bees, allowing colonies to maintain high-level detoxification capacity in exposed workers while minimizing the metabolic costs for protected castes.

4.6. Limitations and Future Directions

While our study provides important insights into caste-specific differences in ABC transporter expression, several limitations should be noted. Gene expression levels do not necessarily correlate directly with protein activity or functional capacity. Future studies should incorporate protein-level measurements and functional bioassays to validate the physiological significance of the expression differences we observed.

Additionally, our analysis focused on baseline expression patterns in unexposed bees. The inducibility of these transporters following chemical exposure may differ among castes and could reveal additional layers of caste-specific adaptation. Environmental factors such as season, nutritional status, and pathogen load may also modulate transporter expression and warrant investigation.

Conclusions

This study provides the first comprehensive analysis of ABC transporter expression across all honey bee castes and developmental stages, with particular focus on 12 MDR-like ABC transporters that are orthologous to human and *Drosophila* MDR transporters. Our findings reveal a clear specialization in chemical defense capacity, with forager bees expressing the highest levels of MDR-like transporters and queens showing consistently low expression. This pattern reflects the social division of labor in honey bee colonies and has important implications for understanding colony-level responses to environmental chemical stressors.

The reduced chemical defense capacity in queens and drones suggests these castes may be particularly vulnerable to xenobiotic exposure, despite their generally protected lifestyle within the hive. As the use of pesticides in agricultural landscapes continues, understanding these caste-specific vulnerabilities will be crucial for developing effective strategies to protect pollinator health and maintain sustainable agricultural systems.

Glossary

ABC transporters – A family of membrane transporter proteins that are conserved in all living organisms and actively transport small molecules across cell membranes.

Agrochemicals – Chemicals used in agricultural ecosystems to promote crop growth and protect against pests and diseases. These include fertilizers, pesticides, herbicides, and other agricultural chemicals.

Chemical defense – Families of genes involved in cellular defense against toxic compounds. These include receptors, metabolic enzymes, heat shock proteins, and transporters.

Downregulated – A decrease in gene expression that can result in reduced production of its encoded protein.

Homologous genes – Genes that share sequence similarity (and often function) due to a common evolutionary origin.

Housekeeping gene (HKG) – A gene that encodes proteins essential for maintaining basic cellular functions. In qPCR analysis, HKGs are selected because of their constant level of expression across cells. This helps normalize the relative expression of target genes and account for variations in RNA content during the experimental process.

Multi-drug resistance (MDR) – The development of tolerance or resistance to multiple drugs/chemicals via genetic changes. This is often observed in cancer cells and microorganisms.

Miticides – Pesticides or chemicals designed to specifically target arachnids, such as mites and ticks.

Orthologous genes – A type of homologous genes that retain the same function across different species and originated from the a common ancestor after a speciation (species splitting) event.

Paralogous genes – A type of homologous genes that may have the same, similar, or different functions within the same species and originated from a common ancestor after a gene duplication event within a lineage.

Phylogenetic analysis – The comparison of DNA, RNA, and protein sequences to construct evolutionary relationships between organisms.

qPCR – Quantitative Polymerase Chain Reaction or Real-Time Polymerase Chain Reaction is a technique used to amplify a target sequence of cDNA via thermal cycles. Unlike regular PCR, quantitative PCR uses fluorescence to monitor the target gene amplification in real time.

Trophallaxis – Food exchange between two individuals that may involve salivary secretions or regurgitation of gut contents. This is the main food-sharing mechanism among honey bee hive members.

Upregulated – An increase in gene expression that can result in increased production of its encoded protein.

Xenobiotic – Chemicals that are not found naturally in living organisms. These include synthetic chemicals, drugs, and environmental pollutants.

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Author Contributions

Angela M. Encerrado-Manriquez: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing - Original draft, Writing - Review & Editing, Visualization,

Supervision, Project administration. **Zeke T. Spooner:** Methodology, Validation, Formal Analysis, Investigation, Resources, Data Curation, Writing - Original draft, Writing - Review & Editing, Visualization. **Tina T. Truong:** Investigation, Resources. **Julia D. Fine:** Validation, Resources, Writing - Review & Editing, Visualization, Supervision, Funding acquisition. **Sascha C.T. Nicklisch:** Validation, Resources, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

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Supplemental Data

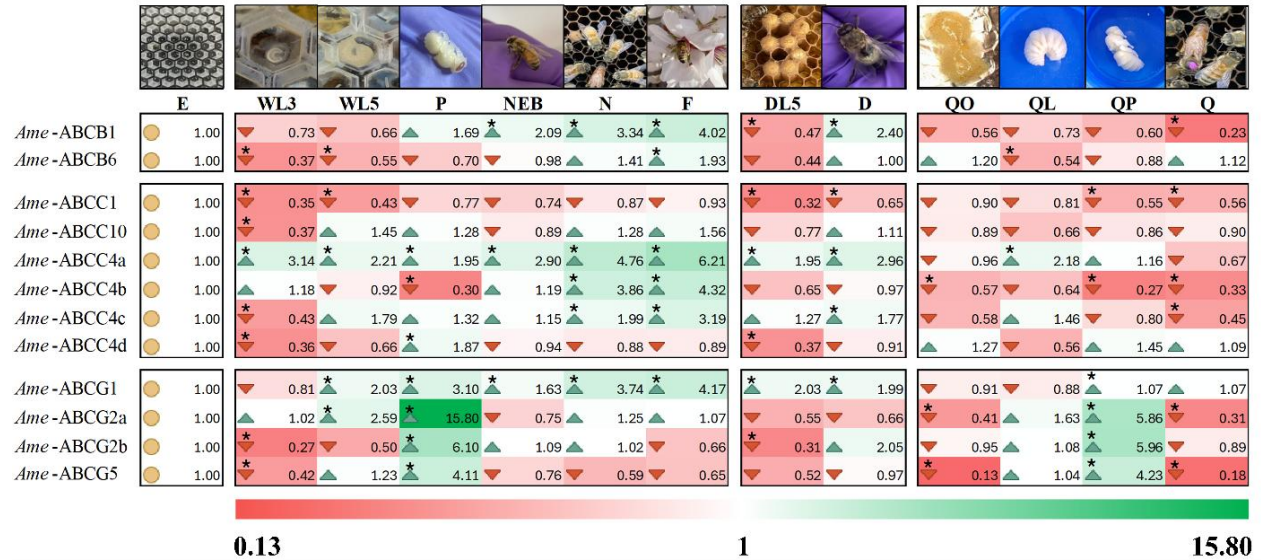


Figure S3.1. Heat map of the relative gene expression (fold change) of twelve MDR-like ABC-type transporters across 13 caste-specific stages and tissues of honey bees. Color intensity reflects the expression of each gene, with green representing relative upregulation (>1) and red representing relative downregulation (<1). The color scale reflects comparisons across all genes and all life stages: E (eggs), WL3 (Worker Larvae 3), WL5 (Worker Larvae 5), P (Pupae), NEB (Newly Emerged Bees), N (Nurses), F (Foragers), DL5 (Drone larvae), D (Drones), QO (Queen ovaries), QL (Queen larvae), QP (Queen pupae), and Q (Queens). The top images show corresponding tissues and caste members at different developmental stages (photographs taken by A. Encerrado). Significant differences between the two samples were validated by one-way ANOVA and are denoted by * ($p < 0.05$).

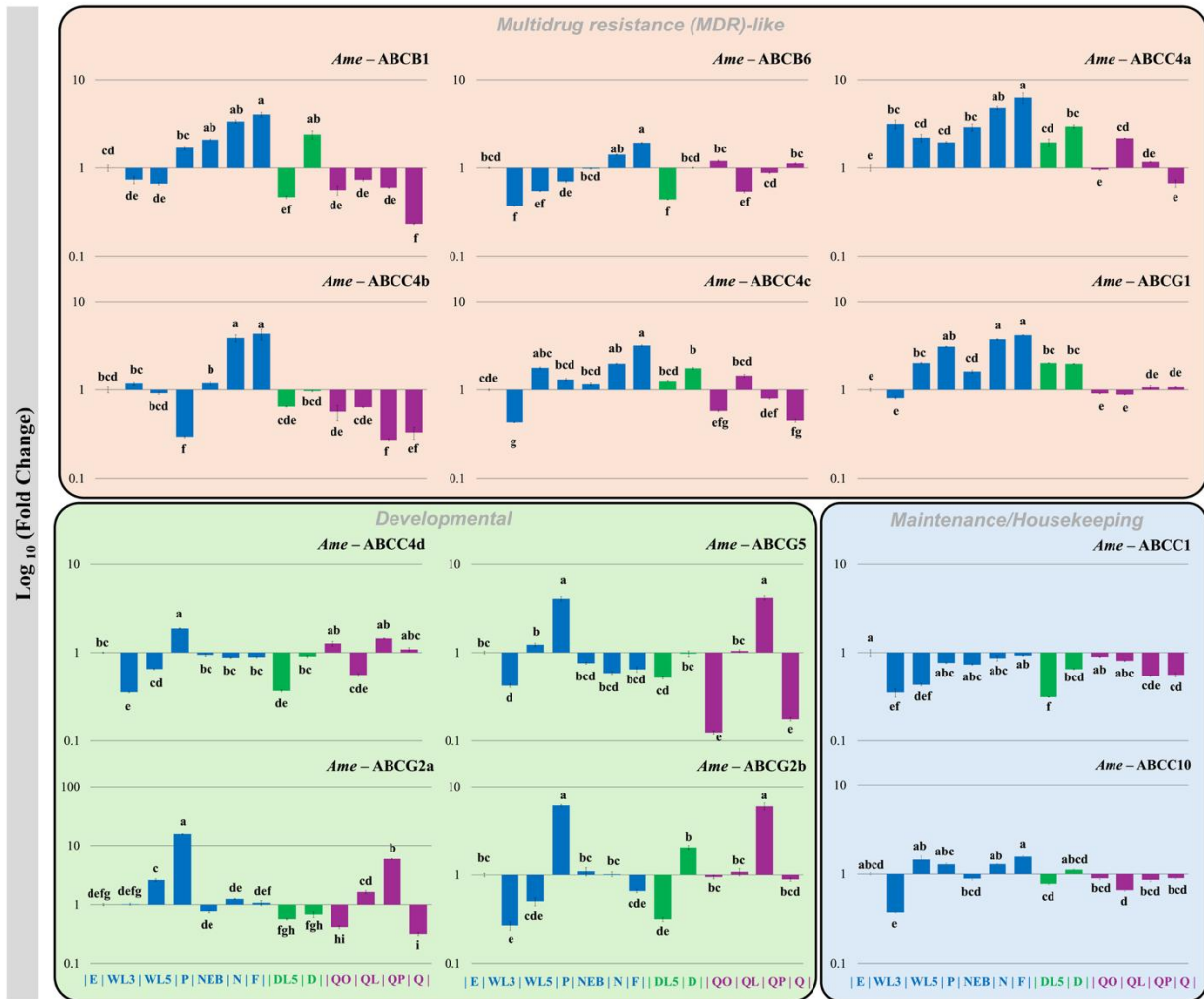


Figure S3.2. ABC transporter expression patterns across honey bee development and castes with functional classification. Expression levels of 12 ABC transporters (Ame-ABCB1, Ame-ABCB6, Ame-ABCC1, Ame-ABCC4a, Ame-ABCC4b, Ame-ABCC4c, Ame-ABCC4d, Ame-ABCC10, Ame-ABCG1, Ame-ABCG2a, Ame-ABCG2b, Ame-ABCG5) measured across 13 developmental stages and tissues: worker development (eggs, larvae 3, larvae 5, pupae, newly emerged bees, nurse bees, foragers), drone development (larvae, adults), and queen/reproductive tissues (ovaries, larvae, pupae, adult queens). Expression values are normalized to worker eggs (= 1.0). Boxes are color-coded by functional classification: light peach family = MDR Function transporters showing forager-specific upregulation for xenobiotic detoxification; light mint green family = Developmental Specialization transporters with pupal-specific expression peaks and caste-specific metamorphosis patterns; light blue family = Housekeeping/Maintenance transporters with stable expression across stages. Significant differences between groups were determined by one-way ANOVA ($p < 0.05$). Groups with different letters differ significantly; groups sharing the same letter are not significantly different.

Table S3.1. Comparative analysis of honey bee ABC transporter orthologs and their functional roles in chemical defense. The table summarizes the orthologous relationships between honey bee (*Apis mellifera*) ABC transporters and their characterized homologs in humans (*Homo sapiens*) and *Drosophila* (*Drosophila melanogaster*), including tissue expression patterns, substrate specificities, and roles in xenobiotic resistance. Gene symbols follow standard nomenclature: Hsa = *Homo sapiens*, Dme = *Drosophila melanogaster*, Ame = *Apis mellifera*.

Honey Bee Protein	Human Ortholog	Reference	Drosophila Ortholog	Reference
Ame-ABCB1	Ortholog: Hsa-ABCB1 Hsa-ABCB1, also known as P-gp or P-glycoprotein, is a full transporter and the most extensively studied member of the ABC family. Its presence in the human intestine, liver, and brain has been well-documented in relation to pumping xenobiotic compounds.	[1,2]	Ortholog: Dme-MRP49 In Dme, there are three homologs of Hsa-ABCB1: MDR49, MDR50, and MDR65. Dme-MRP49 is expressed in the adult head, endodermal anlage, head mesodermal anlage, larval brain, and mesoderm. Dme-MRP49 is a transmembrane transporter that confers insecticide resistance in <i>Drosophila</i>.	[2–4]
Ame-ABCB6	Ortholog: Hsa-ABCB6 Hsa-ABCB6 is a porphyrin transporter located in the outer membrane of mitochondria. Hsa-ABCB6 is a half-transporter that functions as a homodimer and plays a role in protecting against oxidative stress and processing iron and iron-containing biomolecules.	[1]	Ortholog: Dme-Hmt-1 It has been suggested that this transporter can confer resistance against heavy metals in arthropods. Dme-Hmt-1, or heavy metal tolerance factor 1, is involved in transmembrane transport and binding of heme, and cellular detoxification of cadmium ions. Dme-Hmt-1 is expressed in the adult head, adult heart, embryonic/larval midgut, and gastric caecum.	[3,5]
Ame-ABCC1	Ortholog: Hsa-ABCC1 ABCC1 was originally discovered as a cause of multidrug resistance in tumor cells. Further research has shown that it has a broad spectrum of drugs (xenobiotics) and physiological molecules as substrates.	[6]	Ortholog: Dme-MRP Dme-MRP is involved in monoatomic anion transmembrane transport, renal tubular secretion, and transport of toxic substances. Dme-MRP is expressed in the adult head and heart.	[3]
Ame-ABCC10	Ortholog: Hsa-ABCC10 Hsa-ABCC10, also known as MRP7, confers drug resistance to cancer cells and has a broad spectrum of substrates. Hsa-ABCC10 expression is highest in the pancreas, followed	[1,7]	Ortholog: Dme-CG7806 Dme-CG7806 is a protein predicted to be involved in transmembrane transport activity and is highly expressed in the adult head, as well as in the midgut and tubules. Its	[3,8,9]

	by liver, placenta, lungs, kidneys, brain, ovaries, lymph nodes, spleen, heart, leukocytes, and colon.		expression is affected by the molding hormone ecdysone.	
Ame-ABCC4a	Ortholog: Hsa-ABCC4 Hsa-ABCC4 transports nucleosides, endogenous signaling molecules, and a wide range of drugs and xenobiotics.	[1,10]	Ortholog: Dme-Mrp5 Dme-Mrp5 is involved in heme export and is expressed in the adult head and spermatozoon. Furthermore, in lice (<i>Pediculus humanus humanus</i>), ABCC4 was also found to protect against the antiparasitic drug ivermectin.	[3,11]
Ame-ABCC4b				
Ame-ABCC4c				
Ame-ABCC4d				
Ame-ABCG1	Ortholog: Hsa-ABCG1 Hsa-ABCG1 is present in the lungs, heart, spleen, brain, kidney, and adrenal gland. Its function has been linked to cholesterol transport.	[1]	Ortholog: Dme-CG5853 The predicted function of Dme-CG5853 is to actively transport sterols outside the plasma membrane and it is involved in tolerance against insecticides. Dme-CG5853 is expressed in the adult head and amnioserosa, embryonic/larval dorsal vessel, plasmatocyte primordium, and yolk nucleus.	[3,12,13]
Ame-ABCG2a	Ortholog: Hsa-ABCG2 Hsa-ABCG2 is present in the placenta, blood-brain barrier, liver, colon, intestine, mammary gland, and stem cells. Its function has been linked to xenobiotic defense and multidrug resistance for a broad range of substrates. Hsa-ABCG2 also transports steroids, certain chlorophyll metabolites, and organic anions.	[1,14]	Ortholog: Dme-E23 Dme-E23 encodes an ABCG transporter of ecdysone (a steroid hormone) that regulates reproduction, molting, and metamorphosis.	[15]
Ame-ABCG2b			Ortholog: Dme-st Dme-st (Protein Scarlet) is expressed in the Bolwig organ, Malpighian tubule main body primordium, Malpighian tubule primordium, adult head, and embryonic Malpighian tubule. Dme-st transports bioamines, neurotransmitters, and metabolic intermediates.	[3]
Ame-ABCG5	Ortholog: Hsa-ABCG5 Hsa-ABCG5 is expressed in the liver and intestine. Its function is to limit absorption of sterols and promote their biliary excretion. Human ABCG5 (as heteromer with ABCG8) has also been shown to contribute to active extrusion of plant-derived toxic sterols (phytosterols).	[1,14,16]	Ortholog: Dme-CG11069 Dme-CG11069 is predicted to actively transport sterols outside the plasma membrane.	[3]

Table S3.2. Sample collection metadata for honey bee developmental stages, reproductive castes, and tissues.

Life Stage	Collection Period	Location
Eggs (E)	Samples 1-2: midseason 2022; samples 3-5: late season 2023	Collected from hives and queen monitoring cages
Worker Larvae Instar 3 (WL3)	Samples 1-2: midseason 2022; samples 3-5: late-season 2023	Collected directly from hives
Worker Larvae Instar 5 (WL5)	Samples 1-3: midseason 2023; samples 4-5: late-season 2023	Collected directly from hives
Drone Larvae Instar 5 (DL5)	Samples 1-3: midseason 2023; samples 4-5: late-season 2023	Collected directly from hives
Worker Pupae (P)	Samples 1-2: midseason 2022; samples 3-5: late-season 2023	Collected directly from hives
Newly-Emerged Bees (NEB)	Samples 1-2: midseason 2022; samples 3-5: late-season 2023	Collected directly from hives
Nurses (N)	Samples 1-2: midseason 2022; samples 3-5: late-season 2023	Collected directly from hives
Foragers (F)	Samples 1-2: midseason 2022; samples 3-5: late-season 2023	Collected upon return to hives
Drones (D)	Samples 1-2: midseason 2022; samples 3-5: late-season 2023	Collected directly from hives
Queens (Q)	All samples: from healthy hives at the beginning of pollination season 2022	Collected directly from hives during requeening at the beginning of the pollination season
Queen Ovaries (QO)	Subset of the queen samples (see above)	Dissected from queen samples (at the Nicklisch Lab, UC Davis)
Queen Larvae (QL)	Samples 1-5: late-season 2024	Collected directly from hives
Queen Pupae (QP)	Samples 1-5: late-season 2024	Collected directly from hives

Table S3.3. Summary of honey bee ABC transporter metadata and primer sequences for qPCR analysis. Protein names for each ABC transporter and their associated NCBI gene ID are shown. All primers were designed using [Primer Blast](#) or [Primer3 Plus](#). The qPCR amplification efficiencies were calculated for each primer pair (please refer to Section 2.4 in the Main Manuscript for details).

Protein	Gene ID	Primer	Sequence (5'→3')	Length (bp)	Efficiency
ABCB1	551167	Forward	CAGCAGGATCACCGAGGATT	20	114%
		Reverse	GGGTCAGCTTCCATCCGTAG	20	
ABCB6	726867	Forward	GGCCTCAAAGCTCCAGGAAT	20	120%
		Reverse	TCTCGACTGTAAAGCTGGACC	21	
ABCC1	412217	Forward	GAACAATACGCGCTTCCGTG	20	109%
		Reverse	AGAACGCAGTATGGATGGCA	20	
ABCC4a	413959	Forward	TTCGGGAACAGTGCCTAACA	20	103%
		Reverse	TTCTTGCTCGTTGTCCACCA	20	
ABCC4b	551061	Forward	GCACTTCCTGCTAATGCACC	20	120%
		Reverse	TGCTCCTGTCCTTCCCACTA	20	
ABCC4c	725051	Forward	TTGCCAAAAGAAGCCTCGTG	20	122%
		Reverse	CAGTTCTGCCTACGATGCCA	20	
ABCC4d	410269	Forward	GCCCATCGTCTCAATACCGT	20	72%
		Reverse	TCGTAGCTTAGGCGTGCAAT	20	
ABCC10	725993	Forward	TGTGGGAAGATCACTTTTTGCG	22	111%
		Reverse	AGACAGGTGTTGTTGCCCAA	20	
ABCG1	412748	Forward	GCTGTTACACTTGCCTTTCCATT	23	108%
		Reverse	GGTTGCTGCACAAGATGCC	19	
ABCG2a	410967	Forward	CGCACTCTCCAGTTTTCTC	20	96%
		Reverse	GGAAGCCCAACATCGGGTAA	20	
ABCG2b	726508	Forward	AACTCGGGATTCAAGCTGCA	20	138%
		Reverse	ATTCTGCGGTATCAAGGCT	20	
ABCG5	726513	Forward	GCTTGCTGCAATTTCCACCA	20	87%
		Reverse	TCCTGGGCATGTGAAACTCTC	21	
GAPDH	410122	Forward	CACCTTCTGCAAAATTATGGCG	22	98%
		Reverse	ACCTTTGCCAAGTCTAACTGTAA	24	
RS5	409728	Forward	GATGTTTCTCCGTTACGACGAGT	23	121%
		Reverse	GAGTTCATCGGCTAAACATTCGG	23	
RAD1A†	102654987	Forward	CTTAGAGTGGGTCCTCCATC	20	108%
		Reverse	CAGCAGCATCCAGATTTAGAGG	22	

†[17,18]

Table S3.4. Comparative analysis of *Apis mellifera* ABC transporter sequences against *Drosophila melanogaster* and *Homo sapiens*. *Apis mellifera* (Ame) sequences that were blasted against *Drosophila melanogaster* (Dme) and *Homo Sapiens* (Hsa). Results in red represent the first NP (Nucleotide Protein) sequence found in the BLAST results, while results in black indicate the first sequence listed in the BLAST results for that species (not necessarily the first NP sequence). Some sequences were both first found and NP. Therefore, most results show only three sequences per gene of interest.

Ame Gene Symbol	Ame Protein Accession #	Ame Gene ID	Reference Gene Symbol	Reference Species	Reference Accession #	Reference Gene ID	E-value	Length (aa)
Mdr49	XP_623564	551167	Mdr49	Dme	NP_523724.2	36428	0	1302
			ABCB1	Hsa	AAI30425.1	5243	0	1280
			ABCB1	Hsa	NP_001335874.1	5243	0	1350
Hmt-1	XP_001122583	726867	Hmt-1	Dme	NP_650503.1	41925	0	866
			PRP	Hsa	AAG33617.1	N/A	0	896
			ABCB6	Hsa	NP_005680.1	10058	0	842
MRP	XP_006558751	412217	MRP	Dme	NP_995704.1	34686	0	1548
			ABCC1	Hsa	EAWS3939.1	4363	0	1522
			ABCC1	Hsa	NP_004987.2	4363	0	1531
LOC725993	XP_016767210	725993	CG7806	Dme	NP_609207.1	34140	0	1487
			ABCC10	Hsa	EAX04180.1	89845	0	1324
			ABCC10	Hsa	NP_001185863.1	89845	0	1492
LOC413959	XP_006563569	413959	CG7627	Dme	NP_001245943.1	34148	0	1355
			CDS	Hsa	BAD92357.1	N/A	0	1439
			ABCC11	Hsa	NP_001357426.1	85320	0	1382
LOC551061	XP_006566761	551061	CG7627	Dme	NP_001245943.1	34148	0	1355
			KI723_130593	Hsa	KAI2569693.1	N/A	0	1325
			ABCC4	Hsa	NP_005836.2	10257	0	1325
LOC725051	XP_026299794	725051	Mrp5	Dme	NP_001262747.1	42362	0	1408
			MOAT-B	Hsa	AAC27076.1	N/A	0	1325
			ABCC4	Hsa	NP_005836.2	10257	0	1325
LOC410269	XP_393750	410269	CG7627	Dme	NP_001245943.1	34148	0	1355
			ABCC4	Hsa	AAL88745.1	N/A	0	1325
			ABCC4	Hsa	NP_005836.2	10257	0	1325
LOC412748	XP_026301943	412748	ABCG1	Hsa	NP_997057.1	9619	2e-145	677
			CG5853	Dme	AAK93537.1	N/A	1e-135	689
			CG5853	Dme	NP_001260309.1	34322	2e-135	689
E23	XP_026295526	410967	E23	Dme	NP_524648.3	43895	0	1017
			white	Hsa	CAA62631.1	N/A	4e-77	674
			ABCG1	Hsa	NP_004906.3	9619	1e-76	678
LOC726513	XP_006564576	726513	st	Dme	NP_524108.1	39836	0	666
			ABCG2	Hsa	UQX14429.1	N/A	9e-78	655
			ABCG2	Hsa	NP_001335916.1	9429	8e-77	653
LOC726508	NP_001403445	726508	CG11069	Dme	NP_651307.2	42976	8e-155	604
			KI723_020513	Hsa	KAI2523202.1	N/A	7e-67	651
			ABCG5	Hsa	NP_071881.1	64240	4e-66	651

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Chapter 4. From primers to pipettes: An immersive course introducing high school students to qPCR for quantifying chemical defense gene expression.

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Abstract

We created a two-week, dual-module summer course introducing high school students to environmental toxicology by teaching them qPCR (quantitative polymerase chain reaction) as a way to quantify gene expression of chemical defense proteins in response to exposure to environmental pollutants. During the course, students are guided through the various stages of a successful qPCR experiment: *in silico* primer design & quality control, total RNA extraction and isolation, cDNA conversion, primer test PCR, and evaluation of results via agarose gel electrophoresis or UV/Vis spectra. The course combines lectures, discussions, and demonstrations with dry and wet laboratory sections to give students a thorough understanding of the scope, utility, and chemical principles of qPCR. At the end of the course, the students are taught how to analyze qPCR data and are encouraged to discuss their findings with other classmates to evaluate their hypotheses and assess possible sources of error. This course was designed to be easily adaptable to multiple test species, chemical exposures, and genes of interest. To explore both terrestrial and aquatic toxicology, the students use honey bees (*Apis mellifera*) and mosquitofish (*Gambusia affinis*) as test organisms, as well as ABC-type efflux transporters, antioxidant enzymes, and cytochrome P450 enzymes as endpoints for assessing gene expression. We share this course setup and applied protocols to encourage others to design and offer similar courses that give high school students a hands-on introduction to a broad swath of environmental toxicology research and an opportunity to develop scientific skills necessary for university-level research.

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Introduction

Environmental toxicology is a multidisciplinary field of study at the interface of chemistry, biology, and pharmacology that is rarely given attention in secondary education in the United States, and is typically reserved for higher education settings.¹ Perhaps one of the main challenges educators dread when attempting to introduce high school students to toxicology is the lack of previous laboratory experience the students may have, putting them at risk of toxic chemical exposures.¹ Nevertheless, its multidisciplinary nature provides a unique opportunity for students to connect the concepts of analytical chemistry and molecular biology, developing their critical thinking skills in the study of toxic chemicals and their effects on people and the environment. Furthermore, environmental toxicology allows for chemistry and biology concepts learned in class to be applied into real world scenarios, further developing the student's curiosity for science. Given that some students' strengths lean toward biology, while others are more interested in chemistry, and still others are keen about both fields, and considering the safety of the students, we selected a field of study within environmental toxicology for the course—chemical stress-induced gene expression—that we believed would interest all types of students through a focus on chemical detoxification and the biochemical principles underlying gene expression, while providing a safe space for students to take their first steps into applied laboratory sciences.

Chemical stress-induced gene expression not only brings these broad fields together in the context of environmental toxicology but allows for the use of quantitative molecular biology techniques suitable for a high school level of instruction. The quantitative polymerase chain reaction (qPCR) is a popular, simple, and rapid method of quantifying nucleic acids.² Because it is easy to learn, minimally hazardous, technically simple, and broadly applicable, it is an ideal method for giving hands-on experience to secondary students new to the field. Historically, qPCR has been used in the field of toxicology to examine toxicant-induced changes in gene expression.^{3,4} Many studies have shown that environmental exposure to a multitude of xenobiotic chemicals, including pesticides⁵, household chemicals⁶, natural biotoxins⁷, and

even transportation pollutants (such as emissions from cars⁸ and oil from maritime activity⁹) can cause changes in gene expression of exposed organisms.

Although a wide variety of organisms are affected by unwanted chemical exposures in their environment, this course will focus on two model species: the Western Mosquitofish (*Gambusia affinis*) and the Western honey bee (*Apis mellifera*). As its name suggests, the mosquitofish is widely used to control mosquitoes in small bodies of water such as agricultural and urban ponds.¹⁰ Living in these environments can expose these fish to industrial, agricultural, and household chemicals that could affect gene expression. For this course, we use mosquitofish caught from the UC Davis Wastewater Treatment Plant (WWTP), where they are used for mosquito control in semi-treated sedimentation tanks. This wastewater may contain urban runoff, household chemicals, and other contaminants related to human waste. This potentially harmful chemical environment could elicit a change in gene expression in these fish to better fend off chemical accumulation and toxicity. Unexposed control fish will be obtained from a UC Davis IACUC-approved biological specimen supply company (Carolina Biological Supply Co., Burlington, NC). Our second model species is widely familiar to high school students. Honey bees are essential pollinators worldwide and the primary commercially used pollinator in the United States, adding billions of dollars in revenue to crop production yearly.¹¹ Although mitigation strategies are in place during commercial pollination, honey bees are often exposed to multiple pesticides simultaneously. These pesticides (spanning insecticides, herbicides, fungicides, and miticides) are potentially toxic to individual bees and the general hive. They also could affect the detoxification mechanisms of the hive, resulting in a change in gene expression to lessen toxicity.¹² We thought the billion-dollar almond industry in the United States would offer students a real-life example, given that they are one of the main users of pollination services. We identified three pesticides currently used to control pest populations in almond orchards: Diflubenzuron, Methoxyfenozide, and Pyriproxyfen (**Figure 4.1**). The bees used in this class had been previously exposed to these pesticides in a controlled laboratory setting to study their effects on detoxification gene expression.

The goal of this course is to introduce university-level research in environmental toxicology to high school students. The use of environmentally relevant and easily accessible model species is key to achieving this aim. At its core, this course is an environmental toxicology research project that uses qPCR as its main tool to understand chemical exposure effects on detoxification gene expression. Throughout the course, students are taught the chemical principles behind qPCR and chemical detoxification and how to design a qPCR experiment and perform the steps to quantify the gene expression of ABC-type transporter proteins, cytochrome P450 enzymes, and antioxidant enzymes in mosquitofish and honey bees. However, the flexibility of qPCR allows for the easy adjustment of which genes and organisms are studied. In this article, we present the methods we used for administering this course and an organizational map that can be adapted to the design of other courses targeting specific genes, chemical detoxification pathways, and organisms of interest.

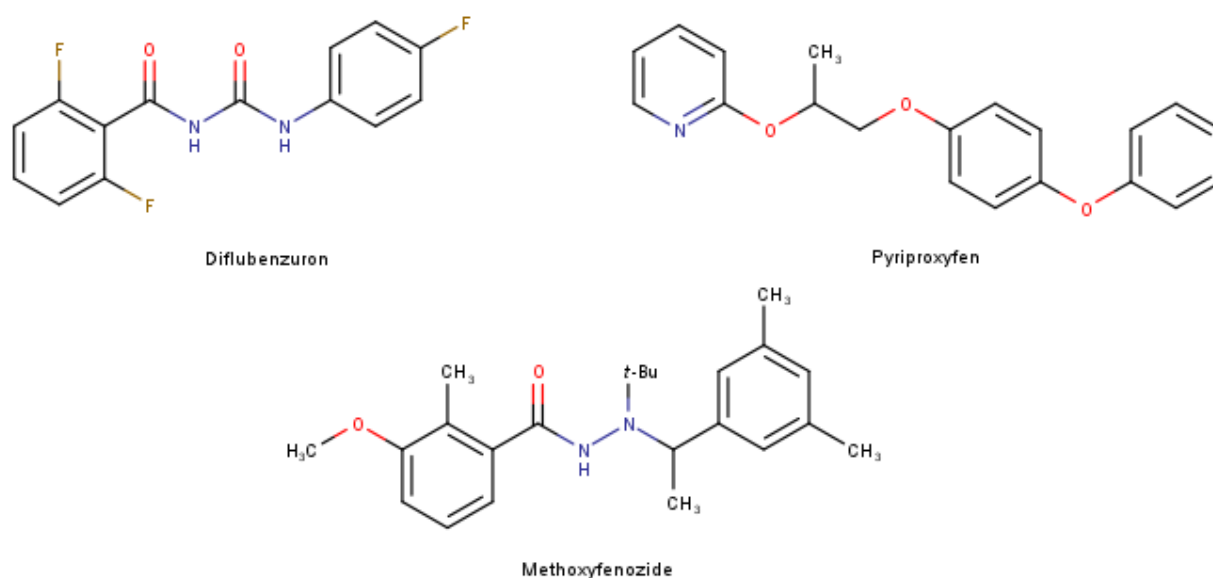


Figure 4.1. Chemical structures of three different pesticides used to expose honey bees studied in this course. All three pesticides belong to the group of insect growth regulators (IGR) that are used to selectively inhibit the life cycle of pest insects.

1.1 | Learning objectives

We created a set of learning objectives that reflected our expectations for students after completing the two-module course. These objectives reflect our goal of creating a low-stakes classroom environment where students can explore their budding interest in environmental toxicology. These learning goals emphasize not only detoxification, but a deep understanding of the methods used in the course to study it. Learning goals were designed around key chemical principles, with students reinforcing their comprehension through lectures, a laboratory manual (see Supporting Information A), and collaborative discussions, culminating in their application in hands-on laboratory work.

1. Students will demonstrate proficiency in the use of bioinformatic software and modern databases through a series of guided assignments (see Supporting Information A).
2. Students will demonstrate a grasp of the scientific method and its application to toxicological research through oral discussion and hypothesis development and evaluation.
3. Students will demonstrate knowledge of the basic chemical and biochemical principles of detoxification through in-class oral discussions of important detoxification proteins that correspond to the genes analyzed in the class laboratory project.
4. Students will demonstrate an understanding of the theory, applications, and practice of qPCR through laboratory exercises, guided assignments, and oral discussion. Examples of emphasized topics include nucleic acid chemistry and the chemical and biochemical differences between DNA and RNA, the thermodynamics of nucleic acid binding and extension as it relates to primer binding and the phases of PCR, and the physical chemistry behind fluorescence and why this is important for qPCR.
5. Students will demonstrate key laboratory skills (including safety procedures and pipette work) with an emphasis on developing and improving technique and safe handling of chemicals through laboratory exercises.

6. Students will further demonstrate the ability to ensure high-quality, quantifiable data through the implementation of standards (such as nucleic acid ladders in gels and reference genes in qPCR) and quality control at each step of the qPCR process.

Methods

2.1 | Experimental overview

Figure 4.2 shows the modular setup and individual tasks of the experimental workflow for the course. This two-module course can be adapted for 16 to 24 high school students, divided into groups of two or three, that meet for six hours of class time per day. These six hours are generally divided into one-hour lectures, discussions, and walkthroughs in the mornings, with three-hour hands-on activities and guided assignments in the afternoons. The first module takes place in a dry lab setting, where students receive lectures on the chemical and biochemical principles behind cellular detoxification and PCR and familiarize themselves with modern bioinformatics databases and software used for sequence analysis and primer design. The second module moves to the wet lab, where students are trained to carry out the steps of a qPCR experiment, ensure the quality of their samples at each step, and analyze their qPCR data. Before starting any type of wet lab activities, a class is specially designated to give students instruction on laboratory safety and etiquette, as well as an introduction to micro-pipetting.

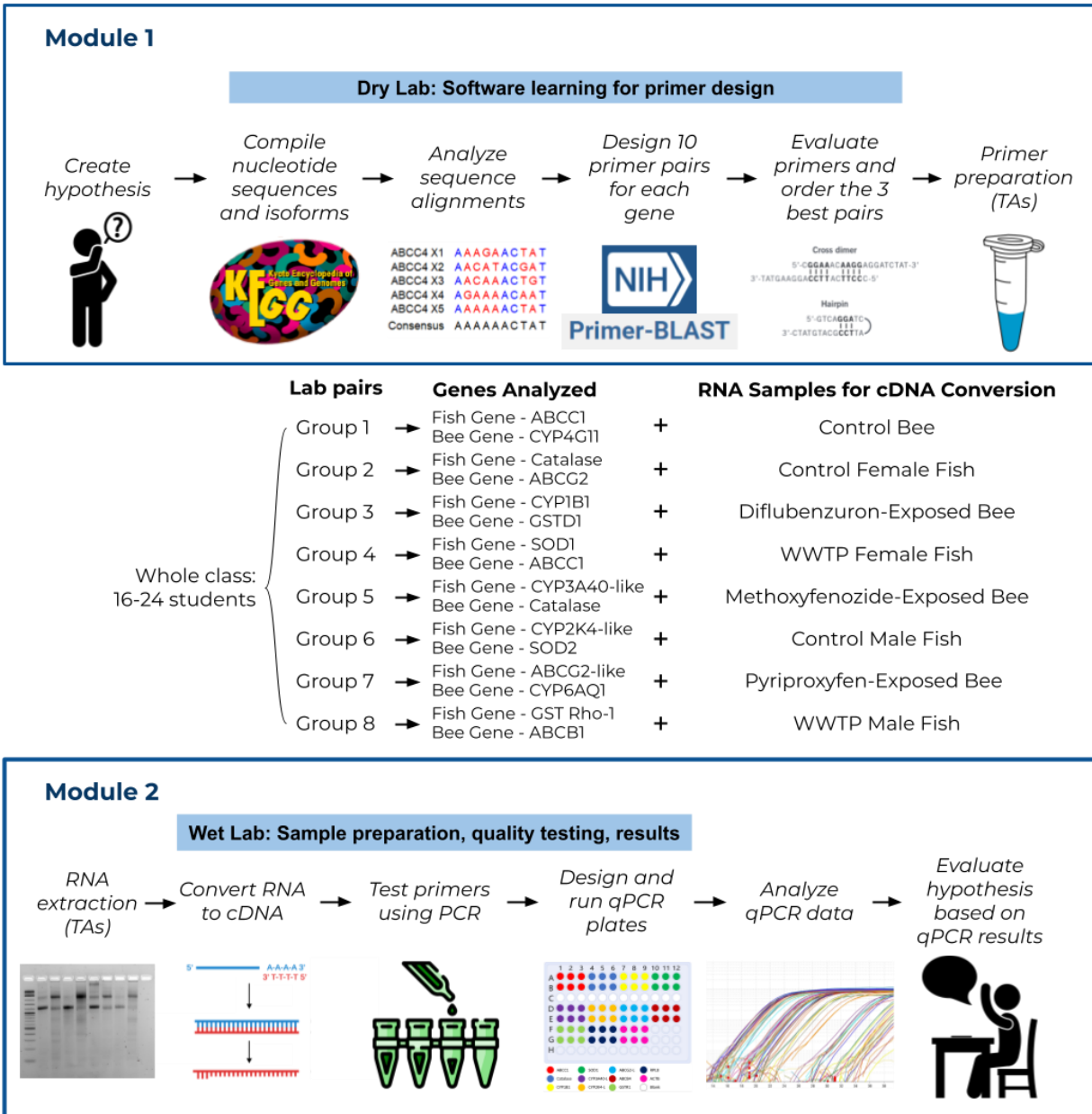


Figure 4.2. Organizational map showing course workflow and class assignment distribution. Days began with lectures and discussions based on the course content of that day, followed by TA walkthrough demonstrations of software use and laboratory techniques, and ended with students applying their knowledge in various activities and assignments. During **Module 1**, students become familiar with open-access modern databases and software commonly used in academic fields to study genes of interest and create qPCR primers. During **Module 2**, students move to a wet lab environment where they practice their laboratory skills by executing a qPCR protocol from start to finish. The middle diagram shows how we divided students into groups to distribute work, including how we allocated genes for them to research and RNA samples to perform cDNA conversion.

In collaboration with the UC Davis Pre-College Program, this course has been successfully run in person and online for three consecutive summers (2021–2023). In addition to the instructor, three TAs provide instructional and laboratory support during the course. In the interest of time and conservation of materials, students are divided into groups of two or three to perform laboratory procedures and computational analysis so that the work is equally divided. The class was designed to be informative, interactive, and minimally stressful, so assignments are not graded, and coursework can be completed entirely during class time (i.e., no homework). However, students are expected to engage in a class discussion at the end of the course, sharing their research project results and their interpretation of them.

2.2 | Specimen acquisition

Prior to the course, honey bees (nurse bees) are collected with the help of our collaborators at the laboratory of Dr. Julia Fine from the USDA-ARS facility (Davis, CA), while mosquitofish are collected from two sources – unexposed fish from the Carolina Biological Supply Company (Burlington, NC) and exposed fish from the UC Davis Wastewater Treatment Plant (Davis, CA). Fish are euthanized in accordance with an approved IACUC-approved protocol (#23388) by use of 1 g/L Buffered Tricaine Methanesulfonate (Spectrum, New Brunswick, NJ, USA). Honey bees are euthanized by flash freezing in liquid nitrogen. Upon euthanasia all frozen tissue is stored at -80°C until homogenization.

2.3 | MODULE 1: Bioinformatic databases and software for sequence analysis and primer design

2.3.1 | Generating research hypotheses

One of the first lessons in the first module is designed to give students the necessary background information on both species and exposures. As a result, each group of students is encouraged to develop a hypothesis about the relative expression of detoxification genes in each of their experimental systems. Here are some examples of hypotheses that students created for their research project:

- *“There will be increased expression of defense proteins in honey bees exposed to almond-crop pesticides than those not exposed.”*

- *“There will be increased expression of some defense proteins, like xenobiotic efflux transporters, and decreased expression in others, like antioxidant enzymes, in honey bees exposed to almond-crop pesticides relative to unexposed bees.”*
- *“There will be increased expression of defense proteins in mosquitofish from wastewater sedimentation tanks relative to store-bought mosquitofish.”*
- *“There will be a difference in expression of defense proteins in male and female mosquitofish from wastewater sedimentation tanks.”*
- *“There will be a difference in expression of defense proteins in male and female mosquitofish, regardless of chemical environment.”*

2.3.2 | Bioinformatics and primer design

The first module involves hands-on assignments that teach students how to begin a qPCR project by compiling information from databases and analyzing it using modern software. To properly analyze and create primers for their assigned ABC-type transporter, students must first learn how to search for and extract relevant information from genetic databases. For each software application and database, students are given an in-class assignment (see Supporting Information A). All assignments serve as an intermediate tool to assess what students have learned on a particular day and allow students to practice their skills and apply their knowledge to their research project.

1. *Kyoto Encyclopedia of Genes and Genomes (KEGG) and the National Center for Biotechnology Information (NCBI):* Databases on genes and proteins used to extract names, sequences, accession numbers, gene IDs and nucleotide and protein lengths.¹³ The goal is for students to use these databases to compile a list of genes and isoforms for their assigned defense genes in the organisms of interest. In Assignment 1A (see Supporting Information A), students fill out a table listing relevant metadata for all isoforms of their genes of interest.

2. *NCBI Basic Local Alignment Search Tool (BLAST)*: An online tool that searches the NCBI database for similar nucleotide or amino acid sequences to the search query.¹⁴ The goal is for students to ensure that all assigned genes and isoforms were found by running the genes they retrieved from KEGG and NCBI through this tool. Students then tabulate this information for use in isoform analysis and primer design. In Assignment 1B (see Supporting Information A), students upload a printout of the top 100 BLAST results for their genes of interest.
3. *CLC Main Workbench 23.0.4 (Qiagen, Aarhus, Denmark)*: Software for nucleic acid and protein sequence comparison and analysis. The goal with CLC is for students to create alignments of all isoforms for a given gene and see how different the isoforms are. If the isoforms are very different, then different primers need to be designed for them; if they are largely the same, then one primer pair can be used to capture all the isoforms. In Assignment 2 (see Supporting Information A), students upload printouts of their isoform alignments and log any gaps or areas of non-consensus in a spreadsheet.
4. *NCBI Primer BLAST and Beacon Designer Free Edition (Premier Biosoft International, Palo Alto, CA)*: Two different types of software used to design PCR primers¹⁵ and analyze the quality of those primers, respectively. The goal is for students to design three primer pairs for their given genes. Using Primer BLAST, 10 qPCR primer pairs are generated for each gene. These primers were between 18-25 nucleotides in length. The targeted amplicon sizes (products of qPCR amplification) were between 80-200 nucleotides. The students are then tasked with picking the optimal three primer pairs from the ten contenders by evaluating their ability to dimerize and form hairpins (as estimated by Beacon Designer). The open-source software we use for the class is recommended for qPCR primer design for SYBR® Green assays.¹⁶ In Assignment 3A (see Supporting Information A), students complete a spreadsheet that documents the sequences, properties, and Beacon Designer analysis of their ten contenders and highlight their top three picks.
5. *SnapGene® Viewer (from Domatics; available at snapgene.com)*: Software used for the visualization and annotation of genes. The goal for students is to ensure that their primers will bind to the gene

sequence of interest and produce the predicted amplicon by annotating their genes with their primers. In Assignment 3B (see Supporting Information A), students use SnapGene® Viewer to perform restriction enzyme digestions on their genes of interest and a plasmid and virtually predict how their resulting fragments would visualize on an agarose gel after the digestion.

After deciding on the top three primers for each gene, the results are compiled, and the instructor and TAs order these primers from an online sequencing service, such as Eurofins Genomics (<https://eurofinsgenomics.com>).

2.4 | MODULE 2: Sample preparation, quality checks, qPCR run, and data analysis

2.4.1 | Lab and Chemical Safety, Etiquette, and Micro-pipetting Techniques

For high school students with little to no experience in the laboratory setting, learning good safety habits is of utmost importance. A two-hour safety lecture was developed in which we instructed students on proper techniques of donning and doffing PPE with an emphasis on glove usage, principles of safe laboratory practices and chemical handling, and proper micropipette operation. Students are required to wear lab coats, gloves, and safety glasses at all times in the lab. The reagents used are non-hazardous and there are only a few risks associated with their use (these include the use of the flammable liquid ethanol and handling very hot agarose solution); nonetheless, students are instructed on proper chemical handling in order to minimize risk of exposure and spills. Students are required to use heat-resistant gloves while handling hot glassware during the gel electrophoresis lessons.

2.4.2 | Agarose gel electrophoresis

Throughout the course, students are trained in using agarose gel electrophoresis to evaluate the quality of their obtained tRNA and generated cDNA and primer pairs. Students are taught about agarose polymerization and fluorescent compounds (i.e., stain and loading dye) used for visualizing bands, then create two gels: a “real” gel containing stain and a “mock” gel that did not contain stain. This is so every

group has a chance to cast and load an RNA or cDNA gel (despite there being more groups than necessary gels). The students in this course create a total of 4 mock gels, 2 tRNA gels, 2 cDNA gels, and 4 test PCR gels (Figure 4.3).

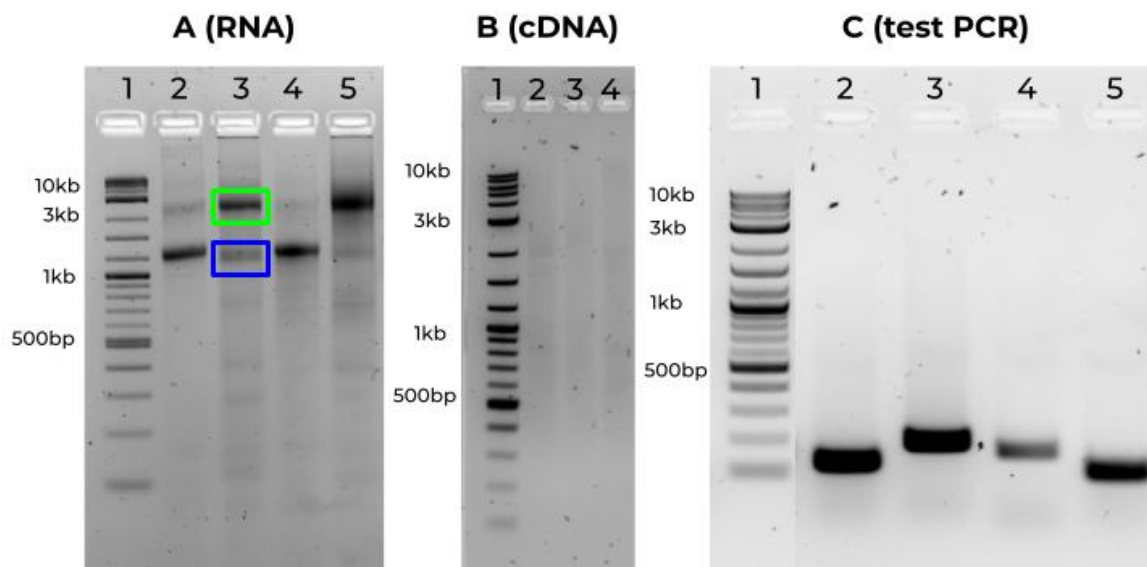


Figure 4.3. Agarose gel electrophoresis to evaluate quality of RNA and DNA products. The agarose gel is 2% agarose in TAE buffer. The first lane (1) in each gel contains a 1kb Plus DNA ladder #N3200S from NEB. Individual ladder bands below 1kb are in steps of 100bp. (A) Honey bee and mosquitofish RNA gel with honey bee RNA samples in lanes 2 and 4 and mosquitofish RNA samples in lanes 3 and 5. The green box indicates the ubiquitous 28S RNA, and the blue box indicates 18S RNA to better evaluate total RNA extraction efficiency and quality. (B) Honey bee cDNA gel with cDNA samples in lanes 2-4. (C) Primer test PCR for honey bee qPCR primers, demonstrating successful and clean amplification of ABCB1 (lanes 2-3) and ABCG4 gene fragments (lanes 4-5).

Gels are 2% agarose (Bio-Rad, Hercules, CA, USA) in 1x TAE buffer (Bio-Rad, Hercules, CA, USA) stained with SYBR® Safe stain (Bio-Rad, Hercules, CA, USA) by spiking 5 μ L per 50 mL of gel into the hot solution before pouring. Before the students test real samples, they are trained in creating mock gels (without stain) and loading store-bought food coloring dye to practice the gel loading procedure. When loading the dyes with sample, students mix 3 μ L of sample with 0.6 μ L of Monarch 6x Purple Loading Dye (New England Biolabs, Ipswich, MA, USA) on parafilm and load the resulting mixture into the gel lanes.

Marker lanes are used as internal standards for all gels, so students are instructed to load 3 μ L of Monarch 1kb DNA ladder (New England Biolabs, Ipswich, MA, USA). A Mini-Sub Cell GT System with 75W PowerPac power supply (Bio-Rad, Hercules, CA, USA) is used for running agarose gels for 60 minutes at 100V before viewing them on a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). A preliminary written gel loading design of each gel is encouraged to help the students keep track of their samples during the loading process of their research project.

2.4.3 | Pre-lab preparation: total RNA (tRNA) extraction

Due to time constraints in this 2-week course and the advanced difficulty of the protocol, a total RNA extraction is performed by the TAs beforehand and stored at -80°C until use. Tissues are homogenized using the BeadBug™ 6 Six-Position Homogenizer (Benchmark Scientific, Sayreville, NJ, USA) and 2.8mm steel beads (Omni International, Kennesaw, Georgia, USA). The Monarch® Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA, USA) is used to obtain high-quality RNA from the honey bee and mosquitofish samples. Whole bees weighing approximately 100 mg and whole fish weighing approximately 200 mg are used in this protocol, and samples are homogenized in buffer provided with the kit for three cycles of 30 seconds at 6 m/s with 45-minute dwell periods. Four different RNA samples are extracted for each model species: for bees, one unexposed control sample and three samples exposed to different pesticides (pyriproxyfen, diflubenzuron, and methoxyfenozide); and for fish, two control samples and two wastewater-exposed samples. Only nurse bees are used, while one male and one female mosquitofish are used for each treatment.

RNA quality is important because it can affect the validity of all downstream processes, and poor extractions of RNA could prevent students from obtaining viable results. Therefore, RNA yield and possible protein contamination are assessed before the class using a DS-11+ Nanovolume and Cuvette Mode Spectrophotometer (DeNovix, Wilmington, DE, USA), while RNA quality and integrity is assessed using agarose gel electrophoresis (see **Figure 4.3A**) by the TAs prior to any student wet lab activities.

2.4.4 | Total RNA (tRNA) to complementary DNA (cDNA) conversion

After obtaining total RNA, including messenger or mRNA, which describes the transcriptional variance in the genes of interest, students must convert the RNA into a form suitable for PCR amplification through cDNA conversion (**Table 4.1**). The cDNA for the qPCR reaction is made using previously prepared tRNA and SuperScript IV First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific, Waltham, MA). Students are guided through the process of calculating the amount of RNA to use in order to yield 1000 ng of cDNA for each reaction according to the following equation:

$$[RNA] (ng/\mu L) = \frac{1000 \text{ ng}}{V_{RNA} (\mu L)} \rightarrow V_{RNA} (\mu L) = \frac{1000 \text{ ng}}{[RNA] (ng/\mu L)}$$
$$V_{H_2O} = 11 \mu L - V_{RNA}$$

The above equation details the calculations needed for determining the volumes of RNA and water necessary to synthesize 1000 ng of cDNA during cDNA conversion. Students were guided through these calculations prior to the cDNA conversion protocol. The calculations assume 100% conversion from RNA to cDNA, and the company protocol for 20 μ L reactions is used. The only modification made concerned the primers used for the reverse transcriptase. Rather than 1 μ L of Oligo d(T)₂₀ or 1 μ L of random hexamers, our protocol uses 0.5 μ L of each (additionally, the optional RNA removal step is not performed).

The students then get additional practice with agarose gel electrophoresis by analyzing the quality of their converted cDNA in another set of gels (**Figure 4.3B**).

Table 4.1. Tabulated volumes of reagents necessary to carry out cDNA conversion using the SuperScript IV First-Strand Synthesis System from Invitrogen.

Reagent	Volume
DEPC-treated water	$V_{\text{H}_2\text{O}}$ (as calculated above)
5× SSIV buffer	4 μL
10 mM dNTP mix	1 μL
100 mM DTT	1 μL
Rnase inhibitor	1 μL
Prepared total RNA	V_{RNA} (as calculated above) up to 11 μL
SuperScript IV reverse transcriptase	1 μL
Oligo d(T) ₂₀ primer	0.5 μL
Random hexamers primer	0.5 μL
Total	20 μL

2.4.5 | Primer test PCR

In order to maximize the quality and consistency of our primers and the generated cDNA, all three primer pairs ordered for each gene are tested using a test PCR reaction. Test PCR is carried out using GoTaq® Green Master Mix (Promega, Madison, WI, USA). The company protocol outlines a 25 μL reaction, but the protocol used scales this down to 20 μL but keeps the proportions of reagents consistent. In a PCR tube, the students combine 10 μL of GoTaq master mix with 7 μL of water and 1 μL each of cDNA, forward primer, and reverse primer. The steps for the PCR incubation conditions are as follows:

- 2 minutes at 95°C (“hot start”)
- 30 cycles of: 30 seconds at 95°C; 30 seconds at 64°C; 1 minute at 72°C, and
- 5 minutes at 72°C for the final elongation of products.

After incubation, 4 μL of the same 6x Purple Loading Dye is combined with the completed PCR reaction, and 20 μL of the resulting mix is loaded into a 2% agarose gel (created using the same method

described above) and run for 30 minutes at 100V. The students are instructed to evaluate the quality of their primers by examining the gel and determining whether one clear, strong band appears at the correct amplicon length. Students look for one strong band at the predicted amplicon size on the resulting gel (**Figure 2.3C**). A lack of bands, a very faint band, the presence of multiple bands, or dense primer clouds could indicate a poor primer pair.

Results

3.1 | Product and data quality assessments

3.1.1 | RNA quality assessment

As previously mentioned, the quality of reagents and products is tested through various means for each step of the qPCR protocol. Our main method of validation is through using agarose gel electrophoresis, a widely-accepted way of evaluating RNA quality.^{17,18} Most of the extracted total RNA is typically ribosomal RNA, which is primarily visible as two clear bands representing the two ribosomal subunits 28S (green box, **Figure 4.3A**) and 18S (blue box, **Figure 4.3A**). Students are taught to first look at the ladder spread to evaluate the quality of the run (M, **Figure 4.3A**), checking for good separation between bands and no misshapen bands. Then, students look for prominent bands in their sample wells corresponding to these two indicator bands in their gels as a quick and easy way to evaluate the quality of their provided total RNA samples. A secondary method to determine RNA quality is using the DeNovix Nanodrop. This instrument allows us to determine the RNA's yield and contamination levels by the absorbance ratio at 260 nm (which corresponds to nucleotides in DNA and RNA) with contaminant absorbing at either 280 nm (e.g., proteins, detergents) and 230 nm (e.g., solvents, salts). The RNA samples are deemed high-quality if the 260/280 and 260/230 ratios are above an accepted threshold of 1.7 and ideally close to 2.0 for pure RNA.

3.1.2 | cDNA quality assessment

As seen in **Figure 4.3B**, an agarose gel is also used to evaluate the quality of the cDNA that the students create. As seen by the figure, the cDNA gels should always show a consistent smear from high molecular weight to low molecular weight, indicative of full conversion of small to large mRNA templates into cDNA.

3.1.3 | Primer quality assessment

As seen in **Figure 4.3C**, an agarose gel is used to assess the results of the test PCR as well. The qPCR amplicons vary in length but are generally between 80 and 200 bp. A strong band is expected to be seen at the predicted amplicon length for each primer pair.

3.2 | qPCR: Experimental setup

Once optimal primers and high-quality cDNA are obtained, the students assess the relative expression of their genes of interest using qPCR. Prior to running qPCR plates, 96-well diagrams are made during class time. Reaction sizes are set to 20 μ L to keep volumes at a reasonable amount. Samples are loaded into each well following the company protocol recommendations: 10 μ L of SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad), 7 μ L of PCR-grade water, 1 μ L of the 10 μ M solutions of forward and reverse primer, and 1 μ L of the 10 ng/ μ L prepared cDNA. This is incubated in a QuantStudio 3 qPCR system (Thermo Fisher Scientific, Waltham, MA, USA) in three cycles:

- A hold stage for 30 seconds at 98°C,
- A 40-cycle PCR stage of 15 seconds at 98°C followed by 30 seconds at 60°C,
- A melt curve stage of 15 seconds at 95°C followed by 1 minute at 60°C,
- And a final 15 seconds at 95°C.

Multiple reference genes (stable, highly expressed genes used to account for differences in expression unrelated to the treatment) are used for both honey bees and mosquitofish. The pre-selected honey bee reference genes are glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein S5 (RS5). Mosquitofish reference genes are Beta Actin 1 (ACTB1) and ribosomal protein L8 (RPL5).

3.3 | qPCR: Data quality assessment

A qualitative way to assess whether amplification has occurred in any given well in a qPCR plate is by examining the amplification plots (**Figure 4.4A**). Generally, successful qPCR reactions display a sigmoidal amplification curve.¹⁹ Seeing an array of sigmoidal curves indicates that the qPCR was successful in most (if not all) of the wells. Two examples of good-quality and poor-quality amplification plots from the students' qPCR experiments are depicted in **Figures 4.4B and 4.4D**, respectively. During the analysis, students are shown how to assess wells for potential contamination and/or nonspecific amplification using the melt curve data (**Figures 4.4C and 4.4E**).²⁰ If a given well has more than one melting point, then there is a possibility that the well was contaminated. Students are encouraged to observe how their results change when including or excluding the data that is deemed untrustworthy by the melt curve analysis. Oftentimes, the wells with multiple melting temperatures have a vastly different cycle threshold (C_T) value than those with a single melting temperature. Including this in the averaged cycle threshold across their three primer sets can alter the results significantly, especially for contaminated wells in control gene plates, as those plates are used for comparison with all of the other plates.

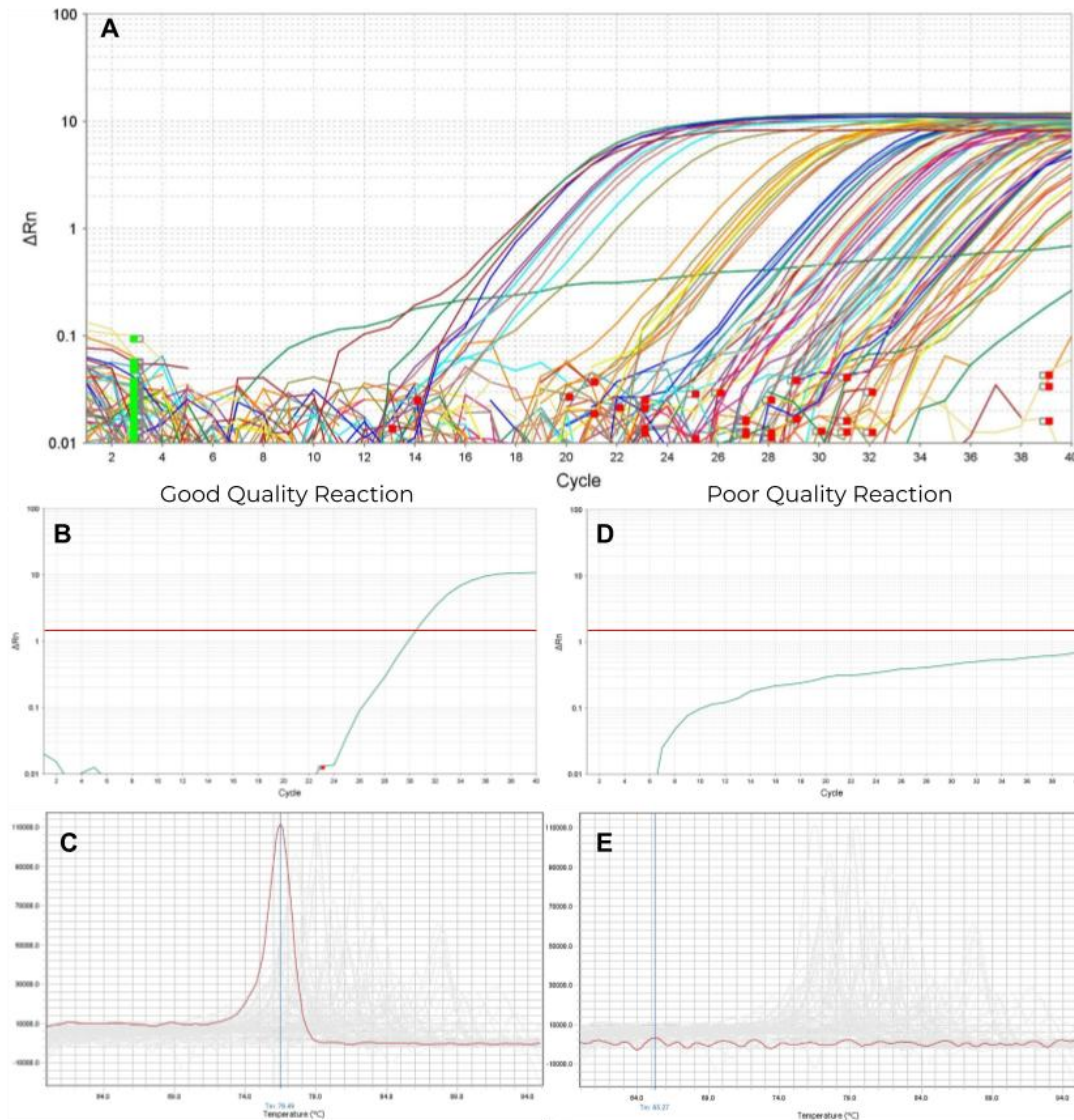


Figure 4.4. Amplification plot of honey bee control samples and examples of good and poor-quality qPCR reactions based on gene amplification and melt curve profiles. These plots were generated by the qPCR machine analyzing the qPCR plate that students loaded using primers they designed and tested with their converted cDNA. (A) Full amplification plots for all genes analyzed in this course shows the variance in expression of different genes. (B) A successful qPCR reaction for gene SOD2, with the signal crossing the threshold value of 1.418 as indicated by the red line; this is validated by (C) the melt curve plot for the same well, which has a single, strong peak. (D) An unsuccessful qPCR reaction for gene SOD2, with the signal failing to cross the threshold value; this is further demonstrated by (E) the melt curve plot for the same well, which has several weak peaks and melting temperatures.

3.4 | qPCR: Data Analysis and Reporting

The final qPCR data is given to students for in-class discussion and analysis. Students are guided through the use of the Livak Method for analyzing the data they obtain.¹⁹ They are taught to identify C_T values, eliminate erroneous data points based on melt curve analysis, and calculate ΔC_T and $\Delta\Delta C_T$ values. The ΔC_T and $\Delta\Delta C_T$ calculations are as follows:

$$\Delta C_T = C_T(\text{chemical defense gene}) - \text{geometric mean of all } C_T(\text{reference gene})$$

$$\Delta\Delta C_T = \Delta C_T(\text{treated sample}) - \Delta C_T(\text{control sample})$$

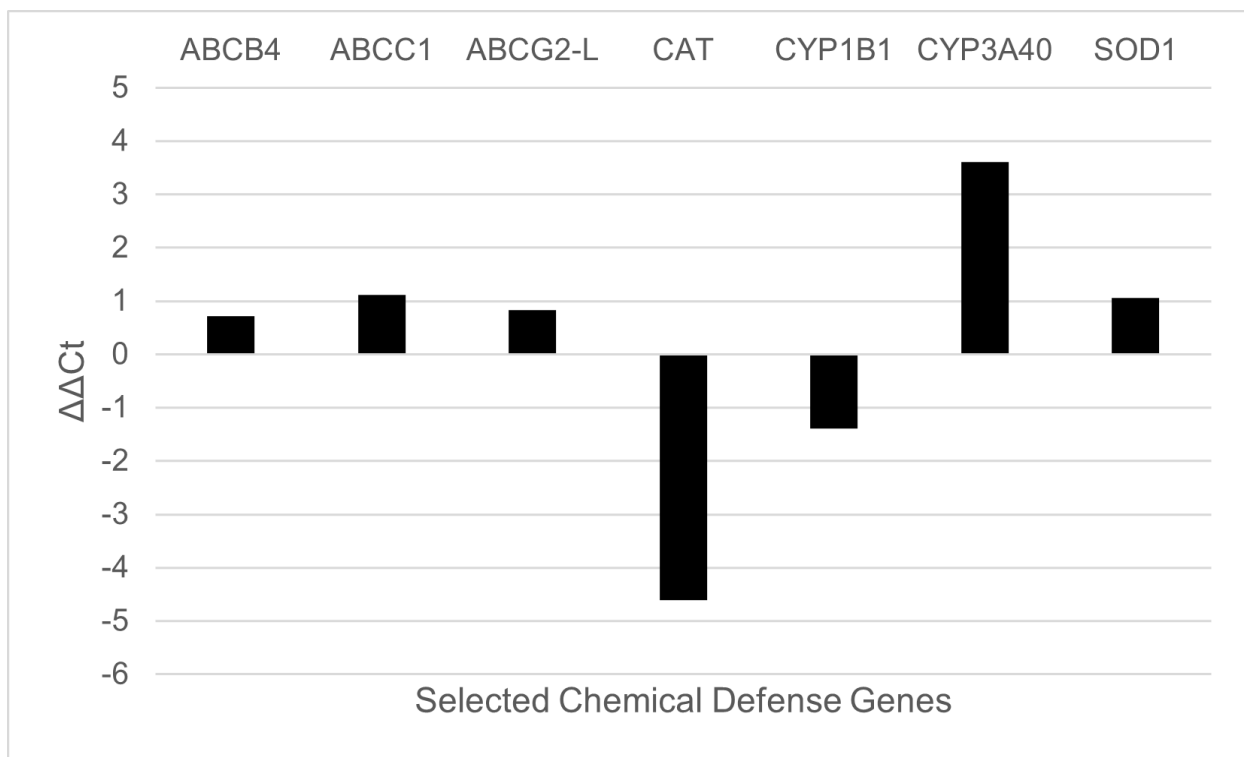


Figure 4.5. An example bar graph displaying the $\Delta\Delta C_T$ values for relative expression of chemical defense genes in exposed versus unexposed mosquitofish. Results are displayed for selected genes of the female wastewater-exposed fish versus the female control fish. Negative $\Delta\Delta C_T$ values indicate relative upregulation of a gene (in the exposed versus unexposed treatments) while positive $\Delta\Delta C_T$ values indicate relative downregulation of a gene. These values are on a logarithmic scale (every time the $\Delta\Delta C_T$ increases by 1, the gene expression doubles). Here, the $\Delta\Delta C_T$ values show a slight downregulation of the ABCB4, ABCC1, ABCG2-L, and SOD1 genes, a slight upregulation of the CYP1B1 gene, a strong downregulation of the CYP3A40 gene, and a strong upregulation of the Catalase gene.

Students are then instructed to find C_T values in their data sheets and to calculate ΔC_T (for comparing the expression of their gene of interest relative to a reference gene), $\Delta\Delta C_T$ (for comparing relative expression of treatment and control organisms), and $2^{-\Delta\Delta C_T}$ (to understand the fold change in expression across treatments).²¹ An example of some of the students' $\Delta\Delta C_T$ values is displayed in **Figure 4.5**.

Student Learning Assessment

All results from this two-week course are reviewed and discussed in an open group discussion session at the end of the second week. The analyzed data from each group is displayed in class for better relative comparison of gene expression profiles, stratified by species, sex, and gene of interest. Students were encouraged to discuss their research project data and relate it to their initial hypothesis. In addition, students can peer-evaluate data from other groups (and previous year data) that could inform their results and help reject or confirm their hypothesis.

4.1 | Intermediate learning assessments via discussions and in-class assignments

Our discussions and in-class assignments help us understand to what extent students are achieving the learning goals presented above. In-class discussions are focused on the first three learning goals. These discussions go in-depth on topics such as detoxification proteins, the PCR process, and qPCR data analysis. They give students an opportunity to share what they have learned and ask questions and gives us an idea of what concepts need further reinforcement. The primary focus of in-class assignments is on the fourth learning goal, bioinformatics, as well as the fifth learning goal, hypothesis testing. The assignments were designed to give students an opportunity to demonstrate their ability to use modern databases and their command of state-of-the-art bioinformatics software in the context of answering their hypotheses. Additionally, these assignments emphasize chemical principles underlying the first three learning goals. For example, the primer design assignment (Assignment 2 in Supporting Information A) has students

practice determining which primers are the highest quality for a qPCR reaction by using software to analyze the thermodynamics of primer byproduct formation reactions and using the resulting free energy values to evaluate a given primer pair. Both discussions and assignments are reviewed by the instructor and TAs when planning lectures and improving the course for future years.

An additional intermediate learning assessment is conducted during the laboratory introduction to determine if students demonstrate proficiency in basic laboratory safety principles and skills (see Supporting Information A). Throughout the course, the instructors examine data from students' successful completion of assignments and their demonstration of conceptual understanding during oral discussions to measure progress toward the learning objectives. To illustrate the effectiveness of the course, some of the student assessment data is provided demonstrating achievement of the specific learning objectives:

- During the final discussion, all (100%) of the students successfully evaluated their hypotheses based on their results, showing the achievement of Learning Objective #2 (*evaluating hypotheses*) and Learning Objective #3 (*applying chemical detoxification knowledge and critical thinking*).
- In the primer design activities, all (100%) students in the 2022 and 81% in 2023 designed successful primers for their assigned genes, indicating progress on Learning Objective #1 (*bioinformatics proficiency*) and Learning Objective #4 (*PCR laboratory skills*).
- During laboratory days, zero safety incidents were registered, and all (100%) students demonstrated their ability to follow laboratory safety guidelines and wear appropriate PPE. Furthermore, a notable improvement in pipetting ability of all (100%) students throughout the course demonstrates achievement of Learning Objective #5 (*lab safety and pipetting skills*).
- During the agarose gel and qPCR activities, all (100%) students included appropriate DNA ladders, controls, and reference genes, satisfying Learning Objective #6 (*understanding the importance of standards and controls*).

4.2 | Summary, interpretation, and discussion of individual results

Students' mosquitofish qPCR was quite successful each of the three times the course has been offered. When comparing female exposed and control mosquitofish, many genes had $\Delta\Delta C_T$ values of roughly ± 1 or ± 2 , indicating minimal differences in exposure across treatments. This was fairly consistent across all mosquitofish comparisons. However, the difference in chemical environment was correlated with changes in two genes—Cytochrome P450 3A40-like (CYP3A40-L) and Catalase. CYP3A40-L had a $\Delta\Delta C_T$ equal to 3.6, indicating that there is a fold change of 0.082, or 12.1 times decrease in expression of this gene relative to the control. On the other hand, Catalase experienced the opposite effect, with a $\Delta\Delta C_T$ value of -4.6, indicating that there is a fold change of 24.3. This would indicate that CYP3A40-L is not related to mosquitofish's defense against wastewater contaminants, while Catalase may play a role.

The C_T values for a given gene from students' qPCR in this class could vary dramatically, leading to inconsistent results. Honey bee results, in particular, experienced high deviation in C_T values and ΔC_T values, especially due to extreme variance in reference gene values. Some genes had differences of 10-15 in C_T values across treatments, with a value for one plate being ~ 20 and others being >30 . This is especially problematic for reference genes, which are supposed to be stable across treatments. Additionally, some results were inconclusive due to a lack of amplification or excessive contamination for all technical replicates of a gene on a given plate. This is an important learning experience for students, since it illustrates how technical errors associated with lab inexperience can lead to poor-quality data that is difficult to salvage for later analysis and interpretation.

4.3 | Hypothesis testing and error analysis

With these varied results, students are tasked with a multifaceted analysis of the complex results of their experiments on the final day of this course. Students are introduced to different sources of error in science, including systematic, random, and personal errors, and are instructed to consider these while evaluating their hypothesis.²² Additionally, students are asked to come up with scientific reasons that could

have led to their inconsistent results or results that disagree with their initial hypothesis. For example, some of the more inconsistent results may have been caused by operator error (a thorough discussion of the prominence of error in this kind of teaching laboratory is discussed below), but a variety of reasons may be responsible for causing downregulation of a certain gene instead of upregulation. Students came up with thoughtful explanations for some of their research project results, such as:

- *“Lower expression of a certain gene in exposed bees may be due to that gene not being involved in the defense against that particular chemical. It is then downregulated so other genes can be upregulated.”*
- *“Higher expression of a certain gene in female fish over male fish may be due to the female fish being pregnant and needing more of that gene to help protect her young.”*
- *“Variance in gene expression could be due to sample differences, such as non-homogenous exposure to a certain chemical.”*

4.4 | Self-reflection and troubleshooting

As an additional exercise incorporated into the final discussion, students were asked what steps could be taken to improve results were the experiment to be repeated. This was an opportunity for students to demonstrate their knowledge of the experimental process and what factors impact the quality of the results. Students were encouraged to apply the concepts they had learned in lecture to this reflection. Students brought up various potential improvements, such as refreshing ice baths more often to maintain temperature-sensitive reagents during long lab days and improving the organization of qPCR workflow to minimize chances of user error. Students also explained how they would avoid errors by applying their knowledge from lectures and discussions to their project. Some of the students’ main reflections and troubleshooting ideas were:

- *“Do a test run of all installed software for this course on my computer/tablet to make sure that it will start.”*
- *“Practice pipetting of small volumes to get more familiar with it before doing the real samples.”*

- *“Attend an Excel software crash course to practice basic spreadsheet functions and formulas.”*

4.5 | Exit questionnaire

Students were provided with the opportunity to give critical feedback for improvement of the course upon completion. In addition to gaining an understanding of how much students enjoyed the course, this exit survey allowed us to better understand what big-picture takeaways the students took from the course, and how the course influenced them as budding scientists. Details on the questions asked in the survey are provided in the Supporting Information B.

Educational Impacts

5.1 | College and career preparation

One of the great strengths of this course is that it gives students a broad introduction to environmental toxicology as a field and illuminates the different career paths that stem from a degree in environmental toxicology. In addition to completing the main project of the class, which focuses on chemical detoxification and qPCR applications, we invited several other faculty members involved in toxicology research as guest lecturers, took the students on field trips to other environmental toxicology laboratories and testing facilities, and included an hour-long lecture dedicated to toxicology career paths at the end of the course. The goal of these activities is to help students think about their newfound knowledge and interests, give them an idea of other types of toxicological research on and off campus, and gain new perspectives on the different careers a toxicologist can pursue. Additionally, students are encouraged to ask questions about their specific interests so we can provide personalized advice for finding the right field for them.

5.2 | Software and bioinformatics proficiency

Computer proficiency and the ability to use and troubleshoot software is vital even for students not planning on going into toxicology research. Many students entered the class not having a working knowledge of basic computer functions, such as navigating through files and using Microsoft Office. Through this class, students can practice their basic computer skills while applying them to a field they are interested in and learning advanced skills in software usage and database proficiency. Students were much more comfortable using programs like MS Excel and PowerPoint by the end of the course and left having software skills transferable to other scientific fields.

5.3 | Interdisciplinary critical scientific thinking

Students gained an advanced understanding of the scientific methods and techniques applied in environmental toxicology through this course. We emphasized forming hypotheses, testing them in the lab, and coming up with conclusions based on their obtained results. By the end of the research project, students were not only able to arrive at conclusions but also think of alternative explanations for their results and create new scientific questions based on them.

Common Pitfalls and Practical Challenges

6.1 | User and Technical Errors

The most prominent sources of error in the experiments described above revolve around students lacking laboratory experience. Even in college, undergraduate students often struggle with pipetting as a skill and keeping track of which reagents go in which tubes.²³ This is one of the biggest challenges of teaching the wet lab part of this course. As such, at the beginning of the second module, before any experiments begin, students are introduced to laboratory safety and proper micropipette operation, including an opportunity to practice pipetting and weighing water samples. During this time, students receive one-on-one help with their pipetting by the TAs so that they can understand basic pipet operation,

such as first and second stops, as well as proper form and hand positioning for maximum accuracy. Nevertheless, developing mastery in pipetting technique comes from practice over time.

Additionally, PCR and qPCR are organizationally challenging protocols to run. Because of this, instructors pipette especially sensitive and/or expensive reagents. Additionally, all reagents and buffers used in the lab course are previously tested for contamination and expiration by the TAs. With the students doing most of the pipetting for cDNA conversion, primer test PCR, and qPCR, many errors still occur, including adding incorrect volumes and forgetting to add primers or template cDNA. Some of these errors are weeded out through the primer test PCR and melting curve analysis, but even the need to remove some of these data points decreases the overall data quality. Despite the course's focus on quality assessment, we recognize that qPCR is difficult to master, and errors will occur while learning it. With this in mind, we emphasize student improvement in technical laboratory skills over the course's duration in addition to the quality of their data. For young adults enthusiastic about a potential career in science, this is arguably more important than having consistent results. That said, at least one of the samples of every student's qPCR plate ran successfully in our courses and students were able to perform a complete data analysis.

6.2 | Limited Knowledge in Fundamental Science

Another significant challenge comes from the lack of background coursework in molecular biology and biochemistry. Environmental toxicology is conceptually challenging, and most students have limited background in this field. Surveying students showed varying levels of experience in biology and chemistry, the two most important courses in secondary education for understanding this course's content. Some students had yet to take a chemistry course in high school, and others had not taken any biology course. This proved to be an immense challenge since PCR and qPCR requires significant background knowledge to understand. It is also where TAs come into play; while the instructor can focus on teaching about environmental toxicology and PCR, TAs can help students grasp new and confusing terms so they

understand fundamental biological and chemical concepts. The emphasis on teamwork also allows students to help fill in each other's gaps in knowledge.

Conclusion

This two-module course provides motivated and advanced high school students with an introduction to environmental toxicology through the lens of gene expression and molecular biology. The core of the course is the execution of a complete qPCR experiment from start to finish, taking students through two modules: a dry lab focusing on understanding gene expression and designing primers through modern databases and software, and a wet lab focusing on carrying out the steps of a qPCR reaction, including cDNA conversion, primer test PCR, qPCR, and qPCR data analysis. Through the course, students gain a thorough understanding of molecular biology techniques for application in environmental toxicology and develop important laboratory skills. The course is designed to encourage students to work hard, challenge them with difficult content, and be an enjoyable experience that motivates them to pursue their scientific interests. As a pre-college class, it gives students a taste of university-level science research and prepares them for the rigors of college-level scientific coursework.

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Supplemental Data

Supplemental Information A & B can be found in: *Spooner ZT, Encerrado-Manriquez AM, Truong TT, Nicklisch SCT. From primers to pipettes: An immersive course introducing high school students to qPCR for quantifying chemical defense gene expression. Biochem Mol Biol Educ. 2024;52(6):633–647. <https://doi.org/10.1002/bmb.21851>*

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