**UNIVERSIDADE FEDERAL DE VIÇOSA**

# Toxicity of the fungicide difenoconazole and the herbicide tebuthiuron on honey bees and stingless bees across exposure pathways and multiple endpoints

Iury Dias de Oliveira
*Magister Scientiae*

**VIÇOSA - MINAS GERAIS**
**2025**

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Dissertation submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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**VIÇOSA - MINAS GERAIS**
**2025**

**Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa**

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2025

Oliveira, Iury Dias de, 1999-
Toxicity of the fungicide difenoconazole and the herbicide tebuthiuron on honey bees and stingless bees across exposure pathways and multiple endpoints / Iury Dias de Oliveira. – Viçosa, MG, 2025.
1 dissertação eletrônica (44 f.): il. (algumas color.).

Texto em inglês.
Orientador: Gustavo Ferreira Martins.
Dissertação (mestrado) - Universidade Federal de Viçosa, Departamento de Entomologia, 2025.
Referências bibliográficas: f. 39-44.
DOI: https://doi.org/10.47328/ufvbbt.2025.699
Modo de acesso: World Wide Web.

1. Toxicologia ambiental. 2. Abelhas. 3. Fungicidas. 4. Herbicidas. 5. Produtos químicos agrícolas - Aspectos ambientais. I. Martins, Gustavo Ferreira, 1980-. II. Universidade Federal de Viçosa. Departamento de Entomologia. Programa de Pós-Graduação em Entomologia. III. Título.

CDD 22. ed. 571.95

Bibliotecário(a) responsável: Bruna Silva CRB-6/2552

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APPROVED: July 28, 2025.

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## ACKNOWLEDGMENTS

This work has been sponsored by the following Brazilian research agencies: Coordination for the Improvement of Higher Education Personnel (CAPES; Financing code 001), Minas Gerais State Foundation for Research Aid (FAPEMIG) and National Council of Scientific and Technological Development (CNPq).

To my advisor, Gustavo, for welcoming me into his lab, trusting my independence, and always being available to discuss ideas. To my co-advisor, Lisbetd, for her patient guidance, motivation, and friendship.

To the Laboratory of Molecular Biology of Insects, for fostering a stimulating and supportive environment. To all my wonderful friends, your laughter, encouragement and companionship made this journey meaningful.

To my friends Álvaro, Dayvson, João Paulo, João Victor, Julia, and Matheus, for making this journey easier and more pleasant. I would also like to extend a special thanks to Letícia, whose encouragement redirected my academic path and whose friendship kept me grounded.

To my parents, Ilda and José Sebastião, for their sacrifices to ensure my education and the values they taught me. And also, to my siblings, Aline, Igor, and Sabrina, for their endless support and love.

## ABSTRACT

OLIVEIRA, Iury Dias de, M.Sc., Universidade Federal de Viçosa, July, 2025. **Toxicity of the fungicide difenoconazole and the herbicide tebuthiuron on honey bees and stingless bees across exposure pathways and multiple endpoints**. Adviser: Gustavo Ferreira Martins. Co-adviser: Lorena Lisbetd Botina Jojoa.

Bees are key pollinators of native flora and agricultural crops, contributing significantly to global food security and ecosystem functioning. The extensive application of agrochemicals has been associated with population declines in multiple bee species in recent decades. While bee diversity is considerable, toxicological research has largely centered on the honeybee (Apis mellifera), resulting in limited understanding of the ecotoxicological effects on other bees, like stingless bees (Meliponini). This study aimed to evaluate the effects of the agrochemicals difenoconazole and tebuthiuron exposure on different parameters on bees, including the Africanized honey bee A. mellifera and the native stingless bees Scaptotrigona xanthotricha and Partamona helleri. Oral and topical exposure were performed, followed by assessments of mortality, food consumption, oxidative stress, through biochemical markers, and histological analyses of the midgut to better understand the impact of these compounds on pollinator health and physiology. Tebuthiuron reduced P. helleri survival both oral and topical exposure. A. mellifera showed reduced diet intake during exposure to tebuthiuron via both exposure routes. However, no change in intake was observed after six hours after topical exposure to tebuthiuron or during the six ours-oral exposure to difenoconazole. P. helleri exhibited reduced diet intake only after oral exposure to tebuthiuron, but showed increased intake following exposure to difenoconazole. In contrast, S. xanthotricha demonstrated increased diet intake after both oral and topical exposure to difenoconazole, although no increase was observed during the initial six hours-oral exposure. Dose-response relationships followed power functions, with tebuthiuron consistently showing higher baseline toxicity but shallower slopes than difenoconazole. Enzyme-activity markers exhibited exposure-dependent responses. In A. mellifera, catalase (CAT) decreased after both exposures, while superoxide dismutase (SOD) and glutathione-S-transferase (GST) decreased after topical exposure. S. xanthotricha showed increased SOD and CAT but decreased GST after oral exposure; topical exposure increased SOD and decreased GST. P. helleri had increased SOD levels after oral exposure and elevated Malondialdehyde (MDA) and reduced nitric oxide after topical exposure. Midgut morphology was altered only in P. helleri, with tebuthiuron

reducing microvilli height and both agrochemicals increasing nuclear area. Our study reveals striking interspecific differences in pesticide sensitivity among bees. Feeding behavior and biochemical markers further highlighted species-specific responses. A. mellifera displayed pesticide avoidance for tebuthiuron and CAT suppression, whereas S. xanthotricha upregulated detoxification enzymes. P. helleri suffered midgut damage, underscoring its risk. These findings challenge the use of A. mellifera as the sole model for pesticide risk assessment, particularly for Neotropical stingless bees. The results highlight the importance of incorporating multiple variables such as different exposure routes, concentrations, and species-specific responses, in agrochemical risk assessments to evaluate their effects on pollinators, including native species.

Keywords: ecotoxicology; bees; sublethal effects; fungicide; herbicide; agrochemicals

## RESUMO

OLIVEIRA, Iury Dias de, M.Sc., Universidade Federal de Viçosa, julho de 2025. **Toxicidade do fungicida difenoconazol e do herbicida tebutiuron nas abelhas melíferas e nas abelhas sem ferrão após as exposições oral e tópica**. Orientador: Gustavo Ferreira Martins. Coorientadora: Lorena Lisbetd Botina Jojoa.

As abelhas são polinizadores fundamentais da flora nativa e das culturas agrícolas, contribuindo significativamente para a segurança alimentar e para o funcionamento dos ecossistemas. A aplicação extensiva de agroquímicos tem sido associada ao declínio populacional de várias espécies de abelhas nas últimas décadas. Embora a diversidade de abelhas seja considerável, a investigação toxicológica é centrada na abelha melífera (Apis mellifera), resultando numa compreensão limitada dos efeitos ecotoxicológicos em outras abelhas, incluindo as sem ferrão (Meliponini). Este trabalho teve como objetivo avaliar os efeitos da exposição (tópica ou oral) dos agroquímicos difenoconazol e tebutiuron em diferentes parâmetros em abelhas, incluindo A. mellifera e as abelhas nativas Partamona helleri e Scaptotrigona xanthotricha. Foram efetuadas exposições orais e tópicas, seguidas de avaliações de mortalidade, quantidade de ingestão de dieta, de atividade de enzimas responsivas a estresse e morfologia do intestino médio para compreender o impacto dos dois ingredientes ativos na saúde e fisiologia das abelhas. O tebutiuron reduziu a sobrevivência de P. helleri tanto por via oral como tópica. A. mellifera apresentou uma redução na ingestão de alimentos durante a exposição ao tebutiuron por ambas as vias de exposição. No entanto, não foram observadas tais alterações seis horas após a exposição tópica ao tebutiuron ou durante as seis horas de exposição oral ao difenoconazol P. helleri apresentou uma redução da ingestão apenas após a exposição oral ao tebutiuron, mas registrou um aumento da ingestão após a exposição ao difenoconazol. Em contrapartida, S. xanthotricha ingeriu mais dieta após exposição oral e tópica ao difenoconazol, embora não tenha sido observado qualquer aumento de ingestão durante as seis horas iniciais de exposição oral. As relações dose-resposta seguiram funções de potência, com o tebutiuron apresentando uma toxicidade de base mais elevada, mas declives menos acentuados do que o difenoconazol. Os marcadores de atividade enzimática apresentaram respostas dependentes da exposição. Em A. mellifera, a atividade da catalase (CAT) diminuiu após ambas as exposições, enquanto as atividades da superóxido dismutase (SOD) e a glutationa-S-transferase (GST) diminuíram após a exposição tópica. S. xanthotricha apresentou aumento de atividade de SOD e CAT e uma diminuição da atividade de GST após exposição oral. A exposição

tópica aumentou a atividade de SOD e diminuiu a de GST. P. helleri registou um aumento dos níveis de SOD após exposição oral e um aumento do malondialdeído (MDA) e uma redução dos níveis de óxido nítrico após exposição tópica. A morfologia do intestino médio foi alterada apenas em P. helleri, com o tebutiuron reduzindo a altura das microvilosidades. Porém, a exposição a ambos agroquímicos aumentou a área nuclear das células digestivas. Nosso estudo revela diferenças interespecíficas marcantes na sensibilidade aos agroquímicos entre as abelhas. O comportamento alimentar e os marcadores bioquímicos evidenciaram ainda mais as respostas específicas de cada espécie. A. mellifera reduziu a ingestão de dieta contaminada com tebutiuron e supressão da CAT, enquanto a S. xanthotricha aumentou a regulação das enzimas de desintoxicação. P. helleri sofreu danos no intestino médio, evidenciando o seu risco. Estes resultados mostram as limitações quanto à utilização de A. mellifera como único modelo para a avaliação de risco a agroquímicos, particularmente para as abelhas sem ferrão. Os resultados reforçam a importância de incorporar múltiplas variáveis, como diferentes rotas de exposição, concentrações e respostas específicas de espécies, nas avaliações de risco para avaliar com mais precisão os efeitos dos agroquímicos nos polinizadores, incluindo espécies nativas.

Palavras-chave: ecotoxicologia; abelhas; efeitos subletais; fungicida; herbicida; agroquímicos

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# 1. INTRODUCTION

Global studies reveal alarming declines in insect populations. Due to habitat loss, agrochemicals, and climate change, some regions have lost over 75% of biomass in recent decades, threatening ecosystems and pollination services (Wagner, 2019). This biodiversity crisis is particularly evident in bee populations. Since the 1990s, global data show a 25% reduction in bee species richness, highlighting the urgent need for conservation efforts (Zattara & Aizen, 2021). Bee decline is especially concerning due to its cascading effects on plant reproduction. Evidence suggests that wild plants and their bee pollinators are declining in parallel (Goulson et al., 2015; Grab et al., 2019; Nottebrok et al., 2022). Intensive agriculture is a major driver of this decline, disrupting ecosystems and the critical services bees provide. Furthermore, pollination services and pollinator populations are dwindling in biodiversity hotspots and endangered biomes, exacerbating ecological instability (Vamosi et al., 2006; Brosi, 2009; Brown & Oliveira, 2014; Carvalho & Del Lama, 2015).

The intensive use of pesticides has emerged as a critical environmental concern, with concentrations lethal to target pests frequently causing unintended ecological impacts on non-target species (Desneux et al., 2007; Guedes et al., 2016). While residues degrade over time, their persistence in ecosystems leads to prolonged sublethal exposure across species (Guedes et al., 2016). In bees, this exposure disrupts antioxidant defenses and elevates reactive oxygen species (Bernardes et al., 2021a; Farder-Gomes et al., 2021a,b). These effects are exacerbated by synergistic interactions in pesticide mixtures, where even low fungicide concentrations create disproportionate risks that often evade regulatory scrutiny (Brigante et al., 2021).

Pollinators face multiple exposure routes to agrochemicals, creating complex risks to their populations. Contamination occurs through direct contact with treated plants and soil, as well as indirect pathways including pesticide-laden pollen and nectar, spray drift, contaminated water sources, and residues in hive materials (Rondeau & Raine, 2021). Exposure risk is shaped by several interacting factors: the persistence, species-specific nesting behaviors and foraging patterns, and the application timing (Schmolke et al., 2022). These multifaceted exposure routes combine to create significant threats to pollinator health across agricultural landscapes.

Although insecticides have long been identified as major contributors to pollinator decline, recent research has broadened this perspective to encompass a wider range of agrochemicals (Bernardes et al., 2022; Botina et al., 2024). Emerging evidence points to the harmful effects of other groups including fungicides, herbicides, agrochemicals of natural origin, adjuvants, and fertilizers, as well as the compounded risks associated with chemical mixtures and their potential synergistic interactions (Johnson, 2015; Mullin et al., 2015;

Rodrigues et al., 2016).

Difenoconazole, a systemic triazole fungicide, is widely used in agriculture due to its broad-spectrum efficacy, targeting ergosterol biosynthesis in fungi (FRAC, 2024; Vanden et al., 1990; Buchenauer, 1995). With global sales reaching $553 million in 2021 (Agrochemical Information Net, 2024), its persistence in the environment is concerning: residues endure in soil for up to 1,000 days (Thom et al., 1997; Man et al., 2021). Difenoconazole have been detected in water (2.91 mg/L), air (430 ng/m³), and food crops (4.74 mg/kg) (Pan et al., 2019; Dong and Hu, 2023; Fang et al., 2024). Studies highlight its toxicological risks, including oxidative stress, endocrine disruption, and metabolic disturbances in non-target species (Jiang et al., 2021; Li et al., 2022a). As difenoconazole residues accumulate in flowers and pollen (up to 456 ng/g in strawberries), impairing honey bee health through sublethal effects like reduced body weight and altered detoxification genes (Han et al., 2023; Burgarelli et al., 2023). Regardless its widespread detection and ecological persistence, gaps remain in understanding its effects on diverse pollinator species (Araújo, 2025).

Another important pesticide used in Brazil is Tebuthiuron, a leading phenylurea herbicide, is extensively used to control broadleaf and grassy weeds in sugarcane cultivation. However, its high-water solubility (2.50 g $L^{-1}$) facilitates rapid environmental dispersion, increasing the risk of ecosystem contamination. As a result, once leached into surrounding environments, tebuthiuron residues persist and accumulate, exposing non-target organisms to xenobiotic. This residual carryover can lead to bioaccumulation through food chains, ultimately threatening biodiversity and ecosystem stability (Qian et al., 2017; Almeida et al., 2018; Jonsson et al., 2019; Sarıgöl Kılıç et al., 2018; Kapsi et al., 2019).

Stingless bees (Meliponini) represent one of the most significant bee groups in the Neotropics in terms of ecology and agriculture. With diverse colony sizes ranging from a few dozen to thousands of workers, comprising over 460 species in this biogeographic region (Camargo & Pedro, 2013).; Nogueira, 2023) and over 550 species globally (Ascher & Pickering, 2025). These pollinators play a vital role in tropical ecosystems, enhancing crop yields and maintaining native plant biodiversity (Giannini et al., 2015a; Kremen et al., 2002; Barbosa et al., 2015a). Certain species, such as *Trigona spinipes* (Fabricius, 1793) (Hymenoptera: Apidae: Meliponini), are particularly valuable for pollination in degraded habitats (Jaffé et al., 2016). However, despite their ecological importance an increasing number of species facing endangerment, though data gaps often obscure their precise extinction risks (ICMBIO, 2014).

Like honey bees, stingless bees are vulnerable to agrochemicals through oral and

topical routes, whether via soil absorption and systemic translocation or aerial spraying, which can contaminate pollen and nectar. Notably, the impacts of these chemicals extend beyond acute lethality to sublethal effects, such as impaired foraging, reproduction, and colony health, which are often overlooked in risk assessments despite their profound ecological consequences (Desneux et al., 2007; Johnson, 2015; Guedes et al., 2016). This underscores the urgent need to evaluate the full spectrum of agrochemical threats to stingless bees, particularly in tropical regions where their conservation is pivotal to biodiversity and food security. The situation is particularly concerning in Brazil, which accounts for approximately 20% of global pesticide consumption, the highest worldwide (dos Santos et al., 2018; Oliveira et al., 2023).

Stingless bees play a crucial role as pollinators across Neotropical ecosystems, demonstrating particular importance for key plant families. They frequently visit species from key botanical families such as Sapindaceae (42% of genera), Arecaceae (43%), and Myrtaceae (52%), which include ecologically dominant trees and economically important fruit species (Bueno et al., 2023). Consequently, their pollination services are vital for several mass-blooming crops, including coffee (*Coffea* spp.), mango (*Mangifera* spp.), and passion fruit (*Passiflora* spp.). This broad visitation pattern underscores the potential to integrate stingless bees into local pollination management programs worldwide, either through meliponiculture or targeted assisted pollination. These findings highlight stingless bees as both ecologically significant pollinators and valuable agricultural allies. Consequently, this suggests important opportunities for their conservation and utilization in sustainable farming practices across tropical and subtropical regions.

*Partamona helleri* (Friese, 1900) and *Scaptotrigona xanthotricha* (Moure, 1950) are two species that represent this tribe in Brazil and have an important role in pollination of native species and wide distribution. For example, cotton, cashew, beans, and sunflower are commonly visited by *P. helleri* (MouraMoraes et al. 2021). *S. xanthotricha* has one of the widest geographic distributions among the Brazilian species of its genus, occurring in areas of Atlantic Rainforest and ranging from the northeastern to southern Brazil (Michener 2000; Camargo and Pedro 2012). Both species occur in Brazilian states such as Bahia, Espírito Santo, Minas Gerais, Paraná, Rio de Janeiro, Santa Catarina, and São Paulo (Nogueira, 2023).

The present study aims to generate species-specific toxicity data to assess whether *Apis mellifera* can serve as a suitable surrogate for predicting the ecological risk to Neotropical stingless bees when exposed to two different agrochemicals separately. We investigated the sublethal effects of difenoconazole and tebuthiuron (active ingredients) on *A. mellifera* compared to two native Brazilian stingless bee species, *P. helleri* and *S.*

*xanthotricha*. We hypothesized that the exotic *A. mellifera* would exhibit greater resilience compared to the native species, and that the physiological responses would be contingent upon the exposure pathway (oral vs. topical). Forager bees were exposed both orally and topically to field-recommended doses (75 mg/L for difenoconazole and 285 mg/L for tebuthiuron) as well as two dilutions (1/10 and 1/100). Assessments included survival probability, food consumption, enzymatic activity, and midgut histological analysis. Such research is fundamental for developing more inclusive pesticide regulation policies and ensuring the long-term sustainability of pollination services in tropical ecosystems.

## 2. GENERAL OBJECTIVE

This study investigated the ecotoxicological effects of the fungicide difenoconazole and the herbicide tebuthiuron on the exotic honey bee *A. mellifera* and the native stingless bees *P. helleri* and *S. xanthotricha* through oral and topical exposure pathways, to assess species-specific sensitivities and refine pesticide risk assessment for pollinators.

### 2.1 SPECIFIC OBJECTIVES

- Compare survival rates among the three species following oral or topical exposure to difenoconazole or tebuthiuron at varying concentrations.
- Analyze alterations in diet intake in response to exposure over short (0–6 h) and long (6–96 h) intervals.
- Quantify biochemical markers related to oxidative stress (SOD, CAT, MDA), detoxification (GST), and nitrosative stress (NO) to determine species and route-specific physiological disruptions.
- Evaluate morphological changes on midgut as indicators of sublethal toxicity.

## 3. MATERIALS AND METHODS

### 3.1 AGROCHEMICALS

Bees were exposed topically or orally to the active ingredients of the fungicide difenoconazole (Pestanal TM, Sigma-Aldrich Corp., St. Louis, MO, USA) or the herbicide tebuthiuron (SigmaAldrich, São Paulo, SP, Brazil). The doses for both exposure methods were determined based on the commercial formulation guidelines provided by CCAB AGRO S.A. (São Paulo, SP, Brazil), using the maximum recommended field application rate for difenoconazole (75 mg a.i. $L^{-1}$) and for tebuthiuron (285 mg a.i. $L^{-1}$).

Stock solutions were prepared separately for oral and topical exposures. For oral administration, difenoconazole (6 μL) or tebuthiuron (228 μL) was diluted in 4.994 mL or 4.772

mL respectively of a 1:1 sucrose solution (Êxodo Científica, Sumaré, SP, Brazil) and deionized water mixture to create the 1X concentration. For topical application, difenoconazole (3.6 µL) or tebuthiuron (91.42 µL) was first diluted in 2.996 µL or 1.908 µL of $H_2O$ respectively, then mixed with acetone (2,500 µL for difenoconazole or 1,500 µL for tebuthiuron) to achieve the 1X dose. These stock solutions represented the 1X dose, from which 10X and 100X concentrations were subsequently prepared.

### 3.2 BEES

Toxicity assays were conducted with permission from the Chico Mendes Institute for Biodiversity Conservation (SISBIO, ID 85749), using foragers from two species of stingless bees (Meliponini) and *Apis mellifera* (Apini). The bees were collected from five distinct locations within the Federal University of Viçosa (UFV), as described in (UFV, MG, 20°45'38.8"S 42°52'12.7"W; the Central Apiary of UFV, Viçosa, MG, 20°45'31.0"S 42°52'05.6"W). Additionally, bees were collected in the municipality of Coimbra, MG, at the Sossego Condominium (20°48'58.5"S 42°49'53.9"W). Bees from three colonies of each species were collected at the nest entrances using glass vials. Subsequently, the bees were transported to the UFV Insect Molecular Biology Laboratory and housed in Biochemical Oxygen Demand (B.O.D.) incubators under controlled temperature and complete darkness. The temperature was adjusted according to the specific requirements of each species, as found in the literature, 32±1 °C for *A. mellifera* and 28±1 °C for *P. helleri* and *S. xanthotricha*.

Before exposure, bees from the same colony were anesthetized with carbon dioxide ($CO_2$) and grouped into 500 mL plastic containers (for bees weighing over 70 mg) or 250 mL containers (for bees weighing under 70 mg). The containers were pre-prepared following the methodology described in Botina et al. (2020). All bee species were subjected to serial dilutions of agrochemicals, and doses were determined through mortality bioassays. Each treatment consisted of three replicates (three colonies), each pot containing 10 bees from same colonies.

The bees were fasted for one hour to stimulate the consumption of the contaminated diet (Botina et al. 2020). The diet was provided during the experiment using a 2 µL centrifuge microtube with a perforated cap, fitted into a hole (~13 mm in diameter) at the bottom of the plastic container. Species identification was based on nest characteristics, including the entrance, as well as the phenotype of worker bees (Menezes et al. 2023).

### 3.3 TOXICITY ASSAYS

Exposure to the uncontaminated sucrose solution was used to assess natural mortality (control). Mortality was evaluated 24 hours after exposure (6 hours of agrochemical exposure + 18 hours after replacing the agrochemical-laced food) and monitored daily until 96 hours.

Completely immobile insects were considered dead.

Diet consumption was measured by weighing the feeder containing the contaminated diet before and after exposure using a precision scale (AG 200 – Gehaka, São Paulo, Brazil). To calculate the average food consumption per bee, three plastic containers with feeders containing the sucrose solution, but without bees, were placed in the incubator during the experiment to estimate the evaporation rate. This evaporation rate was then subtracted from the bees' diet consumption rate to determine the average food consumption per bee (Botina et al., 2020).

### 3.3.1 ORAL EXPOSURE

For oral exposure, the insecticides were diluted in a sucrose solution. Preliminary tests were conducted to establish a mortality range between 1% and 100%. Each dilution of the agrochemicals was provided for 6 h, while the control group received only the sucrose solution throughout the experiment. Food consumption was estimated for both the treated and control groups by weighing the feeder before and after exposure. After the 6-hour exposure, the feeder was re-weighed and replaced with one containing sucrose solution (without insecticide), which was made available *ad libitum* until the end of the experiment. The dose per bee was calculated based on the average insecticide consumption per bee.

### 3.3.2 TOPICAL EXPOSURE

The insecticide solution was applied to the dorsal thorax of each bee using a micropipette with a maximum volume of 10 µL. The volume administered varied depending on the body size of each species (1 µL for *P. helleri* and *S. xanthotricha* and 2 µL for *A. mellifera*). The control group received only acetone. Throughout the experiment, the bees were provided with a 50% sucrose solution as food (Ribas et al. 2024). Bee survival was monitored daily for up to 96 hours.

## 3.4 MIDGUT MORPHOLOGY

Bees that were orally exposed to difenoconazole and tebuthiuron (not previously used in other assays; n = 10 per species) or to the control diet (n = 5 ) were dissected in an insect saline solution (0.1 M NaCl + 0.2 M $KH_2PO_4$ + 0.2 M $Na_2HPO_4$) with a pH of 7.2. The midguts were fixed in Zamboni's fixative solution (2% paraformaldehyde + 15% picric acid in 0.1 M sodium phosphate buffer [PBS]) for 90 minutes, followed by three washes (of 10 min) in PBS.

The midguts were then dehydrated through a graded ethanol series (70%, 80%, 90%, 95%, and 99%) for 10 minutes at each concentration. After dehydration, the samples were embedded in glycol methacrylate historesin (Leica; Heidelberg, BW, Germany) and sectioned into 5 µm thick slices using a Reichert-Jung 2050 rotary microtome (Germany). The sections

were stained with hematoxylin for 15 minutes and eosin for 1 minute, and then analyzed and photographed under a light microscope (Olympus BX60). For this analysis, a total of 45 bees (5 bees × 3 treatments × 3 colonies) per species were used.

### 3.5 Enzyme activity

The selection of biochemical markers was based on their established roles in the detoxification and oxidative stress pathways in bees. Superoxide dismutase (SOD) and catalase (CAT) were chosen as they constitute the primary enzymatic antioxidant defense, responsible for neutralizing superoxide radicals and hydrogen peroxide, respectively (Liu et al. 2025). Glutathione S-transferase (GST) was selected for its critical function in Phase II detoxification, facilitating the conjugation and excretion of xenobiotics (Berenbaum and Johnson, 2015; du Rand et al., 2015). To directly assess the consequent oxidative damage, malondialdehyde (MDA) levels were measured as a reliable indicator of lipid peroxidation (Liu et al. 2025). Finally, nitric oxide (NO) was quantified due to its dual role in cellular signaling and potential to contribute to nitrosative stress (Bartling et al. 2021). Together, this panel provides a comprehensive assessment of the physiological impacts of pesticide exposure.

For each treatment, 10 bees (orally or topical exposed) were individually transferred to 2 mL tubes containing 0,5 mL of PBS (0.1 M; pH 7.6) and homogenized whole under ice-cold conditions using a tissue homogenizer (BioSpec 985370–04 Tissue-Tearor, US). The homogenates were then centrifuged at 10,000 × g for 10 min at 4 °C (Eppendorf Centrifuge 5418 R, Eppendorf AG, Hamburg, Germany), and the resulting supernatant was collected and stored at −80 °C, in triplicate, for subsequent biochemical analyses, including antioxidant enzyme activities, lipid peroxidation, and NO levels.

Superoxide dismutase (SOD) activity was evaluated based on the enzyme's ability to catalyze the dismutation of superoxide (O2−) into $H_2O_2$ and $O_2$, following the pyrogallol autoxidation method (Marlund and Marlund 1974). The reaction mixture included 10 µL of the supernatant, 170 µL of PBS (50 mM, pH 7.8), and 20 µL of pyrogallol (100 µmol/L). Absorbance was measured at 320 nm after a 30-min incubation using a microplate spectrophotometer (Thermo Scientific—Multiskan GO).

Catalase (CAT) activity was determined by monitoring the decomposition of $H_2O_2$ into O2 and $H_2O$ (Hadwan and Abed 2016). The reaction mixture consisted of 10 µL of the supernatant and 190 µL of the substrate solution (50 mM PBS, pH 7.4, 20 mM $H_2O_2$). After a 3-min incubation, the reaction was halted with an ammonium molybdate solution (32.4 mmol/L), and absorbance was measured at 374 nm.

Glutathione S-transferase (GST) activity was assessed based on thioester formation (Habig et al. 1974). The reaction mixture contained 5 µL of the supernatant, 485 µL of PBS (50 mM, pH 7.4), 5 µL of reduced glutathione (0.1 M), and 5 µL of 1-chloro-2,4-dinitrobenzene (CDNB, 0.1 M). The increase in absorbance at 340 nm was monitored over 90 s. Activities of SOD, CAT, and GST were expressed in U/mg.

Lipid peroxidation was measured by quantifying malondialdehyde (MDA) levels, using thiobarbituric acid reactive substances (TBARS) (Buege and Aust 1978). The reaction mixture included trichloroacetic acid (15%), thiobarbituric acid (0.375%), and HCl (0.25 M) combined with the sample supernatant. TBARS formation was detected spectrophotometrically at 535 nm, and MDA levels were calculated using the molar extinction coefficient of TBA ($1.56 \times 10–5$ M cm−1), expressed as µmol/mg.

Nitric oxide (NO) levels were estimated by quantifying nitrite content (NaNO2) (Grisham et al. 1996). The reaction mixture included 50 µL of the supernatant, 50 µL of 1% sulfanilamide, and 50 µL of 0.1% N-(1-naphthyl) ethylenediamine (NED) in 5% phosphoric acid. Absorbance was measured at 540 nm, and NO levels were expressed as µmol/mg.

### 3.6 STATISTICS

The survival, food consumption, uptake dose, and enzymatic activity of three bee species were evaluated following oral and topical exposure to the fungicide difenoconazole, the herbicide tebuthiuron, and a control treatment. Statistical analyses were conducted in R (R Core Team, 2025) using RStudio (Posit Team, 2025).

Bee longevity, recorded in hours and censored at 96 hours post-exposure, was analyzed using Log-Rank tests within the “survival” package (Therneau, 2024; Therneau & Grambsch, 2000). Kaplan-Meier survival curves were created with the “survminer” package (Kassambara et al., 2024), and p-values for pairwise comparisons were adjusted using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995) at a 5% significance level.

To analyze diet intake, we fitted generalized linear mixed-effects models in the “glmmTMB” package (Brooks et al., 2017) with the dilution factor as the main fixed effect and colony of origin as a random effect. Colony of origin was included as a random effect to account for variability, but was excluded if non-significant ($p > 0.05$) via likelihood ratio tests. The dilution factor was log-transformed, with a constant ($\eta = 0.0001$) added for model stability. Polynomial models up to the third degree were tested, and significance was assessed using the “car” package’s analysis of deviance (Fox & Weisberg, 2019) at a 10% threshold due to limited replicates used in the experiments. The “ggeffects” package (Lüdecke, 2018) was used to estimate diet intake values along the dilution factors, including 95% confidence intervals (CI).

For models with $p > 0.1$, a first-degree model was selected, and marginal means with 95% CIs were computed using the "emmeans" package (Length, 2025). Model diagnostics, including residuals, uniformity, outliers, and dispersion, were validated using the "DHARMa" package (Hartig, 2024).

Pesticide uptake was modeled with a power function, $y = a \cdot x^b$, was employed, where $y$ represents the ingested dose, $x$ denotes the dilution factor, $a$ is the scaling coefficient, and $b$ determines the rate of dose change. Nonlinear mixed-effects models were used to fit pesticide uptake using the "nlme" package, with colony used as a random effect to account for variability in the origin of the different bee species. Three random effect structures were evaluated: on $a$, $b$, or both, assuming uncorrelated effects with heterogeneous variances. The model with the lowest Bayesian Information Criterion (BIC) was selected for each species-pesticide combination.

Enzymatic activity (CAT, SOD, GST, MDA, NO) was analyzed using linear models (LM) with transformations (e.g., logarithmic or Box-Cox) as needed, detailed in Supplementary Tables S2-S4. For *S. xanthotricha* (oral exposure, CAT), a generalized linear model (GLM) with a log-normal distribution was used via "glmmTMB" due to non-normal data. Diagnostics were performed with the "DHARMa" package. Treatment effects were assessed with Type II ANOVA in the "car" package, with significant effects ($p < 0.05$) followed by Tukey's pairwise comparisons using the "emmeans" (Lenth, 2025) and "multcomp" (Hothorn et al., 2008) packages. Graphical representations used back-transformed estimates if data were previously transformed for model adjustment. Results, including F-statistics, Chi-square statistics, degrees of freedom, and p-values, are summarized in Supplementary Tables S2-S4.

The statistical analysis of the microvilli-lined border length and nuclear area of midgut cells for the three bee species was performed using the glmmTMB package, employing Gaussian or Log-Normal distributions as necessary to optimize model fit. The models incorporated random effects of individual worker bee identity nested within the colony. Random effects were tested for significance using a likelihood ratio test, and non-significant effects were removed from the models. Model diagnostics were also performed with the "DHARMa" package. Final models are detailed in Supplementary Table S5. The effect of treatments was evaluated using Type II ANOVA from the "car" package, with significant effects ($p < 0.05$) further analyzed through Tukey's pairwise comparisons using the "emmeans" and "multcomp" packages.

# 4. RESULTS

## 4.1 SURVIVAL

In oral exposure assays (Figure 1), *A. mellifera* showed no significant survival differences for difenoconazole ($\chi^2 = 2.17$, $p = 0.5375$) or tebuthiuron ($\chi^2 = 3.29$, $p = 0.3497$), with gradual declines across treatments (including the control). *P. helleri* maintained high survival across all groups, precluding statistical analysis. Tebuthiuron significantly reduced *P. helleri* survival at a dilution factor of 1 ($\chi^2 = 41.90$, $p < 0.0001$; Table 1). *S. xanthotricha* showed no significant survival effects for difenoconazole ($\chi^2 = 6.27$, $p = 0.0992$) or tebuthiuron ($\chi^2 = 7.77$, $p = 0.5282$).

In topical exposure assays (Figure 2), *A. mellifera* survival was unaffected by difenoconazole ($\chi^2 = 2.23$, $p = 0.5269$) or tebuthiuron ($\chi^2 = 5.08$, $p = 0.1663$). *P. helleri* showed no effect from difenoconazole ($\chi^2 = 1.30$, $p = 0.7296$) but a significant survival reduction with tebuthiuron at dilution factor of 1 ($\chi^2 = 32.28$, $p < 0.0001$; Table 1). *S. xanthotricha* exhibited an outperformed survival when subject to difenoconazole ($\chi^2 = 14.89$, $p = 0.0019$) at dilution factors of 0.01 and 0.1 (Table 1). Tebuthiuron had no significant effect ($\chi^2 = 7.77$, $p = 0.5282$), with slight decreases in survival for most of the treatments.

## 4.2 DIET INTAKE

For oral exposure to difenoconazole, *A. mellifera* ($\chi^2 = 0.65$, $df = 1$, $p = 0.42$) and *P. helleri* ($\chi^2 = 0.52$, $df = 1$, $p = 0.47$) showed non-significant diet intake trends (0-6 hours), estimated at 39.3 µL and 8.46 µL, respectively (Figure 3). *S. xanthotricha* exhibited increased intake with higher concentrations ($\chi^2 = 7.67$, $df = 1$, $p = 0.006$). Over 6-96 hours, *A. mellifera* showed reduced intake ($\chi^2 = 3.69$, $df = 1$, $p = 0.05$), while *P. helleri* ($\chi^2 = 0.06$, $df = 1$, $p = 0.80$) and *S. xanthotricha* ($\chi^2 = 2.88$, $df = 1$, $p = 0.09$) showed marginal significant increases.

For oral exposure to tebuthiuron (Figure 4), *A. mellifera* exhibited a significant decrease of diet intake (0-6 hours; $\chi^2 = 18.73$, $df = 1$, $p < 0.0001$), while *P. helleri* ($\chi^2 = 0.22$, $df = 1$, $p = 0.64$) and *S. xanthotricha* ($\chi^2 = 1.35$, $df = 1$, $p = 0.25$) showed non-significant trends. Over 6-96 hours, *A. mellifera* ($\chi^2 = 9.38$, $df = 2$, $p = 0.009$) and *P. helleri* ($\chi^2 = 23.57$, $df = 2$, $p < 0.0001$) displayed quadratic trends, with intake decreasing after a 0.01 dilution. *S. xanthotricha* showed non-significant trends ($\chi^2 = 3.38$, $df = 1$, $p = 0.07$).

For topical exposure to difenoconazole, *A. mellifera* showed a complex third-order trend (0-6 hours; $\chi^2 = 13.11$, $df = 3$, $p = 0.004$), and *S. xanthotricha* exhibited increased intake with higher dilutions ($\chi^2 = 4.24$, $df = 1$, $p = 0.04$) (Figure 5). *P. helleri* showed no significant

trend ($p > 0.1$), with an intake at 6.22 µL. Over 6-96 hours, *S. xanthotricha* showed reduced intake at 0.01 dilution ($\chi^2 = 22.22$, df = 2, $p < 0.0001$), while *A. mellifera* ($\chi^2 = 0.03$, df = 1, $p = 0.86$) and *P. helleri* ($\chi^2 = 2.60$, df = 1, $p = 0.11$) showed non-significant trends, leading to first-degree models with food ingestion estimated in 1,271.04 and 120.22 µL, respectively.

For topical exposure to tebuthiuron within the 0-6 interval hour, all species showed non-significant trends (*A. mellifera*: $\chi^2 = 0.006$, df = 1, $p = 0.98$; *P. helleri*: $\chi^2 = 0.22$, df = 1, $p = 0.64$; *S. xanthotricha*: $\chi^2 = 1.35$, df = 1, $p = 0.25$) (Figure 6). Over 6–96 hours, *A. mellifera* exhibited a significant second-degree trend ($\chi^2 = 8.11$, df = 2, $p = 0.02$), with reduced intake after 0.01 dilution, while *P. helleri* ($\chi^2 = 0.09$, df = 2, $p = 0.77$) and *S. xanthotricha* ($\chi^2 = 0.52$, df = 1, $p = 0.47$) showed non-significant trends. Dose-dilution relationships, depicted in Figure 7, adhered to the power function ($y = a \cdot x^b$), revealing a nonlinear increase in ingested pesticide dose as the dilution factor rose from 0.01 to one. For *A. mellifera*, tebuthiuron exhibited a substantially higher baseline dose ($a = 38.4568$) but a less pronounced response slope ($b = 0.7798$) compared to difenoconazole ($a = 3.1937$, $b = 1.7798$). Similarly, in *P. helleri*, tebuthiuron showed a higher baseline ($a = 11.7049$) and a lower response slope, indicating a less pronounced increase in ingested dose ($b = 0.6736$) with dilution compared to difenoconazole ($a = 0.8006$, $b = 1.2950$). For *S. xanthotricha*, tebuthiuron maintained a higher baseline ($a = 13.8216$) with a slightly flatter response ($b = 0.9072$) relative to difenoconazole ($a = 0.5569$, $b = 1.1152$). Inter-colony variability significantly influenced model parameters $a$ and $b$, as detailed in Supplementary Table S1, highlighting colony-specific differences in pesticide uptake.

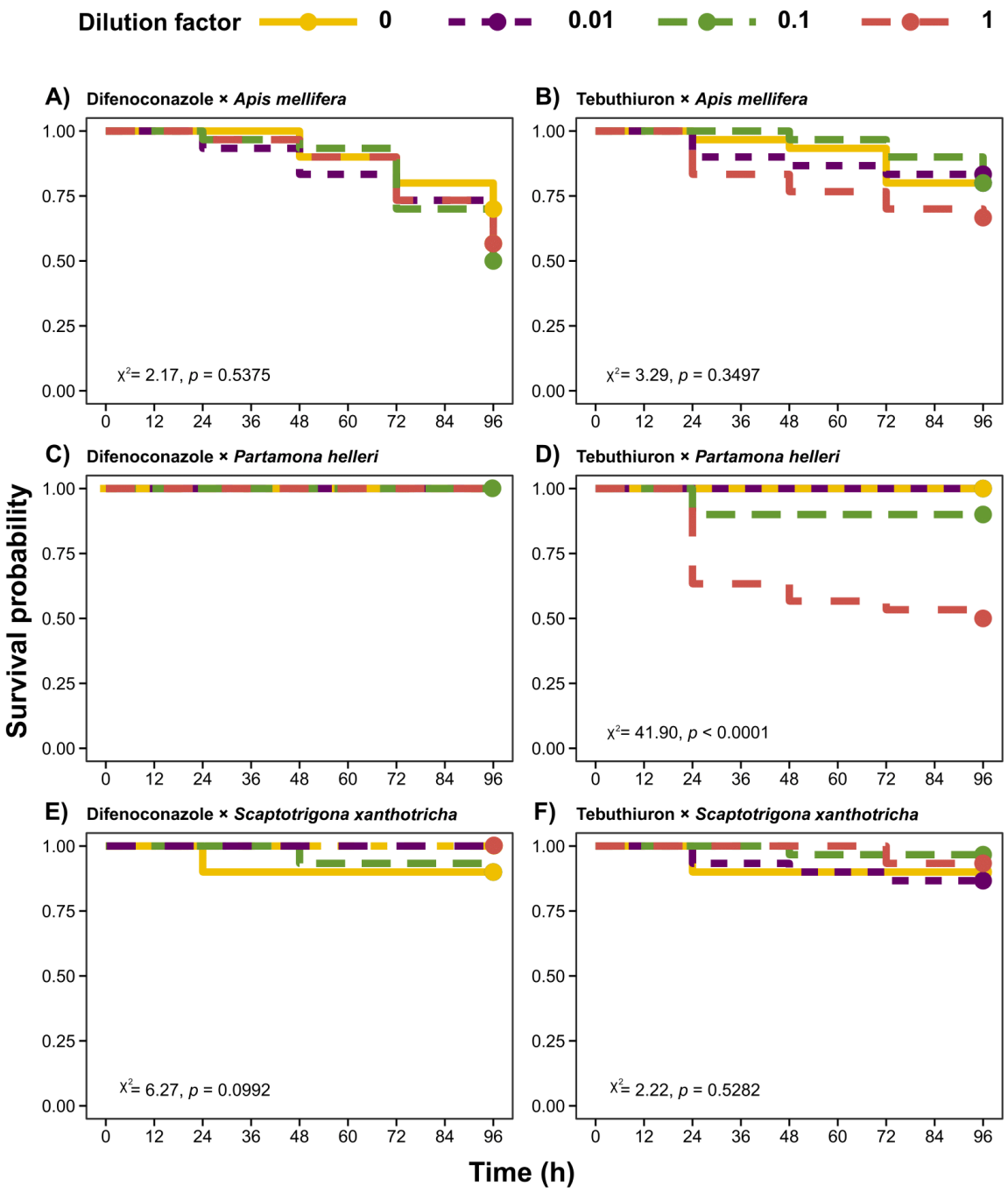


**Figure 1**. Effects of oral exposure to difenoconazole and tebuthiuron on longevity (survival over time) of *Apis mellifera* (A, B), *Partamona helleri* (C, D) and, *Scaptotrigona xanthotricha* (E, F). Dilution factors of agrochemicals are denoted by 0 (control), 0.01, 0.1, and 1.

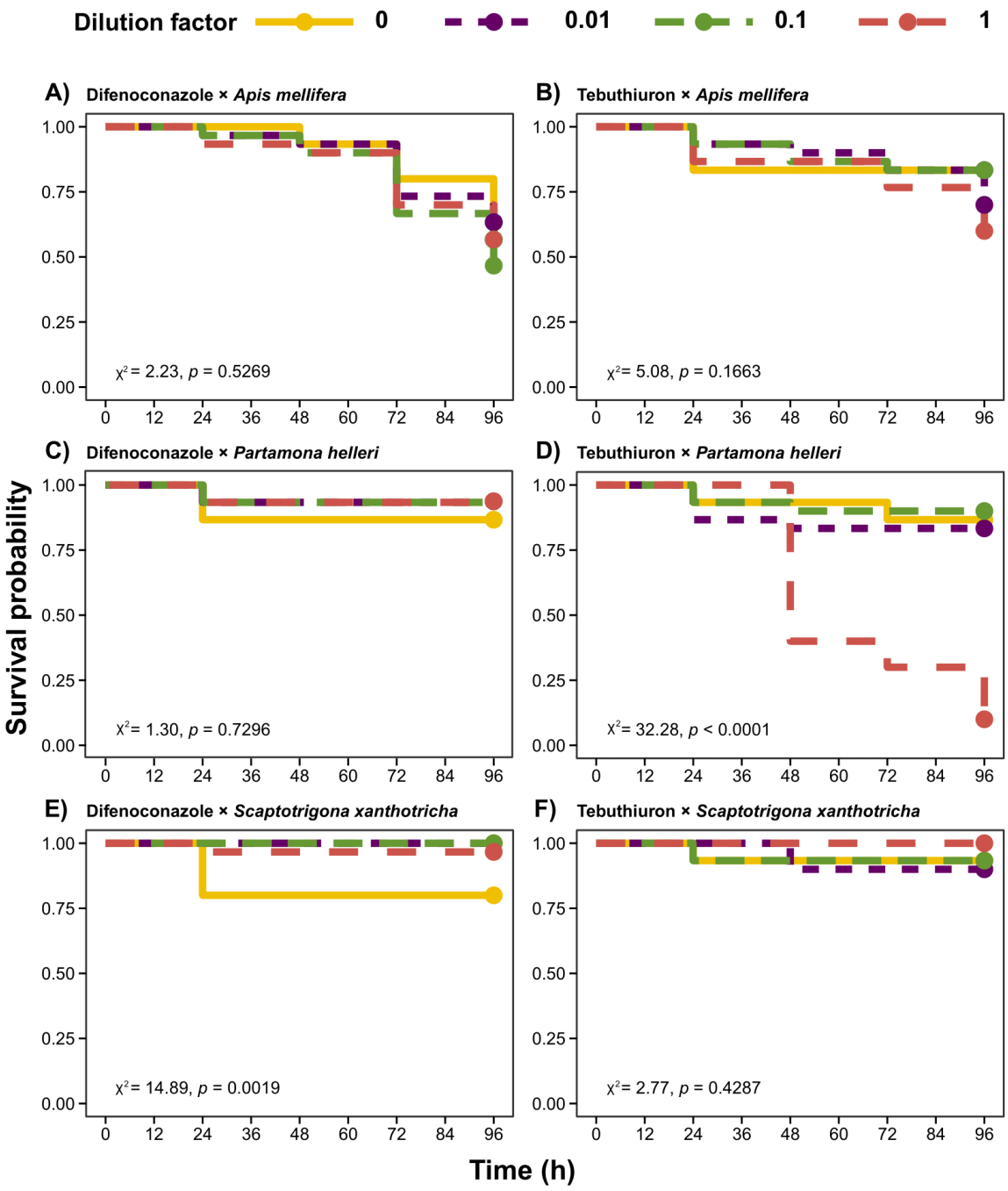


**Figure 2**. Effects of topical exposure to difenoconazole and tebuthiuron on longevity (survival over time) of *Apis mellifera* (A, B), *Partamona helleri* (C, D) and, *Scaptotrigona xanthotricha* (E, F). Dilution factors of agrochemicals are denoted by 0 (control), 0.01, 0.1, and 1.

**Table 1**. Adjusted p-values Benjamini-Hochberg method from pairwise comparisons of survival curves using Log-Rank test. Pairwise comparisons were conducted only when the overall Log-Rank test was statistically significant ($p < 0.05$).

| **Species** | **a.i.** | **Exposure** | **Dilution factor** | **Dilution factor** | | |
|---|---|---|---|---|---|---|
| | | | | **0.01** | **0.1** | **1** |
| *Partamona helleri* | tebuthiuron | oral | **0** | 1.0000 | 0.0936 | < 0.0001 |
| | | | **0.01** | | 0.0936 | < 0.0001 |
| | | | **0.1** | | | 0.0016 |
| | | topical | **0** | 0.7109 | 0.7109 | < 0.0001 |
| | | | **0.01** | | 0.6710 | 0.0002 |
| | | | **0.1** | | | < 0.0001 |
| *Scaptotrigona xanthotricha* | difeconazole | topical | **0** | 0.0314 | 0.0314 | 0.0923 |
| | | | **0.01** | | 1.0000 | 0.3808 |
| | | | **0.1** | | | 0.3808 |

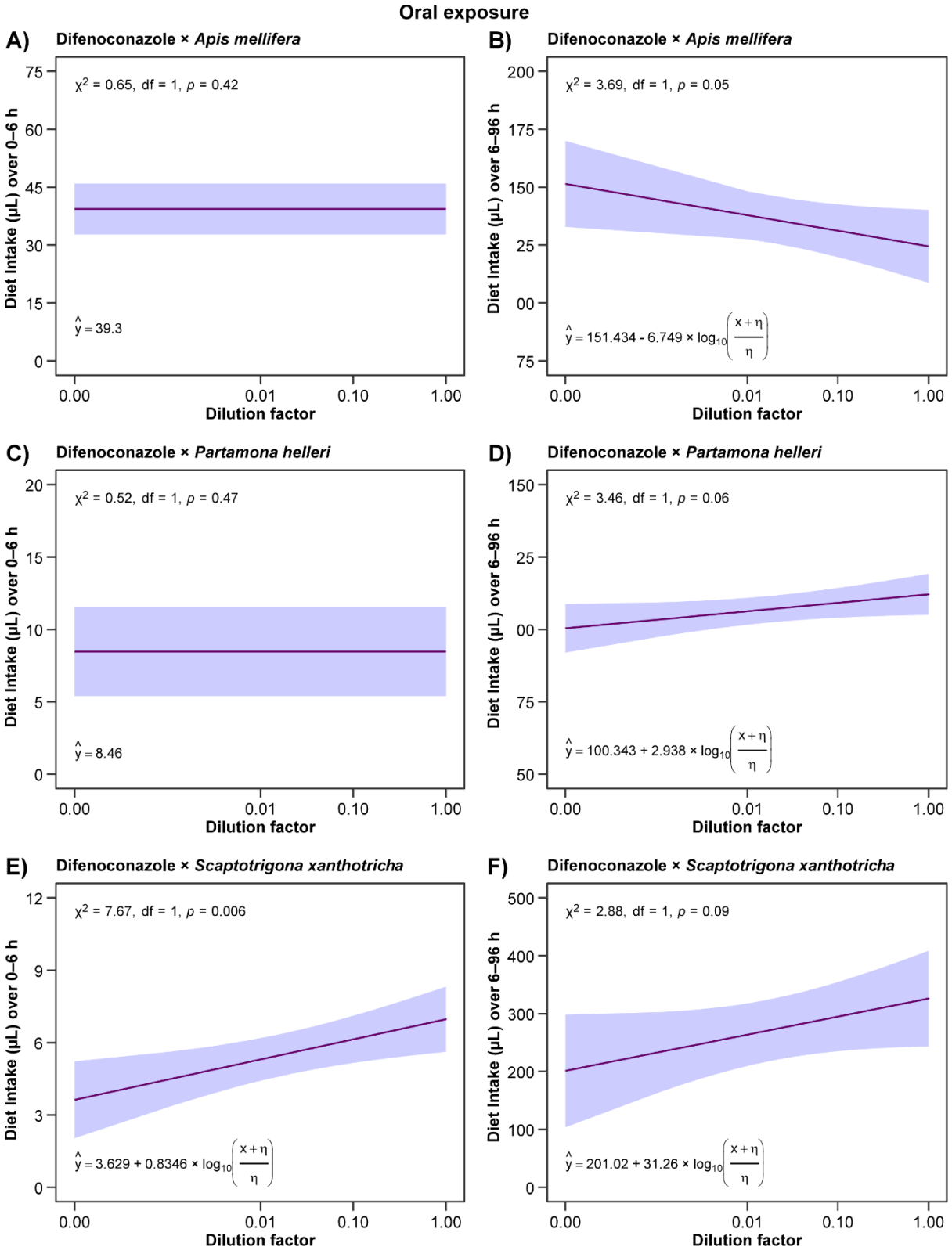


**Figure 3:** Diet intake (µL/bee) by *Apis mellifera*, *Partamona helleri*, and *Scaptotrigona xanthotricha* orally exposed to difenoconazole. Panels A, C, and E show intake over 0-6 hours, while panels B, D, and F show intake over 6-96 hours for the respective species. Solid lines represent fitted models, and shaded areas indicate 95% confidence intervals. Statistical significance is reported as $\chi^2$ and p-values, with a 10% significance threshold. The increase in the dilution factor (x-axis) corresponds to an increase

in agrochemical concentration.

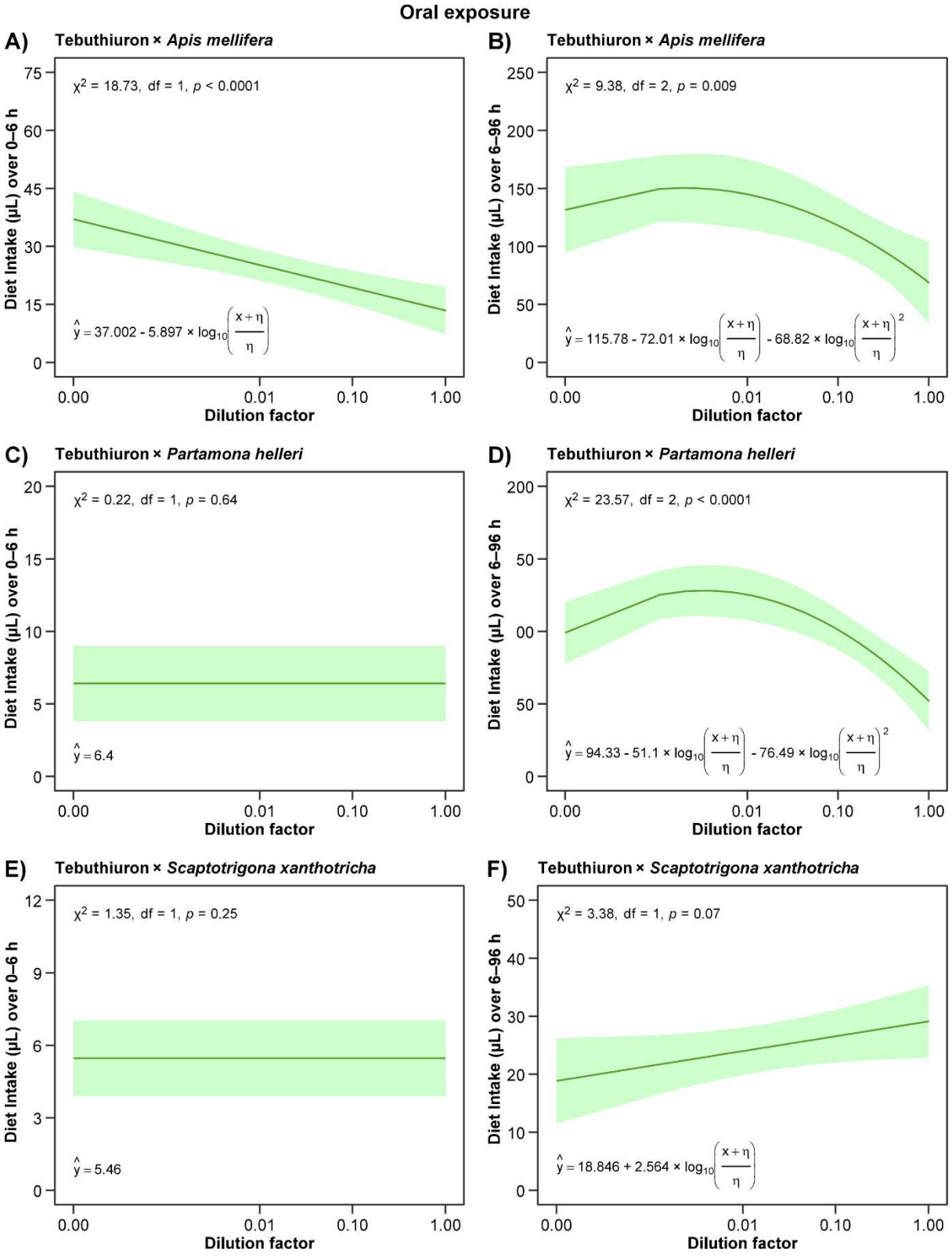


**Figure 4:** Diet intake (µL/bee) by *Apis mellifera*, *Partamona helleri*, and *Scaptotrigona xanthotricha* orally exposed to tebuthiuron. Panels A, C, and E show intake over 0-6 hours, while panels B, D, and F show intake over 6-96 hours for the respective species. Solid lines represent fitted models, and shaded areas indicate 95% confidence intervals. Statistical significance is reported as $\chi 2$ and p-values, with a

10% significance threshold. The increase of the dilution factor (x-axis) corresponds to an increase in agrochemical concentration.

## 4.3

### Contact exposure

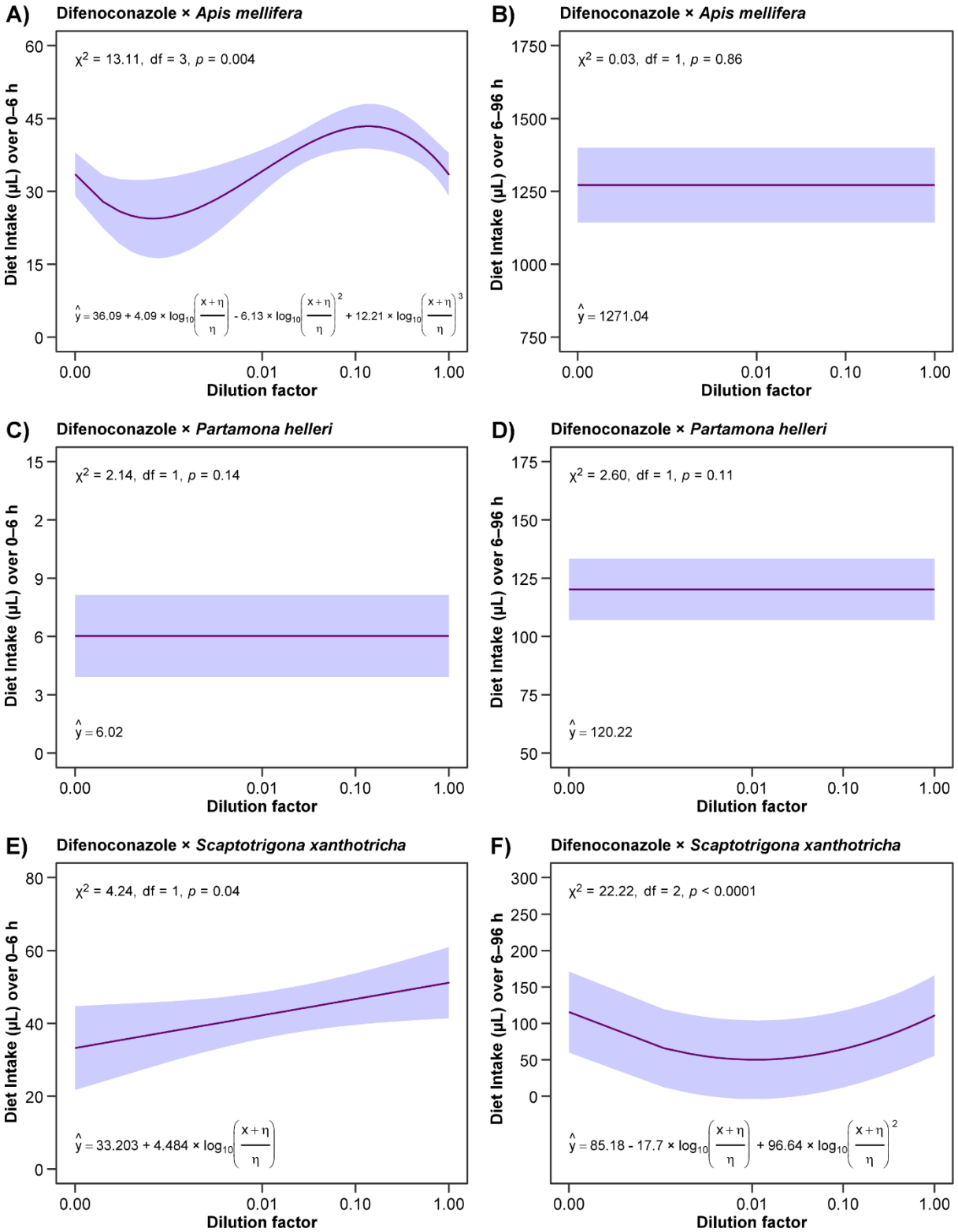


**Figure 5:** Diet intake (µL/bee) by *Apis mellifera*, *Partamona helleri*, and *Scaptotrigona xanthotricha* topically exposed to difenoconazole. Panels A, C, and E show intake over 0-6 hours, while panels B, D,

and F show intake over 6-96 hours for the respective species. Solid lines represent fitted models, and shaded areas indicate 95% confidence intervals. Statistical significance is reported as $\chi^2$ and p-values, with a 10% significance threshold. The increase in the dilution factor (x-axis) corresponds to an increase in agrochemical concentration.

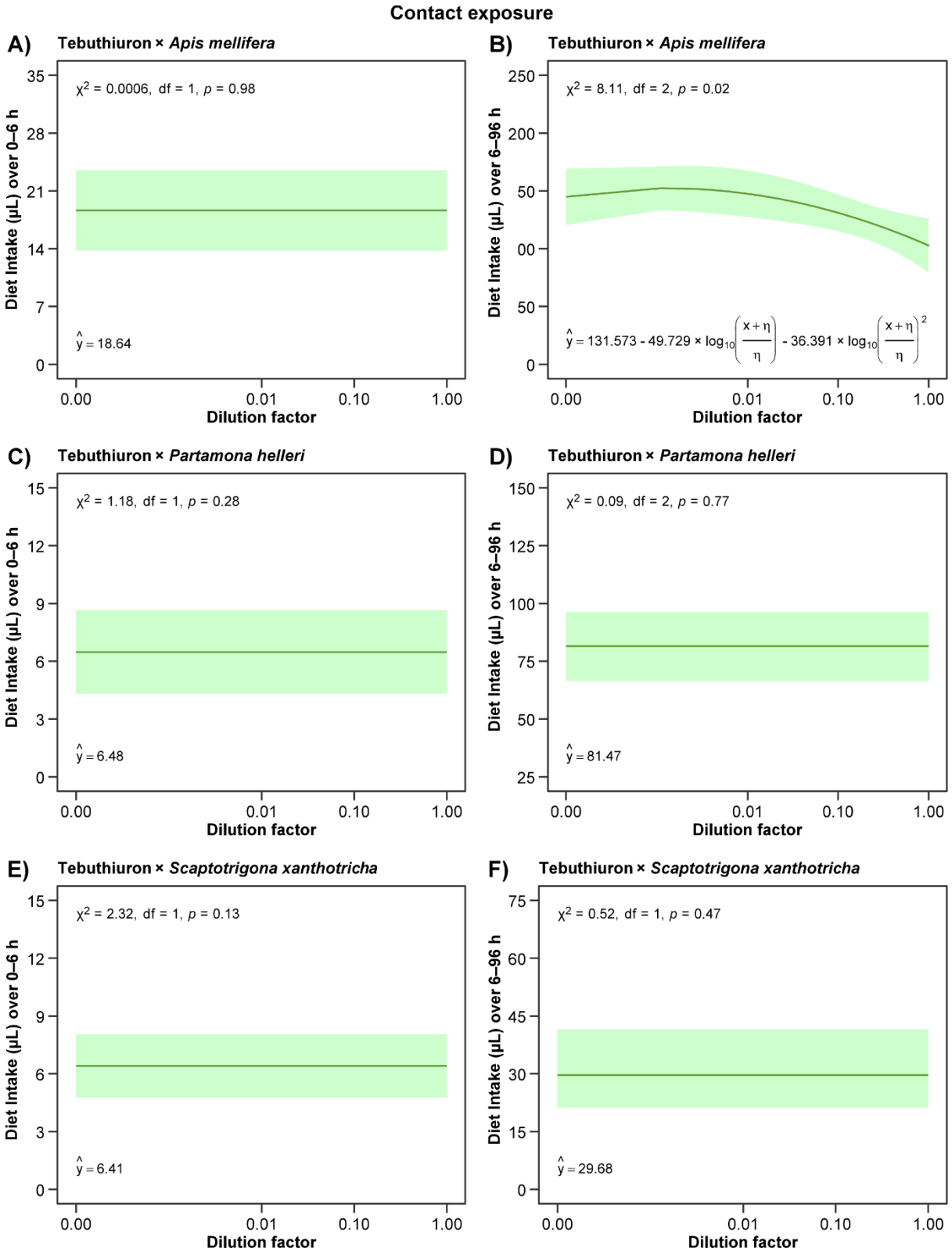

**Figure 6:** Diet intake (µL/bee) by *Apis mellifera*, *Partamona helleri*, and *Scaptotrigona xanthotricha* topically exposed to tebuthiuron. Panels A, C, and E show intake over 0-6 hours, while panels B, D, and F show intake over 6-96 hours for the respective species. Solid lines represent fitted models, and shaded areas indicate 95% confidence intervals. Statistical significance is reported as $\chi^2$ and p-values, with a 10% significance threshold. The increase in the dilution factor (x-axis) corresponds to an increase in agrochemical concentration.

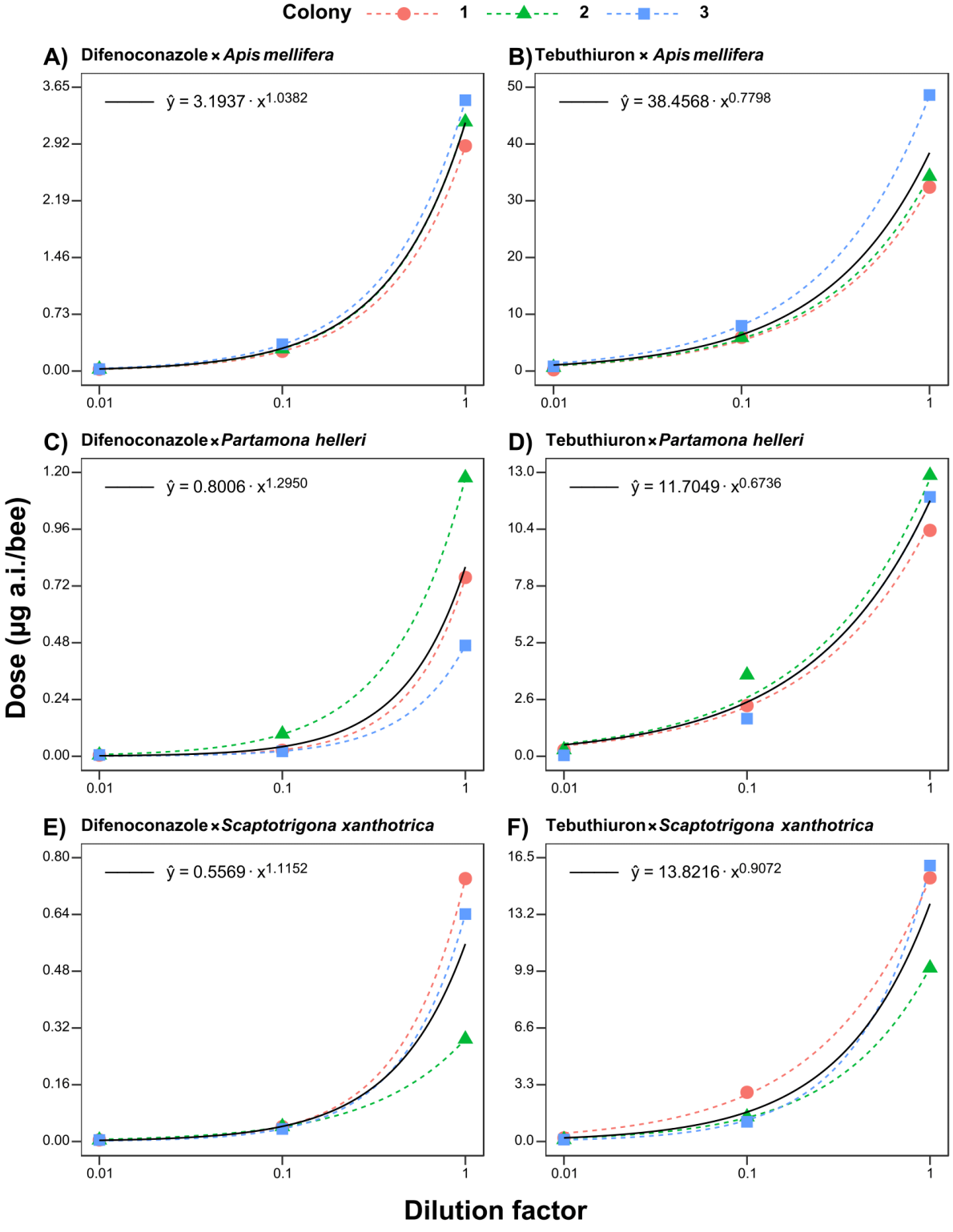

**Figure 7.** Dose ingested by bees (µg a.i./bee) as a function of the factor dilution for different combinations of pesticide and bee species. Panels A, C, and E show the dose of difenoconazole for *Apis mellifera*, *Partamona helleri*, and *Scaptotrigona xanthotricha*, respectively, while panels B, D, and F show the dose of tebuthiuron for the same species. The fitted curves follow a power function of the form $y = a \cdot x^b$, with the specific equation provided in each panel. The x-axis (dilution factor) is on a logarithmic scale ranging from 0.01 to one (where an increase in the dilution factor corresponds to an increase in the concentration of the agrochemical), and the y-axis (dose) varies across the magnitude of the ingested agrochemical dilution.

### 4.1 MIDGUT MORPHOLOGY

Effects of exposure on the brush border height and nuclear area of midgut cells for the three species are depicted in Figure 8. For *A. mellifera* and *S. xanthotricha* neither difenoconazole nor tebuthiuron significantly affected the brush border height ($\chi^2 = 5.0600$, df = 2, p = 0.0797) ($\chi^2 = 5.3898$, df = 2, p = 0.0675) or nuclear area ($\chi^2 = 2.7209$, df = 2, p = 0.2565) ($\chi^2 = 4.6213$, df = 2, p = 0.0992). For *P. helleri*, both the brush border height ($\chi^2 = 13.5495$, df = 2, p = 0.0011) and nuclear area ($\chi^2 = 18.9110$, df = 2, p = 0.0001) were affected by treatments, with tebuthiuron significantly decreasing the height, and both agrochemicals increasing the area compared to the control.

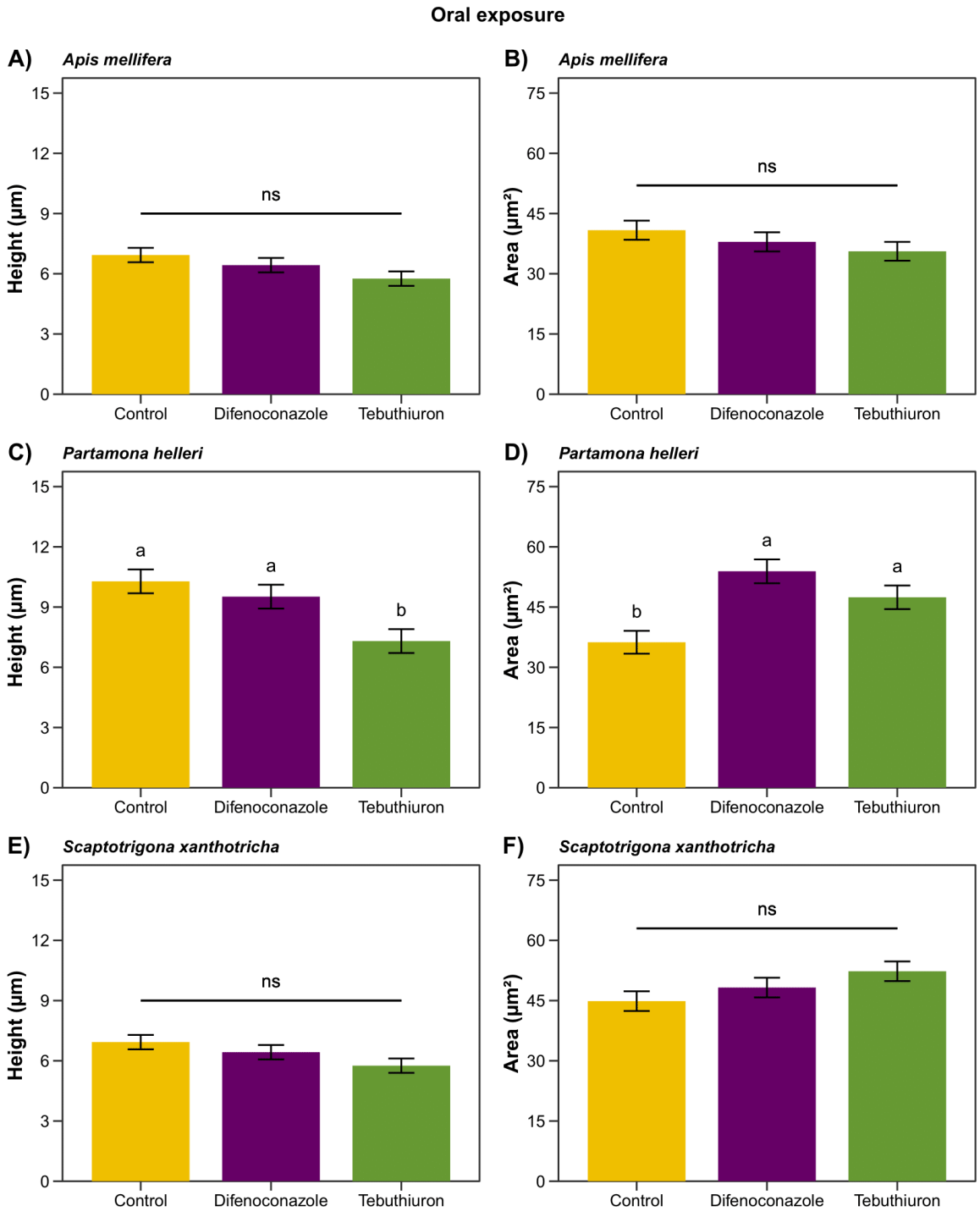


**Figure 8**. Effects of oral exposure to difenoconazole or tebuthiuron on the border height (µm) (Panels A, C, E) and nuclear area (µm²) (Panels B, D, F) of midgut cells in *Apis mellifera*, *Partamona helleri*, and *Scaptotrigona xanthotricha*. Different letters indicate statistically significant differences ($p < 0.05$) between treatments (Tukey's test); "ns" indicates non-significant differences.

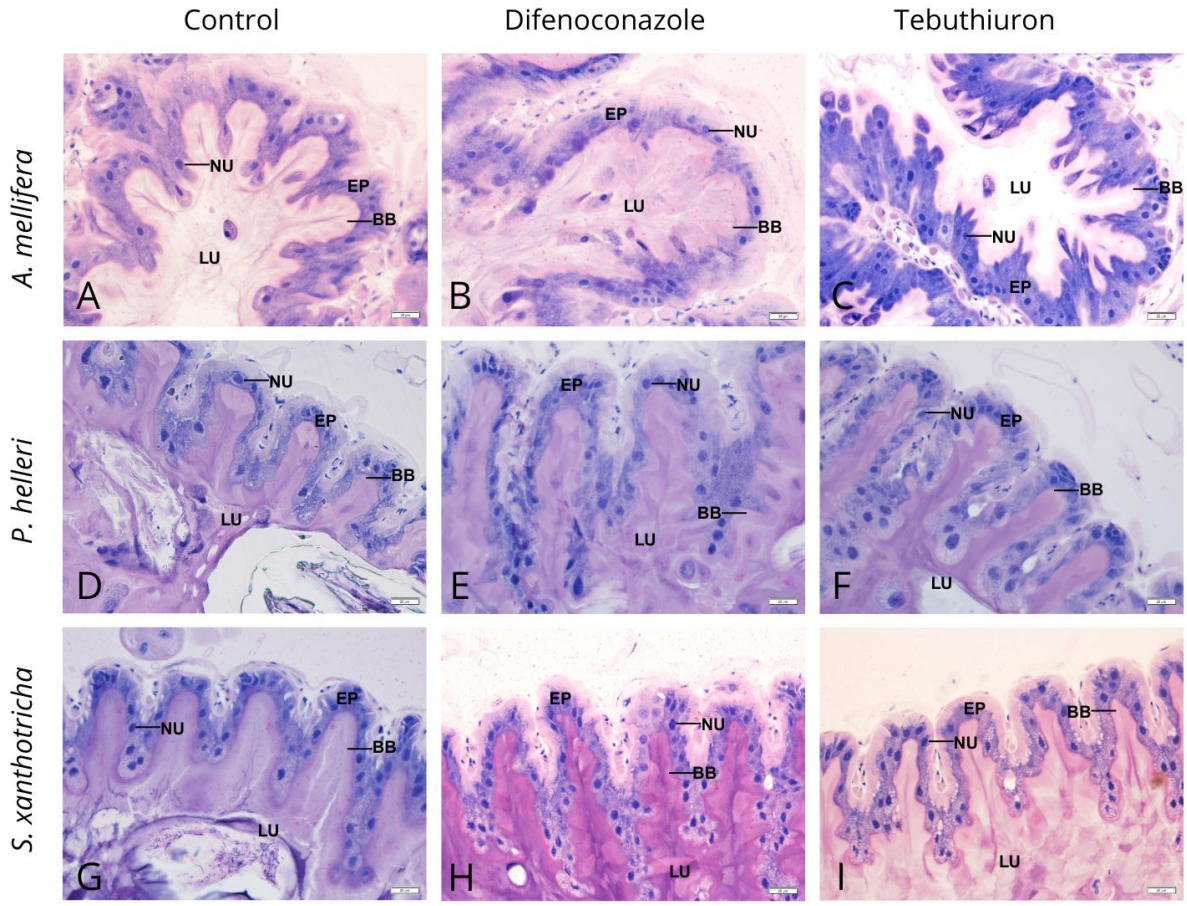


**Figure 9**. Histological sections of midgut of *Apis mellifera*, *Partamona helleri* and *Scaptotrigina xanthotricha* 24 hours post-exposure to difenoconazole, tebuthiuron and control. Midgut lumen (LU), epithelium (EP), brush border (BB) and digestive cell nucleus (NU).

### 4.1 Enzymatic Activity

Enzymatic activity for *A. mellifera* showed no significant SOD differences under oral exposure ($F_{2,18} = 1.74$, $p = 0.2043$) but a decrease under topical exposure to both agrochemicals ($F_{2,18} = 6.63$, $p = 0.0070$) (Figure 10). GST was unaffected orally ($F_{2,18} = 0.79$, $p = 0.4704$) but higher in control under contact exposure ($F_{2,17} = 28.43$, $p < 0.0001$). MDA increased with difenoconazole orally compared to difenoconazole ($F_{2,18} = 5.13$, $p = 0.0172$), but no significant effect was found after topical exposure ($F_{2,18} = 3.14$, $p = 0.0677$). CAT decreased in oral ($F_{2,12} = 9.71$, $p = 0.0031$) or topical exposure ($F_{2,12} = 21.90$, $p = 0.0001$) to both agrochemicals. NO levels were unaffected by the agrochemicals (oral: $F_{2,12} = 0.41$, $p = 0.6736$; topical: $F_{2,12} = 0.06$, $p = 0.9463$).

For *P. helleri*, SOD increased after oral exposure to tebuthiuron ($F_{2,15} = 4.04$, $p = 0.0396$) but not after topical exposure ($F_{2,16} = 2.48$, $p = 0.1155$) (Figure 11). GST ($F_{2,17} = 2.44$, $p = 0.1171$) and CAT ($F_{2,18} = 1.24$, $p = 0.3139$) showed no significant differences. MDA increased under contact exposure to difeconazole or tebuthiuron ($F_{2,18} = 10.30$, $p = 0.0010$).

Did not change after oral exposure ($F_{2,12} = 1.05$, $p = 0.3811$) but increased with difenoconazole after topical exposure compared to tebuthiuron ($F_{2,12} = 4.67$, $p = 0.0317$).

For *S. xanthotricha*, SOD increased with difenoconazole under oral ($F_{2,18} = 4.26$, $p = 0.0306$) and topical exposure ($F_{2,16} = 5.73$, $p = 0.0133$) compared to control (Figure 12). GST increased with tebuthiuron compared to difeconazole under both exposures (oral: $F_{2,18} = 4.91$, $p = 0.0199$; topical: $F_{2,17} = 4.42$, $p = 0.0284$). MDA was unaffected orally ($F_{2,18} = 2.22$, $p = 0.1374$) but increased with tebuthiuron after topical exposure ($F_{2,17} = 8.68$, $p = 0.0025$). CAT increased treatments after oral exposure to the two agrochemicals ($\chi^2 = 12.94$, $df = 2$, $p = 0.0016$) but was unaffected after topical exposure ($F_{2,12} = 1.24$, $p = 0.3244$). NO levels were unaffected by both topical ($F_{2,12} = 2.21$, $p = 0.1525$) and topical ($F_{2,12} = 0.21$, $p = 0.8127$) exposures.

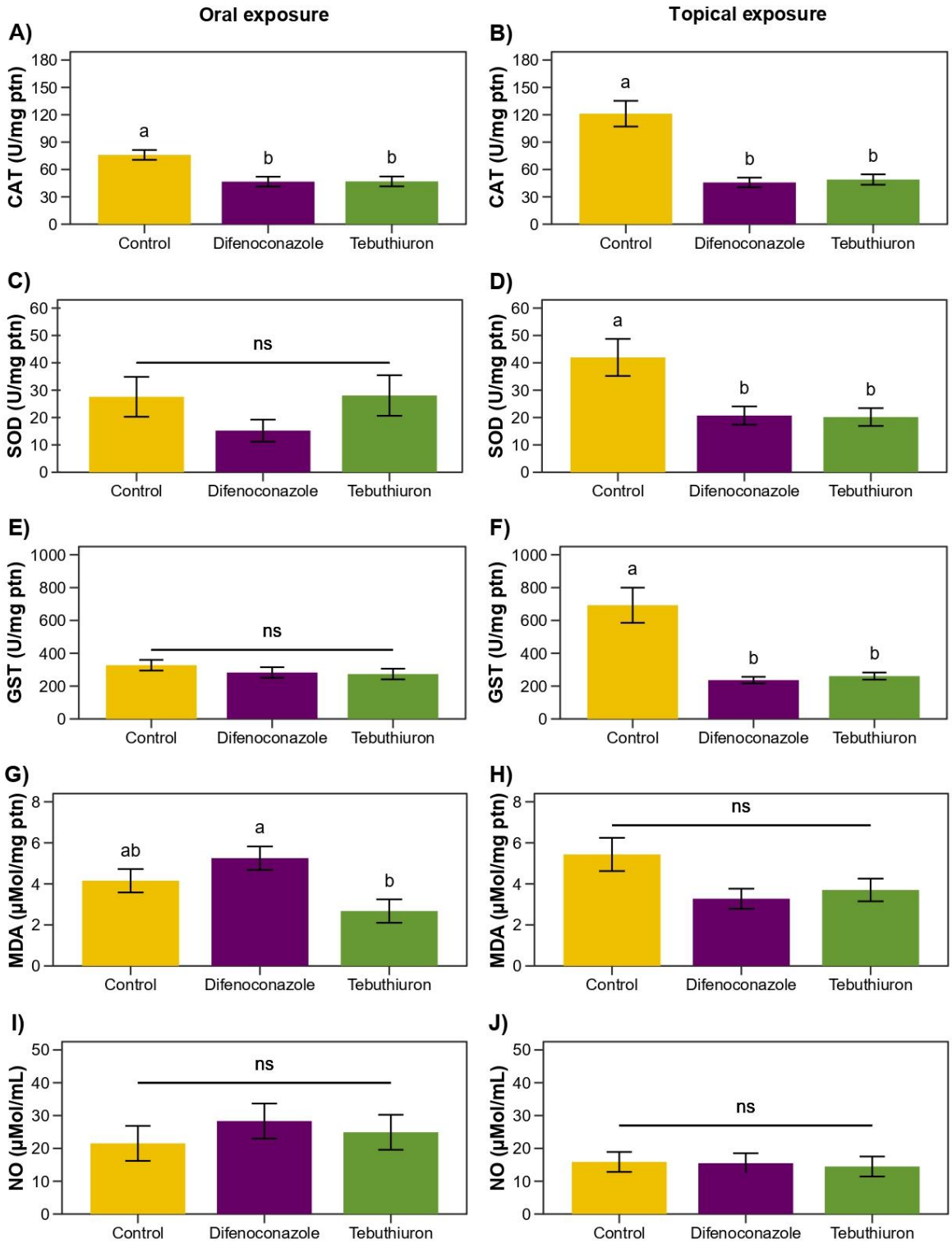


**Figure 10**. Effects of oral or topical exposures to difenoconazole or tebuthiuron on enzymatic activity in *Apis mellifera*. (A, B) CAT activity (µM $min^{-1}$ $mg^{-1}$ protein); (C, D) SOD activity (µM $min^{-1}$ $mg^{-1}$ protein); (E, F) GST activity (µM $mg^{-1}$ protein); (G, H) MDA levels (µM $min^{-1}$ $mg^{-1}$ protein); (I, J) NO levels (µM $mL^{-1}$). Panels A, C, E, G, and I represent oral exposure; panels B, D, F, H, and J represent topical exposure. Different letters indicate significant differences ($p < 0.05$) between treatments (Tukey's test); "ns" denotes non-significant differences.

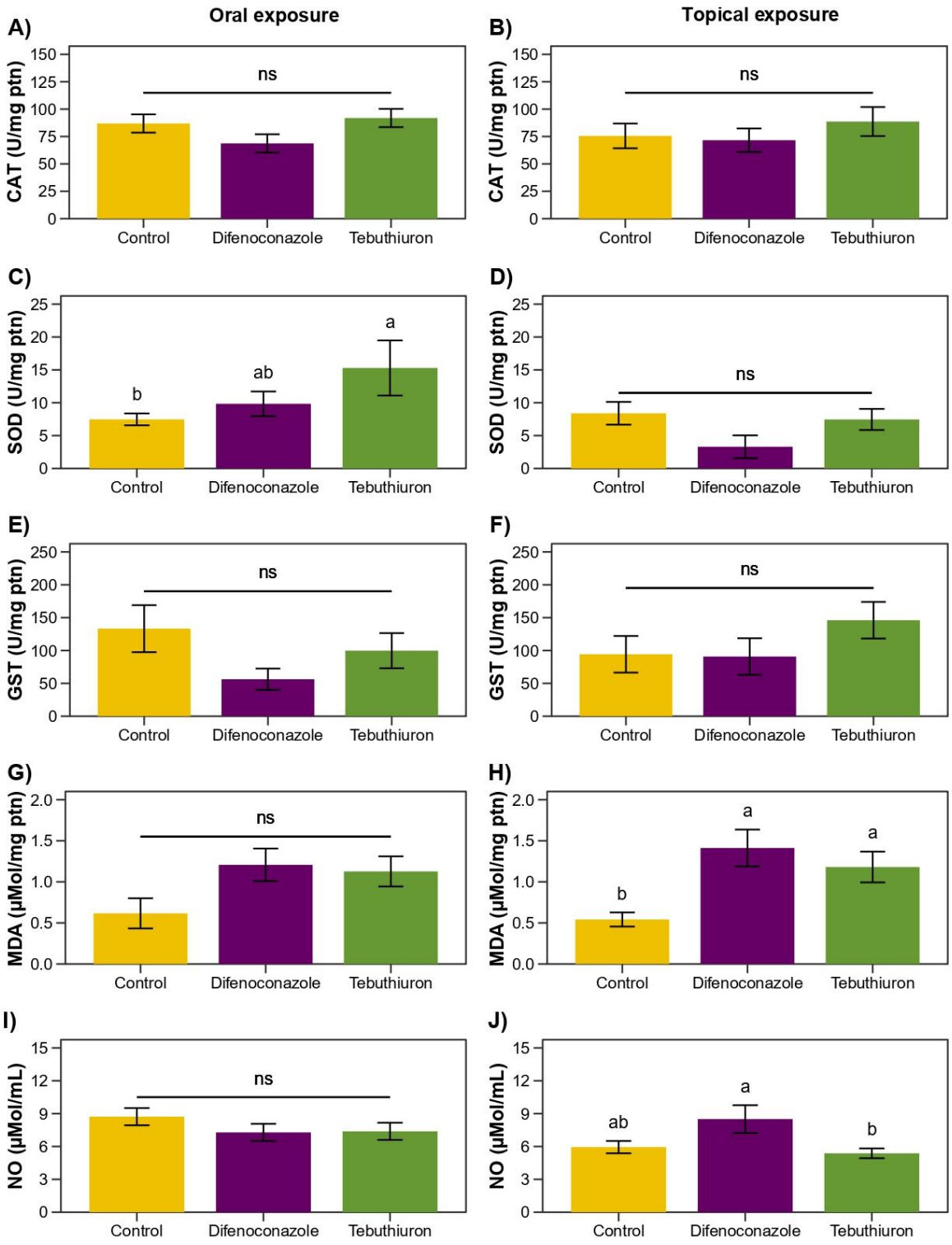


**Figure 11**. Effects of oral or topical exposures to difenoconazole or tebuthiuron on enzymatic activity in *Partamona helleri*. (A, B) CAT activity (µM $min^{-1}$ $mg^{-1}$ protein); (C, D) SOD activity (µM $min^{-1}$ $mg^{-1}$ protein); (E, F) GST activity (µM $mg^{-1}$ protein); (G, H) MDA levels (µM $min^{-1}$ $mg^{-1}$ protein); (I, J) NO levels (µM $mL^{-1}$). Panels A, C, E, G, and I represent oral exposure; panels B, D, F, H, and J represent topical exposure. Different letters indicate significant differences ($p < 0.05$) between treatments (Tukey's test); "ns" denotes non-significant differences.

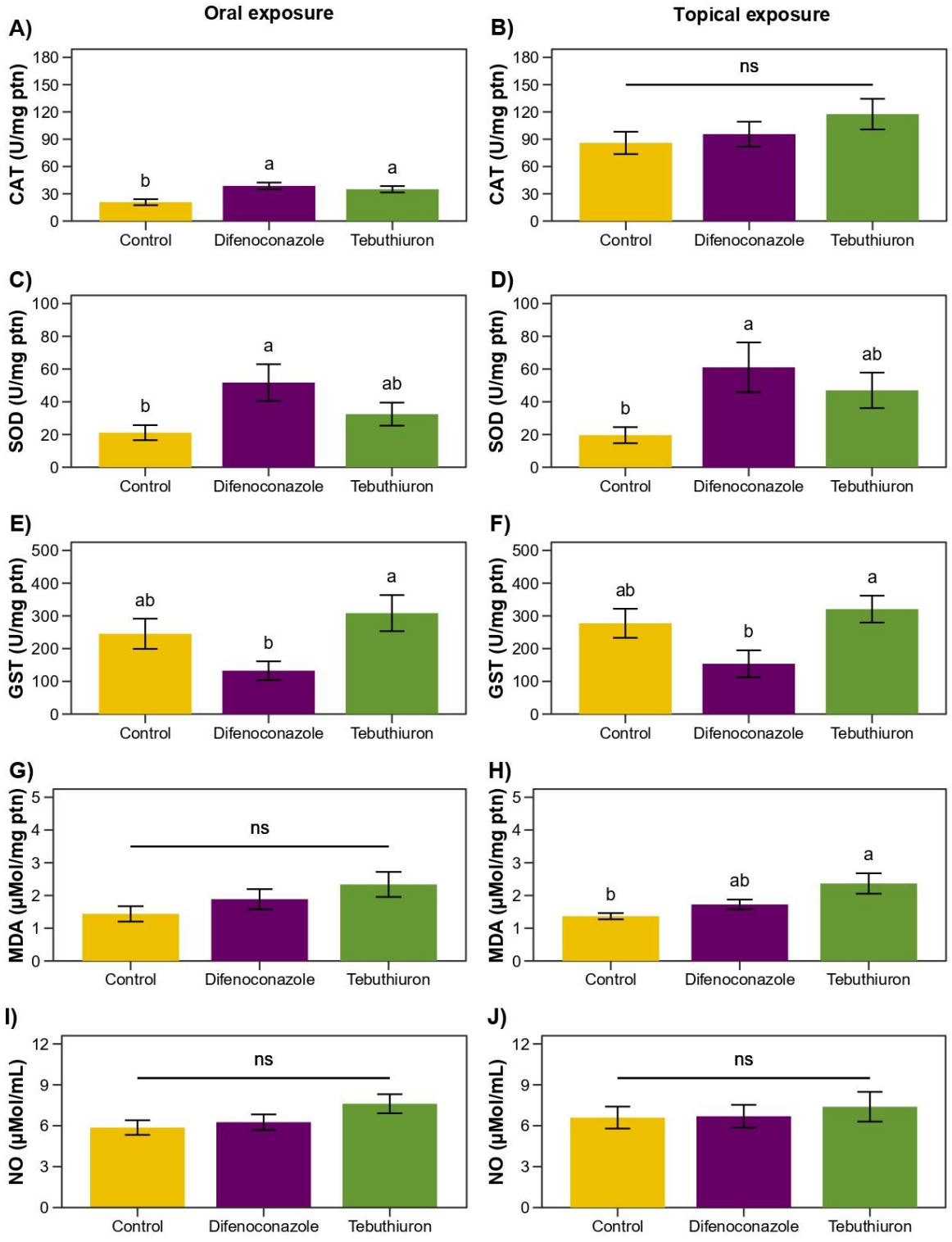


**Figure 12**. Effects of oral or topical exposures to difenoconazole or tebuthiuron on enzymatic activity in *Scaptotrigona xanthotricha*. (A, B) CAT activity (µM $min^{-1}$ $mg^{-1}$ protein); (C, D) SOD activity (µM $min^{-1}$ $mg^{-1}$ protein); (E, F) GST activity (µM $mg^{-1}$ protein); (G, H) MDA levels (µM $min^{-1}$ $mg^{-1}$ protein); (I, J) NO levels (µM $mL^{-1}$). Panels A, C, E, G, and I represent oral exposure; panels B, D, F, H, and J represent topical exposure. Different letters indicate significant differences ($p < 0.05$) between treatments (Tukey's test); "ns" denotes non-significant differences.

## 5. DISCUSSION

Our findings reveal significant interspecific differences in how honey bees and stingless bees respond to difenoconazole or tebuthiuron exposure. While *A. mellifera* showed remarkable resilience to both agrochemicals. *P. helleri* exhibited severe sensitivity to tebuthiuron, particularly at higher concentrations. This aligns with previous work showing *P. helleri*'s vulnerability to this agrochemical (Farder-Gomes et al., 2024), and contrasts with the unexpected survival of *S. xanthotricha* when exposed to difenoconazole. However, to our knowledge, there are no previous reports on the effects of these agrochemicals on *S. xanthotricha*, limiting direct comparisons regarding lethality. Field-recommended doses generally showed low toxic potential, except for *P. helleri*, emphasizing the need to include multiple stingless bee species in ecotoxicological assessments, as responses can vary greatly between species. Additionally, both exposure routes, oral and topical, should be considered. These species-specific differences may be attributed to variations in detoxification capacity and physiological sensitivity, as seen in *A. cerana* and *A. mellifera* where survival was not affected (Han et al., 2023) and affected (Almasri et al., 2022) by difenoconazole exposure, respectively.

The observed patterns of diet consumption provide further evidence of physiological trade-offs in energy allocation, where resources were likely diverted from feeding behavior to cellular detoxification processes. *A. mellifera*'s reduced intake under tebuthiuron in our study is similar to the reduction found in exposures to glyphosate, difenoconazole, imidacloprid, and mixture of them. In this case, repellency was suggested (Almasri et al. 2020). Conversely, *S. xanthotricha*'s increased consumption of difenoconazole-treated diet parallels reports of compensatory feeding in pesticide-exposed bees (Almasri et al., 2022), though without the associated mortality seen in *M. scutellaris* at similar concentrations (Prado et al., 2020). This dichotomy, avoidance in A. mellifera versus compensatory ingestion in S. xanthotricha, highlights how closely-related species may employ fundamentally different strategies when facing chemical stressors (Ribas et al. 2024).

The effect of difenoconazole on *S. xanthotricha* survival warrants particular attention. While hormetic responses to fungicides have been documented in *A. mellifera* larvae (Kaur et al., 2023), our study appears to be the first to show this phenomenon in adult stingless bees. This finding challenges the prevailing view of triazoles as uniformly harmful and suggests that some native species may have evolved tolerance mechanisms, a hypothesis supported by the enzymatic activity profiles discussed below. (Han et al. 2023).

Our dose-response models revealed nonlinear pesticide uptake patterns. The power

function (y=a·x^b) showed tebuthiuron's higher baseline uptake (38.4568 for *A. mellifera*) but shallower slope compared to difenoconazole, suggesting distinct pharmacokinetics. This corroborates findings where *A. mellifera* preferred solutions with imidacloprid (IMD) at low concentrations (1-10 nM), but reduced total food consumption, suggesting a disconnection between attraction and toxicity. The non-linearity in accumulation reinforces the need for dynamic models that integrate food preference, non-linear pharmacokinetics and sublethal effects in risk assessments (Kessler et al. 2015). The observed inter-colony variability underscores the importance of considering genetic diversity in toxicity testing, a point emphasized by recent work on bee populations (Rondeau & Raine, 2024).

The contrasts in survival and consumption among the species pointed to underlying differences in physiological resilience. To unravel these mechanisms, we assessed key enzymatic biomarkers, which confirmed that the distinct species-level outcomes were rooted in fundamentally different strategies for managing chemical stress: from a broad suppression of defense systems in *A. mellifera* to a robust enzymatic induction in *S. xanthotricha* and a failed defensive response in *P. helleri*.

*A. mellifera* showed a reduction in CAT activity after both oral and topical exposure, suggesting a weakened ability to neutralize hydrogen peroxide. The basis for such enzymatic suppression is supported by molecular evidence demonstrating that pesticides can directly bind to and inhibit key antioxidant enzymes like CAT and SOD (Liu et al., 2025). The increase in MDA levels and decrease in CAT activity after oral exposure further reinforce the alteration in enzymatic activity after exposure to agrochemicals in *A. mellifera*, even in the absence of significant mortality. Altered antioxidant defenses were reported in *A. mellifera*, in which GPX upregulation rather than CAT suppression occurred, suggesting route- or pesticide-specific modulation of oxidative stress pathways (Decio et al. 2021). The reduction in CAT and GST activity in *A. mellifera* mirrors findings, where the insecticide triflumezopyrim (TFM) or triadimefon (TAD) directly bound to CAT and SOD, impairing their function (Liu et al. 2025). The authors also reported elevated MDA levels under co-exposure, reinforcing that pesticide mixtures exacerbate oxidative stress despite sublethal doses. Similar to our findings, altered antioxidant defenses in *A. mellifera*, though their study reported GPX upregulation rather than CAT suppression, suggesting route- or pesticide-specific modulation of response pathways (Decio et al. 2021).

*S. xanthotricha* exhibited increased SOD and CAT activity after oral exposure, suggesting a more effective antioxidant defense mechanism. Despite a decrease in GST activity,

the absence of elevated MDA or altered NO levels indicates that this species was able to mitigate damage. This physiological response may explain the high survival rates observed in *S. xanthotricha* under both oral and topical exposure to the tested agrochemicals. While *S. xanthotricha* maintained oxidative balance (no MDA increase), *P. helleri* in our study and in a previous showed elevated MDA and mortality, underscoring interspecific differences in pesticide tolerance (Farder-Gomes et al. 2024).

*P. helleri* was the most sensitive species in terms of survival, particularly at higher concentrations. The observed increase in SOD activity after oral exposure in this species indicates an initial attempt to counteract oxidative stress, but the elevated MDA levels and reduced NO content following topical exposure suggest that the antioxidant defenses were overwhelmed. This is further supported by the significant mortality observed in *P. helleri*, highlighting its vulnerability and limited capacity to manage pesticide-induced stress. The significant mortality in *P. helleri* aligns with the capacity of tebuthiuron to induce midgut damage and locomotor deficits, indicating that this species' antioxidant defenses are easily overwhelmed (Farder-Gomes et al. 2024). *P. helleri's* elevated MDA and SOD activity indicate a failed antioxidant response, wich is similar to *A. mellifera* exposed to the insecticide triflumezopyrim and the triazole fungicide triadimefon mixtures, the authors found that pesticide mixtures inhibit critical detox genes (e.g., CYP4G11), that could explain *P. helleri*'s inability to mitigate oxidative damage, leading to high mortality (Liu et al., 2025).

The morphological impacts on *P. helleri*'s midgut, particularly the reduced brush border height ($\chi^2 = 13.5495$, df = 2, $p = 0.0011$) and nuclear enlargement, provide anatomical correlates, as results, to the observed physiological stress, as are reported for *A. mellifera* exposed to imidacloprid (Gregorc et al., 2018). These findings expand upon documentation of tebuthiuron-induced gut damage and suggest that even sublethal exposure may impair nutrient absorption (Farder-Gomes et al., 2024). The absence of similar effects in *A. mellifera* reinforces the ecological concept of "superorganism resilience" in honey bees, where colony buffering may compensate for individual impairments (Almasri et al., 2022).

The integration of feeding behavior and midgut histological data revealed species-specific responses to pesticide exposure. In *A. mellifera*, neither difenoconazole nor tebuthiuron caused alterations in the midgut, which may explain the lack of major changes in diet intake, especially during exposure to difenoconazole. This resilience aligns with findings that honeybee midguts exhibit high regenerative capacity even after neonicotinoid exposure (Gregorc et al., 2018). The reduced food intake observed with tebuthiuron exposure, in *A.*

*mellifera* for oral and topical exposure and *P. helleri* for oral exposure, may be more closely linked to physiological or sensory avoidance mechanisms (Li et al., 2020) rather than structural gut damage. In contrast, *P. helleri* exhibited both decreased food intake after oral exposure to tebuthiuron and significant histological alterations, including a reduction in brush border height and an increase in nuclear area. These findings mirror midgut degeneration patterns in stingless bees exposed to fipronil (Farder-Gomes et al., 2022). But this relation needs more studies to be better comprehended. For *S. xanthotricha*, increased food intake was observed following difenoconazole exposure, yet no significant histological changes were detected. This parallels the compensatory feeding behavior documented in bumblebees (Tosi & Nieh, 2019).

From a conservation perspective, our results found a strong warning about tebuthiuron's impacts on *P. helleri* while offering indications of *S. xanthotricha*'s resilience. The stark differences between these sympatric species emphasize that stingless bees cannot be treated as a uniform group in risk assessments. Current regulatory frameworks based on *A. mellifera* data clearly fail to protect more sensitive native species (Burgarelli et al., 2023; Silva et al., 2025). We echo their call for species-specific testing protocols and highlight the urgent need to investigate how pesticide formulations, which often prove more toxic than active ingredients alone, affect tropical pollinators (Prado et al., 2020).

Future research should prioritize longitudinal studies of colony-level impacts, as our 96-hour exposure window may miss chronic effects evident in previous works with A. mellifera and midgut health after exposure to pesticides (Almasri et al. 2022). Investigating the molecular basis of *S. xanthotricha*'s apparent tolerance could yield insights into evolutionary adaptations to fungicide exposure. Finally, field monitoring of pesticide residues in stingless bee habitats, combined with controlled studies of mixture toxicity, will be essential for developing evidence-based conservation strategies in agricultural landscapes.

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