UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(BIOLOGIA CELULAR, MOLECULAR E MICROBIOLOGIA)

EXPRESSÃO GÊNICA DIFERENCIAL DOS TRANSCRIPTOMAS DE TÚBULOS DE MALPIGHI DE *Melipona scutellaris* EXPOSTOS AO TIAMETOXAM

LUCAS MIOTELO

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Orientador: Prof. Dr. Osmar Malaspina
Co-orientadora: Dra. Milene Ferro

Dissertação apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Biologia Celular, Molecular e Microbiologia.

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TÍTULO DA DISSERTAÇÃO: **EXPRESSÃO GÊNICA DIFERENCIAL DOS TRANSCRIPTOMAS DE TÚBULOS DE MALPIGHI DE Melipona scutellaris EXPOSTOS AO TIAMETOXAM**

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RESUMO

No Brasil o consumo de inseticidas neonicotinóides, como o tiametoxam (TMX), é uma prática comum em culturas agrícolas. O uso indiscriminado de TMX afeta insetos não alvo, como as abelhas. *Melipona scutellaris* é uma espécie de abelha sem ferrão que foi inserida em uma lista do IBAMA, juntamente com o TMX, para que sejam realizados estudos toxicológicos. O presente estudo realizou uma análise de expressão gênica diferencial dos túbulos de Malpighi (Mt) de *M. scutellaris* expostas à $CL_{50/100}$ do TMX por meio da análise transcriptômica. Além disso, foi avaliada a imunomarcação das proteínas de choque térmico (HSP) 70 e 90, juntamente com a busca por HSPs expressas no transcriptoma e a aplicação do método de TUNEL para detectar morte celular. Após a montagem *de novo* do transcriptoma, foram identificados 237 genes diferencialmente expressos (DEGs) e agrupados em nove clusters. Foram encontrados DEGs envolvidos em processos de destoxificação, excreção, regeneração tecidual, respiração celular, estresse oxidativo, apoptose, sinalização celular e sistema imune. A imunomarcação de HSP70 aumentou em um dia e diminuiu em oito dias de exposição ao TMX. Enquanto a HSP90 diminuiu em um dia e aumentou em oito. Já o método de TUNEL detectou fragmentação de DNA apenas em oito dias. Além disso, não existem DEGs relacionados a HSPs e por meio da análise de TPM (*trascrits per milion*) foi identificada que uma isoforma de HSP70 foi a proteína de choque térmico mais expressa. Considerando os resultados do transcriptoma, o metabolismo celular em Mt foi afetado principalmente após oito dias de exposição. Nove genes foram selecionados de diferentes *clusters* e validados por RT-qPCR. De acordo com nossos resultados, o TMX promove vários tipos de danos nas células dos Mt à nível molecular. Portanto, a interferência em diferentes processos celulares pode afetar diretamente a saúde de *M. scutellaris*, comprometendo funções essenciais dos Mt. Já os dados obtidos pela imunomarcação e análise do transcriptoma são conflitantes uma vez que não há nenhum DEG e a imunomarcação evidencia diferenças entre os grupos controle e exposto. Essas diferenças podem ser resultantes da alta sensibilidade desses imunomarcadores. O presente estudo foi o primeiro a avaliar os efeitos de um neonicotinóide na expressão gênica de uma abelha sem ferrão e evidencia a importância de análises moleculares para estudos ecotoxicológicos.

Palavras-chave: Abelha sem ferrão, destoxificação, neonicotinóides, montagem *de novo*, sequenciamento, HSP70, HSP90, TUNEL.

ABSTRACT

In Brazil, using neonicotinoid insecticides, such as thiamethoxam (TMX), is common in crops. The indiscriminate use of TMX affects non-target insects such as bees. For example, *Melipona scutellaris* is a species of stingless bee that was included in an IBAMA list, along with TMX, for toxicological studies to be carried out. The present study analyzed differential gene expression of Malpighian tubules (Mt) of foragers bees of *M. scutellaris* exposed to $LC_{50/100}$ of TMX through transcriptomic analysis. In addition, the immunostaining of heat shock proteins (HSP) 70 and 90 were evaluated, searching for HSPs expressed in the transcriptome and applying the TUNEL method to detect cell death. After de novo assembly, 237 differentially expressed genes (DEGs) were identified and grouped into nine clusters. DEGs involved in detoxification, excretion, tissue regeneration, cellular respiration, oxidative stress, apoptosis, cell signaling, and the immune system were found. HSP70 immunostaining increased within one day and decreased within eight days of exposure to TMX. At the same time, HSP90 decreased by one day and increased by eight. The TUNEL method detected DNA fragmentation only in eight days. In addition, there are no DEGs related to HSPs, and through the analysis of TPM (transcripts per million), it was identified that HSP70 was the most expressed heat shock protein. Regarding the transcriptome results, cell metabolism in Mt was mainly affected after eight days of exposure. Nine genes were selected from different clusters and validated by RT-qPCR. According to our results, TMX promotes several types of damage in Mt cells at the molecular level. Therefore, interference in different cellular processes directly affects the health of *M. scutellaris*, compromising essential functions of Mt. The data obtained for HSPs (immunostaining and transcriptome analysis) are conflicting since there are no DEGs on the transcriptome. However, the immunostaining showed differences between the control and exposed groups. These differences may result from the high sensitivity of this immunomarkers. The present study was the first to evaluate the effects of a neonicotinoid on the gene expression of stingless bees and highlighted the importance of molecular analyzes for ecotoxicological studies.

Keywords: *De novo* assembly, neonicotinoid, stingless bee, detoxification, sequencing, HSP70, HSP90, TUNEL.

Sumário

INTRODUÇÃO GERAL

Estima-se que existam cerca de 20.000 espécies de abelhas descritas no mundo (MICHENER, 2007); essa diversidade contribui para a realização de serviços ecossistêmicos, como a polinização, em diferentes ambientes (POTTS et al., 2016). No Brasil são descritas 244 espécies de abelhas sem ferrão (PEDRO, 2014) e segundo Roubik (2006), formam um grupo 50 vezes mais diverso do que o grupo das abelhas melíferas (e.x.: *Apis mellifera* africanizada Linnaeus, 1758).

Dentre as abelhas sem ferrão, algumas espécies vêm sendo visadas para a exploração de mel (ALVES, 2013) ou para a inserção de colônias em sistemas agrícolas e/ou naturais a fim de utilizá-las como agentes polinizadores (CHAM et al., 2018). Em plantações agrícolas de interesse econômico, as abelhas sem ferrão podem efetivamente polinizar as seguintes culturas: morango, citrus, abacate, pêssego, girassol, açaí, dentre outras (KLEIN et al., 2020). Especificamente em culturas de tomate, urucum, berinjela e pimentão é necessário que o agente polinizador seja capaz de realizar a polinização por vibração (DE LUCA; VALLEJO-MARÍN, 2013). Ao contrário de *A. mellifera*, incapaz de efetuar esse tipo de polinização (DE LUCA; VALLEJO-MARÍN, 2013), as abelhas sem ferrão, como *Melipona scutellaris* Latreille, 1811, podem polinizar com sucesso plantas com deiscência poricida por meio da vibração da musculatura do tórax (KLEIN et al., 2020).

M. scutellaris, pertencente ao gênero *Melipona*, é uma abelha sem ferrão nativa da região nordeste do Brasil, no entanto, é bem adaptada as condições ecológicas e climáticas do estado de São Paulo (PEDRO; CAMARGO, 1999). Além disso, é uma espécie de fácil manejo na qual a produção e comércio de alguns produtos de alto valor econômico, como o mel, própolis e o pólen vem sendo cada vez mais explorados (COSTA et al., 2015). Os meliponíneos, tribo a qual a espécie *M. scutellaris* pertence, polinizam cerca de 66% das 1.500 espécies cultivadas em áreas agrícolas no mundo (KREMEN; WILLIAMS; THORP, 2002).

Mesmo não sendo o alvo dos agrotóxicos, as abelhas são expostas a essas moléculas por meio de diferentes rotas, como: néctar, pólen, lama/solo, cera, água, superfícies de plantas, dentre outras (BOYLE et al., 2019). Neste contexto, é necessário refletir como as mudanças antropogênicas afetam a saúde, a abundância e a diversidade das abelhas (BIESMEIJER et al., 2006; GOULSON et al., 2015; IMPERATRIZ-FONSECA; SARAIVA; JONG, 2006). Devido a expansão agrícola e o uso indiscriminado de agrotóxicos, estudos que visam entender como é a ação de agrotóxicos na vida destes organismos vêm sendo realizados pelo nosso grupo de

pesquisa e por grupos associados (COSTA et al., 2015; DOMINGUES et al., 2020; MIOTELO et al., 2021, 2022). No entanto, para *M. scutellaris* estudos ecotoxicológicos ainda são escassos.

Tiametoxam - TMX ($C_8H_{10}ClN_5O_3S$) é um inseticida neonicotinóide derivado da nicotina, classificado como N-nitroguanidina (BASS; FIELD, 2018; TOMIZAWA; CASIDA, 2005); que age no sistema nervoso central como agonista nos receptores nicotínicos de acetilcolina. Em condições normais, a acetilcolina é facilmente hidrolisada pela enzima acetilcolinesterase (BASS; FIELD, 2018; FAIRBROTHER et al., 2014), no entanto, os neonicotinóides não são imediatamente degradados, promovendo a transmissão de impulsos nervosos de forma contínua e descontrolada, o que causa hiperexcitação do sistema nervoso central dos insetos, podendo resultar na morte do indivíduo (BASS; FIELD, 2018; EL HASSANI et al., 2008). Além disso, o tiametoxam apresenta características sistêmicas, portanto, mesmo quando usado no tratamento de sementes é possível encontrar resíduos no néctar e no pólen das plantas (FORD; CASIDA, 2008; GOULSON, 2013; THOMPSON et al., 2018). Como consequência da exposição a concentrações subletais do TMX, importantes funções para a sobrevivência das abelhas podem ser comprometidas, como: alimentação, forrageamento, mobilidade, orientação e aprendizado (DECOURTYE et al., 2009; FISCHER et al., 2014; GILL; RAMOS-RODRIGUEZ; RAINE, 2012; HENRY et al., 2012; LAYCOCK et al., 2012).

Uma vez que o TMX é um inseticida neurotóxico, os estudos toxicológicos tendem a focar o cérebro como órgão alvo para análises. No entanto, quando as abelhas são expostas por via oral, o alimento contaminado percorre a rota de metabolização e excreção do composto no organismo, podendo atingir órgãos não alvo como o intestino e os túbulos de Malpighi (CRUZ-LANDIM, 2009; MIOTELO et al., 2022).

Em abelhas, os túbulos de Malpighi são estruturas finas e alongadas responsáveis pela excreção; constituídos por uma faixa de células epiteliais que estão sobre uma lâmina basal. Liberam as excretas na região pilórica do tubo digestório e organizam-se livremente na cavidade abdominal (CRUZ-LANDIM, 2009). Este órgão entra em contato com o inseticida, com metabólitos do inseticida e com moléculas não metabolizadas, durante a rota de metabolização do composto. Além disso, segundo Miotelo e colaboradores (2022), baixas concentrações de TMX apresentam efeitos citotóxicos significativos para os túbulos de Malpighi de *M. scutellaris*. Os autores reportaram que a exposição subletal do TMX em um e oito dias causam os seguintes danos celulares: aumento de esferocristais, perca de microvilosidades na porção apical das células, desorganização do labirinto basal, perda de

material citoplasmático, núcleos com formato irregular e cromatina condensada. Esses danos podem comprometer processos essenciais para a sobrevivência de *M. scutellaris*, como a excreção e destoxificação.

A função primária do sistema excretor está relacionada com a regulação da homeostase, consistindo em manter o meio interno de um organismo nas melhores condições possíveis para que ocorra um bom funcionamento celular (CRUZ-LANDIM, 2009). Para garantir a homeostase da célula é preciso manter constante os níveis de sais, água, pH, pressão osmótica e eliminar produtos potencialmente tóxicos (CRUZ-LANDIM, 2009). Por se tratar de um órgão pequeno e delicado, geralmente são realizados estudos para compreender como a morfologia das células é afetada quando expostas a algum agrotóxico. Em alguns casos os pesquisadores optam por análises utilizando microscopia de luz (FERREIRA et al., 2013; GRELLA et al., 2019; ROSSI et al., 2013) e/ou microscopia eletrônica de transmissão (CATAE et al., 2014; FERREIRA et al., 2013; FRIOL et al., 2017; MIOTELO et al., 2022).

Em contraste com os efeitos morfológicos e fisiológicos, incluindo características relevantes sobre a população, pouco se sabe sobre os efeitos moleculares dos neonicotinóides em abelhas (CHRISTEN et al., 2018). Estudos que aplicam abordagens moleculares usam, quase que exclusivamente, a espécie modelo *A. mellifera* pois foi a primeira espécie de abelha a ter o genoma sequenciado (GROZINGER; ZAYED, 2020). Apesar disso, considerando o território nacional, ainda são escassos estudos moleculares até mesmo para *A. mellifera*. Por outro lado, existem estudos na Europa que elucidam os efeitos de concentrações ambientalmente relevantes de neonicotinóides, demonstrando que esses induzem efeitos transcricionais significativos em vários genes-chave associados à neurotoxicidade, formação de memória, respostas ao estresse, metabolismo e expectativa de vida, bem como a regulação do sistema imunológico em exposições de laboratório e de campo (CHRISTEN et al., 2018; CHRISTEN; BACHOFER; FENT, 2017; FENT et al., 2020; SHI et al., 2017).

Os avanços nas tecnologias e ferramentas de sequenciamento de nova geração (NGS) superaram diversas limitações. As abordagens ômicas, como genômica populacional, transcriptômica e metagenômica, podem fornecer a resolução e a eficiência necessárias para compreender melhor o declínio nas populações de abelhas (GROZINGER; ZAYED, 2020). Os avanços das técnicas moleculares permitem identificar genes centrais, vias metabólicas e comunidades microbianas associadas a abelhas sob condições de estresse (GROZINGER; ZAYED, 2020). Estudos que visam analisar o transcriptoma (conjunto de transcritos expressos numa determinada condição) de um determinado organismo passaram a ser impulsionados a

partir da introdução do NGS (METZKER, 2009). Essa técnica permite avaliar sequencias de RNA convertidas em cDNA em larga escala, além de analisar fragmentos maiores de RNA com alto nível de precisão e sensibilidade (MARTIN; WANG, 2011; OZSOLAK; MILOS, 2010).

A transcriptômica pode facilitar a compreensão das respostas moleculares, fisiológicas e comportamentais de abelhas sob condições de estresse, permitindo o desenvolvimento de abordagens que podem aumentar a resistência e resiliência dessas abelhas (GROZINGER; ZAYED, 2020). Estudos transcriptômicos utilizando NGS têm sido fundamentais na caracterização dos mecanismos moleculares e fisiológicos pelos quais as abelhas respondem a diversos estressores, além de fornecerem possíveis ferramentas para um diagnóstico rápido da saúde das abelhas (GROZINGER; ZAYED, 2020). Recentemente, os estudos que utilizaram análises de sequenciamento de RNA para avaliar mudanças nos padrões de expressão gênica foram conduzidos em abelhas parasitadas (ZANNI et al., 2017), infectadas com vírus (BRUTSCHER; DAUGHENBAUGH; FLENNIKEN, 2017), expostas a agrotóxicos (CHRISTEN; BACHOFER; FENT, 2017; FENT; SCHMID; CHRISTEN, 2020; SHI et al., 2017), induzidas a estresse nutricional (CORBY-HARRIS et al., 2014), expostas a múltiplos estressores (AZZOUZ-OLDEN; HUNT; DEGRANDI-HOFFMAN, 2018) e/ou submetidas a diferentes temperaturas (AMSALEM et al., 2015; DURANT et al., 2016; TORSON et al., 2017). No entanto, todos esses estudos foram realizados com diferentes subespécies de *A. mellifera*.

Quando se trata da análise de organismos não-modelo é comum utilizar uma abordagem de montagem *de novo* do transcriptoma, uma vez que não há um genoma de referência para realizar a montagem das bibliotecas de transcritos (CHRISTEN et al., 2018). Dessa forma o método *de novo* configura uma boa estratégia para estudos de expressão gênica com a espécie de abelha *M. scutellaris*, uma vez que apenas o genoma mitocondrial da espécie está disponível (SILVERIO et al., 2014). A expressão gênica de um organismo pode variar com as condições ambientais, estado de desenvolvimento e tecido de origem (RUDD, 2003) e a avaliação da abundância dos diferentes transcritos pode estimar a expressão gênica de determinada célula ou tecido (BOUCK; VISION, 2007), permitindo a identificação de genes relacionados aos processos de intoxicação por neonicotinóides ou ainda reconhecer os genes relacionados aos processos de destoxificação em abelhas.

Além disso, acessar os níveis de expressão de HSP e morte celular por apoptose nos túbulos de Malpighi de abelhas contaminadas a agrotóxicos e abelhas não contaminadas pode representar uma abordagem promissora para detectar e diagnosticar rapidamente insetos

contaminados com TMX (MALASPINA; SILVA-ZACARIN, 2006a). Quando as células são expostas à ambientes quentes, acima de suas temperaturas normais de crescimento, são produzidas chaperonas moleculares denominadas proteínas de choque térmico (HSP) (ALBERTS et al., 2015; CANDIDO, 2001). Essas proteínas foram descobertas em *Drosophila melanogaster*, porém, atualmente a síntese dessas proteínas é descrita como um fenômeno universal, uma vez que, ocorre em todas as espécies de animais e plantas estudadas (CANDIDO, 2001; ELEKONICH, 2009; ZHAO et al., 2010). São caracterizadas e nomeadas de acordo com seu peso molecular (SCHLESINGER, 1990) e, em insetos, são descritas quatro principais famílias de HSPs, sendo elas: HSP90, HSP70, HSP60 e as sHSPs de baixo peso molecular (16 – 27 kDa) (COLLIER; BENESCH, 2020; DUBREZ et al., 2020; FEDER; HOFMANN, 1999; KING; MACRAE, 2015).

Também podem ser chamadas de proteínas de estresse pois células expostas a produtos químicos prejudiciais ou eventos que proporcionem estresse celular tendem a diminuir a síntese da maioria das proteínas, no entanto, a presença das HSPs aumenta (CANDIDO, 2001; KING; MACRAE, 2015). Este aumento pode ocorrer em resposta à estressores ambientais, infecções, luz UV, agrotóxicos, frio, calor, hipóxia, fome, dentre outros (DUBREZ et al., 2020; ZHANG; OHASHI; RIKIHISA, 1998). As HSPs são chaperonas dependentes de ATP, com exceção das sHSPs que são independentes e representam a primeira linha de defesa celular, prevenindo a desnaturação irreversível de proteínas enquanto as células estão submetidas a condições de estresse (BASHA; O'NEILL; VIERLING, 2012; KING; MACRAE, 2015). Neste contexto, sua principal função é manter as proteínas em um estado de conformação funcional, além de auxiliar no dobramento de novas proteínas sintetizadas (DUBREZ et al., 2020; KING; MACRAE, 2015).

Embora as chaperonas dependentes de ATP também sejam capazes de redobrar e desagregar proteínas desnaturadas, algumas HSPs atuam como potentes proteínas antiapoptóticas. Após se associar e bloquear as principais proteínas apoptóticas, as HSPs mantem as células vivas durante eventos de estresse junto com processos de desenvolvimento e diferenciação (DUBREZ et al., 2020). A interação de HSPs com outras proteínas também pode influenciar processos essenciais como: síntese de proteínas, sinalização celular, transcrição e metabolismo (KING; MACRAE, 2015; SOTTILE; NADIN, 2018). Em resumo, as funções mais importantes atribuídas às HSPs são: transporte de proteínas para compartimentos celulares; dobramento de proteínas no citosol, mitocôndrias e retículo endoplasmático; prevenção de agregações de proteínas; controle de proteínas regulatórias;

degradação de proteínas instáveis; redobramento de proteínas mal dobradas e dissolução de complexos de proteínas (BUKAU; HORWICH, 1998).

Dentre as principais famílias de HSPs, considerando a regulação da apoptose, HSP27 e HSP70 são denominadas antiapoptóticas (GARRIDO et al., 2001). A família da HSP70 é considerada o grupo mais conservado dentre todas as HSPs (BEERE; GREEN, 2001) e atuam em vários pontos nas rotas de sinalização de apoptose, sugerindo que sua ação determinará o destino das células sob estresse (BEERE et al., 2000; KURASHOVA; MADAEVA; KOLESNIKOVA, 2020; MOSSER et al., 2000; RAVAGNAN et al., 2001). Além disso, corrigem mudanças conformacionais em proteínas e contribuem com respostas primárias ao estresse oxidativo, protegendo proteínas e enzimas dos efeitos de EROs (KURASHOVA; MADAEVA; KOLESNIKOVA, 2020). Já as HSP90, embora também sejam produzidas durante eventos de estresse, ainda não existe um consenso se atuam de forma anti ou pró apoptose uma vez que respondem de maneiras diferentes à estresse (KING; MACRAE, 2015).

Uma das técnicas amplamente utilizadas para avaliar a morte celular é o método TUNEL (*Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling*) (GAVRIELI; SHERMAN; BEN-SASSON, 1992). Quando as células estão em processo de morte as endonucleases clivam o DNA em muitos fragmentos, gerando várias extremidades livres de DNA (ALBERTS et al., 2015). Consequentemente, o método TUNEL é capaz de detectar o dano usando a capacidade da enzima desoxinucleotidil transferase terminal (TdT) que transfere cadeias de desoxinucleotídeo marcados (dUTP) para os terminais 3'-hidroxil (3'-OH) dos fragmentos (GAVRIELI; SHERMAN; BEN-SASSON, 1992). Assim, o método TUNEL aliado as HSPs podem ser utilizados para entender como as células estão enfrentando os danos moleculares causados por concentrações ambientalmente relevantes de agrotóxicos e avaliar seu nível de severidade para as abelhas, pois ambos representam uma ótima alternativa para diagnóstico biológico rápido (MALASPINA; SILVA-ZACARIN, 2006b).

OBJETIVOS

Objetivo geral

O presente estudo visou realizar a análise dos genes diferencialmente expressos nos túbulos de Malpighi da abelha sem ferrão *M. scutellaris* submetidos à $CL_{50/100}$ do inseticida TMX em dois períodos de tempo (um e oito dias). Além de identificar as possíveis alterações morfofisiológicas causadas pelo TMX nos túbulos de Malpighi de *M. scutellaris*, por meio da imunomarcação de proteínas de estresse celular e morte celular por fragmentação do DNA e correlacionar com os dados dos transcriptomas.

Objetivos específicos

- Identificar os genes diferencialmente expressos entre os grupos controle e TMX em um e oito dias de exposição a uma concentração subletal.
- Identificar as vias metabólicas putativamente afetadas pelo TMX, com especial atenção às funções primárias dos túbulos de Malpighi: destoxificação, excreção e osmorregulação.
- Avaliar genes que possam estar associados a estresse oxidativo e morte celular.
- Analisar as possíveis alterações morfofisiológicas causadas pelo TMX nos túbulos de Malpighi por meio das técnicas de imunomarcação de HSP70 e HSP90.
- Selecionar, no transcriptoma, apenas genes associados com HSPs e por meio da análise de TPM verificar quais HSPs estão sendo mais expressas.
- Detectar indícios de morte celular por meio da técnica de TUNEL.

Capítulo 1

O artigo referente a este capítulo foi submetido para publicação na revista **Science of the Total Environment**. Portanto, está editado conforme as normas da revista.

Transcriptomic analysis of Malpighian tubules from the stingless bee *Melipona scutellaris* reveals thiamethoxam-induced damages

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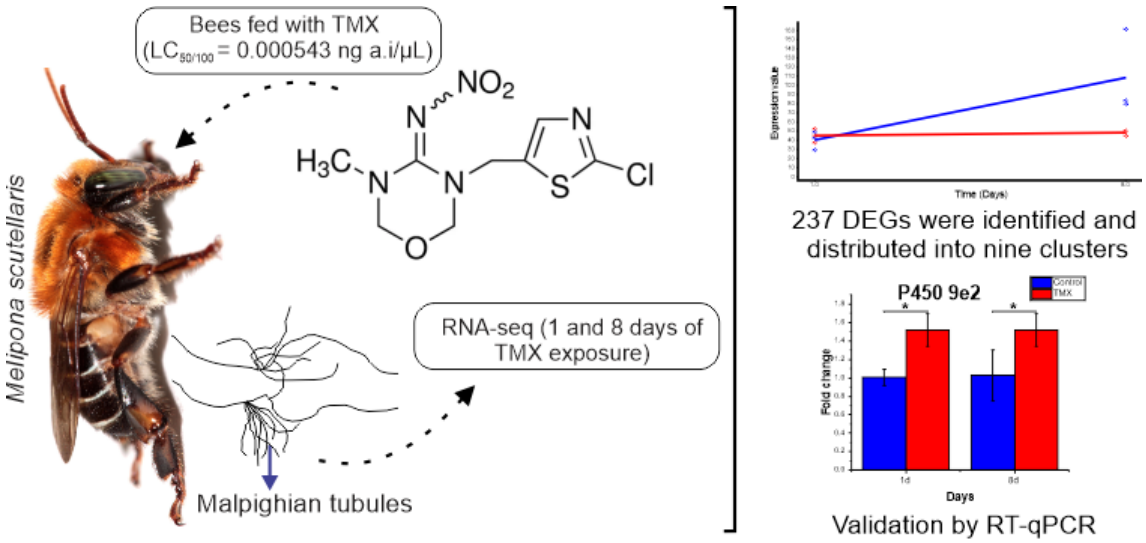
Graphical Abstract



Highlights

Melipona scutellaris was exposed to a sublethal concentration of thiamethoxam.

More the 200 genes were differentially expressed in the presence of TMX.

Malpighian tubules were selected as non-target organs for transcriptome analyses.

Oxidative stress, energy metabolism, and cell death are pathways triggered by TMX.

Abstract

The concern about pesticide exposure to neotropical bees has been increasing in the last few years, and knowledge gaps have been identified. Although stingless bees, (e.g.: *Melipona scutellaris*), are more diverse than honeybees and they stand out in the pollination of several valuable economical crops, toxicity assessments with stingless bees are still scarce. Nowadays new approaches in ecotoxicological studies, such as omic analysis, were pointed out as a strategy to reveal mechanisms of how bees deal with these stressors. To date, no molecular techniques have been applied for the evaluation of target and/or non-target organs in stingless bees, such as the Malpighian tubules (Mt). Therefore, in the present study, we evaluated the differentially expressed genes (DEGs) in the Mt of *M. scutellaris* after one and eight days of thiamethoxam (TMX) exposure. Through functional annotation analysis of four transcriptome libraries, the time course line approach revealed 237 DEGs (nine clusters) associated with carbon/energy metabolism and cellular processes (lysosomes, autophagy, and glycan degradation). The expression profiles of Mt were altered by TMX in processes, such as detoxification, excretion, tissue regeneration, oxidative stress, apoptosis, and DNA repair. Transcriptome analysis showed that cell metabolism in Mt was mainly affected after 8 days of exposure. Nine genes were selected from different clusters and validated by RT-qPCR. According to our findings, TMX promotes several types of damage in Mt cells at the molecular level. Therefore, interference of different cellular processes directly affects the health of *M. scutellaris* by compromising the function of Mt.

Keywords: *De novo* assembly, differentially gene expressed, excretion organ, neonicotinoid, sublethal effects.

Introduction

Pollination by insects is a valuable ecosystem service predominantly provided by bees (Potts et al., 2016). Among the 20,000 bee species reported (Michener, 2007), approximately 244 stingless bees species are found in Brazil (Pedro, 2014). *Melipona scutellaris* (Latreille, 1811), a stingless bee species from northeast Brazil, can perform buzz pollination (De Luca and Vallejo-Marín, 2013). Buzz pollination is characterized by vibration resulting from contractions of the bee's body muscles, thereby, releasing pollen grains (De Luca and Vallejo-Marín, 2013). Although *Apis mellifera* (Linnaeus, 1758) cannot perform buzz pollination, many stingless bee species significantly contribute to flowers with poricidal anthers, such as tomato, annatto, eggplant, and sweet pepper (De Luca and Vallejo-Marín, 2013; Klein et al., 2020). In fact, this bee species is especially relevant for Neotropical regions owing to its great potential as a commercial pollinator; this is because it is easily managed (Cham et al., 2018), and the production and commercialization of propolis, honey, and pollen can be of high economic value (Costa et al., 2015).

In the last few decades, there have been reports of significant decline in bees diversity (Potts et al., 2016; Zattara and Aizen, 2021). Consequently, plant species diversity may decline, compromising food security (Biesmeijer et al., 2006; Potts et al., 2016). One of the causes of this decrease in bee diversity is related to agricultural landscapes and practices (Kremen et al., 2002; Potts et al., 2010). In this scenario, bees are exposed to several agricultural pesticides (Boyle et al., 2019), such as those within the neonicotinoid class (Bass and Field, 2018). Thiamethoxam (TMX) is a highly active neurotoxin that acts as an agonist of nicotinic acetylcholine receptors (AChRs) (Bass and Field, 2018), and affects the nervous system, compromising orientation, learning, and memory (Decourtye et al., 2004; Roat et al., 2020). Owing to the systemic characteristics of TMX, even if used in seed treatment, residues can be found in the nectar and pollen of flowers (Ford and Casida, 2008; Thompson et al., 2018). Sublethal exposure of TMX has been reported to affect the brain (Christen et al., 2018; Miotelo et al., 2022, 2021; Roat et al., 2020) and non-target organs, such as the midgut and Malpighian tubules (Mt) (Miotelo et al., 2022).

Bees can come into contact with insecticides through different routes, such as particles in the air, mud/soil, water, plant surfaces, propolis/resin, nectar, and pollen (Boyle et al., 2019). Upon oral exposure, TMX can affect organs that are not their target, such as the Mt. Accordingly, the organs related to the routes of absorption, metabolism of pesticide residues, and excretion must be evaluated (Miotelo et al., 2022). Mt is responsible for osmoregulation,

excretion, and detoxification (Nocelli et al., 2016). Few studies have evaluated the cytotoxic effects of pesticides on Mt using light microscopy (Ferreira et al., 2013; Rossi et al., 2013) or transmission electron microscopy (Catae et al., 2014; Ferreira et al., 2013; Friol et al., 2017; Miotelo et al., 2022). However, to date, there is no available information regarding the molecular effects of TMX on this organ.

Studies that apply molecular approaches almost exclusively use the model species, *Apis mellifera* Linnaeus 1758 (Grozinger and Zayed, 2020). These studies revealed the effects of environmentally relevant concentrations of neonicotinoids and the significant transcriptional changes in several key genes associated with neurotoxicity, memory formation, stress responses, metabolism, and lifespan (Christen et al., 2018, 2017; Fent et al., 2020; Shi et al., 2017). Next-generation sequencing and *de novo* transcriptome assembly enable advances in knowledge of non-model organisms (Grozinger and Zayed, 2020). The transcriptome helps to understand how bees respond to environmental stressors, showing molecular, physiological, and behavioral responses (Christen et al., 2018; Grozinger and Zayed, 2020).

Since 2017, the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA) has stimulated research on stingless bees and neonicotinoids (IBAMA, 2017). An omics approach can improve the comprehension of stressors that undermine bee health. Further, the transcriptome allows the evaluation of mechanisms and pathways by which bees deal with these stressors (Grozinger and Zayed, 2020). According to Miotelo et al. (2022), the Mt of *M. scutellaris* displays ultrastructural damage when exposed to an environmentally relevant concentration of TMX. Therefore, the present study aimed to analyze the differentially expressed genes (DEGs) in the Mt of the stingless bee, *M. scutellaris*. Further, by using RNA-sequencing (RNA-seq) and time course analysis, we evaluated how TMX influenced gene expression and molecular pathways at two different times of exposure while promoting several damages in Mt cells. To our knowledge, this is the first study to employ transcriptome analyses to investigate the molecular effects of pesticides on stingless bees and non-target organs.

Material and methods

2.1 Oral exposure to TMX

Forager bees of *M. scutellaris* were collected from three healthy colonies in the meliponary of São Paulo State University (UNESP), Institute of Biosciences, Rio Claro, SP, Brazil. Bees were collected in 250 mL plastic cages containing holes side-drilled for air exchange. The experimental group consisted of 220 bees divided into two groups: a control group (110 bees) and a TMX group (110 bees). Thiamethoxam PESTANAL®

($C_8H_{10}ClN_5O_3S$), an analytical standard (purity ≥98.0%), was purchased from Sigma-Aldrich and solubilized in water. The sublethal concentration of TMX was based on the mean lethal concentration of *M. scutellaris* (LC_{50} = 0.0543 ng a.i./μL), as determined by Miotelo et al. (2021). A stock solution of 1000 ng of TMX was prepared (10 mg of TMX in 10 mL of deionized water) and subjected to serial dilution to achieve the desired concentration ($LC_{50/100}$ = 0.000543 ng a.i./μL). A sugar solution (water and sugar: 50% w/v) was used to perform the serial dilutions. Of note, the control group only received a sugar solution without any contaminants. Microtubes (2 mL) containing side-drilled holes were provided as feeders, and syrup was provided *ad libitum*. After exposure to TMX, the experimental cages were kept in an incubator (chamber of biochemical oxygen demand, B.O.D) at 28 ± 2 ºC, relative humidity of 70 ± 05%, and constant darkness. The exposure assay was carried out according to the recommendations of the Organization for Economic Cooperation and Development (OECD) (1998), with adaptation to stingless bees. Based on the mean lethal time determined by Miotelo et al. (2022), the bees were exposed to TMX for eight days and the molecular influence of the insecticide was evaluated after one and eight days of exposure.

2.2 RNA extraction

Mts were dissected after one and eight days of exposure to TMX. On each day of the dissection, 27 bees from each group were employed. The control and TMX groups had three microtubes containing a pool of nine bees each (three bees per colony). The organs were stored in RNAlater throughout the dissection process. Total RNA was extracted using the QIAGEN® RNeasy Mini Kit, according to the manufacturer's instructions. RNA integrity was verified via agarose gel electrophoresis (1% w/v). Samples with total RNA were sent to Macrogen (South Korea) for purity and concentration quality control. Macrogen also constructed a cDNA library (TruSeq stranded mRNA library). Thereafter, sequencing was performed using Illumina NovaSeq6000, paired-end 2 × 100 bp, according to the manufacturer's protocol (Macrogen, South Korea).

2.3 Preprocessing reads, *de novo* assembly, and functional annotation

The FastQC tool provided by Babraham Bioinformatics was used for read control (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). A common phenomenon, known as deviation in the GC content, was observed at the beginning of the reads; this was derived from sequencing on the Illumina platform (Conesa et al., 2016; Hansen et al., 2010). Briefly, cuts were made in the first 15 nucleotides of all reads; this removal was performed using the fastx_trimmer tool available in the FASTXToolkit

(http://hannonlab.cshl.edu/fastx_toolkit/index.html). Low-quality raw reads (Phred Q score <30) were trimmed using the *SeqyClean* software (https://github.com/ibest/seqyclean) (Zhbannikov et al., 2017). The normalization protocol incorporated in Trinity (version 2.2.0) (Grabherr et al., 2013) was applied to increase the efficiency of assembly reads (Brown et al., 2012). The libraries were *de novo* assembled using Trinity default parameters. At the end of *de novo* assembly, the completeness of the assembly was evaluated using BUSCO v.5 (Waterhouse et al., 2018) based on gene databases containing universal single-copy orthologs (OrthoDB) for the order, Hymenoptera_odb10.

2.4 Differential gene expression analysis

Bowtie2 tool (Langmead and Salzberg, 2012) and RSEM (Li and Dewey, 2011) were used to map and quantify the reads of each library in the assembled reference transcriptome. This process was performed using the OmicsBox software (BioBam, Valencia, Spain) (Götz et al., 2008). Differential expression analysis was performed using the maSigPro tool in the OmicsBox software to verify related changes over the time of exposure to the insecticide and between the experimental groups using the time-course expression approach (Nueda et al., 2014; Pradhan et al., 2020). DEGs were grouped into nine clusters. Gene annotation was performed on OmicsBox using BlastX (Altschul et al., 1990) with an E-value of 1e-03 against the NCBI nonredundant (NR) database. Gene Ontology (GO) functional classifications into the three main categories were carried out: Biological Processes (BP), Molecular Function (MF), and Cell Component (CC). Domain proteins were found with InterProScan (Zdobnov and Apweiler, 2001), followed by the attribution of Enzyme Commission (EC) terms. Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation was performed to obtain pathway annotations for the DEGs (Christen et al., 2018).

2.5 Gene validation by RT-qPCR

The DEGs selected for validation by qRT-PCR were *P450 9e2*, *SUN1*, *BAI1*, *BolA*, *D3PD*, *AQP*, *UDP*, and *ADAM-17* (Supplementary table 1). The previously extracted RNA (see the section on RNA extraction) was used for cDNA synthesis using the RevertAid H minus First-Strand cDNA Synthesis Kit (Thermo Scientific, USA). Samples containing 1646 ng of total RNA were used to validate the RNA sequencing data. Each selected gene was applied in quadruplicate (technical replicates), and no template control (NTC) was used. The reaction mixture (20 µL) was added to each well. This mixture contained 10 µL Power Track SYBR Green Master Mix (Applied Biosystems), 1 µL of each gene-specific primer (Forward and Reverse), 0.6 µL of cDNA, and 0.5 µL of 40x inert yellow dye (SYBR Kit). Gene-specific

primers were designed using the Gene Runner software (Version 6.5.52). The qRT-PCR cycling conditions were as follows: holding stage of 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, and annealing/extension at 60 °C for 30 s. Melt curve analysis of each primer was performed following PCR amplification to rule out the possibility of primer-dimers and non-specific product formation. Ribosomal protein s5 was used as the reference gene for data normalization. The comparative $2^{-\Delta\Delta Ct}$ method (based on C_T values) was used to determine the expression level of each gene. Differences between treatments were assessed using one-way ANOVA followed by Tukey's test to compare treatment means with respective controls. Differences were considered statistically significant at $p < 0.05$.

2.6 Accession numbers

The raw sequence data from the transcriptome of Mts of *M. scutellaris* are available in the Short Read Archive (SRA) GenBank database: BioProject (PRJNA836917), BioSample (SAMN28188996–SAMN28188998, SAMN28189016–SAMN28189021, SAMN28189023–SAMN28189025), and SRA (SRR19178717–SRR19178728).

Results

3.1 Transcriptome assembly

After cleaning raw reads, normalizing, and performing *de novo* assembly, 97,706 contigs and 58,208 genes with a GC content of 37.16 were identified. In addition, an N50 value of 3736 was obtained. BUSCO software revealed the completeness and integrity of the assembled transcriptome against the Hymenoptera ortholog bank, indicating that 84% of the genes were complete (single-copy and duplicated).

3.2 DEGs

Using time course analysis, 237 DEGs with significant temporal expression changes and differences between treatments (control and TMX group) were identified and divided into nine clusters (Figure 1). Genes in clusters two, three, four, six, and nine were upregulated after one day of exposure to TMX, and those in clusters four, six, eight, and nine were upregulated after eight days. Although clusters five and seven were downregulated after one day of exposure to TMX, clusters one, two, three, and five were downregulated after eight days. There was no difference in the expression of the genes in clusters one and eight after one day of exposure, and cluster seven after eight days of exposure. To perform further analysis, all non-annotated (NA) genes were removed, and 74 DEGs were selected as genes of interest, as shown in Table 1.

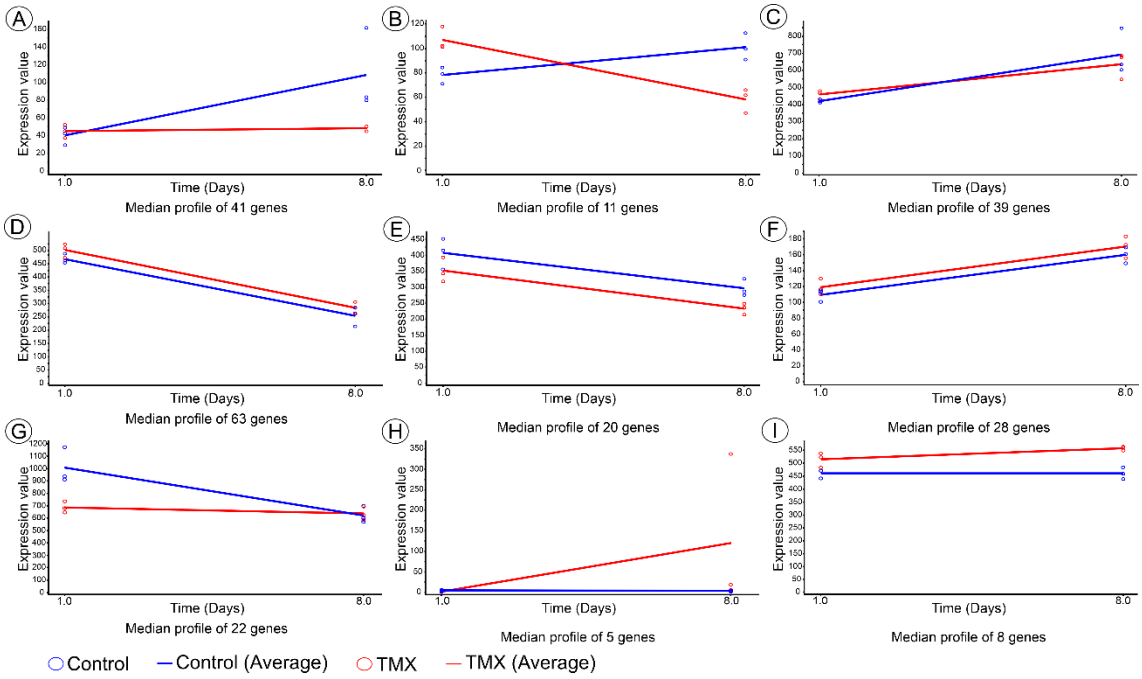


Figure 1 - Time course analysis showing differentially expressed genes grouped in clusters. Each plot represents a cluster of genes according to their average expression profile. Dots show the average expression values for each sample (blue and red dots and lines represent the control and TMX groups, respectively). Lines connect the average value of the gene expression at each time point. **A** – cluster one, **B** – cluster two, **C**- cluster three, **D** – cluster four, **E** – cluster five, **F** – cluster six, **G** - cluster seven, **H** – cluster eight and **I** – cluster nine.

Cluster 1						
Gene ID	**Description**	**E-value**	**1 day**	**8 days**	**Function**	**Abbreviation**
TRINITY_DN9117_c0_g1_i1	DNA replication complex GINS protein SLD5	3,80E-155	*	-	Tissue regeneration	*GINs*
TRINITY_DN7900_c0_g3_i1	BolA-like protein 3	1,73E-61	*	-	Detoxification	*BolA*
TRINITY_DN4348_c0_g1_i1	Longitudinals lacking protein, isoforms N/O/W/X/Y	2,55E-55	*	-	Signaling	*Lola1*
TRINITY_DN10521_c0_g1_i1	NADH dehydrogenase subunit 5	1,61E-102	*	-	Cellular Respiration/Oxidative stress	*NADH5*
TRINITY_DN17751_c1_g1_i1	Brain-specific angiogenesis inhibitor 1	4,26E-118	*	-	Phagocytosis/Apoptosis	*BAI1*
Cluster 2						
TRINITY_DN6431_c0_g3_i1	THO complex subunit 3	0	+	-	Oxidative stress/Apoptosis	*THO*
TRINITY_DN12574_c1_g1_i1	NADH dehydrogenase subunit 4	7,40E-93	+	-	Cellular Respiration/Apoptosis	*NADH4*
TRINITY_DN9947_c0_g2_i1	Homocysteine S-methyltransferase YbgG-like	0	+	-	Osmoregulation/Stress	*BHMT*
TRINITY_DN8571_c0_g1_i1	Vacuolar protein sorting-associated protein 37B	5,95E-56	+	-	Protein degradation	*Vps37*
Cluster 3						
TRINITY_DN17485_c0_g5_i8	mpv17-like protein isoform X1	7,04E-114	+	-	Oxidative stress	*Mpv17*
TRINITY_DN13120_c0_g1_i1	TGF-beta-activated kinase 1 and MAP3K7-binding protein 1-like isoform X1	0	+	-	Tissue regeneration	*TGFbeta*
TRINITY_DN17065_c0_g1_i4	Mitochondrial glycine transporter isoform X1	1,57E-167	+	-	Oxidative stress	*MGT*
TRINITY_DN16063_c1_g1_i10	SUN domain-containing protein 1	0	+	-	Nuclear morphology	*SUN1*
TRINITY_DN12548_c0_g1_i3	Equilibrative nucleoside transporter 3	0	+	-	Immune system	*ENT*
TRINITY_DN17904_c1_g1_i13	Tyrosine kinase receptor Cad96Ca	0	+	-	Tissue regeneration	*Cad96Ca*
TRINITY_DN17904_c1_g1_i4	Non-homologous end-joining factor 1	1,76E-85	+	-	DNA repair	*NHEJ*
TRINITY_DN14444_c0_g1_i1	Monocarboxylate transporter 2-like	0	+	-	Energy metabolism	*MCT2*
TRINITY_DN16944_c0_g1_i3	Spermatogenesis-associated protein 20 isoform X1	0	+	-	Oxidative stress	*SPATA20*
TRINITY_DN15630_c0_g1_i3	Spindle pole body component 110-like	0	+	-	Cell division	*SPB*
TRINITY_DN17366_c0_g1_i8	Dynein regulatory complex protein 1 isoform X2	0	+	-	Cell division	*CD*
TRINITY_DN14214_c0_g1_i1	Tektin-4	0	+	-	Cell division	*Tektin-4*
TRINITY_DN16528_c0_g1_i4	Inositol polyphosphate 1-phosphatase	0	+	-	Signaling/Stress	*INPP*

TRINITY_DN17537_c0_g1_i21	Gastric triacylglycerol lipase-like	0	+	-	Energy/Resistance	*GTL*
TRINITY_DN16112_c0_g1_i1	Tetratricopeptide repeat protein 7B	0	+	-	Nuclear morphology	*TTC*
TRINITY_DN15105_c0_g1_i2	Cyclin-dependent kinase 12-like	0	+	-	Tissue regeneration	*CDK*
Cluster 4						
TRINITY_DN17433_c0_g1_i1	UDP-glucuronosyltransferase 2B17-like	2,76E-34	+	+	Detoxification	*UDP*
TRINITY_DN17472_c2_g1_i4	Flocculation protein FLO11-like isoform X2	0	+	+	Cell adhesion	*FLO*
TRINITY_DN17474_c1_g1_i1	Facilitated trehalose transporter Tret1-like	0	+	+	Energy metabolism/Sugar source	*Tret1*
TRINITY_DN18028_c2_g1_i5	EGF domain-specific O-linked N-acetylglucosamine transferase isoform X1	0	+	+	Cell adhesion	*EOGT*
TRINITY_DN9586_c0_g1_i1	AF4/FMR2 family member 4 isoform X1	0	+	+	Gene transcription/Splicing	*AF4*
TRINITY_DN17043_c1_g1_i5	Insulin-like growth factor-binding protein complex acid labile subunit	0	+	+	Energy metabolism	*IGFs*
TRINITY_DN17982_c0_g2_i5	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	0	+	+	Energy metabolism	*SDH*
TRINITY_DN16465_c0_g1_i4	Glucose dehydrogenase [FAD, quinone]-like	0	+	+	Energy metabolism	*GDH-FAD*
TRINITY_DN13313_c1_g1_i3	Interferon-related developmental regulator 1-like	0	+	+	DNA Regulation and repair	*IFRD1*
TRINITY_DN16684_c0_g2_i1	Collagen alpha-1(IV) chain	0	+	+	Tissue regeneration	*Col4A1*
TRINITY_DN11297_c0_g1_i1	Defensin-2 precursor	5,18E-58	+	+	Immune system	*Defensin*
TRINITY_DN18434_c1_g1_i2	Titin isoform X2	0	+	+	Hemolymph filtration	*Titin*
TRINITY_DN17683_c1_g1_i1	ATPase H (+)-transporting accessory protein 2 isoform X3	8,08E-76	+	+	DNA repair	*Atp6ap2*
TRINITY_DN17303_c0_g1_i8	Hamartin isoform X1	0	+	+	Tissue regeneration	*TSC1*
TRINITY_DN17375_c0_g1_i4	ATP-binding cassette sub-family G member 5	0	+	+	Excretion	*ABCG5*
TRINITY_DN17375_c0_g1_i11	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial	0	+	+	Energy metabolism	*SCOT*
TRINITY_DN15526_c0_g1_i2	Monocarboxylate transporter 9	0	+	+	Excretion	*MCT9*
TRINITY_DN15526_c0_g1_i6	Carboxypeptidase M	0	+	+	Removal of dead cells	*CPM*
TRINITY_DN14329_c0_g1_i1	Carboxypeptidase B-like	0	+	+	Removal of dead cells	*CPB*
TRINITY_DN15039_c0_g1_i2	Sodium-coupled monocarboxylate transporter 1	0	+	+	Energy metabolism	*MCT1*
TRINITY_DN16559_c0_g1_i1	Protein NDRG3 isoform X2	3,60E-12	+	+	Oxidative stress	*NDRG3*
TRINITY_DN16560_c0_g1_i2	MAGUK p55 subfamily member 7 isoform X1	0	+	+	Cell junction	*MPP7*

TRINITY_DN12422_c0_g2_i1	ATP-binding cassette sub-family G member 4	0	+	+	Apoptosis	*ABCG4*
TRINITY_DN16739_c0_g1_i1	Peroxidasin-like isoform X1	0	+	+	Tissue regeneration	*PXDN*
TRINITY_DN5497_c0_g1_i1	Regucalcin-like	1,95E-30	+	+	Anti-apoptosis.	*Regucalcin*
TRINITY_DN15325_c0_g1_i3	Esterase FE4 isoform X1	0	+	+	Detoxification/Oxidative stress	*Esterase FE4*
TRINITY_DN10411_c0_g1_i1	Excitatory amino acid transporter 1	0	+	+	Signaling/Cytotoxicity	*EAAT1*
TRINITY_DN16383_c2_g4_i2	Cytochrome P450 9e2	1,40E-33	+	+	Resistance to insecticides	*P450 9e2*
Cluster 5						
TRINITY_DN14745_c3_g1_i1	Protein NDRG3	2,5073E-13	-	-	Oxidative stress	*NDRG3*
TRINITY_DN16163_c0_g1_i1	D-3-phosphoglycerate dehydrogenase	0	-	-	Protein synthesis	*D3PD*
Cluster 6						
TRINITY_DN8149_c0_g1_i1	Protein phosphatase 1B isoform X1	1,97E-102	+	+	Apoptosis	*PTP-1B*
TRINITY_DN16819_c0_g1_i1	Longitudinals lacking protein, isoforms A/B/D/L-like isoform X9	1,39E-59	+	+	Signaling	*Lola*
TRINITY_DN13740_c0_g1_i4	ADAM 17-like protease	0	+	+	Tissue regeneration	*ADAM-17*
TRINITY_DN14442_c0_g1_i2	tRNA guanosine-2'-O-methyltransferase TRM13	4,82E-93	+	+	Transcription regulation	*TRM13*
TRINITY_DN18179_c3_g2_i1	Protein flightless-1 isoform X1	0	+	+	Tissue regeneration	*Flii*
TRINITY_DN18179_c3_g2_i10	Mitochondrial import receptor subunit TOM22 homolog	2,44E-49	+	+	Apoptosis	*TOM22*
TRINITY_DN13818_c0_g1_i6	Procollagen-lysine,2-oxoglutarate 5-dioxygenase isoform X2	0	+	+	Tissue regeneration	*PLOD2*
Cluster 7						
TRINITY_DN18305_c0_g1_i1	Origin recognition complex subunit 3	8,43E-26	-	*	Tissue regeneration	*ORC*
TRINITY_DN18305_c0_g1_i2	Signal-induced proliferation-associated 1-like protein 2 isoform X1	0	-	*	Signaling	*SIPA*
TRINITY_DN18339_c2_g1_i2	Protein TAR1	1,22E-80	-	*	Oxidative stress	*TAR1*
TRINITY_DN17944_c1_g4_i2	Tumor protein p53-inducible nuclear protein 2 isoform X1	7,31E-45	-	*	Protein degradation	*TP53INP2*
TRINITY_DN6865_c0_g1_i1	Aquaporin isoform X1	1,65E-29	-	*	Excretion	*AQP*
TRINITY_DN16716_c1_g3_i1	Omega-conotoxin-like protein 1	1,07603E-12	-	*	Immune system	*ICK*
Cluster 8						
TRINITY_DN12014_c0_g1_i1	ATP-dependent RNA helicase DDX51	1,97E-23	*	+	DNA repair	*DDX51*
TRINITY_DN38669_c0_g1_i1	Alcohol dehydrogenase	1,18E-142	*	+	Oxidative stress	*ADN*
TRINITY_DN33225_c0_g1_i1	Glyceraldehyde-3-phosphate dehydrogenase	0	*	+	Oxidative stress	*GAPDH*

Cluster 9						
TRINITY_DN16635_c0_g1_i7	G-protein coupled receptor	0	+	+	Immune system	*GPCRs*
TRINITY_DN17254_c0_g1_i1	Codanin-1	8,60238E-09	+	+	Cell division	*Cod1*
TRINITY_DN17254_c0_g1_i2	Cathepsin L	5,53E-33	+	+	Apoptosis	*Cat1*

Table 1: Expression profile and putative function of the 74 DEGs of interest. Consider the abbreviations listed in this table for the results and discussion section. +: upregulation. -: downregulation. *****: no difference.

3.3 Functional analysis of DEGs (GO term attribution)

The potential function relevance of the DEGs was evaluated through function annotation by GO term attribution. The GO terms were classified in three categories: biological processes (BP), molecular functions (MF) and cellular components (CC) (Figure 2). In the nine clusters, the GO terms attribution revealed genes annotated mainly to 17 BP terms, 10 CC terms, and 5 MF terms.

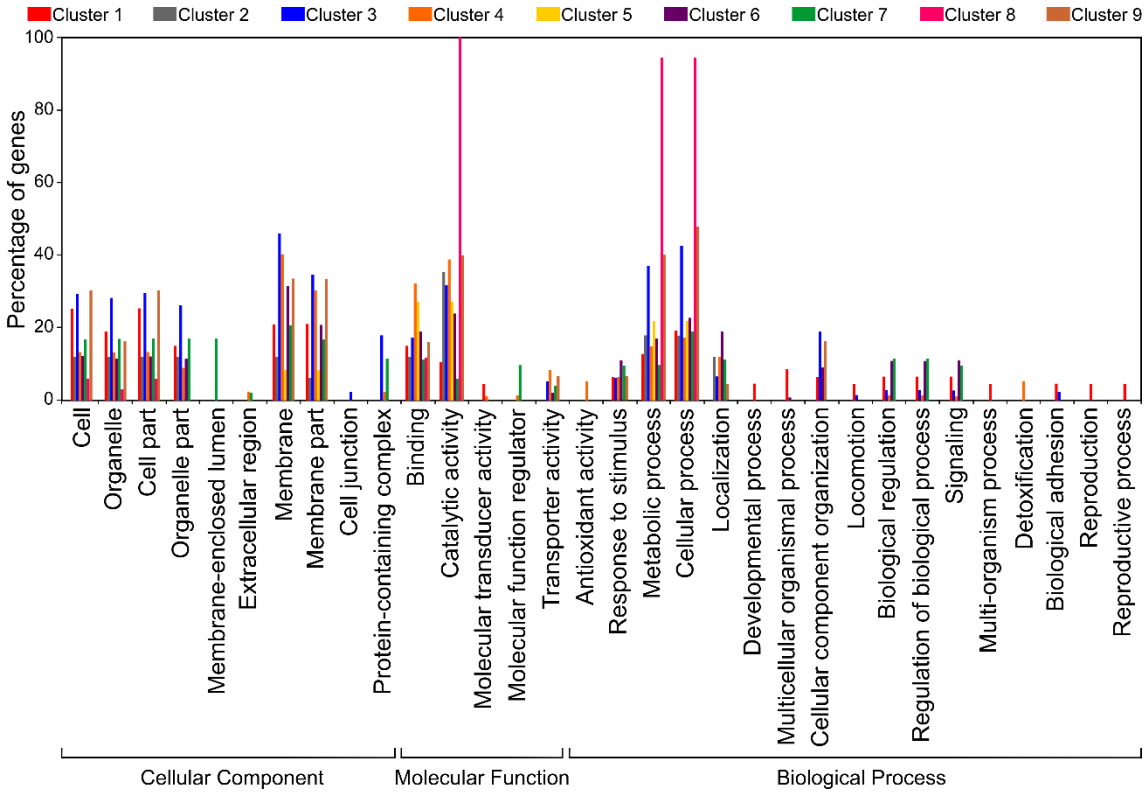


Figure 2 - Functional enrichment analysis of all time-course DEGs of *M. scutellaris* for each cluster. The GO terms include biological process, cellular component, and molecular function. The group of enzymes most expressed through the DEGs was hydrolases, followed by transferases, oxidoreductases, translocases, ligases, isomerases, and lyases (Figure 3).

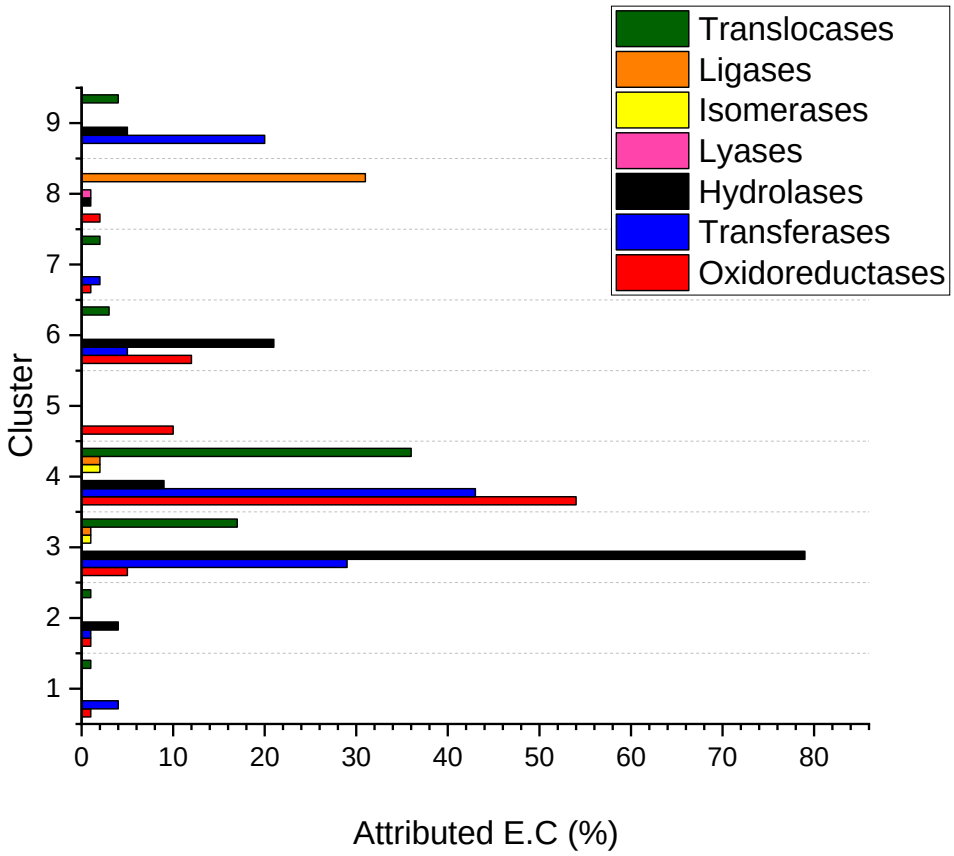


Figure 3 – Enzyme Commission number (EC number) attributed to DEGs, showing the chemical reactions that they catalyze in each cluster.

3.4 KEGG

To address the potential pathways in which the DEGs were involved, annotation was performed in the KEGG database. A total of 142 pathways were assigned to 52 unigenes. Table 2 shows the pathways with the highest number of attributed unigenes. The KEGG pathways mainly belonged to carbon metabolism, energy, signaling, and cellular processes. Several unigenes were also assigned to the immune system, amino acid metabolism, xenobiotic degradation, nervous system disorders, genotoxicity, and/or mutagenesis pathways.

Pathways	Sequence	Protein/Enzyme
Signaling		
AGE-RAGE signaling pathway in diabetic complications	TRINITY_DN17606_c0_g1_i1	Tyrosine-protein kinase hopscotch isoform X1
	TRINITY_DN16684_c0_g2_i1	Collagen alpha-1(IV) chain
Glucagon signaling pathway	TRINITY_DN17472_c2_g1_i4	Flocculation protein FLO11-like isoform X2
	TRINITY_DN7320_c0_g1_i1	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial-like

Insulin signaling pathway	TRINITY_DN17472_c2_g1_i4	Flocculation protein FLO11-like isoform X2
	TRINITY_DN17303_c0_g1_i8	Hamartin isoform X1
Inositol phosphate metabolism	TRINITY_DN16528_c0_g1_i4	Inositol polyphosphate 1-phosphatase
	TRINITY_DN15157_c1_g1_i1	Type II inositol 1,4,5-trisphosphate 5-phosphatase
Phosphatidylinositol signaling system	TRINITY_DN16528_c0_g1_i4	Inositol polyphosphate 1-phosphatase
	TRINITY_DN15157_c1_g1_i1	Type II inositol 1,4,5-trisphosphate 5-phosphatase
PI3K-Akt signaling pathway	TRINITY_DN17303_c0_g1_i8	Hamartin isoform X1
	TRINITY_DN16684_c0_g2_i1	Collagen alpha-1(IV) chain
	TRINITY_DN17606_c0_g1_i4	Tyrosine-protein kinase hopscotch isoform X1
Imune system		
Hepatitis B	TRINITY_DN13120_c0_g1_i1	TGF-beta-activated kinase 1 and MAP3K7-binding protein 1-like isoform X1
	TRINITY_DN17606_c0_g1_i1	Tyrosine-protein kinase hopscotch isoform X1
Herpes simplex virus 1 infection	TRINITY_DN13120_c0_g1_i1	TGF-beta-activated kinase 1 and MAP3K7-binding protein 1-like isoform X1
	TRINITY_DN17303_c0_g1_i8	Hamartin isoform X1
	TRINITY_DN17606_c0_g1_i1	Tyrosine-protein kinase hopscotch isoform X1
Human papillomavirus infection	TRINITY_DN17303_c0_g1_i8	Hamartin isoform X1
	TRINITY_DN16684_c0_g2_i1	Collagen alpha-1(IV) chain
Leishmaniasis	TRINITY_DN13120_c0_g1_i1	TGF-beta-activated kinase 1 and MAP3K7-binding protein 1-like isoform X1
	TRINITY_DN17606_c0_g1_i1	Tyrosine-protein kinase hopscotch isoform X1
Toxoplasmosis	TRINITY_DN13120_c0_g1_i2	TGF-beta-activated kinase 1 and MAP3K7-binding protein 1-like isoform X1
	TRINITY_DN17606_c0_g1_i1	Tyrosine-protein kinase hopscotch isoform X1
Xenobiotic degradation/detoxification		
Drug metabolism - cytochrome P450	TRINITY_DN17433_c0_g1_i1	UDP-glucuronosyltransferase 2B17-like
	TRINITY_DN38669_c0_g1_i1	Alcohol dehydrogenase
Drug metabolism - other enzymes	TRINITY_DN15325_c0_g1_i3	Esterase FE4 isoform X1
	TRINITY_DN5497_c0_g1_i1	Regucalcin-like
	TRINITY_DN17433_c0_g1_i1	UDP-glucuronosyltransferase 2B17-like
Metabolism of xenobiotics by cytochrome P450	TRINITY_DN17433_c0_g1_i1	UDP-glucuronosyltransferase 2B17-like
	TRINITY_DN38669_c0_g1_i1	Alcohol dehydrogenase
Genotoxicity/mutagenesis		
Pathways in cancer	TRINITY_DN16684_c0_g2_i1	Collagen alpha-1(IV) chain

	TRINITY_DN17606_c0_g1_i1	Tyrosine-protein kinase hopscotch isoform X1
Carbon and/or energy metabolism		
Citrate cycle (TCA cycle)	TRINITY_DN17982_c0_g2_i5	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial-like
	TRINITY_DN7320_c0_g1_i1	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial-like
Glycerolipid metabolism	TRINITY_DN14880_c0_g1_i1	Dynein intermediate chain 2, axonemal
	TRINITY_DN16525_c0_g2_i1	Alpha-amylase-related protein
Glycolysis / Gluconeogenesis	TRINITY_DN7320_c0_g1_i1	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial-like
	TRINITY_DN33225_c0_g1_i1	Glyceraldehyde-3-phosphate dehydrogenase
	TRINITY_DN18693_c0_g1_i1	Class II fructose-bisphosphate aldolase
	TRINITY_DN38669_c0_g1_i1	Alcohol dehydrogenase
Methane metabolism	TRINITY_DN18094_c0_g2_i1	Serine-pyruvate aminotransferase, mitochondrial
	TRINITY_DN16163_c0_g1_i1	D-3-phosphoglycerate dehydrogenase
	TRINITY_DN18693_c0_g1_i1	Class II fructose-bisphosphate aldolase
Oxidative phosphorylation	TRINITY_DN12574_c1_g1_i1	NADH dehydrogenase subunit 4
	TRINITY_DN17982_c0_g2_i5	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial-like
	TRINITY_DN41596_c0_g1_i1	NADH dehydrogenase subunit 3
	TRINITY_DN10521_c0_g1_i1	NADH dehydrogenase subunit 5
Pentose phosphate pathway	TRINITY_DN5497_c0_g1_i1	Regucalcin-like
	TRINITY_DN18693_c0_g1_i1	Class II fructose-bisphosphate aldolase
Protein digestion and absorption	TRINITY_DN16684_c0_g2_i1	Collagen alpha-1(IV) chain
	TRINITY_DN14329_c0_g1_i1	Carboxypeptidase B-like
Pyruvate metabolism	TRINITY_DN7320_c0_g1_i1	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial-like
	TRINITY_DN38669_c0_g1_i1	Alcohol dehydrogenase
Ubiquinone and other terpenoid-quinone biosynthesis	TRINITY_DN10521_c0_g1_i1	NADH dehydrogenase subunit 5
	TRINITY_DN12574_c1_g1_i1	NADH dehydrogenase subunit 4
	TRINITY_DN41596_c0_g1_i1	NADH dehydrogenase subunit 3
Aminoacids metabolism		
Cysteine and methionine metabolism	TRINITY_DN9947_c0_g2_i1	Homocysteine S-methyltransferase YbgG-like
	TRINITY_DN18094_c0_g2_i1	Serine-pyruvate aminotransferase, mitochondrial
	TRINITY_DN16163_c0_g1_i1	D-3-phosphoglycerate dehydrogenase

Glycine, serine and threonine metabolism	TRINITY_DN9947_c0_g2_i1	Homocysteine S-methyltransferase YbgG-like
	TRINITY_DN18094_c0_g2_i1	Serine-pyruvate aminotransferase, mitochondrial
	TRINITY_DN16163_c0_g1_i1	D-3-phosphoglycerate dehydrogenase
	TRINITY_DN38669_c0_g1_i1	Alcohol dehydrogenase
Valine, leucine and isoleucine degradation	TRINITY_DN16545_c0_g1_i1	Methylglutaconyl-CoA hydratase, mitochondrial
	TRINITY_DN17375_c0_g1_i11	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial
Cellular process		
Autophagy - animal	TRINITY_DN17303_c0_g1_i8	Hamartin isoform X1
	TRINITY_DN17944_c1_g4_i2	Tumor protein p53-inducible nuclear protein 2 isoform X1
Lysosome	TRINITY_DN17254_c0_g1_i2	Cathepsin L
	TRINITY_DN17537_c0_g1_i21	Gastric triacylglycerol lipase-like
	TRINITY_DN26441_c0_g1_i1	Lysosomal acid glucosylceramidase-like isoform X3
	TRINITY_DN17844_c2_g1_i2	Beta-galactosidase-like
	TRINITY_DN18693_c0_g1_i1	Class II fructose-bisphosphate aldolase
Other glycan degradation	TRINITY_DN17254_c0_g1_i2	Cathepsin L
	TRINITY_DN26441_c0_g1_i1	Lysosomal acid glucosylceramidase-like isoform X3
	TRINITY_DN17844_c2_g1_i2	Beta-galactosidase-like
	TRINITY_DN18693_c0_g1_i1	Class II fructose-bisphosphate aldolase
Peroxisome	TRINITY_DN17485_c0_g5_i8	mpv17-like protein isoform X1
	TRINITY_DN18094_c0_g2_i1	Serine-pyruvate aminotransferase, mitochondrial
Retinol metabolism	TRINITY_DN17433_c0_g1_i1	UDP-glucuronosyltransferase 2B17-like
	TRINITY_DN38669_c0_g1_i1	Alcohol dehydrogenase
Sphingolipid metabolism	TRINITY_DN26441_c0_g1_i1	Lysosomal acid glucosylceramidase-like isoform X3
	TRINITY_DN17844_c2_g1_i2	Beta-galactosidase-like
Thermogenesis	TRINITY_DN17303_c0_g1_i8	Hamartin isoform X1
	TRINITY_DN17982_c0_g2_i5	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial-like
Nervous system disorders		
Alzheimer disease	TRINITY_DN17982_c0_g2_i5	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial-like
	TRINITY_DN13740_c0_g1_i4	ADAM 17-like protease
Cholinergic synapse	TRINITY_DN15325_c0_g1_i3	Esterase FE4 isoform X1

TRINITY_DN17606_c0_g1_i1	Tyrosine-protein kinase hopscotch isoform X1

Table 2: The main KEGG pathways assigned to the differentially expressed unigenes

3.5 qPCR

RT-qPCR was used to validate nine randomly selected DEGs (Figure 4). The *P450 9e2*, *BAI1*, and *BolA* genes were validated by qRT-PCR, and the results were concordant with data from the time-course analyses. The *D3PD* and *AQA* genes were validated after one day of TMX exposure, but did not show significant alterations after eight days of exposure based on qPCR. The *UDP*, *ADAM-17*, and *Flii* genes showed the same tendency of transcriptional changes between the transcriptome and RT-qPCR, although the alterations were not significant based on qPCR. The results for *SUN1* (one day) and *UDP* and Flii (eight days) were not consistent with those of RNA-Seq analysis. The expression of the housekeeping gene (ribosomal protein s5) was unaltered after one and eight days of TMX exposure.

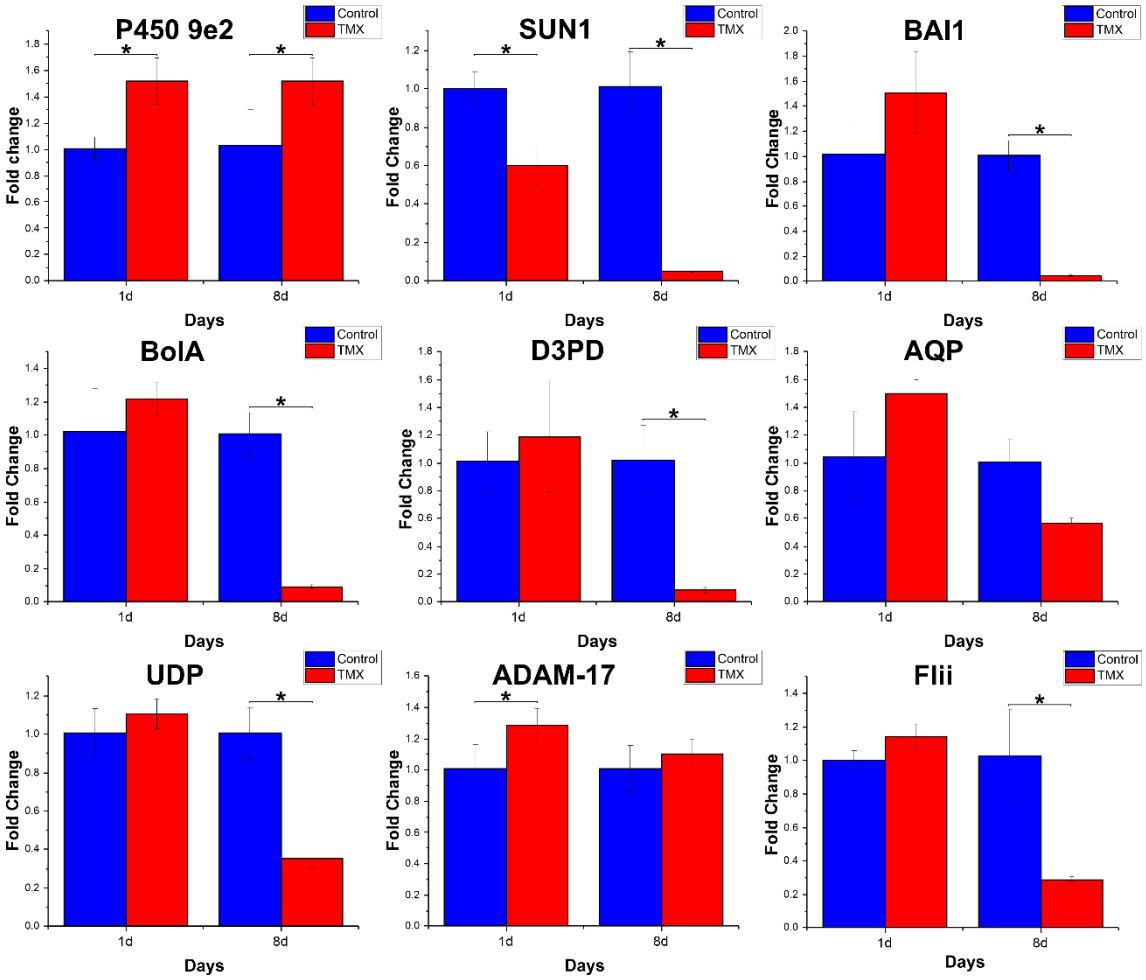


Figure 4: Validation of the selected differentially expressed genes in the Malpighian tubules of the stingless bee, *M. scutellaris*, using qPCR. Gene expression levels were normalized to

those of ribosomal protein s5 and are presented as relative fold change compared with the respective levels in the control group after 1 and 8 days of exposure to TMX. The asterisk indicates a statistically significant difference ($p < 0.05$) between the control (blue) and TMX (red) groups.

Discussion

In 2012, the use of TMX was restricted by the European Union to crops that were attractive to bees, but was completely banned from all crops in 2018 (Bass and Field, 2018). Nonetheless, TMX is commercialized in North America and Latin America. In Brazil, this pesticide has been under environmental risk reassessment since 2017 by the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA) (IBAMA, 2017). Since then, researchers have been encouraged to investigate the effects of this molecule on stingless bees, a native South American bee group. Studies carried out with the model species, *A. mellifera*, revealed different effects induced by TMX using the following approaches: cytotoxic studies (Catae et al., 2014; Friol et al., 2017; Miotelo et al., 2021; Oliveira et al., 2013; Tavares et al., 2015), proteomic expression profile (Roat et al., 2020), alternative splicing (Decio et al., 2019, 2021b), enzymatic activity (Decio et al., 2021a; Tavares et al., 2017), immunohistochemistry (Tavares et al., 2019), and other approaches. However, studies on *M. scutellaris* are scarce (Miotelo et al., 2022, 2021). Further, no studies employed molecular techniques.

In the present study, more than 200 genes were differentially expressed in *M. scutellaris* when the stingless bee was exposed to TMX. These DEGs were found to be involved in detoxification, excretion, tissue regeneration, cellular respiration, energy metabolism, oxidative stress, apoptosis, cell signaling, cell division, DNA repair, immune system, cell-cell adhesion, and nuclear morphology. To date, no study has investigated the molecular influence of TMX on non-target organs; however, Christen et al. (2018) evaluated the molecular effects of different neonicotinoids (including TMX) in the brain. Among the pesticides tested by these researchers, TMX was found to have the least effect on the gene expression profile. By considering the highest concentration tested, only 25 genes were found to have altered expression profiles owing to the influence of TMX, and these genes were mainly related to glucose metabolism.

Excretion and detoxification are among the main functions of the Mt (Cruz-Landim, 1998). The transcriptome of *M. scutellaris* revealed three DEGs associated with detoxification (*BolA*, *UDP*, and esterase *FE4*). UDP belongs to an enzymatic family responsible for the

detoxification of xenobiotic compounds, making them more water-soluble (King et al., 2000; Tephly and Burchell, 1990). According to Pan et al. (2018), the upregulation of different members of this family may contribute to the detoxification of TMX in the cotton aphid, *A. gossypii*. The esterase FE4 has antioxidant properties in the presence of hydrogen peroxide or imidacloprid exposure (neonicotinoid insecticide). They also prevent damage caused by reactive oxygen species (ROS) (Ma et al., 2018). Thus, the upregulation of *UDP* and esterase FE4 after one and eight days of TMX exposure may represent a response of the Mt to detoxify the insecticide and protect cells from oxidative stress. However, *BolA* expression was downregulated after eight days. As *BolA* acts on responses to stressors (Cameron et al., 2011; Mil-homens et al., 2018), TMX could negatively interfere with other detoxification pathways. Regarding excretion, transcriptome analysis revealed three DEGs (*ABCG5*, *MCT9*, and *AQP*). *ABCG5* aids in sterol excretion and is located in the apical area of cells, which is lined with the brush border (Cserepes et al., 2004; Klett et al., 2004; Nomura et al., 2021). *ABCG5* promotes the excretion of xenobiotic compounds from cells into the lumen (Williams et al., 2021). *MCT9* acts on the excretion of uric acid in healthy epithelial cells of the intestine and kidneys (Nakayama et al., 2013). As Mt produce a filtrate from the hemolymph called primary urine (Cruz-Landim, 1998), the upregulation of *MCT9* and *ABCG5* in one and eight days of TMX exposure can indicate a higher excretion level by the Mt.

Although the Mt can recover from injury (Singh et al., 2007), the present study found 11 DEGs associated with tissue regeneration, including: *GINs*, *TGFbeta*, *Cad96Ca*, *CDK*, *Col4A1*, *PXDN*, *TSC1*, *ADAM-17*, *Flii*, *PLOD2*, and *ORC*. Tissue regeneration is also characterized by the formation and modulation of the basal membrane (Kovács et al., 2021). In *Drosophila*, Col4a1 is associated with the basal membrane of the Mt and acts on the maintenance of epithelial integrity (Kiss et al., 2016). *PDXN* participates in the synthesis of extracellular matrix and basal membranes (Kovács et al., 2021; Péterfi and Geiszt, 2014). *ADAM-17* is the first defense against injury, and acts on the repair and regulation of tissue regeneration (Matthews et al., 2017; Scheller et al., 2011). *PLOD2* is involved in post-transcriptional collagen modifications, is indispensable for the stability of the extracellular matrix, and can alter the arrangement of extracellular matrix fibers, and a consequently, affect cell morphology, adhesion, migration, and proliferation capacity (Cao et al., 2021; Du et al., 2017; Neyazi et al., 2017). These genes were upregulated at one and eight days of TMX exposure, possibly acting to regenerate on the Mts to recover from damage caused by the insecticide. *TGB-beta* and *Cad96Ca* exhibited the same pattern on day one and were

downregulated after eight days. In the kidneys, *TGF-beta* induces the production of collagen and fibronectin (Ma et al., 2022). Activation of *Cad96Ca* is essential to the stimulation of epithelial regeneration responses, as it promotes the assembly of actin filaments during tissue regeneration (Tsarouhas et al., 2014). This downregulation may represent failures in some regeneration pathways of the Mt. This event on day eight aligns with the results reported by Miotelo et al. (2022), as TMX is more harmful to the Mt cells after eight days of exposure.

The transcriptome revealed two DEGs related to cellular respiration, NADH dehydrogenase subunits 5 and 4. Mitochondria generate energy for cells through the oxidative phosphorylation system. Complex I (NADH-dehydrogenase) belongs to this system and is the entry point for electrons in the respiratory chain (Grba and Hirst, 2020; Xue et al., 2019). Damage to complex I can compromise the ability of the respiratory chain to oxidize NADH to NAD (Seo et al., 2006). According to Xue et al. (2019), blocking cellular respiration causes an increase in the NADH/NAD+ ratio, which consequently increases the rates of O_2 and other free radicals. Once complex I activity is disrupted, intracellular ROS levels increase (Murphy, 2009; Xue et al., 2019). The downregulation of NADH dehydrogenase subunits 4 and 5 after eight days of exposure to TMX can decrease cellular respiration, thereby increasing ROS levels in Mt cells, which corroborates the upregulation of UDP and esterase FE4 discussed previously. However, the overexpression of subunit 4 of NADH dehydrogenase is associated with its anti-apoptotic function (Ghosh and Girigoswami, 2008). Thus, the upregulation of subunit 4 after one day of exposure may represent a response of the organ to combat cell death. According to the literature, the Mt cells of *M. scutellaris* increase the number of mitochondria in the apical portion when exposed to TMX (Miotelo et al., 2022). Based on the results related to cellular respiration genes, the increase in mitochondria observed by Miotelo et al. (2022) may be a compensatory strategy of the cell induced by the energy deficit.

Other genes also showed that the Mt cells were experiencing an energy deficit due to the upregulation of genes, such as *MCT1* and *MCT2*, at one and eight days of TMX exposure. MCTs are membrane proteins that transport lactate, pyruvate, and ketone bodies (Pierre and Pellerin, 2009; Visser et al., 2007). A high concentration of lactate stimulates the upregulation of *MCT2*, which provides lactate to organs that require a greater energy demand (Medin et al., 2019). *MCT1* overexpression is associated with glycolysis in cells with high energy demand (Kobayashi et al., 2021). *Tret1*, *IGFs*, *SDH*, *GDH-FAD*, and *SCOT* were found to be correlated with energy metabolism. Trehalose is the main sugar present in insect hemolymph and is a source of energy and carbon (Kikuta et al., 2012). In insects, the Mt have high concentrations

of soluble trehalose, which facilitates their conversion (regulated by Tret1) into glucose and its incorporation into cells, which requires energy (Kanamori et al., 2010; Kikuta et al., 2012). *IGFs* regulate circulating levels of trehalose and glucose by transferring these sugars from the hemolymph to the muscle or organ (Chowański et al., 2021). *SDH* and *GDH-FAD* are involved in oxidative phosphorylation, glycolysis, and carbohydrate homeostasis processes that generate energy (Moosavi et al., 2019; Okuda-Shimazaki et al., 2020). According to Acevedo et al. (2013), SDH is upregulated in organisms exposed to environmental stressors. *SCOT* catalyzes the conversion of acetoacetate to acetoacetyl-CoA, which is further metabolized via the citric acid cycle for energy production (Grünert et al., 2021). The upregulation of these genes at one and eight days could represent a response of the organ to increase energy production to compensate for the deficit caused by TMX cytotoxicity.

The following nine DEGs were associated with oxidative stress: *THO*, *Mpv17*, *MGT*, *SPATA20*, *NDRG3*, *TAR1*, *AND*, and *GAPDH*. In insects, the *THO* complex responds to environmental stressors (Kim et al., 2011), specifically cellular stress (Moon and Chung, 2013). *Mpv17* participates in the cellular antioxidant defense system (Iida et al., 2010; Krick et al., 2008). The overexpression of *Mpv17* is related to cellular ROS levels (Iida et al., 2010). *MGT* facilitates the import of glycine into cells (Howard et al., 2010). Glycine has antioxidant properties and acts against ROS by increasing antioxidant enzymes, such as glutathione, catalase, and superoxide dismutase (Chen et al., 2018). *SPATA20* is involved in compensatory oxidative stress responses and is upregulated under these conditions (Jarvis et al., 2020). The above genes were upregulated after one day and downregulated after eight days of TMX exposure. Thus, the Mts could increase the responses to oxidative stress in one day, and after continuous exposure for eight days, this organ may decrease these responses. *Tar1* is downregulated when cellular respiration is defective, thereby avoiding severe consequences related to the presence of ROS (Bonawitz and Shadel, 2009). Therefore, considering the upregulation of *Mpv17*, *MGT*, and *SPATA20* after one day, and the upregulation of *UDP* and esterase *FE4* genes after one and eight days of exposure, TMX and/or its metabolites promote oxidative stress in the Mt cells.

The following six DEGs were related to apoptosis: *BAI1*, *ABCG4*, *TOM22*, regucalcin, *PTP-1B*, and *Cat1*. The upregulation of *PTP-1B* induces cell death by apoptosis through the activity of cellular caspases (Takada et al., 2002) while the upregulation of *ABCG4* is associated with apoptosis (Hegyi and Homolya, 2016). The upregulation of these genes on days one and eight may indicate that TMX promoted cell death. These results corroborate those of our

previous studies in which the morphological changes in the Mts caused by the same concentration of TMX were evaluated, which culminated in cell death (Miotelo et al., 2022). In contrast, regucalcin (upregulated on day one and eight) presents antiapoptotic effects by suppressing the signaling pathways that regulate apoptosis (Yamaguchi, 2013). Therefore, the regulation of apoptosis in the Mts is very complex, and different pathways may be involved.

The transcriptome revealed five DEGs related to the signaling process: *Lola1*, *INPP*, *EAAT1*, *Lola*, and *SIPA*. *Lola* is a transcription factor involved in various axonal guidance decisions in the Drosophila nervous system. According to Goeke (Goeke et al., 2003), when the *Lola* isoform is compromised, damage occurs at a specific point on the axon. *SIPA* regulates synaptic signaling (Dolnik et al., 2016) and is essential for synaptic vesicle trafficking (Andres-Alonso et al., 2019). The downregulation of these genes at one and eight days can compromise cell signaling by preventing the reception of stimuli through neurotransmitters and result in the loss of communication between the Mts and neurons. In contrast, *EAATs* capture glutamate to aid in cell signaling processes and prevent cytotoxicity. The upregulation of *EAATs* on days one and eight may represent an increase in cell signaling to assist in events that may be cytotoxic to Mt cells. Among the few studies that assessed the possible molecular changes caused by TMX in *A. mellifera*, Roat et al. (2020) reported that sublethal exposure could affect cell signaling.

Four DEGs, namely *SPB*, *CD*, *Tektin-4*, and *Cod1*, were associated with the cell division process. *SPBs* are important microtubule-organizing centers that are crucial for mitotic spindle assembly (Rüthnick and Schiebel, 2018). *CD* also acts on mitotic spindle assembly (Htet et al., 2020), and *Tektin-4* is involved in the stability and structural complexity of microtubules in axonemes (Amos, 2008). The upregulation of these genes on day one could be an indication of the active process of cell division. However, as other genes indicate cell death and a decrease in tissue regeneration on day eight, the downregulation of *SPB*, *CD*, and *Tektin-4* on day eight emphasizes the hypothesis that TMX compromises this process.

Analysis of DEGs and KEGG pathways demonstrated that TMX and its derivative metabolites can affect DNA metabolism. Regarding DNA repair, the following DEGs were identified: *Atp6ap2*, *DDX*, and *NHEJ*. The overexpression of *Atp6ap2* can compromise replication and DNA repair (Shibayama, 2020). *DDX* contributes to DNA repair by recruiting p53 (Nicol et al., 2013). During DNA damage, p53 triggers events, such as cell cycle arrest, DNA repair, or apoptosis (Zebian et al., 2022). The upregulation of *Atp6ap2* on day one and eight can negatively affect DNA repair, and *DDX* can be an indication that TMX causes DNA

damage (genotoxicity). *NHEJ* is the main pathway for DNA damage repair and is triggered upon exposure to agents that culminate in DNA damage (Hefferin and Tomkinson, 2005). In the present study, this gene was upregulated after one day and could represent a response of the Mts to repair DNA damage caused by TMX. However, decreased *NHEJ* expression impairs DNA repair (Li et al., 2021). Thus, *NHEJ* downregulation on day eight is insufficient to correct the damage, which can also lead to cell death.

Miotelo et al. (2022) reported that Mts changed the morphology of cells and nuclei after TMX exposure. In the present study, transcriptome analysis revealed two DEGs related to nuclear morphology (*TCC* and *SUN1*). *TCC* is an important factor in chromatin compaction and nuclear organization (El-Daher et al., 2018). The downregulation of *TCC* impacts nuclear morphology and destabilizes collagen adhesion (Jardine et al., 2019). *SUN1* is localized in the inner nuclear membrane and is responsible for establishing physical connections between the nuclear envelope and cytoskeleton (Haque et al., 2006). These DEGs were upregulated on day one, indicating responses of Malpighian cells to maintain a stable nuclear morphology. However, after eight days, these DEGs were downregulated, possibly showing one of the origins that could destabilize the nuclear shape. Furthermore, changes in nuclear morphology can alter chromatin arrangement and trigger cell death, as reported by Miotelo et al. (2022).

Different infection pathways were triggered in the presence of TMX according to KEGG analysis. Four DEGs, namely *Defensin-2*, *ENT3*, *ICK*, and *GPCRs*, were related to the immune system. *Defensin-2* is responsible for individual immunity in bees (Ilyasov et al., 2012). Stress factors activate defense response pathways that induce defensin gene expression (Contreras et al., 2020). *ENT3* assists in the development and proliferation of immune system cells (Wei et al., 2018). *GPCRs* recognize extracellular stimuli and act on responses related to infections or engulfing cells in apoptosis/necrosis. The upregulation of these genes on days one and eight indicated that *M. scutellaris* activated immune system responses against xenobiotic stressors.

As the mean lethal time (LT_{50}) of the $LC_{50/100}$ (0.000543 ng a.i./μL syrup) is seven days for *M. scutellaris*, which indicates a 50% chance of bees dying in seven days, TMX is more harmful after eight days of exposure [19]. Our results show that oxidative stress was high on day one and continued to stress the cells after eight days. In addition, cell death triggered by *PTP-1B* and *ABCG4* is associated with continuous exposure to TMX. Therefore, our results demonstrate that the highest cytotoxicity was achieved after eight days of continuous exposure to TMX.

Conclusion

TMX affects the gene expression profiles in the Mts of *M. scutellaris* exposed to an environmentally relevant sublethal concentration of TMX. Among the 237 DEGs, TMX affected important pathways and promoted oxidative stress, cell damage and death, genotoxicity, and recovery impairment. Different cellular processes can be related to TMX exposure, such as detoxification, excretion, tissue regeneration, cellular respiration, energy metabolism, oxidative stress, apoptosis, cell signaling, cell division, DNA repair, immune system, and nuclear morphology. The eighth day of exposure resulted in changes in the gene expression profile, which is critical for the organ. Interference in these processes compromises the functioning of the Mts and can directly interfere with organ function, thereby directly affecting the health of the organism.

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Authorship contributions

Lucas Miotelo: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, and Visualization. **Milene Ferro:** Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Data Curation, Writing - Original Draft, and Visualization. **Geovana Maloni:** Validation, Investigation, and Writing - Review & Editing. **Igor V. R. Otero:** Conceptualization, Formal analysis, Data Curation, Writing - Original Draft, Investigation, and Visualization. **Roberta C. F. Nocelli:** Resources, Writing - Review & Editing, and Supervision. **Mauricio Bacci:** Resources, Writing - Review & Editing, and Supervision. **Osmar Malaspina:** Conceptualization, Resources, Writing - Review & Editing, and Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Material

		Primers			
B	Actin	Fw TGCCAACACTGTCCTTTCTG Re AGAATTGACCCACCAATCCA		TRINITY_DN96_c0_g1_i1	
C	efl-α	Fw TGTCCATCTTGTTCACGCCGACG Re ATCTCGAAGAACGGACAAACGCGC	91 pb	TRINITY_DN11312_c0_g1_i1	
D	Ribosomal protein s5	Fw ATTTCGACCAGATGCAATTTTTTC Re TGCATGTTAAAGTTGAAGGATC	124 pb	TRINITY_DN16907_c0_g1_i1_28S	
E	NADH4	Fw TGAGTCTAGATTATTATTAATTTT Re AGGTAAAGAAAAAATTAAAGTATA	112 pb	TRINITY_DN12574_c1_g1_i1	Cluster 2
F	P450 9e2	Fw ATGTGCAGAGTGATGTTGTTCTAC Re CATCCCTCCTTCGCCATCAGCAC	119pb	TRINITY_DN16596_c0_g1_i2	Cluster 4
G	SUN1	Fw ATTTCTTCATTAGGCAAAATTG Re CAACCAGGTGCTTGCTGGGCATTTC	128pb	TRINITY_DN16063_c1_g1_i10	Cluster 3
H	Brain	Fw TTCCCAACGATGGTTCCGATCTG Re ATACTTTTACATTGCTGCACGCC	121pb	TRINITY_DN17751_c1_g1_i1	Cluster 1
I	Bola-like	Fw ATATATTCTTACTCCATGCATA Re TGTGGTGCAATGTTTGAGATAAATG	129pb	TRINITY_DN7900_c0_g3_i1	Cluster 1
J	D 3-phospho	Fw TCTGCAGTCTGGCGCGAAACGTTC Re ATTCCGAGCACCGCCAGTGTTTTC	118pb	TRINITY_DN16163_c0_g1_i1	Cluster 5
K	Aquaporin	Fw ACCAAACGTACTAAATGCTAAAG Re TGAACAGCGTCGCCACCCAGAAG	125pb	TRINITY_DN6865_c0_g1_i1	Cluster 7
L	UDP	Fw CGTGTTCCGTCCACCAAGCGAG Re GACAACGTACGTAACAGTTTCTAAC	138pb	TRINITY_DN17433_c0_g1_i1	Cluster 4
M	ADAM	Fw ATCTCTTGATAAAAGCGATAGT Re ATTAGATAAACATTCCAGGACAAAG	111pb	TRINITY_DN13740_c0_g1_i4	Cluster 6
N	Glyceraldehyde	Fw CCAGGGGATCTGAGAGGGCTCAC Re TGTTCAAGTACGACTCTGCCCAC	125pb	1TRINITY_DN33225_c0_g1_i1	Cluster 8

O	Flii	Fw Re	CGGTCAAATGAATGAACAACGT ACAATTTGAAGGAAGTGCCAGAGGG	114pb	TRINITY_DN18179_c3_g2_i1	Cluster 6

Capítulo 2

O artigo referente a este capítulo será submetido para publicação na revista *Chemosphere*. Portanto, está editado conforme as normas da revista.

HSPs of *Melipona scutellaris* under thiamethoxam exposure: immunolabeling, transcriptome analysis, and in silico characterization

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Abstract

Bees belong to a diverse insect group that stands out during pollination services in managed and wild ecosystems. Some bee groups, such as stingless bees, significantly contribute to the pollination in tropical and subtropical ecosystems, providing and improving pollination services for economically important crops. Stingless bees, such as *Melipona scutellaris*, have been exposed to neonicotinoids during foraging activity. Therefore, the present study analyzed foragers bees of *M. scutellaris* exposed to a sublethal concentration of thiamethoxam (TMX). The possible effects of TMX on Malpighian tubules, the organ responsible for excretion and detoxification, were evaluated through cellular biomarkers of stress (Heat Shock Proteins (HSPs) and silico characterization) and cell death (TUNEL method - Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling). The immunolabeling of HSP70 exhibited an increase in one day and a decrease in eight days. However, the immunostaining of HSP90 decreased in one day and increased in eight days. The transcriptome analyses, using the transcripts per million (TPM) values, revealed that an isoform of HSP70 was the most expressed HSP in the Malpighian tubules. Furthermore, it has no differentially expressed genes (DEGs) from HSPs. The results from immunolabeling of HSP70 and HSP90 are in discordance with those from transcriptome since there is no DEGs related to HSPs. Considering the high sensitivity of HSPs and the possibility of changes in the expression profile of HSPs without any stressor during the same day, perhaps applying the immunostaining method alone is not enough to investigate the effects of insecticides. We can consider the same premise for the TUNEL method since only two cells showed DNA fragmentation. As this method opens up early stages of cell death, it is essential that this technique be combined with others to improve the quality of the results.

Keywords: HSP70, HSP90, cell death, stress markers, stingless bee

Introduction

Human well-being is extremely related to pollinators' health and diversity as they are responsible for ecosystem maintenance, wild and crop plant reproduction, consequently ensuring food security (Potts et al., 2016). Bees are considered the most important insect pollinators, they visit more than 90% of the main global crops (Klein et al., 2007). In the world, there are more than 20,000 species of bees described (Michener, 2007) and Brazil houses a big diversity of them, including 244 valid species of stingless bees (Pedro, 2014). The stingless bees are 50~fold more diverse than honeybees (Roubik, 2006). They pollinate crops of global importance, like coffee, apple, citrus, strawberry, and cotton, and some of them, such as *Melipona scutellaris* Latreille, 1811, are able to buzz pollinate crops like tomato, sweet pepper, annatto, and flowers with poricidal anthers (De Luca and Vallejo-Marín, 2013; Klein et al., 2020). Recently *M. scutellaris* have been recognized as a producer of products with high economic value, such as propolis, honey, and pollen (Cavalcante da Silva et al., 2018).

The threats to bees' health and their decline are topics of current concern, as bees have been suffering mainly from environmental impacts caused by humans (Campbell et al., 2018; Drossart and Gérard, 2020). The intense and inadequate use of pesticides in agricultural lands has been studied (Potts et al., 2016) since, even though bees aren't the target of pesticides, they can be exposed by several routes (Boyle et al., 2019; Cham et al., 2018). Bees may come into oral contact with pesticide residues in nectar and pollen and stingless bees specifically may come into topic contact with mud, resins, soil, petals, or leaves contaminated, because they use these materials to build their nests (Boyle et al., 2019; Cham et al., 2018; Roubik, 2006). Hence, one of the major concerns is related to the systemic characteristics of neonicotinoids that allow the passage of residues through plant tissues, reaching pollen and nectar (Bass and Field, 2018).

The neonicotinoids, such as thiamethoxam (TMX), can impact the central nervous system of insects, mimicking the action of neurotransmitters of acetylcholine (Ach) (Bass and Field, 2018; Tomizawa and Casida, 2005). When they are present, the enzyme acetylcholinesterase (AChE) isn't able to degrade their molecules on the cholinergic receptors, leading to a hyperexcitation of neurons, impairing the cholinergic synapses, resulting in several harmful effects, such as loss of flying ability and death (Buszewski et al., 2019; Tomizawa and Casida, 2005). Furthermore, the European Union completely banned TMX from all crops in 2018, but is still in revaluation in Brazil since 2017 (Bass and Field, 2018; IBAMA, 2017). Despite its current use, several studies reveal that its

sublethal concentration impairs bee development (Tavares et al., 2017, 2015), causes molecular effects (Christen et al., 2018; Roat et al., 2020), increases oxidative damages (Decio et al., 2021; Gauthier et al., 2018), affects enzymatic activities (Almasri et al., 2020; Pal et al., 2022), and alters brain, midgut, and Malpighian tubules morphology (Catae et al., 2014; Friol et al., 2017; Miotelo et al., 2022, 2021). Even though Malpighian tubules aren't de target of TMX, they are responsible for the detoxification process of xenobiotics, thus, representing a potential organ for evaluation of exposure to neonicotinoids insecticides (Miotelo et al., 2022; Nocelli et al., 2016).

When exposed to adverse conditions, like environmental stressor as pesticides, cells can produce molecular chaperones known as heat shock proteins (HSPs) (Alberts et al., 2015; Candido, 2001). HSPs are one of the first lines of cellular defense involved in a variety of physiological processes preventing the organism from the irreversible denaturation of proteins (Dubrez et al., 2020; Guedes et al., 2020; Zhang et al., 1998). Although they are capable of refolding and desegregating denatured proteins, HSPs are also involved in apoptotic signaling and some HSPs act as potent anti-apoptotic proteins by associating with and blocking major apoptotic proteins, keeping cells alive during stress events (Dubrez et al., 2020). Among the main families of HSPs, regarding to apoptosis, HSP27 and HSP70 are called anti-apoptotic, while HSP60 and HSP10 are pro-apoptotic (Garrido et al., 2001). HSP90 is also produced during stress events, but its functions aren't so clear since they respond in different ways depending on the stress (Garrido et al., 2001; King and Macrae, 2015). HSPs action might determine the fate of stressed cells and for this reason, they are considered great ecotoxicological and cytoprotective biomarkers (Agus et al., 2015; Malaspina and Silva-Zacarin, 2006b), including biomarkers for bees exposed to pesticides (Silva-Zacarin et al., 2006).

One of the widely techniques used to evaluate cellular death is the TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) method (Gavrieli et al., 1992). When cells are in the death process, endonucleases cleave the DNA into many fragments, generating several DNA free ends (Alberts et al., 2015). Consequently, the TUNEL method is able to detect the damage using the ability of the enzyme terminal deoxynucleotidyl transferase (TdT) that transfers chains of labeled deoxynucleotide (dUTP) to the 3'-hydroxyl (3'-OH) termini of the fragments (Alberts et al., 2015; Gavrieli et al., 1992). Hence, the TUNEL method allied to HSPs biomarkers can be used to understand how cells are facing the molecular damage to environmentally relevant concentrations of pesticides and evaluate its level of severity for the bees, as both

represent a great alternative for rapid biological diagnosis (Malaspina and Silva-Zacarin, 2006b; Silva-Zacarin et al., 2006). Recently, applying genomics approaches has been stimulated to improve the comprehension of identification, monitoring, and predictions of the effects of stressors in bees (Grozinger and Zayed, 2020). Transcriptomes represent great tools to evaluate the damage caused by the sublethal concentrations of pesticides and are a robust method to evaluate cell impairment. For this reason, this study aimed to evaluate the toxicological effects of a sublethal concentration of TMX in the Malpighian tubules of *M. scutellaris* throughout the TUNEL method and biomarkers HSPs 70 and 90. In addition, the present study also searched for HSPs genes on the transcriptome of *M. scutellaris* exposed to the same sublethal concentration.

Material and methods

Forager's bees of *M. scutellaris* were collected from three healthy colonies placed on the meliponary of the São Paulo State University (UNESP), Institute of Biosciences, Rio Claro campus, SP, Brazil. Collections were made in 250 mL plastic cages containing holes side-drilled for air exchange. The experimental group was composed of 90 bees divided into the control group (45 bees) and the TMX group (45 bees). The insecticide used was thiamethoxam PESTANAL® ($C_8H_{10}ClN_5O_3S$), Sigma-Aldrich analytical standard, purity ≥98.0%, and soluble in water. The sublethal concentration ($LC_{50/100}$ = 0.000543 ng a.i./µL) was based on Miotelo et al. (2021). It was prepared a stock solution of 1000 ng of TMX and cascade dilutions were made until reach the desiderated sublethal concentration. A sugar solution (water and sugar: 50% v/w) was used for the cascade dilutions and to feed the control. Microtubes (2mL) containing side-drilled holes were provided as feeders, and the syrup was provided *ad libitum*. The experimental cages were kept in an incubator (chamber of biochemical oxygen demand – D.B.O) at 28 ± 2 ◦C, relative humidity of 70 ± 05%, and constant darkness. The exposition assay was carried out following the recommendations of the Organization for Economic Cooperation and Development – OECD (1998), with adaptation to stingless bees.

Detection of HSP70 and HSP90 by immunofluorescence

For the collection of the Malpighian tubules, six bees from each treatment (Control and TMX group) were dissected after 1 and 8 days of the assay. For that, bees were anesthetized at -20 ºC for 70 s. The organs were dissected using a stereomicroscope EZ4 (Leica) in a 4% paraformaldehyde fixative solution. After two hours in 4%

paraformaldehyde at room temperature, they were kept in 0.1 M sodium phosphate buffer solution (PBS) pH 7.4 for 24 h at 4 ºC. The organs were placed in agarose and submitted to the vibratome (VT 1000S – Leica) to obtain 100µm histological thick sections. Starfrost® microscope slides were used to collect the histological sections containing the Malpighian tubules.

The method was performed according to the manufacturer's instructions. A solution of triton 0.5% v/v X-100 was added above each of the histological sections for ten minutes at room temperature and then they were washed with PBS. The fetal bovine serum (BSA 3%) was added for 30 minutes to block nonspecific reactions from the primary antibody and washed again with PBS. For the immunolabeling of HSP70, its specific primary antibody (monoclonal anti-heat shock protein 70, antibody produced in mouse from Sigma-Aldrich) was diluted in PBS (1:100). The primary antibody was added to the histological sections that were kept in a humid chamber (D.B.O) at 37 ºC for one hour and a half in constant darkness and washed with PBS. For the immunolabeling of HSP90, the primary antibody monoclonal anti-heat shock protein 90 (ThermoFischer) was diluted in PBS (1:100). The material was kept in a humid chamber (D.B.O) at 37 ºC for one hour in constant darkness and then washed with PBS. Forward, all the steps were performed in a dark chamber. For both HSP70 and 90, the second antibody (IgG) conjugated with Cy5® was diluted in PBS (1:500, Molecular Probes) and applied on the histological sections. The incubation period was performed at room temperature for one hour. By the end the histological sections were washed with PBS and the slides were mounted with Prolong® Gold Antifade (Molecular Probes). To ensure the specificity of the technique, it was performed a negative control following all the steps, except the one with the primary antibody. In this case, it was replaced by PBS and the cells received only the second antibody. The positive control was assigned for counterstaining reaction with DAPI (4′,6-diamidino-2-phenylindole, Molecular Probes) to allow visualized nuclei in blue.

The immunofluorescence of HSP70 and HSP90 was visualized at a Confocal Laser Scanning Microscopy (Leica® TCS-SP5). The images were acquired using the software Leica Application Suite-AF, with the following parameters: *Smart Gain* (1000-1200 V); *Smart Offset* (1%); *Pinhole* (67,90); *Line* (8); *Frame* (2); *Resolution* (1024x1024); *Speed* (400Hz); *Lazer* (30% Standy); *Number of steps* (10); *Z size* (30µm) and objective (40x). For each experimental group, 18 images were analyzed trough the software Leica Application Suite-AF. Five Regions of Interest (ROIs, 100µm diameter

circles) were delimited in each image. From that, the level of intensity in each ROI was quantified, and one average per image was calculated (Guedes et al., 2020). The results were submitted to the tests of homogeneity (*Bartlett*) and normality (*Shapiro-Wilk*). For multiple comparison of means, the *T test* with *Bonferroni* protection was used. All statistical analyzes were performed in the R software (R Core Team, 2019).

TUNEL reaction for DNA fragmentation immunostaining

Nine bees of each treatment (control and TMX group) were dissected after 1 and 8 days of the assay, and their Malpighian tubules were removed. The bees were previously anesthetized at -20 ºC for 70 s and the organs were dissected using a stereomicroscope EZ4 (Leica) in a 4% paraformaldehyde fixative solution. After dissection, the organs were maintained for two hours in 4% paraformaldehyde at room temperature. They were kept in 0.1 M sodium PBS (pH 7.4) for 24 h at 4 ºC. Then the organs were placed in agarose and submitted to the vibratome (VT 1000S – Leica) to obtain 100 µm histological thick sections. Starfrost® microscope slides were used to collect the histological sections containing the Malpighian tubules. In order to identify cell death, the TUNEL method (Gavrieli et al., 1992) was conducted using the In Situ Cell Death Detection Kit, AP (Roche®, Sigma-Aldrich). According to the manufacturer's instructions, the kit's reaction identifies cell death in the early stages through the detection of single and double stranded DNA breaks.

The histological sections were treated with 20 µg/mL proteinase K in 10Mm Tris-HCl (pH 7,5), incubated at 37 ºC for 15 minutes and then washed with PBS. Afterwards the TUNEL method was applied in all histological sections following the manufacturer's instructions. A positive and negative control were used to ensure the functionality of the TUNEL technique. The first one by applying DNase 1 (3000 U/mL in Tris-HCl buffer, pH 7.5 - 10mM MgCl2, 1mg/mL bovine serum albumin - BSA) at room temperature for ten minutes, followed by washes with PBS. The second one received only the antibody without DNase 1. All samples with or no TUNEL reaction mixtures were kept in a humid chamber (D.B.O) at 37 ºC for one hour in constant darkness. The TUNEL reaction was stopped by washing the samples with PBS. The slides were mounted with Prolong Gold Prolong® Gold Antifade (Molecular Probes). To visualize the immunolabeling of the TUNEL reaction the Laser Scanning Confocal Microscopy was used with wavelength of 488 nm, *Smart Gain* between 800 to 1000, *Smart Offset* of 1% and 10% of *Laser*.

Transcripts per million – TPM and in silico characterization of HSPs

Based on the raw sequence data from the transcriptome of the Malpighian tubules of *M. scutellaris* available in the Short Read Archive (SRA) GenBank database: BioProject (PRJNA836917), BioSample (SAMN28188996–SAMN28188998, SAMN28189016–SAMN28189021, SAMN28189023–SAMN28189025), and SRA (SRR19178717–SRR19178728), reads from RNA sequencing (RNA-seq) at each experimental group were mapped to the corresponding HSP genes and normalized to TPM.

All sequences that matched the keywords HSP and heat shock proteins were selected and submitted to silico characterization as described by Otero et al. (2017). Peptide composition analysis was performed using GeneRunner software (Version 5.0.66). Molecular weight and isoelectric point prediction was performed using ProtParam (http://web.expasy.org/protparam/). Peptide cleavage site prediction was performed using SignalP 4.1 software (http://www.cbs.dtu.dk/services/SignalP/). The prediction of N-glycosylation sites was made using NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). Peptide chain similarity analysis was performed using the BLASTP tool (NCBI) that searched the Swiss-Prot data bank (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Immunolabeling of HSP70 and HSP90

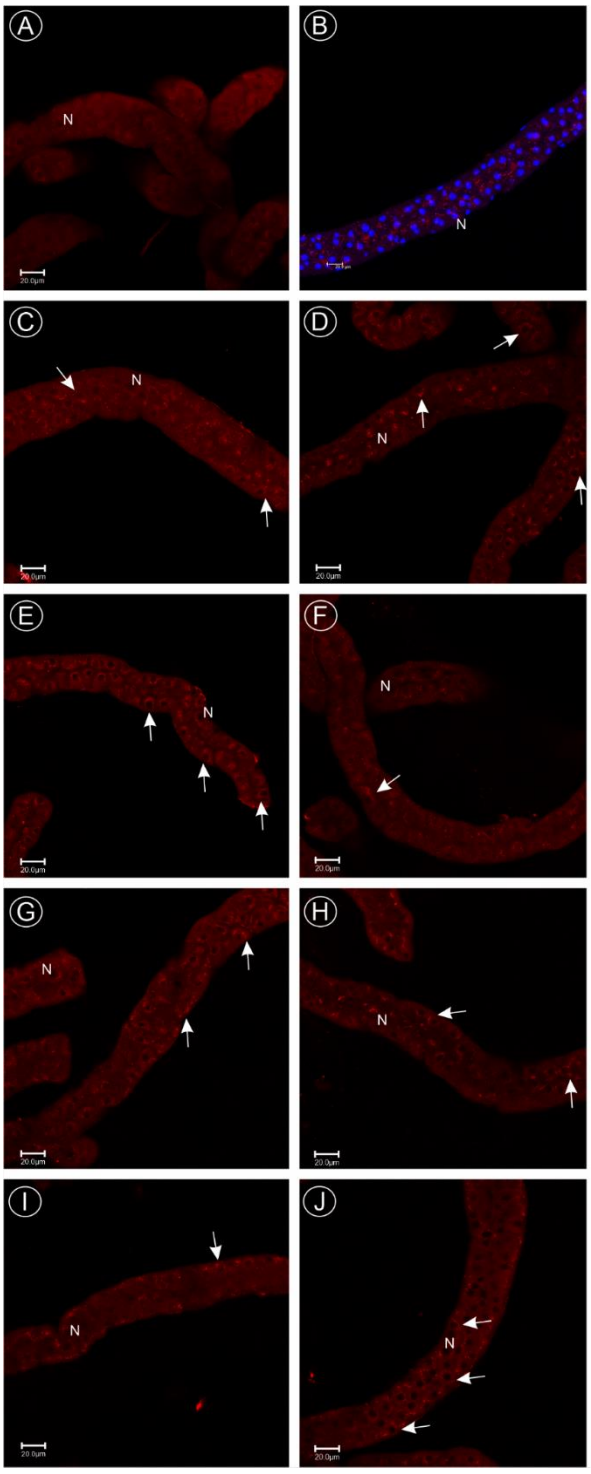
The evaluation of cell stress reveals that the sublethal exposure to TMX significantly increased the intensity of the immunolabeling of HSP70 in one day (Figure 1C and D). While it significantly decreased the intensity of immunolabeling in eight days of exposure to the insecticide (Figure 1G and H). The intensity of HSP90 immunolabeling in one day was higher in the control group than in the TMX group (Figure 1E and F). In eight days, there was a significantly increase in the immunolabeling of HSP90 in the TMX group (Figure 1I and J) (Table 1).

Table 1: Malpighian tubules of the stingless bee *M. scutellaris* exposed to TMX (one and eight days) showing the expression of immunolabeling of HSP70 and HSP90.

Treatment		Time (days)	
		1	8
HSP70	Control	66.54 ±1.22a	47.47±1.25a
	TMX	73.02±2.04b	40.49±0.82b
HSP90	Control	71.66±1.50a	36.06±0.92a
	TMX	58.84±1.17b	53.50±1.45b

Mean values ± SD. followed by different letters (comparing columns only between the control group and TMX group in each day analyzed) indicates a significant difference according to the T-test ($p < 0.05$).

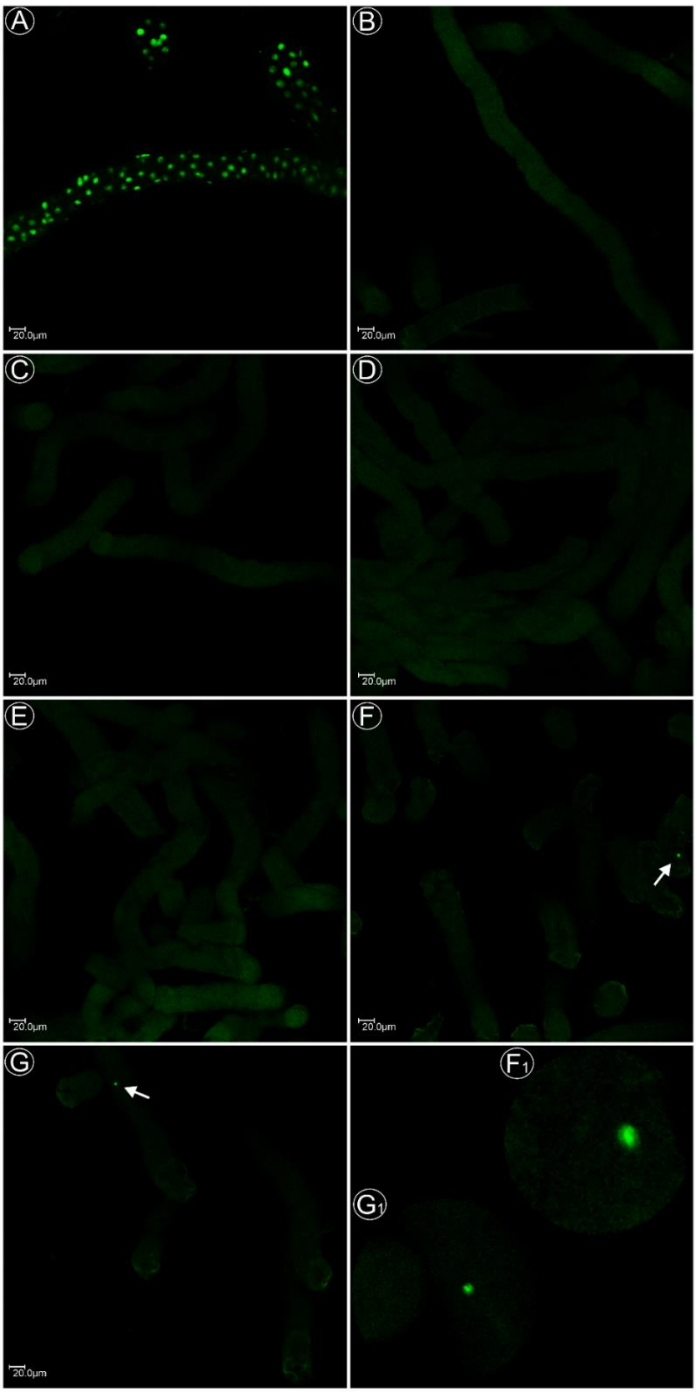
Figure 1: Immunolabeling of HSP70 and HSP90 of *M. scutellaris* in the Malpighian tubules exposed to TMX. **A** – Negative control without immunostaining. **B -** Primary and secondary fluorophore-conjugated antibodies revealed immunolabeling of HSPs in red, and the overlap with DAPI showed the nuclei in blue in the positive control. **C** and **G** – HSP70 of the control group after 1 and 8 days; **D** and **H** – HSP70 of the TMX group after 1 and 8 days; **E** and **I** – HSP90 of the control group after 1 and 8 days respectively; **F** and **J** – HSP90 of the TMX group after 1 and 8 days respectively. **N** – nuclei. **Arrows** indicate the immunostaining of the HSPs.



TUNEL reaction

The cells from the Malpighian tubules showed minimal DNA fragmentation only on the eight-day of exposure according to the TUNEL reaction. Any cells presented negative immunolabeling of the TUNEL reaction in the control group (one and eight days) and TMX group after one day. Positive immunostaining of the nuclei with DNA fragmentation was observed only in the cells of the Malpighian tubules exposed to TMX after eight days.

Figure 2: TUNEL method was applied to detect DNA fragmentation in the Malpighian tubule's cells of *M. scutellaris* exposed to TMX. **A** - Positive control of the TUNEL method showing cells with DNA fragmentation. **B** - Negative control. **C** and **D**- Control group and TMX group after one day, respectively. **E** – Control group after eight days. **F** and **G** – Group TMX revealing positive marking TUNEL reaction after eight days of exposure to TMX. **F_1** and **G_1** represent the focus on the cells from Malpighian tubules that are under DNA fragmentation. **White arrow** - positive marking of the TUNEL reaction.



TPM expression from HSPs and in silico characterization

Based on the *de novo* assembly of the transcriptome of *M. scutellaris* exposed to TMX, the HSPs expressed were: HSP60A, different isoforms of HSP70, HSP97, isoforms of HSP factors, and activator of 90 kDa HSP (Table 2). The HSP most expressed was HSP70 (Figure 3B). Considering the discrepant differences in TPM values between this HSP70 (300 thousand) and the second most expressed HSP (HSP60A with TPM value = 5000), were created two plots to represent them. Figure 3A shows TPM expression values of HSPs below 5 thousand.

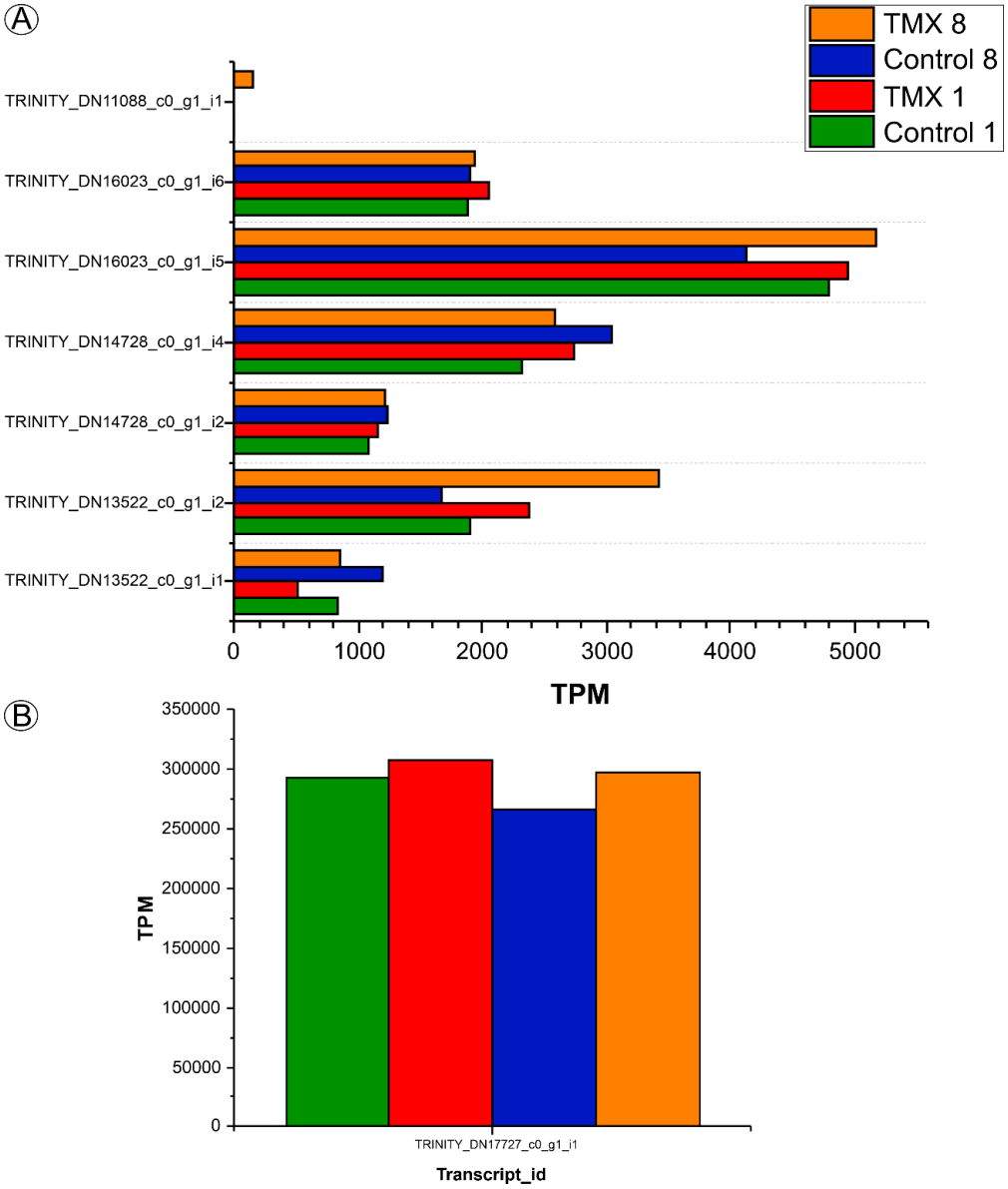


Figure: Expression values of TPM (Transcripts per million) from HSPs, based on the transcriptome of *M. scutellaris* exposed to TMX. **A** – Different HSPs expressed on the transcriptome. **B** – The HSP mostly expressed (HSP 70 kDa). The numbers after the group name are related with the day of exposition (1 = one day and 8 = eight days).

Sequence ID	Size (bp)	Size (aa)	Predicted protein size (kDa)	Theoretical pI	Similarity
TRINITY_DN16023_c0_g1_i5	1605	535	56.54	5.29	98.84% with the HSP 60A-like from *Frieseomelitta varia*
TRINITY_DN16023_c0_g1_i6	1707	569	60.37	5.83	98.91% with the HSP 60A-like from *Frieseomelitta varia*
TRINITY_DN13522_c0_g1_i1	1665	555	61.00	5.54	99.64% with the HSP 70 kDa cognate 4-like from *Frieseomelitta varia*
TRINITY_DN13522_c0_g1_i2	1923	641	70.23	5.55	99.69% with the HSP 70 kDa cognate 4-like from *Frieseomelitta varia*
TRINITY_DN14728_c0_g1_i2	1029	343	39.08	5.05	99.71% with the HSP 70 kDa 4L from the *Melipona quadrifasciata*
TRINITY_DN14728_c0_g1_i4	921	307	35.26	4.95	98.05% with the HSP 97 kDa isoform X2 from *Frieseomelitta varia*
TRINITY_DN11088_c0_g1_i1	1011	337	36.19	4.71	87.46% with the HSP 70 kDa from *Theileria ovis*
TRINITY_DN17727_c0_g1_i1	360	120	13.00	5.00	96.87% with the HSP 70 kDa from *Ectomyelois ceratoniae*
TRINITY_DN16037_c0_g2_i2	525	173	18.92	4.20	95.32% with the HSP factor from *Melipona quadrifasciata*
TRINITY_DN16037_c0_g2_i5	1488	496	55.91	5.42	94.77% with the HSP factor 1-like isoform X8 from *Frieseomelitta varia*
TRINITY_DN16037_c0_g2_i7	1914	638	71.46	4.78	94.14% with the HSP factor 1-like isoform X8 from *Frieseomelitta varia*
TRINITY_DN16037_c0_g2_i8	1116	372	42.63	6.58	87.13% with the HSP factor 1-like isoform X7 from *Osmia bicornis bicornis*
TRINITY_DN16494_c0_g1_i1	714	238	27.50	6.4	99.14% with the activator of 90 kDa HSP (ATPase-like protein 1) from *Melipona quadrifasciata*
TRINITY_DN16494_c0_g1_i3	1014	338	39.18	6.01	99.11% with the activator of 90 kDa HSP (ATPase-like protein 1) from *Melipona quadrifasciata*
TRINITY_DN16494_c0_g1_i6	687	229	26.40	7.64	98.20% with the activator of 90 kDa HSP (ATPase-like protein 1) from *Melipona quadrifasciata*

Table 2: In silico characterization of the HSPs expressed on the transcriptome of *M. scutellaris* exposed to TMX. **Size (bp)** shows the size of the HSPs ORFs (Open reading frame) found on the transcriptome. The similarity exhibits the HSP and the species name, and the percentage of similarity between the ORF from the transcriptome and data presented on the NCBI.

Discussion

Insects can be used as bioindicators in ecotoxicological studies. Different organs also can be investigated to access the toxicity of xenobiotics (Malaspina and Silva-Zacarin, 2006a). Although the Malpighian tubules are responsible for the detoxification of xenobiotics, they rarely are chosen in these studies, prevailing only on morphological analyses (Catae et al., 2014; Miotelo et al., 2022). Considering that bees are wide-ranging foragers, applying other approaches in the Malpighian tubules of stingless bees could improve our knowledge about the effects of pesticides used in crops or released into the environment. The increase of HSPs expression has been associated with cytotoxic effects of pesticides in bees, such as: fipronil (Zaluski et al., 2020) and imidacloprid (Koo et al., 2015; Škerl and Gregorc, 2010). In the present study, the immunolabeling of HSP70 showed the following pattern: an increase in one day and a decrease in eight days of TMX exposure. While for HSP90, this pattern was the opposite, with a decrease in one day and an increase in eight days.

Some authors hypothesized that the differences in the HSP expression can be related with oxidative stress caused by pesticides (Zaluski et al., 2020). Furthermore, considering that HSP70 contributes in the maintenance of cellular functions under stress situations (Beckmann et al., 1992), the increase of HSP70 reported in one day it was expected since HPS70 has a cytoprotective role against harmful effects on cells (Moreira-de-Sousa et al., 2018). The protective ability of HSP70 occurs due their anti-oxidant and anti-inflammatory properties that acts regulating the production of ROS (Dubrez et al., 2020). Miotelo et al. (2022) showed that TMX affects the expression of genes related with oxidative stress and the Malpighian tubules may increase the responses to oxidative stress in one day, however after eight days these responses could decrease. If HSP70 is contributing with responses to oxidative stress, the increase of HSP70 in one day and the decrease and eight days are in accordance.

The HSP90 is also recognized as a biomarker, and cells under stress can increase HSP90 expression levels to protect them and avoid cell death (Bierkens, 2000). Although there are uncertainties as to whether HSP90 exhibits anti-apoptotic or pro-apoptotic characteristics, evidence shows that the HSP90 can be predominantly anti-apoptotic depending on the apoptotic stimuli (Garrido et al., 2001; Schopf et al., 2017). However, specific functions and catalytic mechanism still remain unclear (Mader et al., 2020). Considering that the pattern of HSP90 reported in the present study it was opposite of the HSP70, this could represent a compensatory response of HSP90 to protect Malpighian tubule cells from the stress caused by TMX.

Although the immunolabeling of HSP70 and HSP90 exhibited differences between the control and TMX groups, Miotelo et al. (2022) did not find any gene of HSP differently expressed (DEGs). One of the reasons between the differences in the DEGs and immunolabeling of HSP70 and HSP90 results could be related to the assays performed to collect the organs since collections on different days/months could result in differences in the levels of HSPs expression. According to Hranitz et al. (2010), when bees were analyzed without any stressor, the discrepancies in HSP70 expression may be related to the following factors: months and methods of collection, the environmental conditions, or the transport of the experimental cages to the laboratory. Furthermore, HSP70 expression in *Apis mellifera meda* does not exhibit a stable pattern at the same day, showing the lowest HSP70 gene transcription in the morning and the highest at midday (Morammazi and Shokrollahi, 2020). Thus, considering that HSPs are abundant and highly sensitive (Moreira-de-Sousa et al., 2018), the immunolabeling of HSP not necessarily is related to TMX since there is no DEGs on the transcriptome. Furthermore, Koo et al. (2015) emphasizes that HSPs of bees responds in different ways and highly specific depending on the stressor.

Searching for HSPs on the transcriptome, we visualized all HSPs that were expressed in one and eight days of TMX exposure. Here, it is important to highlight that the reagents used to perform the immunolabeling do not recognize all HSPs isoforms. According to the manufacturer's instructions, the monoclonal Anti-Heat Shock Protein 70 antibody has reactivity with species of rat, plant, Drosophila and others (Brocchieri et al., 2008; Wang et al., 2014). Although the reactivity with insects is not a problem, the antibody does not recognize all possible isoforms of HSP70. Heat shock protein 90 alpha antibody is indicated to detect 90 kDa from mice, humans, rats, and chickens (Silverstein et al., 1999). No further information about HSP90 isoforms is provided. Thus, we believe that data from transcriptome is more reliable since is based on sequencing of thousands of transcripts.

In silico characterization revealed the expression of different isoforms of the HSP factor 1-like (HSF1). HSF1 is a heat shock-induced transcription factor responsible for the regulation of HSPs expression (Barna et al., 2018). However, in recent years was discovered that HSF1 acts by regulating the transcription of genes encoding other proteins, associated with physiological and stress-induced cellular processes and molecular mechanisms such as apoptosis, cell cycle, chromatin remodeling, and others (Barna et al., 2018). The presence of HSF1 on the transcriptome of *M. scutellaris* may not be related only to HSPs regulation since

there is no DEG of HSPs or transcription factors on the transcriptome. Furthermore, HSF1 was not differently expressed, showing that TMX does not affect their expression.

TUNEL reaction exhibit positive immunolabeling for DNA fragmentation only in eight days. DNA fragmentation has been associated with cell death in *A. mellifera* in salivary glands (Silva-Zacarin et al., 2008) and midgut (Gregorc et al., 2018, 2016). Although the present study shows only two cells with positive immunolabeling for DNA fragmentation, we cannot rule out the hypothesis that TMX causes cell death. According to Silva-Zacarin et al. (2008), the DNA fragmentation detected by TUNEL reaction is one of the first symptoms of cell death. Considering that it is a very specific marker and represents an early stage of cell death, the results of the TUNEL reaction do not exclude the hypothesis that TMX could cause cell death. Miotelo et al. (2022) show that TMX impacts DEGs associated with apoptosis in *M. scutellaris* and the regulation of apoptosis in the Malpighian tubules can occur through different pathways. Thus, performing analyzes with the TUNEL method alone is not enough to state whether cell death has occurred since different cell death pathways can be triggered.

Conclusion

The data obtained for the immunostaining and the transcriptome of *M. scutellaris* exposed to a sublethal concentration of TMX are divergent. While immunolabeling indicates that TMX altered the expression profile of HSPs, transcriptome analysis does not reveal any differentially expressed HSP gene. We believe that performing only the HSP technique (immunolabeling) is not enough to make a statement about whether TMX is toxic or not since different metabolic pathways can be triggered by TMX. The most expressed HSP was an isoform of HSP70, and only families of HSP60 and HSP90 were expressed. The TUNEL reaction exhibited DNA fragmentation only in eight days of TMX exposure. However, only two cells were immunostained. Once again, based on the TUNEL technique alone, it is not possible to conclude that TMX does not cause cell death. We strongly recommend that other methods be combined with HSP immunolabeling and the TUNEL method to provide more robust data and conclusions.

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Authorship contributions

Lucas Miotelo: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, and Visualization. Milene Ferro: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Data Curation, Writing - Original Draft, and Visualization. Geovana Maloni: Validation, Investigation, and Writing - Review & Editing. Igor V. R. Otero: Conceptualization, Formal analysis, Data Curation, Writing - Original Draft, Investigation, and Visualization. Roberta C. F. Nocelli: Resources, Writing - Review & Editing, and Supervision. Mauricio Bacci: Resources, Writing - Review & Editing, and Supervision. Osmar Malaspina: Conceptualization, Resources, Writing - Review & Editing, and Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CONSIDERAÇÕES FINAIS

O TMX afeta os perfis de expressão gênica nos túbulos de Malpighi de *M. scutellaris* expostos a uma concentração subletal, ambientalmente relevante.

Diferentes vias metabólicas, fundamentais para o bom funcionamento dos túbulos, são afetadas pelo TMX, levando ao comprometimento do órgão. Com maior prejuízo após o oitavo dia de exposição.

Embora a imunomarcação indique que o TMX alterou o perfil de expressão de HSPs, a análise do transcriptoma não revelou nenhum gene de HSP diferencialmente expresso.

Os dados obtidos para a imunomarcação de HSP70 e HSP90 são divergentes com os dados obtidos no transcriptoma. Evidenciando que os resultados de imunomarcação precisam ser avaliados com mais cuidado.

Realizar apenas a técnica de imunomarcação para HSP70, HSP90 e TUNEL pode gerar resultados inconclusivos e/ou tendenciosos. Seja pela alta sensibilidade do imunomarcador, no caso das HSPs, ou pela indicação da morte celular baseado numa etapa muito restrita, como o TUNEL.

Devido a importância ecossistêmica das abelhas sem ferrão, novos estudos que combinem diferentes formas de análise de toxicidade precisam ser realizados, visando não só expandir a área do conhecimento, mas também fornecer dados que subsidiem novas políticas públicas de regulamentação de agrotóxicos e a preservação de polinizadores.

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