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ARTIGO ORIGINAL

EFEITO DE PESTICIDAS NO NÍVEL DE DANOS AO MTDNA EM CABEÇAS DE ABELHAS (*BOMBUS TERRESTRIS L.*)

PESTICIDES EFFECT ON THE LEVEL OF MTDNA DAMAGE IN BUMBLEBEES HEADS (*BOMBUS TERRESTRIS* L.)

ВЛИЯНИЕ ПЕСТИЦИДОВ НА УРОВЕНЬ ПОВРЕЖДЕНИЯ мтДНК В ГОЛОВЕ ШМЕЛЕЙ (*BOMBUS TERRESTRIS* L.)

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RESUMO

O zangão é um dos polinizadores mais importantes para plantas consumidas por seres humanos. Atualmente, existe um risco significativo de extinção para vários polinizadores, incluindo abelhas. Uma das causas mais prováveis é o efeito tóxico dos pesticidas. O efeito mutagênico de pesticidas no DNA de abelhas não foi estudado. O objetivo deste trabalho foi estudar a genotoxicidade de pesticidas para DNA em cabeças de abelhas *Bombus terrestris*. Os autores descobriram que, se adicionados às mitocôndrias isoladas das abelhas, os pesticidas direcionados às mitocôndrias causavam menos danos que os pesticidas de amplo espectro. A maior quantidade de dano ao mtDNA foi causada pela adição de Malathion e Difenoconazole às mitocôndrias isoladas. Além disso, os insetos que consumiram xarope com pesticidas apresentaram mais danos à cabeça do que quando o pesticida foi adicionado às mitocôndrias isoladas. Malathion e Cypermethrin demonstraram efeitos genotóxicos significativos *in vivo*. O difenoconazol causou danos graves ao mtDNA, enquanto a deltametrina não teve nenhum efeito genotóxico. Entre os pesticidas direcionados às mitocôndrias, Fenazaquin e Pyridaben demonstraram a maior genotoxicidade. O clorfenapir, o hidrametilnona e o tolfenpiradado não apresentaram genotoxicidade do mtDNA. Foi observado um aumento no número de cópias do mtDNA em insetos que consumiram xarope de açúcar com Deltametrina e Tolfenpirita. Esse aumento é provavelmente um efeito compensatório em resposta à inibição da respiração mitocondrial. Este estudo constatou que, em geral, pesticidas de amplo espectro (Difenoconazol, Deltametrina, Esfenvalerato, Malathion e Cipermetrina) demonstram maior genotoxicidade do mtDNA em cabeças de abelhas em comparação com pesticidas direcionados às mitocôndrias (Fenazaquina, Clorfenapir, Hydramethylnon, Pyridaben e Pyridaben) .

Palavras-chave: *pesticidas, abelhas, cabeça, mtDNA, danos, genotoxicidade..*

ABSTRACT

Bumblebees are one of the most important pollinators for plants consumed by humans. Currently, there is a significant risk of extinction for several pollinators, including bumblebees. One of the most likely causes is the toxic effect of pesticides. The mutagenic effect of pesticides on the DNA of bumblebees has not been studied. The aim of this work was to study the genotoxicity of pesticides for DNA in *Bombus terrestris* heads. The authors found that if added to bumblebees' isolated mitochondria, mitochondrial-directed pesticides caused less damage than broad-spectrum pesticides. The greatest amount of mtDNA damage was caused by adding Malathion and Difenoconazole to isolated mitochondria. Moreover, insects that consumed syrup with pesticides displayed more damage to the head than when the pesticide was added to isolated mitochondria. Malathion and

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Cypermethrin demonstrated significant genotoxic effects *in vivo*. Difenoconazole caused severe damage to mtDNA, while Deltamethrin did not have any genotoxic effect. Among mitochondria-targeted pesticides, Fenazaquin and Pyridaben demonstrated the highest genotoxicity. Chlorfenapyr, Hydramethylnone, and Tolfenpyrad did not show mtDNA genotoxicity. An increase in the number of copies of mtDNA was observed in insects that consumed sugar syrup with Deltamethrin and Tolfenpyrad. This increase is probably a compensatory effect in response to inhibition of mitochondrial respiration. This study found that, in general, broad-spectrum pesticides (Difenoconazole, Deltamethrin, Esfenvalerate, Malathion, and Cypermethrin) demonstrate greater mtDNA genotoxicity in bumblebees' heads compared with mitochondria-targeted pesticides (Fenazaquin, Chlorfenapyr, Hydramethylnon, Pyridaben, and Tolfenpyrad).

Keywords: *pesticides, bumblebees, head, mtDNA, damage, genotoxicity.*

АННОТАЦИЯ

Шмели являются одним из важнейших опылителей растений, потребляемых в пищу человеком. В настоящее время в мире наблюдается тенденция вымирания опылителей, в том числе шмелей. Одной из наиболее вероятных причин такого явления – токсическое действие пестицидов. Мутагенное действие пестицидов на ДНК шмелей практически не изучено. Целью данной работы явилось изучение генотоксичности пестицидов для ДНК в головах шмелей *Bombus terrestris*. Выявлено, что при добавлении к изолированным митохондриям шмелей митохондриально-направленные пестициды вызывали меньшее количество повреждений, чем пестициды широкого спектра действия. Наибольшее количество повреждений мтДНК вызывало добавление к изолированным митохондриям малатиона и дифеноконозола. При этом потребление насекомыми сиропа с пестицидами индуцирует большее количество повреждений в голове, чем добавление пестицида к изолированным митохондриям. Значительный генотоксический эффект *in vivo* демонстрировали малатион и циперметрин. Сильные повреждения мтДНК вызывал дифеноконозол, в то время как дельтаметрин не оказывал никакого генотоксического эффекта. Среди митохондриально-направленных пестицидов наибольшую генотоксичность проявляли феназахин и пиридабен. Хлорфенапир, гидраметилнон и толфенпирад не проявляли генотоксичность в отношении мтДНК. Увеличение количества копий мтДНК наблюдалось у насекомых, которые потребляли сахарный сироп с дельтаметрином и толфенпирадом. Увеличение количества копий мтДНК, возможно, является компенсаторным эффектом в ответ на ингибирование митохондриального дыхания. Установлено, что в целом пестициды широкого спектра действия (дифеноконозол, дельтаметрин, эсфенвалерат, малатион, циперметрин) демонстрируют большую генотоксичность в отношении мтДНК в головах шмелей по сравнению с митохондриально-направленными пестицидами (феназахин, хлорфенапир, гидраметилнон, пиридабен, толфенпирад).

Ключевые слова: пестициды, шмели, голова, мтДНК, повреждения, генотоксичность.

1. INTRODUCTION

Bumblebees are the most important pollinators of entomophilous crops, both in the open and closed ground, capable of pollinating plants even in cool and windy weather (Berger *et al.*, 1998; Goodell & Thomson, 2007; Thomson & Goodell, 2002). Bumblebees for commercial use have been produced since the mid-1980 (Velthuis & van Doorn, 2006). Commercial bumblebee farming is currently undergoing intensive development (Lye *et al.*, 2011). Bumblebees and bees are becoming a popular research subject in light of the threatening crisis of declining pollinator populations in the world (Biesmeijer *et al.*, 2006; Potts *et al.*, 2010; Thomann *et al.*, 2013; Connelly *et al.*, 2015; Rhodes, 2018) , which is already a matter of food security for humans (Klein *et al.*, 2007). One of the main reasons for this decline is the widespread use of pesticides (Rortaisa *et al.,* 2005). At the same time, the simultaneous effect of pathogens on pollinators leads to a synergistic increase in the harmful effect of pesticides on insects (Grassl *et al.*, 2018).

Pesticides may affect the nervous systems of organisms. A number of pesticides, such as carbamates, organophosphates, etc., influence the nervous system (Keifer & Firestone, 2007). There is a study that has shown the association between exposure to pesticides and neurological dysfunction (Kamel & Hoppin, 2004). The neurotoxicity of pesticides has also been proved for bees, so it was found that exposure to pesticides can impair a bee's spatial working memory (Samuelson *et al.*, 2016). Exposure to cholinergic pesticides impairs olfactory learning and memory in bees (Williamson & Wright, 2013). Moreover, the toxic effect of pesticides can be mediated by mitochondria (Singh *et al.*, 2018; Salama *et al.*, 2014).

There are a number of pesticides whose

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mechanism of action is associated with the inhibition of the electron transport chain (ETC) of mitochondria. Chlorfenapyr is an insecticide that disrupts oxidative phosphorylation in mitochondria. Chlorfenapyr inhibits the production of adenosine triphosphate (ATP), causing the death of the target organism (Raghavendra *et al.*, 2011). Tolfenpyrad inhibits ETC complex I (NADH dehydrogenase) in mitochondria (Simon, 2015). Hydramethylnone also acts as a mitochondrial respiration inhibitor (Hollingshaus, 1987). It is believed that it inhibits the flow of electrons in mitochondria to the levels of ETC complex III (Simon, 2008). Pyridaben is also a mitochondria-directed pesticide, and its target is mitochondrial ETC complex I (Navarro *et al.*, 2010; Sherer *et al.*, 2007). Fenazaquin, like Pyridaben, inhibits mitochondrial ETC complex I (Lümmen, 1998). The authors have previously shown that fungicides can affect isolated bumblebee mitochondria (Syromyatnikov *et al.*, 2017).

The mutagenic effect of pesticides on the DNA of bees and bumblebees has been poorly studied. The genotoxicity of pesticides on bumblebees has not been studied. Meanwhile, it is known that exposure to pesticides may lead to various forms of DNA damage (Marcelino *et al.*, 2019; Bolognesi, 2003). Of particular interest is the study of the genotoxicity of pesticides of various classes, including mitochondria-targeted, on the level of DNA damage in bumblebee heads. The centers of the bumblebee's nervous system and sense organs are located in its head. Therefore, the aim of this work was to study the genotoxicity of pesticides on the DNA in bumblebees' heads using long-range PCR, which is based on the amplification of long fragments of mitochondrial DNA.

2. MATERIALS AND METHODS

2.1. The object of the study

Tekhnologii Shmelevodstva LLC (Voronezh, Russia) provided male bumblebees (*Bombus terrestris L.*). The bumblebees were kept in cylindrical cages (diameter: 14 cm, height: 7 cm) with a mesh bottom and a lid, at a temperature of 27 °C, and at a relative humidity of 55%, and there were no more than ten bumblebees in each cage.

All experimental procedures with insects were performed in accordance with the rules set by Voronezh State University Ethical Committee on Biomedical Research (Section of Animal Care and Use, protocol 42-04 dated September 16, 2019).

2.2. In vitro pesticide toxicity study

The toxicity of the following commonly used, commercially available broad-spectrum pesticides were studied: Difenoconazole, Deltamethrin, Esfenvalerate, Malathion, Cypermethrin, and some mitochondria-targeted pesticides (Fenazaquin, Chlorfenapyr, Hydramethylnon, Pyridaben, and Tolfenpyrad). All by Sigma-Aldrich, USA.

Mitochondria from bumblebees were isolated according to the protocol described previously (Syromyatnikov *et al.*, 2013). Intact mitochondria used a mixture of five mmol/L of malate and five mmol/L of pyruvate as a substrate for respiration. Each pesticide, at a concentration of 50 μM, was added to tubes with intact mitochondria. Mitochondria with respiratory substrates and pesticides were incubated for an hour at a temperature of 37 °C. Afterward, the total DNA was isolated from mitochondria by DiaGene DNA isolation kit (Dia-M, Russia).

2.3. The study of the pesticides' toxicity on bumblebees

The concentration of pesticide added to the syrup fed to the bumblebees was preliminarily selected empirically (data not published) so that the mortality of the bumblebees did not exceed 10%. As a result, 1 mg of the investigated substances was dissolved in 0.5 ml of 99% DMSO; after which the resulting solution was added to 10 ml of 60% sugar syrup. The pesticide concentration of 1 mg / 10 ml is the concentration that the bumblebee can potentially come into contact with on plants (Girolami *et al.*, 2009). The control solution contained 10 ml of 60% sugar syrup with 0.5 ml of DMSO. The bumblebees were placed in cylindrical cages (diameter: 14 cm, height: 7 cm) with a mesh bottom (so that the bumblebees would meet the feeder filled with syrup) and a lid; there were 10 bumblebees in each cage. The bumblebees were kept at a temperature of 27–28.5 °C with an air humidity of 55%–68%. DNA was extracted from the bumblebees' heads 24 hours after the experiment.

2.4. DNA isolation and long-range PCR

Afterward, the total DNA was isolated from mitochondria by DiaGene DNA isolation kit (Dia-M, Russia). The amount of oxidative damage was assessed using long-chain PCR on a CFX96 Touch™ Real-Time PCR Detection System thermocycler (made by Bio-Rad, USA) using the Encyclo polymerase kit (Evrogen,

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Russia). The reaction conditions were as follows: initial denaturation at 95 °C for five minutes, 35 cycles with denaturation at 95 °C for 10 seconds, annealing the primers at 59 °C for 30 seconds, and elongation at 66 °C for five minutes. The number of lesions was evaluated in fragments corresponding to mitochondrial genes *Cox1* and *Cox2* F: 5'-CCCCAGATATAGCTTTTCCTC-3'; R: 5'-CCAGGAATTGCATCAACTTT-3' (product length is 2,083 bps) and *Nad6, Nad1, CybB* F: 5'-CGCTATTGCTGGCACTAATTT-3'; R: 5'-AAATTATTCAGAAACAAAATGGAAA-3' (fragment length is 2,013 bps). A short fragment (99 bps) was used as a reference, which was amplified using primers: F: 5'-TCCATGGGATTCATGTTCTT-3'; R: 5'-CAAAATTAATATGATGAATTGAAGAG-3'.

The amount of DNA damage per 10,000 bps was calculated by Equation 1:

$$X = (1 - 2^{-(\Delta\Delta Cq)}) * 10,000 \text{ bp/fragment length, bp.}$$

Where, X - the amount of damage/10 kbp; $\Delta\Delta Cq$ - ΔCq between the control and the experimental long fragments divided by ΔCq between the control and the experimental short fragments.

2.5. Estimated number of mtDNA copies

The number of mtDNA copies was estimated using real-time quantitative PCR using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) using the qPCRmix-HS SYBR + LowROX kit (Evrogen, Russia). The following mtDNA fragments were amplified using the following pair of primers: F: 5'-TCCATGGGATTCATGTTCTT-3'; R: 5'-CAAAATTAATATGATGAATTGAAGAG-3' and genomic DNA using the following pair of primers: F: 5'-AGAACCTCCGTATCCCCTTCG-3'; R: 5'-AGCCTACCCAFTGCTGAAC-3'.

The reaction conditions were as follows: initial denaturation at 95 °C for 5 minutes, then 35 cycles with denaturation at 95 °C for 10 seconds, annealing the primers at 59 °C for 30 seconds, and elongation at 66 °C for 30 seconds. Normalized mtDNA level relative to nuclear DNA was calculated using standard formula $2^{(-\Delta\Delta Cq)}$ (Yuan *et al.*, 2006).

2.6. Statistical processing

Statistical processing of the results was carried out using the software package Statistica 10 (StatSoft.Inc, USA). The normality of data distribution was determined using the Shapiro-Wilk test. The distribution quantity value is presented as the median (Q1 and Q3 quartiles). A comparison of the number of injuries was carried out using the nonparametric Mann–Whitney U-test. The value of the number of mtDNA copies is presented as the mean ± standard error of the mean. The number of mtDNA copies was compared using the parametric Student's T-test. The work discusses statistically significant differences (p <0.05).

3. RESULTS:

3.1. In vitro pesticide toxicity

The greatest amount of mtDNA damage was caused by the addition of Malathion (median 1.63 (0.71; 2.60), p <0.05) and Difenoconazole (median 1.52 (0.52; 2.30), p < 0.05) compared with the control (median -0.07 (-0.10; 0.06)) (Figure 1).
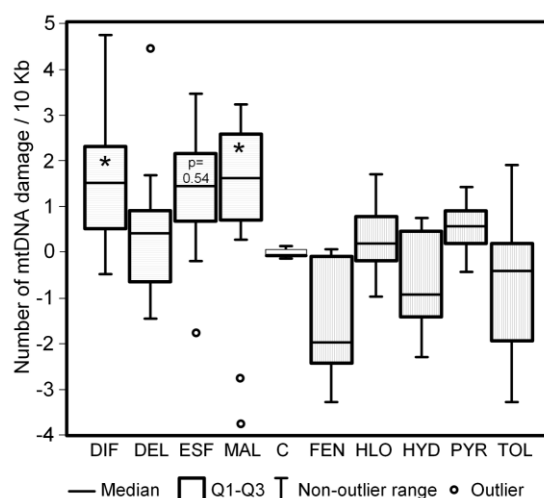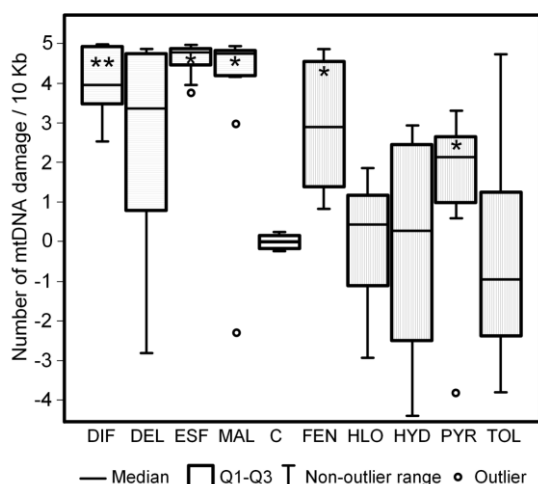

Figure 1. *The amount of damage to mtDNA/10 kbp when pesticides are added to isolated mitochondria in vitro. *p <0.05 differences compared with the control are statistically significant according to the Mann-Whitney test. Dif – Difenoconazole; Del – Deltamethrin; Esf – Esfenvalerate; Mal – Malathion; C – Control; Fen – Fenazaquin; Hlo – Chlorfenapyr; Hyd – Hydramethylnon; Pyr – Pyridaben; Tol – Tolfenpyrad.*

The addition of Esfenvalerate caused damage in intact mitochondria (median (1.45 (0.70; 2.17)), but the data are statistically insignificant (p = 0.58). Deltamethrin, as well as Fenazaquin, Chlorfenapyr, Hydramethylnone, Pyridaben, and Tolfenpyrad, did not cause damage to mtDNA when added to intact mitochondria.

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3.2. In vivo pesticide toxicity

When bumblebees consumed pesticides, Esfenvalerate caused the largest amount of mtDNA damage (median 4.80 (4.47; 4.92), p <0.05). Significant genotoxic effect was demonstrated by Malathion (median 4.78 (4.20; 4.84), p <0.05) and Cypermethrin (median 4.78 (3.31; 4.84), p <0.05). Difenoconazole caused severe mtDNA damage (median 3.98 (3.49; 4.97), p <0.01), while Deltamethrin also had no genotoxic effect. Among mitochondria-targeted pesticides, Fenazaquin (median 2.92 (1.37; 4.54), p <0.05) and Pyridaben (median 2.18 (0.97; 2.69 p <0.05) showed the highest genotoxicity. Chlorfenapyr, Hydramethylnon, and Tolfenpyrad did not show genotoxicity with respect to mtDNA (Figure 2).

*Figure 2. The amount of damage to mtDNA/10 kbp when bumblebees consumed pesticides. *p <0.05; **p <0.01 differences compared with the control are statistically significant according to the Mann-Whitney test. Dif – Difenoconazole; Del – Deltamethrin; Esf – Esfenvalerate; Mal – Malathion; C – Control; Fen – Fenazaquin; Hlo – Chlorfenapyr; Hyd – Hydramethylnon; Pyr – Pyridaben; Tol – Tolfenpyrad.*

3.3. Change in the number of copies of mtDNA in bumblebees heads when consuming pesticides

The effect of pesticides on the number of mtDNA copies was not the same. Deltamethrin increased the number of copies of mtDNA by 37%, and Tolfenpyrad by 74% (both p <0.05). Esfenvalerate caused a decrease in the number of mtDNA copies by 34%, and Pyridaben by 38% (both p <0.05) (Fig. 3).
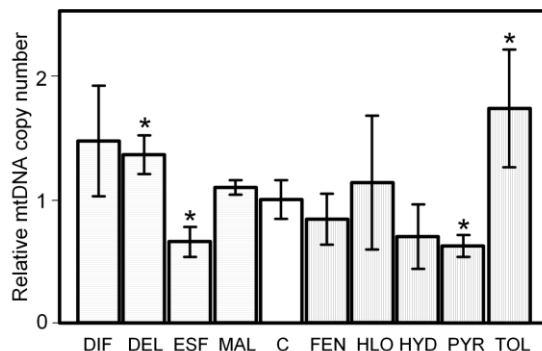
*Figure 3. The number of mtDNA copies when bumblebees consumed pesticides. *p <0.05 differences compared with the control are statistically significant according to Student's T-Test. Dif – Difenoconazole; Del – Deltamethrin; Esf – Esfenvalerate; Mal – Malathion; C – Control; Fen – Fenazaquin; Hlo – Chlorfenapyr; Hyd – Hydramethylnon; Pyr – Pyridaben; Tol – Tolfenpyrad.*

Difenoconazole, Malathion, Fenazaquin, Chlorfenapyr, and Hydramethylnon did not show a statistically significant effect on the number of mtDNA copies.

4. DISCUSSION:

The results showed that mitochondrial-directed pesticides cause less damage than pesticides with a wide exposure profile if added to bumblebees' isolated mitochondria (Fig. 1). Mitochondria-directed pesticides, mainly blocking the respiration of mitochondria, can explain this. In turn, a decrease in the respiration rate caused, for example, by dissociation of the inner mitochondrial membrane, may be associated with a decrease in the rate of production of reactive oxygen species (ROS), which are the main damaging factors (Cadenas *et al.*, 2018).

Insect consumption of pesticide syrup induces more damage to the head than adding pesticide to isolated mitochondria (Figures 1 and 2). This may be due to the characteristics of the nervous tissue, which is one of the most metabolically active tissues in the body (Herculano-Houzel, 2011). Among mitochondria-targeted pesticides, Pyridaben, and Fenazaquin (Figure 2) cause mtDNA damage. Pyridaben is known to increase oxidative stress due to the selective inhibition of ETC complex I (Navarro *et al.*, 2010), which causes serious damage to the DNA in mammals (Ebadi Manas *et al.*, 2013). Therefore, its toxicity to insect mtDNA is obvious. Fenazaquin is also an inhibitor of mitochondria

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ETC complex I (Lümmen, 1998), and its mechanism of genotoxicity is probably similar to Pyridaben.

A change in the number of mtDNA copies can be a compensatory effect of mitochondria in response to stressful conditions induced by the consumption of pesticides. It is noteworthy that an increase in the number of copies of mtDNA was observed in insects that consumed sugar syrup with Deltamethrin and Tolfenpyrad (Figure 3). Deltamethrin is the only broad-spectrum pesticide that did not cause a statistically significant increase in mtDNA damage, and Tolfenpyrad is the only pesticide whose damage value (median -0.96 (-2.43; 1.24)) was lower than that of the control, though the differences are not statistically significant (Figure 3). An increase in the number of mtDNA copies is probably a compensatory effect in response to inhibition of mitochondrial respiration. It has been shown previously that many cytotoxic agents, such as Hexavalent Chromium (VI) (Zhong et al., 2017), Methamphetamine (Valian et al., 2016), Tert-Butyl Hydroperoxide (Rasbach & Schnellmann, 2007), Buthionine Sulfoximine (Lee et al., 2000), Doxorubicin, Mitoxantrone (Kluza et al., 2004) and ROS (Gutsaeva et al., 2006) are able to activate mitochondrial biogenesis as a compensatory reaction in mammalian cells. It is likely that some pesticides have a similar effect on insects, but this issue needs further study.

In contrast, Esfenvalerate and Pyridaben caused a decrease in the number of mtDNA copies (Figure 3). Esfenvalerate is the most genotoxic of the studied pesticides in vivo and one of the most toxic when added in vitro. Pyridaben and Fenazaquin, mitochondria-targeted pesticides that showed genotoxicity when bumblebees consumed the syrup, also led to a decrease in the number of mtDNA copies, though the data were not statistically significant for Fenazaquin.

5. CONCLUSIONS:

Broad-spectrum pesticides demonstrate greater mtDNA genotoxicity in bumblebee heads compared to mitochondria-targeted pesticides. We have shown that broad-spectrum pesticides on the whole cause 4.9 times more mtDNA damage than mitochondria-targeted pesticides. In this case, the most toxic pesticides caused a decrease in the number of copies of mtDNA, while when exposed to less toxic pesticides, it was observed an increase in the number of mtDNA, which is probably a compensatory response of the cell in response to inhibition of mitochondrial respiration.

6. ACKNOWLEDGMENTS:

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