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Method for detection of mtDNA damages for evaluating of pesticides toxicity for bumblebees (*Bombus terrestris* L.)

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ABSTRACT

Bumblebees are important for crop pollination. Currently, the number of pollinators is decreasing worldwide, which is attributed mostly to the widespread use of pesticides. The aim of this work was to develop a method for assessing the genotoxicity of pesticides for the *Bombus terrestris* L. bumblebee using long-range PCR of mitochondrial DNA fragments. We have developed a panel of primers and assessed the genotoxicity of the following pesticides: imidacloprid, rotenone, deltamethrin, difenocanozole, malathion, metribuzin, penconazole, esfenvalerate, and dithianon. All pesticides (except imidacloprid) inhibited mitochondrial respiration fueled by pyruvate + malate; the strongest effect was observed for rotenone and difenocanozole. Three pesticides (dithianon, rotenone, and difenocanozole) affected the rate of H_2O_2 production. To study the pesticide-induced DNA damage *in vitro* and *in vivo*, we used three different mtDNA. The mtDNA damage was observed for all studied pesticides. Most of the studied pesticides caused significant damage to mtDNA *in vitro* and *in vivo* when ingested. Our results indicate that all tested pesticides, including herbicides and fungicides, can have a toxic effect on pollinators. However, the extent of pesticide-induced mtDNA damage in the flight muscles was significantly less upon the contact compared to the oral administration.

1. Introduction

Currently, the number of insect pollinators is decreasing worldwide (Potts et al., 2010; Biesmeijer et al., 2006; Rhodes, 2018; Thomann et al., 2013; Connelly et al., 2015). About 35% crops directly dependent on pollinators (Klein et al., 2007), with the cost approximately 153 billion euros per year (Gallai et al., 2009). Parasites and pesticides are among the factors most commonly associated with the death of bees (Guzman-Novoa, 2016). Pesticides, especially neonicotinoids, are often blamed for the loss of bee populations (Van der Sluijs et al., 2013). Insect pollinators mediate pollen exchange between flowers and contribute to fruit and seed production in about 88% flowering plants (Ollerton et al., 2011). As suggested by several studies, parasites, pesticides, or a combination of these factors, may be responsible for the health damage of honeybees (Vanengelsdorp et al., 2009).

Bumblebees are important pollinators of many wildflowers critical to terrestrial ecosystems (Goulson, 2010). Pollinators, such as *Bombus terrestris*, *Bombus impatiens*, and *Bombus ignitus* are used commercially (Velthuis and van Doorn, 2006). A decline in the populations of bumblebees has been observed in North America, Europe, and other world regions (Goulson, 2010; Cameron et al., 2011; Grixti et al., 2009; Colla and Packer, 2008; Goulson et al., 2008). At the same time, there has been a sharp increase in the systemic use of neonicotinoids for seed treatment. Neonicotinoids are currently world's most widely used class of insecticides (Goulson, 2013). Other insecticides, such as carbamates, commonly used in managed ecosystems, can also affect the health of honeybees (Johnson et al., 2010). Fungicides can synergistically enhance the effect of neonicotinoids on pollinators (Fisher et al., 2017; Raimets et al., 2018; Zhu et al., 2017; Sgolastra et al., 2017). The use of pesticides is also considered a serious threat to wild bees. Both laboratory and field studies have identified negative effects of pesticides on bumblebee behavior, reproduction, and colony development (Baron et al., 2017). The synergistic action of pesticides and diseases on pollinators could be the major cause of the decline of these insects (Lopez et al., 2017; Grassl et al., 2018; O'Neal et al., 2018; Aufauvre et al., 2012). Since we have previously excluded pathogens as the main cause

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of decline of bumblebee micro-colonies (Syromyatnikov et al., 2019), suggesting that pesticides are the main stress factor in the development of bumblebees.

Exposure to pesticides can lead to DNA damage and formation of DNA-protein cross-links (Marcelino et al., 2019). For example, exposure to organophosphate leads to abnormal sperm development, fetal death, birth defects, hormonal changes, DNA damage, and changes in the ovaries and eggs (Arshad et al., 2016). Most pesticides have been tested for the induction of gene mutations, changes in chromosomal structure, and DNA damage. Experimental data revealed that many agrochemical ingredients possess mutagenic properties (Bolognesi, 2003). Various methods have been developed for assessing the genotoxicity of pesticides that typically evaluate DNA damage, chromosomal aberrations, and point mutations. These tests include *in vitro* analysis of pesticide genotoxicity, the micronucleus test, mammalian bone marrow cytogenetic tests, chromosomal analysis, mammalian germ cell cytogenetic analysis, and mouse hereditary translocation tests (Raghavendra et al., 2015).

The aim of this work was to develop a method for assessing the genotoxicity of pesticides in *B. terrestris* L. bumblebees using long-range PCR of mitochondrial DNA (mtDNA) fragments and to assess the genotoxic effect of common pesticides with the developed method.

2. Materials and methods

2.1. Animals and pesticides

Bombus terrestris L. males were obtained from the Technology of Bumblebee Rearing Ltd. (Voronezh, Russia). The bumblebees were kept at a temperature of 27–28.5°C and humidity of 55–68%.

The following pesticides were used in the study: imidacloprid, rotenone, deltamethrin, difenocanozole, malathion, metribuzin, penconazole, esfenvalerate, and dithianon (Sigma Aldrich, USA).

The damage of mtDNA was studied on the next day after exposure of bumblebees to the pesticides.

2.2. Contact administration of pesticides

The pesticides were dissolved in 500 μl DMSO, and the resulting solution was diluted with 9.5 ml of distilled water; distilled water (9.5 ml) containing 500 μl of DMSO was used as a control. The concentration of the pesticide solution was brought to the desired value by multiple dilutions. The bumblebees were gently placed for 1 s in the test tube with the pesticide solution and then kept for 2 h in a specialized cage with a filter paper at the bottom to dry the insects. Next, the bumblebees were placed in cylindrical cages (diameter, 14 cm; height 7 cm) with a mesh bottom and a lid (10 bumblebees per cage). Inverted sugar syrup (60%) was used as a feed.

2.3. Oral administration of pesticides

The pesticides were dissolved in 500 μl DMSO, and the resulting solution was diluted with 9.5 ml of inverted sugar syrup (60%). The concentration of the pesticide solution was brought to the desired value by multiple dilutions. The bumblebees were placed in cylindrical cages (diameter, 14 cm; height, 7 cm) with a mesh bottom and a lid (10 bumblebees in each cage) and fed with the pesticide-containing syrup; sugar syrup without the pesticide was used as a control.

2.4. Evaluation of the pesticide effect on the respiration rate in the flight muscle mitochondria

Mitochondria were isolated from the flight muscles of bumblebees as described earlier (Syromyatnikov et al., 2013). The thorax was separated from the body; nine thoraces were placed in 15 ml of ice-cold isolation medium (100 mM sucrose, 220 mM mannitol, 1 mM EGTA, 2 mg/ml fatty acid-free bovine serum albumin (BSA), and 20 mM HEPES, pH 7.4). The thoraces were homogenized with a Dounce type homogenizer (Thermo Fisher Scientific, USA), and the homogenate was centrifuged at 600*g* for 5 min. The supernatant was collected and centrifuged at 10,000*g* for 10 min. The pellet was resuspended in 1 ml of washing medium containing the same ingredients as the isolation medium except BSA. The resulting suspension was diluted to 35 ml with the washing medium and centrifuged at 10,000*g* for 10 min. The pellet was resuspended in 0.1 ml of the washing medium and stored on ice. The rate of oxygen consumption by the isolated mitochondria was recorded with an Oxygraph system (Hansatech Instruments, UK). The measurements were performed at 24 °C in 1 ml of incubation medium containing 220 mM mannitol, 100 mM sucrose, 1 mM EGTA, 4 mM KH_2PO_4, 20 mM HEPES (pH 7.3), and 5 mM of respiratory substrates. The pesticides were added at 50 μM final concentration.

2.5. Effect of pesticides on the reactive oxygen species (ROS) production by the flight muscles mitochondria

The rate of H_2O_2 generation was measured with the fluorescent dye Amplex Red Ultra (Sigma, USA) as described early (Starkov, 2010) in the incubation medium (1 ml) containing 100 mM sucrose, 220 mM mannitol, 4 mM KH_2PO_4, 1 mM EGTA, 20 mM HEPES (pH 7.4), 2 μM Amplex Red, 0.1–0.2 mg of mitochondrial protein, and 1 mg/ ml horseradish peroxidase. Fluorescence was registered at 581 nm with a Hitachi F-7000 spectrofluorometer (excitation at 568 nm). Protein concentration was measured with a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, USA).

2.6. Pesticides toxicity in vitro

Mitochondria were isolated from the bumblebee flight muscles as described above. Pesticides at a concentration of 100 μM were added to intact flight muscle mitochondria (0.1–0.2 mg) and incubated for 30 min in the presence of respiratory substrates (5 mM malate + 5 mM pyruvate). The control sample contained mitochondria and respiratory substrate at the same concentration without the pesticide. mtDNA was isolated from the treated and control mitochondria using a diaGene DNA extraction kit (Dia-M, Russia).

2.7. Long-range PCR

The primers to assess the genotoxicity of pesticides from the extent of mtDNA damage were designed using the primer3 software (Table 1).

The extent of mtDNA damage was evaluated in three mtDNA fragments. Fragment 1 included *Cox1, Cox2*, *tRNA Leu,* and *tRNA Phe* genes.

Table 1
Primers for detection of mtDNA damage.

Fragment	Forward 5′ – 3′	Reverse 5′ – 3′	Fragment length
1	CCCCAGATATAGCTTTTCCTC	CCAGGAATTGCATCAACTTT	2083
2	CTTCAATTCACCTTTAAAACAA	GTATTACCACGAATTCGATATG	2013
3	CGCTATTGCTGGCACTAATTT	AAATTATTCAGAAACAAAATGGAAA	2113
Ref	TCCATGGGATTCATGTTCTT	CAAAATTAATATGATGAATTGAAGAG	99

Fragment 2 included *Nad6, Nad1, CybB, tRNA Ser, tRNA Gln,* and *tRNA Met* genes. Fragment 3 included ribosomal genes, D-loop region, and *tRNA Asn* and *tRNA Val* genes. For the long-range PCR, we used a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) and Encyclo polymerase kit (Evrogen, Russia) according to the manufacturer's protocol. The reaction conditions were: 5 min at 95 °C, followed by 35 cycles of denaturation (95 °C for 10 s), primer annealing (59 °C for 30 s), and elongation (66 °C for 5 min). The temperature gradient from 56 to 72 °C for the elongation step was used during the method optimization. The size of the PCR products was determined by electrophoresis in 2% agarose gel in 1 × TAE buffer. The extent of mtDNA damage induced by the pesticide exposure was estimated using the ΔΔCq method as described earlier (Gureev et al., 2017).

2.8. Statistical analysis

Statistical analysis was performed with the STADIA software (Moscow State University, Russia). The results were expressed as mean ± SEM. The differences among the experimental groups were analyzed using the one-way analysis of variance (ANOVA); the differences were considered statistically significant at $p < 0.05$. Correlation analysis was performed using the Spearman's rank correlation coefficient (R_s).

3. Results

3.1. The choice of pesticide dose for studying pesticide genotoxicity

To assess the genotoxicity of pesticides upon the contact with the bumblebees, we investigated the mortality of bumblebees exposed to the pesticide aqueous solutions of varying concentrations from 0.0001 to 0.1% (data not shown). The concentrations of pesticides that caused less than 10% mortality in the bumblebees were chosen for the following studies on the contact action of these compounds: 0.0001% for deltamethrin, malathion, esfenvalerate, imidacloprid, and penconazole; 0.001% for dithianon, rotenone, and difenocanozole; and 0.01% for metribuzin. The same concentrations were used in the studies of pesticide genotoxicity upon oral administration, except the pesticide solutions were prepared in sugar syrup.

3.2. The effect of pesticides on the respiration in the bumblebee flight muscles

Mitochondria were isolated from the bumblebee flight muscles and the rate of mitochondrial respiration on malate + pyruvate, *i.e.*, respiration mediated by the electron transport chain (ETC) complex I, was estimated in the presence and absence of pesticides. The components of the reaction mixture were added to the oxygraph cell in the following order: mitochondria; ADP; pesticide. The obtained results on the effect of pesticides on the mitochondrial respiration are presented in Table 2.

All pesticides except imidacloprid inhibited mitochondrial respiration on pyruvate + malate, the strongest effect being exhibited by rotenone. At the same time, it was shown by us earlier that respiration on α-glycerophosphate was inhibited only by dithianon (Syromyatnikov et al., 2017). Only three pesticides affected the rate of H_2O_2 production: dithianon (1.4-fold decrease), rotenone (3.4-fold increase), and difenocanozole (4.1-fold increase).

3.3. Pesticide toxicity in vitro

Most of the studied pesticides caused significant damage to mtDNA upon addition to intact mitochondria (Fig. 1). Deltamethrin did not cause a statistically significant increase in the extent of oxidative damage in the mtDNA fragments 1 and 2, but induced 1.36 ± 0.41 lesions/10 kb in fragment 3 ($F(1,14) = 10.79$, $p < 0.01$). Difenocanozole caused lesions in fragment 1 (1.4 ± 0.31 lesions/10 kb; $F(1,14) = 20.01$, $p < 0.01$), fragment 2 (1.92 ± 0.7 lesions/10 kb; $F(1,14) = 7.61$, $p < 0.05$), and fragment 3 (2.22 ± 0.38 lesions/10 kb; $F(1,14) = 33.39$, $p < 0.001$). Dithianon did not cause statistically significant increase in the number of lesions in any of the studied fragments; however, there was a trend toward the increase in the number of lesions in fragment 3 (0.67 ± 0.39 lesions/10 kb; $p = 0.063$). Esfenvalerate did not affect the number of lesions in any of the studied fragments. Imidacloprid caused mtDNA damage in fragment 1 (0.86 ± 0.27 lesions/10 kb, $F(1,14) = 10.23$, $p < 0.01$), fragment 2 (2.7 ± 0.48 lesions/10 kb; $F(1,14) = 31.33$, $p < 0.001$), and fragment 3 (1.98 ± 0.49 lesions/10 kb; $F(1,14) = 16.58$, $p < 0.01$). Malathion increased the number of lesions in fragment 1 (1.23 ± 0.23 lesions/10 kb; $F(1,14) = 30.85$, $p < 0.001$) and fragment 3 (2.97 ± 0.65 lesions/10 kb; $F(1,14) = 20.73$, $p < 0.01$), but not in fragment 2. Metribuzin caused the most pronounced damage: 1.61 ± 0.39 lesions/10 kb ($F(1,14) = 16.74$, $p < 0.01$) in fragment 1; 2.97 ± 0.4 lesions/10 kb in fragment 2 ($F(1,14) = 55.54$, $p < 0.001$); and 2.35 ± 0.43 lesions/10 kb in fragment 3 ($F(1,14) = 29.56$, $p < 0.001$). Significant genotoxic effect was demonstrated by rotenone and penconazole, which caused damage in fragment 1 (2.19 ± 0.21 lesions/10 kb; $F(1,14) = 109.8$, $p < 0.01$), fragment 2 (1.86 ± 0.62 lesions/10 kb; $F(1,14) = 8.87$, $p < 0.05$), and fragment 3 (2.45 ± 0.17 lesions/10 kb, $F(1,14) = 212.5$, $p < 0.001$). For rotenone, mtDNA damage was 1.72 ± 0.46 lesions/10 kb in fragment 1 ($F(1,14) = 14.01$, $p < 0.01$), 2.21 ± 0.61 lesions/10 kb in fragment 2 ($F(1,14) = 13.27$, $p < 0.01$), and 1.88 ± 0.27 lesions/10 kb in fragment (($F(1,14) = 48.37$, $p < 0.001$)).

Table 2
Effect of pesticides on the respiration of bumblebee flight muscle mitochondria in the presence of pyruvate + malate as substrates.

Pesticide	Respiration rate		H_2O_2 production	
	respiratory substrate and ADP (nmol O_2/min/mg protein)	+ pesticide (nmol O_2/min/mg protein)	respiratory substrate (nmol H_2O_2/min/mg protein)	+ pesticide (nmol H_2O_2/min/mg protein)
Imidacloprid	124,1 ± 7,1	118,4 ± 7,5	0,27 ± 0,04	0,26 ± 0,04
Rotenone	133,3 ± 7,9	7,7 ± 1,9**	0,27 ± 0,04	0,92 ± 0,02**
Deltamethrin	128,3 ± 6,7	29,4 ± 5,9*	0,26 ± 0,05	0,28 ± 0,05
Difenocanozole	121,6 ± 6,8	14,7 ± 3,7**	0,28 ± 0,04	1,15 ± 0,11**
Malathion	125,7 ± 7,1	21,3 ± 5,1*	0,27 ± 0,03	0,28 ± 0,04
Metribuzin	123,9 ± 6,4	28,4 ± 6,1*	0,28 ± 0,05	0,28 ± 0,05
Penconazole	120,8 ± 7,3	17,1 ± 4,0*	0,26 ± 0,03	0,28 ± 0,04
Esfenvalerate	122,4 ± 6,2	21,4 ± 3,9*	0,27 ± 0,03	0,29 ± 0,05
Dithianon	121,5 ± 7,2	4,7 ± 1,2**	0,26 ± 0,03	0,18 ± 0,02*

* $p < 0.05$.
** $p < 0.01$.

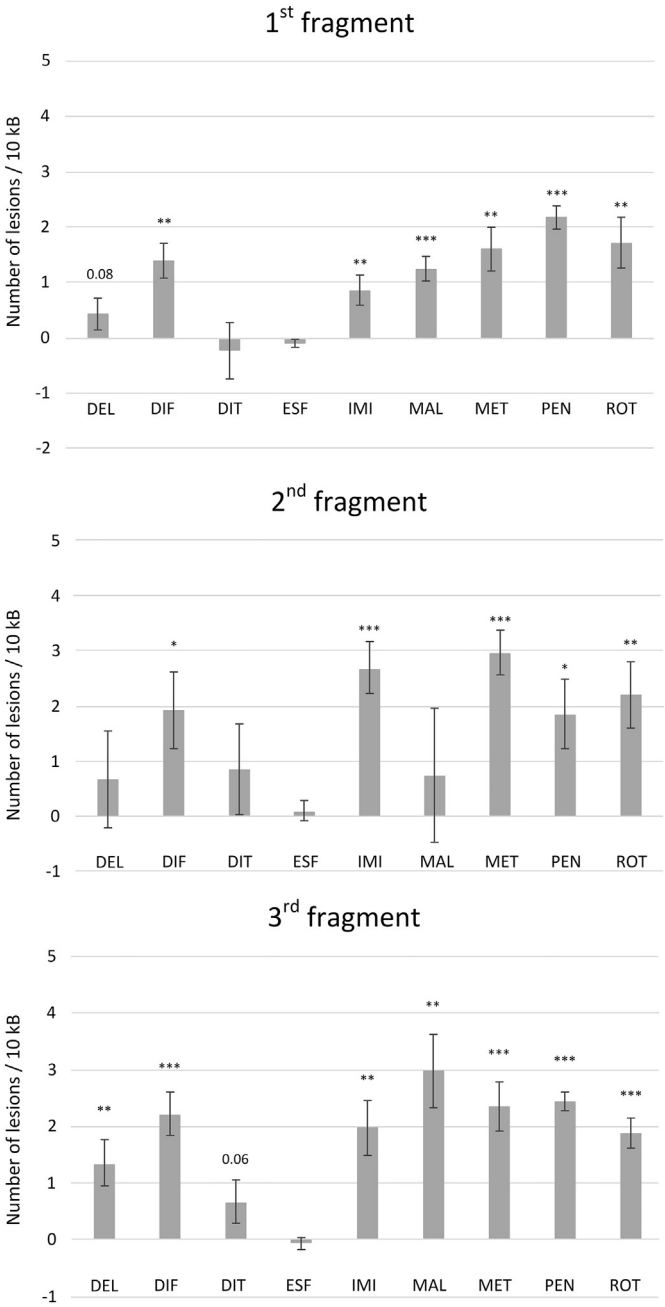

Fig. 1. Number of mtDNA lesions caused by pesticides *in vitro*. Deltamethrin (DEL), malathion (MAL), rotenone (ROT), esfenvalerate (ESF), imidocloprid (IMI), dithianone (DIT), penconazole (PEN), difenoconazole (DIF), metribuzin (MET). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

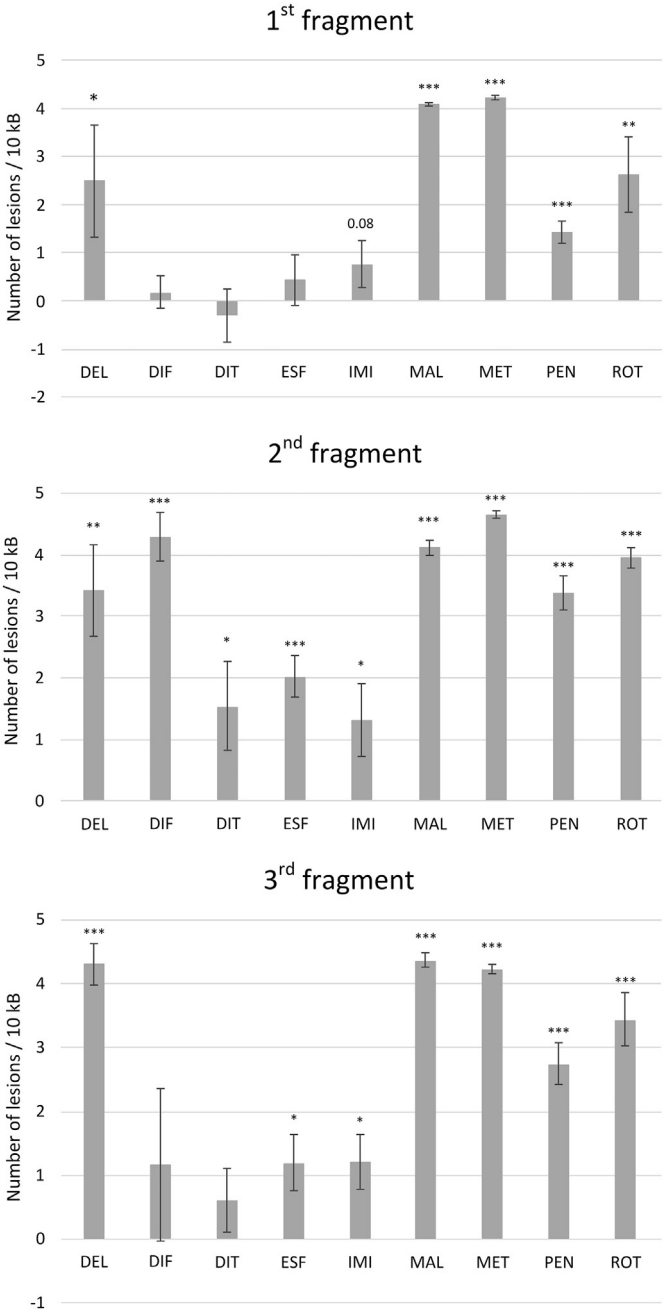

Fig. 2. Number of mtDNA lesions caused by pesticides upon ingestion. Deltamethrin (DEL), malathion (MAL), rotenone (ROT), esfenvalerate (ESF), imidocloprid (IMI), dithianone (DIT), penconazole (PEN), difenoconazole (DIF), metribuzin (MET). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.4. Pesticide toxicity upon ingestion

Most of the studied pesticides caused significant mtDNA damage upon oral administration (Fig. 2). Deltamethrin caused 2.49 ± 1.17 lesions/10 kb in fragment 1 ($F(1,14) = 4.52$, $p < 0.05$)), 3.42 ± 0.75 lesions/10 kb in fragment 2 ($F(1,14) = 20.9$, $p < 0.01$), and 4.31 ± 0.32 lesions/10 kb in fragment 3 ($F(1,14) = 177.01$, $p < 0.001$). Difenocanozole caused damage only in fragment 2 (4.29 ± 0.40 lesions/10 kb; $F(1,14) = 115.2$, $p < 0.001$). Dithianon also damaged mtDNA in fragment 2 only (1.54 ± 0.72 lesions/10 kb; $F(1,14) = 4.58$, $p < 0.05$). Esfenvalerate increased the number of lesions in fragment 2 (2.02 ± 0.35 lesions/10 kb; $F(1,14) = 32.24$, $p < 0.001$) and fragment 3 (1.20 ± 0.43 lesions/10 kb; $F(1,14) = 8.83$, $p < 0.01$), but not in fragment 1. Imidacloprid caused an increased number of lesions in fragment 2 (1.32 ± 0.59 lesions/10 kb; $F(1,14) = 4.96$, $p < 0.05$) and fragment 3 (1.22 ± 0.42 lesions/10 kb; $F(1,14) = 8.28$, $p < 0.01$); however, its effect on the number of lesions in fragment 1 was statistically insignificant (0.76 ± 0.49 lesions/10 kb; $p = 0.08$). Malathion and metribuzin caused the most pronounced damage in all the studied fragments (> 4 lesions/10 kb; $p < 0.001$). Penconazole and rotenone caused less mtDNA damage. The number of lesions induced by penconazole was 1.43 ± 0.24 lesions/10 kb in fragment 1 ($F(1,14) = 37.12$, $p < 0.001$), 3.38 ± 0.29 lesions/10 kb in fragment 2 ($F(1,14) = 140.2$, $p < 0.001$), and 2.75 ± 0.32 lesions/10 kb in fragment 3 ($F(1,14) = 71.58$, $p < 0.001$). Rotenone caused

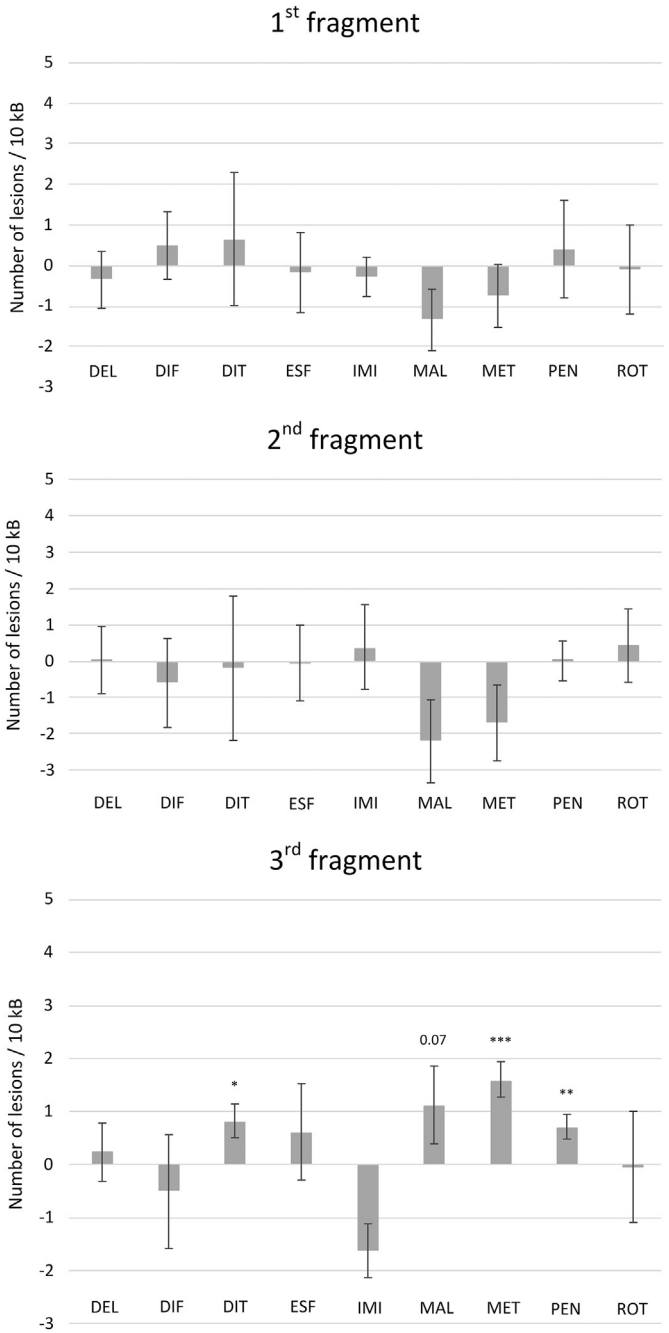

Fig. 3. Number of mtDNA lesions caused by pesticides upon contact exposure. Deltamethrin (DEL), malathion (MAL), rotenone (ROT), esfenvalerate (ESF), imidocloprid (IMI), dithianone (DIT), penconazole (PEN), difenoconazole (DIF), metribuzin (MET). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

2.62 ± 0.78 lesions/10 kb in fragment 1 ($F(1,14) = 11.34$, $p < 0.01$), 3.49 ± 0.16 lesions/10 kb in fragment 2 ($F(1,14) = 606.8$, $p < 0.001$), and 3.45 ± 0.42 lesions/10 kb in fragment 3 ($F(1,14) = 66.44$, $p < 0.001$).

3.5. Pesticide toxicity upon contact exposure

The contact exposure of bumblebees to the pesticide did not significantly increase the amount of mtDNA damage in the flight muscles (Fig. 3). Statistically significant increase in the number of mtDNA damage in fragment 3 was demonstrated for only for dithianon (0.82 ± 0.31 lesions/10 kb; $F(1,14) = 7.15$, $p < 0.05$), metribuzin (1.61 ± 0.33 lesions/10 kb; $F(1,14) = 15.38$, $p < 0.001$), and penconazole (0.71 ± 0.23 lesions/10 kb; $F(1,14) = 11.14$, $p < 0.05$)). There was a trend toward the increase in the number of damages for malathion (1.12 ± 0.74 lesions/10 kb), but the data were not statistically significant ($p = 0.074$). None of the studied pesticides caused damage in mtDNA fragments 1 and 2.

4. Discussion

Since the purpose of the study was to develop a method for detecting the toxic effects of pesticides at the concentrations that do not cause massive death of bumblebees, the pesticides were used in doses that less than 10% mortality in bumblebees upon contact exposurecaused. The following classes of pesticides were studied: neonicatininoids (imidacloprid), pyrethroids (deltamethrin, esfenvalerate), organophosphorus compounds (malathion), triazoles (penconazole, difenoconazole), rotenones (rotenone), and triazines (metribuzin). Neonicotinoids (Matsuda et al., 2001) and pyrethroids (Burns and Pastoor, 2018) are commonly used for insect pest control. Organophosphate pesticides are applied for controlling insects, diseases, and weeds, and may be toxic to non-target organisms (Gurpreet et al., 2019). Triazoles are fungicides (Peyton et al., 2015), and triazines are herbicides (Good, 1961; Tischer and Strotmann, 1977). Dithianon does not belong to any large class of pesticides and is used against fungi, but it can also affect non-target organisms (Yang et al., 2011; Scariot et al., 2018). Rotenone is a well-known insecticide; it is also toxic to fish (Rayner and Creese, 2006).

The studies of the pesticide toxicity in honeybees and/or bumblebees showed that the LD50 of imidacloprid is 4–104 ng/insect in honeybees (Blacquière et al., 2012) and 40 ng/insect in bumblebees (Patetta et al., 2003). The LD50 of malathion in honeybees is 0.47 μg/insect upon contact administration and 9.2 μg/insect upon ingestion (Sanchez-Bayo and Goka, 2016). The data on the toxicity of this pesticide in bumblebees are absent. The LD50 of deltamethrin in bumblebees is 0.9 μg/insect upon contact administration and 0.6 μg/insect upon ingestion. The LD50 of esfenvalerate is 0.015 μg/bee (Abbassy et al., 2020). The LD50 for penconazole in bees ranges from > 112 to > 178 μg /insect upon oral administration and over 30 μg/insect upon contact (EFSA, 2008). The LD50 of difenoconazole in honeybees is over 100 μg/insect (Ostiguy et al., 2019) upon oral and contact administration. The LD50 values for rotenone and metribuzin in honeybees are over 60 μg/insect (Ostiguy et al., 2019). Therefore, the most toxic pesticides in honeybees and bumblebees are, as expected, imidacloprid, malathion, deltamethrin, and esfenvalerate, which was also confirmed in our study. Imidacloprid is one of the most toxic pesticides for bees, as well as other neonicotinoids such as thiamethoxam and clothianidin (Ostiguy et al., 2019). Pyrethroids are slightly less toxic for bees than neonicotinoids.

We found that all pesticides (except imidacloprid) inhibited respiration mediated by the ETC complex 1. Pesticides from different classes (pyrethroids, organophosphorus compounds, triazoles, rotenones, and triazines) significantly reduced the respiratory rate of mitochondria in flight muscles, which can potentially be dangerous for the physiology of pollinators. Inhibition of mitochondrial respiration might decrease the flight and pollinating activities of bumblebees and probably other beneficial insects, since respiration provides energy to the insects. Rotenone, difenocanozole, and dithianon increased the rate of ROS production by the mitochondria, which is dangerous for cell compartments in the bumblebee flight muscles. Interestingly, imidacloprid produced no significant effect on the respiration. This pesticide is known to be an agonist of the nicotinic acetylcholine receptor (Crossthwaite et al., 2017), which should affect the general respiration of insects. Our data show that this pesticide does not inhibit mitochondrial respiration *in vitro*. However, imidacloprid is likely to reduce insect respiration *in vivo via* mechanisms not associated with the mitochondria.

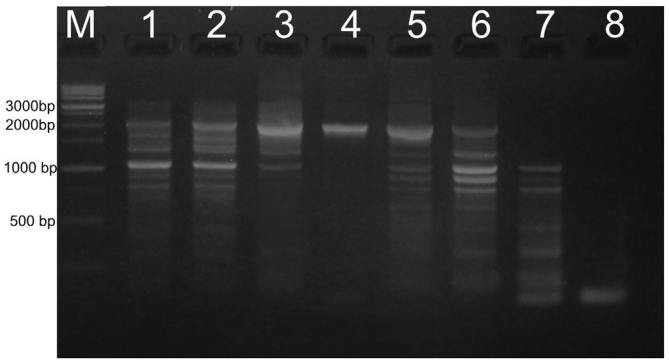

Fig. 4. Electrophoregram of long-range PCR products obtained at different elongation temperatures: 1–72°C; 2–71.1°C; 3–69.3°C; 4–66.1°C; 5–62.3°C; 6–59.2°C; 7–57.1°C; 8–56°C.

mtDNA is located in close proximity to the mitochondrial ETC, a significant source of ROS. It is not protected by histones and has less powerful repair systems in comparison with the nuclear genome. For this reason, mtDNA can be used as an indicator for evaluating the genotoxicity of various compounds (Yakes and Van Houten, 1997). The application of long-range PCR for detecting DNA lesion is based on the assumption that DNA damage inhibits DNA polymerase and slows down the rate of DNA amplification. Therefore, the rate of accumulation of PCR product would be inversely proportional to the number of damaged DNA molecules (Furda et al., 2012). Previously, we found that 2 kb is the optimal PCR product length for mtDNA amplification with Encyclo polymerase. Hence, Encyclo polymerase-catalyzed amplification of mtDNA fragments much longer than 2 kb will be inefficient and produce no valid results (Gureev et al., 2017). Here, we developed a panel of primers for the amplification of three fragments of bumblebee mtDNA (Table 1). However, a standard PCR protocol with an elongation temperature of 72 °C yielded a large number of non-specific products (Fig. 4), presumably, due to the structural features of insect mtDNA. In insects, an average GC content of mtDNA varies between 31.5 and 15.1%, while in vertebrates, it varies between 47 and 34% (Arunkumar and Nagaraju, 2006) (Fig. 5). The mitochondrial genome of *B. terrestris* is 17,400 bp in length and has the GC content of 15% (Du et al., 2016). It is possible that this structural feature makes correct amplification of long mtDNA fragments at a standard elongation temperature impossible. Earlier, Su et al. (1994) recommended lowering the temperature of elongation for the amplification of extremely AT-rich DNA fragments (Su et al., 1996). Using this approach, we found that the optimal elongation temperature for the bumblebee mtDNA amplification is 66 °C. At this temperature, a product of about 2 kb in length can be amplified without formation of non-specific PCR products (Fig. 4).

We investigated different types of pesticides, such as acaricides (deltamethrin, malathion, rotenone, esfenvalerate, imidocloprid), fungicides (dithianone, penconazole, difenoconazole), and a herbicide (metribuzin). All the studied pesticides induced mtDNA damage in both target and non-target action. This suggests that all pesticides, including herbicides and fungicides, can be toxic for pollinators, with metribuzin (herbicide) exhibiting the highest toxicity. When added to intact mitochondria, metribuzin caused 2.31 ± 0.25 lesions/10 kB (mean number of lesions for all fragments), which was significantly higher ($F(1,130) = 6.14$, $p < 0.05$) than the average number of lesions caused by other insecticides (1.41 ± 0.14 lesions/10 kB). The number of lesions induced by the fungicides (1.57 ± 0.24 lesions/10 kb) was not statistically different from the number of lesions caused by the insecticides.

The fungicides caused the least damage in mtDNA (1.67 ± 0.34 lesions/10 kB) when ingested, compared to insecticides (2.70 ± 0.15 lesions/10 kB) ($F(1,188) = 18.67$, $p < 0.001$). The highest toxicity was observed for metribuzin (4.36 ± 0.07 lesions/10 kB *in vivo* and 2.31 ± 0.25 lesions/10 kB *in vitro*). In plants, metribuzin inhibits all Hill reactions that utilize water as an electron donor, but not photoreduction by photosystem I using an artificial electron donor (Trebst and Wietoska, 1975). However, it has been shown that metribuzin promotes oxidative stress and impairs development in mammals (Samir et al., 2019) and fish (Plhalova et al., 2012; Hostovsky et al., 2012). This is consistent with our data showing acute toxic effect of metribuzin in insects. It was found that metribuzin does not exhibit a mutagenic effect in *Drosophila melanogaster* (Kaya et al., 2000). However, the authors of this work studied mutations only in wing somatic cells. It is possible that the high toxicity of metribuzin is due to the fact that it was used in a higher concentration than other pesticides, because it caused the lowest mortality among the bumblebees.

The extent of mtDNA damage by the pesticides was different in the investigated mtDNA fragments both *in vitro* and *in vivo*. Fragment 1 (1.08 ± 0.14 lesions/10 kB *in vitro* and 1.66 ± 0.27 lesions/10 kB *in vivo*) contained *Cox1, Cox2*, *tRNA Leu,* and tRNA *Phe* genes. Fragment 2 (1.64 ± 0.26 lesions/10 kB *in vitro* and 3.10 ± 0.21 lesions/10 kB *in vivo*) contained *Nad6, Nad1, CybB*, *tRNA Ser, tRNA Gln,* and *tRNA Met* genes. Fragment 3 (1.86 ± 0.16 lesions/10 kB *in vitro* and 2.51 ± 0.25 lesions/10 kB *in vivo*) contained ribosomal genes, D-loop region, and *tRNA Asn* and *tRNA Val* genes.

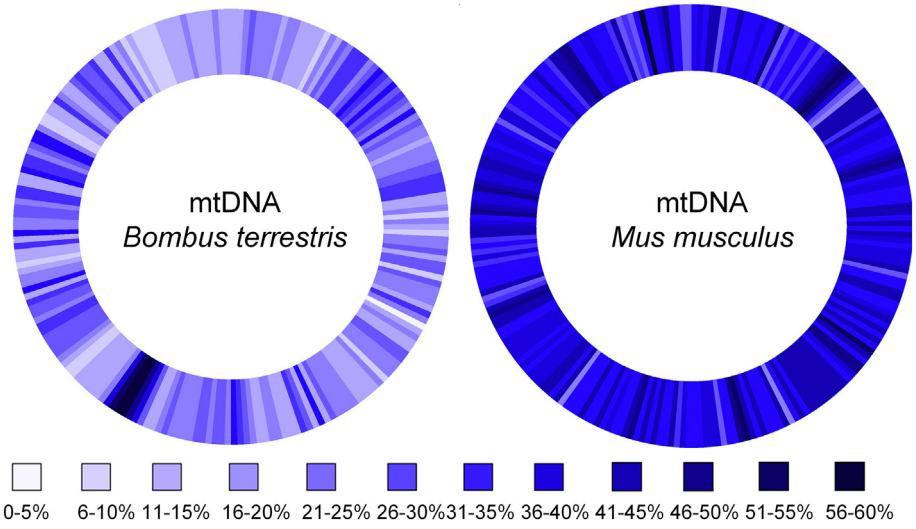

Fig. 5. The GC content of mtDNA in bumblebees (*B. terrestris*) and mice (*Mus musculus*).

Similar results were obtained earlier for intact mouse mtDNA (Gureev et al., 2017). Exogenous addition of H_2O_2 to intact mitochondria caused severe damage to mtDNA fragments that encode the D-loop and ribosomal genes, as well as *Nad1*, while the fragments encoding COX subunits were almost insusceptible to the damage (Gureev et al., 2017). Previously, we attributed this to the fact that the most damaged fragments contained a greater number of GTGR sequences that are able to concentrate Fe^{2+} ions capable of catalyzing the Fenton reaction. Inactive H_2O_2 is converted to OH^*, which causes severe damage to mtDNA (Henle et al., 1999). Iron is an important component for magnetoreception in eusocial insects, such as bees and bumblebees (Wajnberg et al., 2010). It was shown previously that honeybee trophocytes have iron granules with a density of 1.25 g/cm^3, which are necessary for magnetizing bees to the atmosphere for the proper flight memory (Hsu et al., 2007). Such an essential role of iron in the vital activity of bees suggests its possible negative effect due to increased risk of OH^* formation on the integrity of insect genome.

We revealed no correlation between the pesticide-induced damage in bumblebee mtDNA and the number of GTGR sequences in the studied fragments. A negative correlation was found between the GC content and the extent of mtDNA damage in the *in vivo* experiments ($r_s = -0.97$, $p < 0.05$).

Surprisingly, no correlation was found between the number of pesticides-induced lesions *in vitro* and *in vivo*. H_2O_2 production did not exert a noticeable effect on the mtDNA damage. A significant increase in the level of H_2O_2 production due to the ETC inhibition was observed only for rotenone and difenoconazole (Table 2), as it was described in our previous studies (Syromyatnikov et al., 2017; Syromyatnikov et al., 2017). However, other pesticides did not increase the rate of H_2O_2 production. This suggests an existence of two different mechanisms of genotoxicity: first, ROS can be generated at the sites other than the ETC; second, the genotoxic effect of pesticides may not be necessarily mediated by an increase in the ROS production, although this hypothesis requires further investigation. Both rotenone and difenoconazole caused mtDNA damage *in vitro*. Rotenone added to the nutrient syrup also caused significant mtDNA damage, unlike difenoconazole, which damaged mtDNA only in fragment 2. Deltamethrin (pyrethroid) caused only slight mtDNA damage *in vitro*, but induced significant mtDNA *in vivo*, probably, due to the inhibition of the sodium channel activation gate, which results in prolonged permeability of the nerve cells to sodium and produces a series of repetitive nerve signals in sensory organs, sensory nerves, and muscles (WHO, 1990). Similar effect was showed for esfenvalerate (pyrethroid), which caused mtDNA lesions *in vivo*, but not *in vitro*.

Malathion is an inhibitor of acetylcholinesterase. It irreversibly binds to several random serine residues in the enzyme, and the resulting phosphoester group inactivates acetylcholinesterase (Colović et al., 2013). For this reason, it is not surprising that the effect of malathion on mtDNA was 5 times stronger when this pesticide was added to the nutrient syrup than in intact mitochondria. The opposite result was shown for imidocloprid. Imidocloprid did not inhibit ETC and did not cause an increase in ROS production, but damaged mtDNA *in vitro* more than *in vivo*. The mechanism of mtDNA damage for this pesticide is not fully understood, since imidocloprid acts mostly on the central nervous system by interfering with the transmission of stimuli by competing with the natural neurotransmitter acetylcholine (Yamamoto, 1999). However, imidocloprid also causes DNA damage in fish (Iturburu et al., 2018; Ge et al., 2015) and mammals (Hassan et al., 2019; Bal et al., 2012), including human cell lines (Guo et al., 2018). Among fungicides, the highest *in vivo* damage was caused by penconazole, which also causes DNA fragmentation in rat heart (Chaâbane et al., 2016).

The pesticides caused minor damage to the mtDNA upon contact exposure of the bumblebees, but caused significant toxicity when added to the nutritious syrup. Previous studies have also shown that pesticides are more toxic upon ingestion than upon the contact exposure (Damalas and Eleftherohorinos, 2011).

The developed method for assessing the genotoxicity of pesticides in bumblebees using long-range PCR can be applied to investigate the mutagenic effect of xenobiotics. The tests should be carried out by adding pesticides to the nutrient syrup, since pesticides exhibit higher toxicity upon ingestion compared to contact exposure.

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