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Biomonitoring of genotoxicity of industrial fertilizer pollutants in *Aiolopus thalassinus* (Orthoptera: Acrididae) using alkaline comet assay

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HIGHLIGHTS

- Comet assay allows a potential biomonitoring of detection the DNA damage in different tissue of grasshoppers subjected to environmental pollutants.
- A highly significant correlation was confirmed in brain, thoracic muscles, and gut of *A. thalassinus* collected at polluted and control sites.
- A strong negative correlation was found between percentage of cells with visible DNA damage (% severed cells) and distance from fertilizer industry.
- Specific pollution from fertilizer industry cause comparable adverse effects in organisms inhabiting areas up to 6 km.

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ABSTRACT

Phosphate fertilizer industry is considered as one of the main sources of environmental pollutants. Besides solid waste products, e.g. phosphates, sulphates, and heavy metals, also atmospheric pollutants, such as hydrofluoric acid fumes (HF), sulphur dioxide (SO_2), nitrogen oxides (NO_2), and particulate matter with diameter up to 10 μm (PM_{10}) can be dangerous. Genotoxic effect of these pollutants was monitored by assessing the DNA damage using alkaline comet assay on cells from brain, thoracic muscles and gut of *Aiolopus thalassinus* collected at three sites (A-C) located at 1, 3, and 6 km away from Abu-Zaabal Company for Fertilizers and Chemical Industries. Control site was established 32 km from the source of pollution, at the Cairo University Campus. The level of the DNA damage was significantly higher in insects from polluted sites comparing to that from the control site. A strong negative correlation between percentage of cells with visible DNA damage (% of severed cells) and the distance of the sites from Abu-Zaabal Company was found. The best parameter for monitoring of fertilizer pollutants is % of severed cells. Possible impact of Abu-Zaabal Company (extremely high concentration of phosphates and sulphates in all the polluted sites) on DNA integrity in *A. thalassinus* tissues was discussed. The potential use of the comet assay as a biomonitoring method of the environmental pollution caused by fertilizer industry was proposed. Specific pollution resulting from the activity of the fertilizer industry can cause comparable adverse effects in the organisms inhabiting areas up to 6 km from the source of contamination.

1. Introduction

Dust particles emitted from phosphate fertilizer industries form a complex of organic compounds and minerals. Transport and deposition of such pollutants may have a hazardous effect on the environment, particularly soil, and air. Phosphorus species are the principal carriers of heavy metals such as Zn, and Cd in soils, therefore the phosphate industry is considered as one of the soil pollution hazards. These contaminants can be potentially hazardous to terrestrial organisms and groundwater (Kassir et al., 2012). The main pollutants from phosphate fertilizer industries are: hydrofluoric acid (HF), sulphur dioxide (SO_2) (29 μg/m^3, comparing to the Egyptian maximum limits; 60 μg/m^3 in the air), nitrogen dioxide (NO_2) (32 μg/m^3, comparing to the Egyptian maximum limits; 60 μg/m^3 in the air), heavy metals, phosphates, sulphates,

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and particulate matter of 10 μm diameter (PM_{10}) (ESE, 2014).

The ability of an organism to inhabit strongly contaminated areas involves a kind of trade-off. Usually, such an option needs expenditure of energy for self-defense processes instead of allocating it toward individual growth or/and reproduction. There are several costs of self-defense: smaller size, lower fertility and bigger sensitivity to additional environmental stressors, such as pesticides or food deprivation (Stone et al., 2001; Walker et al., 2001). Changes in the organism which allow to minimize the negative effects of pollutants are beneficial for an individual, and can be based on phenotypic plasticity and other forms of nongenetic inheritance, or/and epigenetic, or/and genetic modifications in the cells. In case of animals inhabiting polluted areas, a natural selection can occur. However, the time that is needed for a genetic adaptation is usually long and can last for hundreds of years (Peck, 2011). Therefore, in case of anthropogenic pollutants most of the changes in an organism physiology should be perceived as temporary and reversible. Thus, insects common in terrestrial ecosystems, such as grasshoppers, can be seen, as sensitive to environmental changes. They can be considered as an interesting subject of ecotoxicological research, and a biomonitor of environmental pollutants, including heavy metals, near an industrial region (Chen et al., 2005; Azam et al., 2015). Moreover, grasshoppers are widespread in strongly industrialized areas (Augustyniak and Migula, 1996, 2000).

Total genotoxic potential in the environment can be measured using single cell gel electrophoresis (SCGE), also known as the comet assay. This method is considered as one of the simplest, most sensitive and reliable methods for detecting DNA strand breakages. High sensitivity of the comet assay allows early detection of the deleterious effect of pollutants (Jha, 2008; Al-Shami et al., 2012; Guanggang et al., 2013). DNA damage can be measured in the cells of particular organisms (Rojas et al., 1999; Dhawan et al., 2009). For DNA oxidative damage, the values of tail length (TL), tail moment (TM), and % DNA in tail (TDNA) are the most informative (Tice et al., 2000; Lovell and Omori, 2008; Carmona et al., 2011; Augustyniak et al., 2016a). In the assessment of this oxidative DNA damage, the percentage of severed cells may be considered as a useful supplementary parameter (Mourón et al., 2001; Bilbao et al., 2002; Augustyniak et al., 2016b). DNA damage may be considered as a signal of disturbances occurring at the molecular level that may lead to chromosomal instability and subsequent pathological changes in the cells and tissues, e.g. morphological abnormalities, cancer diseases, reduction in gamete production, and finally, population extinction (Jha, 2008; Dhawan et al., 2009).

The comet assay applications involve genotoxicity studies, biomonitoring, ecotoxicology, as well as basic research on DNA damage and its repair (Rojas et al., 1999; Jha, 2008; Dhawan et al., 2009; Collins et al., 2014). Recently, the comet assay has become more popular as a tool to study genotoxic effects of environmental pollutants in different animals, and in the last decade, also in insects (Mukhopadhyay et al., 2004; Siddique et al., 2005; Yousef et al., 2010; Carmona et al., 2011; Sharma et al., 2011; Shukla et al., 2011; Guanggang et al., 2013; Lucas et al., 2017).

In the present study, we want to answer the question if continuous contact of *A. thallasinus* individuals with specific pollutants, resulting from fertilizer industry activity, influences DNA stability in different tissues of the insect. Another important task of the study is to check the relation between the level of DNA damage in insect cells and distance (up to 6 km) from the source of contamination: Abu-Zaabal Company for Fertilizers and Chemical Industries. Therefore, the main aim of the present work is the measurement of DNA damage in *Aiolopus thalassinus* individuals inhabiting sites 1, 3 and 6 km away from the fertilizer industry and comparison to DNA damage of insects from control site, located 32 km from the source of pollution.

2. Materials and methods

2.1. Study area

Animals were collected at four sites located at various distances from Abu-Zaabal Company for Fertilizers and Chemical Industries – the main source of contamination. Specific conditions in the area allowed to set experimental plot along a pollution gradient. Three polluted sites (A-C) of the cultivated spots of this area were located nearly ≤ 1, 3, and 6 km away from Abu-Zaabal Company, respectively (along a branch of the Nile river). Control site was established about 32 km from the source of pollution – at the Cairo University Campus (Fig. 1). The level of heavy metals, as well as PO_4^{3-} and SO_4^{2-}, in the soil samples was analyzed using the inductively coupled spectrometry plasma atomic emission spectroscopy (ICP-AES – Model Ultima 2 – Jobin Yvon) according to Boon and Soltanpour (1991). The analysis revealed the highest concentrations of Zn, Cu, Cd, and Pb as well as sulphates and phosphates in the soil samples collected at site A, placed the closest to Abu-Zaabal Company. These values were always significantly higher comparing control and other polluted sites. The level of sulphates and phosphates was extremely high at all the polluted sites. Moreover, the level of phosphates at each of the sites reflected the distance from the source of contamination (Fig. 2).

Preliminary insect sampling was performed to check the quantitative structure of the grasshopper population so as proper material for further studies would be accessible. Adult males and females of grasshoppers were collected with a sweep-net in March, 2016. Insects were transported to the laboratory in small

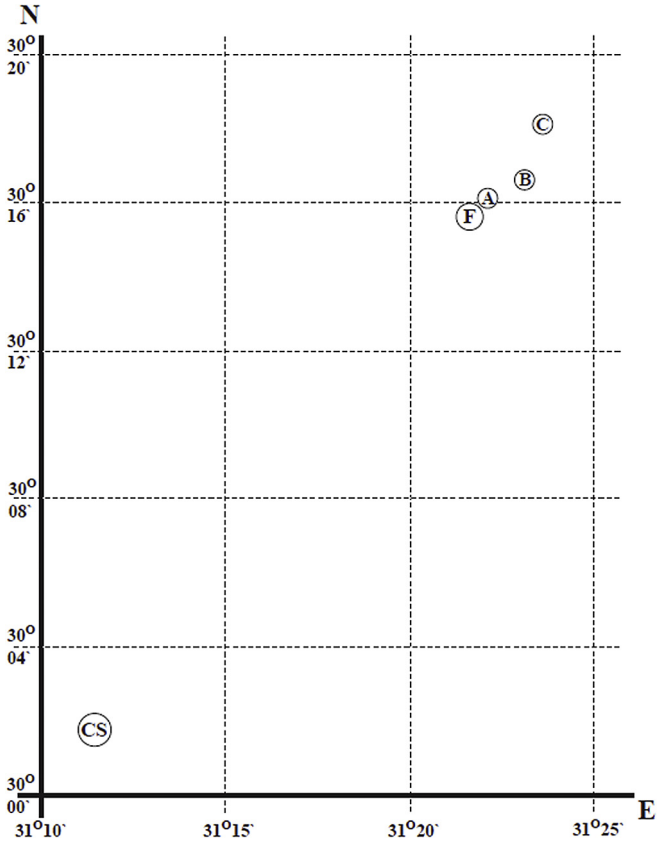

Fig. 1. Location of sampling sites. Abbreviations: CS – Control site (30 1′48″N, 31 11′23″E); F – Factory (30 15′49″N, 31 21′39″E); A-C – polluted sites: A – Site A (30 16′03″N, 31 22′06″E); B – Site B (30 16′33″N, 31 23′04″E); C – Site C (30 18′04″N, 31 23′39″E).

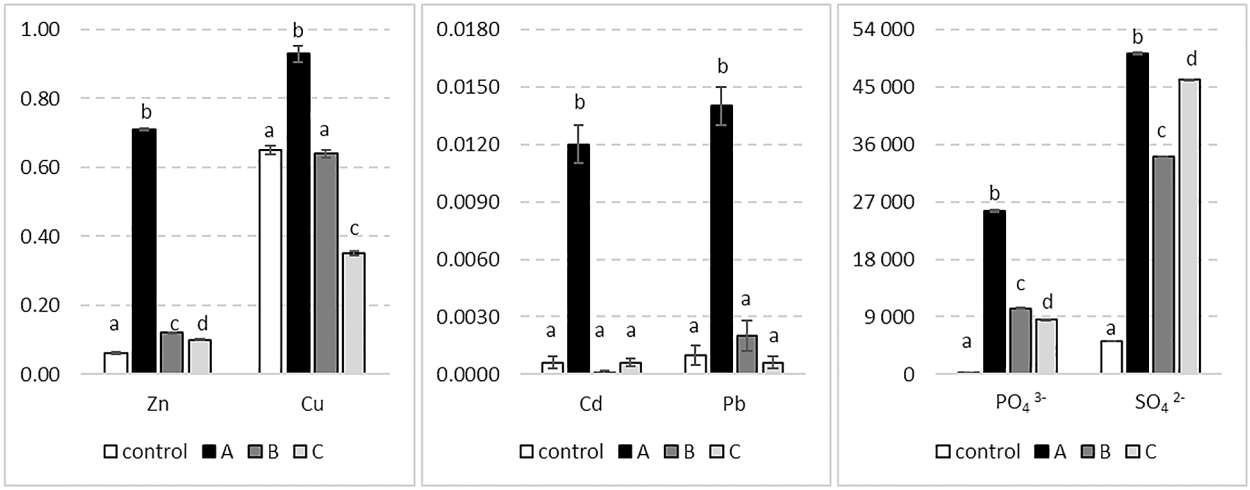

Fig. 2. Concentrations of heavy metals (Zn, Cu, Cd, and Pb), PO_4^{3-}, and SO_4^{2-} (mean ± SE; mg/kg dry weight) in soil samples collected at control and polluted sites (A–C) located at different distances from Abu-Zaabal Fertilizer Company. Abbreviations: the same letters denotes homogeneous groups. Statistical analysis was made for each pollutant separately ($p < 0.05$).

30 cm × 30 cm × 30 cm cages (approximately 25 insects per cage). Insects were immediately transported to the laboratory, and, after exact determination to the species, individuals were dissected and the tissues were used for further DNA damage analyses. For each experimental groups, from each site, and both sexes a number of three slides were prepared. A pool of 5 females and 5 males tissues suspension was used to prepare of each sample. Total number of 15 females and 15 males were dissected from each site.

Positive control (with H_2O_2, 50 μM) for selected samples was done in order to validate the procedures on each stage of the measurement. The parameter tail DNA after induction with H_2O_2 reached about 50% in all tested slides, proving correctness of the experiments.

As a negative control the individuals from laboratory breading population were used. The insect where captured in the environment (at Abu Rawash site - 8 km to the north of Giza Governorate), transferred to the laboratory, and reared under laboratory condition (temperature: 25 ± 2 °C; photoperiod L:D 12:12; humidity: 50% ± 5%) for three generations.

2.2. DNA damage – comet assay

The alkaline Single Cell Gel Electrophoresis assay (SCGE), known as the Comet assay was used to assess the DNA strand breaks according to Yousef et al. (2010) with minor modifications. Insects were slightly anesthetized on ice, and then brain, thoracic muscles, and gut were isolated. Immediately after dissecting, tissues were macerated in 0.5 mL of 1x PBS with a teasing needle for 30 s. The PBS composition was as follows: 0.8 g NaCl, 0.02 g KCl, 0.144 g Na_2HPO_4, 0.024 g KH_2PO_4 diluted in 100 mL of distilled water; pH was adjusted to 7.4 by adding 2 M HCl or NaOH. The macerates were resuspended in 3 mL of 1x PBS and centrifuged (1500 rpm, 4 °C, 5 min), and then pellet was again suspended in 0.5 mL of 1x PBS (Martínez-Paz et al., 2013; Morales et al., 2013). The average number of cells in the suspension was in a range 10^4 to 10^5 cells/mL. Cell suspensions (60 μl) were mixed with 1% low melting-point agarose (60 μl), and 110 μl of the mixture was spread on a microscopic slide previously covered with a layer of 0.8% regular melting-point agarose. After the layer with the cells solidified at 4 °C, the slides were immersed for 24 h (Tice and Vásquez, 1999) in a fresh lysis solution (164 g NaCl, 37 g of ethylene-diamine tetracetic acid (EDTA), 1 g Tris base merged into 890 mL of distilled water and stirred before adding 8 g of NaOH; pH 10.0; 4 °C. Then 10 mL of TritonX-100 and 100 mL of dimethyl sulfoxide was added before use).

After lysis, the slides were washed two times with distilled water, and immersed for 5 min at 4 °C in a freshly prepared alkaline electrophoresis buffer (30 mL of 10 N NaOH, 0.5 mL of 200 mM EDTA mixed with 1000 mL of distilled water; pH was adjusted to 13.0 using 2 M HCl or NaOH). Electrophoresis was performed at 20 V, 0.3 A for 20 min. Next, the slides were immersed in neutralization buffer for 15 min (Tris base; pH was adjusted to 7.5 using 2 M HCl), drained by immersing in cold absolute ethanol for 5 min, and stored under dry conditions.

Before the analyses, slides were stained with 40 μl of ethidium bromide solution (2 μg/mL). An analysis of DNA damage was performed using OPTIKA B-350 fluorescent microscope (OPTIKA, Ponteranica, Italy), which was linked with a CCD camera. An image analysis system (Comet IV) was used to measure DNA damage level. The percentage of DNA in the comet tail (TDNA; defined as the total comet tail intensity divided by the total comet intensity, and multiplied by 100), the length of the comet tail (TL; described as the comet head diameter subtracted from the overall comet length), as well as percentage of severed cells (% severed cells; the number of cells with DNA damage) were recorded. Olive Moment (OM; defined as product of tail DNA% and the distance between the intensity-weighted centroids of head and tail) and Tail Moment (TM; defined as Tail length times Tail DNA%) were also estimated and included into the statistical analysis (Gyori et al., 2014; Comet Score Tutorial). For each site, sex, and tissue 3 slides and 50 cells per slide were analyzed. A total number of 72 slides were analyzed.

2.3. Statistical analysis

Results of DNA damage were presented as median and quartile deviation (P25, and P75). Nonparametric tests were carried out using either Mann-Whitney test or Kruskal-Wallis test to compare the medians of data. Generalized Estimating Equation (GEE) was used to examine the effect of distance from the fertilizer company, types of tissues, sex and the interactions of the variables on comet parameters (TL, TDNA, OM, TM and % of severed cells). Correlation between distance from the fertilizer company and DNA damage parameters was performed basing on Pearson's regression analysis

Table 1
Comet parameters: tail length (μm), tail moment, % DNA in tail, olive moment, and % of severed cells expressed as median and percentile deviation (P25 and P75) of comets obtained from brain, thoracic muscles, and gut cells of males and females of *A. thalassinus* which were reared for 3 generations under laboratory conditions (negative control).

Sex	Tissue	Brain			Thoracic muscles			Gut		
	Comet Parameters	Median	P25	P75	Median	P25	P75	Median	P25	P75
Male	Tail length	**0.33***	0.30	0.35	**0.14**	0.12	0.17	**0.37***	0.34	0.39
	Tail moment	**0.70**	0.60	0.81	**0.39**	0.37	0.44	**1.20**	1.11	1.41
	% DNA in tail	**8.40**	7.91	9.82	**6.11**	5.85	6.21	**17.12**	16.82	17.50
	Olive moment	**0.49***	0.47	0.51	**0.51***	0.41	0.56	**0.95**	0.81	1.02
	% of severed cells	**5**	4	6	**11**	10	12	**17**	16	18
Female	Tail length	**0.22***	0.21	0.19	**0.25***	0.22	0.28	**0.28***	0.24	0.29
	Tail moment	**0.14**	0.11	0.15	**0.49**	0.39	0.52	**0.87**	0.85	0.90z
	% DNA in tail	**3.72**	3.52	3.81	**8.21**	7.10	9.12	**10.00**	9.81	10.21
	Olive moment	**0.29**	0.27	0.31	**0.61***	0.51	0.68	**0.61***	0.52	0.71
	% of severed cells	**6**	5	7	**9***	8	10	**10***	9	11

Abbreviations: In the same row: * indicate no significantly different among tissues (Kruskal-Wallis test, $p > 0.05$). There are no significant differences between comet parameters in negative control insect and insect which were collected from control site in each case separately (Mann-Whitney test, $p > 0.05$). There is a significant difference between male and female in each case separately (Mann-Whitney test, $p < 0.05$).

using the multiple regression module. All the statistical analyses were performed using IBM SPSS Statistics for Windows (Version 17.0. Armonk, NY: IBM Corp.).

2.4. Cluster analysis

Hierarchical Cluster Analysis (HACA) based on agglomerative statistics using Ward's Method was calculated for DNA damage parameters at each of the sampling sites. The goal of HACA is finding possible clusters or groups among the observational units, based on level of similarities and differentiations (Azam et al., 2015). At each stage the average similarity of the cluster is measured. The difference between each case within a cluster and that average similarity is calculated.

3. Results

The level of DNA damage in the tissues of untreated individuals reared under controlled laboratory conditions (negative control) was the lowest in the brain. The median value of TDNA in male and female reached 8.40% and 3.72%, respectively. The highest damage of DNA was stated in the gut, where median value of TDNA for male and female was 17.12% and 10.00%, respectively. All the tested tissues differed among each other significantly in term of TDNA, both in males and females (Table 1). There were no significant differences between comet parameters in negative control insects and individuals collected from control site in each tissue, and both sexes separately (Mann-Whitney test, $p > 0.05$).

The level of the DNA damage was higher in insect cells from polluted sites comparing to animals from control site. Median TDNA in the brain cells of females (5.85%) and males (8.15%) from control site were the lowest. In the gut of males from site C the highest values of TL and TDNA were found (Tables 2 and 3). Significant differences between sexes regarding TDNA parameter in brain were visible in individuals from all the polluted sites (Table 2).

In the control groups the DNA damage, expressed as TL, was significantly lower in cells of thoracic muscles than in brain and gut of males of *A. thalassinus*, while in females no significant differences were observed in the analyzed tissues. Both in males and females, median values of TL parameter were significantly higher in insects from the polluted sites than in those from control site ($p < 0.05$), and did not have a close relationship with the distance from the fertilizer company (Table 3).

The values of Olive Moment parameter were also the lowest in tissues of insects collected at control site. However, a statistical analysis revealed significant differences between males and females from all the sites, and in almost all tissues. Median OM values were homogenous only in thoracic muscles of males and females collected at site B, and gut of both sexes from site C (Fig. 3). Olive Moment had the highest value in the gut of males from sites C and B. The highest OM value in females was also detected in the gut of individuals collected at site A – located closest to Abu-Zaabal Fertilizer Company (Fig. 3).

Tail Moment had the highest values in the gut of males from sites A and C. In case of females the highest TM value was observed in the gut of individuals inhabiting site A (Fig. 4).

Table 2
TDNA (Tail % DNA), expressed as median and percentile deviation (P25 and P75), of comets obtained from brain, thoracic muscles, and gut cells of males and females of *A. thalassinus,* which were collected at different distances from Abu-Zaabal Fertilizer Company.

Sex	Tissue	Brain				Thoracic muscles				Gut			
	Site	Median		P25	P75	Median		P25	P75	Median		P25	P75
Male	Control	**8.15**	aA	6.36	9.94	**8.82**	aA	7.51	10.31	**16.50**	aC *	14.27	18.73
	Site A	**13.27**	bA *	11.87	14.67	**19.70**	bB *	16.90	22.50	**18.19**	aB	14.69	21.69
	Site B	**12.70**	bA *	11.10	14.30	**12.10**	acA	9.90	14.30	**17.10**	aA	14.90	19.30
	Site C	**10.60**	abA *	8.70	12.50	**13.30**	cA *	11.10	15.50	**26.50**	bC *	21.70	31.30
Female	Control	**5.85**	aA	4.83	6.87	**8.82**	aB	7.16	10.48	**11.41**	aB *	9.85	12.97
	Site A	**17.50**	bA *	15.80	19.20	**9.63**	aB *	7.13	12.13	**19.24**	bA	16.14	22.34
	Site B	**17.30**	bA *	14.50	20.10	**15.20**	bA	12.90	17.50	**12.61**	aA	10.50	14.70
	Site C	**15.60**	bA *	12.80	18.40	**19.90**	bA *	16.20	23.60	**14.30**	abA *	11.80	16.80

Abbreviations: In the same column, median values marked with different small letters are significantly different between control and polluted sites located at different distances from the fertilizer company. In the same row, median values marked with different capital letters are significantly different among tissues (Kruskal-Wallis test, $p < 0.05$). Stars denote significant differences between males and females in each case separately (Mann-Whitney test, $p < 0.05$).

Table 3
Tail length (TL; μm), expressed as median and percentile deviation (P25 and P75), of comets obtained from brain, thoracic muscles, and gut cells of males and females of *A. thalassinus*, which were collected at different distances from Abu-Zaabal Fertilizer Company.

Sex	Tissue	Brain				Thoracic muscles				Gut			
	Site	Median		P25	P75	Median		P25	P75	Median		P25	P75
Male	Control	**3.25**	aA	3.05	3.44	**1.79**	aB	1.67	1.90	**3.84**	aA *	3.63	4.04
	Site A	**7.69**	bA	7.30	8.08	**5.04**	bB	4.61	5.46	**13.83**	bC	11.55	16.10
	Site B	**15.02**	cA *	14.04	15.99	**5.48**	bB *	4.88	6.07	**5.61**	aB	5.09	6.13
	Site C	**8.01**	bA	7.11	8.90	**12.06**	cA	11.07	13.04	**20.74**	bB *	18.37	23.10
Female	Control	**2.36**	aA	2.10	2.62	**2.02**	aA	1.90	2.13	**2.60**	aA *	2.47	2.73
	Site A	**7.87**	bA	7.48	8.26	**6.13**	bA	5.35	6.91	**12.16**	bB	10.48	13.93
	Site B	**9.28**	bA *	8.24	10.32	**9.93**	cA *	9.02	10.84	**5.74**	cB	5.08	6.40
	Site C	**7.98**	bAB	7.08	8.87	**11.71**	cA	10.54	12.88	**8.71**	acB *	7.90	9.51

Abbreviations: In the same column, median values marked with different small letters are significantly different between control and polluted sites located at different distances from the fertilizer company. In the same row, median values marked with different capital letters are significantly different among tissues (Kruskal-Wallis test, $p < 0.05$). Stars denote significant differences between males and females in each case separately (Mann-Whitney test, $p < 0.05$).

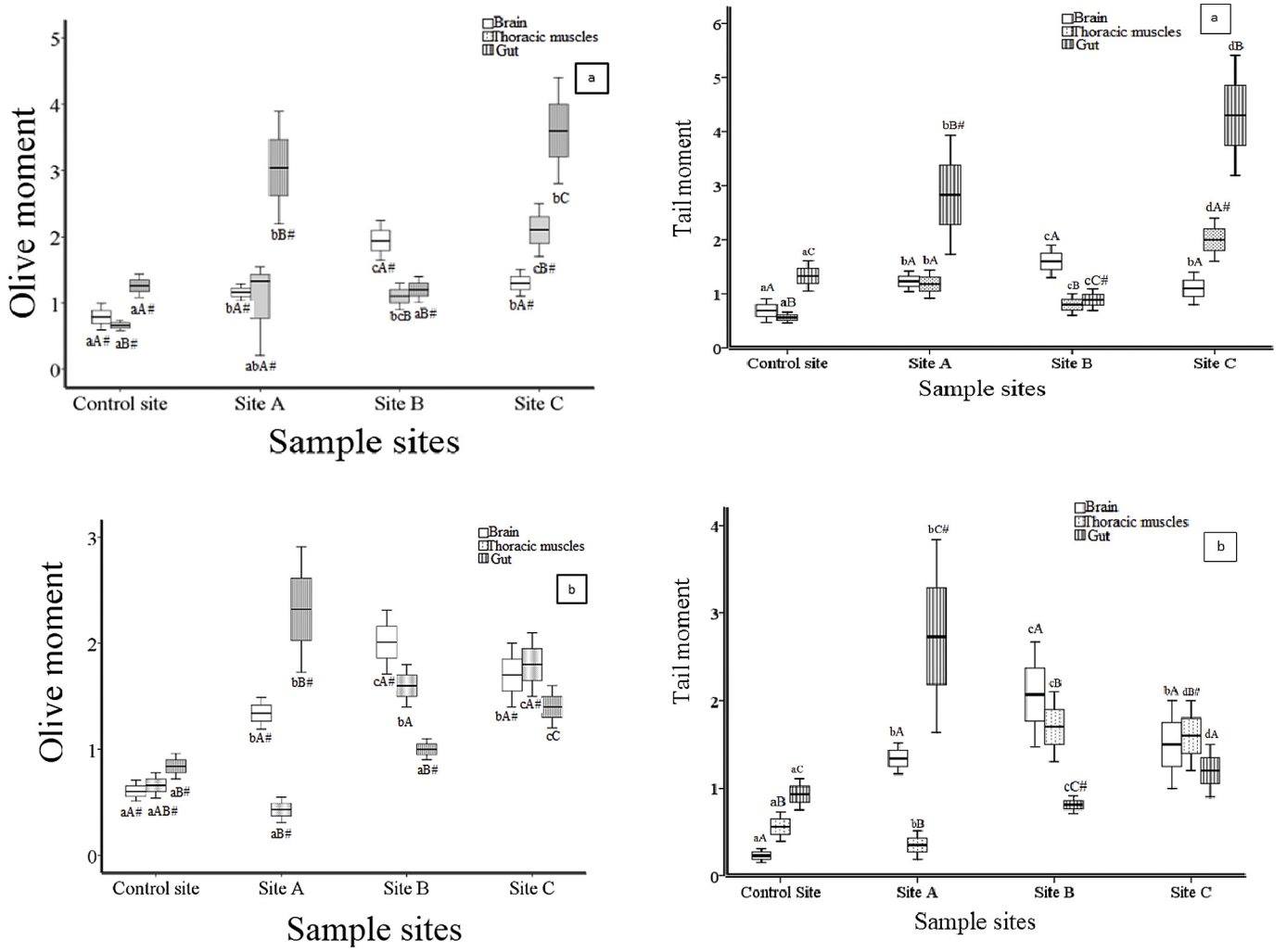

Fig. 3. Olive moment (OM), expressed as median, percentile deviation (P25 and P75 - boxes), and min-max values of comets obtained from brain, thoracic muscles, and gut cells of males (a) and females (b) of *A. thalassinus* collected at different distances from Abu-Zaabal Fertilizer Company. Abbreviations: median values marked with different small letters are significantly different between control and polluted sites (A–C) located at different distances from the fertilizer company. Median values marked with different capital letters are significantly different among tissues (Kruskal-Wallis test, $p < 0.05$). # denote no significant differences between males and females in each case separately (Mann-Whitney test, $p < 0.05$).

Fig. 4. Tail moment (TM), expressed as median, percentile deviation (P25 and P75 - boxes), and min-max values of comets obtained from brain, thoracic muscles, and gut cells of males (a) and females (b) of *A. thalassinus* collected at different distances from Abu-Zaabal Fertilizer Company. Abbreviations: median values marked with different small letters are significantly different between control and polluted sites (A–C) located at different distances from the fertilizer company. Median values marked with different capital letters are significantly different among tissues (Kruskal-Wallis test, $p < 0.05$). # denote no significant differences between males and females in each case separately (Mann-Whitney test, $p < 0.05$).

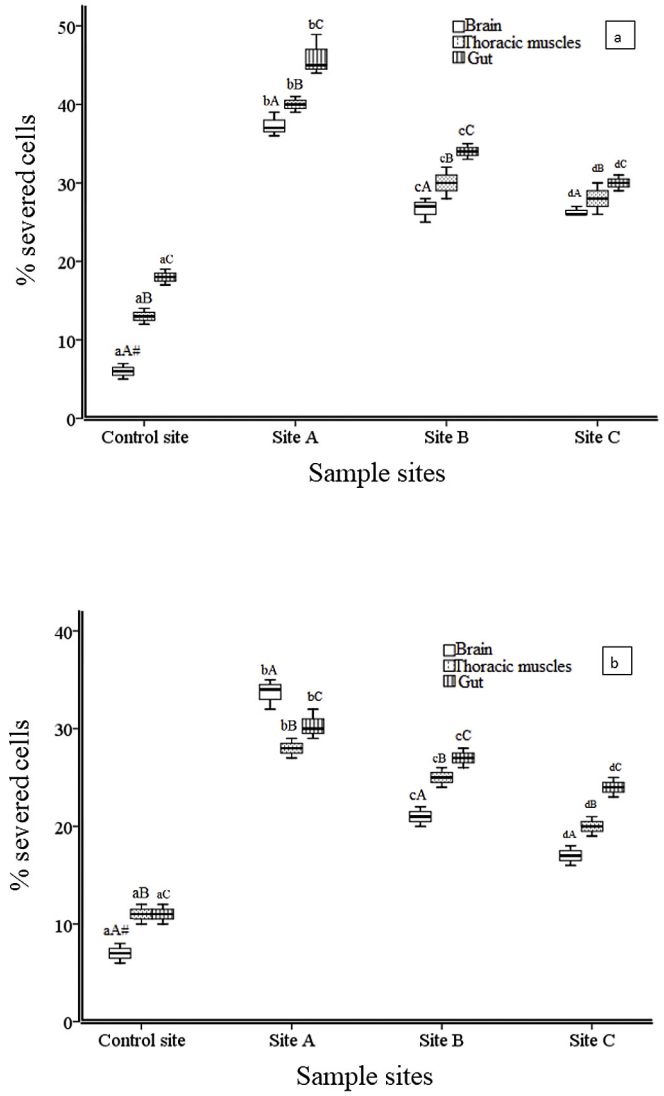

Fig. 5. The % of severed cells expressed as median, percentile deviation (P25 and P75 - boxes), and min-max values of comets obtained from brain, thoracic muscles, and gut cells of males (a) and females (b) of *A. thalassinus* collected at different distances from Abu-Zaabal Fertilizer Company. Abbreviations: median values marked with different small letters are significantly different between control and polluted sites (A–C) located at different distances from the fertilizer company. Median values marked with different capital letters are significantly different among tissues (Kruskal-Wallis test, $p < 0.05$). # denote no significant differences between males and females in each case separately (Mann-Whitney test, $p < 0.05$).

The highest percentage of cells with visible DNA damage (% of severed cells) was noticed, both in males and females collected at site A. The number of cells with the damage was higher in males than in females (Fig. 5).

A correlation analysis between comet parameters and the distance from Abu-Zaabal Fertilizer Company, revealed the most significant relationships in thoracic muscles (Table 4). In female thoracic muscles a correlation at $p < 0.001$ between the distance from the source of contamination and TL or TDNA or OM or % of severed cells parameters were shown. In the case of male thoracic muscles, a significant correlation at $p < 0.001$ was observed only for % of severed cells parameter. Moreover, significant relationships between the distance from Abu-Zaabal Company and TDNA or OM in thoracic muscles of males were found at $p < 0.05$. A highly significant negative correlation (at $p < 0.001$) between the distance and % of severed cells in all tissues of both sexes was revealed. This parameter seems to be the most useful for biomonitoring of genotoxicity of industrial fertilizer pollutants (Table 4). However, the interaction analysis showed significant influence of distance, sex and tissue on all comet parameters (Table 5).

A cluster analysis using Ward's method revealed slightly dissimilar patterns for males and females, however, the general tendency was similar (Figs. 6 and 7). The level of DNA damage was highly similar in brain as well as in thoracic muscle of males collected at sites B and C. The level of DNA damage in this tissue of males from site A was also linked with those from sites B and C. Males from control site created a separate cluster. Control site cluster and polluted sites cluster had relatively high distances (Fig. 6).

A hierarchical cluster analysis of DNA damage in female tissues also enabled to create separate clusters (Fig. 7). The level of DNA damage in the brain as well as in the gut of females from sites B and C was almost the same. The DNA damage in the female thoracic muscles was almost the same in the individuals from sites A and C. Females from control site created a separate cluster unlike the cluster of polluted sites (Fig. 7).

4. Discussion

In the present work, the alkaline comet assay was used to estimate genotoxic effects of environmental pollutants emitted by the fertilizer industry. Grasshoppers, *Aiolopus thalassinus*, were collected at three sites located at different distances from Abu-Zaabal Company for Fertilizers and Chemical Industries (Fig. 1) – a serious source of contamination in the vicinity of Cairo, Egypt (ESE, 2014). The distances of the sites from Abu-Zaabal Company were chosen following other authors who designed and performed

Table 4
Pearson's correlation coefficient among comet parameters (tail length, %DNA in tail, olive moment, tail moment, and % of severed cells) from brain, thoracic muscles, and gut cells of males and females of *A. thalassinus* and the distance from Abu-Zaabal Fertilizer Company.

Comet parameters	Tissue	Brain		Thoracic muscles		Gut	
	Sex	Regression analysis	r	Regression analysis	r	Regression analysis	r
Tail length	Male	$y = -0.71x^2 + 2.86x - 1.37$	**+0.008**	$y = 0.31x^2 - 0.89x + 1.09$	**+0.50**	$y = 1.24x^2 - 4.63x + 4.79$	**+0.38**
	Female	$y = -0.12x^2 + 0.49x + 0.43$	**−0.02**	$y = 0.09x^2 + 0.66x + 0.05$	**+0.81****	$y = 0.45x^2 - 2.06x + 2.84$	**−0.65**
%DNA in tail	Male	$y = -0.76x^2 + 1.72x + 12.31$	**−0.61**	$y = 4.40x^2 - 20.80x + 36.10$	**−0.67***	$y = 5.24x^2 - 16.82x - 29.70$	**+0.54**
	Female	$y = -0.75x^2 + 2.05x + 16.20$	**−0.35**	$y = -0.43x^2 + 6.87x + 3.19$	**+0.88****	$y = 4.16x^2 - 19.11x + 34.19$	**−0.57**
Olive moment	Male	$y = -0.71x^2 + 2.91x - 1.04$	**+0.14**	$y = 0.61x^2$ - 2.07x + 2.79	**+0.69***	$y = 2.12x^2 - 8.20x + 9.12$	**+0.19**
	Female	$y = -0.49x^2 + 2.14x - 0.31$	**+0.42**	$y = -0.48x^2 + 2.62x - 1.71$	**+0.88****	$y = 0.86x^2 - 3.90x + 5.36$	**−0.59**
Tail moment	Male	$y = -0.19x^2 + 0.75x + 0.86$	**−0.17**	$y = 0.72x^2 - 2.61x + 3.32$	**+0.60**	$y = 3.78x^2 - 14.38x + 14.53$	**+0.37**
	Female	$y = -x^2 + 4x - 1$	**+0.13**	$y = 1.39 \ln(x) + 0.57$	**+0.75***	$y = 1.46x^2 - 7.32x + 9.71$	**−0.63**
% Severed cells	Male	$y = 3x^2 - 17x + 50$	**−0.86****	$y = 4x^2 - 22x + 58$	**−0.90****	$y = -12.92 \ln(x) + 43.71$	**−0.94****
	Female	$y = 3x^2$ - 19x + 48	**−0.94****	$y = -2x^2 + 4x + 26$	**−0.96****	$y = -x^2 + x + 30$	**−0.93****

Abbreviations: * significant at $p < 0.05$; ** significant at $p < 0.001$.

Table 5
Generalized Estimating Equation to analyze the interactions among the distance from Abu-Zaabal Fertilizer Company, types of tissues, sex on comet parameters (Tail length, Tail DNA, and Olive Tail Moment) from brain, thoracic muscles, and gut cells of males, and females of *A. thalassinus* collected at different sites located at various distances from Abu-Zaabal fertilizer company.

Source	Chi-square (χ^2)	*df*	*p* value
Distance – sex interaction			
Tail length	6.43	2	<0.05
Tail DNA	10.43	2	<0.05
Olive moment	19.81	2	<0.0001
Tail moment	32.16	2	<0.0001
% severed cells	35.50	2	<0.0001
Distance – tissue interaction			
Tail length	106.29	4	<0.0001
Tail DNA	27.18	4	<0.0001
Olive moment	133.05	4	<0.0001
Tail moment	94.90	4	<0.0001
% severed cells	37.41	4	<0.0001
Sex – tissue interaction			
Tail length	23.67	2	<0.0001
Tail DNA	48.88	2	<0.0001
Olive moment	30.22	2	<0.0001
Tail moment	18.21	2	<0.0001
% severed cells	22.17	2	<0.0001
Distance – tissue – sex interaction			
Tail length	60.12	4	<0.0001
Tail DNA	72.30	4	<0.0001
Olive moment	28.36	4	<0.0001
Tail moment	25.76	4	<0.0001
% severed cells	83.28	4	<0.0001

studies on invertebrates inhabiting a gradient of pollution (Stone et al., 2001, 2002; Łaszczyca et al., 2004; Migula et al., 2004; Wilczek et al., 2004; Augustyniak et al., 2005). In the quoted works, all carried out in the vicinity of Olkusz, Poland; the sites were located every few kilometers from the smelter. The reference site, like in present work, was located approximately 30 km from the source of contamination. However, the fundamental difference between the present and the quoted studies was the main source of contamination. In Olkusz, pollution appeared as a result of mining and metallurgical activity of human, while in Cairo the main contaminants are the result of fertilizer production spreading through the air and water irrigation.

DNA damage in brain, thoracic muscles and gut of *A. thalassinus* (both male and female) inhabiting all the selected sites at different

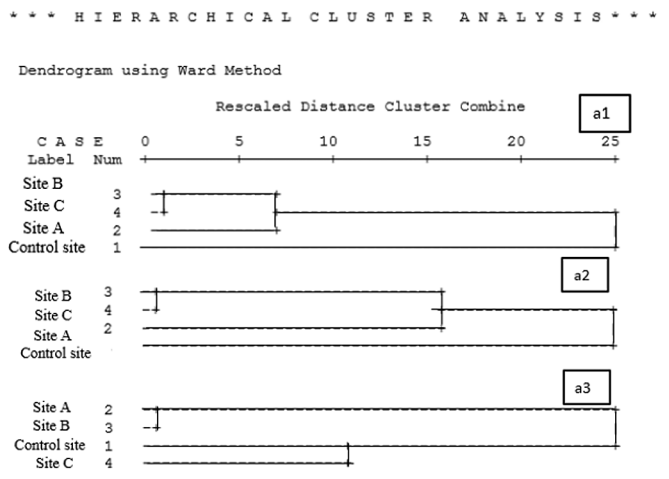

Fig. 6. Dendrogram of the cluster analysis (using Ward's Method) applied for biomarkers (DNA damage analysis) in brain (a1), thoracic muscles (a2), and gut (a3) cells of males *Aiolopus thalassinus*, which were collected at control site and polluted sites (A–C) located at different distances from Abu-Zaabal Fertilizer Company.

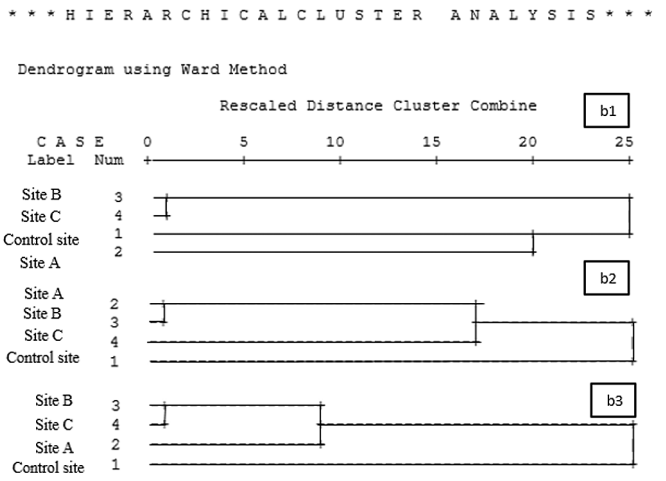

Fig. 7. Dendrogram of the cluster analysis (using Ward's Method) applied for biomarkers (DNA damage analysis) in brain (b1), thoracic muscles (b2), and gut (b3) cells of females of *Aiolopus thalassinus*, which were collected at control site and polluted sites (A–C) located at different distances from Abu-Zaabal Fertilizer Company.

distances from Abu-Zaabal Company was measured to evaluate the level of environmental stress, reported previously by Yousef et al. (2017).

Generally, the level of DNA damage in tissues of *A. thalassinus* from the polluted sites was significantly higher than in individuals from control sites (Tables 1–5; Figs. 3–7). However, no single parameter of the comet did reflect the level of contamination to the same extent as a comprehensive analysis of all DNA damage parameters. The obtained results indicated a possible impact of Abu-Zaabal Company on DNA integrity in *A. thalassinus* tissues. Therefore, the potential use of the comet assay as a biomonitoring method of the environmental pollution, also caused by fertilizer industry, should be considered (Lovell and Omori, 2008; Valverde and Rojas, 2009; Augustyniak et al., 2016a).

Analysis of TDNA and TL parameters (Tables 1 and 2) revealed that brain of females could be consider as a good responding cell system. The level of damage in this tissue reflected well the level of pollution on each site. However, it should be stressed here that DNA damage varied among analyzed tissue. Surely, the difference are derived from both the function of tissue and additional stressing factors. Cells of gut have direct contact with all damaging factors that are ingested through the life in polluted environment. On the other hand, muscle are characterized by high metabolism rate and extensive consumption of oxygen (Klowden, 2007). Reactive oxygen species (ROS) are generated routinely during metabolic processes in the cells. In mitochondrial respiration, it was revealed that up to 2% of the consumed oxygen can be transformed into superoxide anion that initiates cascade of reactions leading to production of highly reactive radicals that exert its destructive effects on various biomolecules within the cell, including DNA. Numerous environmental factors may increase the level of ROS in the cell, which is equipped with various enzymatic and non-enzymatic responses to counteract the damage (Ahmad, 1995; Boesch et al., 2011; Yousef et al., 2017). The final effect is a result of all processes.

Control and polluted sites differed in the soil content of heavy metals (Pb, Cd, Zn, and Cd), but above all, they significantly varied in PO_4^{3-} as well as SO_4^{2-} concentration in the soil (Fig. 2). Some interaction among all pollutant should be also considered as it can influence the final hazard to plants, groundwater, and animals (Kassir et al., 2012). Atmospheric pollutants such as hydrofluoric acid fumes (HF), sulphur dioxide (SO_2), nitrogen oxides (NO_2), and particulate matter of up to 10 μm diameter (PM_{10}) also differ

around Abu-Zaabal Company for Fertilizer Industry (Egypt State of Environment ESE, 2014). These pollutants can increase the production of reactive oxygen species (ROS) in the cells of an individual exposed to them, and therefore can cause oxidative stress with all the adverse consequences for an organism (Farahat et al., 2010; Okamoto et al., 2014; Zhu et al., 2014; Shinkai et al., 2015; Yousef et al., 2017). This might explain the fluctuation of results among the different sites.

The present results are in compliance with the data presented by Lobo et al. (2010), who reported that DNA is one of the most important targets of free radical attack in the cells. Molecular mechanisms causing DNA damage may include: activation of nucleases, direct reaction of free radicals such as hydroxyl radicals ($^{\bullet}OH$) with the DNA, or breaks resulting from free radical reaction of deoxyribose residues (Halliwell and Aruoma, 1991; Hegde et al., 2008). Moreover, DNA damage can involve: formation of a basic site that finally leads to strand breaks (Halliwell, 1999; Friedberg et al., 2005; Cakmakoglu et al., 2011); modifications and degradations of nitrogenous bases; damage to sugar moiety; formation of DNA-DNA and DNA-protein cross links, and damage to the repairing system of DNA (Kohen and Nyska, 2002; Birben et al., 2012).

Heavy metals have a tendency to accumulate in an organism. They can indirectly increase the production of ROS in the cells by affecting the rate of respiratory metabolism for which oxygen is the most essential factor. It was proved that metals such as Zn caused DNA damage in cells isolated from the brain of *Chorthippus brunneus*, but this effect was not proportional to the metal dose (Augustyniak et al., 2006). Copper, another essential trace element, can induce oxidative damage to macromolecules such as DNA, proteins and lipids (Shukla et al., 2011). In the presence of the reducing agents, Cu can catalyze the production of ROS, such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and $^{\bullet}OH$, through Fenton and Haber-Weiss reactions. Previous studies showed that DNA breaks are caused by a site-specific reaction of Cu ions, both *in vitro* and *in vivo* (Hayashi et al., 2000).

Yousef et al. (2010) found that the genotoxicity of heavy metals, cadmium and lead, in *Schistocerca gregaria* was very high. Hence, this may reflect the role played by *S. gregaria* as a valuable bioindicator of environmental genotoxic pollutants. Joseph (2009) hypothesized that genotoxic effect of Cd may also result from generation of ROS, and lead to oxidative stress that is associated with generation of 7,8-dihydro-8-oxoguanine (8-oxoGua) commonly used to monitor DNA damage (Shukla et al., 2011).

The relationship between comet parameters and distance of sites from Abu-Zaabal Fertilizer Company in the present research has no clearly definite pattern. However, we described a strong negative correlation between % of severed cells and the distance of the sites from Abu-Zaabal Company. A highly significant correlation was confirmed in case of brain, thoracic muscles, and gut of males and females of *A. thalassinus* collected at polluted and control sites (Table 4). Also, a cluster analysis of DNA damage using Ward's method revealed a high level of similarity of insects from all the polluted sites (Figs. 6 and 7). We postulate that specific pollution resulting from the activity of the fertilizer industry can cause comparable adverse effects in the organisms inhabiting areas up to 6 km from the source of contamination. The best parameter for monitoring of fertilizer pollutants is percentage of the cells with visible DNA damage (% of severed cells). It is probably connected with an extremely high concentration of phosphates and sulphates in all the polluted sites (Fig. 2).

Conflict of interest

Authors declare that they have no conflict of interest.

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