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Effects of a Fungicide Fosetyl Al 50% + Folpet 25% (Mikal-Flash) on Biochemical and Histological Aspects of *Lumbricus terresteris* Earthworms

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ABSTRACT

Most studies related to the effects of pesticides on soil communities are devoted to earthworms due to their role as soil pollution indicator. Thus, the present study was aimed to evaluate the effects of a fungicide (fosetyl Al 50% + folpet 25%) named Mikal-Flash on two biochemical parameters in *Lumbricus terresteris.* Physico-chemical analysis of soil showed it as a very convenient environment for *L. terresteris* life due to its richness in organic matters (16.12%), phosphorus (up of 20 ppm), total carbon level (9.37 %), in addition to an average richness in calcium carbonate ($CaCO_3$; 0-25%). Additionally, soil pH was slightly alkaline where pH water and pHKCl were respectively 7,1 and 8.0, soil humidity ranged from 45 – 60% and soil texture was found silty clayey. The results showed significant differences in total proteins, and the malondialdehyde (MDA) contents between controls and treated earthworms with 12.5, 25,50 mg/500 mg of Mikal-Flash during 24 h, 48 h and 72 h. These adverse effects were supported by the histological observations of the digestive tract, showing marked alterations in epithelial cells and muscle fibers.

Keywords *Lumbricus terresteris,* Bioindicators, Mikal flash, Total proteins, MDA.

INTRODUCTION

World-wide of millions of tons of pesticides, including herbicides, fungicides, and insecticides are annually world-widely used in agriculture to protect crops from yield losses, there by boost the production (Al-Assiuty *et al.* 2014). Excessive pesticide use has posed a serious threat to human health and environment, through deteriorating the quality of groundwater and coastal waters, as well as reducing the terrestrial biodiversity. Overall, pesticides residues could be mixed into the top 15 cm layer of soil, considered a region of the greatest microbial and soil faunal activity (Blasco et Pico 2009). The earthworms are one of the major components of the soil fauna proving thus, their presence in all terrestrial ecosystems, their effects inducing changes in the soil

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biological and physico-chemical properties, and their likely effective role in the soil formation, structure, and fertility (Freita 2012, Julia clause 2014). Previous studies investigating the effects of pesticides effects on the soil community have mainly focused on using earthworms whose distribution and ecological importance make them effective bio-indicators of soil chemical contamination by providing early detection of the soil deterioration process (Al-Assiuty *et al.* 2014). Recently, more efficient methods based on using the biological and biochemical parameters of living organisms have filled the gaps inherent in strictly chemical analyses, or they have appeared necessary for a good assessment of the environmental risks caused by substances present in the living environment (Mathias 2011, Zeriri 2014). In this regard and on the basis of the related previous works. The present work was aimed to evaluate the biochemical and histological effects of a fungicide fosetyl Al 50% + folpet 25%, (MIKAL-FLASH), largely used in the Souk-Ahras city (Northwest Algeria) on the common abundant soil biological model, earthworm *Lumbricus terresteris.*

MATERIALS AND METHODS

Sampling sites

Our specimens were obtained from a house garden located in Sidi Fredj town, about 40 km from Souk-Ahras city (North-East Algeria) at the limit of the Tunisian borders. The specimens region has astrological coordinates of 36° 09' 13' N and 8°11'43'' E, and are characterized by a cold climate in winter and a hot summer (Fig. 01).

Soil physico-chemical analysis

A soil sample taken from the study site was used for the evaluation of some physico-chemical parameters. The sample was prepared by separating the fine earth, which were then dried in the open air. The soil pH was determined as described elsewhere (Baize and Jabiole 1995) by weighing 10 g of soil and then mixed with 20 mL of distilled water and left to stand for 24 h.

The pH of KCl (pHKCl) of soil samples was determined by mixing 20 g of fine earth with 50 ml of KCl, and the mixture was stirred for 5 min, and kept standing for 30 min. The measurement of water and KCl pH can be performed by a pH meter.

The dosage of organic matter (OM) content in the soil was determined by measuring of the weight loss of the soil samples after calcination at 375°C for 16 h, and subsequently, the proportions of the organic matter can be known:

OM level = Soil dry weight (g) - the weight of incinerated soil /weight of dry soil×100

The soil texture and carbonate content were determined as per the method of Guesmia (2019). A mixture of 10 ml, dilute HCl, 30 ml distilled water and 10 g fine soil was agitated and left as much to stand. The carbonate content (%) was determined as follows:

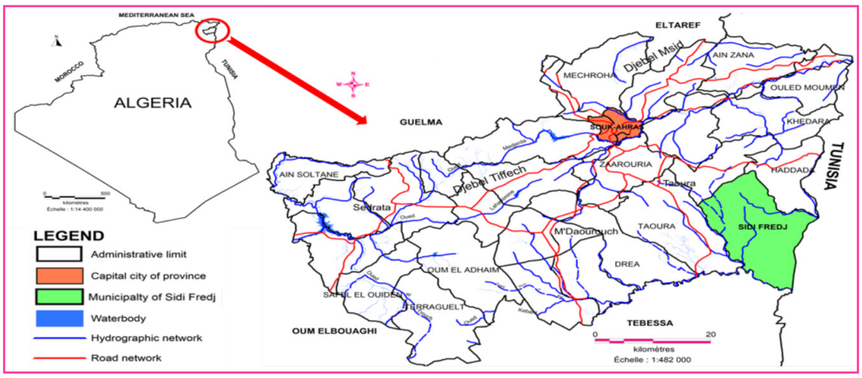

Fig. 1. Geographical location of Sidi-Fredj , Souk-Ahras city (North-East-Algeria).

$CaCO_2$ (%) = Weight of released carbon dioxide × 2.274/soil weight × 100.

Where the weight of released $CaCO_2$ is determined by subtracting the initial weight of beaker + acid (W1) and 10 g soil (W1+10 g soil=W2) from the final weight of beaker, acid and soil (W3)

Soil moisture was studied according to the method of Ita (1975) based on the drying process of a soil sample at 105 ^{0}C for 24 h until constant weight, and the difference in weight before and after the drying process corresponds to the quantity of water.

The dosage of total phosphorus was performed according to the method of Gharoucha (1995) with the aim to know the quantity and the state of phosphorus in the soil sample.

Biological materials

Lumbricus terresteris are commonly used as biological models due to their low costs and simple maintenance and handling. Individual earthworms were collected by manual digging in the surface layer of the soil up to 30 cm deep and then were placed in plastic beaks filled with the original soil and debris of plants to better acclimatize them for a period of 14-21 days (Bekker and Bouzned 2014).

Chemical material

MIKAL-FLASH is preventive systemic fungicide acting actively against excoriosis and downy mildew of the vine. The chemical belongs to the family of phthalemides consisting of two active ingredients; 50% fosetyl aluminum and 25% folpel. The first act indirectly by stimulating the natural defense of the plant, while the second inhibit the germination of mildew spores (Bayer 2014).

Experimental design

MIKAL-FLASH at 12.5, 25.5, 50 mg/500 g soil doses were directly sprayed on the spouts containing the original soil samples, then 120 juvenile individuals of *L. terresteris* were distributed in equal numbers (30 individuals) per beak, including the untreated control beak sprayed with distilled water. After the treatment periods (24, 48, and 72 h), nine (09) individuals were collected from each beak to perform the two biochemical parameters (3 repetitions to test the dose/period effect) (Lordache and Borza 2011). Here, the earthworm samples were washed with distilled water, weighed with a precision balance, placed in small plastic tubes each containing 1ml of phosphate buffer, and stored in the freezer at -32°C until the biochemical analysis of total proteins and of MDA. Of note the earthworms were crushed manually using a mortar prior to the biochemical analysis.

Biochemical analysis

Total protein contents were determined according to Bradford (1976) with dye Coomassie Blue (BBC) using bovine serum albumin BSA as standard substrate ?the absorbance was recorded at a wavelength of 595 nm.

The Malondialdehyde (MDA) level was quantified according to the method of Draper and Hadley (1990) and the absorbance was recorded at 532 nm.

Histopathological evaluation

The histological studies of earthworm's tissues were performed at the end of experiment in Anatomy Pathology Laboratory of Souk-Ahras Hospital. One earthworm from each replicate (three per treatment) were recovered from soil, washed with distilled water and put in jars containing 1% agar jelly, kept standing for 96 h to facilitate the removal of soil from digestive tract and dehydrated with upgrading ethanol (70-100%) for 15 min before cutting them into two parts. The earthworms were placed in specimen bottles and fixed with Bouin's fluid for 12 h before subjecting it to histological procedures of embedding in paraffin wax, sectioning and staining with Haemtoxylin-Eosin for microscopic observation. The tissue section were observed at 40X under a binocular microscope and photographed with the help of a microscope-mounted camera (Oluah *et al.* 2010, Berrouk *et al.* 2021, Berrouk *et al.* 2022)

Statistical analysis

The mortality data were represented by the means of the dead individuals and were statistically tested by analysis of variance with two classification criteria (concentration-time) with Student t test -The statistical tests were performed using specialized statistical software (Statistica Ver 8.0,). Our statistical analysis is based on the following points:

> A statistical description was assigned for the two studied variables (total protein and MDA) by calculating the mean, the median, and the two minimum values X_{min} and maximum X_{max}

> A comparison between several groups was attributed by the Kruskal Wallis non-parametric test to test the "period" and effect relating to "MIKEL-FLASH"

> A Spearman correlation coefficient analysis of the linear relationships between all the used treatment periods.

RESULTS

Soil physico-chemical analysis

The physicochemical analysis of the soil showed that *L. terresteris* earthworms live in a slightly alkaline soil where the water pH and KCl pH values range between 7.1 and 8.0.

The total carbon level is 9.37%, and soil organic matter contents are 16.12%. Thus the soil is moderately rich in $CaCO_3$, and hence the contents were between 10 and 25%. In addition, total phosphorus content in the soil was very high with a rate greater than 20 ppm, while the soil humidity (50.90%) varies from 45 to 60% and consequently, the soil appears a clay-silty texture.

Biochemical studies

Effects of MIKEL-FLASH fungicide on *L. terresteris* in the function of exposure time

MIKEL-FLASH fungicide exposed *L. terresteris* showed that the relationship dose/period effect (24 hours) is remarkable for the two studied parameters (total proteins and MDA), and this could be proved by using the Kruskal-Wallis non-parametric test. Results of H and p are reported in Table 1.

Table 1. Kruskal Wallis results of the relationship dose-period effect of MIKAL-FLASH.

Biochemical parameters	*H*	*p*
Total proteins content	20.04054	0.000
MDA content	0.8663664	0.6484

Effect of MIKEL-FLASH on total protein level

The total protein level (µg / individual) in the controls and the MIKEL-FLASH treated *L. terresteris* with the doses 12.5, 25, 50 mg/g of soil showed a significant increase (p=0.000) in the protein level in the treated earthworms compared with controls during the three treatment periods (24, 48.72 h) (Fig 2).

Effect of MIKEL-FLASH on MDA content

Figure 3 depicts that the MDA level in the treated earthworms with the 12.5 mg dose of MIKEL-FLASH is lower than that of controls after 24 h of treatment since the level of MDA increased in the treated earthworms with the 12.5 mg of MIKEL-FLASH compared to the controls and those treated after 48 h and 72 h. Same Fig. 3, shows no significant differences in MDA content between the treated and untreated earthworms (p=0.6484).

Histological evaluation

Histological observations of the digestive tract tissues of treated earthworms revealed marked dose – de-

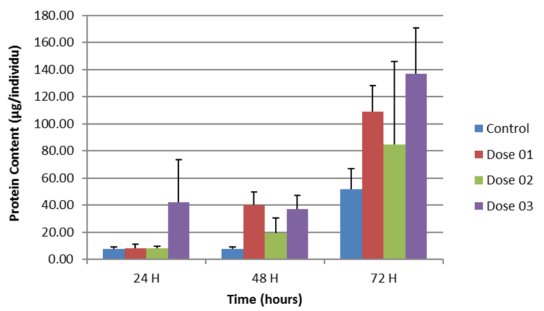

Fig. 2. Effect of MIKEL-FLASH treatment on total protein content in *L.terresteris.*

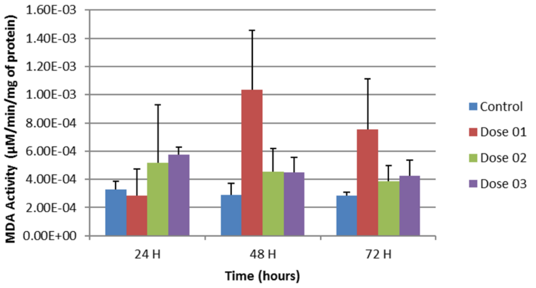

Fig. 3. Effect of MIKEL-FLASH treatment on the MDA content in *L. terresteris.*

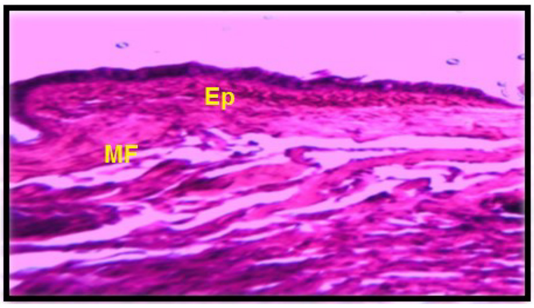

Fig. 4. Light microscopic cross-section of digestive tract tissue from control *L.terrestris* (magnification X40), showing normal appearance of the epithelial layer (Ep) and muscle fibers (MF).

pendent histological changes in the epidermis, the first fungicide-target tissue, circular and longitudinal muscle fibers and the intestinal cells following the ingestion of pollutant contaminated soil (Figs 5, 6 and 7) when compared with controls showing normal tissue structures of the digestive tract (Fig. 4).

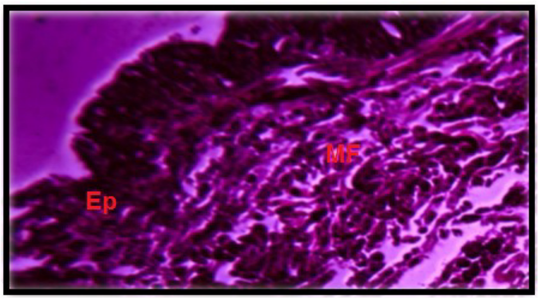

Fig. 5. Light microscopic cross-section of digestive tract tissue from 12,5 mg /500 g sol treated *L. terrestris* after 72 h of exposure time (magnification X40), showing alterations in the epithelial layer (Ep) and muscle fibers (MF).

Fig. 6. Light microscopic cross-section of digestive tract tissue from 25 mg /500 g sol treated *L. terrestris* after 72 h of exposure time (magnification X40), showing chloragogenic tissue (ChT) and vacuolated cells (V).

DISCUSSION

Soil analysis

The soil physicochemical and biological properties, as well as the variability in the environment and pedoclimatic conditions, can strongly affect the spatial distribution, density, and biomass of earthworms (Kanianska *et al.* 2016). In this regard, Lairammuana *et al.* (2020) have reported a relationship between the soil pH and precipitation variations, and acidity with a deficiency in some menials, including phosphate, calcium, magnesium, and potassium. Unlike acidic soils, neutral and alkaline soils provide favorable conditions for the earthworms proliferation (Yesguer 2015). Bazari (2015) reported that the distribution of *Aporrectodea caliginosa* and *Aporrectodea monticola*

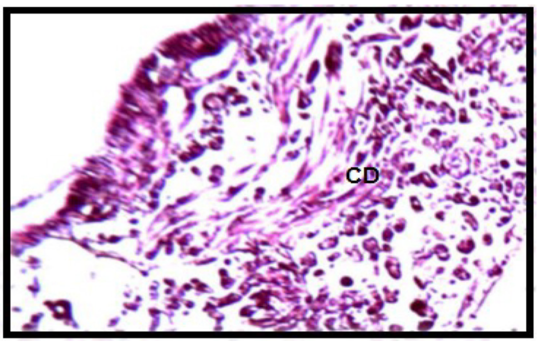

Fig. 7. Light microscopic cross-section of digestive tract tissue from 50 mg /500 g sol treated *L. terrestris* after 72 h of exposure time (magnification X40), showing a total loss of longitudinal, circular muscle cell connectivity, and cell dispersions (CD).

is related to the parameters of $CaCO_3$ and Ca++, and that six species of earthworms among the 18 identified species have a tendency towards the high contents of organic matter and clay. Another identified earthworms group used at the same period showed obvious preference to loamy soil and poor in organic matter, Zerrouk *et al.* (2022) reported that the texture range in chrea (Algeria) from sity clay to sitysand. Furthermore, Mamadou *et al.* (2012) have proved that the contribution of organic matter results in an increase in the number of earthworm individuals per unit area, meanwhile several authors have reported the sandy texture as the less favorable texture for earthworm survival. Hence, the availability of organic matter in the soil stimulates the propagation of earthworms which consequently play an important role in soil decomposition (Cesarz 2016, Frousz 2017, Singh 2018). Chauhan (2014) was reported that humidity, texture, and the high contents in organic matter, phosphorus and total nitrogen of the soil can affect the number and distribution of earthworms in the soil, Houda *et al.* (2022) showed that the organic matter level was 8.93% (higher than 3) in Ouled –driss city (Northeast of Algeria) Mamadou *et al.* (2012) and Bazari (2015) also found that the supplementation with nitrogen increases the number of earthworm individuals per unit area, such as *Aporrectodea rosea* and *Aporrectodea trapezoides* which were found in sandy and nitrogen-rich environments. On top of that, Alice (2014) has found that a disturbance in the earthworms environment can have significant consequences at various scales (populations, organisms and even cellular and molecular). Here, Sri Minarsih *et al.* (2021) have confirmed that the distribution of some earthworm species, including *P.hamayana P. californica, E. tetraeda,* and *E. eugeniae* is positively correlated with the physico- chemical properties of soil (from the Merapi forest in Indonesia).

Biochemical study

Under pollution conditions, earthworms can decrease the toxic effects of chemicals by regulating their internal biochemical responses before their growth are affected and their life is threatened. Herein, Wang *et al.* (2012) have reported that the use of pesticides for crop protection can expose earthworms through direct contact, either with treated granules or seeds or through ingestion of contaminated soil particles. Interestingly, the terrestrial invertebrates can accumulate pesticides and heavy metals following uptake, distribution and storage processes (Guessasma *et al,* 2020). In this study, MIKAL-FLASH fungicide caused a marked effect in a dose-exposure time-dependent manner on the total proteins content of *L. terresteris* and similarly, several previous studies have proved this finding. Among them, the study of Matthias (2010) investigating the effect of Cd and Pb on standard parameters (growth, lethality and reproduction) in *Eisenia andrei* based on the determination of metallothionein (MT) level, a ubiquitous protein involved in the bioaccumulation, metabolism and detoxification of metals, has reported that Cd caused an accumulation of MT, in association with a decrease in reproduction, since Pb did not produce a significant effect on the studied parameters. In addition, Bekkar and Bouzned (2014) have found that the soil treated with a standard herbicide showed a significant increase in the contents of total proteins, total carbohydrates and total lipids in *Lumbricus terresteris* during different exposure periods. Also, Khaldi *et al.* (2014) showed a significant increase in total protein after treatment of Saccharomyces cerevisiae (yeast) with cadmium and similarly, Zeriri (2014) has found an increased level of total proteins following treatment with increased concentrations of methomyl. Dedeke *et al.* (2016) have reported a positive correlation between the accumulation of pollutants like heavy metals and the increase in MT proteins in different species of earthworms. Additionally, Dehamnia and Amamra (2019) have found a significant increase in total proteins and total carbohydrate levels in metal trace element treated terrestrial gastropod *Helix aspersa.* Atanu *et al*. (2020) have reported a rapid response of MT (biomarkers of oxidative stress) following exposure to lead and nickel, and subsequently increased their concentrations in the tissues. Moreover, MDA content is effectively correlated with the reactive oxygen species (ROS)-inducing peroxidation of membrane lipids (Zhang *et al.* 2012), considered a good indicator of oxidative level and resulted from the reaction between free radicals and unsaturated fatty acids. Chui *et al.* (2013), Grara (2011) and Ait hamlate *et al.* (2021) confirmed that MDA is a bioindicator of lipid peroxidation in terrestrial gastropods. As reported, the level of lipid peroxidation is an important parameter evaluating the oxidative effort in living organisms, and the MDA level is a bioindicator of lipid peroxidation in earthworm *L.terresteris* (Zeriri 2014). In our study, MIKAL-FLASH treatment showed a significant increase in MDA levels in the treated earthworms compared to the controls, and accordingly with several recent scientific studies, including the study of Lin *et al*. (2010) and Liu (2011) reporting increased MDA level in pesticide exposed earthworms. Likewise, Azouz (2012) has found a marked lipid peroxidation in relation to an increase in MDA levels in a pesticide exposed Paramecia. Emilie (2011) has found that the TiO2 nanoparticles used in a large number of manufactured products accumulated in the tissues of different species of earthworms, in addition to changes in the enzymatic activity of glutathione S transferase (GST) and catalase (CAT) and MT protein level. Another study has reported that the level of MDA was significantly increased in *Eisenia fetidea* treated with Cu for 28 days compared to the controls Chui *et al.* (2013). Also, Zeriri (2014) has reported that different concentrations of Methomyl (2.4, 6.8 mg/500 g soil) increased the MDA level in earthworms, *Octodrilus complanatus* as a function of time. Mekahlia *et al.* (2016) have found that a mixture of Sekator (herbicide) and a phosphate fertilizer caused significant changes in the enzymatic activity of GST, GSH and AchE in *L. terresteris*. Further, Otmani *et al.* (2018) have found that cadmium increased the levels of total proteins and MDA, leading to suggest the involvement of ROS induced severe peroxidation of membrane lipids. In this context, Soumaya *et al.* (2018) found that high concentrations (10%,15% and 20%) of phosphogypsum (PG) caused an increase in the enzymatic activity of ChE, LDH, and lipid peroxidation levels in an earthworm, *Glyphidrillus tuberosus.* What's more, Sulata *et al.* (2018) have reported that the different concentrations of heavy metals, including Fe, Co, Ni, Cu, Zn, Cd, Pb and Hg in the soil caused significant changes in the enzymatic activity of glutathione peroxidase, glutathione-1 reductase and glutathione transferase in *Aporrectodea caliginosa* earthworms.

Histopathological study

Contaminants enter the body of earthworms through the permeable skin, or through the digestive tract by mixing with soil particles, distributing themselves afterward to all tissues of the organism Atanu *et al.* (2020). Our results revealed that fungicide MIKAL-FLASH treated was able to induce severe histological changes in the epidermis, muscle tissue, and cells of earthworms. This result concords with those previously reported, including the study of Oluah *et al.* (2010) reporting that the herbicide (atrazine) as an environmental hazard caused severe tissue damage and intense mortalities in *Nuskkadrillus mbae*, the study of Dittbrenner *et al.* (2011) reporting marked histological changes in the body mass of *L. terresteri, Eisenia fetida* and *Aporrectodea caliginosa* after treatment with an insecticide, and the study of Goa *et al.* (2013) showing cell necrosis in a fongicide (triazole) exposed *E. foetida.* Also, Zeriri (2014) has reported serious alterations in the epidermis following the toxicity of Methomyl in *Octadrilus complanatus* and similar results have been found in zinc and lead treated juvenile *Aporrectodea* spp., Houda *et al.* (2022). Soumaya *et al*. (2018) have found that the 10% concentration of phosphogypsum caused damage in the muscle fibers of *G.tuberosus,* along with loss of connectivity of muscle tissue and the structural integrity of intestinal cells at the higher doses of the pollutant. Importantly, chemicals exposure can cause severe histological damages and can accumulate mainly in the circular muscles of earthworms exposed to xenobiotics (Kilic 2011). sIn this context, Berrouk *et al.* (2021) have reported that the acetamiprid (insecticide) caused harmful effects on the circular muscles, the epidermis and the cells of the digestive system in *Aporrectodea giardi.*

CONCLUSION

Earthworms, in particular, *L. terresteris* have been used as the subject of numerous studies, both ecologically and biologically, or as bio-indicators of soil quality through the analysis of enzymatic biomarkers of physiological disturbances. Our results showed a highly significant increase in total proteins in earthworms treated with MIKAL-FLASH compared to controls. The lipid peroxidation biomarker, malondialdehyde (MDA level) showed a non-significant increase in individuals treated with different doses of MIKAL-FLASH during different exposure periods. These adverse effects of MIKAL-FLASH on earthworms were confirmed by the histopathological observations of the digestive tract tissues, showing alterations and abnormalities in epithelial cells, chloragogenic cells, and muscle fibers.

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