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Can insecticide-free clean water regenerate the midgut epithelium of the freshwater shrimp after dimethoate treatment?

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ABSTRACT

Insecticides such as dimethoate persist for a long time in freshwater environments, influencing the physiology of the animals inhabiting such environments. In aquatic organisms, toxic substances can enter the body through the epidermis and the digestive system. The midgut is part of this system in which intense processes constitute a barrier against the effects of toxic substances on the body. The aim of this study was to evaluate the toxic potential of dimethoate in the midgut epithelium of the freshwater shrimp *Neocaridina davidi*, emphasizing ultrastructural alterations. However, the additional and main purpose was to determine whether the midgut epithelium can regenerate after placing animals in insecticide-free clean water after various periods of exposure to dimethoate. *N. davidi* originates from Asia, but it has also been described in European rivers. This species is of particular interest among breeders worldwide due to its ease of breeding and reproduction. The animals were treated with dimethoate for 1, 2, and 3 weeks and then placed in clean water for 1, 2, and 3 weeks. The qualitative and quantitative analysis revealed different sensitivity of organs forming the midgut in freshwater crustaceans and the possibility for midgut regeneration after insecticide exposure. We concluded that different processes were triggered in the intestine and hepatopancreas to regenerate cells after damage, and mitochondria were the first organelles to respond to the appearance of a stressor in the living environment.

1. Introduction

The development of industry and agriculture leads to an increase in pollution of the natural environment, especially the freshwater environment, in which numerous chemical substances accumulate. These include, among others, organophosphorus pesticides, over 100 types of which have been recorded and which are used mainly for agricultural purposes (Binelli and Provini, 2004; Ciglasch et al., 2005; El-Saeid and Selim, 2016; El-Saeid et al., 2016; Pan et al., 2021). They can damage the functioning of organisms by affecting their organs, tissues, and cells in the body (Amalin et al., 2000; Hanazato, 2001; Nyffeler and Sunderland, 2003; Wilczek et al., 2014; Hasenbein et al., 2016, 2017). One such organophosphorus insecticide is dimethoate, belonging to the acetylcholinesterase inhibitors. Dimethoate ([O,O-dimethyl S-(*N*-methylcarbamoylmethyl) phosphorodithioate]) is common in agriculture and non-agricultural applications due to its high water solubility (Van Scoy et al., 2016). In freshwater organisms, it can enter the body through the epidermis and the epithelium covering the digestive system. The middle region of the digestive system, which is of endodermal origin, takes part in homeostasis maintenance (Malagoli et al., 2010; Wilczek et al., 2014; Włodarczyk et al., 2017; Rost-Roszkowska et al., 2018, 2019). From the midgut through the body fluids, xenobiotics (e.g., heavy metals, pesticides) can be distributed throughout the body (Kumar et al., 2010; Wilczek et al., 2014; Stalmach et al., 2015). Histological and ultrastructural changes are regarded as useful markers of the direct effect of various xenobiotics on organisms, so such alterations are good markers of environmental stressors (Ansoar-Rodríguez et al., 2016; Rost-Roszkowska et al., 2020a, 2020b).

Aquatic invertebrates have been shown to exhibit a wide range of sensitivity to xenobiotic compounds such as organophosphorus pesticides and could be used as biomonitors for pesticide contamination. Toxicity of several pesticides (e.g., dimethoate, chlorpyrifos, fenarimol, cypermethrin) to freshwater invertebrates has been studied in order to present physiological properties, survival, mortality, reproduction, and changes in population (Roast et al., 1999; Hanazato, 2001; Kumar et al., 2010; Srivastava et al., 2013; Chen et al., 2014; Hasenbein et al., 2015, 2016, 2017; Van Scoy et al., 2016; Pan et al., 2021). As it turns out, crustaceans are one of the first invertebrates whose population size

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strongly decreases or all die under the effect of pesticides (Roast et al., 1999; Kumar et al., 2010; Negro et al., 2011; Srivastava et al., 2013). As a species for research, we chose the exotic (and widespread in Asia) freshwater shrimp *Neocaridina davidi*, which is adapted to cold freshwater environments (Klotz et al., 2013). Due to the high tolerance of low temperatures, this species may spread in cold waters and become invasive (Jabłońska et al., 2018). To date, papers connected with studies on *N. davidi* have been published on the impact of fasting on survival in the larval stages (Pantaleão et al., 2015) and the structure of the digestive system (Włodarczyk et al., 2017, 2019a, 2019b), as well as the impact of reproductive frequency on biochemical composition and egg size (Tropea et al., 2015), and water quality on survival and growth of specimens (Viau et al., 2015). This species is of particular interest among breeders worldwide due to its ease of breeding in aquarium conditions. *N. davidi* is appreciated by scientists for its ease of preparation for analyses and for the possibility of conducting comparative studies for other freshwater organisms. The *N. davidi* midgut is composed of two organs: the tube-shaped intestine (the midgut tract) and lobular hepatopancreas (the midgut gland) formed by two large diverticula divided into blind-end tubules. Within the intestine, the anterior intestine lined with an epithelium containing D-cells (digestive cells) and E-cells (embryonic cells, regenerative cells), and the posterior intestine with an epithelium composed only of D-cells can be distinguished. The hepatopancreas is a lobular gland that contains numerous blind diverticula. Its epithelium can be composed of various types of cells depending on their location in these diverticula. Therefore, three distinct regions have been distinguished: distal (with E-cells), median (with differentiating cells), and proximal (with F-, B- and R-cells) (Sonakowska et al., 2015, 2016; Włodarczyk et al., 2017, 2019a, 2019b; Sonakowska-Czajka et al., 2020). Thus, this study aimed to evaluate the toxic potential of dimethoate in the middle region of the digestive system (the midgut) of freshwater shrimp, emphasizing ultrastructural alterations. The study's additional aim was to determine whether the midgut epithelium can regenerate after placing animals in insecticide-free clean water after various periods of exposure to dimethoate. It was important to observe whether any changes, visible at the ultrastructural level, appeared.

2. Material and methods

2.1. Material

The material for studies (adult males and females of the freshwater shrimp N. davidi) was obtained from local shrimp breeders and kept in a 40 L shrimp tank equipped with a heater with a thermostat and a mechanical filtration system. The water temperature was set to 21 °C, pH to 7, and total water hardness was equal to 10°GH. The N. davidi shrimp were fed with JBL Novo Prawn.

2.2. Experiment

To evaluate the effect of dimethoate on the midgut of *N. davidi* the value of the median lethal dose (LD_{50}) was established. According to the literature (Kumar et al., 2010) and our studies, the concentration of 70 μg/l of dimethoate was chosen. The value of LD_{50} for 70 μg/l in the *N. davidi* population is equal to 21 days (50 % of analyzed specimens died after 21 days). Shrimps were divided into the following groups: the **control group** – animals cultured as described above; **1D, 2D, 3D** experimental groups – animals exposed to 70 μg/l of dimethoate for 1, 2 and 3 weeks respectively. To determine whether the changes caused by dimethoate were reversible, after the exposure to 70 μg/l of dimethoate, animals were restored to the clean water for 1, 2, and 3 weeks. The combination of dimethoate treatments with the restoration treatments (D x W) resulted in nine combined experimental groups: **1D1W, 1D2W, 1D3W, 2D1W, 2D2W, 2D3W, 3D1W, 3D2W, 3D3W** (Table 1, Fig. 1). During the experiment, shrimps were separately cultured in plastic cups. All the specimens were fed *ad libitum* as animals from the control group.

Table 1
Experimental groups. D – water supplied with 70 μg/l of dimethoate, W – clean water.

EXPERIMENTAL GROUPS	DIMETHOATE TREATMENT (70 μg/l)	CLEAN WATER
CONTROL	–	Continuously
1D	1 week	–
1D1W	1 week	1 week
1D2W	1 week	2 weeks
1D3W	1 week	3 weeks
2D	2 weeks	–
2D1W	2 weeks	1 week
2D2W	2 weeks	2 weeks
2D3W	2 weeks	3 weeks
3D	3 weeks	–
3D1W	3 weeks	1 week
3D2W	3 weeks	2 weeks
3D3W	3 weeks	3 weeks

2.3. Methods

The animals were anesthetized with chloroform, and the midgut, composed of two organs – the intestine and hepatopancreas – was isolated from their body. The organs were prepared for the analysis using histological and histochemical methods.

2.3.1. Light and transmission electron microscopy

Adult specimens of *N. davidi* (10 individuals from each experimental group) were decapitated and fixed with 2.5 % glutaraldehyde in a 0.1 M sodium phosphate buffer (pH 7.4, 4 °C, 2 h) and postfixed in 2 % osmium tetroxide in a 0.1 M phosphate buffer (4 °C, 1.5 h). Dehydration and embedding were conducted according to the standard procedure described previously for this species (Sonakowska et al., 2015, 2016; Włodarczyk et al., 2017; Sonakowska-Czajka et al., 2020). The material embedded in epoxy resin (Epoxy Embedding Medium Kit; Sigma) was cut on a Leica Ultracut UCT25 ultramicrotome. Semi-thin sections (0.8 μm thick) after staining with 1 % methylene blue in 0.5 % borax were examined using an Olympus BX60 light microscope. Ultra-thin sections (70 nm) were stained with uranyl acetate and lead citrate and eventually analyzed using a Hitachi H500 transmission electron microscope.

2.3.2. Histochemistry

Semi-thin sections from each experimental group were prepared for histochemical analysis according to protocols described previously: detection of glycogen and polysaccharides (PAS method), detection of proteins (bromophenol blue staining) and detection of lipids (Sudan Black B staining) (Sonakowska et al., 2015; Sonakowska-Czajka et al., 2020). The slides were examined using an Olympus BX60 light microscope.

2.3.3. Statistical analysis

The sections stained with 1 % methylene blue were used in the quantitative analysis of autophagy and necrosis. Thus, cells with signs of autophagy or signs of necrosis were counted for 100 cells of the intestine and hepatopancreas separately. All assays were based on 5 samples, performed in duplicate. The results were reported as mean values ± SD. Normality was checked by the Shapiro – Wilk test. The data were tested for homogeneity of variance using Levene's test of equality of error variances. Significance of differences in the levels of analyzed parameters was assessed by one-way analysis of variance, followed by the Tukey's HSD test, at significance level $p < 0.05$.

3. Results

3.1. Intestine

Within the intestine, we can distinguish the anterior intestine lined with an epithelium containing D-cells (digestive cells) and E-cells

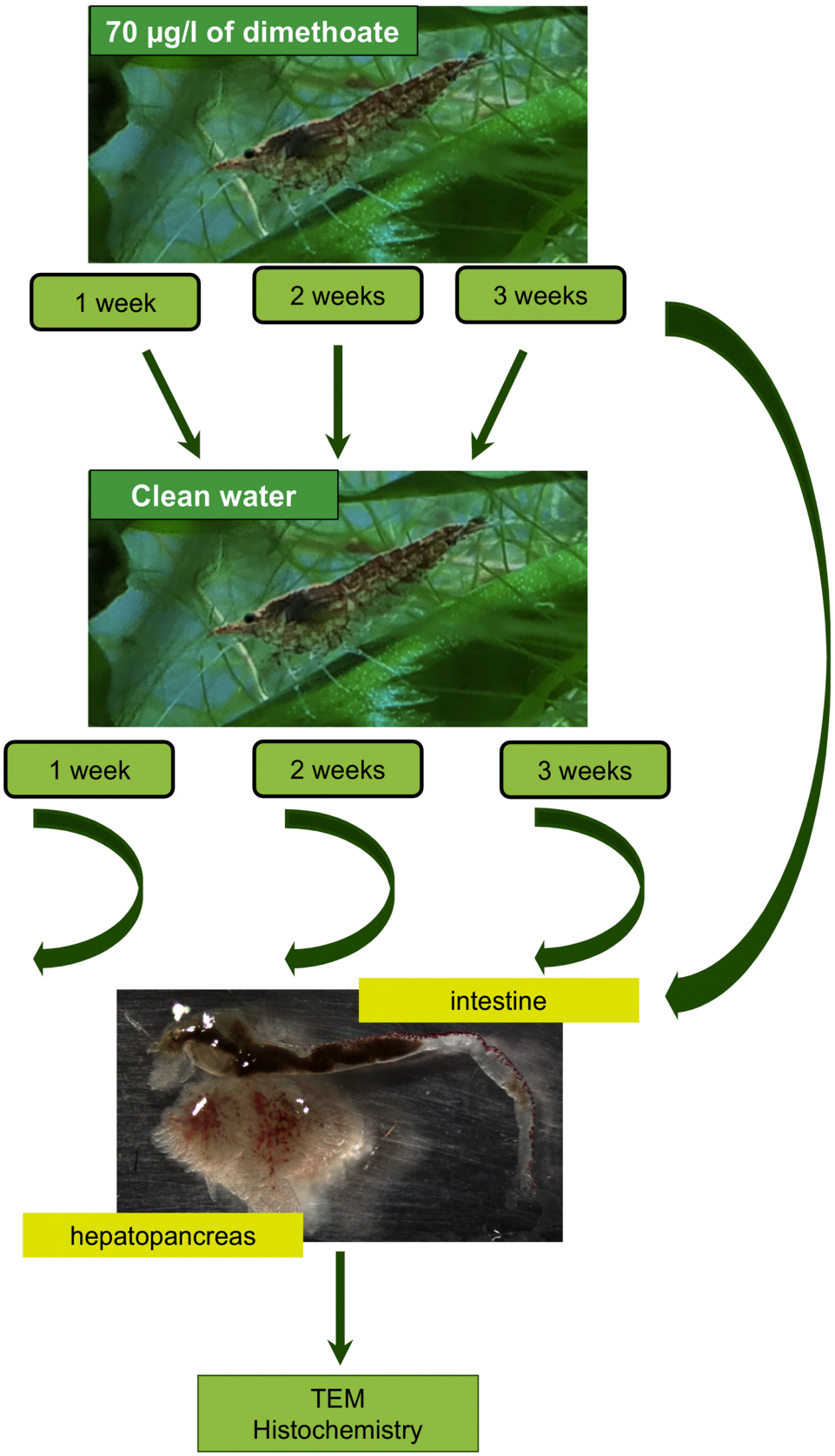

Fig. 1. Scheme of experimental setup.

(embryonic, regenerative cells), and the posterior intestine with an epithelium composed only of D-cells as the principal cells of this epithelium. The digestive cells' cytoplasm shows three cytoplasmic regions: basal, perinuclear and apical. The basal cytoplasm with the strongly folded basal cell membrane is abundant in endoplasmic reticulum cisternae and some Golgi complexes. A similar accumulation of endoplasmic reticulum cisternae was detected in the neighborhood of the nucleus. However, more mitochondria were stored in the basal than in the perinuclear cytoplasm. The apical cytoplasm, together with numerous mitochondria and endoplasmic reticulum cisternae, possesses some multivesicular bodies and vesicles with the content of different electron density. Their structure and ultrastructure have been described in our previous papers (Sonakowska et al., 2015; Sonakowska-Czajka et al., 2020).

3.1.1. Exposure to dimethoate

Exposure of shrimps to dimethoate affected the ultrastructure of D-cells that made up the intestine epithelium. Degenerative changes increased with the prolongation of dimethoate treatment. E-cells did not show any alterations in any of the experimental groups (Fig. 2A).

The cytoplasm of digestive cells in the **1D** experimental group possessed swollen mitochondria with an electron-lucent matrix and devoid of cristae, but mitochondria with the proper structure were also observed (Fig. 2B). No changes of the cisterns of RER, SER, (Fig. 2C) Golgi complexes, or the nuclei were detected. Degenerated and distended mitochondria with an electron-lucent matrix and strongly reduced mitochondrial cristae were detected in the digestive cells of specimens from experimental group **2D** (Fig. 2D). The process of mitochondrial fission was also observed (Fig. 2D). Single spheres of reserve

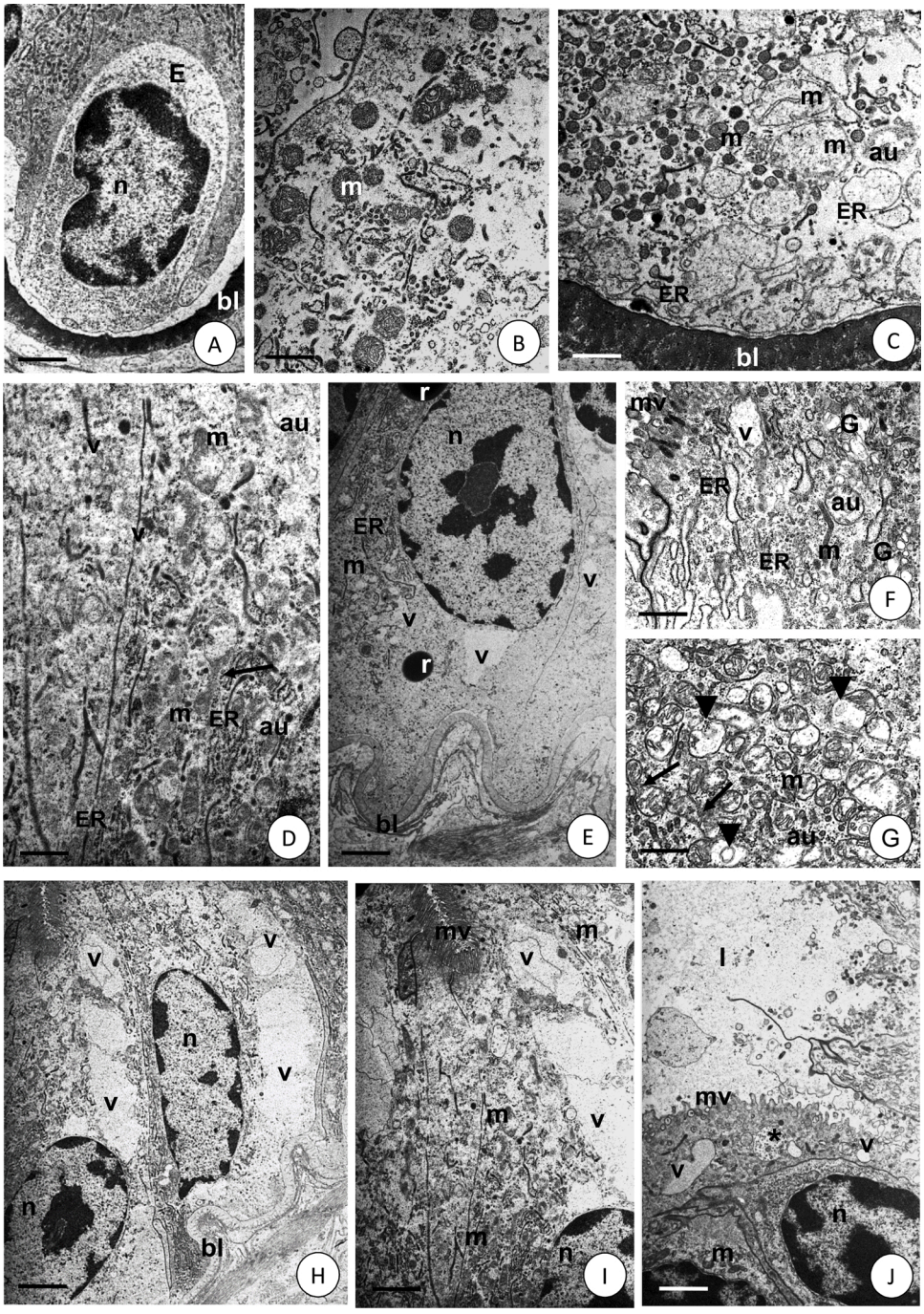

Fig. 2. Ultrastructural changes in the intestine epithelium of animals treated with dimethoate for 1 week (experimental group 1D) **(A–C)**, 2 weeks (experimental group 2D) **(D** and **E**, **H** and **I)** and 3 weeks (experimental group 3D) **(F** and **G**, **J)**. Regenerative cell (E), nuclei (n), autophagosome (au), midgut lumen (l), microvilli (mv), endoplasmic reticulum cisternae (ER), Golgi complexes (G), mitochondria (m), mitochondrial fission (arrows), lamellar structures (arrowheads), basal lamina (bl), vacuoles (v), multivesicular bodies (stars), reserve material (r), midgut lumen (l). TEM. **(A)** Scale bar = 1 μm. **(B)** Scale bar = 0.9 μm. **(C)** Scale bar = 0.7 μm. **(D)** Scale bar = 0.55 μm. **(E)** Scale bar = 1.6 μm. **(F)** Scale bar = 0.9 μm. **(G)** Scale bar = 0.6 μm. **(H)** Scale bar = 1.85 μm. **(I)** Bar = 1 μm. **(J)** Scale bar = 1.3 μm.

materials (lipid droplets) were detected in the cytoplasm of some of the digestive cells (Fig. 2E). No changes in the structure of RER, SER, and Golgi complexes occurred in the digestive cells. In experimental group **3D**, the cytoplasm of all the D-cells had numerous mitochondria with signs of transformation/degeneration. Their electron-lucent mitochondrial matrix was observed together with a reduced number of mitochondrial cristae. Lamellar structures were visible inside transforming mitochondria and mitochondrial fission occurred (Fig. 2G). Numerous swollen cisterns of RER, SER, and Golgi complexes were observed in the D-cells of animals from experimental group **3D** (Fig. 2F).

Autophagosomes and autolysosomes were detected in the cytoplasm of D-cells in animals from experimental group **1D**. Autophagy was intensified in animals from experimental group **2D** (Fig. 2D), while it was sporadic in experimental group **3D**. In group **2D**, intensive mitophagy occurred. With the increase in exposure to dimethoate, the level and frequency of necrotic changes in the digestive epithelial cells increased. Necrosis was sporadically observed in the digestive cells of the intestine in animals that originated from experimental group **1D**. Within the epithelial cells of group **2D**, necrosis was observed frequently. Necrotic digestive cells were highly vacuolated (Fig. 2H and I). Their electron-lucent cytoplasm contained a reduced number of organelles (Fig. 2H). Cell membranes of neighboring necrotic digestive cells lost their continuity, and granules with high electron density occurred in the cortical layer. Within group **3D**, necrotic changes affected numerous digestive cells (Fig. 2J). Strong vacuolization of the cytoplasm, many vesicles, and a vacuole with an electron-lucent interior were observed. The histochemical methods confirmed the lack of storage material in the intestinal cells of animals exposed to dimethoate for 1 (Fig. 9A) and 3 (Fig. 9I) weeks, while they revealed the appearance of lipids after two weeks of the experiment (Fig. 9E).

3.1.2. *Restoration of clean water after exposure to dimethoate*

The cytoplasm of D-cells in animals from **1D1W** showed no changes compared to animals from experimental group **1D**, so individual degenerated mitochondria were detected. At the same time, the majority of these organelles revealed no ultrastructural alterations (Fig. 3A). The

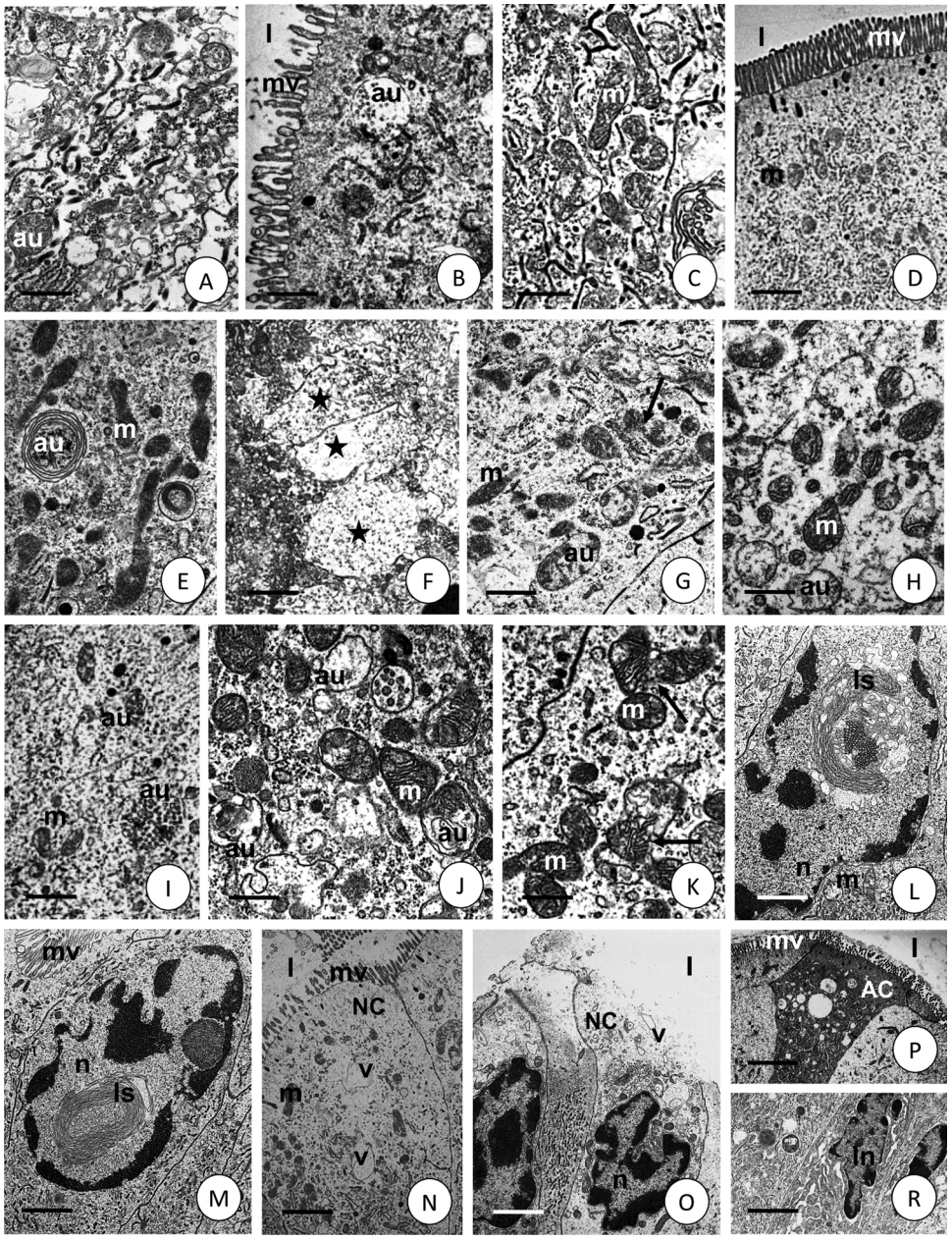

Fig. 3. Ultrastructural changes in the intestine epithelium of animals treated with dimethoate for 1 week and transferred to clean water for 1 week (experimental group 1D1W) **(A, F)**, 2 weeks (experimental group 1D2W) **(B** and **C)** and 3 weeks (experimental group 1D3W) **(D** and **E)**, treated with dimethoate for 2 weeks and transferred to clean water for 1 week (experimental group 2D1W) **(G, N, P–R)**, 2 weeks (experimental group 2D2W) **(H** and **I)** and 3 weeks (experimental group 2D3W) **(J–M, O)**. Nucleus (n), autophagosome (au), midgut lumen (l), microvilli (mv), mitochondria (m), lamellar structures (ls), necrotic cell (NC), apoptotic cell (AC), lobular nucleus (ln), vacuolated cell (stars), mitochondrial fission (arrows). TEM. **(A)** Scale bar = 0.6 μm. **(B)** Scale bar = 0.7 μm. **(C)** Scale bar = 0.65 μm. **(D)** Scale bar = 1.1 μm. **(E)** Scale bar = 0.7 μm. **(F)** Scale bar = 1.3 μm. **(G)** Scale bar = 0.8 μm. **(H)** Scale bar = 0.4 μm. **(I)** Scale bar = 0.75 μm. **(J)** Scale bar = 0.4 μm. **(K)** Scale bar = 0.5 μm. **(L)** Scale bar = 1.3 μm. **(M)** Scale bar = 1.5 μm. **(N)** Scale bar = 2 μm. **(O)** Scale bar = 1.6 μm. **(P)** Scale bar = 1.85 μm. **(R)** Scale bar = 2.4 μm.

digestive cells in experimental group **1D2W** contained sporadic autophagosomes (Fig. 3B) and degenerated mitochondria. However, most mitochondria presented the fine structure characteristic for these organelles (Fig. 3C) (compared to group **1D1W**). The ultrastructure of digestive cells in **1D3W** closely resembled that of the control group. Correctly developed microvilli, electron-dense granules in the cortical layer (Fig. 3D), electron-dense mitochondria, and single autophagosomes could be seen (Fig. 3E). Necrosis in all animals restored to the clean water was sporadically observed in the D-cells, as was described for animals from experimental group **1D** (Fig. 3F).

The D-cells of animals from groups **2D1W** and **2D2W** showed an ultrastructure similar to experimental group **2D**, with numerous degenerated mitochondria and autophagosomes that mainly accumulated near the degenerated mitochondria (Fig. 3G–I). Mitochondrial fission was detected (Fig. 3G). While the ultrastructure of D-cells that composed the intestine epithelium in animals from experimental group **2D3W** did not exhibit any changes in comparison to **2D, 2D1W**, and **2D2W**, we detected intensive autophagy. Autophagosomes occurred close to the damaged mitochondria (Fig. 3J). The mitochondria showed fission (Fig. 3K). However, the most important observation was the presence of numerous lamellar/membranous structures within the nuclei of D-cells (Fig. 3L–M). Necrosis was frequently observed in D-cells of animals originating from experimental groups **2D1W**, **2D2W**, and **2D3W** (Fig. 3N–O). Additionally, several D-cells showed signs of apoptosis: electron-dense cytoplasm, swollen endoplasmic reticulum cisternae, and lobular nuclei with patches of electron-dense chromatin occurred (Fig. 3P–R).

In experimental groups **3D1W**, **3D2W**, and **3D3W**, no changes compared to experimental group **3D** were observed. Degenerated mitochondria and autophagosomes were abundant within the cytoplasm of D-cells (Fig. 4A and B). Processes such as mitochondrial fission (Fig. 4C–E) and mitophagy were observed (Fig. 4A). Despite being returned to clean water, necrosis (Fig. 4F) and apoptosis (Fig. 4G and H) still occurred in digestive cells after three weeks of dimethoate

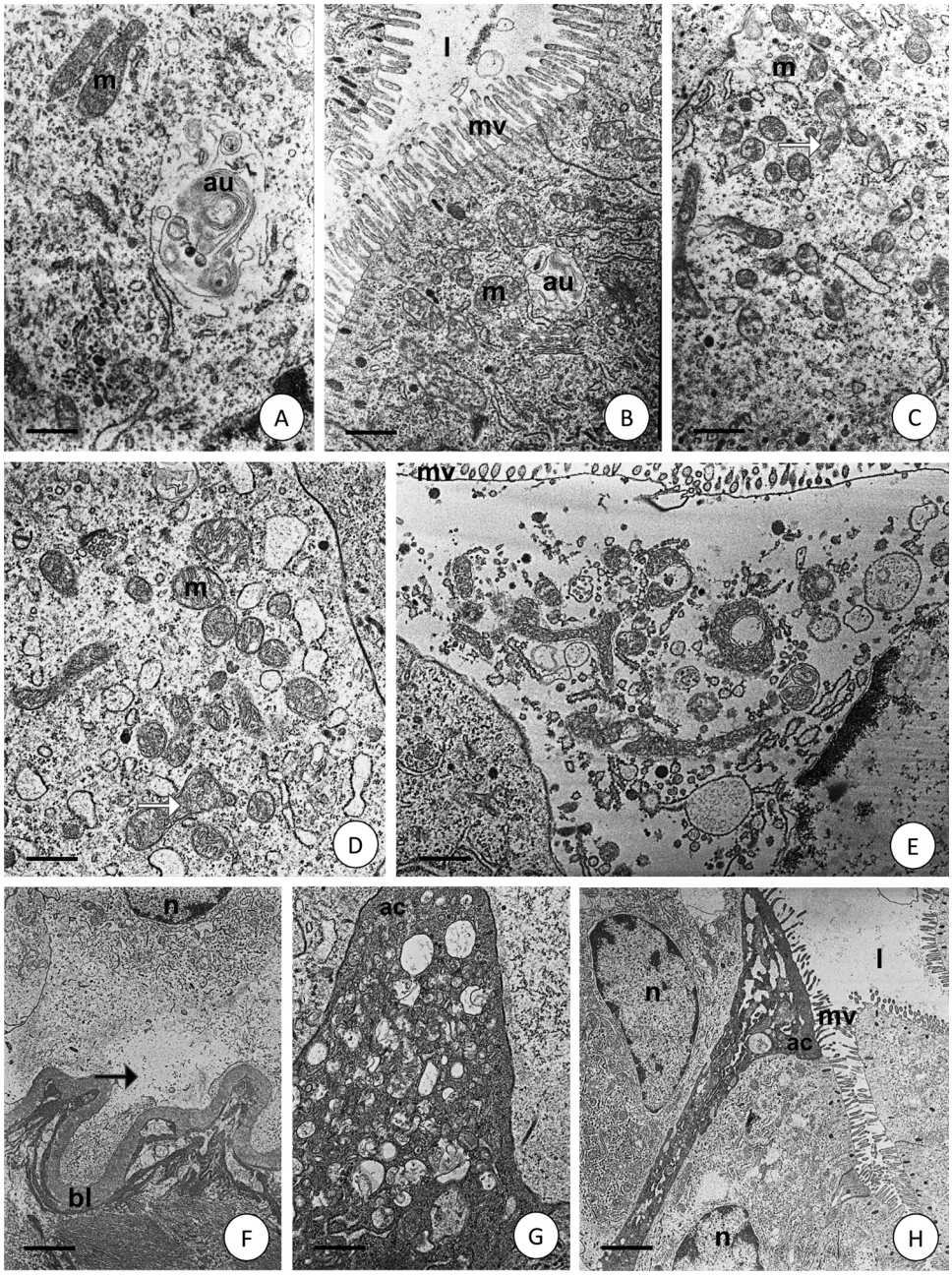

Fig. 4. Ultrastructural changes in the intestine epithelium of animals treated with dimethoate for 1 week and transferred to clean water for 1 week **(A** and **B)**, 2 weeks (**C** and **D**, **F** and **G)** and 3 weeks **(E**, **H)**. Autophagosome (au), mitochondria (m), midgut lumen (l), microvilli (mv), mitochondrial fission (white arrow), vacuolated cell (black arrow), basal lamina (bl), apoptotic cell (AC). TEM. **(A)** Scale bar = 1 μm. **(B)** Scale bar = 0.7 μm. **(C)** Scale bar = 0.6 μm. **(D)** Scale bar = 0.55 μm. **(E)** Scale bar = 0.7 μm. **(F)** Scale bar = 2.35 μm. **(G)** Scale bar = 0.5 μm. **(H)** Scale bar = 2 μm.

treatment. The intestine analysis with the detection of transmission electron microscopy and histochemical methods showed lipids in the cytoplasm of D-cells in groups 1D1W (Fig. 9B), 1D2W (Fig. 9C), and 2D2W (Fig. 9G). Lipids were absent in the other experimental groups (Fig. 9D, F, H, J–L)

3.2. Hepatopancreas

The hepatopancreas is a lobular organ formed by numerous tubules. Each tubule is lined with simple epithelium composed of several types of cells: E-cells in the distal region, differentiating cells in the median region, and R-, F- and B-cells in the proximal region. The cytoplasm of R-cells is abundant in endoplasmic reticulum cisternae, Golgi complexes and medium-electron dense vesicles located mainly in the apical cytoplasm. B-cells have small amounts of endoplasmic reticulum cisternae in their cytoplasm, but it is rich in vacuoles of different size, mitochondria and spheres composed of reserve material. The cytoplasm of F-cells is devoid of storage material, but it possesses numerous well-developed endoplasmic reticulum cisternae and Golgi complexes. For the precise ultrastructure of these cells see Sonakowska et al. (2015) and Sonakowska-Czajka et al. (2020).

3.2.1. Exposure to dimethoate

Dimethoate affected cells that form the epithelium of the proximal region of each hepatopancreatic tubule: R-, F- and B-cells. No changes were observed in the E-cells or differentiating cells.

After one week of treating animals with dimethoate (experimental group **1D**), the cytoplasm of all cells forming the tubules' proximal

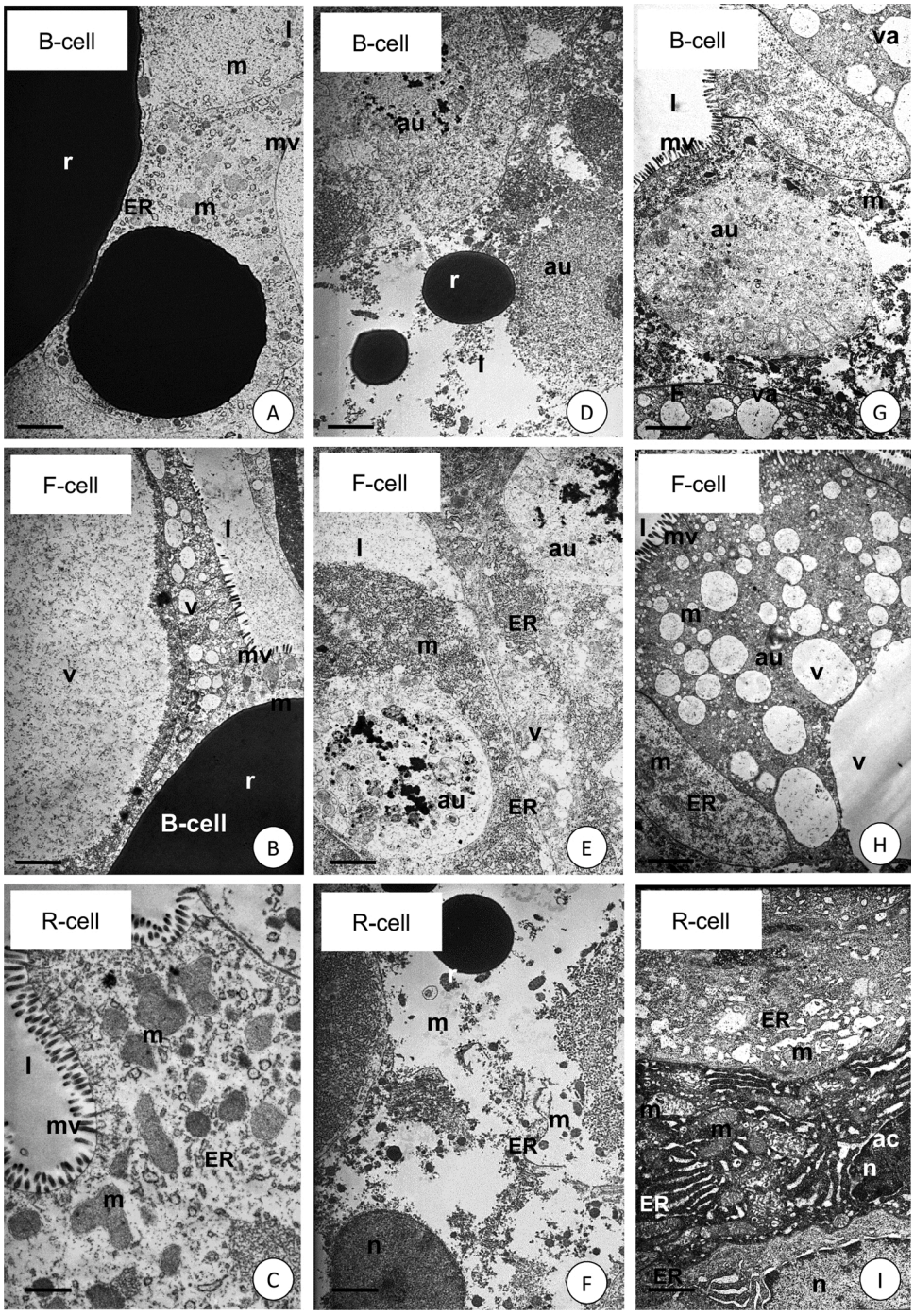

Fig. 5. Ultrastructural changes in hepatopancreas of animals treated with dimethoate for 1 week (experimental group 1D) **(A–C)**, 2 weeks (experimental group 2D) **(D–F)** and 3 weeks (experimental group 3D) **(G–I)**. Endoplasmic reticulum cisternae (ER), mitochondria (m), nuclei (n), reserve material (r), microvilli (mv), vacuoles (v), autophagosomes (au), apoptotic cell (ac), midgut lumen (l). TEM. **(A)** Scale bar = 2 μm. **(B)** Scale bar = 1.7 μm. **(C)** Scale bar = 0.8 μm. **(D)** Scale bar = 2.1 μm. **(E)** Scale bar = 14 μm. **(F)** Scale bar = 1.7 μm. **(G)** Scale bar = 1.4 μm. **(H)** Scale bar = 1.85 μm. **(I)** Scale bar = 1.3 μm.

region showed strong degenerative changes resembled necrotic ones. These changes also persisted after 2 (experimental group **2D**) and 3 (experimental group **3D**) weeks of the experiment. The vacuolated cytoplasm became electron-lucent, cisterns of RER, SER, Golgi complexes and mitochondria became distended in F-, B- and R cells (Fig. 5A–I). The mitochondria had an electron-lucent matrix, and the number of their cristae decreased (Fig. 5A–I). After two weeks of the experiment (experimental group **2D**), a reduction in reserve material in B cells was also observed (Fig. 5D). B-cells in group **3D** showed only some small spheres of storage material (Fig. 5G). Autophagy appeared in all types of cells after 1, 2, and 3 weeks of dimethoate exposure (Fig. 5D, E, G). In all specimens from experimental groups **1D, 2D,** and **3D**, apoptotic changes occurred (Fig. 5I). Histochemical studies revealed the accumulation of lipids in B cells in animals exposed to dimethoate (Figs. 10A, E, I).

3.2.2. *Restoration of clean water after exposure to dimethoate*

In animals placed in clear water after one week of dimethoate treatment, degenerative changes at the ultrastructural level were still observed in B-cells of the hepatopancreas, after 1, 2 and 3 weeks of life in clean water (experimental groups **1D1W, 1D2W,** and **1D3W**). Thus, the electron-lucent cytoplasm with vacuoles and the bloated organelles resembled necrotic changes (Fig. 6A, D, G). In the hepatopancreas of animals from experimental group **1D1W**, the cytoplasm of F- and R-cells presented features characteristic for the control animals with properly formed structures and organelles (Fig. 6B and C). However, in animals from experimental groups **1D2W** and **1D3W**, F-cells in hepatopancreatic tubules presented the ultrastructure with proper endoplasmic reticulum

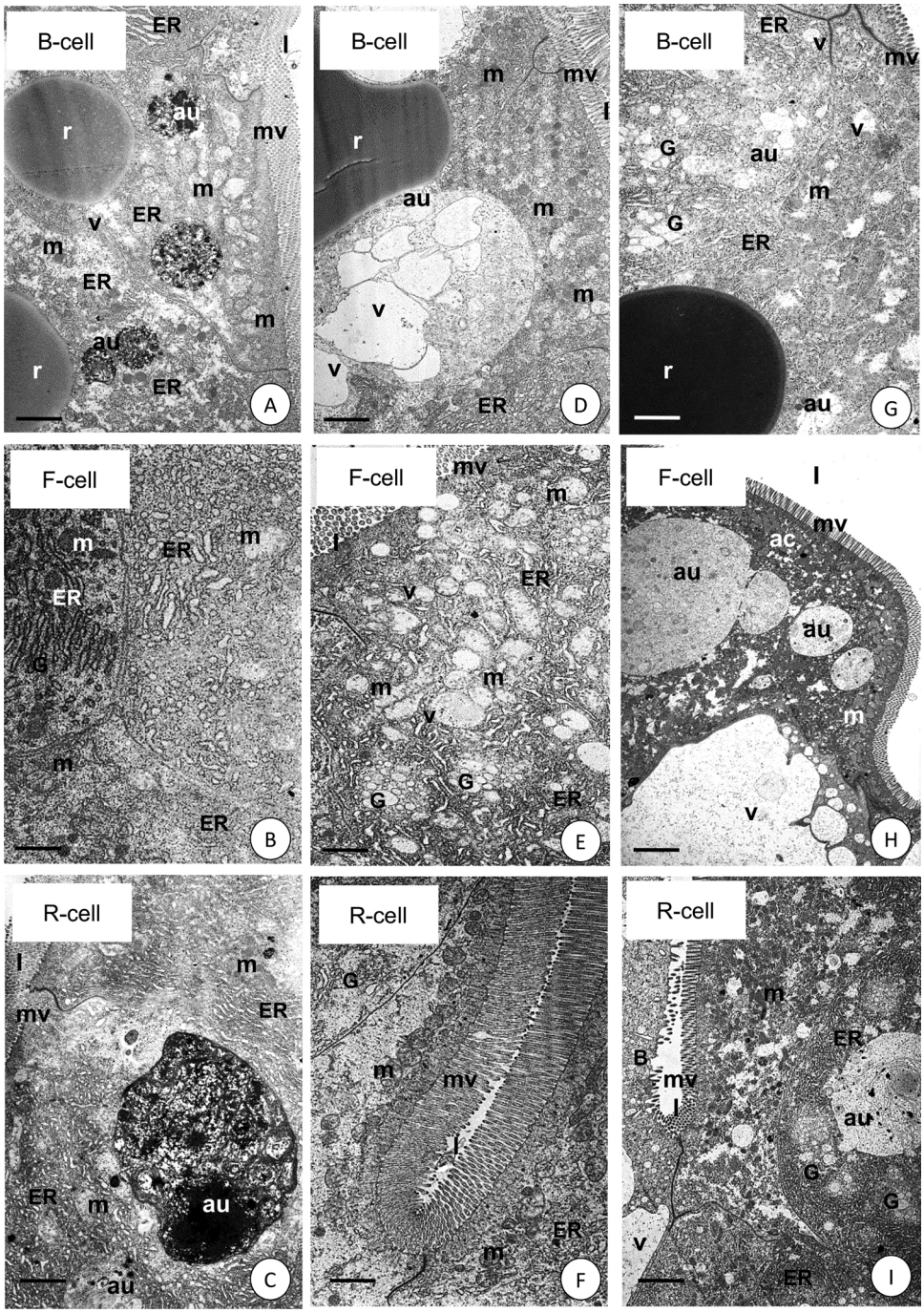

Fig. 6. Ultrastructural changes in hepatopancreas of animals kept in water with dimethoate for 1 week and transferred to clean water for 1 week (experimental group 1D1W) **(A–C)**, 2 weeks (experimental group 1D2W) **(D–F)** and 3 weeks (experimental group 1D3W) **(G–I)**. Endoplasmic reticulum cisternae (ER), Golgi complexes (G), mitochondria (m), reserve material (r), microvilli (mv), vacuoles (v), autophagosomes (au), midgut lumen (l). TEM. **(A)** Scale bar = 1.7 μm. **(B)** Scale bar = 1.04 μm. **(C)** Scale bar = 2.1 μm. **(D)** Scale bar = 1.7 μm. **(E)** Scale bar = 0.8 μm. **(F)** Scale bar = 1.2 μm. **(G)** Scale bar = 0.8 μm. **(H)** Scale bar = 2.4 μm. **(I)** Scale bar = 1.9 μm.

cisternae, mitochondria and vacuoles characteristic for this type of cell (Fig. 6E, H), but some necrotic changes were still detected in R-cells: degenerated mitochondria and bloated endoplasmic reticulum cisternae and Golgi complexes were visible (Fig. 6F, I). Intensive autophagy (Fig. 6A, C, G–I) occurred in the hepatopancreatic epithelial cells of all animals placed in clean water after 1 week of dimethoate treatment (experimental groups **1D1W, 1D2W, 1D3W**), while apoptosis (Fig. 6H) and necrosis (Fig. 6A, D, F, G, I) were reduced.

Placing the animals in clean water after two weeks of dimethoate treatment did not lead to the appearance of any changes. Thus, in group **2D1W**, the ultrastructure of B-, F-, and R-cells resembled that of animals treated with dimethoate for two weeks (Fig. 7A–C). Storage material began to accumulate in B-cells (Fig. 7A). Most of the cells were still apoptotic (Fig. 7B) or necrotic. Single autophagosomes were observed (Fig. 7A, C). Meanwhile, in group **2D2W**, autophagy was intensified, and the reserve material accumulated in B-cells (Fig. 7D). The remaining organelles, such as the endoplasmic reticulum cisternae and Golgi complexes, were still distended, and most of the mitochondria degenerated in B- and R-cells. The organelles in F-cells showed typical structure (Fig. 7D–F). Necrosis of R-cells was still observed (Fig. 7F). In group **2D3W**, B-cells accumulated reserve material in the cytoplasm (Fig. 7G). However, most organelles in the cytoplasm of these cells showed degenerative features (Fig. 7G). Similarly, degenerative changes were also observed in the cytoplasm of R-cells (Fig. 7I), while organelles such as endoplasmic reticulum cisternae, Golgi complexes, or mitochondria in F-cells did not show degenerative features (Fig. 7H). Intensive autophagy occurred in B- and R-cells (Fig. 7G, I), while necrosis was a sporadic process.

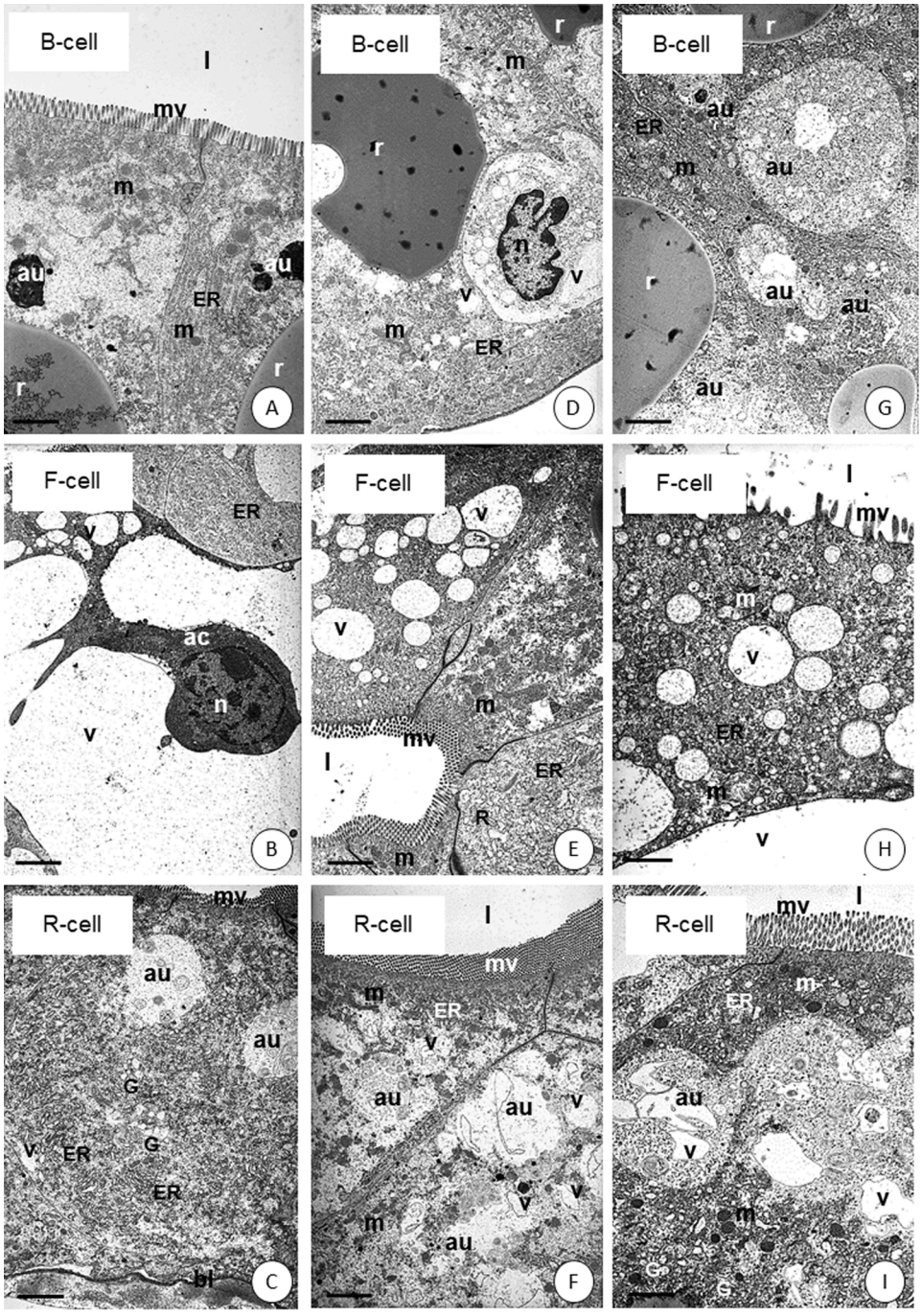

Fig. 7. Ultrastructural changes in hepatopancreas of animals kept in water with dimethoate for 2 weeks and transferred to clean water for 1 week (experimental group 2D1W) **(A–C)**, 2 weeks (experimental group 2D2W) **(D–F)** and 3 weeks (experimental group 2D3W) **(G–I)**. Endoplasmic reticulum cisternae (ER), Golgi complexes (G), mitochondria (m), nuclei (n), reserve material (r), microvilli (mv), vacuoles (v), autophagosomes (au), apoptotic cell (ap), midgut lumen (l). TEM. **(A)** Scale bar = 1.25 μm. **(B)** Scale bar = 2.85 μm. **(C)** Scale bar = 1.7 μm. **(D)** Scale bar = 2.1 μm. **(E)** Scale bar = 1.4 μm. **(F)** Scale bar = 2.2 μm. **(G)** Scale bar = 2.1 μm. **(H)** Scale bar = 0.85 μm. **(I)** Scale bar = 1.85 μm.

In animals treated with dimethoate for three weeks and clean water for one week (experimental group 3D1W), the cytoplasm of numerous B-cells presented degenerative alterations with many bloated mitochondria and endoplasmic reticulum cisternae. However, the only change in the cytoplasm of these cells was the accumulation of storage materials in the form of large spheres (Fig. 8A). On the other hand, the cytoplasm of F- and R-cells regenerated, so all organelles showed the structure characteristic of animals of the control group (Fig. 8B and C). In the case of animals which after three weeks of treatment with dimethoate were restored to clean water for 2 (**3D2W**) and 3 (**3D3W**) weeks, all cells (B-, F-, and R-cells) regenerated and showed no signs of necrosis (Fig. 8B–I). Thus, their cytoplasm possessed properly formed mitochondria, endoplasmic reticulum cisternae or Golgi complexes. It was also devoid of vacuoles characteristic for necrotic cells (the only vacuoles were those that are characteristic features of F-cells). Autophagy was a process frequently observed mostly in R-cells (Fig. 8C, F, I), and necrosis and apoptosis occurred sporadically. Histochemical studies showed that lipids were the only material collected in B-cells due to placing animals in clean water (Fig. 10B–D, F–H, J–L).

3.3. *Quantitative analysis*

The use of dimethoate for *N. davidi* intestine and hepatopancreas revealed a significantly increased percentage od necrotic and autophagic cells, compared with the control group (Figs. 11 and 12).

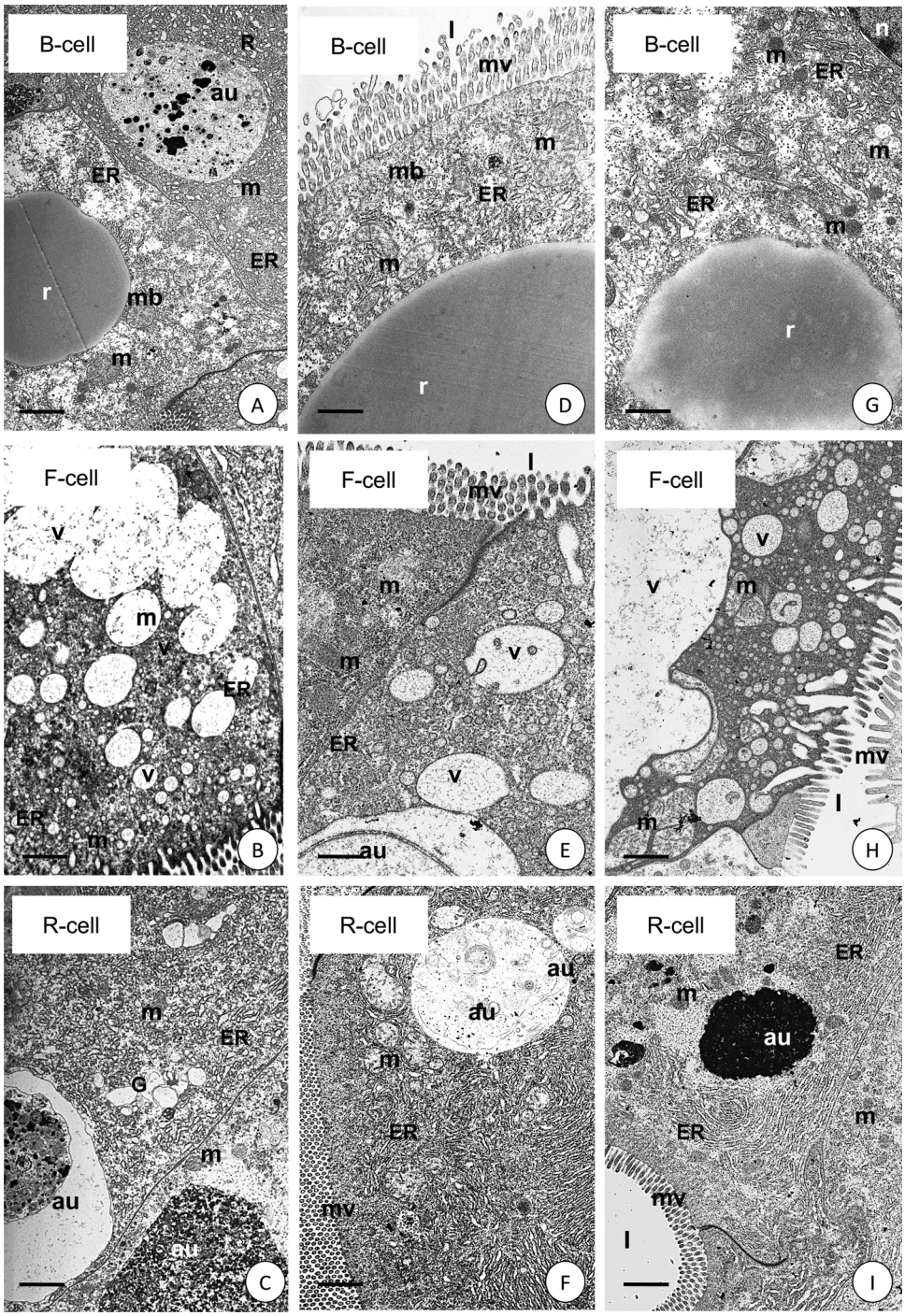

Fig. 8. Ultrastructural changes in hepatopancreas of animals kept in water with dimethoate for 3 weeks and transferred to clean water for 1 week (experimental group 3D1W) **(A–C)**, 2 weeks (experimental group 3D2W) **(D–F)** and 3 weeks (experimental group 3D3W) **(G–I)**. Endoplasmic reticulum cisternae (ER), Golgi complexes (G), mitochondria (m), reserve material (r), microvilli (mv), vacuoles (v), autophagosomes (au), midgut lumen (l), multivesicular bodies (mb). TEM. **(A)** Scale bar = 1.4 μm. **(B)** Scale bar = 0.9 μm. **(C)** Scale bar = 1.1 μm. **(D)** Scale bar = 0.8 μm. **(E)** Scale bar = 0.5 μm. **(F)** Scale bar = 0.9 μm. **(G)** Scale bar = 1.4 μm. **(H)** Scale bar = 0.7 μm. **(I)** Scale bar = 1 μm.

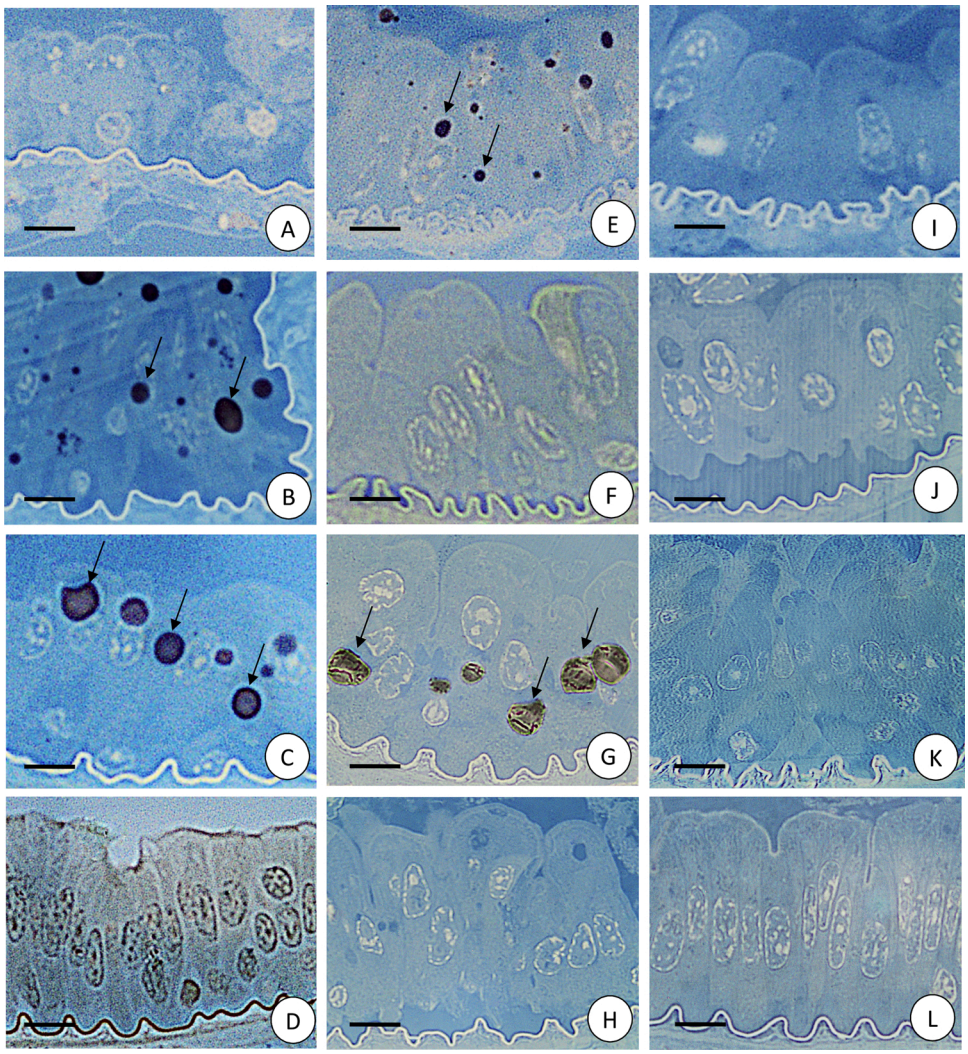

Fig. 9. Histochemical staining of the intestine epithelium of *N. davidi*. Light microscopy. Sudan Black B staining. Localization of lipids (arrows). **(A)** Experimental group 1D. Scale bar = 8 μm. **(B)** Experimental group 1D1W. Scale bar = 7 μm. **(C)** Experimental group 1D2W. Scale bar = 7 μm. **(D)** Experimental group 1D3W. Scale bar = 8 μm. **(E)** Experimental group 2D. Scale bar = 7 μm. **(F)** Experimental group 2D1W. Scale bar = 7 μm. **(G)** Experimental group 2D2W. Scale bar = 7 μm. **(H)** Experimental group 2D3W. Scale bar = 8 μm. **(I)** Experimental group 3D. Scale bar = 7 μm. **(J)** Experimental group 3D1W. Scale bar = 7 μm. **(K)** Experimental group 3D2W. Scale bar = 7 μm. **(L)** Experimental group 3D3W. Scale bar = 8 μm.

3.3.1. Quantitative analysis of necrosis in analyzed organs

The quantitative analysis showed more necrotic cells in the intestine than the hepatopancreas independently of the length of exposure to this chemical. After the different periods of exposure the differences in mean percentage of necrotic cells in the intestine were 10.4 %, 21.8 %, 45.6 % respectively for groups D1, D2 and D3, while in the hepatopancreas the percentages were smaller by nearly half in group D1 and 39 % and 33 % respectively in groups D2 and D3 (Fig. 11A and B). The largest increase in the number of necrotic cells in both organs was after 21 days exposure to dimethoate (group D3). After the return to clean water, percentages of necrotic cells in analyzed organs of *N. davidi* did not change with the duration of exposure (W1, W2, W3) (Fig. 11A and B).

3.3.2. Quantitative analysis of autophagic cells of analyzed organs

The quantitative analysis showed that the number of autophagic cells was similar in the intestine and hepatopancreas after the selected period of exposure to dimethoate. Additionally, independently of organ, the pattern of changes in percentage of autophagic cells was similar for successive lengths of time that shrimps remained in clean water, in relation to the earlier condition of exposure to dimethoate (Fig. 12A and B). After one week of exposure to dimethoate (group D1), both in intestine and hepatopancreas, the percentage of autophagic cells was low and similar to the control. The level of this parameter also did not change when shrimps were kept in uncontaminated water.

The highest number of autophagic cells was observed after shrimps remained for three weeks in clean water (W3), after having been exposed to dimethoate for 2 weeks (2D). In this group the average percentage of cells with autophagy symptoms was 55.2 % in the hepatopancreas, while it was 64 % in the intestine (Fig. 12A and B). After three weeks (3D) exposure to dimethoate the number of autophagic cells decreased in both organs, but this change was significant compared to the control only in the hepatopancreas (Fig. 12B). Staying in clean water caused a gradual and progressive decrease in the number of autophagic cells in the intestine (to 31.4 % in W3) (Fig. 12A), while in the hepatopancreas their number decreased after one week of staying in uncontaminated water (to 26.8 %), then after two and three weeks in these conditions increased to the control level (38.8 %) (Fig. 12B).

4. Discussion

The freshwater environment is perfect for dissolving various toxic substances, originating either from natural processes or from human activities. The latter include numerous pesticides used to protect crop plants against pests or pathogens. Such pesticides include dimethoate (Ciglasch et al., 2005; El-Saeid and Selim, 2016; El-Saeid et al., 2016; Pan et al., 2021). Changes in the body under the influence of various xenobiotics damage cells, tissues, and organs. One of the organs exposed to any stressors from the external environment is the midgut. This middle region of the digestive system in Crustacea comprises a tubular midgut, lobular hepatopancreas or long intestine with lobes of hepatopancreas (Herrera-Alvarez et al., 2000; Sousa and Petriella, 2001, 2006; Symonova, 2007; Rost-Roszkowska et al., 2012; von Vaupel Klein et al.,

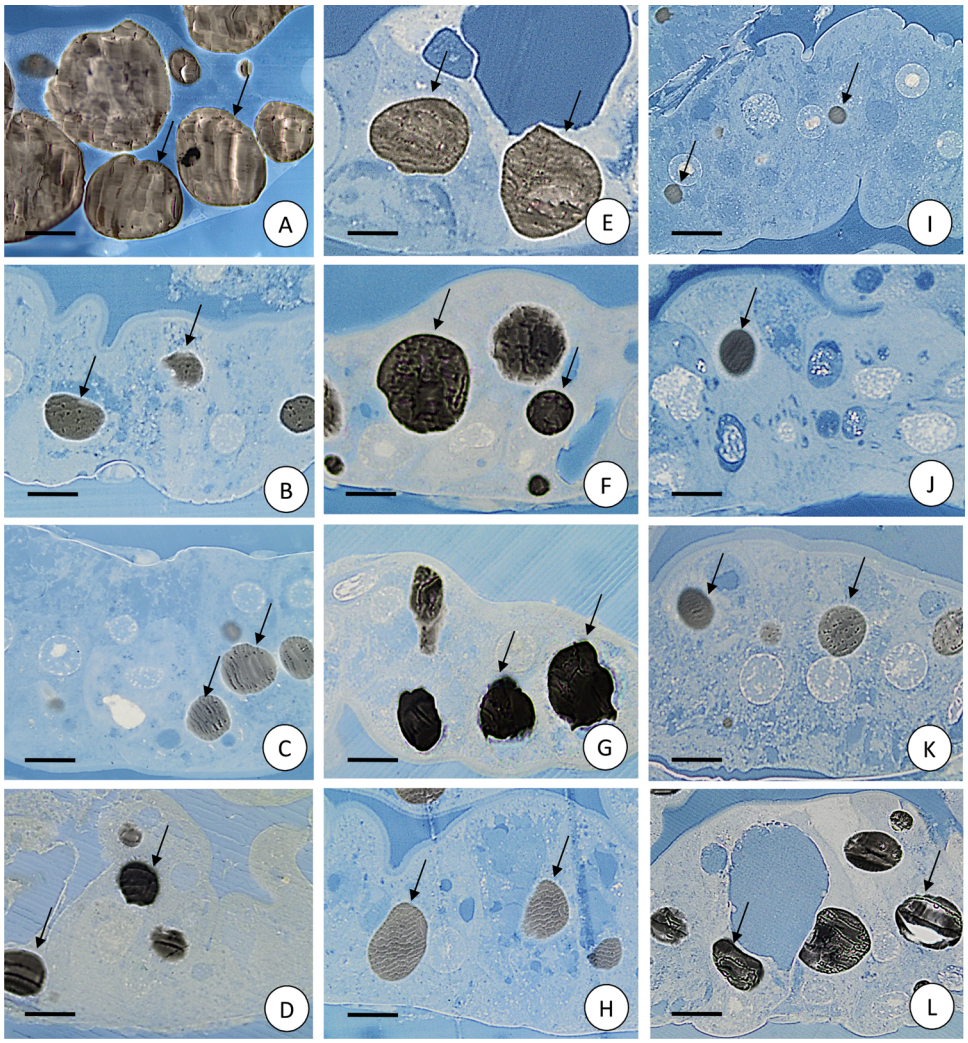

Fig. 10. Histochemical staining of the hepatopancreatic epithelium of *N. davidi*. Light microscopy. Sudan Black B staining. Localization of lipids (arrows). **(A)** Experimental group 1D. Scale bar = 12 μm. **(B)** Experimental group 1D1W. Scale bar = 12 μm. **(C)** Experimental group 1D2W. Scale bar = 12 μm. **(D)** Experimental group 1D3W. Scale bar = 15 μm. **(E)** Experimental group 2D. Scale bar = 12 μm. **(F)** Experimental group 2D1W. Scale bar = 12 μm. **(G)** Experimental group 2D2W. Scale bar = 12 μm. **(H)** Experimental group 2D3W. Scale bar = 15 μm. **(I)** Experimental group 3D. Scale bar = 12 μm. **(J)** Experimental group 3D1W. Scale bar = 12 μm. **(K)** Experimental group 3D2W. Scale bar = 12 μm. **(L)** Experimental group 3D3W. Scale bar = 15 μm.

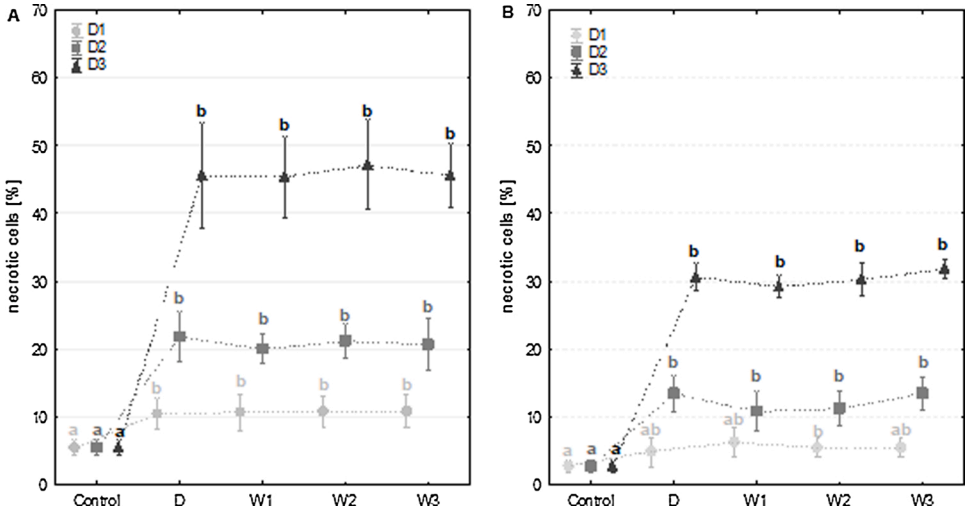

Fig. 11. Percentage of necrotic cells (mean ± SD) in intestine **(A)** and hepatopancreas **(B)** of *N. davidi* after exposure to dimethoate for different periods of time (D1 – one week; D2 – two weeks; D3 – three weeks) and after different lengths of time spent in uncontaminated water (W1 – one week; W2 – two weeks; W3 – three weeks). Different letters (a, b) indicate significant differences among groups at the same point of dimethoate exposure (Tukey test, $p < 0.05$).

2015; Sonakowska et al., 2015). The *N. davidi* midgut is composed of the tubular intestine and lobular hepatopancreas (Sonakowska et al., 2015; Sonakowska-Czajka et al., 2020) and is sensitive to stressors such as fasting (Włodarczyk et al., 2017, 2019a, 2019b). Because this species is a perfect model for environmental research, we decided to check what changes in its midgut epithelium occur under the influence of a popular

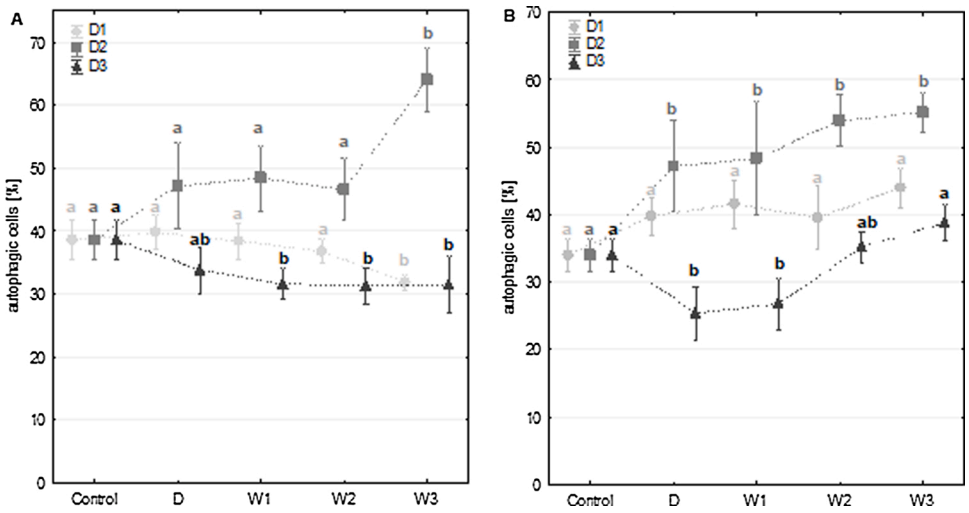

Fig. 12. Percentage of autophagic cells (mean ± SD) in intestine **(A)** and hepatopancreas **(B)** of *N. davidi* after exposure to dimethoate for different periods of time (D1 – one week; D2 – two weeks; D3 – three weeks) and after different lengths of time spent in uncontaminated water (W1 – one week; W2 – two weeks; W3 – three weeks). Different letters (a, b) indicate significant differences among groups at the same point of dimethoate exposure (Tukey test, $p < 0.05$).

insecticide. In our study, apart from describing the changes that occur due to the animal's life in a freshwater environment with a pesticide, it was essential to check whether any regenerative changes might appear after placing the animals in clean water. If they did occur, it was important to check whether they were visible at the ultrastructural level.

In individuals exposed to dimethoate, it has been observed that with increasing duration of exposure to the pesticide, changes in the ultrastructure of cells forming the intestinal epithelium and hepatopancreas gradually appear. The degenerative changes that appeared in the cells were necrotic changes, when the cytoplasm became electron-lucent and proceeded vacuolization, and the number of bloated organelles gradually decreases (Wilczek et al., 2014; Rost-Roszkowska et al., 2020b). First, mitochondria degenerate, while other organelles, such as endoplasmic reticulum cisternae, Golgi complexes, or nuclei, show no changes. In the intestine, these organelles began to degenerate only in individuals exposed to dimethoate for three weeks, which turns out to be the period when most individuals die. Meanwhile, in the hepatopancreas, these changes were visible after the first week of exposure to the pesticide and persisted regardless of the duration of exposure to dimethoate. It suggests that the hepatopancreas is more sensitive to the effects of a toxic substance than the intestine. One week of pesticide treatment of animals is too short for the xenobiotic to induce irreversible changes in epithelial cells, as indicated by regeneration of D-cells in the intestine and most of the hepatopancreatic epithelial cells after placing the animals in clean water. Meanwhile, putting the animals in clean water after two weeks of exposure to the pesticide indicates that degenerative changes in the intestine are still present. In addition, the hepatopancreas gradually regenerates F-cells, and the B-cell cytoplasm begins to accumulate storage material. A similar observation was made in individuals treated with the pesticide for three weeks until half of the individuals died. The intestinal epithelium does not tend to regenerate even after the animals stay in clean water for three weeks. On the other hand, the epithelium with all cell types (F-, B, R-cells) was regenerated, including the accumulation of storage material in B-cells. These changes were accompanied by intense autophagy, not observed to such a degree in control subjects (Sonakowska et al., 2016), or after treating animals with dimethoate. As it turns out, the hepatopancreas is an organ more sensitive to the effects of stressors (in this case, a pesticide), but it has faster processes aimed at balancing homeostasis and returning to the self-renewal of cells.

The results of our previous studies revealed that long-term fasting of *N. davidi* activates the increase of non-active mitochondria in both endodermal regions of the digestive system. Mitochondria underwent drastic changes of their ultrastructure: swollen mitochondria with a decreased number of short cristae were observed (Włodarczyk et al., 2017, 2019a, 2019b). Our research confirms that mitochondria are the first organelles exposed in a freshwater organism to the stressor (Cano et al., 2014; Faron et al., 2015), which in this case is dimethoate. Changes in mitochondrial ultrastructure could be associated with the activation of cell death in different tissues and organs (Orrenius, 2004; Manella, 2008; Martin and Mentel, 2010; Sonakowska et al., 2016; Karpeta-Kaczmarek et al., 2016; Włodarczyk et al., 2017), but autophagy as a pro-survival process enabling organisms to maintain homeostasis in organs could be activated (Malagoli et al., 2010; Romanelli et al., 2016; Tettamanti et al., 2019; Rost-Roszkowska et al., 2020a, 2020b). Mitochondria were swollen, the number of mitochondrial cristae decreased, and their membranes were damaged after the first week of exposure to dimethoate. The fission of these organelles and the accumulation of visible lamellar structures inside were also detected. Mitochondria are responsible for proper physiology, cell death, regeneration, and aging, but they are also treated as one of the first responders to different stressors, including xenobiotics (Sanni et al., 2008; Cannino et al., 2009; Hödl et al., 2010; Westermann, 2010; Wilczek et al., 2014, 2018; Stalmach et al., 2015; Bednarska and Świątek, 2016; Włodarczyk et al., 2017; Rost-Roszkowska et al., 2020a). Their functioning is also connected with the production of reactive oxygen species (Wilczek et al., 2014, 2018; Włodarczyk et al., 2019a). Changes in their membrane potential are treated as good markers of all changes leading to cell death (Wilczek et al., 2014). However, their number, morphology, and intracellular distribution are controlled by their fusion and fission (Westermann, 2010). Mitochondria originate from the proliferation and growth of pre-existing mitochondria, while their excess could be utilized by autophagy (Neupert and Herrmann, 2007; Westermann, 2010). When many mitochondria are damaged, they are degraded in autophagosomes (mitophagy), but for the energy budget to be balanced in the cell, new mitochondria must be generated. Therefore, intensive mitochondrial fission should be observed (Detmer and Chan, 2007; Westermann, 2010). In the case of *N. davidi* exposed to the insecticide, intensive autophagy (including mitophagy) and mitochondrial fission occurred in the intestine in all experimental groups. Mitochondrial fission in hepatopancreatic epithelial cells was not observed. In addition, under the influence of dimethoate in *N. davidi*, autophagy is activated in both intestinal and hepatopancreatic epithelial cells (non-selective autophagy), including mitophagy, which is a type of selective autophagy (Kim et al., 2007; Klionsky et al., 2016, 2021; Wang et al., 2019). In this case, degenerated/damaged mitochondria are degraded to block the

activation of cell death and enable proper cellular functioning and differentiation (Kim et al., 2007; Youle and Narendra, 2001; Wang et al., 2019). The excess of unhealthy mitochondria causes ROS production and activates numerous proapoptotic proteins, leading to cell death (Zorov et al., 2014; Wang et al., 2019). Similar cross-talk between autophagy and apoptosis/necrosis has been described in the midgut epithelia of some invertebrates (Hödl et al., 2010; Franzetti et al., 2012; Amachree et al., 2013; Park et al., 2013; Sheir et al., 2013; Włostowski et al., 2014; Romanelli et al., 2016; Rost-Roszkowska et al., 2018, 2019, 2020a). If the freshwater shrimp is exposed to the pesticide for too long, autophagy in intestinal epithelial cells will be inhibited. Meanwhile, in the hepatopancreas, autophagy occurs intensely regardless of the duration of exposure to the pesticide. It is accompanied by necrosis and apoptosis. It suggests that in the epithelial cells, triggered autophagy is a mechanism that compensates for organ homeostasis. The intensification of this process, together with the prolonged stay in pesticide-free water, confirms this fact. Thus, we suspect that different mechanisms are involved in cell survival in the intestine and hepatopancreas. Autophagy (including mitophagy) associated with mitochondrial fission is involved in the intestine's survival processes. However, when the stressor lasts too long, autophagy is blocked in this organ. Meanwhile, in the hepatopancreas, intensive autophagy is not associated with mitochondrial fission, and it continues regardless of the duration of the stressor.

A clear correlation between the autophagy and the accumulation of reserve materials has been described in different arthropods' organs (Rost-Roszkowska et al., 2018; Tettamanti et al., 2019) as well as in *N. davidi*. It should also be noted that in individuals exposed to dimethoate, lipids accumulated in the cytoplasm of D-cells of the intestine. Reserve material is not accumulated in control individuals (Sonakowska et al., 2015). Meanwhile, in B-cells of pesticide-treated individuals, a gradual reduction of the reserve material accumulated in their cytoplasm was observed. The storage of reserve materials may be related to the fact that in organisms living in a polluted environment or exposed to any stressors, the animal's energy expenditure increases, related to the detoxification and excretion of harmful substances (Sibly and Calow, 1989). It can lead to the accumulation of storage materials in organs (Bednarska et al., 2013), which can be used in the maintenance of energy homeostasis in cells (Stone et al., 2001; De Coen and Janssen, 2003a; Moolman et al., 2007; Lipovšek et al., 2011, 2014, 2018). Some analyses connected with energy budgets (accumulation of lipids, proteins, and polysaccharides) have been conducted on several aquatic (De Coen and Janssen, 1997, 2003a, 2003b; Verslycke et al., 2004a; Erk et al., 2008; Smolders et al., 2003) and terrestrial (Donker, 1992; Bednarska et al., 2013) specimens. Thus, the accumulation of reserve materials in the D-cells of the intestine, where they are not stored, with the increasing number of degenerated mitochondria and their fission in *N. davidi* suggests coping with stress due to energy expenditure and balancing the energy budget (Calow, 1991; Bednarska et al., 2013). Changes occurring in cells and tissues under the influence of toxic substances are more easily regenerated in the case of increased energy expenditure (Rowe et al., 1998; Calow, 1991; Maryański et al., 2002; Pook et al., 2009; Bednarska et al., 2013; Bednarska and Stachowicz, 2013). The consumption of reserve material in the B-cells of the hepatopancreas and the D-cells of the intestine in *N. davidi* individuals exposed to the pesticide confirms this fact. The accumulation of storage material in cells not intended for this purpose suggests greatly increased energy consumption by the organism. Different and individual energy sources and energy distribution have been described in animals (De Coen and Janssen, 1997; Knigge and Köhler, 2000; Arrese and Soulages, 2010; Bednarska et al., 2013). In *N. davidi*, the material accumulated in B- and D-cells is lipids, which suggests increased lipogenesis and energy efficiency (Arrese and Soulages, 2010; Bednarska et al., 2013). We can conclude that under the influence of a stressor and intense energy consumption (e.g., autophagy, mitochondrial fission), reserve materials can be accumulated in cells that are not typical storage cells. The analysis of changes caused by the presence of a toxic substance in the environment clearly shows that the processes related to the functioning of the mitochondria, the accumulation of reserve materials constituting an energy reservoir, and cell death were activated. It is also essential to determine how the mitochondrial membrane potentials changed and whether any enzymes were activated to maintain homeostasis. Mitochondrial dismutases described in crustaceans (cytMnSOD and mtMnSOD) are useful markers of mitochondrial activity (Brouwer et al., 2003; Lin et al., 2010; Gómez-Anduro et al., 2012).

5. Conclusions

Based on the results of our research related to the effect of a common insecticide on the digestive system of a freshwater organism, we can conclude that: (1) the various organs that make up the midgut show different sensitivity to the stressor; (2) other processes are triggered in the intestine and hepatopancreas to regenerate cells after an insecticide damages them; (3) mitochondria are the first organelles that respond to the appearance of a stressor in the living environment; (4) the accumulation of spare materials (lipids) is associated with high energy utility related to pro-survival processes (e.g., autophagy, mitophagy, mitochondrial fission), which means that they can accumulate even in organs not intended for this.

Ethical approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. This article does not concern any studies with human participants that were performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no conflict of interests.

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