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Autophagy and apoptosis in starved and re-fed *Neocaridina davidi*
(Crustacea, Malacostraca) midgut

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Abstract

Adult specimens of the freshwater shrimp *Neocaridina davidi* Bouvier, 1904 (Crustacea) were starved for 7, 14 and 21 days. Specimens from first and second experimental group were collected for the studies. The majority of animals starved for 21 days died. Additionally, some specimens from each group were re-fed for 4, 7, and 14 days. The epithelium of the midgut, which is composed of the intestine and hepatopancreas, was analyzed. While the epithelium of the intestine is formed by D- and R-cells, the epithelium of hepatopancreas has R-, B- and F-cells. Autophagy and apoptosis in the midgut epithelium were analyzed using TEM and immunohistochemical methods. These processes were only observed in the D-cells of the intestine and the F- and B-cells of the hepatopancreas. Starvation led to a reduction in the amount of reserve material in the B-cells. Although this process activated autophagy in both regions of the midgut, the intestine and hepatopancreas, after re-feeding the level of autophagy decreased. Starvation caused an increase in the apoptotic cells in both organs, while the re-feeding caused a decrease in the number of apoptotic cells in both organs analysed. Re-feeding after periods of starvation caused an accumulation of reserve material in the hepatopancreas.

Key words: ultrastructure, digestive system, apoptosis, autophagy, crustacean, starvation, *Neocaridina davidi*

Introduction

The epithelial cells of the digestive system perform an important function in defending the body against the effects of various types of stressors that originate from the external environment such as toxic substances, metals derived from food, pathogens or even a lack of food (Munafo and Colombo 2001; Suzuki et al. 2011; Lipovšek et al. 2014, 2018; Romanelli et al. 2014; Wilczek et al. 2014). Therefore, when under the influence of many stressors, various mechanisms are activated that are designed to maintain homeostasis in the cells, tissues and the entire body. One such mechanism is the activation of cell death, thanks to which the dying cells will not cause an inflammation or will not lead to the degeneration of the entire organ (Rathmell and Thompson 2011). Three main types of cell death have been distinguished in the digestive system of invertebrates: apoptosis, necrosis and autophagy (Klionsky and Emr 2000; Orrenius 2004; Kourtis and Tavernarakis 2009; Rost-Roszkowska et al. 2010; Franzetti et al. 2012). The relationships between them can be established due to experimental data. Therefore, we decided to use the experimental procedure that was presented in our previous paper (Włodarczyk et al. 2017) in order to determine how starvation and re-feeding after different periods of starvation affect apoptosis and autophagy.

A lack of food leads to a deficiency of energy, which in turn initiates the catabolic process of autophagy (self-digestion). Autophagy is a type of cell death because it is directed towards energy recovery and is a fundamental part of the survival strategy of organisms in unfavorable environmental conditions (Klionsky and Emr 2000; Kourtis and Tavernarakis 2009). Although the strategy for the survival of organisms in the absence of food is not fully understood, it can be assumed that an increase in apoptosis during starvation is characteristic

for invertebrates. Unlike necrosis, apoptosis makes it possible to remove unnecessary, damaged or even infected cells without initiating local inflammation (Savill et al. 2000; Orrenius 2004; Maghsoudi et al. 2012). Experiments with starvation or analyses of changes that occur during natural periods of starvation (hibernation, hibernation) are widely used in order to present such relationships (Lipovšek et al. 2014, 2018; Lipovšek and Novak 2015). However, studies that involve re-feeding after different periods of starvation can present a wider field of view on a given topic.

As the object of our studies connected with changes which appear in tissues/organs after starvation and re-feeding we chose the freshwater shrimp *Neocaridina davidi* Bouvier, 1904 (formerly known as *Neocaridina heteropoda*). It originates from Taiwan and is famous all over the world thanks to its easy breeding. The laboratory cultures of this species show great economic potential of this species (Tropea et al. 2015). The middle region of its digestive system, which is called as the midgut, is formed by two organs: the tube-shaped intestine and a lobular organ – the hepatopancreas, which are lined with a simple epithelium that rests on the basal lamina. The epithelium of the intestine has two types of cells: D-cells (digestive cells, principal cells) and R-cells (regenerative cells), which are located in the anterior region of the intestine. The hepatopancreas is formed by two large diverticles, which are secondarily divided into blind-end tubules. Three regions were distinguished in each tubule: distal, median and proximal. The R-cells form the distal region, while the F- (fibrillar cells) and B-cells (storage cells) form the functional epithelium (Sonakowska et al. 2015, 2016).

The survival of the first and third larval stage of *N. davidi* has been analyzed with reference to starvation as an important stressor (Pantaleo et al. 2015). Changes in the ultrastructure and the activation of mitochondria after different periods of starvation and re-feeding has also been presented. During these studies on *N. davidi* we calculated the point of

no-return (PNR₅₀) that was equal to 24.72 ± 0.08 (Włodarczyk et al. 2017). Therefore, using the experimental data from mentioned studies, we had the new aims connected with the cell death's activation/deactivation: (a) to determine whether starvation activates autophagy and/or apoptosis; (b) to determine whether re-feeding after starvation activates autophagy and/or apoptosis; (c) to describe apoptosis and autophagy at the ultrastructural level, and finally, (d) to compare the activation of autophagy/apoptosis and any changes in the mitochondrial activity after starvation and re-feeding.

Material and Methods

The research was conducted on adult males and females of the freshwater shrimp *N.* (formerly known as *N. heteropoda*) (Crustacea, Malacostraca, Decapoda). The specimens were obtained from local shrimp breeders and kept in a main laboratory breeder, i.e. 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 10⁰d. The *N. davidi* shrimp were fed with JBL Novo Prawn.

Experiment. The starvation experiment was performed by placing the shrimp in individual plastic (250 mL) containers. Adult shrimp with a cephalothorax length of more than 2.5 mm were selected for the experiment,. Each day 10% of water was replaced and the plastic containers were cleared of excrement and cuticle exoskeletons. The containers were kept in a shaded room in order to avoid the development of algae. The shrimp were starved for 7, 14 and 21 days. Specimens from each experimental group were collected for the studies. Additionally, some specimens from each experimental group were re-fed for 4, 7, 14 and 21 days. The number of specimens from each experimental group that were collected for the experiment and all of the techniques that were used are presented in Table 1. The results for

the control group, the non-starved *N. davidi*, were presented in our previous paper (Sonakowska et al. 2016).

Most of animals starved for 21 days were dying (PNR₅₀) – the process of re-feeding did not increase their survival, so we did not conduct the quantitative analysis for this experimental group.

Light and transmission electron microscopy. Adult specimens of *N. davidi* were decapitated and fixed with 2.5% glutaraldehyde in a 0.1 M sodium phosphate buffer (pH 7.4, 4 °C, 2 h), post-fixed in 2% osmium tetroxide in a 0.1 M phosphate buffer (4 °C, 1.5 h) and dehydrated in a graded series of concentrations of ethanol (50, 70, 90, 95 and 4 x 100% each for 15 min) and acetone (15 min). Afterwards, the material was embedded in epoxy resin (Epoxy Embedding Medium Kit; Sigma-Aldrich, St. Louis, MO, USA). Semi- (0.8 µm thick) and ultra-thin (70 nm) sections were cut on a Leica Ultracut UCT25 ultramicrotome. 100 sections were cut from each individual. The semi-thin sections were stained with 1% methylene blue in 0.5% borax and observed using an Olympus BX60 light microscope. After staining with uranyl acetate and lead citrate, the ultra-thin sections were examined using a Hitachi H500 transmission electron microscope. The ultrathin sections were used in order to count the number of midgut epithelial cells with autophagosomes in relation to the total number of cells. The percentage of cells with autophagosomes was determined randomly by counting the cells in the two organs of the midgut of five adult specimens. The significance of differences in the percentage of cells with autophagosomes was assessed using the Student's test, $p < 0.05$.

TUNEL assay – detecting DNA fragmentation during apoptosis. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is the method that is used to

investigate the DNA fragmentation that occurs during apoptosis. The prepared intestine and hepatopancreas were washed in TBS (3 x 5 min) and stained with a TUNEL reaction mixture (*In Situ* Cell Death Detection Kit, TMR red, Roche, Basel, Switzerland) (60 min at 37 °C in the dark). After washing in TBS, the material was stained with 1 mg/ml Hoechst 33342 for the detection of nuclei (10 min at RT). Finally, the material was analyzed using an Olympus FluoView FV1000 confocal microscope with 40×/NA 0.95 objective. Z-stack images were generated using a 405nm laser for the Hoechst 33342 dyes and 559nm for the TMR red dye (TUNEL reaction mixture). Image sets were deconvolved in AutoQuant X3 (custom software developed by Bitplane Scientific Software, Zurich, Switzerland) using blind deconvolution. Three-D data sets were analyzed as volume-rendered data sets using Imaris (Bitplane, Zurich, Switzerland). For statistical analysis all of the cells were segmented using the surface modeling available in Imaris software. For each segmented cell, the mean fluorescence value was calculated for the TMR red dye. An arbitrary mean intensity threshold for the TMR red dye was used to determine the number of stained cells. As the result, we calculated the mean percentage of the stained cell population. Negative controls without terminal deoxynucleotidyl transferase (TdT) were prepared according to the labeling protocol (*In Situ* Cell Death Detection Kit, TMR red, Roche, Basel, Switzerland).

LysoTracker Red (LTR) staining – labeling autophagosomes and autolysosomes.

LysoTracker Red (LTR) staining selectively accumulates in the acidic organelles and can be used to investigate lysosomes and autolysosomes. The isolated intestine and hepatopancreas were incubated in the dark for 15 min in 2.5 mM LysoTracker Red DND-99 (Molecular Probes, L 7528, Thermo Fisher Scientific, Waltham, MA, USA) diluted in 500 ml of PBS (Phosphate-buffered saline) at RT. Next, the material was washed several times with PBS. The nuclei were stained for 10 min in 1 mg/ml Hoechst 33342 diluted in PBS and washed

several times in PBS. The slides were analyzed using an Olympus FluoView FV1000 confocal microscope. Excitation at 559 nm was provided by a DPSS laser.

Caspase 3 (apoptosis)

Caspase-3 is the primary activator of apoptotic DNA fragmentation. The isolated organs (intestine and hepatopancreas) were fixed with PFA (20 min, RT). After washing the material with TBS (2 x 5 min), it was incubated with 3% BSA in 0.1% Triton in TBS (90 min). Then, the organs were incubated with the anti-caspase 3 primary antibody (Life Technologies, Carlsbad, CA, USA) (12 h, RT). After washing with TBS, the material was stained with anti-goat secondary antibody conjugated with Alexa Fluor 488 (Life Technologies, Carlsbad, CA, USA). Then the material was washed with TBS (2 x 5 min) and stained for 10 min in 1 mg/ml Hoechst 33342 diluted in TBS and washed several times in TBS. The slides were analyzed using an Olympus FluoView FV1000 confocal microscope. Excitation at 488 was provided by an Argon laser.

Results

The endodermal midgut of *N. davidi* is composed of two distinct structures: the intestine and the hepatopancreas. The precise ultrastructure of the D- and R-cells of the intestine and the R-, B- and F-cells of the hepatopancreas was presented in our previous paper (Sonakowska et al. 2015). Because the process of autophagy and apoptosis has also been observed as a common process in D-, B- and F-cells, the description of the processes of cell death only concern these cells, while they were absent in the regenerative (R-cells) and differentiating cells. Additionally, no differences were observed among the males and females of the species that were analyzed, and therefore the results presented here relate to both sexes (Figs. 1a-e, 2a-d, 3a-e, 4a-d). The non-starved *N. davidi* as the control specimens were analyzed during

our previous studies (Sonakowska et al. 2016) so the results presented below will be discussed according to them.

After 7 days of starvation no changes were detected in the cytoplasm of the D-cells in the intestine and the B- and F-cells in the proximal zone of the hepatopancreatic tubules at the ultrastructural level (Figs. 1c-e). However, after 14 days of starvation, the number of organelles (e.g., cisterns of the rough and smooth endoplasmic reticulum, Golgi complexes) in the cytoplasm of the D-cells in the intestine and the B- and F-cells in the hepatopancreas gradually decreased (Fig. 2e). Nuclei began to be folded and the heterochromatin became electron lucent (Fig. 2e). The amount of reserve material in the B-cells in the hepatopancreas gradually decreased (Fig. 2d). After 21 days of starvation, the cytoplasm of all described cells was electron lucent and had only small number of organelles showing the signs of necrosis (the majority of animals were dying).

Animals starved for 7 days were re-fed, however because there were no changes after starvation for this period, no changes were also described after re-feeding animals for 4, 7 and 14 days. After four days of re-feeding the animals starved for 14 days, the cytoplasm of the D-cells in the intestine and the B- and F-cells in the hepatopancreas was still poor in organelles (Fig. 3a). However, the reserve material in the cytoplasm of the B-cells in the proximal region of each hepatopancreatic tubule began to accumulate (Fig. 3c). After seven days of re-feeding the animals that were starved for 14 days, the cytoplasm changed its electron density and the number of organelles and the amount of the reserve material gradually increased (Figs. 3d-e). However, the period of 14 days of re-feeding for sufficient for cytoplasm regeneration in D-, B- and F-cells: it became electron dense, the number of organelles increased gradually (Figs. 4a-b, 4d) and eventually, the cytoplasm resembled that of the animals in the control group. The cytoplasm of the B-cells had numerous spheres of the reserve material (Fig. 4c).

1 **Autophagy**

2 The transformed and degenerating organelles formed distinct agglomerations (Fig. 2e), which
3 were gradually surrounded by a double-membranous structure (Figs. 1d, 2b, 3b), which is
4 called a phagophore, and is formed by cisterns of the endoplasmic reticulum (Figs. 2e and
5 3b). The membrane of the phagophore expanded due to its fusion with numerous small
6 vesicles and gradually enclosed further organelles. Eventually, the blind ends of the
7 phagophore (Fig. 2b) connected and an autophagosome was formed (Figs. 1b, 1c, 1e, 2b and
8 2d). Cisterns of the endoplasmic reticulum, vesicles that had an electron-lucent content,
9 vacuoles, lamellar bodies, lipids and electron-dense granules, fragments of nucleus and
10 mitochondria could be observed inside autophagosomes (Figs. 1c, 1e, 2b, 2d, 4b). The
11 autophagosomes fused with electron-dense lysosomes and eventually, the autolysosomes were
12 formed. The digestion of the autolysosomal interior caused the formation of residual bodies
13 (Fig. 2e). Eventually, the residual bodies were discharged into the midgut lumen in a manner
14 that resembled apocrine secretion. Therefore, the apical cell membrane formed an evagination
15 into the intestinal or hepatopancreatic lumen. The cytoplasm with some organelles and
16 autophagosomes/autolysosomes/residual bodies penetrated the evagination. The evagination
17 was finally separated from the entire cell and the autophagosomal structures were digested
18 inside the midgut lumen (Figs. 1e, 2e).

19 Qualitative analysis using LysoTracker Red revealed the presence of strongly acid
20 organelles in the cytoplasm of the D-cells in the intestine and the B- and F-cells in the
21 hepatopancreas (Figs. 5a-b). A quantitative analysis showed that 27% of the cells in
22 hepatopancreas and 24% in the intestine had autophagosomes, autolysosomes and residual
23 bodies in their cytoplasm (Tab. 2). The results presented here differ from the results presented
24 in our previous studies (Sonakowska et al. 2016) because only completely formed autophagic
25 structures were taken into consideration here. During our previous studies, the early stages of

1 autophagy, when degenerated organelles were aggregating and the phagophore was formed,
2 were also counted.

3 A qualitative analysis using LysoTracker Red showed the presence of strongly acid
4 organelles in the D-cells of the intestine and the F- and B-cells in the hepatopancreas in all
5 animals that were starved and re-fed after starvation (Figs. 5c-f, 6a-f).

6 A quantitative analysis revealed an increase of number of in D-, B- and F-cells with
7 signs of autophagy according to periods of starvation. Seven and 14 days of starvation caused
8 the autophagy in 40%/41% of cells in hepatopancreas, and 46%/56% of cells in intestine. The
9 quantitative analysis for animals starved for 7 days and re-fed for 4, 7 and 14 days showed
10 that the number of autophagic cells decreased gradually: 24%, 23%, 28% in hepatopancreas
11 and 22%, 25%, 22% in intestine respectively. Four days of re-feeding after 14 days of
12 starvation showed a drastic reduction in the number of autophagic cells in both organs
13 examined here. 7/14 days of re-feeding after 14 days of starvation revealed that 27%/26% of
14 the F- and B-cells in the hepatopancreas and 30%/23% of the D-cells in the intestine had
15 autophagic structures (Tab. 2).

17 **Apoptosis**

18 The cell began to shrink at the beginning of apoptosis. The cytoplasm became electron dense
19 (Fig. 2c) and distinct extracellular spaces appeared between the apoptotic cell and adjacent
20 cells (Fig. 4d). The nucleus of the apoptotic cell shrank and developed a lobular shape. Its
21 chromatin formed electron-dense patches that gathered near the nuclear envelope (Figs. 2a,
22 2c, 4c-d). Numerous mitochondria degenerated and lost their cristae (Figs. 2c, 4c). Cisterns of
23 the rough and smooth endoplasmic reticulum became swollen (Fig. 2c) and numerous small
24 vacuoles with an electron-lucent content occurred in the cytoplasm of the apoptotic cell (Fig.
25 2c). The apoptotic cell gradually lost contact with the basal lamina (Fig. 4d) and eventually
26 the entire cell was discharged into the midgut lumen (Fig. 4c) where it was digested.

Confocal microscopy revealed the activity of active caspase-3 in the cytoplasm of the epithelial cells in the hepatopancreas and intestine in animals from all experimental groups (Figs. 7a-f, 8a-f). A quantitative analysis revealed the presence of apoptosis in 18% of the F- and B-cells of the hepatopancreas and in 23% of the D-cells in the intestine in animals from control group (Sonakowska et al. 2016) (Tab. 3, Figs. 9a-b).

A quantitative analysis revealed that in animals starved for 7 days, only 0.17% of the cells in the proximal region of each hepatopancreatic tubule in the hepatopancreas were apoptotic, while the percentage of apoptotic cells in the intestine was higher – 32.34% (Tab. 3, Figs. 9c-d). In animals starved for 14 days, the difference between intestine and hepatopancreas appeared: 28.15% of cells in the proximal region of each hepatopancreatic tubule in the hepatopancreas were apoptotic, while the percentage of apoptotic cells in the intestine was higher – 44.88% (Tab. 3, Figs. 9e-f).

The quantitative analysis for animals starved for 7 days and re-fed for 4, 7 and 14 days showed that the number of apoptotic cells decreased gradually. Re-feeding for 4, 7 and 14 days after 7 days of starvation caused the reduction in number of apoptotic cells in the hepatopancreas (0%, 0%, 0% respectively) and intestine (2.93% ,0,28% ,0% respectively). Re-feeding for 4 and 7 days after 14 days of starvation caused the reduction in number of apoptotic cells in the hepatopancreas (6.69%, 1.23% respectively) and in the intestine (33.58%, 18% respectively) (Tab. 3, Figs. 10a-d). A quantitative analysis of animals re-fed for 14 days after starvation did not reveal any apoptotic cells in the proximal region of the hepatopancreatic tubules, while the percentage of apoptotic cells in the intestine was very low: 3.35% (Tab. 3, Figs. 10e, 10f).

Discussion

Autophagy and apoptosis, which are mechanisms activated by many external factors (Lipovšek et al. 2014, 2018; Romanelli et al. 2014; Wilczek et al. 2014), are widely studied nowadays with a special emphasis on the relationship between these two processes and their roles in the body. To date, *N. davidii* has been the object of experimental studies in which starvation was used as the stressor (Włodarczyk et al. 2017).

The process of autophagy in this species was described in our previous paper (Sonakowska et al. 2016), while here we present additional quantitative analyses that are connected with the activation of this process depending on the stressor – starvation and re-feeding. In *N. davidii*, autophagy was only detected in the D-cell of the intestine and the B- and F- cells of the hepatopancreas, whereas it was absent in the R-cells and differentiating cells. This may be caused by the fact that these cells do not come into contact with the midgut lumen so they are not exposed to any stressors (Sonakowska et al. 2016). Additionally, the R-cells are responsible for the self-renewal of the entire midgut epithelium after its degeneration. Therefore, they should not be exposed at the stressors as the midgut stem cells (Rost-Roszkowska et al. 2011; Sonakowska et al. 2015, 2016; Hyra et al. 2016). Because the D-, B- and F-cells are the functional cells of the midgut epithelium they will be the first to be affected by autophagy. A similar relationship between the periods of starvation/re-feeding and the intensity of autophagy in the intestine and hepatopancreas occurred, thereby suggesting that both of these organs participate in maintaining homeostasis.

The midgut of invertebrates is the organ which enables to accumulate the reserve material utilized during natural periods of starvation such as hibernation. During this period the metabolism, and all biochemical and physiological processes in animals body slow down (Lipovšek et al., 2011, 2014, 2016, 2017; Rost-Roszkowska et al. 2013; Lipovšek and Novak 2015; Hyra et al. 2016). Therefore, the reserve material after digestion (e.g., due to autophagy) supply the energy for the proper functioning of the organism (Ryan et al. 2004;

1 Lipovšek et al. 2011, 2014, 2016, 2017). In *N. davidi*, a reduction in the amount of reserve
2 material occurred after starvation. After re-feeding, the reserve material began to accumulate
3 in the B-cells of the hepatopancreas. Additionally, autophagy has been observed as a process
4 that is activated after starvation – the longer the period of starvation was, the higher the
5 number of autophagic structures that were observed in the cytoplasm. Two weeks of
6 starvation were treated as the critical time because for the shrimp that were starved
7 continuously for 14 days, the regeneration starts after three days after re-feeding (animals stop
8 dying), while the majority of shrimps that were starved for 21 days (close to the PNR50) died,
9 the regeneration period was extended to seven days but regeneration was still possible.
10 Therefore, we could conclude that the animals that were re-fed after 21 days of starvation,
11 almost all died, while after 14 days of starvation, the individual died for three more days, but
12 after this period they stopped dying (Włodarczyk et al. 2017). Autophagy is activated during
13 natural periods of starvation such as hibernation (Lipovšek et al. 2014, 2015, 2018; Lipovšek
14 and Novak 2016), when it protects a cell against its death, which is caused by the degradation
15 of proteins and organelles (Kourtis and Tavernarakis 2009; Wilczek et al. 2014; Lipovšek and
16 Novak 2016). Additionally, it is treated as a survival factor in degradation of crustacean
17 midgut epithelium, which is devoid of regenerative cells, e.g. *Eubbranchipus grubii* (Rost-
18 Roszkowska et al. 2012). Our experiment revealed that re-feeding of animals which were
19 starved causes the decrease in number of autophagic structures (autophagosomes,
20 autolysosomes, residual bodies) in midgut epithelial cells together with the increase in the
21 number of organelles. It suggests that not all of the organelles such as rough and smooth
22 endoplasmic reticulum or Golgi complexes have been disrupted and after returning to the
23 feeding, they could act their functions combined with the synthesis of proteins, lipids and/or
24 polysaccharides. During our previous studies (Włodarczyk et al. 2017) we showed that
25 starvation caused the degeneration of mitochondrial ultrastructure and an increase of cells

1 with depolarized (non-active) mitochondria. However, after re-feeding the proper
2 ultrastructure of mitochondria has been restored and the number of active mitochondria
3 increased (Włodarczyk et al. 2017). Changes in the structure and number of active
4 mitochondria correspond to changes in the number of the remaining organelles presented
5 here. Our studies showed that the number of autophagic structures gradually decreased when
6 animals returned to feeding after different periods of starvation: 7 and 14 days, so their
7 number is similar to the number of autophagic structures in control group.

8 Unlike necrosis, apoptosis is responsible for removing unnecessary, destroyed or
9 damaged cells without initiating local inflammation (Savilli et al. 2000; Orrenius 2004;
10 Maghsoudi et al. 2012). The main features of an apoptotic cell are changes in the electron
11 density, fragmentation of the nucleus, chromatin condensation, transformation of the
12 organelles, cell shrinkage, etc. (Rost-Roszkowska et al. 2008, 2010; Sonakowska 2016). In
13 the midgut epithelium of invertebrates, an apoptotic cell does not fragment, but is discharged
14 into the gut lumen (Rost-Roszkowska et al. 2010). Cell death in the midgut epithelium of
15 crustacean species has been described as a degenerative desquamation when a strong
16 vacuolization is correlated with the lysis of cells and degradation of the nucleus (Vogt 1990;
17 Masson 2001; Cuartas et al. 2003). However, it is difficult to state whether this description is
18 connected with apoptotic or necrotic cell death. Meanwhile, the precise ultrastructural and
19 immunohistochemical analysis revealed that the process of apoptosis occurs in *N. davidii*
20 (Sonakowska et al. 2016). Our studies showed that the course of apoptosis after starvation and
21 re-feeding is similar to the one described in a control group (Sonakowska et al. 2016), while
22 the changes were connected with the intensity of this process. Starvation causes an increase in
23 the number of apoptotic cells. Studies on other groups of arthropods has proven that starvation
24 activates apoptosis in the digestive cells of the midgut epithelium (Wilczek et al. 2014;
25 Lipovšek and Novak 2016; Lipovšek et al. 2018). In *N. davidii*, a high number of apoptotic

cells was observed after two weeks of starvation, which correlated with an increase in the number of cells with depolarized (non-active) mitochondria (Włodarczyk et al. 2017). Mitochondria, which are responsible for energy synthesis, generate a high number of reactive oxygen species (ROS) (Ramalho-Santos et al. 2009) and their increase in the cell activates cell death (Yang et al. 1997; Liu et al. 2000; Fernandez-Checa 2003; Grad and Lemire 2004; Orrenius 2004; Ramalho-Santos et al, 2009). After re-feeding of animals starved for 7 days or 14 days the number of apoptotic cells decreased significantly below the level in the control group in the intestine and hepatopancreas of *N. davidi*. This correlates with the our previous observation – re-feeding induces the regeneration of the mitochondrial ultrastructure and causes an increase in the number of cells with active (polarized) mitochondria (Włodarczyk et al. 2017). However, the intensity of apoptosis lower than in the control group after re-feeding can be connected with the fact that the number of mitochondria with an altered membrane potential was still greater at this time. This may suggest that two weeks is not a sufficient period of time for mitochondrial regeneration (Włodarczyk et al. 2017). A similar increasing amount of mitochondria with an altered membrane potential in the early stages of starvation was associated with an increasing number of apoptotic cells in the digestive system of spiders (Wilczek et al. 2014). Therefore, it is probable that long periods of starvation activate mutations and dysfunctions in the proteins that are responsible for activating apoptosis, which results in a decrease in the level of apoptosis in cells (McGivern et al. 2009) or even the transformation of mitochondrial cristae (Frezza et al. 2006).

Apoptosis and autophagy can occur at the same time and in the same tissue. In this case, autophagy mediates the induction of apoptosis (Eisenberg et al. 2009; Franzetti et al. 2012). Our results confirmed the relationship between these two processes. They are intensified after starvation, while re-feeding inhibits their course. After 14 days of re-feeding apoptosis was detected in its early stages (a positive reaction with anti-caspase 3 antibodies)

as has been described in the literature (Porter and Jänicke 1999). We were not able to prepare the experiment and collect the same number of specimens which were starved for 21 days as from all experimental groups, because the majority of specimens died, what correlates with our observation that strong necrosis was observed in the intestine and hepatopancreas.

Conclusions

The results of these studies showed that in the freshwater shrimp *N. davidii* that were analyzed: (a) starvation causes a reduction in the amount of reserve material in the hepatopancreas; (b) starvation activates autophagy in the intestine and hepatopancreas; (c) starvation causes an increase of apoptotic cells in the intestine and hepatopancreas; (d) re-feeding after starvation enables the accumulation of reserve material in the hepatopancreas; (e) re-feeding inhibits autophagy in the intestine and hepatopancreas; (f) re-feeding causes a decrease in the number of apoptotic cells in the intestine and hepatopancreas.

Compliance with ethical standards

Ethical approval. This article does not contain any studies with human participants or animals performed by any of the authors.

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Table legends

Table 1. Number of adult specimens of *N. davidi* used in the each part of the experiment.

Table 2. Percentage [%] of cells in the entire intestine and proximal zone of hepatopancreatic epithelium in *N. davidi* with signs of autophagy.

Table 3. Percentage [%] of apoptotic cells in the entire intestine and proximal zone of hepatopancreatic epithelium in *N. davidi*.

Figure legend

Fig. 1. Ultrastructure of the midgut epithelium in *N. davidi*. TEM. Mitochondria (m), midgut lumen (l), microvilli (mv), nucleus (n), electron-dense granules (eg), autophagosomes (au), cisterns of RER (RER), phagophore (ph), vacuoles (v). **a** Hepatopancreas in non-starved animals. Bar = 1.2 µm. **b** Intestine in non-starved animals. Bar = 0.2 µm. **c** Hepatopancreas in animals starved for 7 days. Bar = 1.7 µm. **d** Intestine in animals starved for 7 days. Bar = 1 µm. **e** Intestine in animals starved for 7 days. Bar = 0.2 µm.

Fig. 2. Ultrastructure of the midgut epithelium in *N. davidi*. TEM. Mitochondria (m), midgut lumen (l), microvilli (mv), nucleus (n), , autophagosomes (au), cisterns of RER (RER), vacuoles (v), apoptotic cell (ac), lamellar body (lb), phagophore (ph), residual bodies (rb), electron dense granules (eg), blind ends of phagophore (arrows), degenerating organelles (do). **a** Hepatopancreas in animals starved for 14 days. Bar = 1.3µm. **b** Intestine in animals starved for 14 days. Bar = 0.8 µm. **c** intestine in animals starved for 14 days. Bar = 1 µm. **d** intestine in animals starved for 14 days. Bar = 0.3 µm. **e** intestine in animals starved for 14 days. Bar = 1,2 µm.

Fig. 3. Ultrastructure of the midgut epithelium in *N. davidi*. TEM. Mitochondria (m), microvilli (mv), nucleus (n), phagophore (ph), blind ends of phagophore (arrows), cisterns of RER (RER), vacuoles (v), reserve material (rm). **a** Hepatopancreas in animals re-fed for 4 days after 14 days of starvation Bar = 1.5 µm. **b** Intestine in animals re-fed for 4 days after 14 days of starvation Bar = 0.4 µm. **c** Hepatopancreas in animals re-fed for 4 days after 14 days of starvation Bar = 0.5 µm. **d** Hepatopancreas in animals re-fed for 7 days after 14 days of starvation Bar = 1.4 µm. **e** Intestine in animals re-fed for 7 days after 14 days of starvation Bar = 0.6 µm.

Fig. 4. Ultrastructure of the midgut epithelium in *N. davidi*. TEM. Mitochondria (m), midgut lumen (l), microvilli (mv), nucleus (n), autophagosomes (au), cisterns of RER (RER), apoptotic cell (ac), reserve material (rm). **a** Hepatopancreas in animals re-fed for 14 days after 14 days of starvation Bar = 0.6 µm. **b** Intestine in animals re-fed for 14 days after 14 days of starvation Bar = 0.4 µm. **c** Hepatopancreas in animals re-fed for 14 days after 14 days of starvation Bar = 1 µm. **d** Hepatopancreas in animals re-fed for 14 days after 14 days of starvation Bar = 0.8 µm.

Fig. 5. Autolysosomes and acid phosphatase localization in the *N. davidi* midgut. 3D representation of the accumulation of lysosomes and autolysosomes (red signals). Nuclei

(blue signals). LysoTracker Red and Hoechst 33342 staining. Confocal microscope. **a** fragment of the hepatopancreas in non-starved animals Bar = 20 μ m. **b** fragment of the intestine in non-starved animals Bar = 20 μ m. **c** fragment of the hepatopancreas in animals starved for 7 days. Bar = 20 μ m. **d** fragment of the intestine in animals starved for 7 days. Bar = 20 μ m. **e** fragment of the hepatopancreas in animals starved for 14 days. Bar = 30 μ m. **f** fragment of the intestine in animals starved for 14 days. Bar = 20 μ m

Fig. 6. Autolysosomes and acid phosphatase localization in the *N. davidi* midgut. 3D representation of the accumulation of lysosomes and autolysosomes (red signals). Nuclei (blue signals). LysoTracker Red and Hoechst 33342 staining. Confocal microscope. **a** fragment of the hepatopancreas in animals re-fed for 4 days after 14 days of starvation. Bar = 20 μ m. **b** fragment of the intestine in animals re-fed for 4 days after 14 days of starvation. Bar = 20 μ m. **c** fragment of the hepatopancreas in animals re-fed for 7 days after 14 days of starvation. Bar = 30 μ m. **d** fragment of the intestine in animals re-fed for 7 days after 14 days of starvation. Bar = 30 μ m. **e** fragment of the hepatopancreas in animals re-fed for 14 days after 14 days of starvation. Bar = 30 μ m. **f** fragment of the intestine in animals re-fed for 14 days after 14 days of starvation. Bar = 20 μ m.

Fig. 7. 3D representation of caspase 3 in the *N. davidi* midgut. Caspase 3 (green signals), nuclei (blue signals) anti-caspase 3 primary antibody and anti-goat secondary antibody conjugated with Alexa Fluor 488 (Life technologies). Hoechst 33342 staining. Confocal microscope **a** fragment of the hepatopancreas in non-starved animals Bar = 20 μ m. **b** fragment of the intestine in non-starved animals Bar = 15 μ m. **c** fragment of the hepatopancreas in animals starved for 7 days. Bar = 20 μ m. **d** fragment of the intestine in animals starved for 7 days. Bar = 20 μ m. **e** fragment of the hepatopancreas in animals starved for 14 days. Bar = 20 μ m. **f** fragment of the intestine in animals starved for 14 days. Bar = 15 μ m

1 **Fig. 8.** 3D representation of caspase 3 in the *N. davidi* midgut. Caspase 3 (green signals),
2 nuclei (blue signals) anti-caspase 3 primary antibody and anti-goat secondary antibody
3 conjugated with Alexa Fluor 488 (Life technologies). Hoechst 33342 staining. Confocal
4 microscope. **a** fragment of the hepatopancreas in animals re-fed for 4 days after 14 days of
5 starvation. Bar = 30 μ m. **b** fragment of the intestine in animals re-fed for 4 days after 14 days
6 of starvation. Bar = 20 μ m. **c** fragment of the hepatopancreas in animals re-fed for 7 days after
7 14 days of starvation. Bar = 20 μ m. **d** fragment of the intestine in animals re-fed for 7 days
8 after 14 days of starvation. Bar = 20 μ m. **e** fragment of the hepatopancreas in animals re-fed
9 for 14 days after 14 days of starvation. Bar = 20 μ m. **f** fragment of the intestine in animals re-
10 fed for 14 days after 14 days of starvation. Bar = 20 μ m.

11 **Fig. 9.** 3D representation of the Tunel assay and Hoechst 33342 staining. Nuclei of apoptotic
12 cells (red), nuclei (blue). Confocal microscope. **a** fragment of the hepatopancreas in non-
13 starved animals Bar = 30 μ m. **b** fragment of the intestine in non-starved animals Bar = 30
14 μ m. **c** fragment of the hepatopancreas in animals starved for 7 days. Bar = 30 μ m. **d** fragment
15 of the intestine in animals starved for 7 days. Bar = 30 μ m. **e** fragment of the hepatopancreas
16 in animals starved for 14 days. Bar = 30 μ m. **f** fragment of the intestine in animals starved for
17 14 days. Bar = 30 μ m

18 **Fig. 10.** 3D representation of the Tunel assay and Hoechst 33342 staining. Nuclei of apoptotic
19 cells (red), nuclei (blue). Confocal microscope. **a** Fragment of the hepatopancreas in animals
20 re-fed for 4 days after 14 days of starvation. Bar = 30 μ m. **b** Fragment of the intestine in
21 animals re-fed for 4 days after 14 days of starvation. Bar = 30 μ m. **c** Fragment of the
22 hepatopancreas in animals re-fed for 7 days after 14 days of starvation. Bar = 30 μ m. **d**
23 Fragment of the intestine in animals re-fed for 7 days after 14 days of starvation. Bar = 30
24 μ m. **e** Fragment of the hepatopancreas in animals re-fed for 14 days after 14 days of

- 1 starvation. Bar = 30 μm . **f** Fragment of the intestine in animals re-fed for 14 days after 14
- 2 days of starvation. Bar = 30 μm .
- 3

Draft

Tab. 1. The number of adult specimens of *N. davidi* that were used in the each part of the experiment.

Number of days of starvation		Number of specimens analyzed			
		Part 1: starvation			
		TEM	Confocal microscopy		
Caspase 3	TUNEL		LysoTracker		
7 days	Males	5	4	4	4
	Females	5	4	4	4
14 days	Males	5	4	4	4
	Females	5	4	4	4
21 days	Males	5	0	0	0
	Females	5	0	0	0

Number of days of re-feeding after 7 days of starvation		Number of specimens analyzed			
		Part 2: re-feeding after 7 days of starvation			
		TEM	Confocal microscopy		
Caspase 3	TUNEL		Lysotracker		
4 days	Males	5	4	4	4
	Females	5	4	4	4
7 days	Males	5	4	4	4
	Females	5	4	4	4
14 days	Males	5	4	4	4
	Females	5	4	4	4

Number of days of re-feeding after 14 days of starvation		Number of specimens analyzed			
		Part 3: re-feeding after 14 days of starvation			
		TEM	Confocal microscopy		
Caspase 3	TUNEL		LysoTracker		
4 days	Males	5	4	4	4
	Females	5	4	4	4
7 days	Males	5	4	4	4
	Females	5	4	4	4
14 days	Males	5	4	4	4
	Females	5	4	4	4

Number of days of re-feeding after 21 days of starvation		Number of specimens analyzed			
		Part 4: re-feeding after 21 days of starvation			
		TEM	Confocal microscopy		
Caspase 3	TUNEL		LysoTracker		
4 days	Males	5			
	Females	5			
7 days	Males	5			
	Females	5			
14 days	Males	5			
	Females	5			

Tab. 2. Mean ($\bar{x}$) $\pm$ standard deviation (SD) of cells with signs of autophagy in the entire intestine and proximal zone of hepatopancreatic epithelium in *N. davidi* (Student *t*-test, $p < 0.05$; $n = 5$).

	hepatopancreas	intestine
Control group (Sonakowska et al. 2016)	27 $\pm$ 10	24 $\pm$ 8
7 days of starvation	40 $\pm$ 10	46 $\pm$ 11
14 days of starvation	41 $\pm$ 15	56 $\pm$ 17
4 days of re-feeding after 7 days of starvation	24 $\pm$ 8	22 $\pm$ 10
7 days of re-feeding after 7 days of starvation	23 $\pm$ 7	25 $\pm$ 8
14 days of re-feeding after 7 days of starvation	28 $\pm$ 3	22 $\pm$ 5
4 days of re-feeding after 14 days of starvation	20 $\pm$ 7	23 $\pm$ 5
7 days of re-feeding after 14 days of starvation	27 $\pm$ 8	30 $\pm$ 6
14 days of re-feeding after 14 days of starvation	26 $\pm$ 3	23 $\pm$ 4

Tab. 3. Mean ($\bar{x}$) $\pm$ standard deviation (SD) of apoptotic cells in the entire intestine and proximal zone of hepatopancreatic epithelium in *N. davidi* (Student *t*-test, $p < 0.05$; $n = 5$).

	hepatopancreas	intestine
Control group (Sonakowska et al. 2016)	18 $\pm$ 8	23 $\pm$ 10
7 days of starvation	0.17 $\pm$ 4	32.34 $\pm$ 10
14 days of starvation	28.15 $\pm$ 15	44.88 $\pm$ 14
4 days of re-feeding after 7 days of starvation	0 $\pm$ 6	2.93 $\pm$ 10
7 days of re-feeding after 7 days of starvation	0 $\pm$ 5	0.28 $\pm$ 8
14 days of re-feeding after 7 days of starvation	0 $\pm$ 7	0 $\pm$ 10
4 days of re-feeding after 14 days of starvation	6.69 $\pm$ 3	33.58 $\pm$ 12
7 days of re-feeding after 14 days of starvation	1.23 $\pm$ 5	18 $\pm$ 8
14 days of re-feeding after 14 days of starvation	0 $\pm$ 2.4	3.35 $\pm$ 4

Fig. 1. Ultrastructure of the midgut epithelium in *N. davidi*. TEM. Mitochondria (m), midgut lumen (l), microvilli (mv), nucleus (n), electron-dense granules (eg), autophagosomes (au), cisterns of RER (RER), phagophore (ph), vacuoles (v). a Hepatopancreas in non-starved animals. Bar = 1.2 μ m. b Intestine in non-starved animals. Bar = 0.2 μ m. c Hepatopancreas in animals starved for 7 days. Bar = 1.7 μ m. d Intestine in animals starved for 7 days. Bar = 1 μ m. e Intestine in animals starved for 7 days. Bar = 0.2 μ m.

74x106mm (300 x 300 DPI)

Fig. 2. Ultrastructure of the midgut epithelium in *N. davidi*. TEM. Mitochondria (m), midgut lumen (l), microvilli (mv), nucleus (n), , autophagosomes (au), cisterns of RER (RER), vacuoles (v), apoptotic cell (ac), lamellar body (lb), phagophore (ph), residual bodies (rb), electron dense granules (eg), blind ends of phagophore (arrows), degenerating organelles (do). a Hepatopancreas in animals starved for 14 days. Bar = 1.3 μ m. b Intestine in animals starved for 14 days. Bar = 0.8 μ m. c intestine in animals starved for 14 days. Bar = 1 μ m. d intestine in animals starved for 14 days. Bar = 0.3 μ m. e intestine in animals starved for 14 days. Bar = 1,2 μ m.

72x105mm (300 x 300 DPI)

Fig. 3. Ultrastructure of the midgut epithelium in *N. davidi*. TEM. Mitochondria (m), microvilli (mv), nucleus (n), phagophore (ph), blind ends of phagophore (arrows), cisterns of RER (RER), vacuoles (v), reserve material (rm). a Hepatopancreas in animals re-fed for 4 days after 14 days of starvation Bar = 1.5 μ m. b Intestine in animals re-fed for 4 days after 14 days of starvation Bar = 0.4 μ m. c Hepatopancreas in animals re-fed for 4 days after 14 days of starvation Bar = 0.5 μ m. d Hepatopancreas in animals re-fed for 7 days after 14 days of starvation Bar = 1.4 μ m. e Intestine in animals re-fed for 7 days after 14 days of starvation Bar = 0.6 μ m.

72x93mm (300 x 300 DPI)

Fig. 4. Ultrastructure of the midgut epithelium in *N. davidi*. TEM. Mitochondria (m), midgut lumen (l), microvilli (mv), nucleus (n), autophagosomes (au), cisterns of RER (RER), apoptotic cell (ac), reserve material (rm). a Hepatopancreas in animals re-fed for 14 days after 14 days of starvation Bar = 0.6 μm . b Intestine in animals re-fed for 14 days after 14 days of starvation Bar = 0.4 μm . c Hepatopancreas in animals re-fed for 14 days after 14 days of starvation Bar = 1 μm . d Hepatopancreas in animals re-fed for 14 days after 14 days of starvation Bar = 0.8 μm .

74x69mm (300 x 300 DPI)

Fig. 5. Autolysosomes and acid phosphatase localization in the *N. davidi* midgut. 3D representation of the accumulation of lysosomes and autolysosomes (red signals). Nuclei (blue signals). LysoTracker Red and Hoechst 33342 staining. Confocal microscope. a fragment of the hepatopancreas in non-starved animals Bar = 20 μ m. b fragment of the intestine in non-starved animals Bar = 20 μ m. c fragment of the hepatopancreas in animals starved for 7 days. Bar = 20 μ m. d fragment of the intestine in animals starved for 7 days. Bar = 20 μ m. e fragment of the hepatopancreas in animals starved for 14 days. Bar = 30 μ m. f fragment of the intestine in animals starved for 14 days. Bar = 20 μ m

74x78mm (300 x 300 DPI)

Fig. 6. Autolysosomes and acid phosphatase localization in the *N. davidi* midgut. 3D representation of the accumulation of lysosomes and autolysosomes (red signals). Nuclei (blue signals). LysoTracker Red and Hoechst 33342 staining. Confocal microscope. a fragment of the hepatopancreas in animals re-fed for 4 days after 14 days of starvation. Bar = 20 μ m. b fragment of the intestine in animals re-fed for 4 days after 14 days of starvation. Bar = 20 μ m. c fragment of the hepatopancreas in animals re-fed for 7 days after 14 days of starvation. Bar = 30 μ m. d fragment of the intestine in animals re-fed for 7 days after 14 days of starvation. Bar = 30 μ m. e fragment of the hepatopancreas in animals re-fed for 14 days after 14 days of starvation. Bar = 30 μ m. f fragment of the intestine in animals re-fed for 14 days after 14 days of starvation. Bar = 20 μ m.

73x71mm (300 x 300 DPI)

Fig. 7. 3D representation of caspase 3 in the *N. davidi* midgut. Caspase 3 (green signals), nuclei (blue signals) anti-caspase 3 primary antibody and anti-goat secondary antibody conjugated with Alexa Fluor 488 (Life technologies). Hoechst 33342 staining. Confocal microscope a fragment of the hepatopancreas in non-starved animals Bar = 20 µm. b fragment of the intestine in non-starved animals Bar = 15 µm. c fragment of the hepatopancreas in animals starved for 7 days. Bar =20 µm. d fragment of the intestine in animals starved for 7 days. Bar = 20 µm. e fragment of the hepatopancreas in animals starved for 14 days. Bar = 20 µm. f fragment of the intestine in animals starved for 14 days. Bar = 15 µm

73x108mm (300 x 300 DPI)

Fig. 8. 3D representation of caspase 3 in the *N. davidi* midgut. Caspase 3 (green signals), nuclei (blue signals) anti-caspase 3 primary antibody and anti-goat secondary antibody conjugated with Alexa Fluor 488 (Life technologies). Hoechst 33342 staining. Confocal microscope. a fragment of the hepatopancreas in animals re-fed for 4 days after 14 days of starvation. Bar = 30 μm . b fragment of the intestine in animals re-fed for 4 days after 14 days of starvation. Bar = 20 μm . c fragment of the hepatopancreas in animals re-fed for 7 days after 14 days of starvation. Bar = 20 μm . d fragment of the intestine in animals re-fed for 7 days after 14 days of starvation. Bar = 20 μm . e fragment of the hepatopancreas in animals re-fed for 14 days after 14 days of starvation. Bar = 20 μm . f fragment of the intestine in animals re-fed for 14 days after 14 days of starvation. Bar = 20 μm .

73x101mm (300 x 300 DPI)

Fig. 9. 3D representation of the TUNEL assay and Hoechst 33342 staining. Nuclei of apoptotic cells (red), nuclei (blue). Confocal microscope. a fragment of the hepatopancreas in non-starved animals Bar = 30 μ m. b fragment of the intestine in non-starved animals Bar = 30 μ m. c fragment of the hepatopancreas in animals starved for 7 days. Bar = 30 μ m. d fragment of the intestine in animals starved for 7 days. Bar = 30 μ m. e fragment of the hepatopancreas in animals starved for 14 days. Bar = 30 μ m. f fragment of the intestine in animals starved for 14 days. Bar = 30 μ m

74x90mm (300 x 300 DPI)

Fig. 10. 3D representation of the TUNEL assay and Hoechst 33342 staining. Nuclei of apoptotic cells (red), nuclei (blue). Confocal microscope. a Fragment of the hepatopancreas in animals re-fed for 4 days after 14 days of starvation. Bar = 30 μ m. b Fragment of the intestine in animals re-fed for 4 days after 14 days of starvation. Bar = 30 μ m. c Fragment of the hepatopancreas in animals re-fed for 7 days after 14 days of starvation. Bar = 30 μ m. d Fragment of the intestine in animals re-fed for 7 days after 14 days of starvation. Bar = 30 μ m. e Fragment of the hepatopancreas in animals re-fed for 14 days after 14 days of starvation. Bar = 30 μ m. f Fragment of the intestine in animals re-fed for 14 days after 14 days of starvation. Bar = 30 μ m.

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