Midgut remodeling during the metamorphosis of *Chironomus riparius*, Meigen (1804)

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ABSTRACT

The holometabolous insects go through a complete metamorphosis that includes four life stages: egg, larva, pupa, and imago (adult). *Chironomus riparius* is a suggested model organism by the Organization for Economic Cooperation and Development (OECD) that is used in acute and chronic tests of chemicals. Tissue morphology of healthy non-biting midge larval stage was already described but the faith of the midgut digestive cells and tissue organization during the metamorphosis is unknown. We here described histological alterations of the midgut during ecdysis to distinguish them from the ones caused by toxins' negative effects. The present study showed differences in tissue architecture of the midgut in the larval, prepupal, and pupal stages of development of *C. riparius*. During ecdysis, larval digestive cells detached from the midgut epithelium and moved to the lumen. In the pupa, the larval midgut layer was replaced with an adult midgut that had considerably reduced width. These changes in the midgut tissue morphology and organization probably follow changes in the environment and feeding behavior of *C. riparius* at different stages of development.

Keywords: Chironomidae, metamorphosis, midgut, histology, xenobiotics

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Introduction

One of the main characteristics of holometabolous insects is their ability to go through the process of metamorphosis with an intercalated pupal stage between larva and adult. During that phase, the tissues are extensively remodeled while some of them are completely degraded (Rolff et al., 2019). Each of the phases is adapted to the specific roles in the life cycle of the organism (Hall & Martín-Vega, 2019). The family of Chironomidae (Diptera) are widely spread organisms that can adapt to diverse water ecosystems (Hilsenhoff, 2001). The aquatic model organism, suggested by the Organization for Economic Cooperation and Development (OECD) for ecotoxicological testing of chemicals is *Chironomus riparius*, Meigen, 1804 (Diptera, Chironomidae) (OECD, 2004a, b). The larvae of *C. riparius* are aquatic and active with nonselective feeding behavior. They go through 4 larval instars, which are benthic, before entering the prepupal and pupal stadium. Adults are released by eclosion from the pupa. The complete life cycle of *C. riparius* in laboratory conditions (at 20 °C) is completed in 20-28 days (Armitage et al., 1995). During the metamorphosis of chironomids as holometabolous insects, the midgut undergoes remodeling through the proliferation and differentiation of imaginal stem cells and the removal of larval midgut cells (Wu et al., 2006). Generally, the insect alimentary canal is divided into 3 regions: foregut, midgut, and hindgut (Chapman, 2012). A previous study by Stojanović et al. (2021) described the internal morphology of the *C. riparius* larvae digestive system based on histology analyses. Foregut starts with the mouth and ends in the metathorax on the place where the stomodeal valve separates the fore- and midgut. Based on digestive cell morphology, the midgut is divided into 3 regions. The transition between the midgut and hindgut is a place where the Malpighian tubules insert. Hindgut is formed by the ileum, colon, and rectum. There is a lack of information about what happens with the midgut after the metamorphosis of *C. riparius* larvae.

The industrial era has brought novel emerging contaminants to the environment that raised a concern about their ecotoxicity. Emerging contaminants were defined by the United States Environmental Protection Agency (EPA) as chemicals or materials that are a potential or real threat to human health or the environment (United States Environmental Protection Agency, 2011). Because of their extensive production and use, metal-oxide nanomaterials and microplastics have gained global attention as emerging contaminants (Maddela et al., 2022). Xenobiotics that reach waters may be deposited in sediment so detection of their presence and toxic activity is of great

importance (Gonçalves et al., 2012). For this purpose, benthic organisms are extensively used as bioindicators of toxic pollution of the sediment (Richardi et al., 2015). Even though chironomids were ecologically classified by functional feeding groups, the certain flexibility in their feeding behavior has led to the conclusion that they are opportunistic omnivores (Armitage et al., 1995). This way, they ingest xenobiotics along with the food and sediment. It was noticed in a previous study that exposure to the TiO_2 nanoparticles induced changes in the midgut similar to those described in healthy *Drosophila melanogaster* during the prepupal stage of the normal life cycle (Nelliot et al., 2006; Stojanovic et al., 2021). Nanoparticles have the potential to penetrate cells of the midgut, as shown in a study with *Ceriodaphnia dubia* (Dalai et al., 2013). The toxic potential of nanoparticles depends on their characteristics such as size, surface chemistry, and concentration (Yao et al., 2015). The general mechanism that was proposed to be the cause of nanoparticle cytotoxicity is the generation of reactive oxygen species (ROS) that could damage cells. It was described that nanoparticles have the potential to generate electrons that could enter the cells and disrupt the respiratory chain, leading to ROS overexpression and finally cell apoptosis, necrosis, and mutagenesis (Yu et al., 2020). Additionally, the insect larvae can regenerate digestive cells when exposed to xenobiotics, which cause changes in the midgut region (Castagnola & Jurat-Fuentes, 2016; Stojanovic et al., 2021). Given that apoptosis also takes a part, as one of the main processes, in midgut remodeling, it is important to distinguish the cause of the cell death by describing tissue alterations in both cases: cytotoxicity and metamorphosis (Franzetti et al., 2012).

Toxicokinetics describes processes that include absorption, distribution, metabolism, and excretion of toxins which determine the potential of the organism to handle the chemical. Toxicodynamics quantitatively describes the effect that toxin has on biological systems (Arena, 1976). The toxicodynamics and toxicokinetics of xenobiotics in *C. riparius* couldn't be understood without a description of the changes that occur during the life cycle of the healthy organism.

This study aims to describe potential changes in the morphology of the digestive system during the ecdysis of *C. riparius* larvae using histology. By defining them, we could detangle the tissue alterations caused by the direct toxic influence of xenobiotics versus indirect ones that induce ecdysis.

Experimental

Model organism

In this study, *C. riparius* Meigen, 1804, obtained from the stock culture housed at the laboratory of the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Nis, was used. The culture is formed and maintained following OECD guidelines (OECD, 2004a). The population is cultured in glass tanks filled with a mixture of distilled and tap water (1:1) and cellulose sediment. The temperature was adjusted to 23˚C±2˚C, with a 16/8 h photoperiod and constant aeration.

Experimental setup

To detangle histological changes of the midgut during metamorphosis from the ones induced by toxins, the population of *C. riparius* larvae from one egg mass was used. The experiment was conducted in laboratory conditions at the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Nis. A 700ml glass jar was filled with 105 cm^3 of coarse quartz sediment and poured over with a mix of distilled and tap water (50:50). The heater was placed and set to maintain the constant temperature of 23 °C ±2 °C. A 16/8h photoperiod was provided and the setup was left for 24h to stabilize the environment. The egg mass was transferred into the jar and fed regularly until the ecdysis started. After the larvae reached the 4^{th} instar, the collection of the individuals started. 10 individuals were collected each day until the emergence started. The collected 4^{th} instar larvae, prepupa, and pupa individuals were fixed in 70% alcohol and saved for further analyses.

Histology analyses

After fixation, they were dehydrated by increasing the series of alcohol (70%, 80%, 90%, and 96%) and then transferred to toluene for 10 min. The samples were placed in tissue-embedding paraffin and left overnight. The day after, samples were subjected to embedding and sectioning. Five-µm longitudinal sections, made on a Leica® RM 2125RT microtome, were stained using the combination of hematoxylin and eosin (H&E), then analyzed and photographed using Leica® DM 2500 light photomicroscope.

Results and Discussion

Out of total of 50 analysed individuals, 10 were 4th instar larvae, 22 were in the prepupal phase and 18 were in the pupal phase of development. By examining histological slides of the fourth instar larvae, all previously described elements of the midgut were detected (Stojanović et al., 2021). Morphological analyses of prepupa and pupa revealed changes in the tissue architecture of the alimentary canal during the process of ecdysis. The second layer of digestive cells was noticed in midgut region I of the prepupal stage when compared to the midgut region I of the 4th instar larvae (Figure 1A, D).

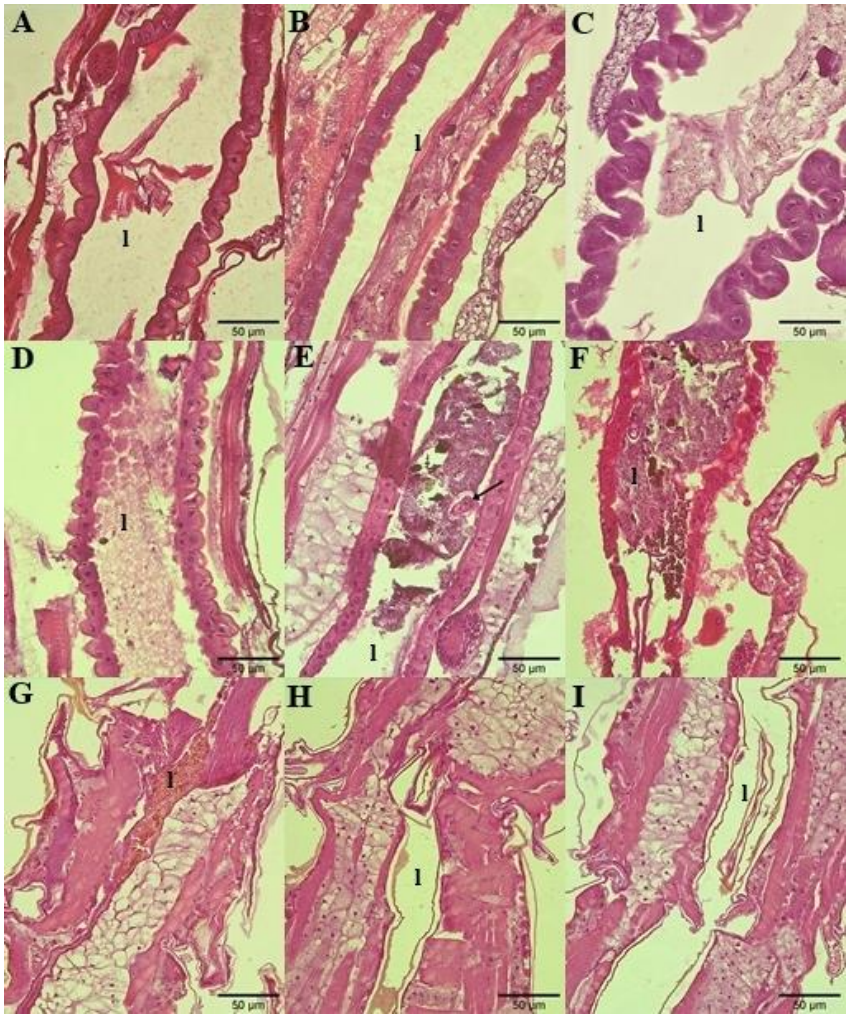

Figure 1. Photomicrographs of *C. riparius* midgut in different developmental stages. **A-C**, midgut regions I, II and III, respectivelly of the 4th larval instar; **D-F**, midgut regions I, II and III, respectively of the prepupal stage; **G-I**, midgut regions I, II and III, respectively of the pupa. Arrow (**E**) shows coated pair of cells projecting to the lumen (**l**).

In region II of the prepupal stage pair of coated cells, detached from the epithelial layer and projected to the lumen of the intestine was detected (Figure 1E). Prepupal midgut region III lost invaginations of the epithelial layer that was apparent in the larval stage (Figure 1C, F).

The midgut of the pupa showed different morphology of digestive cells in all three regions (Figure 1G, H, I). The epithelial layer was noticeably thinner and cells lost their shape that was characteristic for each midgut region (Stojanović et al., 2021). The intestinal lumen of the pupa was narrow and clear when compared to the lumen in earlier stages of development, larvae, and prepupa.

The process of metamorphosis includes certain rearrangements of the midgut of *C. riparius* (Wu et al., 2006). Richardi et al. (2018) described the midgut as one of the most sensitive tissues to the presence of toxins. To distinguish changes in the architecture of the midgut caused by ecdysis from the ones caused by toxins, a description of midgut remodeling during metamorphosis is necessary. Previous studies done on mosquitoes showed that during the prepupal stage, larval digestive cells detach from the pupal digestive cells and move to the midgut lumen completely 12h after ecdysis to the pupal stage. Eventually, pupal midgut cells differentiate and form an adult midgut with considerably reduced width (Wu et al., 2006). This strongly correlates with our study, where a detachment of the midgut cells was observed in midgut region II of the prepupal stage. The study on *Heliothis virescens* described midgut remodeling as a result of two overlapping processes which include degrading the old epithelium and generation of a new one. In the larval stage, the need for intake and accumulation of nutrients is increased so the midgut cells are metabolically active with a well-developed brush border. During the ecdysis, regenerative stem cells take the lead role and replace the larval midgut with the adult midgut (Tettamanti et al., 2007). Cell proliferation was noticed in the present study as well, where the second layer of digestive cells appeared in midgut region I. It was believed that adults of Chironomidae are not feeding because of their non-functional mouthparts, but few studies have shown that they indeed feed on sucrose (Burtt et al., 1986; Foucault et al., 2018). As it was visible on histological sections of the pupa, the lumen was more narrow and empty in midgut regions II and III, while in region I some intestinal content was detectable but distinct from the one in previous stages of development.

Conclusion

The importance of describing the changes that happen during the ecdysis lies in using histopathology as a method for ecotoxicological assessment, where normal remodeling of the midgut could easily be misunderstood as a change caused by toxin exposure (Stojanović et al., 2021). The present study showed that remodeling of the midgut does occur during the metamorphosis of the *C. riparius* where stem cells proliferated and provided new, pupal digestive cells that replaced the larval intestinal epithelium. While the new layer of digestive cells was generated, the larval digestive cells detached from the midgut and moved to the intestinal lumen. As a result, the newly formed epithelial layer of the midgut was thinner with a more narrow intestinal lumen without any visible content in regions II and III. The change of the environment and feeding behavior during different stages of development of *C. riparius* probably demands different organization of the alimentary canal that could satisfy its adult stage requirements.

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Conflict-of-Interest Statement

The authors did not declare any conflict of interest.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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