Entomology and Applied Science Letters
Volume 5, Issue 3, Page No: 35-40

Available Online at: www.easletters.com

ISSN No: 2349-2864

AFM and CT Study of Zophoba Smorio Morphology and Microstructure

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ABSTRACT

In recent years, there has been a growing interest in the use of insects as food and stuff for production of new feeds for farm animals. At the moment, more than 2000 species of edible insects have been described in the literature. The increased interest is based on the potential of using insects to convert low-quality organic materials into high-quality feed and food. At the moment, there are a lot of aspects that should be studied before starting to use insects' biomass in food and feed technologies, including cultivation and industrial processing. Earlier, the authors found that Zophobas morio Fabricius, 1776 (Coleoptera, Tenebrionidae) can change the color in case of melanin-based feeding. To understand the mechanism of this phenomenon, there were carried out researches on the morphology and microstructure of Z. morio larvae, which results have been described in the article.

Keywords: Invasion; Insect; *Zophobas morio*; Larvae, AFM, Computed tomography; Chitin; Protein, Melanin; Chitin and melanin complex.

HOW TO CITE THIS ARTICLE: Pushkin, Sergey Viktorovich, Nagdalian, Andrey Ashotovich, Rzhepakovsky, Igor Vladimirovich, Povetkin, Sergey Nikolaevich, Simonov, Alexander Nikolaevich, Svetlakova, Elena Valentinovna, AFM and CT Study of Zophoba Smorio Morphology and Microstructure, Entomol Appl Sci Lett, 2018, 5 (3): 35-40.

Corresponding author: Pushkin, Sergey Viktorovich
Received: 20/03/2018
Accepted: 28/07/2018

INTRODUCTION

Zophobas morio is one of the most promising insects for cultivation and industrial processing. Initially, this species was cultivated in artificial conditions as feed for exotic animals. The prospect of using this insect in industrial processing has been based on extremely high fertility, short life cycle and high feed conversion. The conversion of feed in the larval stage in these insects reaches 80%, which is several times higher than the similar indicators of any vertebrates [1].

As a part of the implementation of the State Contract 1701GS1/24288 of 23.11.16 "Development of a method for obtaining fodder and food products from insects with high protein content, which have prophylactic and immune-stimulating properties" (START Program, Fund for Assistance to Small Innovative Enterprises in Science and Technology), the phenomenon of change in the color of larvae of Zophobas morio was observed when feeding with melanin-containing feed stuff (figure 1).

The detected phenomenon has not been described earlier in scientific sources and, apparently, it is associated with the complexion property of chitin, which have been manifested even in the process of vital activity. To study this process, it was decided to carry out research on the chitin cover of Z. morio by atomic force microscopy and magnetic resonance imaging methods.

Scientific research based on the State Contract 1701GS1/24288 needed performing new tasks related to the study of morphology and physiology of Zophoba smorio larvae.

A B C

Figure 1 . Zophobas morio color reaction on melanin-containing feed stuff

Type of feed stuff:
A – sawdust
B – red belt fungus
C –Gryllusbimaculatus biomass

MATERIALS AND METHODS

The study of Zophoba smorio surface was carried out by atomic force microscopy (AFM). The larvae were placed on a solid substrate – mica, which was considered as an optimal surface for sample preparation of biological objects. The layered structure of mica allowed the larvae to be applied on a fresh chip with an atomically smooth surface. The prepared larvae were fixed on a substrate of aliquot water as an adhesive agent.

Atomic force microscopy of Z. morio was carried out using the installation of Ntegra-Spectra/NT-MDT in semi-contact mode using the cantilever NSG01. After installation of mica with larvae in a holder of the microscope, a suitable site with the optical system of preliminary search was chosen. Then, a scan field size 60x60 μm^2 was conducted. For more detailed visualization of the surface, individual areas in larger resolution were studied. The obtained images of the larvae surface were processed using the software tools in Nova Px (NT-MDT).

Three-dimensional structural properties of Zophoba smorio were determined using a Skyscan 1176 X-ray microcomputed tomography system (Bruker, Belgium, http://bruker-microct.com/company/newsletter.htm).

The X-ray voltage and current were 65 kV and 380 μA, respectively, at filtration 1 mm Al.

The scanning of the whole sample volume was carried out using 8.77 μm pixel size as resolution, taking about 60 minutes. The scan protocol included rotation through 180° at 0.3° as rotation step, and an exposure time of 1175 ms per frame, at the frame averaging of - 2.

Three-dimensional reconstruction of samples was created using the reconstruction NRecon software (Version 1.7.1.0, Bruker, Belgium). For tomographic reconstruction, the following settings were used: no smoothing, ring artifact correction=14, and beam hardening correction=51%.

CTAn software (version: 1.13.11.0, Bruker microCT, Belgium) was used for the quantitative analyses upon the reconstructed structure. Global thresholding was then used to segment the grayscale images into binary black/white images to facilitate the quantitative analysis and 3D visualization of the structure.

In order to obtain data on the size of the bone inclusions, the reconstructed sample was divided into cylinder disks (10 mm-thick) upon which the quantitative analysis was performed.

RESULTS AND DISCUSSION

The study of Zophobasmorioas bioobject included the analysis of morphological characteristics of larvae.

Cuticle of Zophobasmorio was mainly built of organic substances, and inorganic compounds were less than 1% in relation to its dry weight. The most important organic compounds of the cuticle were chitin, proteins, lipids and phenols [5].

Proteins made up one quarter to one half of the dry cuticle. Using warm water as a solvent, cuticular proteins could be divided into two fractions having a similar amino acid composition [6].

The water-soluble fraction of cuticle proteins were consisted of arthropodan. It contained several protein compounds that could be separated by electrophoretic methods. In combination with chitin, arthropod formed a polymer complex that made up the main component of the procuticula [7].

The insoluble fraction of cuticle proteins in water has not been studied enough. It contains several protein compounds, including the original protein resilin. The mechanical and optical properties of resilin are similar to rubber with only one difference – lack of fluidity. This protein accumulates in the most elastic areas of Z. moriocover. The functional importance of resilin clusters, apparently, is to provide the necessary flexibility and extensibility of the cuticle. Cuticulin is another cuticles protein, which is unable to dissolve in warm water.

Lipids, concentrated mainly in the wax layer of the epicuticula, have been represented by a wide range of chemical compounds. Free fatty acids, fatty acids, hydrocarbons, diene alcohols and sterols were found in the composition of cuticles lipids, but aliphatic alcohols have been usually absent in the cuticle..

Deaminated and nitrogen-containing phenols, which are also a part of the cuticle, have been synthesized from tyrosine and its derivative – dihydroxyphenylalanine[4].

Their atomic force microscopy (AFM) was performed to study the external layer of the larval cuticle. Atomic force microscopy compared to the other microscopy methods has been characterized by the smallest number of artifacts associated with the fixation and staining of the samples. In addition, the height and width of the samples on the AFM images gave more detailed information about of the degree of heterogeneity and polymers association [2].

The results of AFM of larvae cuticle are shown in figure 2. According to the images, chitinous covered Z. morio as an cellular structure with a regular shape, resembling the bee honeycombs (figure 2C). Width of cells was up to 10 μm (figure 2G, 2I), and the height was 120 nm (figure 2I, 2J). Macrocombs represented a cluster of microcells with a hex shape. This form of larvae cuticle was optimal for the external metabolism outside, and the formation of chitin and protein complex inside.

The nature of the forces holding such long molecules in isolated crystalline micelles has not been well studied. Probably, the formation of large fibrils from discrete chitin molecules has been linked with hydrogen bonds, which was emerged as a result of electrostatic interaction between the hydrogen atomic nucleus and electrons of oxygen atoms.

During embryonic development and the formation of the new cuticle (during molting), an internal protein layer has appeared, which was produced by epidermis cells and epidermal oenocytes. The upper part of this layer was consisted of quinone-hardened lipoprotein – cuticulin. The middle part of the layer was dense, homogeneous and thicker than the cuticulin plate. With its high permeability, the protein layer was not effective enough as the barrier limiting the penetration of water molecules or other chemical compounds through the cuticle. The protein layer contained polyphenols and diphenols, taking part in the hardening and cuticle colouring.

On proteins and polyphenols layer, there were lipid compounds of epicuticula in the form of a separate wax layer. The wax forming this layer was synthesized in the epicuticle from water-soluble precursors of the wax, which were allocated to the epidermal cells and reached the surface of the larvae cover through the pore tubules or by direct diffusion through procuticle. The wax layer played the role of a barrier that limited the transpiration and protected the larvae from the water loss.

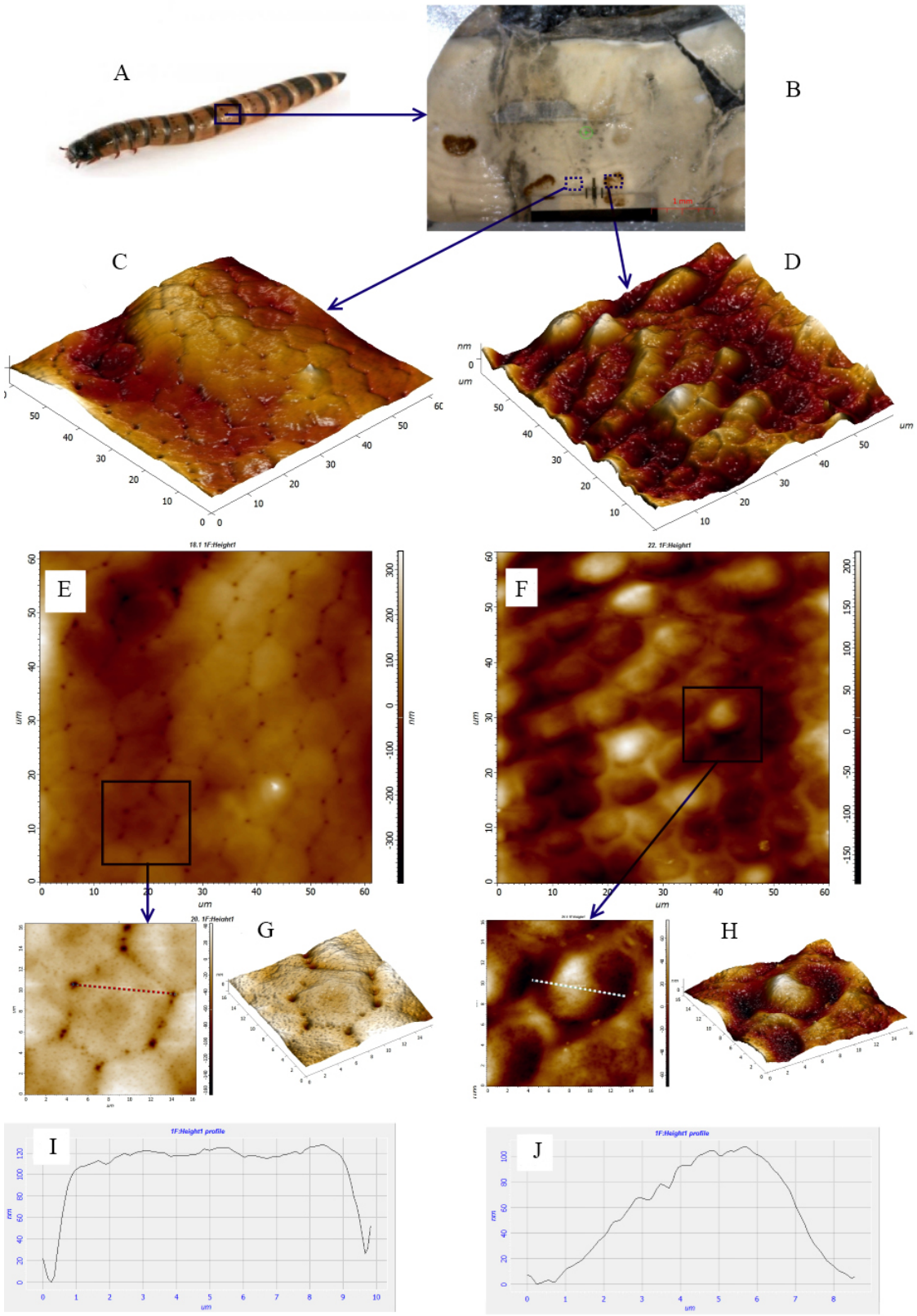

Figure 2 . AFM of Zophobas morio

The molecular chains of chitin in larvae cuticle were combined with strictly organized filamentous or lamellar micelles. Apparently, each polymer molecule of chitin included several hundred N-acetylglucosamine residues.

A more detailed study of Zophobasmorio larvae morphology was carried out by computed tomography (CT) (figure 3).

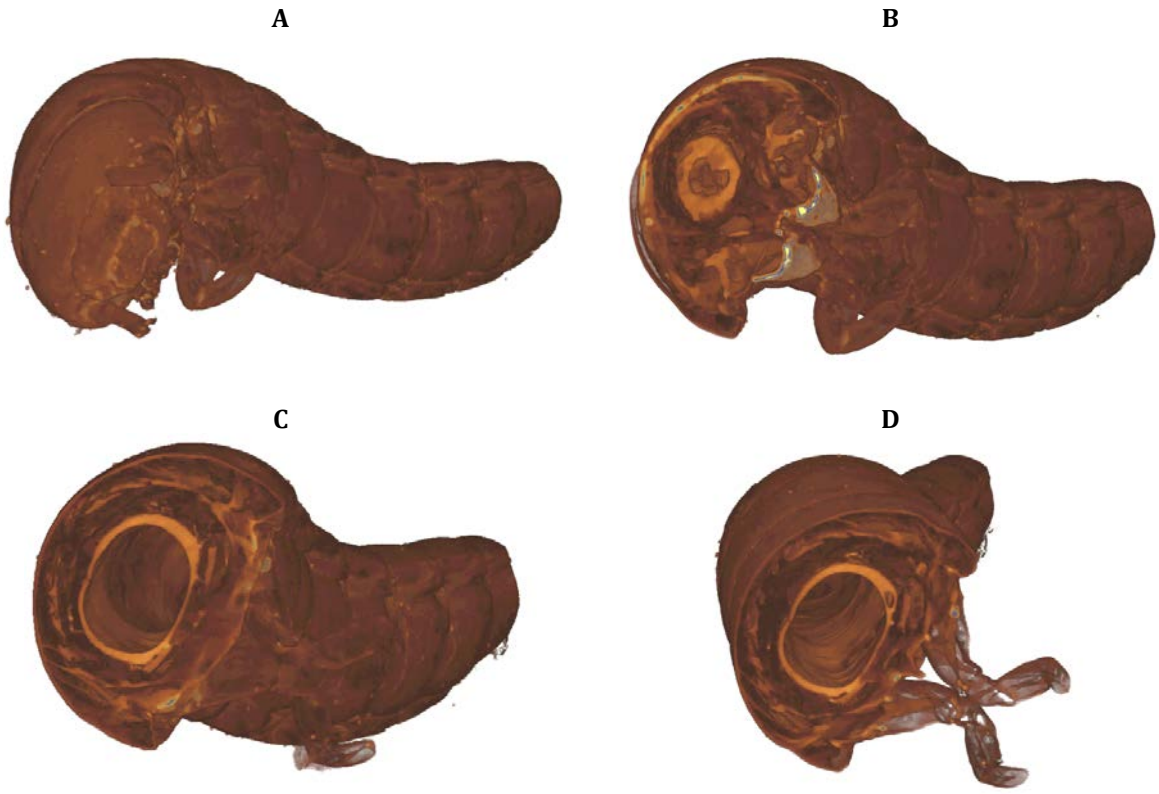

Figure 3 . CT of Zophobas morio

It was detected that the larvae surface represented by chitin, was the thinnest, but shielded and hardly permeable for radiation. Under the chitin layer, large vascular formations with contrasting hemolymph (Figure 3B) were observed. Next to those formations, there were thickened parts presumably of ganglion nature. On the ventral surface, the temporary structures (arthropods) were found. The larvae body was segmented into 10 segments (Figure 3A). Along with the entire length of the larvae body, there was a thorough intestinal cavity which clearly had visible mucous, muscular and serous layers (Figure 3C). Ground substance was characterized by the alternation of muscle fibers and areolar tissue. The intestine was simple, relatively short, without pyloric appendages, crypts or caecum. Procuticula from top to bottom was permeated by pore tubules that extended from the epidermis, and provided communication with the outer surface of the larvae cover (Figure 3D).

CONCLUSION

Thus, the study of the morphology and microstructure of Zophobas morio larvae revealed that the biological object was consisted of a thin but relatively solid chitin reinforced with protein fibers. Protein fibers due to their active centers have been associated with the

main protein mass of the insect. Apparently, the change in larval color in case of melanin-containing stuff feeding was associated with the complex formation of melanin with chitin and proteins. The isolation of chitosan-melanin complex from the resulting poly-complex was possible by homogenization, centrifugation and leaching of the protein [3], which is suggested to be examined by the subsequent studies.

ACKNOWLEDGEMENT

The paper was carried out by the authors jointly in 2017-2018 due to money resources. The authors were grateful to all those who contributed to this study.

Author's contribution:

The article was written at the expense of the authors.

Conflict of interest:

In the article, there has been no information capable of provoking conflicts of interest, with the exception of information contained in previously published articles by the Pushkin, S.V., Naghdalyan, A. A.Rzhepakovsky, I. V., Povetkin, S. N., Simonov, A. N., Svetlakova, E. V.

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