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A new method of staining larvae of Lepidoptera using carbol fuchsin

Новый метод окрашивания гусениц с использованием фукорцина

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KEY WORDS: Phycitini, *Plodia interpunctella*, caterpillars, staining, carbol fuchsin, moths, larvae, Lepidoptera.

КЛЮЧЕВЫЕ СЛОВА: Phycitini, *Plodia interpunctella*, гусеницы, окрашивание, фукорцин, чешуекрылые, личинки, Lepidoptera.

ABSTRACT. A new method of staining caterpillars using carbol fuchsin has been proposed. The recommended time of dipping larvae into carbol fuchsin is more than 2 minutes. Studying of stained specimens was carried out in glass containers filled with eucalyptus oil. Examination of stained specimens in alcohol is difficult because of rapid washout of carbol fuchsin. Further storage of larvae was carried out in alcohol due to the fact that eucalyptus oil damages the covers during prolonged storing.

РЕЗЮМЕ. Предложен новый метод окрашивания гусениц с использованием фукорцина. Рекомендуется погружать гусениц в фукорцин не менее, чем на две минуты и проводить их дальнейшее исследование в стеклянной посуде в эвкалиптовом масле. Изучение окрашенных экземпляров в спирте затруднительно из-за быстрого вымывания фукорцина. Дальнейшее хранение гусениц осуществляли в спирте ввиду того, что эвкалиптовое масло повреждает покровы при длительном воздействии.

Introduction

Staining is widely used in biology to visualize poorly visible or invisible morphological features of different organisms. Various dyes are used in entomology, for example, Evans blue dye [Zabaluev, 2021], fuchsine [Borchsenius, 1950, Danzig, 1993, Sirisena *et al*., 2013 etc.], haematoxylin [Perdoni *et al*., 2014] and eosin [Krupitsky et al., 2017].

The larva is the detrimental life stage of Lepidoptera, so its correct identification is especially important. Species identification by larvae is widely used in phytosanitary practice [Lovtsova, Kamayev, 2019]. Chaetotaxy is one of the main identification characters of the larvae covered only with primary setae. On long-fixed specimens, these structures are poorly visible or not visible at all. In some cases, the setae may be pale on the recent samples. For these reasons, it seems appropriate to use various staining methods. Acetocarmine and Chlorazol Black E are used for the staining of the caterpillar's cuticle [Komai, 1999, Gilligan *et al*., 2008] followed by mounting in Euparal or glycerin.

We propose a new method of staining total larvae with carbol fuchsin, which is used in particular for staining some bacterial spores [Haddad *et al*., 2015]. Carbol fuchsin is available, low-cost, and non-toxic, therefore, using this dye appears convenient. The primary data was published in a repository [Lovtsova *et al*., 2022].

Materials and Methods

In the experiment, 87 larvae of *Plodia interpunctella* (Hübner, 1813) were used (59 were kept for more than 50 years and 28 were kept for one year). The larvae were

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extracted from the fixing liquid, and the control photos were taken. Then, they were dipped into 1.5 ml Eppendorf tubes containing 0.5 ml of carbol fuchsin for time periods from 1 second to 5 hours. Further, the larvae were placed into Eppendorf tubes with eucalyptus oil. After that, final photos of specimens placed in a Petri dish filled with eucalyptus oil were taken (Figs 2–13).

Furthermore, after staining and taking photos some larvae were immediately fixed in eucalyptus oil for 1 day and 45 days, and others were fixed in 70% alcohol for 45 days. Then, photos of these larvae were taken.

Only larvae initially stored for over 50 years were used as a control.

Photography and analysis of photos

Images of the samples were made using Zeiss Stereo Discovery V12 microscope (lighting settings were fixed) with Canon EOS 6D camera. The stereomicroscope was focused on different structures of the prothorax on the lateral view. The camera settings were fixed at ISO 400 and shutter speed of 1/25 s. Dark frames were taken with the same parameters; the lighting of the microscope was turned off. Then, the contrast of setae was analyzed based on these images. Different setae were selected for the analysis. First, we selected images in which the analyzed setae were as sharp as possible. Then, the selected images were converted into linear monochrome 16-bit TIFF files with dark frame subtraction using the open-source Dcraw software [Coffin, 2005]. The color channels were converted with constant color temperature, no debayerization was performed. The resulting images were analyzed in the open-source ImageJ software [Rasband, 2012]. At first, 4*4 binning was performed to sum up color channels and reduce resolution of the image. Two image sections were selected: one with the cuticle near the seta (background), and the other one on the seta (Fig. 1). Using the ImageJ program, the average brightness for both areas was determined in analog-to-digital converter counts. The contrast of the structure under study was calculated as $K=|I_s-I_b|/I_b$, where K stands for contrast, I_s and I_b — for seta and background brightness and || brackets means absolute value. The dependences of the contrast of setae on the time of treatment with carbol fuchsin are shown in Figs 14–16.

Results and discussion

Examination of stained larvae in 70% alcohol without eucalyptus oil was difficult, because carbol fuchsin was washed out too quickly. Therefore, the use of oils is essential.

The contrast of all setae of control specimens before staining (larvae stored for more than 50 years) was less than 0.1, so these setae were invisible.

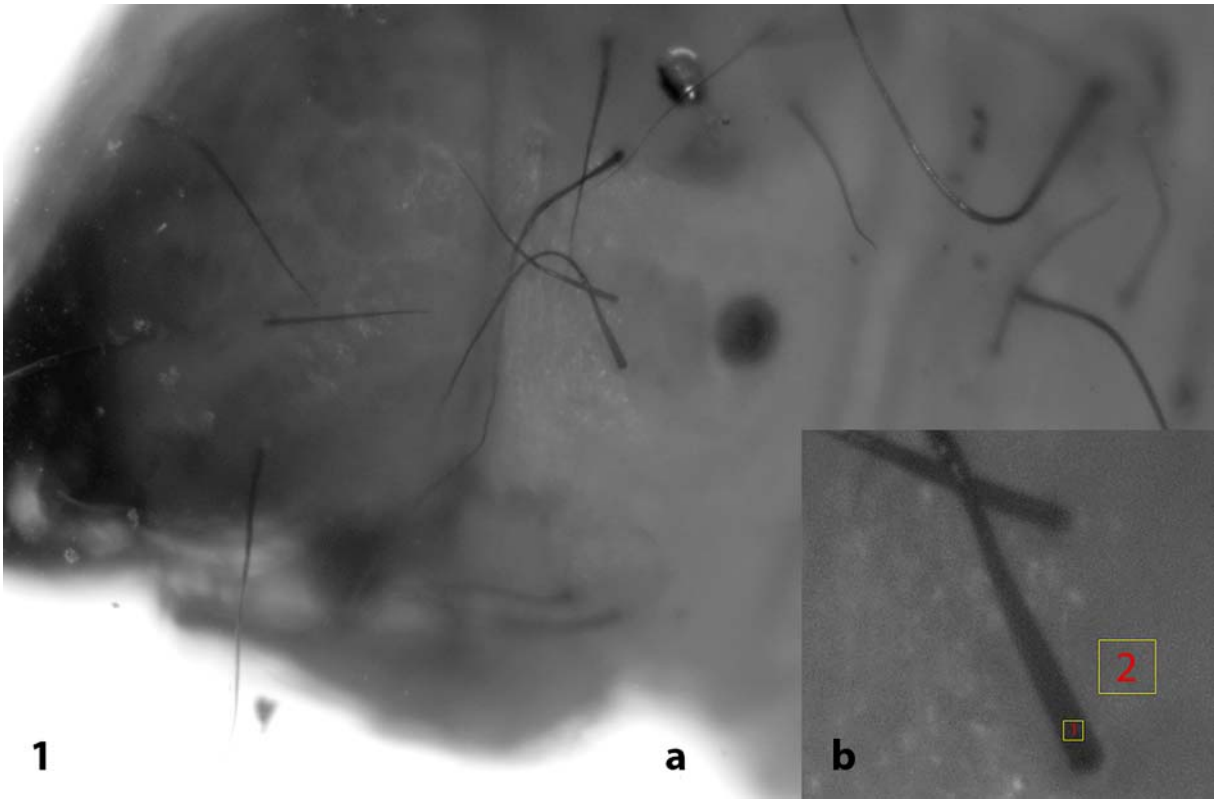
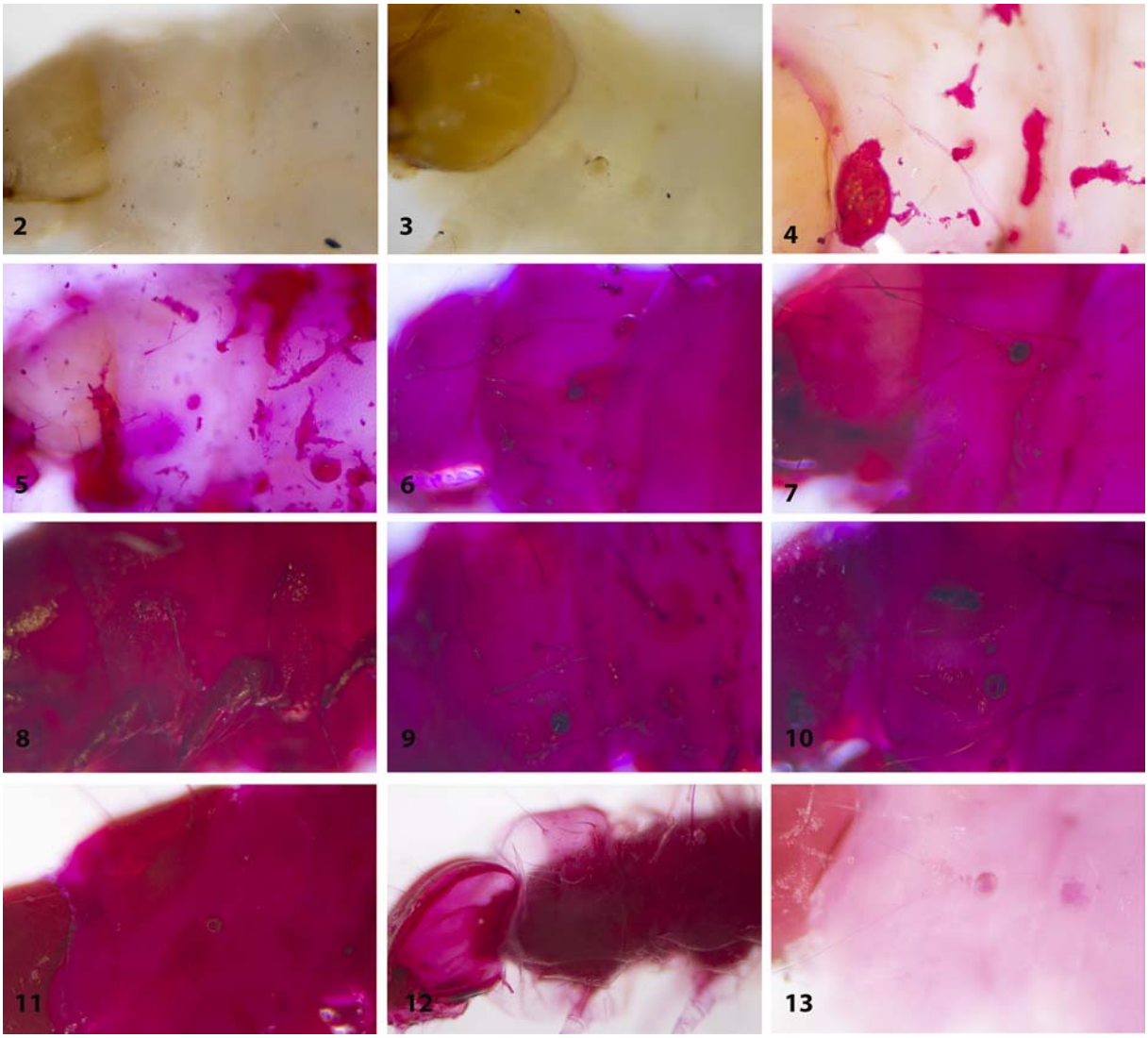


Fig. 1. Linear monochrome 16-bit TIFF files: a — general view of larvae, b — enlarged view, yellow rectangles correspond to areas where brightness for the seta (1) and cuticle near the seta (2) were determined.

Рис. 1. Линейный монохромный TIFF файл: a — общий вид, b — увеличенный вид, желтыми прямоугольниками показаны области измерения яркости щетинки (1) и фона рядом с щетинкой (2).



Figs 2–13. Parts of stained and unstained (control) larvae: 2–3 unstained larvae were kept for more than 50 years; 4–10 — immediately after staining for 5 seconds (4), 2 minutes (5), 10 minutes (6), 15 minutes (7), 30 minutes (8), 2 hours (9), 5 hours (10); 11–13 — larva stained during an hour and fixed in: eucalyptus oil for 1 day (11); in eucalyptus oil for 45 days (12); in 70% alcohol for 45 day (13).

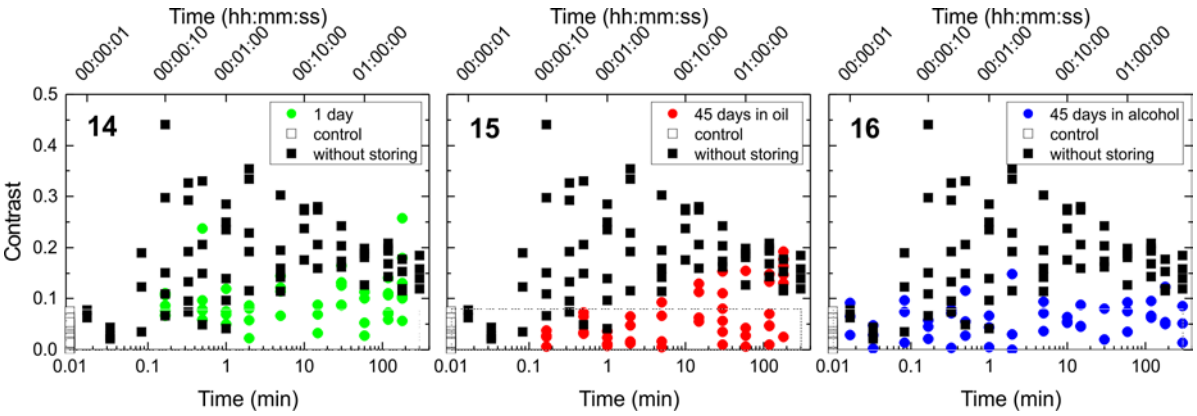
Рис. 2–13. Фрагменты окрашенных и неокрашенных (контрольных) гусениц: 2–3 — неокрашенные гусеницы, хранившиеся более 50 лет; 4–10 — сразу после окрашивания в течение 5 секунд (4), 2 минут (5), 10 минут (6), 15 минут (7), 30 минут (8), 2 часов (9), 5 часов (10); 11–13 — окрашенная в течение часа гусеница, хранившаяся в эфирном масле эвкалипта в течение 1 дня (11); окрашенная в течение часа гусеница, хранившаяся в эфирном масле эвкалипта в течение 45 дней (12); окрашенная в течение часа гусеница, хранившаяся в этиловом спирте в течение 45 дней (13).

Studies have shown (Figs 14–16) that the contrast of setae of larvae dipped into carbol fuchsin for the period from 5 seconds to 1 minute is too variable. The setae were stained extremely irregularly, some were clearly visible, others however remained colorless, while the cuticle was unstained. Starting from the staining time of 2 minutes, the contrast of the setae was consistently greater than 0.1 and different from the control ones, so all the setae were clearly visible. Staining the setae and the cuticle for more than one hour reaches maximum saturation, and the contrast remains stable from one to five hours.

Studies have shown that contrast of setae fixed in eucalyptus oil for 1 day (Fig. 14) and 45 days (Fig. 15), as well as in 70% alcohol for 45 days (Fig. 16) is less than 0.1, so these setae are poorly visible. On the other hand, eucalyptus oil damaged the specimens and they were shriveled or spoiled, while storage in alcohol preserved the quality of the larvae for research.

Conclusion

It is recommended that larvae are dipped into carbol fuchsin for more than 2 minutes and then placed in glass containers with eucalyptus oil (e.g. Petri dishes) for examination. After exploring, the specimen should be flushed in 70% alcohol, and after that put into fixing



Figs 14–16. Contrast of setae before staining (open rectangles), after staining for various time (black rectangles) in log time scale, and after staining and storing for 1 day in eucalyptus oil (14, green circles), for 45 days in eucalyptus oil (15, red circles) and 45 days in alcohol (16, blue circles).

Рис. 14–16. Контраст щетинки до окрашивания (пустые квадраты), после окрашивания в течение различного времени (черные квадраты) с логарифмической шкалой времени, и после окрашивания и хранения 1 день (зеленые кружки), 45 дней в масле эвкалипта (красные кружки) и 45 дней в спирте (синие кружки).

liquid for storage. It may be necessary to renew the fixing liquid because it will be stained.

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