Available online at http://bjas.bajas.edu.iq
https://doi.org/10.37077/25200860.2022.35.2.12
College of Agriculture, University of Basrah

Basrah Journal of Agricultural Sciences

ISSN 1814 – 5868 Basrah J. Agric. Sci., 35(2): 173-184, 2022 E-ISSN: 2520-0860

Identification of some Fourth Instar Larvae of the Mosquito (Diptera, Culicidae) using Scanning Electron Microscope (SEM) in Basrah Province, Iraq

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Received 16th April 2022; Accepted 1st July 2022; Available online 15th October 2022

Abstract: The study was conducted to determine the distinguishing characteristics of the fourth-instar larvae that used to identify the six species of mosquitoes (Diptera: Culicidae) in Basrah Province. Some morphological characteristics, pectin teeth, comb scales, lateral palatine brush filaments, and microspine patterns on the siphon were studied by using the scanning electron microscopy technique. The results showed that there are morphological differences in these micro-structures between the species, *Aedes caspius* (Pallas, 1771), *Culex pipines* (Linnaeus, 1758), *Culex pusillus* Macquart, 1850, *Culex tritaeniorhynchus* Giles,1901, *Culiseta longiareolata* (Mecquart, 1838) and *Uranotaenia unguiculata* Edwards, 1913.

Keywords: Culicidae, Fourth- instar, Larvae, Mosquito, Scanning electron microscopy (SEM).

Introduction

Mosquitoes (Diptera: Culicidae) are blood-sucking arthropods considered dangerous disease human vectors malaria, filariasis, encephalitis, yellow fever, dengue and other diseases. Culicidae is comprised of 41 genera incorporating about 3,500 species (Braack *et al*., 2018; Foster & Walker, 2019). Mosquitoes are holometablous (complete) metamorphosis. Their eggs require water to hatch, and the larvae develop through four instars before transforming into active, non-feeding pupae. The adult's wings, sucking mouthparts, and legs can be seen through the transparent pupal skin; then the adult emerges from the pupal stage to the water's surface (Rutledge, 2008). A proper diagnosis is necessary for mosquitoes control actions (Farajollahi & Price, 2013). Mosquitoes and other insect species can be classified using morphological identification, which heavily relies on various exterior traits (Tewfick *et al*., 2014; Atta *et al*., 2019) (SEM) was used for the identification because it produces a precise 3-dimensional microscopic image of the surface structure of biological materials (SEM) gives information concerning morphology (the shape and size of the particles which make up the object),topography (the surface features of an object), composition (the elements and compounds which make up the object and their relative amounts) and crystallographic information (how the object crystallizes). It has allowed scientists to obtain a better knowledge of microstructure by offering samples with unparalleled optical magnification. Hence, this technique provided an accurate description of biological and non-biological samples at the nanoscale (Sayid *et al*., 2020). SEM usually used in the describing of mosquito eggs (Schaper & Hernandez-Chavarría, 2006), however it has not been frequently used to explain and describe the exterior appearance and microstructures of fourth-instar larvae (Junkum *et al*., 2004), or adult mosquitoes (Kong & Wu, 2010). At the local studies about the mosquito in Basrah Province, Al-Yacoub (2018) determined the effect of environmental factors on *Culex pipines* larvae. However, Scanning electron microscope-Energy dispersive Spectrometer (SEM-EDS) technique was used in the agricultural sector locally to determine and identify the metal elements of date's syrup (Al Hilfi *et al*., 2019). The current study used scanning electron microscopy to identify some species' fourth-instar larvae by examining pectin teeth, comb scales, lateral palatine brush filaments, and siphon patterns (micro spines).

Materials & Methods

The study chose to focus this research on the fourth larval instar because it is an important developmental instar for mosquitoes since it marks the completion of the diagnostic traits of this stage development, which will eventually lead to the active pupa stage and then to adults.During the period of September 2020 to August 2021, mosquito larvae were collected from several sites in Basrah Province using a standard 350-ml plastic dipper. Permanent sampling sites at northern Basrah (Districts of Al-Maddina and Al-Qurna) were used in this study; temporary sampling sites throughout the province included water bodies of water that ranged from ponds to swamps to pits to manufactured ponds to water basins and other mosquito breeding areas. The first larval instars were also collected and transported to the laboratory, where they were grown in breeding cages and the life cycle tracked to corroborate the morphological classification of the larvae. Finally, the fourth-instar larvae were morphologically identified using a local taxonomic identification key designed by Jabbar *et al*. (2018) and worldwide taxonomic identification keys designed by Snell (2005) and Azari-Hamidian & Harbach (2009). SEM was used to study and Identification 4th-instar larvae according to the method published by Adham *et al*. (2013) through the following steps:

1. Fixation

Larvae were fixed in 2.5% glutaraldehyde in 0.1 buffer (7.4 solution) pH for 3 days to ensure tissue stiffness and maintain sample structure and stability.

2. Dehydration

The larvae were passed sequentially to a series of increasing concentrations of ethanol (50%, 70%, 80%, 90%, 96%, and 100%). To remove water from the specimens, the specimens were placed in a second 100% percent ethanol solution. The fourth-instar larvae were soaked in each concentration of ethanol for 5 minutes during each level of dehydration.

3. The specimens were fixed on a special holder using electrically conductive double-sided carbon tape.

4. The specimens were coated with gold inside asputter-coating apparatus to make their surfaces electrically conductive and to introduce contrast and make the sample visible under the microscope.

Fourth-instar larvae were examined using a scanning electron microscope of type 450 FEI Nava Nano SEM, Department of Physics College of Science, University of Basrah. Abdominal segments, including the siphon, (VIII+IX) of fourth-instar larvae were studied for diagnostic structures, such as pectin teeth, comb scales and certain patterns on the siphon, as well as the mouth's lateral palatal filaments.

Results

The results, using a scanning electron microscope, which examined the fourth instar larvae, appeared to indicate to indicate the presence of six species of mosquitoes that belong to the family Culicidae in Basrah Province, included: *Aedes caspius* (Pallas, 1771), *Culex pipiens* (Linnaeus,1758), *C. pusillus* Macquart,1850, *C. tritaeniorhynchus* Giles,1901, *Culiseta longiareolata* (Mecquart, 1838), and *Uranotaenia unguiculata* Edwards, 1913. Pectin teeth, comb scales, lateral palatal brush filaments, and the distribution patterns of microspines on siphon surfaces of the mosquito larvae species were also examined to differ amongst mosquito larvae species in the study, which is illustrated below:

1 -*A. caspius* (Pallas, 1771)

Microscopic examination of this species revealed that pectin teeth are produced in the form of long, thin, almost curved spines with a sharp end. The thorn is small and increases in size toward the apex of the thorn. It has It has five secondary teeth, and the last tooth is long and thin (Fig. 1-A). Comb scales are formed of two rows, each 48m in length, and each scale with a finger or tooth-like structure surrounded by all sides (Fig. 1-B). The lateral palatal filaments of the mouth brush are comprised of two sets of long filaments, and each filament has a row of regular teeth that give the filament a comb-like shape or distal rake-like structure (Fig. 1-C-1). In contrast, the other group has short and wide teeth (Fig. 1-C-2). The pattern of micro spines distribution on siphon surface with a pattern of thorns consisting of two types rows of various lengths ranged between 17.94 -23.99 µm and each row has a group of thorns vary in number according to length row and are long, sharp at the end and vary in length between 3.5-5.7 µm (Fig. 1-D.)

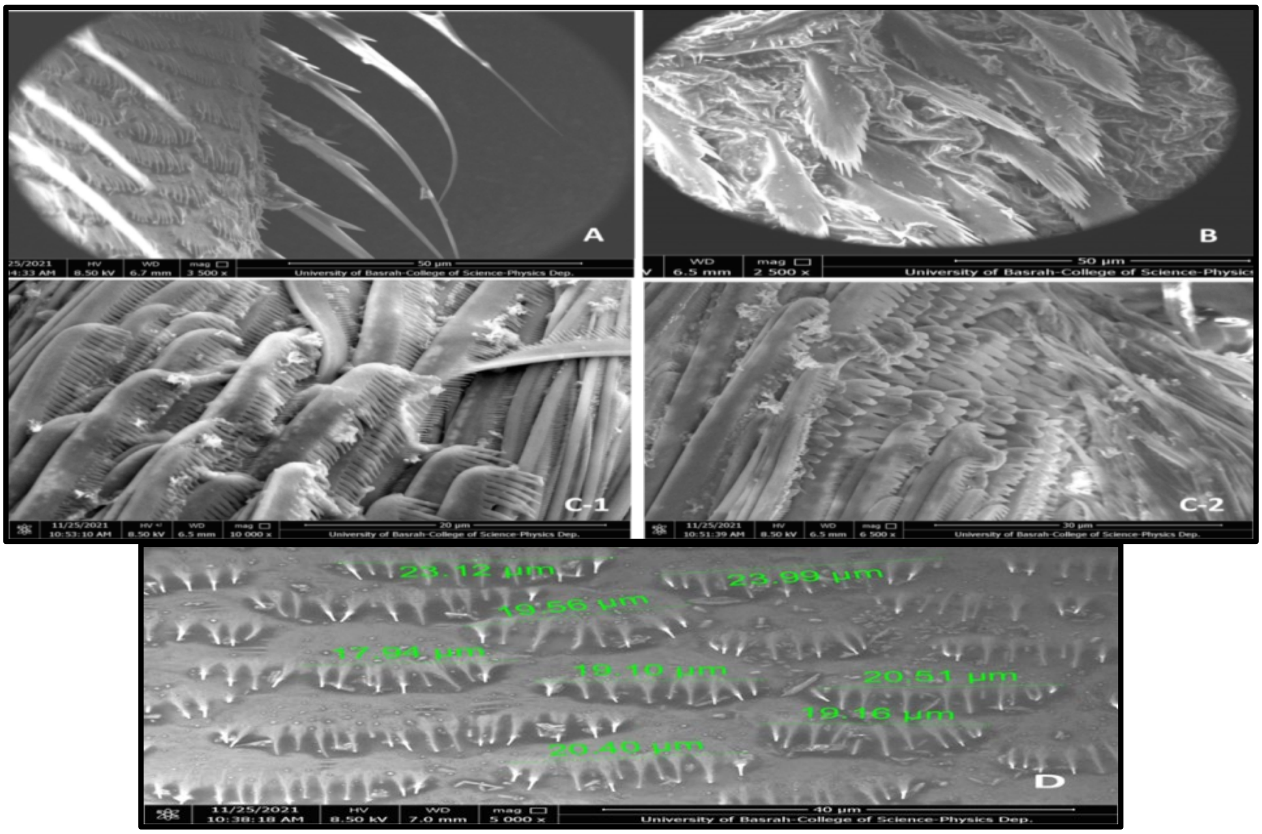

Fig. (1): Scanning electron micrograph of *A. caspius* larva, A- pectin teeth, B - Comb scale, C-1 -The first group of palatal brush filaments, C-2 -The second group of palatal brush filaments, D - Microspines pattern

2 -*Culex pipiens* (Linnaeus, 1758)

Pectin teeth have spines, with each spine length ranging between 61.26 -to 64.62 µm. It is broad at the base and has five short teeth; the last terminal tooth is long and sharp (Fig. 2-A). The comb scales are arranged in irregular rows, with 26.02 µm in length surrounded by many smooth teeth in the fringed form, and are not pointed (Fig. 2-B). The lateral palatal brush filaments have a structure akin to a broom. It also has many simple teeth (Fig. 2-C). The patterns of microspines on the siphon surface are organized in rows of variable lengths ranging from 13.16–18.55µm and take the shape of fish shells, and the fine spines not plainly projecting emerge from all rows, which vary in number in each row between 11–14 spines (Fig. 2-D)

3 -*Cx. pusillus* Macquart, 1850

Pectin teeth long, have sharpened spines ranging in length from 45.01-54.64 µm. Each spine has seven short base teeth and ends with a long, sharp tine (Fig. 3-A). The comb scales are arranged in rows; the length of each one varies from 23.19-to 25.81 µm, consisting of many finger-like structures, protrude from the sides and bottom of the comb scales (Fig. 3-B). The filaments of the lateral palatine brush do not differ much from those of the former species, and the filaments bear long, sharp-ended teeth, giving the filaments the shape of a broom (Fig. 3-C). While the patterns of microspines the siphon surface are also divvied up into rows of close length, ranging from 11.17–13.49µm, small and short spines emerge from them, prominent and slightly pointed at the end (Fig. 3-D).

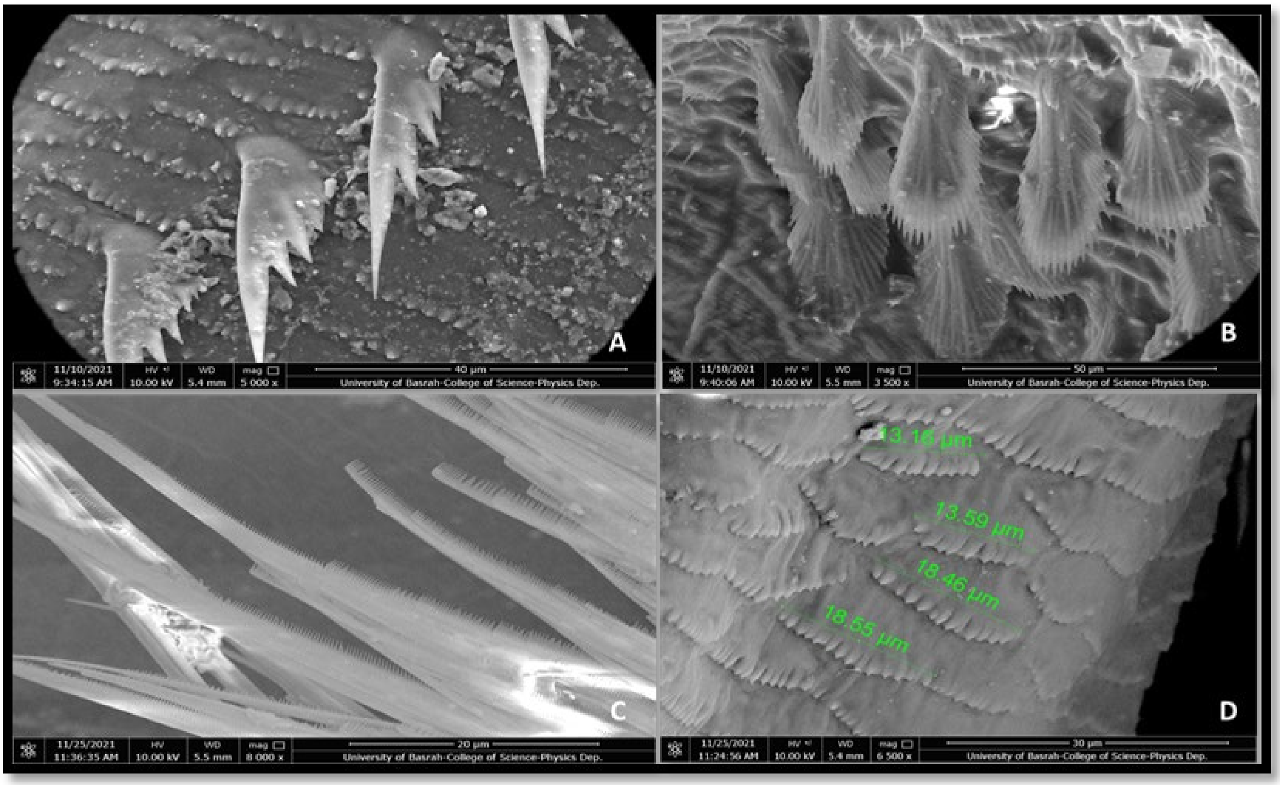

Fig. (2): Scanning electron microscope of *C. pipiens* larva, A- Pectin teeth, B- Comb scale, C- Lateral palatine brush filaments, D- Microspine patterns.

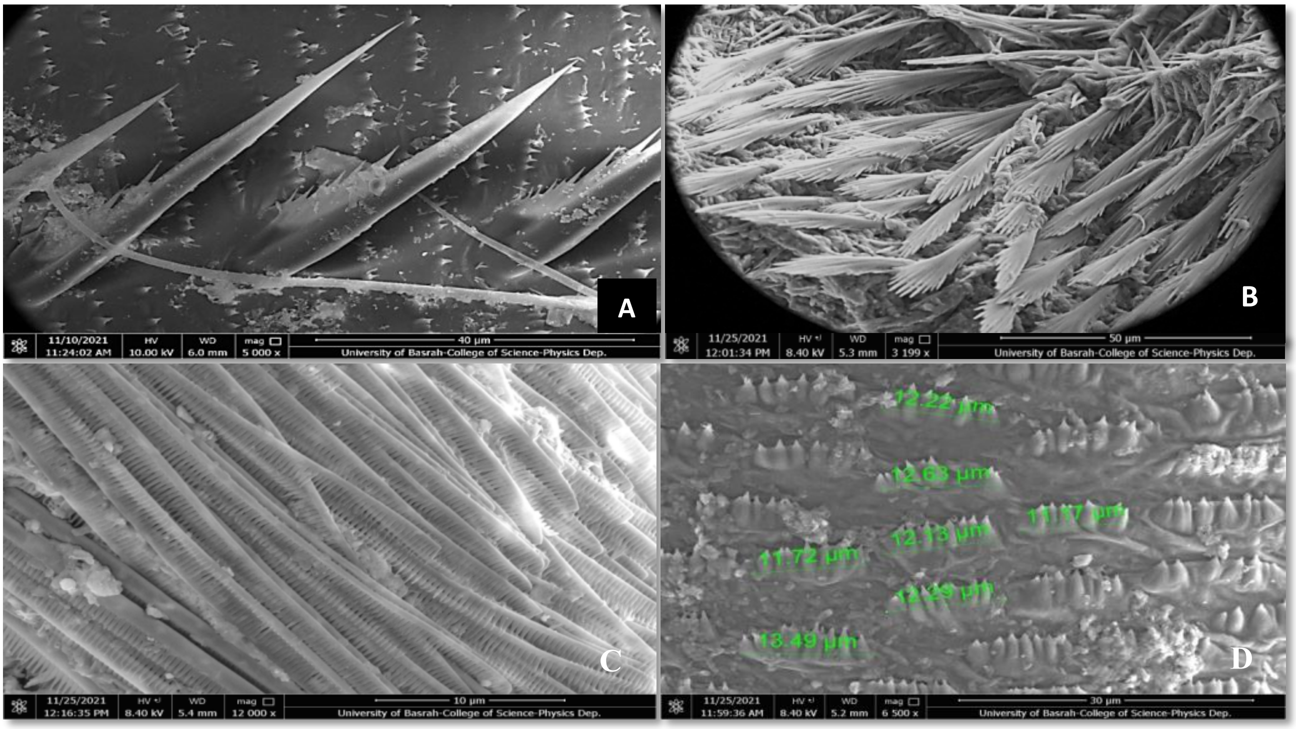

Fig. (3): Scanning electron microscope of *Cx. pusillus* larva, A - Pectin teeth, B-Comb scale , C - Lateral palatine brush filaments , D - Microspine patterns

4 -*Culex tritaeniorhynchus* Giles, 1901

Pectin teeth appeared with long 60.22 µm, pointed ends, and dentated with 10 secondary teeth of varying sizes, gradually increasing in length until they reach the final tooth that is well prominent and sharp (Fig. 4-A). Comb scales are not much different from the comb scales of *Cx. pusillus* larva, with lengths ranging from 27.11-to 36.34 µm (Fig. 4-B). The filament of the lateral palatal brush shows the teeth in a bilateral shape, and the ends of each two teeth join together to form two pairs of teeth along the brush filaments (Fig. 4-C). The surface siphon patterns' microspines are arranged in a form ranging in length from 13.21-to 18.87 µm, from which emerge narrow and conspicuous spicules of similar size, spaced extensively and pointed at the ends, giving the siphon surface a pattern resembling the shape and arrangement of shark teeth (Fig. 4-D).

5 - *Culiseta longiareolata* (Mecquart, 1838)

Fig. (5 -A) is shows long pectin spines and has one short lateral tooth, and the ultimate tooth is longer and sharper, with a length reaching 99.21 µm. The comb scales are arranged in rows and equipped with structures resemble finger. The lateral ones are small and unnoticeable, while the lower ones are long and sharp (Fig. 5-B). The filaments of lateral palatal brush have long and sharp teeth regularly arranged along with the filament (Fig. 5-C), while the patterns of the siphon surface give the appearance of similar to cactus plant leaves, and rows form tripartite groups with spicules of variety of sizes, short, base-wide, and not prominent at the end (Fig. 5-D).

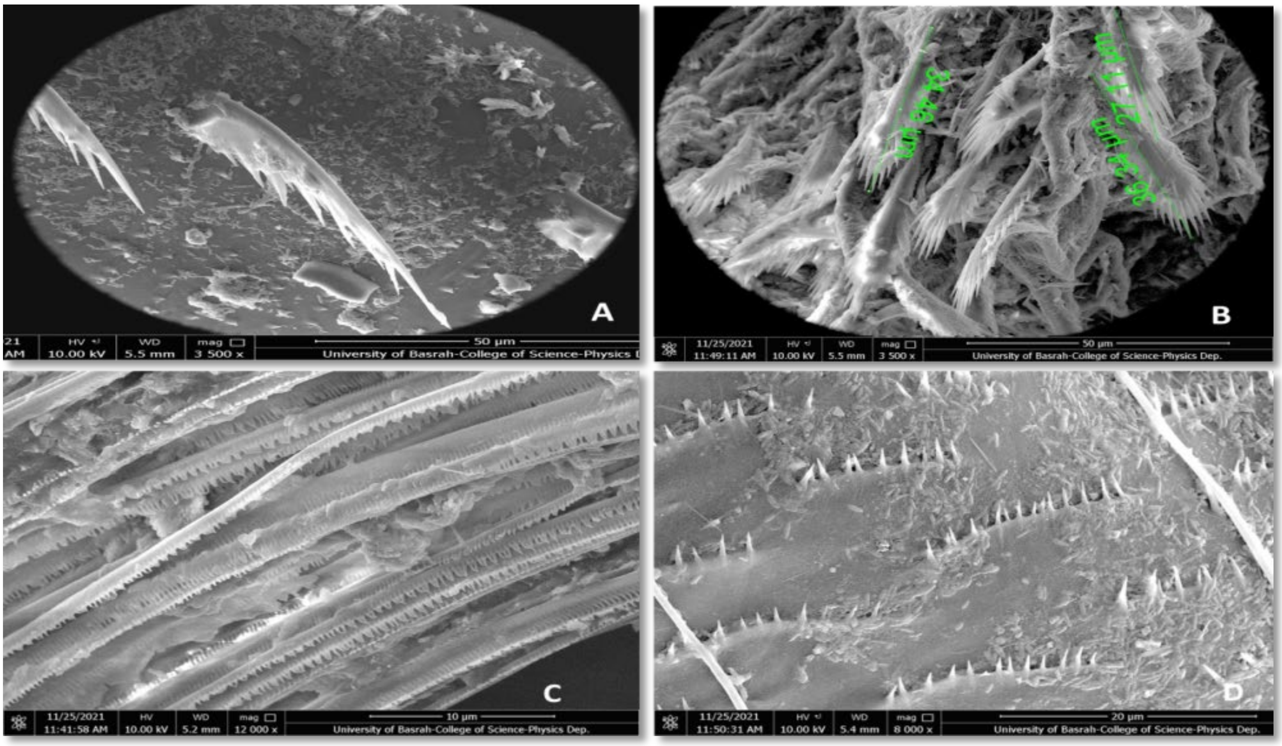

Fig. (4): Scanning electron microscope of *C. tritaeniorhynchus* larva, A- Pectin teeth, B-Comb scale, C- Lateral palatine brush filaments, D- Microspines patterns.

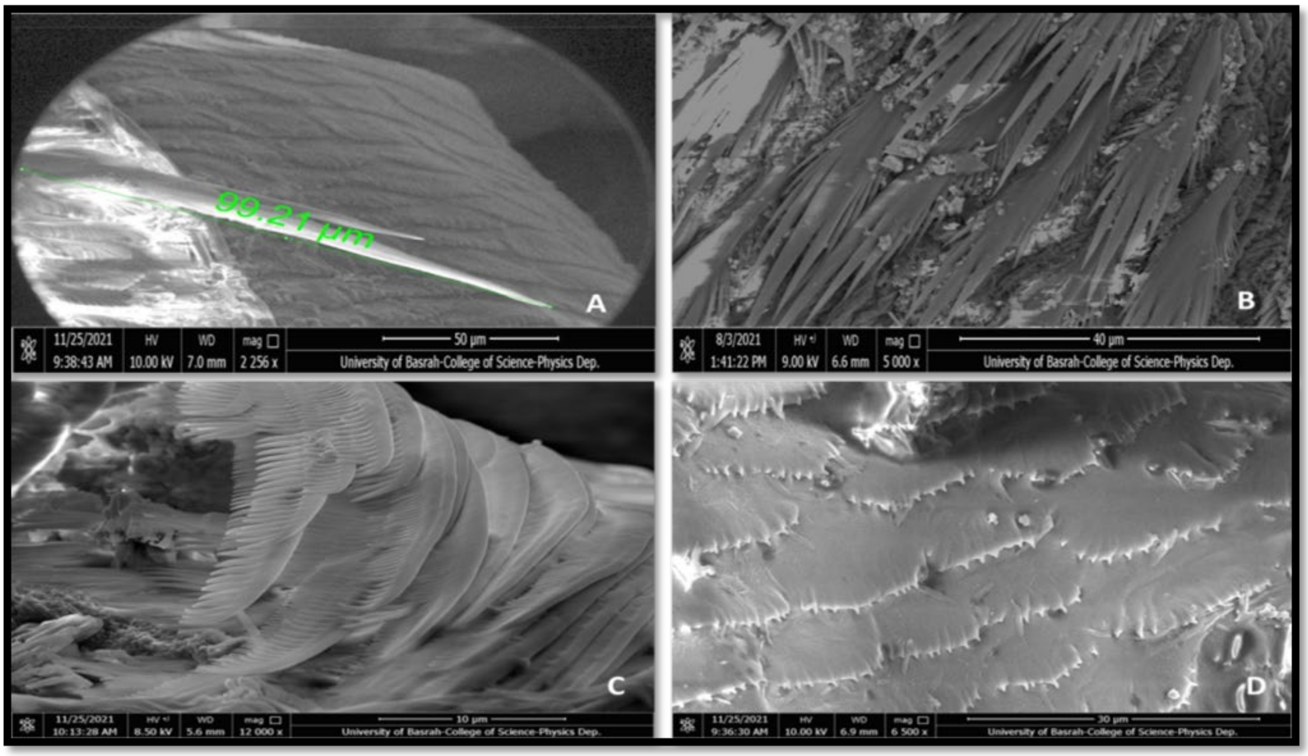

Fig. (5): Scanning electron microscope of the *Cs. longiareolata* larva: A- Pectin teeth, B- Comb scale, C - Lateral palatine brush, D- Microspines patterns.

6 -*Uranotaenia unguiculata* Edwards, 1913

The larva of this species is distinguished by the presence of rectangular plate called sclerotized plate on terminal abdominal segment (VIII) (Fig. 6-A).Pectin teeth differ from all pectin teeth of other species, have a broad base and a narrow end, with small teeth on both sides that increase in size towards the upper of spine and a medial incision that extends from the base to the middle of the spine (Fig. 6-B). Comb scales originate from the sclerotized plate with 6 scales that include large, broad, and strong spines, as well as a sharp end. The middle spine is the longest and is fitted on both sides with other fine spicules that reach the middle of the strong spine (Fig. 6-C). The filaments of lateral palatine brush have broad teeth and others are narrow and long (Fig. 6-D). Microspine patterns have very fine, indistinct, and not sharp spicules arranged rows ranging in length from 17.64 - to 20.17 µm similar to water waves (Fig. 6-E)

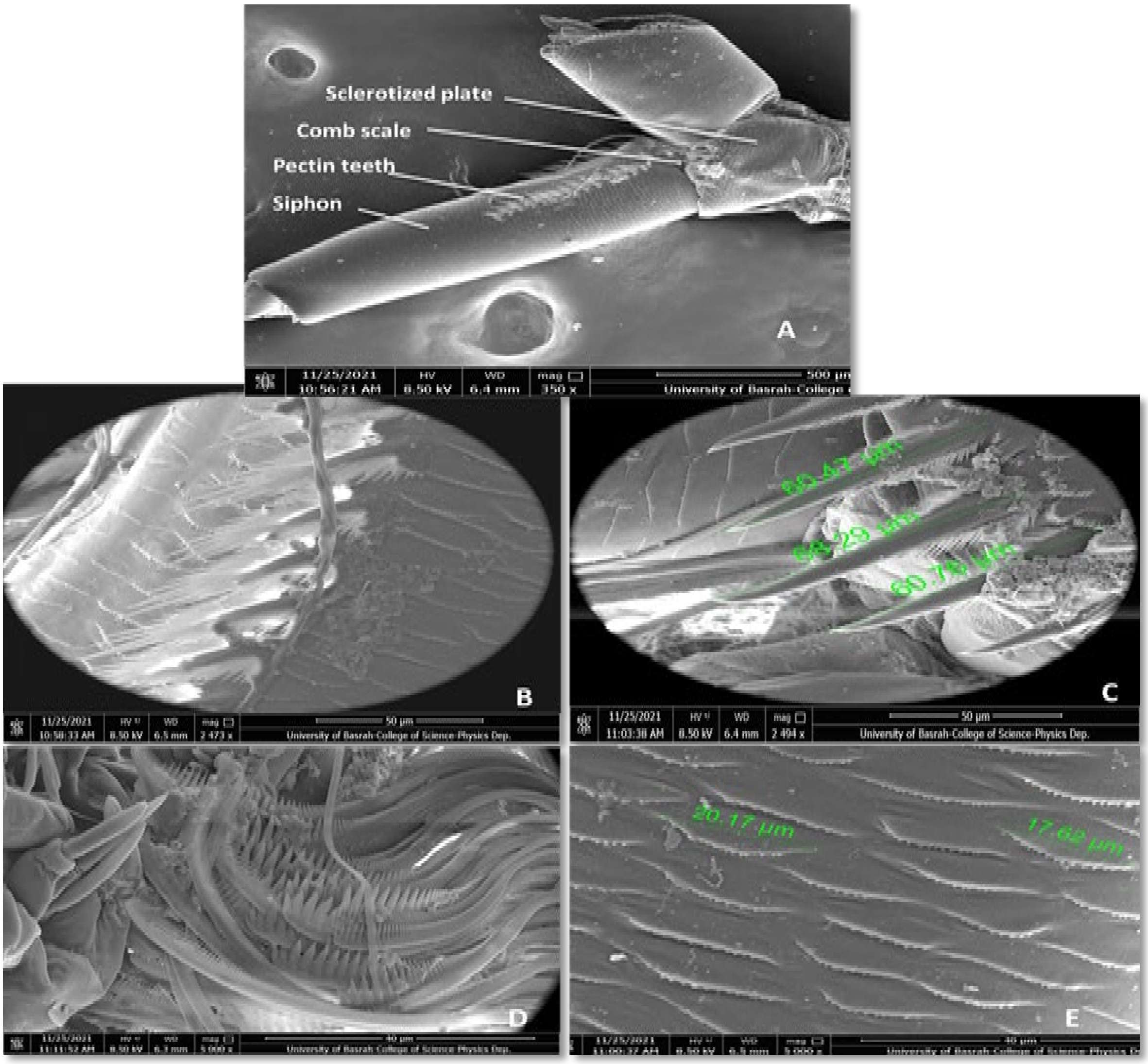

Fig. (6): Scanning electron microscope of *Uranotaenia unguiculata* larva, A- Sclerotized plate B- Pectin teeth, C-Comb scale, D - Lateral palatine brush filaments, E- Microspine patterns.

Discussion

A. caspius is a common species in Iraq and specifically in Basrah Province, the siphon is barrel-shaped with long, sharp-end and curved pectin teeth, with five serrations, and the comb scales arranged in two rows. The description of this species agreed with what has been described by Tewfick *et al*. (2014), in Egypt who indicated that the siphon of this species, which was illustrated by a scanning electron microscope. The pectin teeth are recognizable as they are made up of secondary spicules. The lateral palatine brush is made up of a distal rake-like structure that has pointed teeth; the lateral palatine filaments are one of the filaments which are engaged in the process of aqueous flow (Lacoursière *et al*., 1999). The findings of Dahl (1978), who observed the microspine siphon patterns of six different *Aedes* larvae using taxonomic features for the very first time. The microspines are distributed in a specific type-specific pattern resulting from the larval membrane growth .The comb scales were described as having a clear apical and a medial spine as stated by (Al Ahmad *et al*., 2011).

Cx. pipiens or common house mosquito is the most widely distributed species worldwide (Al-Doaiss *et al*., 2021), and *Cx. pipiens* species have been recognized as important vectors of diseases transmitted by medical and veterinary arthropods (Kasai *et al*., 2008). The larvae of this species are characterized by the fact that the siphon is long and has 4 seta bundles of 6 spicules and bearing 10 pectin teeth with 5 serrations. According to the results of Jabbar *et al*. (2018), the terminal abdomen segment(VIII)possesses comb scales dispersed in rows; each scale have a fringed structure surrounded by numerous cilia that are just not sharp. Additionally, the pectin teeth range from 8 to 6 have 5-6 secondary teeth .The scanning electron microscope showed that the pectin teeth are broad and serrated with secondary teeth; this results were in agreement with Tewfick *et al*. (2014) findings by (SEM) in Egypt about the siphon of this species and pectin teeth; and about the sutures of the lateral palatal brush which was found to have tapered and less pointed teeth by using an electron microscope.

This species' fourth- instar larval morphology was studied by Guntay *et al*., (2018), who focused on the anal segment of the tenth abdominal segment and the pectin teeth on its siphon and spicules; they identified the head seta and the comb scales at the top of the last ventral segment of the larva.

Dehghan *et al.* (2016) studied the shape of larval siphons in Iran and noted that the average number of branches for 1a-S and 1b-S seta was 6-7. In India, comb scales have been observed as fringed on both sides; the apex was equally, and the distal pectin spines have 2-5 teeth of different sizes that emerge close to each other (Moirangthem & Singh, 2018). This results agreed with our current description by (SEM) Mouth brush filaments have simple, comb-like teeth. There may be a link between the sort of food that the larva feeds and its development. Siphon spicules (microspines) are an excellent example of species-specific patterns. Dahl (1978) stated that these patterns arise directly from the membrane or skin of the larva, and it is a form of growth that occurs to the membrane of the larva.

Cx. pusillus was recorded in northern Iraq by Abul-Hab (1967); it distributes in central and southwestern Asia and North Africa, and was first recorded outside the ancient polar region (Harbach, 1998) larvae of this species have medium-sized siphon with many spicules which carry pectin teeth formed of sharp-ended spines with seven small basal teeth and finishing with a long and sharp tooth. Tewfick *et al.* (2014) described that filaments of the palatine brush have less dentition and that the pectin teeth and the comb scales are fat.Azari-Hamidian & Harbach (2009) indicated siphon index (SI) was about 3.0 (2.6–3.2); pecten was at least 0.5 length of siphon; 2 apical setae 1-S (siphonal tufts) were at least as long as width of siphon at point of attachment; 2 pairs of seta 1-S inserted within pecten; anal papillae (gills) about 0.5 length of saddle. Al Ahmad *et al*., (2011) described the larvae of this species, displaying that the seta of the S-1 siphon are arranged in a single row throughout the length of the siphon, with 6-8 seta arising along the posterior midline. The filaments of the palatine brush take the shape of a comb and are equipped with less squishy teeth. The pattern of microspines has short rows of fine spicules due to the nature of the growth of the larval membrane of this species.

The siphon of *C. tritaeniorhynchus* larvae is long and narrow and has 5 seta bundles. The siphon carries sharp-end and serrated pectin teeth with 10 teeth that vary in size and gradually increase in length. The comb scales have finger structures and are arranged in rows. Distal pectin teeth with 7 or more protuberances of almost the same size can be found on the larva's abdominal segment (VIII). (Assany *et al*., 2012).

Al Ahmad *et al.* (2011) recorded it in Saudi Arabia and noted that the Seta 5-C with three branches; seta 1-S longer than the diameter of the siphon at the point of attachment. Mahgoub *et al*. (2020) described the larvae of this species in Sudan and noted that the C-1 vertical spicule appears in the form of adark spine, and the C-5 seta has 3-5 branches and the number of comb scales on the VIII abdominal segment is more than 50. Abul-Hab (1967) described precise structures of the siphon without using electron microscopy; the ciliated comb scales, pectin teeth, palatal brush filaments, microspines patterns with a species-specific pattern were described for the first time and adopted as an important feature in species identification.

The species *Cs. longiareolata* is rare and was recorded in northern Iraq by Abul-Hab (1967). Larvae have a short, thick siphon, one pair of basal seta, and long, pointed pectin teeth. Spiny, pointed comb scales correspond with (Azari-Hamidian & Harbach, 2009). Nabti & Bounechada (2020) mentioned that it is easy to distinguish the larva *Culiseta longiareolata* as the siphon is short and the saddle is incomplete. The pectin teeth are tall with one tiny secondary tine and the last pectin spine seems to be at the top of the siphon, around 0.75 length of the siphon. Some comb scales are pointed and sharp (Azari-Hamidian & Harbach, 2009). Abu El-Hassan *et al*. (2021) found that the siphon has one pair of seta, and the pectin teeth have strong, spaced spines, which is congruent with the local model in our findings. The terminal abdominal segment of *Uranotaenia unguiculata* larva has sclerotized plate from which 6 to 7 comb scales emerge, with sharp-ended spines of varying lengths which are serrated with fine teeth. However, the siphon has pectin teeth including around 15 thorns and has a distinguishing dentated shape on both sides with long secondary teeth; this results was in agreement with Azari-Hamidian & Harbach (2009) who found that the abdominal segment (VIII) has a lateral or lateral dorsal plate. This study's findings were similar to those of Al Ahmad *et al*., (2011) in Saudi Arabia, who found that the pectin teeth have a different shape and the head was much longer. However, results of current study found that the pectin teeth have an unusual shape and that the comb scales are formed from a sclerotized plate.

Conclusions

The results of current study indicated that the scanning electron microscope could be an effective method for identifying the fourth instar larvae of some mosquito species; SEM could be alternative way for the conventional methods of identification. This could be particularly useful for identifying more subtle structures, including comb scales, pectin teeth, lateral palatine brush filaments, and certain siphon patterns (micro spine). The presence of phenotypical differences in these structures, as well as the spicules on the surface of the siphon, could be used to discriminate between the studied mosquito species.

Acknowledgements

The authors thank the Deanship of the College of Education for Pure Sciences for their cooperation, as well as the Deanship of the College of Science Department of Physics, represented by Prof. Dr. Mazen Awni, for providing assistance us in conducting a scanning electron microscope examination of the specimens under study.

Conflicts of interest

The authors declare that they have no conflict of interests.

Contributions to authors

W.H.A.: Sample collection, Data collection, write the manuscript**.**

D.K.K.: Statistical analysis, Read and revise the manuscript.

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تشخيص بعض يرقات البعوض في الطور الرابع (Diptera , Culicidae) باستخدام المجهر الإلكتروني الماسح (SEM) في محافظة البصرة، العراق

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الملخص: أجُريت الدراسة لتحديد الصفات المميزة ليرقات الطور الرابع لاعتمادها كصفة تشخيصية للتعرف على ستة أنواع من البعوض ضمن الأجناس الأربعة (Diptera: Culicidae) في محافظة البصرة وذلك بالاعتماد على التشخيص بواسطة تقنية المجهر الالكتروني الماسح اذ لوحظت بعض الصفات المظهرية لليرقات وهي اسنان البكتين وحراشف المشط وخيوط الفرشاة الحنكية الجانبية وانماط السيفون الشوكية microspine وقد جمعت اليرقات خلال المدة من ايلول 2020 الى اب 2021 وبينت النتائج ان هناك اختلافات مظهرية في هذه التراكيب الدقيقة ما بين الانواع قيد الدراسة التي تضمنت: *Culex pusillus* Macquart,1850 و *Culex pipines* (Linnaeus,1758) و *Aedes caspius* (Pallas,1771) و *Culiseta longiareolata* (Mecquart, 1838) و *Culex tritaeniorhynchus* Giles,1901 و *Uranotaenia unguiculata* Edwards, 1913.

الكلمات المفتاحية: عائلة البعوض، الطور الرابع، اليرقات، البعوض، المجهر الالكتروني الماسح.