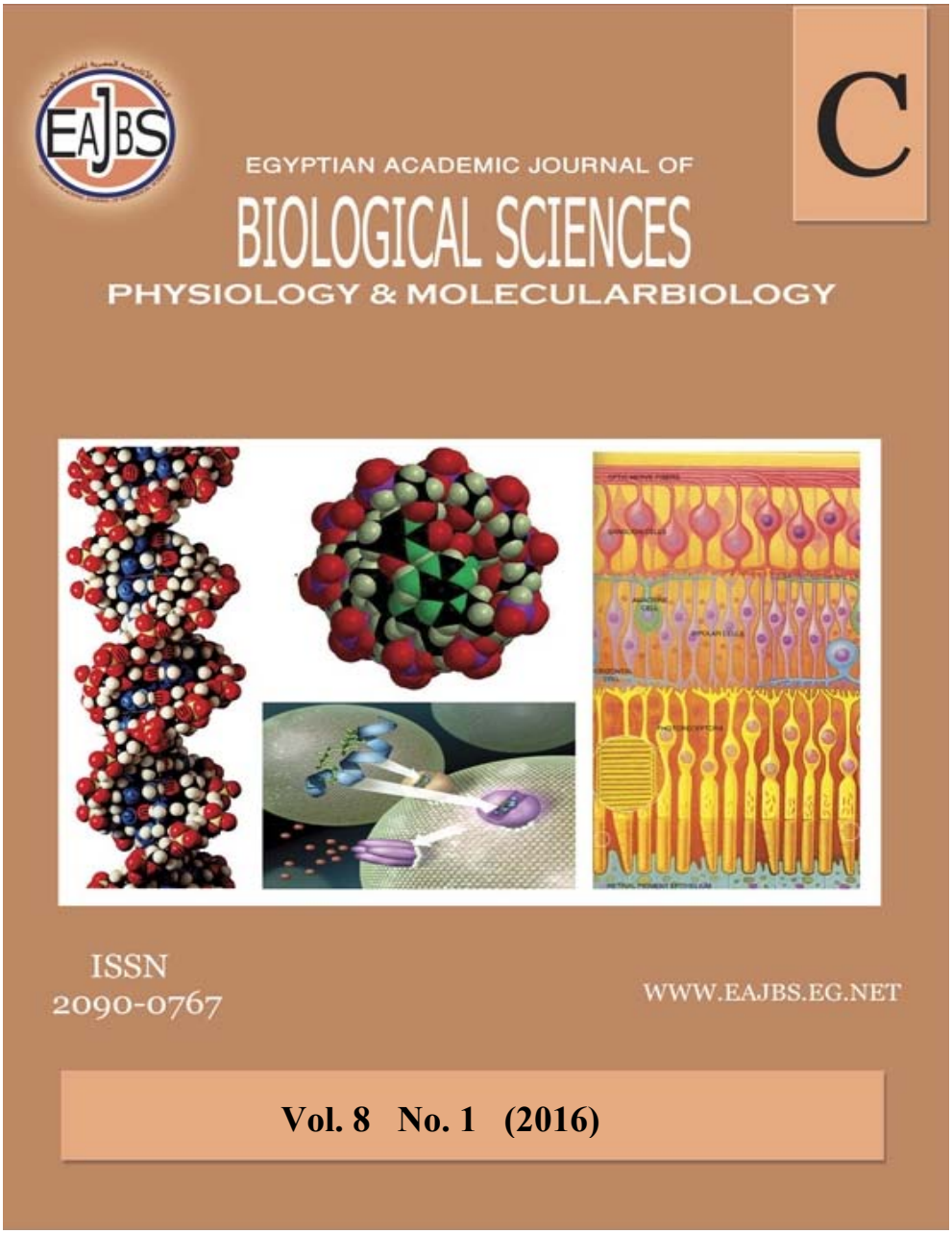

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Electrophoretic Analysis of Salivary Gland Proteins of Adult *Culex antennatus* (Diptera: Culicidae)

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ABSTRACT

Salivary glands of mosquitoes play an important role in food ingestion and digestion as well as transmission of pathogens. Mosquitoes are able to adapt to feed on blood by salivary glands which secrete proteins that work against the haemostasis process. So, the identification of these protein contents in the salivary glands of *Culex antennatus* (males and females) is the aim of this study. In the present study, protein banding pattern (Native and SDS PAGE) of salivary glands of adult *Culex antennatus* (un-fed, sugar-fed, starved and blood-fed) was investigated. Males and females of *Cx. antennatus* were dissected and their salivary glands were collected at un-fed stage, 0-, 3-, 6-, 12- and 24- h after sugar feeding and starved stage. Female salivary glands were additionally collected at different stages of blood feeding (probing time, partial engorgement, full engorgement, 3-, 6-, 12-, 24-, 48- and 72- h. after blood meal and after oviposition. Results of native-PAGE demonstrated that there were differences in the overall protein banding pattern in salivary glands of males and females of *Cx. antennatus* in the cases of un-fed, sugar-fed and starved stages. Differences in salivary gland proteins were observed when comparing males of all stages, too. Differences in salivary gland proteins were also noticed when comparing females of all stages. Furthermore, all blood-feeding stages of females showed differences in salivary gland proteins when compared to sugar-fed and starved females. Results of SDS-PAGE clarified that the molecular weight of the separated proteins (in all stages) ranged from 317.36 to 10.91 KDa. Differences were also observed between males and females in the cases of un-fed, sugar-fed and starved stages of *Cx. antennatus*.

INTRODUCTION

Salivary glands of hematophagous invertebrates have been intensively studied because they possess a variety of substances that are involved in counteracting the homeostatic systems and inflammatory reactions of the vertebrate host (Ribeiro 1987, 1995, Andrade *et al.*, 2005).

Adult mosquito salivary glands are paired organs located on either side of the thorax flanking the oesophagus (Dhar and Kumar, 2003, Jariyapan, *et al.,* 2007). The female gland has three lobes, including two lateral lobes, with distinct proximal and distal portions, and a medial lobe. Salivary glands contain several kinds of protein (Siriyasatien, *et al.,* 2005), but female glands contain approximately 10 times more protein than male glands. Mosquitoes feed on blood as quickly as possible to avoid haemostasis processes consisting of platelet aggregation, vasoconstriction and blood coagulation. Proteins are the most complex compounds and at the same time, the most characteristic components of living matter. Insect proteins are numerous and present in all viable cells (Kyung and Kim, 1990). Each protein is considered as reflect to the activity of specific gene through the production of enzyme which act as catalyst to produce protein responsible for specific biological character (Cersa, 2003).

Polyacrylamide gel electrophoresis (PAGE) has been extensively used as an excellent tool for the separation of proteins from all living organisms (Zacharius *et al.,* 1969). The vast majority of recent studies on insect proteins have used electrophoretic techniques. Polyacrylamide gel, with the advantages of high sensitivity and resolving power, is generally the most efficient medium (Wyatt and Pan, 1978). In the present study, salivary gland proteins of *Cx. antennatus* were electrophoretically separated by native and SDS-PAGE at different time intervals; un-fed stage, 0-, 3-, 6-, 12- and 24- h. after sugar feeding, starved stage and at probing time, partial engorgement, full engorgement, 3-, 6-, 12-, 24-, 48- and 72- h. after blood meal and after oviposition.

Taking all of these considerations into account, it should be of great importance to identify protein contents in the salivary glands of males and females of *Cx. antennatus* qualitative (native and SDS-PAGE).

MATERIALS AND METHODS

Insect colonization:

A laboratory colony of the *Cx. antennatus*, used for experiments was obtained from the field (Shubramunt, Giza, Egypt), and maintained in the insectary of the Department of Zoology, Faculty of Science, Al-Azhar University. This colony was maintained under laboratory conditions of 27 $\pm$ 2 °C, 60-70% RH and 10L: 14D photoperiod for supplying clean adults of known ages, according to the method described by (El-Bokl and Moawad, 1996).

Mosquito Salivary Gland Dissection:

Mosquitoes were anesthetized by subjecting to a temperature of 4°C, until immobilized. Salivary glands of adult mosquitoes (20 pairs of salivary glands were used) were dissected using fine entomological needles under a stereo-microscope at 4X magnification in phosphate-buffered saline [PBS;10 mM Na2SO4, 145 mM NaCl (pH 7.2)] and stored at −80°C prior to SDS-PAGE (Cotama *et al.*, 2013).

Salivary glands were collected at different time intervals; un-fed stage, 0-, 3-, 6- 12- and 24 h after sugar fed, starved stage (from males and females) and blood stages which include probing time, partial-engorged, full-engorged, after 3, 6, 12, 24, 48 and 72 h from blood meal and after oviposition (approximately after 10 days from blood meal).

Native polyacrylamide gel electrophoresis (PAGE):

Native-PAGE was used to analyze protein content of the salivary glands of males and females of *Cx. antennatus* at the above mentioned intervals. 15 % polyacrylamide gels pH 4, using a discontinuous buffer system was

employed (Gabriel, 1971). Acrylamide/ bisacrylamide ratio was 60:0.8. The gels were run at 100 V until the tracker dye (Bromophenol blue) was running off the gel (approximately 4 h).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE was used to analyze protein content of salivary glands of males and females of *Cx. antennatus* at the above mentioned intervals. 15% polyacrylamide gels pH 8.8, in a discontinous buffer system was employed (Maizel and Jr, 1971). Acrylamide/ bisacrylamide ratio was 50: 1. The gels contained no SDS before electrophoresis. Protein samples were pretreated with 1% SDS and 1% β-mercaptoethanol for 5-10 min at 100 °C. The gels were run at 100 V until the tracker dye was leaving the gel (approximately 4 h.). All gels were fixed in 20% 5-sulfosalicylic acid, stained with Coomassie Brilliant Blue R250, destained in 7% acetic acid and photographed using gel-documentation system with a 20-Mp camera.

Data analysis:

The data obtained from the scanning process of each gel were analyzed using Gel pro analyzer (Ver. 31 Media cybernetics, USA) and Alpha Ease FC stand alone for windows 2000/XP.

RESULTS AND DISSCUTION

Native-PAGE :

Figures (1, 2, 3 and 4) illustrated the results of native-PAGE salivary gland proteins of males and females of *Cx. antennatus* at the above-mentioned intervals. It was observed that the total number of protein bands resolved in 15% native gel was 12 bands in un-fed female compared with 10 bands in un-fed male (Fig. 1). 12, 10, 9, 6 and 7 bands were observed in the case of females at 0, 3, 6, 12 and 24 h after sugar feeding, respectively (Figs. 1 and 2). Meanwhile, 12, 11, 9, 7 and 7 bands were observed in the case of males at 0, 3, 6, 12 and 24 h after sugar feeding, respectively (Figs. 1 and 2). Both starved females and males showed 8 bands on native-PAGE (Fig. 2). 9, 9, 8, 8, 7, 8, 8, 8, 8 and 7 bands were observed at probing stage, partially-engorged, fully-engorged, 3, 6, 12, 24, 48, 72 h after blood-fed females and females after oviposition, respectively (Figs. 3 and 4). Generally, native-PAGE demonstrated many changes in the bulk protein content of salivary glands of males and females of *Cx. antennatus*.

SDS-PAGE:

Salivary gland proteins of males and females of *Cx. antennatus* mosquito were electrophoretically separated by SDS-PAGE using 15% acrylamide gel (Figs. 5, 6, 7 and 8). Protein standard (marker) was used to estimate the molecular weights of the separated bands.

Figure (5) showed changes in salivary gland protein banding patterns of males and females of *Cx. antennatus* at un-fed stage and after 0-, 3- and 6 h sugar feeding. The numbers of observed bands were 25 and 22 bands (M.wt. ranged from 289.21 to 10.91 KDa) in the case of un-fed females and un-fed males, respectively. 23 and 29 bands (M.wt. ranged from 317.36 to 10.91 KDa) were observed after 0 h from sugar feeding in females and males, respectively. 22 and 27 bands (M.wt. ranged from 289.21 to 10.91 KDa) were observed after 3 h from sugar feeding in females and males, respectively. 19 and 25 bands (M.wt. ranged from 317.36 to 10.91 KDa) were observed after 6 h sugar feeding in females and males, respectively.

Figure (6) showed protein banding patterns of male and female *Cx. antennatus* at 12, 24 h after sugar feeding and starved stage using 15% SDS gel. 21 and 18 bands (M.wt ranged from 284.81 to 11.35 KDa) were observed in the salivary glands of female and male 12 h after sugar feeding (Fig. 6), 18 and 21 bands (ranged from 284.81 to 12.7 KDa) were recorded in female and male 24 h

after sugar feeding (Fig. 6), 16 and 21 bands (M.wt ranged from 284.81 to 11.35 KDa) in the case of starved female and male mosquitoes (Fig. 6).

Figure (7) demonstrated changes in salivary gland protein patterns of control and female mosquito after blood meal. It was obvious that the females exhibited different number of protein bands after feeding on the host blood. 21 and 16 bands (M.wt. ranged from 243.38 to 11.35 KDa) were detected in females 0 h after sugar feeding and starved stage, respectively (Fig. 7), 16 bands (M.wt. ranged from 169.86 to 15.4 KDa) were present in probing time, 15 bands (M.wt. ranged from 146.24 to 15.4 KDa) were found in partially engorged females, 21 bands (M.wt. ranged from 243.38 to 15.4 and KDa) were distinguished in both fully engorged females and 3 h after blood meal females (Figs. 7 and 8).

Figure (8) showed protein banding of females after blood meal resolved in 15 % SDS gel. It was clear that females exhibited different number of protein bands after feeding on the host blood. 14 bands (M.wt. ranged from 238.55 to 15.4 and KDa) were distinguished in each of females 6, 12 and 24 h after blood meal, 15 bands (M.wt. ranged from 204.11 to 15.4 and KDa) were found in females 48 h after blood meal, 16 bands (M.wt. ranged from 204.11 to 15.4 and KDa) were detected in females 72 h after blood meal and 14 bands (M.wt. ranged from 243.38 to 15.4 KDa) were detected in females after oviposition (Fig. 8).

Native and SDS-PAGE of salivary glands of male and female *Cx. antennatus* at different time intervals demonstrated many changes in bulk and denatured proteins. The appearance of different bands in sugar fed and blood fed may be attributed to the induction of new proteins in a specific feeding stage.

Similar results were recorded on the salivary gland proteins of sugar and normal blood-fed *An. gambiae* mosquitoes. Salivary glands of blood-fed *An. gambiae* showed high and low molecular mass proteins 1 h postfeeding. The most notable difference was expression of 100 and 29 kDa proteins in response to a blood meal when compared to sugar-fed mosquitoes (Brennan, *et al.,* 2000). In addition, salivary gland proteins profile of *Aedes aegypti*, *Armigeres subalbatus* and *Culex quinquefasciatus* mosquito were studied by (Siriyasatien *et al.,* 2005). SDS-PAGE analysis demonstrated 8 major polypeptides of 20, 35, 37, 42, 45, 47, 70 and > 118 kDa in female *Ae. aegypti* pre-blood feeding. After a blood meal, depletion of major peptide bands of 35, 37, 45, 47, 70 kDa and high molecular weight band> 118 kDa was observed. In the case of *Cx. quinquefasciatus*, nine major polypeptides were observed with molecular weights of 20, 25, 36, 38, 45, 47, 49 and two bands of > 118 kDa. The bands of 20, 26, 36 and 38 kDa were depleted after a blood feeding. Similar results were observed in other blood sucking insects e.g. *Thyrsopelma guianense,* the vector of onchocerciasis, in Brazil. The salivary glands of this species begin synthesizing proteins soon after adult emergence, similar to the protein expression observed in mosquitoes. The levels of soluble proteins present in the salivary secretions are comparable to other anthropophilic species already studied, such as *Simulium metallicum* and *Simulium ochraceum* (Cross *et al.*, 1993 and Abebe *et al.,* 1994). Protein level reflects the size of the salivary glands of each species. Electrophoretic analysis of *T. guianense* showed similar profile to other black fly species in regard to the number of polypeptides (10-12 bands). Meanwhile, the expression profile changes with salivary gland maturation (Jariyapan *et al.*, 2006). Cross *et al.* (1993) analyzed four black flies species and found that zoophilic species had more bands (19-20 molecular bands) than anthropophilic species (11-12 molecular bands). This

observation suggested that the difference in salivary protein composition may represent different evolutionary adaptations in anthropophilic species to aid feeding from human hosts.

In addition, the differences in protein level at different developmental stages, as visualized by SDS-PAGE, reflect an increase in the amount of protein in the glands of older black flies. This observation may be associated with the fact that efficient blood feeding occurs mainly during maximum salivary secretions, at 48 h following emergence for this black fly species (Cross *et al.,* 1993). In sand flies, which are pool feeders like black flies, the number of protein components gradually increases with age and depends not only on sex but also on physiological state of the female (Volf *et al.,* 2000). In contrast, our results showed that the protein concentration of the black fly salivary glands did not vary qualitatively during the first three days of female adult life.

Jariyapan *et al.* (2006) showed that all black fly species had a small number of major proteins and some of the major proteins differed in molecular mass between species. The proteins responsible for inhibiting coagulation cascade are secreted soon after emergence. It is possible that the anti-coagulation activity may be even greater than that shown here if the flies are measured at the peak of salivation.

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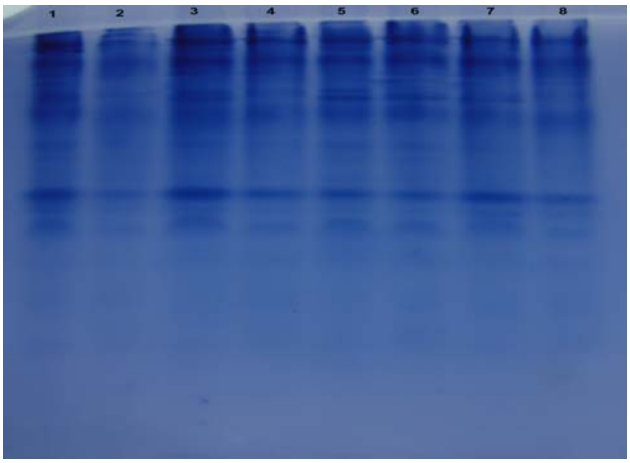

Fig . 1: Electrophoretic native gel (15%) of salivary glands of males and females of *Cx. antennatus* at different times after sugar fed. L1: un-fed female (control), L2: un-fed male (control), L3: after sugar fed female, L4: after sugar fed male, L5: 3 h after sugar fed female, L6: 3 h after sugar fed male, L7: 6 h after sugar fed female, and L8: 6 h after sugar fed male.

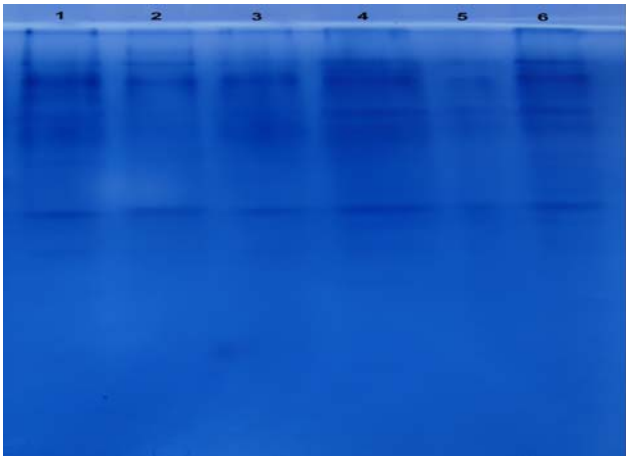

Fig . 2: Electrophoretic native gel (15%) of salivary glands of males and females of *Cx. antennatus* at different times after sugar fed. L1: 12 h after sugar fed female, L2: 12 h after sugar fed male, L3: 24 h after sugar fed female, L4: 24 h after sugar fed male, L5: starved female and L6: starved male.

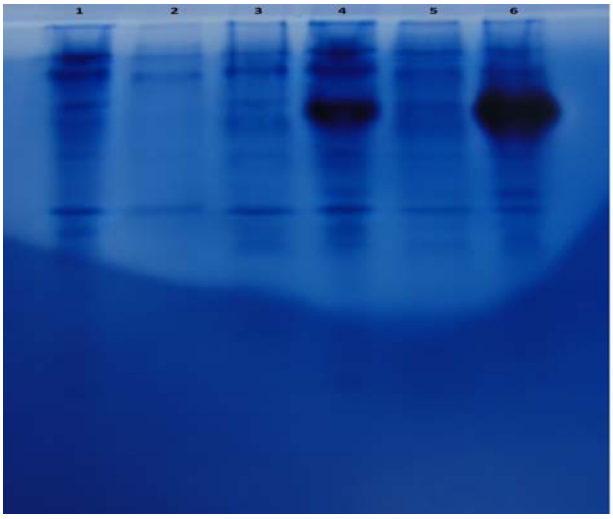

Fig . 3: Electrophoretic native gel (15%) of salivary glands of females of *Cx.antennatus* at different times after sugar fed and blood meal. L1: after sugar fed female, L2: starved female, L3: probing time, L4: partial engorged, L5: full engorged and L6: 3 h after blood meal.

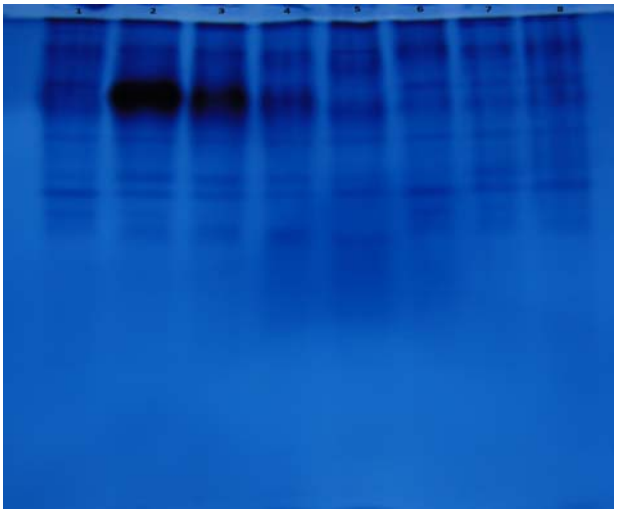

Fig . 4: Electrophoretic native gel (15%) of salivary glands of females of *Cx.antennatus* at different times after blood meal. L 1: full engorged and L 2-7 at 3, 6, 12, 24, 48 and 72 h after blood meal, L8: post oviposition.

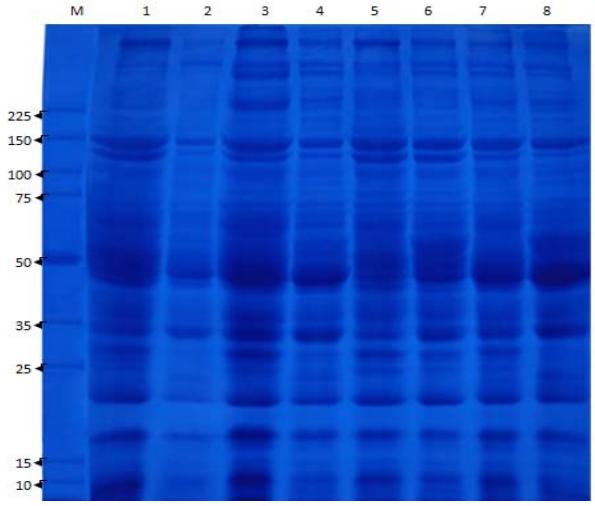

Fig. 5: Changes in the salivary glands protein banding patterns of males and females of *Cx. antennatus* using SDS-PAGE (15%) at different times, L1: un-fed female, L2: un-fed male, L3: after sugar fed female, L4: after sugar fed male, L5: 3 h after sugar fed female, L6: 3 h after sugar fed male, L7: 6 h after sugar fed female, and L8: 6 h after sugar fed male.

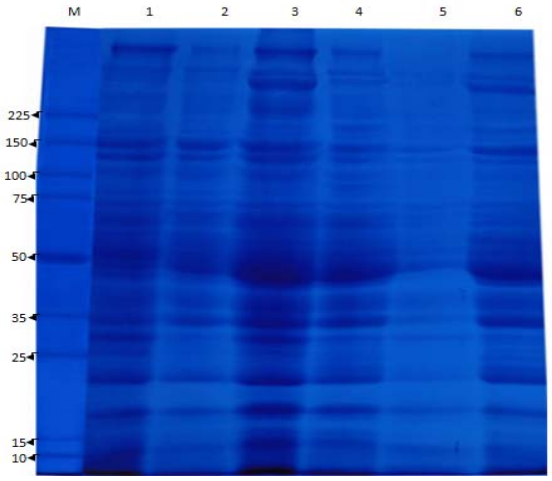

Fig. 6: Changes in the salivary glands of males and females of *Cx. antennatus* using SDS-PAGE (15%) at different times after sugar fed, L1: 12 h after sugar fed female, L2: 12 h after sugar fed male, L3: 24 h after sugar fed female, L4: 24 h after sugar fed male, L5: starved female and L6: starved male.

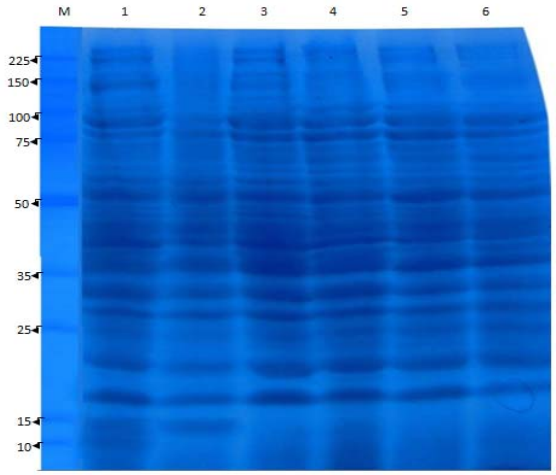

Fig. 7: Changes in the salivary glands of females of *Cx. antennatus* using SDS-PAGE (15%) at different times after blood meal, L1: after sugar fed female, L2: starved stage female, L3: probing time female, L4: partial engorged, L5: full engorged and L6: 3 h after blood meal.

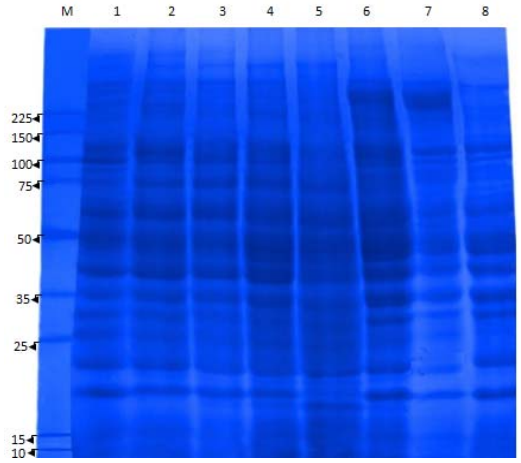

Fig. 8: Changes in the salivary glands of females of *Cx. antennatus* using SDS-PAGE (15%) at different times after blood meal. L1: full engorged, L2: 3 h after blood meal, L3: 6 h after blood meal, L4: 12 h after blood meal, L5: 24 h after blood meal, L6: 48 h after blood meal, L7: 72 h after blood meal and L8: after oviposition.

ARABIC SUMMARY

تحليل أنماط التفريد الكهربائى لبروتينات الغدد اللعابية فى الطور البالغ لبعوضة *Culex antennatus*

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استهدفت الدراسة الحالية فحص التغيرات البروتينية عن طريق الفصل الكهربائى لبروتينات الغدد اللعابية لذكور واناث بعوضة الكيولكس انتيناتس باستخدام طريقتين للفصل على مراحل مختلفة من التغذية على السكر والدم وتتضمن مرحلة عديمة التغذية وبعد 0و 3و 6و 12و24 ساعة من التغذية على السكر ومرحلة التجويع اى بعد التغذية على السكر لمدة يومين وتجويع الحشرة لمدة 12 ساعة واخيرا مرحلة التغذية على الدم وهى تشمل الانثى فقط وتتمثل فى الوقت الذى تستغرقه البعوضة من بداية وصول أجزاء الفم لجلد العائل وقبل التغذية على الدم و بعد اخذ نصف وجبة دم و بعد اخذ وجبة دم كاملة وبعد 3و 6و 12و 24و 48و 72 ساعة من التغذية على الدم وبعد وضع البيض اى بعد 10 أيام من التغذية على الدم. أظهرت النتائج الكلية للفصل الكهربائى للبروتين بالطريقة الاولى عن حدوث تغيرات فى الصورة العامة للبروتينات فى عينات الغدد اللعابية لذكور واناث كل مرحلة بدون تغذية وبعد .و 3و 6و 12و 24 ساعة من التغذية على السكر ومرحلة التجويع ووجود اختلافات ايضا بين ذكور كل المراحل وايضا اختلافات بين اناث كل المراحل اما بالنسبة لمراحل التغذية على الدم يتم مقارنتها بالاناث بعد 0 ساعة من التغذية على السكر وبعد مرحلة التجويع. أيضا أوضحت نتائج الفصل الكهربائى للبروتين بالطريقة الثانية ان الوزن الجزيئى يتراوح مابين 317.36 و 10.91 ك.د. واوضحت ايضا ان عديمة التغذية و التغذية على السكر ومرحلة التجويع للغدد اللعابية لبعوضة الكيولكس أنتيناتس يوجد حزم بروتينية عند المقارنة بين ذكر وانثى كل مرحلة وأيضا مراحل التغذية على الدم يوجد حزم بروتينية جديدة عند مقارنتها باناث البعوض بعد التغذية على السكر ومرحلة التجويع.