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Feeding Behavior - Expression of Certain Salivary Gland Genes Relationship in Females *Culex Pipiens* and *Hyalomma Dromedarii*

Amira F. Hussein[1], Mostafa M. Mokhtar[2*], Walaa A. Moselhy[1], Shaymaa H. Mahmoud[3]

[1]*Zoology and Entomology Department, Faculty of Science, Al-Azhar University (girls branch), Nasr City, Cairo, Egypt*

[2]*Zoology and Entomology Department, Faculty of Science, Al-Azhar University (boys branch), Nasr City, Cairo, Egypt*

[3]*Zoology Department, Faculty of Science, Menoufia University, Shebin El- Kom, Menoufia, Egypt*

Abstract

Salivary glands of hematophagous arthropods are enriched sources of pharmacologically active components that affect their hosts. In this work we compared mRNA expression pattern of salivary allergen gene, serine protease and salivary alpha glucosidase in the salivary gland of female *Culex pipiens* (Diptera: Culicidae) and *Hyalomma dromedarii* (Ixodida: Ixodidae) after sugar and blood feeding as well as electrophoretic protein banding pattern by SDS-PAGE. The results of SDS for salivary glands of female *Cx. pipiens* and *H. dromedarii* after different feeding time intervals demonstrated many changes in the protein pattern. A striking different expression pattern was observed in the two arthropods. A significant increase in allergen gene 3 days post blood feeding compared to unfed females in *Cx. pipiens* and down regulation after sugar feed reflecting that prolonged blood feeding may be a factor involved in allergen activation. A similar pattern was reported in *H. dromedarii*, where a significant up-regulation was recorded in partially-fed and fully-fed females. Additionally, a significant down-regulation of serine protease after blood feeding in both species was observed. α-glucosidase expression exhibited down regulation in *Cx. pipiens,* after blood feeding.These results provide initial information that will lead to further study on the role of salivary genes of *Culex pipiens* and *Hyalomma dromedarii* in hematophagy.

Keywords: Salivary gland, allergen gene, serine protease and salivary α-glucosidase *Culex pipiens*, *Hyalomma dromedarii*

1. Introduction

Many diseases that affect both humans and animals are brought on by pathogens that are transmitted through the salivary glands of blood-feeding arthropods [1]. The saliva of hematophagous arthropods causes allergic reactions in human hosts, manifesting as cutaneous pruritic wheal and flare symptoms at the bite site [2], [3]. Inhaling mosquito allergens can induce asthma and/or allergy rhino-conjunctivitis symptoms in sensitive persons. Allergic responses to mosquito bites are a significant clinical issue [4], [5]. Mosquito saliva contains a cocktail of substances to facilitate blood feeding, including anticoagulants, anti-inflammatory, and immunosuppressive factors [6], [7]. Throughout blood feeding, the salivary glands of female mosquitoes are continually depleted of salivary proteins. Several studies have reported a decline in total salivary gland proteins in various *Anopheles* species after feeding on human blood [8], where specific proteins are secreted with the saliva during probing for feeding, while others before and after blood feeding [9]. *Aedes aegypti* salivary gland proteins, such as apyrase, D7 protein, salivary serpin putative anticoagulant, and putative 30 kDa allergen-like protein, were up-regulated ten days after feeding [10]. Various mosquito species like *Aedes caspius*, *Armigeres subalbatus*, *Culex pipiens*, and *Mansonia*

*Corresponding author e-mail: drmosta80@azhar.edu.eg.; (Mostafa M. Mokhtar).

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uniformis were also documented to have alterations in the quantity of total salivary gland proteins and electrophoretic protein patterns following their first blood meal [11]–[13].

Studying the expression of salivary α-glucosidases and their relation to sugar and blood feeding may provide suppressors targeted mosquito -glucosidases that may be utilized to slow glucose generation, hence impeding mosquito maturation, and thus used as promising insecticides [14]. Ticks have acquired an arsenal of salivary substances, such as anti-hemostatic, anti-inflammatory, and immunomodulatory components, which enable them to securely take a blood meal by eliminating the host's defenses [15]. Furthermore, bioactive compounds released by tick saliva are implicated in infection transmission to hosts [16]. Because ticks are long-term bloodsuckers, the host's defense may disrupt their blood feeding [16]. However, both male and female ticks are hematophagous, and their feeding patterns are vastly distinct. Females' blood-feeding process is longer than males' [17], [18]. Amongst the most promising methodologies for producing anti-tick/mosquito vaccines relies on salivary compounds with immunosuppressive characteristics released by blood-feeding insects [19]. The duration of blood-feeding is an important behavior displayed among hematophagous arthropods, which ranges from a few minutes (Culicidae) [20] to many days (ticks) [21]. In females, salivary glands express different gene products depending on their feeding mode [22].

The long-term blood feeding of hematophagous arthropods may activate or promote salivary substances' expression. To our knowledge, no publication has examined the relationship between the expression of these allergens and salivary-related genes and the period of blood feeding. In the current study, we attempted to identify allergen-suspected genes by examining their differential expression in salivary glands of femlas of *Cx. Pipiens* and *H. dromedarii* at different intervals following blood feeding.

2. Materials and Methods

2.1. Mosquito rearing

In this study, females *Culex pipiens* were reared in Medical Entomology insectary, Animal house Faculty of Science, Al-Azhar University, under controlled conditions of temperature (27±2°C), relative humidity (70±10%) and light-dark regime (12-12 h) according to the methods described by [23]. Females were allowed to receive blood meal from pigeon.

2.2. *Tick collection*

All female ticks were collected from camels' market at latitudes 30°08'57.1"N and longitudes 30°59'42.8"E, Birqash village, Giza Governorate, Egypt. The one-humped camels were sampled for ticks on head, shoulder, belly, and perineal regions using curved forceps. The samples were transported alive to Medical Entomology Laboratory, Animal house Faculty of Science, Al-Azhar University. They were incubated in glass tubes with one folded filter paper at 28±1°C and 75±5% relative humidity. The identification of ticks was confirmed in the laboratory using standard keys [24], [25].

2.3. Dissection of the salivary glands

Mosquito: Female mosquitoes were given 4°C sedative until immobilized, then their salivary glands were removed with fine needles in ice-cold phosphate-buffered saline [PBS; 10 mM (Millimolar) Na_2So_4 ,145 mM NaCl (pH 7.2)], transferred to labeled microcentrifuge tubes containing a small volume of PBS and stored at −80°C until use [26]. Salivary glands were collected from unfed females, sugar fed females, 2 hours post blood feeding (single blood meal) and 3 days post blood feeding (multiple blood meal). Tick: Salivary glands from adult female *H. dromedarii* ticks were removed and preserved at -80 °C after being dissected with a needle dipped in ice-cold, pH 7.2 phosphate-buffered saline (PBS) [27]. Salivary glands were collected from unfed females, partially fed females, and fully fed females.

2.4. Electrophoretic protein analysis of female Cx. pipiens and *H. dromedarii salivary glands*

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by [28] and 12% separating gel was used. The salivary glands from fifty pairs were combined and ground with an equivalent volume of sample buffer. After boiling for 5 min, and letting the mixture cool, 20 µl of the mixture was added to each well of the stacking gel. Standard marker (10–180 kDa) was used. Protein electrophoresis was carried out using a constant voltage setting at 150 volts for 60 min. till the tracking dye reached the bottom of the gel.

2.5. Expression of allergen and salivary-related genes

Total RNA was extracted from 50 salivary glands of female *Cx. Pipiens* (un-fed, sugar fed, 2 hr. and 3 days post blood meal) and *H. dromedarii* (un-fed, partially-fed, and fully-fed) using Thermo Scientific GeneJET RNA Purification Kit (Cat#K0731) according to the manufacturer's protocol. RNA purity at Absorbance 260/280 ratio was estimated using

spectrophotometer (Milton Roy spectrophotometer, Spectronic 1201, Houston, USA).

First-strand cDNA was performed using RevertAid M-MuLV Reverse Transcriptase kit following manufacturer instructions with 2 µg RNA as a template (11µl) was placed in a 0.2ml tube in ice to which 1µl oligo (dt) primer was added in a total volume of 12µl then the mix was incubated in 65^{o}C for 5 min and placed the tube back on ice and the following components were added; 4µl (5x) reaction buffer, 1µl Ribo Lock RNase Inhibitor, 2µl of 10mM dNTP Mix and 1µl RevertAid M-MuLV Reverse Transcriptase in a total volume of 20 µl. Then the components were gently mixed and put it into thermal cycler at 42^{o}C for 60 min and 70^{o}C for 5 min.

2.6. Expression of allergen and salivary related genes

The mRNA expression levels of allergen related genes were measured by quantitative real time PCR (qPCR) Biosystem step one plus instrument using Maxima SYBR Green/ROX qPCR Master Mix (2X) (SABio- sciences™, Applied Biosystems, Foster City, CA, U.S.A.) (2X) Kit (Thermo Scientific no. K0223). Primers were design based on transcriptomic analysis of salivary glands of both *Cx. Pipiens H. dromedarii* using Primer 3 software and their sequences are listed in Table (1). β-actin primer was used as endogenous control [29] and each qRT-PCR was performed with triplicate samples. Reaction was performed in 20 µl total volume of the followings; 10 µl Maxima SYBR Green/ROX qPCR Master Mix (2X), 1 µl Forward Primer, 1 µl Reverse Primer, 2 µl Template cDNA, 6 µl nuclease-free water. Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C, and finally 60 s at 72 °C. The template formats your text by using a Word® feature called 'Styles'. Styles define the format (or appearance) of a paragraph of text as regards letter size, indentation, line spacing, etc. If you're not familiar with using styles, do not worry; the template arranges everything for you in a user-friendly way.

2.7. QPCR Data analysis

Expression level of the target genes was calculated by comparing the cycle threshold value (Ct) to the reference gene β-actin. The relative quantification (comparative method) was calculated using the ΔΔCt method [30]. All samples were normalized to the ΔCt value of a reference gene to obtain a ΔΔCt value (ΔCt target−ΔCt reference). The final relative expression was calculated using the following formula: F=2−(ΔCt target−ΔCt reference). A randomly selected untreated control sample was used as the calibrator for calculating relative expression ratios.

2.8. Statistical analysis

Results were as the mean ± SD. One-way analysis of variance (ANOVA) was applied to determine the statistical significance, using SPSS software version 21.1 (IBM, New York, NY, USA, 2019). The values with $p < 0.05$ were considered.

3. Results

3.1. Electrophoretic protein results

Variation in salivary gland protein banding patterns of females *Cx. pipiens* and *H. dromedarii* after different feeding times was demonstrated in Fig. (1A and 1B). in case of females *Cx. pipiens* salivary glands There were 12, 11, 12 and 13 bands Fig. (1A) and among them 8 common bands with molecular weights of (193.61, 110.50, 89.29, 62.89, 40, 30.2, 20.45, 10 kDa) observed in unfed, sugar fed, 2hr. and 3 days post blood feeding, respectively (Table 2).

Table 2
Molecular weight analysis of protein profile of salivary glands of female *Culex pipiens*

M. Wt.	unfed	sugar fed	2hrs post blood meal	3 days post blood meal
204.73	--	--	+	--
193.61	+	+	+	+
165.71	+	--	--	--
137.14	--	+	+	+
110.50	+	+	+	+
100.00	+	--	--	+
89.29	+	+	+	+
74.29	--	+	--	--
62.89	+	+	+	+
58.55	--	--	--	+
46.72	+	+	--	+
40.00	+	+	+	+
34.091	+	--	+	--
30.20	+	+	+	+
29.55	--	--	+	+
20.45	+	+	+	+
10.00	+	+	+	+

A unique band was detected in each sample with molecular weight of (165.7, 74.29, 204.73 and 58.55 kDa) in salivary glands of unfed, sugar-fed, 2 hrs. and 3 days post blood feeding, respectively (Table 2).

On the other hand, *H. dromedarii* salivary gland showed 11, 10 and 8 bands Fig. (1B) and among them 7 common bands recorded with molecular weights of (201.13, 114.35, 95.71, 74.29, 54.67, 37.62 and 30.5 kDa) in unfed, partially-fed and fully-fed females respectively (Table 3). Two unique bands with molecular weights of 165.71 and 106 kDa were observed in unfed females, while a unique band was observed in both partially-fed and fully-fed females with a molecular weight of 128.31KDa and not found in unfed ones (Table 3).

Table 3
Molecular weight analysis of protein profile of salivary glands of female *H. dromedarii*

M. Wt.	Un-fed	Partially fed	Fully fed
201.13	+	+	+
165.71	+	--	--
128.31	--	+	+
114.35	+	+	+
106.00	+	--	--
95.71	+	+	+
74.29	+	+	+
54.67	+	+	+
50.79	+	+	--
41.12	+	+	--
37.62	+	+	+
30.50	+	+	+

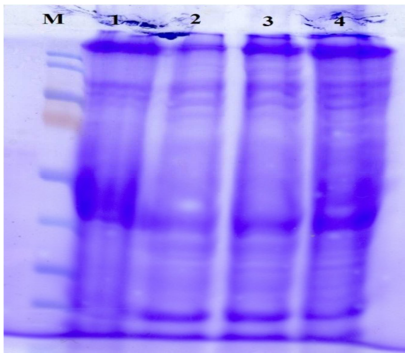
3.2. Tissue-specific expression patterns of allergen and salivary related genes

In qPCR study, the primer pairs for allergen, seine protease and α-glucosidase were used from salivary glands of *Cx. pipiens* and *H. dromedarii* using β-actin as a positive control. The qPCR results showed that allergen, serine protease and α-glucosidase were detected in both tested arthropods.

3.3. Expression profile of allergen gene in salivary glands of Cx. pipiens and H. dromedarii

The transcript level of allergen gene in salivary glands decreased after sugar feeding and 2 hours post blood feeding. However, it significantly up-regulated 3 days post blood feeding compared to unfed females. In *H. dromedarii*, a significant up-regulation was recorded in partially-fed and fully- fed females (Fig. 2 A and B).

A



B

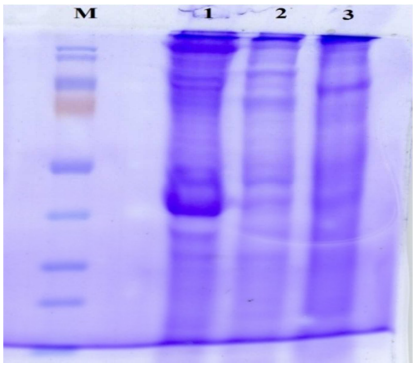


Fig 1: Electrophoretic protein profile of A: salivary glands of female *Culex pipiens* (lane M: 10 to 180 kDa marker, lane 1: un-fed, lane 2: sugar fed, lane 3: 2 hr. post blood meal, and lane 4:3 days post blood meal) and B: salivary glands of female *H. dromedarii* (lane M: marker, lane 1: un- fed, lane 2: partially fed, lane 3: fully fed)

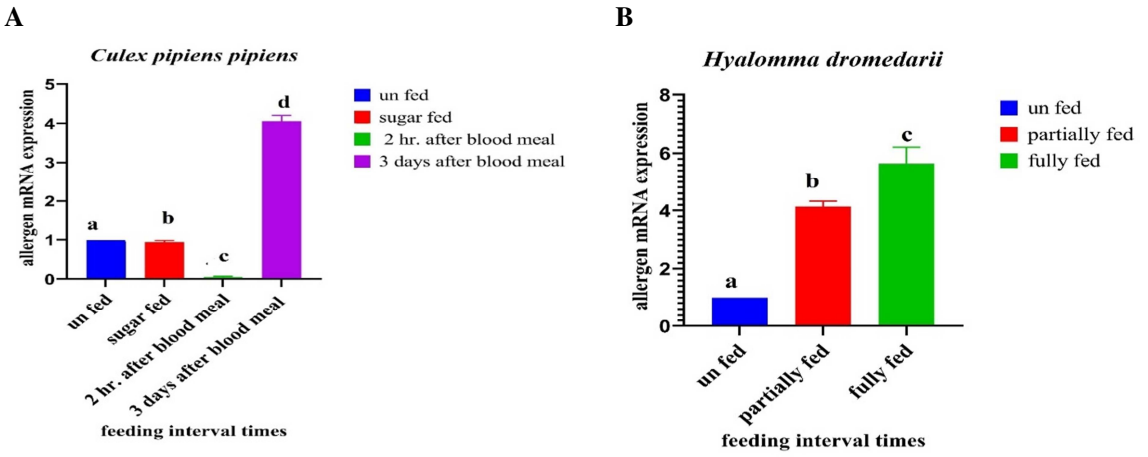


Fig 2: Expression pattern of allergen gene in salivary glands of A: female *Cx. pipiens* and B: female *H. dromedarii*. Different letters indicate significant difference ($p < 0.05$). Values were expressed as mean ± SD. The experiments were done in triplicate ($n = 3$)

Table 1
Primers used in qPCR analysis of female *Cx. pipiens* and *H. dromedarii* salivary allergens and salivary related genes

Primer name	Forward primer 5' - 3'	Reverse primer 5'- 3'
Allergen	TGACAAAGCAGCTGGACTTG	CTTCTCTCCCGGTTGCTTCT
Allergen 2 Sp	CATTGCTTCACCCACACCAA	GTCTGCGTTACTTGCCTCAG
Allergen 3 Ag	GAACTGGGTGCTCGGAAATC	GATTTCCGAGCACCCAGTTC
β-actin	GCGTGAACTGACGGCTCTTG	ACTCGTCGTACTCCTGCTTGG

3.4. Expression profile of serine protease gene in salivary glands of Cx. pipiens and H. dromedarii

In sugar fed Cx. females, serine protease expression in salivary glands was reduced. Also, blood feeding after 2 hours and 3 days significantly affected the expression of serine protease in salivary glands (Fig. 3 A). on the other hand, the blood feeding increased the expression level of serine protease gene in *H. dromedarii* female salivary glands compared to unfed ones (Fig. 3 B).

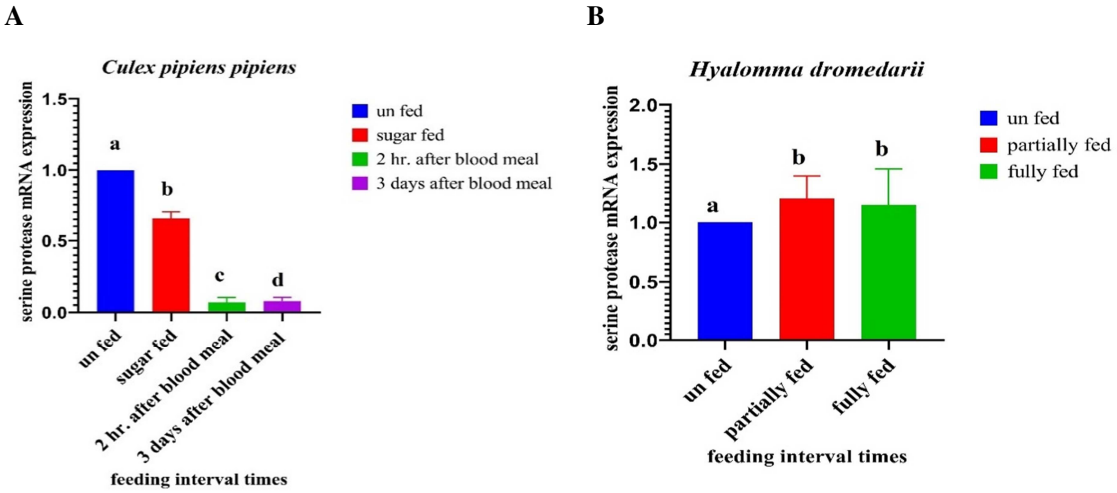


Fig 3: Expression pattern of serine protease gene in salivary glands of A: female *Cx. pipiens* and B: *H. dromedarii*. Different letters indicate significant difference ($p < 0.05$). Values were expressed as mean ± SD. The experiments were done in triplicate ($n = 3$).

3.5. Expression profile of α-glucosidase mRNA gene in salivary glands of Cx. pipiens and H. dromedarii

α-glucosidase mRNA expression followed the same pattern of serine protease in both arthropod species. When adult females *Cx. pipiens* were fed on sugar, a decline in α-glucosidase expression was observed compared to unfed females. A similar decrease in α-glucosidase expression was observed after 2 hours and 3 days post blood feeding. (Fig. 4 A). In contrast, the expression levels of α-glucosidase are higher in salivary glands of partially-fed and fully-fed females of *H. dromedarii* than those of un-fed ones (Fig. 4 B).

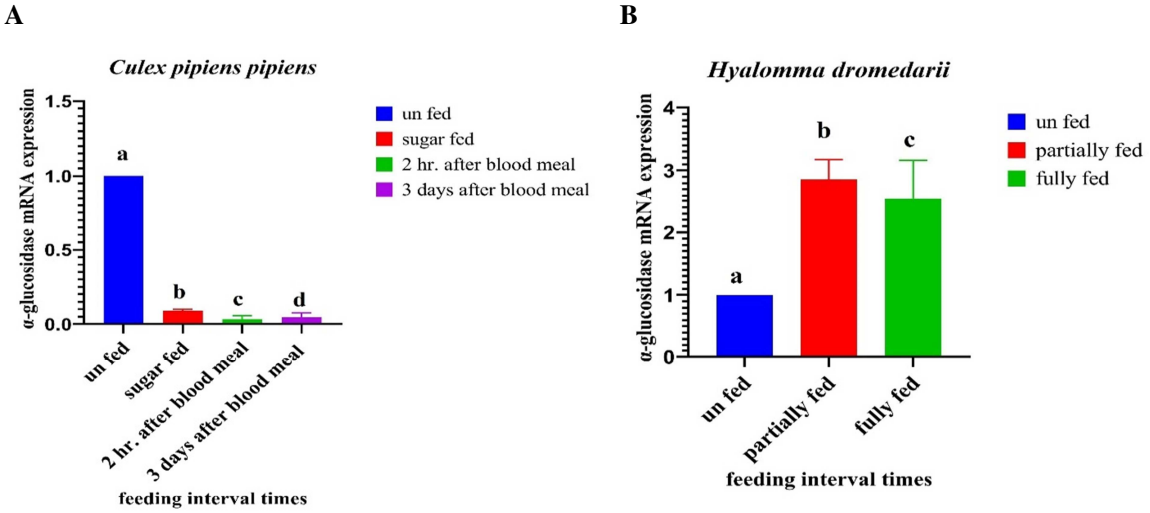


Fig 4. Expression pattern of α-glucosidase gene in salivary glands of A: female *Cx. pipiens* and B: *H. dromedarii*. Different letters indicate significant difference ($p < 0.05$). Values were expressed as mean ± SD. The experiments were done in triplicate ($n = 3$)

4. Discussion

Hematophagous arthropod vectors have medical and veterinary importance due to their blood-feeding leading to transmission of pathogens [31]. To accomplish blood flow, an array of salivary biomolecules is injected into the bite site [32]–[35].

The results of SDS for salivary glands of female *Cx. pipiens* and *H. dromedarii* after different feeding time intervals demonstrated many changes in the protein pattern. The variation in protein bands between sugar fed and blood fed may be attributed to induction of proteins incorporated in digestion and metabolism of blood meal. These results are in consistent with previous reports of salivary glands of different mosquito species using SDS-PAGE [36]–[41] where they demonstrated that there are nearly 12–15 proteins in female salivary glands. Also [8], [11]–[13] detected changes in protein profiles of the salivary gland after a blood meal of several mosquito species, including *Ae. aegypti*, *Ae. caspius*, *Anopheles stephensi*, *An. albimanus*, *An. gambiae*, *An. freeborni*, *Armigeres subalbatus* and *Cx. quinquefasciatus* found that a small fragment of salivary gland transcriptome was dynamically changing two hours post blood feeding and attributed depletion of proteins after blood feeding to their introduction into vertebrate hosts when a mosquito took a blood meal. Similar trend was observed in salivary glands of female *H. dromedarii* after different feeding interval times where depletion in number of protein bands was observed. Similarly, it has been observed that the expression of several proteins alters in the salivary glands of female *Haemaphysalis longicornis* during the transition from an unfed to a fed state [42]. In the present work appearance of unique bands after blood feeding either in salivary glands of partially fed or fully-fed may be due to induction of genes during the feeding process, leading to the expression of new proteins [43]. Additionally, SDS analysis of salivary gland extracts from unfed and 5 days fed adult females of *Ixodes ricinus; Haemaphysalis inermis* and *Dermacentor reticulatus* revealed a relative difference in protein profiles as some proteins were synthesized preferentially and other bands in the unfed ticks were less discernible in that of fed ticks. These proteins may be secreted or converted to other substances during the feeding process[44]. The selected genes in the current study were selected based on transcripts expressed at high levels within salivary gland of mosquitoes and ticks [45]. 30-kDa allergen gene protein is a major mosquito salivary allergen and causes host hypersensitivity responses [1], [46] and expressed in distal-lateral and medial lobes of salivary glands [47]. It was identified as a salivary antigen in *Ae. aegypti* [48] and found in salivary transcriptomes and proteomes of culicine and anopheline mosquitoes [38], [49]–[51] and expressed in female salivary glands of mosquitoes [39], [40], [52]. Here, we reported a significant increase in allergen gene 3 days post blood feeding compared to unfed females in *Cx. pipiens* and down regulation after sugar feed reflecting that prolonged blood feeding may be a factor involved in allergen activation. The same observation can be reported in *H. dromedarii*, where a significant up-regulation was recorded in partially-fed and fully-fed females. No previous reports concerned the relation between allergen and blood feeding in *Cx. pipiens* neither in *H. dromedarii* which is an important issue to be concerned in time-dependent vaccination development against hematophagous arthropods. Proteomic analysis revealed that a 30 kDa allergen-like protein was elevated ten days after *Ae. aegypti* was fed [10]. Similarly, gene expression and salivary chemical synthesis increase when a tick is attached to a host, mainly to promote feeding and prevent tick rejection by the host [53]. Various proteases are involved in digestion of blood meal as energy source for vital processes, ensuring arthropods survival, development and reproduction [54]. The animal genome contains 2–4% of genes encoding proteolytic enzymes [55] and serine proteases are the most abundant and functionally diverse group [56]. Blood-feeding exoparasites are rich sources of protease inhibitors [57]. Protease inhibitor domain-containing peptides are known to be expressed in salivary glands of *H. marginatum rufipes* [58], [59] as well as *Ae. aegypti* [47]. Gene products encoding serine protease contain signal peptides expressed in proximal-lateral lobes. These peptides are required to metabolize low abundance proteins consumed during sugar feeding and may be involved in blood feeding [47]. On the other hand, serine protease inhibitor 6 (AamS6) was released into the host during *Amblyomma americanum* tick feeding, and its mRNA and protein were strongly expressed during the first three days of tick feeding. Which indicates that AamS6 is involved in facilitating blood meal feeding through reducing platelet aggregation and plasma clotting time [60]. The obtained results in the present study indicated a significant down-regulation of serine protease after blood feeding. Similar down regulation in salivary protease transcript has been reported in *Ae. aegypti* for the first three hours after the blood meal [61]. It was hypothesized that knockdown of serine proteases significantly affected blood feeding, survival, fecundity, active trypsin levels in the midgut, and hemoglobin breakdown in completely engorged, replete female ticks, assuring that serine proteases operate as digestive enzymes [60]. Salivary α-glucosidases are less well recognized than their equivalent in the midgut [14]. In the salivary glands of *Ae. aegypti*, *Ae. albopictus*, *An. darling*, *Cx. quinquefasciatus*, and *An. dirus*, alpha-glucosidase accumulates in the distal and proximal parts of the

lateral lobes (regions important for blood feeding) [37], [62]–[65]. It was discovered that the proximal-lateral lobes produce the alpha-glucosidase gene, which correlates to sugar-feeding and nectar-related digestive and bacteriocidal activities [47] which supports the results in the current study for *Cx. pipiens,* where a decrease in α-glucosidase expression was observed after blood feeding. Generally, biochemically active substances involved in blood feeding and/or the blood digesting process may vary amongst mosquito genera. According to our knowledge, no previous reports concern relation between transcriptional changes in Alpha-glucosidase and blood feeding in *H. dromedarii* suggesting further studies for investigation of its potential role in feeding behavior of *H. dromedarii*.

5. Conclusion

The current study's findings provide a basis for further research into the proposed significance of these proteins and underlying physiological processes during blood feeding. In addition, the current findings give information for further research into the involvement of salivary proteins in disease transmission and blood feeding by *Cx. pipiens* and *H. dromedarii*.

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7. Conflict of interest

The authors declare that there is no conflict of interest.

8. References

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