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EFFECT OF 20-HYDROXCY ECDYSONE ON THE SALIVARY GLANDS OF NYMPHAL *HYALOMMA* (*HYALOMMA*) *DROMEDARII* KOCH (ACARI: IXODOIDEA: IXODIDAE) AT DIFFERENT DEVELOPMENTAL STAGES

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ABSTRACT

The exogenous dose of 20-hydroxcy Ecdysone (20-HE) used in the present study induced an increase in the size and the secretory activity of the different types of alveoli of salivary glands in nymphal *Hyalomma dromedarii* during its developmental stages. Also, it causes salivary gland degeneration. The results suggest that the (20-HE) enhance synthesis and release of tick salivary gland degeneration factor' (the degeneration factor), which is probably an ecdysteroid. (20-HE) seems to provide a stimulus for synthesis of protein, lipids and glycogen drates.

INTRODUCTION

Ecdysteroid hormones regulating moulting, vitellogenesis, excretory activity and exciting sex pheromone activity, have been reported in many invertebrate groups including arthropods (Hetru and Horn, 1980). Much work has been devoted to elucidation of the chemistry, biosynthesis, action and metabolism of ecdysteroids in insects and crustaceans as found and review by Hoffman (1980), Downer and Laufer (1983), Hoffman and Porchet (1984) and (Kelly *et al.*, 1986, 1987). However, very little is known about ecdysteroids in ticks. The site of ecdysteroid hormone production in ticks is unclear but substantial evidence of their occurrence in these parasites is accumulating.

Ecdysteroids in nymphs were detected in the ixodid *Amblyomma hebraeum* (Delbecque *et al.*, 1978) and the argasid *Ornithodors moubata* (Diehl *et al.*, 1982a). Germond *et al.* (1980) and (Zhu *et al.*, 1994, Gonzalez *et al.* 2005) performed a detailed study on the temporal correlation between integument structure and ecdysteroid titers in fifth instar nymphs of *O. moubata*. Moulting hormone activity was also found in the haemolymph of fifth instar nymphs of *O. porcinus* (Solomon *et al.* 1982). Ellis and Obenchain (1984) studied *in vivo* and *in vitro* production of ecdysteroids by nymphal *A. variegatum*, whereas Dees *et al.* (1984) reported on ecdysteroids in *Dermacentor variabilis* during different periods of tick development. Recently, Darwish and Mohamed (1995) studied the effect of 20 Hydroxy Ecdysone (20-HE) on the neurohemal – endocrine organs in nymphal *Hyalomma dromedarii* during its developmental stages

The effect of ecdysteroids on ixodid salivary glands of female *Amb. hebraeum* was reported by (Harris and Kaufman, 1981; Kaufman, 1984; Connat *et al.*, 1985; Harris and Kaufman, 1985) and in *A. americanum* (Lindsay and Kaufman, 1988; Shelby *et al.*, 1989).

Generally, the importance is ecdysteroids in ticks was first implicated by Cox (1960) in adult *O. turicata*. Topical application, dipping or injection of α and β-ecdysone terminated larval diapause in *D. alpipictus* (Wright, 1969) and *Rhipicephalus sanguineous* (Sannasi and Subramoniam, 1972) and inhibited oogenesis in Boophilus microplus (Mansingh and Rawlins, 1977) and *O. moubata* (Diehl *et al.* 1982 b). Molting and supermolting were accelerated in *O.moubata* (Kitaoka, 1972; Mango, 1978; Mango *et al.*, 1976) and in *H.dromedarii* (Khalil *et al.*, 1984; Marzouk *et al.*, 1994) by 20-HE. Ticks respond to exogenous ecdysteroids by forming storage ecdysteroids conjugates (Diehl *et al.*, 1985; James and Zhu, 1997).

In the present study the effect induced by topical application of 20-HE on the salivary glands of nymphal stage *H. dromedarii* during different stages of development was described and compared with our previous data.

MATERIALS AND METHODS

Hyalomma dromedarii colony originated from engorged females collected from camels in the Imbaba camel market, Giza Governorate, Egypt were colonized in Faculty of Science, Al-Azhar University for Girls laboratories, at 28 ± 1°C and 75% R.H. using the rabbit *Oryctolagus cuniculus* as a host after the method of Berger *et al.* (1971).

Twenty-Hydroxy Ecdysone (20-HE) (Sigma Chemical Company, St. Louis, Mo., U.S.A.) was dissolved in absolute ethanol and applied topically in aliquots of 1 µl/ tick to 0 day dropped nymphs. The dose applied was 20 µl/1 tick (Dees *et al.*, 1985) control included untreated nymphs and treated with 1 µl ethanol nymphs. All nymphs were kept in the incubator until prepared for histological and histochemical examination.

The effect of 20-HE on the salivary glands of nymphs 1, 3, 5, 7, 9 and 11 days after engorgement, and detachment from the host (referred to herein after as 1ddN, 3ddN, 5ddN, 7ddN, 9ddN, and 11ddN respectively) were investigated.

For histological examination, Bouin's fixed tissues were serial dehydrated, double-embedded in calloidin paraplast, and serial sections, 5-7 µm thick, were stained with Chorme Hematoxylin- phloxine (CHP) (Gomori, 1941). Measurements of salivary glands alveoli and their contents of 7 to 10 specimens from each treated group examined were determined and compared with untreated groups using Student t-test.

To detect lipids and glycogen by histochemical examination, freshly dissected nymphs were fixed in modified formalin fixative. Fixed tissues were washed in running water, then dehydrated and double-embedded in celloidin-paralast. Sections 5 to 7 µm thick, were stained with Sudan Black (SB) (Chiffelle and Putt, 1951) stain. For glycogen demonstration, sections were stained according to the periodic Acid-Schiff (PAS) stain (Lilllie, 1951).

Dissected nymphs were fixed Carnoy fixative for the demonstration of basic proteins. Fixed tissues were dehydrated and embedded as described before. Sections were stained using Mercury Bromophenol Blue (Hg-BPB) method (Chapman, 1975).

The changes observed in the present study, in the size of the different salivary gland's alveoli, their nuclei, secretory product and histochemical components are compared with those previously reported in the untreated nymphs (Mohamed and Darwish, 1999).

RESULTS

I- Histology

Generally, the size of salivary glands alveoli in 1, 3, 5 and 7 ddN treated with 20-HE exhibited a significant increase. On the other hand the size of salivary glands alveoli of 9 and 11ddN remains unchanged when compared with untreated ones. (Table 1-3). Meanwhile, the secretory products observed within the cytoplasm of different cell types increased as indicated by the stronger staining reaction using CHP stain (Figs. 1-4).

1ddN:

Type I alveoli of 1ddN, increased in size ($P < 0.05$) than untreated ticks being 33.6 ± 0.4 x 70.1 ± 0.2 µm and their central nuclei remains unchanged (Table 1). The granules observed are in the form of fine granules densely aggregated at the alveoli periphery (Fig. 1).

Type II alveoli increased markedly in size ($P < 0.001$) than untreated ones, measuring 99.6 ± 0.5 x 111.0 ± 0.3 µm while their nuclei appears unchanged (Table 2). Granular inclusions appears in the form of fine to coarse droplets evenly distributed in the cell cytoplasm (Fig. 1).

Type III alveoli exhibits also a change in size when compared with untreated ones. These alveoli slightly increased in size to 71.8 ± 0.3 x 88.9 ± 0.2 µm, but their nuclei are statistically similar (Table 3). No change is observed in the form of their secretion. However, granular inclusions become densely distributed in some cell types or concentrated at the cell periphery of others (Fig. 1).

3 ddN:

In 3 ddN, **type I** alveoli is comparably larger in size than those in the control ones, measuring 26.4 ± 0.5 x 29.8 ± 0.8 µm. Their central nuclei remain almost unchanged, measuring 4.7 ± 0.2 x 7.1 ± 0.3 µm (Table 1). No change is observed in the from of granular inclusions observed in the cytoplasm, however, these granular inclusions are evenly scattered in some cells and concentrated at the periphery in others (Fig 2).

Type II alveoli also exhibited an increase in size, measuring 56.1 ± 0.7 x 83.0 ± o.2 µm. The nuclei remain almost unchanged (Table 2). The nature of secretory product in the different cell types appears in the form of fine to thick granules with very dark purple droplets and their pattern of distribution changed, being concentrated in the perinuclear area in some cells and remained evenly distributed in the cytoplasm of others (Fig. 2).

Type III alveoli increased in size significantly than that observed in the untreated 3 ddN being 51.6 ± 0.2 x 75.2 ± 0.1 µm. However, no change is

observed in the nuclear size being 5.1 ± 0.3 µm in diameter. The nature of the secretory granules at the different cell types shows fine droplets (Fig. 2).

5 ddN:

Generally, in 5 ddN treated nymphs, all the 3 types of alveoli are markedly increased in size than those of untreated control.

The size of **type I** alveoli is 23.3 ± 0.3 x 30.1 ± o.1 µm and their central nuclei are 4.9 ± o.2 x 6.9 ± 0.2 µm (Table 1). **Type II** alveoli increased to 29.7 ± 0.1 x 66.2 ± 0.1 µm, their nuclei remain almost unchanged, measuring 8.5 ± 0.3 µm (Table 2). **Table III** alveoli increased to 17.8 ± 0.1 µm in diameter ($P < 0.01$). The nuclei measure 5.0 ± 0.3 µm in diameter (Table 3).

No significant change is observed in the form of cytoplasmic secretion of both 3 types alveoli from that observed in untreated ones.

However, the distribution pattern of the secretory product changed in all types. In type I alveoli, the granular inclusions become densely distributed in the cell with heavy aggregates at the cell periphery, whereas in type II, it becomes evenly scattered in the cell cytoplasm. Type III alveoli granular inclusions become distributed within the different types of cells (Fig. 3).

7 ddN:

In treated 7 ddN, **type** I alveoli showed an increase in size, measuring 20.3 ± 0.5 x 32.1 ± 0.2 µm and their central nuclei remain almost unchanged, measuring 5.0 ± 0.2 x 6.3 ± 0.1 µm ($P > 0.05$) (Table 1). No change is observed in the form and distribution of the cytoplasmic inclusions from that observed in the untreated ones.

Type II alveoli significantly increased in size when compared with untreated ones, measuring 16.3 ± 0.1 x 13.0 ± 0.2 µm. Also their nuclei are similarly unchanged measuring 5.1 ± 0.2 µm in diameter (Table 2). No significant change is observed in the form of cytoplasmic secretion from that in the untreated ones. On the other hand, their distribution pattern changed appeared densely scattered in the different types of cells.

Type III alveoli enlarges considerably, measuring 15.7 ± 0.1 µm in diameter ($P < 0.02$). The nuclei of its cells remain almost unchanged, measuring 4.8 ± o.2 µm in diameter ($P > 0.70$) (Table 3). No significant change is observed in the form and distribution pattern of the secretory product in the different cells when compared with untreated ones.

9 ddN:

Type I alveoli, remains almost unchanged measuring 19.5 ± 0.7 x 29.8 ± 0.6 µm, and their central nuclei remain unchanged, measuring 4.8 ± 0.2 x 6.5 ± 0.2) (Table 1). The size of **type II** alveoli is unchanged being 16.1 ± o.5 x 12.1 ± 0.5 µm, and their nuclei 4.8 ± 0.3 µm in diameter (Table 2). **Type III** alveoli also unchanged measuring 13.3 ± o.2 µm and 4.9 ± 0.2 um in diameter, respectively (Table 2). No significant change is observed in the form of secretory product of the different cells of the 3 types of alveoli when compared with untreated ones (Fig. 4).

11 ddN:

In 11 ddN treated with 20 µg β-ecdysone, **types I, II and III** alveoli and their nuclei remain almost unchanged in size measuring 20.3 ± 0.5 x 33.3 ± 0.8 µm, 17.1 ± 0.5 x 19.6 ± 0.3 µm, and 16.2 ± o.2 µm in diameter respectively. Their nuclei are 6.2 ± 0.1 x 7.2 ± o.1 µm, 4.9 ± 0.2 µm and 5.1 ± 0.3 µm in diameter respectively (Tables 1-3). No change is observed in the form of the cytoplasmic secretion of the different cell types, but their distribution pattern changed to be densely distributed in the cells.

II Histochemistry:

Positive reaction to general proteins, lipids and glycogen stains in treated nymphs was observed (Figs. 5-7). In treated engorged nymphs, protein, lipid and glycogen contents of types I, II, and III alveoli of salivary glands increased when compared with untreated nymphsticks at the same developmental stage and this is indicated by a stronger staining reaction using Hg-BPB, SB and PAS stains respectively.

Table 1: Changes in the size of the salivary glands type I alveoli and their nuclei in nymphal *H. dromedarii* at different developmental stages after treatment with 20-Hydroxy Ecdysone on nymphal engorgement day.

Nymphal Stage	Mean alveoli dimensions (µm ± SE).		Mean central nucleus Dimensions (µm ±SE)	
	Untreated	Treated	Untreated	Treated
IddN*	30.9 ± 0.2 x 68.0 ± 0.7 a	33.6 ± 0.4 x 70.1 ± 0.2 b	5.3 ± 0.3 x 7.1 ± 0.2 g	5 3 ±0.3 x 7.1 ± 0.2 g
3 ddN	21.9 ± 0.5 x 26.0 ± 0.5 c	26.4 ± 0.5 x 29.8 ± 0.8 d	4.3 ± 0.3 x 6.8 ± 0.3 g	4.7±0 2 x 7.1 ± 0.3 g
5 ddN	20.4 ± 0.9 x 28.1 ± 0.2 c,e	23.3 ± 0 3 x 30.1 ± 0.1 d	4.9 ± 0.2 x 6.7 ± 0 3 g	4.9±0.2 x 6.9 ± 0.2 g
7 ddN	19 6 ± 0.6 x 28.3 ± 0.5 e	20.3 ±0.5 x 32.1± 0.2 d	4.8 ± 0.3 x 6.1 ± 0.2 g	5.0 ±0.2 x 6.3 ± 0.1 g
9 ddN	18.8 ± 0.5 x 28.6 ± 0.9 e	19.5 ± 0.7 x 29.8 ± 0.6 e	4.8 ± 0.2 x 6.3 ± 0.2 g	4.8 ±0.2 x 6.5 ± 0.2 g
11 ddN	19.9 ± 0.5 x 32.0 ± 0.9 f	20.3 ± 0.5 x 33.3 ± 0.8 f	6.0 ± 0.1 x 7.2 ± 0.1 g	6.2 ±0.1 x 7.2 ± 0.1 g

* I ddN = 1 day after nymphal engorgement and dropping off the host; 3 ddN = 3 days after nymphal engorgement and dropping off the host; etc . . . .

** Figures followed by the same letters are statistically similar (P > 0.05); those followed by different letters are significantly different (P < 0.05 - < 0.001).

Table 2: Changes in the size of the salivary glands type II alveoli and their nuclei in nymphal *H. dromedarii* at different developmental stages after treatment with 20-Hydroxy Ecdysone on nymphal engorgement day.

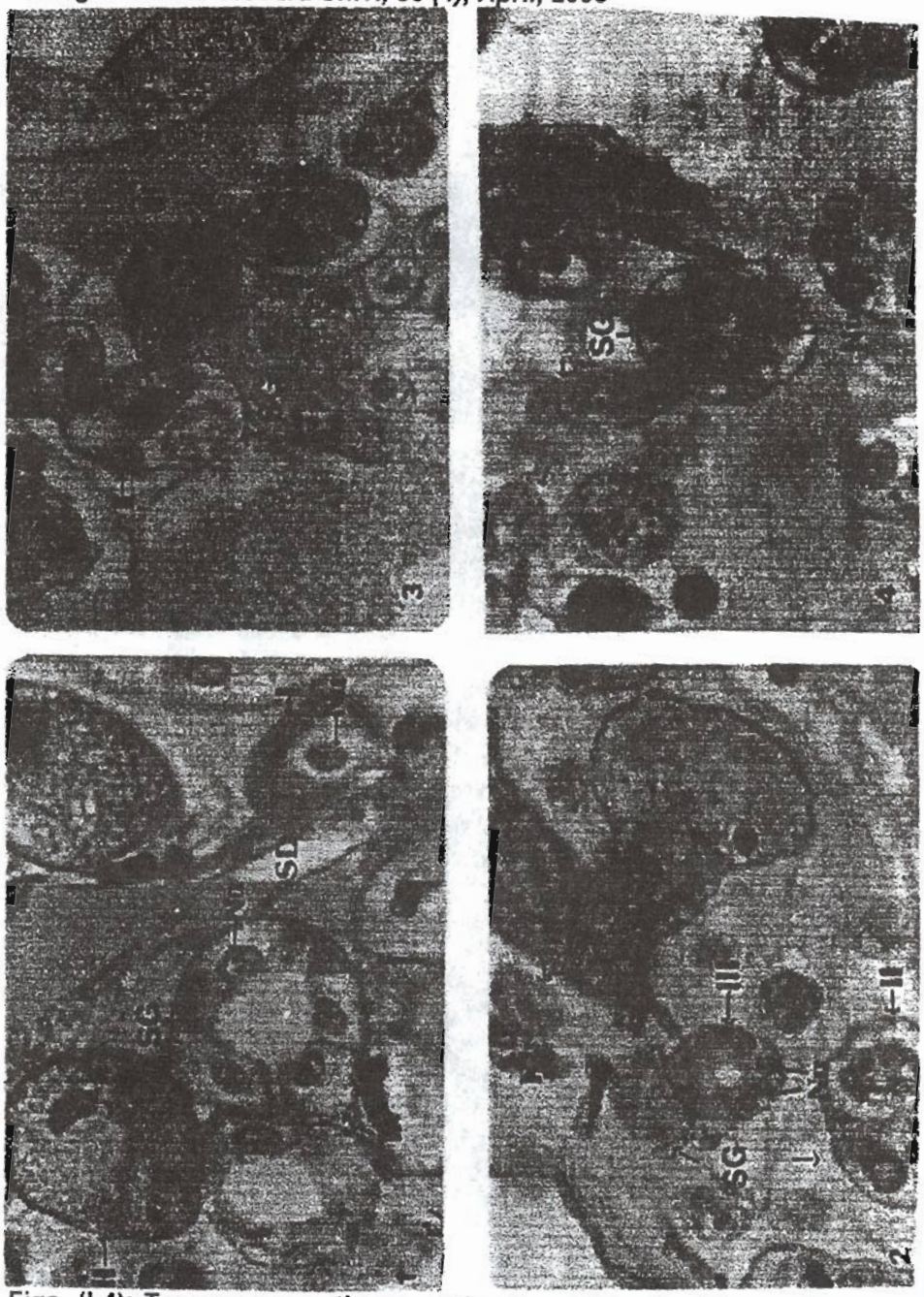
Nymphal Stage	Mean alveoli dimensions (µm ± SE).		Mean* nucleus Diameter (µm ±SE)	
	Untreated	Treated	Untreated	Treated
IddN**	77.1 ± 0.9 x 97.4 ± 0.9a***	99.6 ± 0.5 x 111.0 ± 0.3 b	8.8 ± 0.5 j	8.9 ± 0.3 j
3 ddN	51.3 ± 0.4 x 81.5 ± 0.3 c	65.1 ± 0.7 x 83.0 ± 0.2 d	8.5 ± 0.5 j	8.7 ± 0.3 j
5 ddN	27.3 ± 0.8 x 64.0 ± 0.1 e	29.7 ± 0.1 x 66.2 ± 0.1 f	8.5 ± 0.5 j	8.5 ± 0.3 j
7 ddN	11.5 ± 0.5 x 14.0 ± 0.6 g	16.3 ± 0.1 x 13.0 ± 0.2 h	4.8 ± 0.3 k	5.1 ± 0.2 k
9 ddN	11.5 ± 0.5 x 16.1 ± 0.5 g	16.1 ± 0.5 x 12.1 ± 0.5 g	4.8 ± 0.3 k	4.8 ± 0.3 k
11 ddN	16.9 ± 0.5 x 18.8 ± 0.8 i	17.1 ± 0.5 x 19.6 ± 0.3i	4.8 ± 0.3 k	4.9 ± 0.2 k

* This mean was obtained from the measurements of the nuclei present in the alveolus since cell boundaries disappeared and the alveolus appears as a syncytium.
** 1 ddN = 1 day after nymphal engorgement and dropping off the host; 3 ddN = 3 days after nymphal engorgement and dropping off the host; etc . . . . . .
*** Figures followed by the same letters are statistically similar (P > 0.05); those followed by different letters are significantly different (P < 0.05 - < 0.001).

Table 3: Changes in the size of the salivary glands type III alveoli and their nuclei in nymphal *H. dromedarii* at different developmental stages after treatment with 20-Hydroxy Ecdysone on nymphal engorgement day.

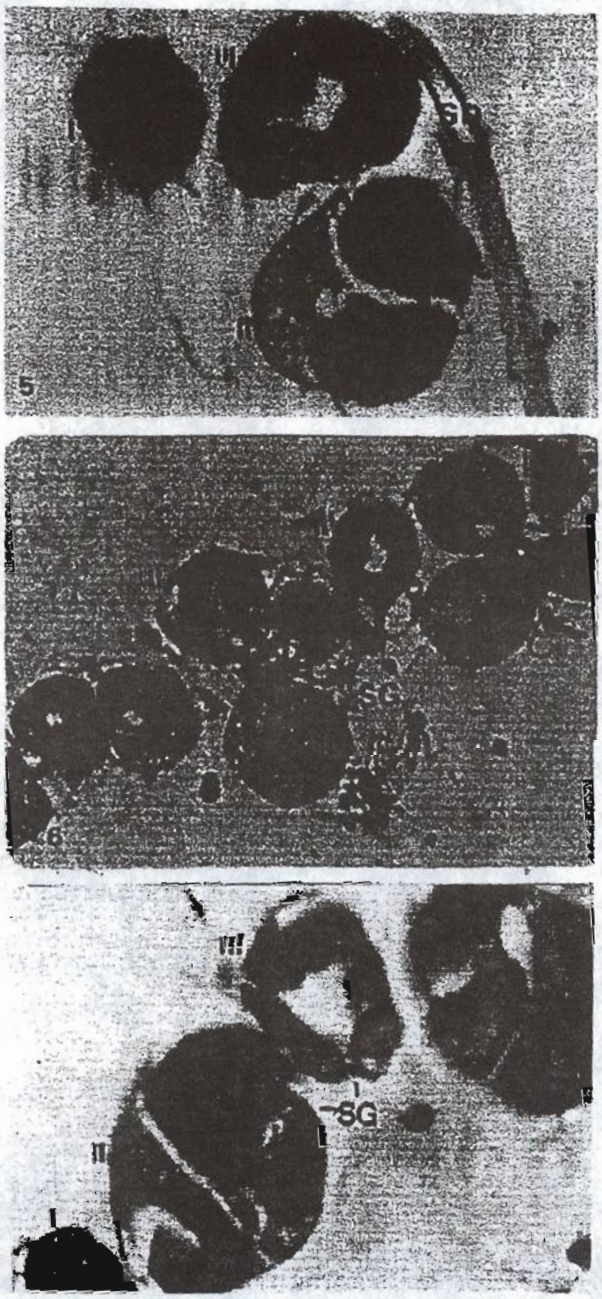
Nymphal Stage	Mean alveoli dimensions (µm ± SE).		Mean* nucleus Diameter (µm ±SE)	
	Untreated	Treated	Untreated	Treated
IddN**	69.6 ± 0.3 x 87.1 ± 0.9a***	71.8 ± 0.3 x 88.9 ± 0.2 b	4.8 ± 0.8 i	5.9 ± 0.5 i
3 ddN	49.0 ± 0.7 x 74.4 ± 0.3 c	51.6 ± 0.2 x 75.2 ± 0.1 d	4.8 ± 0.3 i	5.1 ± 0.3 i
5 ddN	14.3 ± 0.6 e	17.8 ± 0.1 f	4.8 ± 0.3 i	5.0 ± 0.3 i
7 ddN	14.0 ± 0.6 e	15.7 ± 0.1 g	4.8 ± 0.3 i	4.8 ± 0.2 i
9 ddN	12.3 ± 0.3 e	13.3 ± 0.2 e	4.8 ± 0.3 i	4.9 ± 0.2 i
11 ddN	15.9 ± 0.2 h	16.2 ± 0.2 h	4.9 ± 0.1 i	5.1 ± 0.3 i

* This mean was obtained from the measurements of the nuclei present in the alveolus since cell boundaries disappeared and the alveolus appears as a syncytium.
** 1 ddN = 1 day after nymphal engorgement and dropping off the host; 3 ddN = 3 days after nymphal engorgement and dropping off the host; etc . . . . . .
*** Figures followed by the same letters are statistically similar (P > 0.05); those followed by different letters are significantly different (P < 0.05 - < 0.001).



Figs. (I-4): Transverse sections passing through salivary glands alveoli of treated *Hyalomma dromedarii* nymph. I, type I alveoli; II, type II alveoli; III, type III alveoli; Nu, nucleus; SD, salivary duct; SG, salivary gland. Chrome Haematoxylin-Phloxine (X400).

Fig. 1, 1 day dropping treated nymph; Fig. 2, 3 days dropping treated nymph; Fig. 3, 5 days dropping treated nymph; Fig. 4, 9 days dropping treated nymph.



Figs. (5-7): Transverse sections passing through salivary glands alveoli of treated *Hyalomma dromedarii* nymph. I, type I alveoli; II, type II alveoli; III, type III alveoli; SD, salivary duct; SGg, salivary gland. Figs. 5 & 7 (X400), Fig. 6 (X 250).
Fig. 5, 1 day dropping treated nymph stained with Mercury Bromophenol Blue; Fig. 6, 1 day dropping treated nymph stained with Sudan Black; Fig. 7, 1 day dropping treated nymph stained Periodic Acid-Schiff.

DISCUSSION

The effect of the topical application of 20-HE on nymphal stage *H. dromedarii* on dropping day was observed on the salivary glands of nymphs (1 ddN, 3 ddN, 5 ddN, 7 ddN, 9ddN and 11 ddN). It did not alter the normal pattern of salivary gland differentiation at all different developmental stages of nymphs. 20-HE stimulated a significant size increase in types I, II and III alveoli in 1 ddN, 3ddN, 5 ddN, and 7 ddN and these types remain unchanged in size in 9 ddN and 11 ddN when compared with the untreated nymphs (Mohamed and Darwish 1999).

Accompanying the difference in the mean diameter of the salivary gland alveoli noted between treated and untreated nymphs, was observed a stronger staining affinity of secretory inclusions present in the different alveoli types to proteins, lipids and glycogen stains in treated nymphs. It is therefore, possible to suggest that application of 20-HE may stimulate increased synthesis of protein, these results agree with Shelby *et al.* (1989). Also, this activation may further induce increased production of secretory proteins, lipids and glycogen presumably act as energy necessary for the secretion of the attachment cement. Biochemical analysis of cement cones shows that some lipid and carbohydrate is present in the form of lipoprotein and glycoprotein respectively (Binnington and Kemp, 1980; Walker *et al.* 1985, Marzouk, 1988; Marzouk *et al.* 1994). The ecdysteroides probably act directly in the form of an ecdysteroid- receptor complex on the genetic material. In most cases, the hormone increase the rate of biosynthesis of protein, lipids and carbohydrates (Gadallah *et al.*; 1990. Friesen and Kaufman, 2004). These results confirm with the present study. In *Amblyomma americanum* treatment with 20-HE, stimulated virgins to gain additional weight (195%) and total salivary gland protein (144%) (Shelby *et al.*, 1989). Similarly Epstein and Lockshin, 1981, were also reported that 20-HE initiate synthesis of proteins and RNA in insect salivary glands.

In the present study, all alveoli types remain unchanged in size in 9 ddN and 11 ddN when compared with untreated nymphs may be due to 20-HE was used for cuticle synthesis, increase epidermal cell activity and deposition of the endocuticular lamellae in this nymphal stage to begain moult to adult stage especially 20-HE, accelerate moulting in nymphs and play an important role in the humoral control of tick moulting process (Khalil *et al.* 1984; Marzouk *et al.* 1994).

Recent evidence strongly suggests that the degeneration factor is an ecdysteroid. Harris and Kaufman (1985) showed that infusion of 20-hydroxyecdosone over a 24h period into small partially fed ticks (i.e. ticks below the critical weight for release of the degeneration factor) induced degeneration of the salivary glands. Salivary gland degeneration was reported in *A. hebraeum* and *A. americanum* to be initiated by 20-HE, but does not occur in unmated females below a weight threshold (Kaufman, 1986 and Lindsay and Kaufman, 1988). Harris and Kaufman (1981) and Kaufman (1988) suggested that the control of normal salivary gland degenerated in ixodids, is mediated by a tick salivary gland degeneration factor which is

probably an ecdysteroid. It is therefore, possible to assume, that in the present study the application of 20-HE to nymphs may therefore mimic this natural increase in ecdysteroids, leading to increased degeneration. 20-hydroxyecdysone also induces the formation of autophagic vacuoles in the secretory labyrinth of the salivary gland this demonstrated that ecdysteroids can mimic the physiological effects of the degeneration factor (Harris and Kaufman, 1985) this results strengthens my suggestion that the natural degeneration factor is indeed an ecdysteroid. It may be concluded that there are probably other intrinsic physiological differences which regulate the responses of various tick species to exogenous ecdysteroids.

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أثر المعالجة بهرمون ٢٠- هيدروكسي إيكدايسون على الغدد اللعابية في حورية القراد من نوع هيالوما (هيا لوما) دروميدارياي أثناء مراحل تطورها.

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تناولت هذه الدراسة التغيرات التي تحدث في الغدد اللعابية في حورية القراد من نوع هيالوما(*هيالوما*) *دروميدارياي* المعامل بجرعة من هرمون ٢٠ – هيدروكسي إيكدايسيون ومقارنتها بالحوريات غير المعاملة. وقد وجد أن جرعة الهرمون المستعملة لها أثرها الواضح على ازدياد حجم الحويصلات الثلاث المكونة للغدة اللعابية وكذلك ازدياد نشاطها الإفرازي أثناء المراحل المختلفة لتطور الحورية. كما لوحظ ظهور بعض الفجوات في خلايا حويصلات النوع الثاني والثالث في اليوم التاسع والحادي عشر من اغتذاء الحورية وتركها لعائلها. كذلك تمت دراسة مدى ايجابية تفاعل مكونات هذه الحويصلات للصبغات الهستوكيميائية الخاصة بالبروتينات والدهون والكربوهيدرات، وقد دل زيادة هذه المكونات على زيادة النشاط الإفرازي للحويصلات أثناء المراحل المختلفة لتطور الحورية. مما يرجح أن الهرمون قد يؤثر بإيجابية اكثر إذا استعمل بتركيبات أخرى أو إذا اختلفت طرق المعاملة به.