**Cuticular Plasticization Induced by Injection of
Synganglion Extracts, Haemolymph or
Biogenic Amines in Virgin Female
Haemaphysalis longicornis
(Acari: Ixodidae)**

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ABSTRACT

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Synganglion extracts from 5-day fed non-copulated females or haemolymph from 5-day fed copulated females were injected into the haemocoel of partially engorged virgin females, and physiological saline was subsequently infused into the haemocoel at 150 mmHg for 48 hours. These components, the extract and the haemolymph, induced cuticular plasticization or >10-fold increase in relative body volume. Protease digestion did not prevent these components from inducing cuticular plasticization. Biogenic amines, octopamine and noradrenaline ($\geq 10^{-6}$ M in the haemolymph), also induced cuticular plasticization. These results suggest that a neurohormonal factor inducing cuticular plasticization is released from the central nervous system into the haemolymph after receiving copulatory stimuli.

Key words: cuticular plasticization, soft integument, organ extracts, biogenic amines, copulatory stimuli, *Haemaphysalis longicornis*

INTRODUCTION

In adult female ixodid ticks, expansion of the soft integument during feeding is an important event, because the expansion enables a great deal of feeding resulting in production of numerous eggs. The integumentary expansion consists of two phases; pre-copulatory “cuticle synthesis phase”, and post-copulatory “rapid expansion phase” (Hackman and Filshie, 1982).

Recently, Okura *et al.* (1996) revealed that cuticular plasticization induced by copulatory stimuli is necessary for initiation of integumentary rapid expansion. Moreover,

mechanisms of cuticular plasticization have been elucidated: novel “acidophilic epidermal cells” probably make the cuticle acidic, resulting in cuticular plasticization (Okura *et al.*, 1997). Thus, the importance of cuticular plasticization occurring after copulation has been increasingly recognized in metastriate ixodid ticks. However, nothing has been yet determined about neuroendocrinological mechanisms regulating the cuticular plasticization cued by copulatory stimuli in ticks.

We attempted to artificially induce cuticular plasticization in partially fed virgin females of *Haemaphysalis longicornis* Neumann to presume these neuroendocrinological mechanisms.

MATERIALS AND METHODS

Ticks used in the present experiments. Adult *Haemaphysalis longicornis* (bisexual race) used in the present experiments were collected with dragging at pastures in Kuju Highland, Oita Prefecture, Kyushu, Japan. Female and male ticks were separately fed on laboratory rabbits. Then, replete males were allowed to copulate with 5-day fed females. Partially engorged non-copulated (5-day fed) and partially engorged copulated (5-day fed, and 10 hours after copulation) females removed from hosts were used for preparation of haemolymph and organ extracts. Previous data (Okura *et al.*, 1996) indicate that the cuticular plasticization already occurs in the female several hours after copulation. Partially fed (5-day fed) virgin females removed from hosts were used for recipients of inflation experiments.

Chemicals. Dopamine (3-hydroxytyramine hydrochloride), noradrenaline (arterenol hydrochloride), octopamine (DL-octopamine hydrochloride), serotonin (5-hydroxytryptamine hydrochloride), trypsin (type II-S), and chymotrypsin (α -chymotrypsin type II) were purchased from Sigma Chemical Company (U. S. A.). Other chemicals used were analytical grade.

Preparation of haemolymph and organ extracts. A caudal incision was made on the integument of non-copulated and copulated females and 5 μ l of physiological saline (0.8% NaCl) were injected through the scutum into the haemocoel of the ticks, and then $\sim$ 3 μ l of haemolymph flowing out from the incision were collected into Eppendorf tubes with a small quantity of phenylthiourea as a clotting inhibitor at 4°C using capillary pipets. To prevent dilution of haemolymph by the saline, the saline was gently injected and the yellowish color of the haemolymph was monitored during the collection. After centrifugation at 3,000 g for 10 minutes, the haemocyte free haemolymph from non-copulated and copulated females were separately kept at -20°C until injection. Synganglia, salivary glands, midguts, tracheae and ovaries were removed from ticks after collecting haemolymph, and were washed with cold physiological saline. Synganglia, salivary glands, tracheae and ovaries from 50 females, and midguts from 10 were separately homogenized in 50 μ l of cold physiological saline using a microhomogenizer. The homogenates were centrifuged at 3,000 g for 10 minutes. The supernatants were removed and the precipitates were re-extracted with the same volume of cold physiological saline. The extracts were pooled and kept at -20°C until injection.

Protease digestion of haemolymph and synganglion extracts. The haemolymph from copulated females and the synganglion extracts from non-copulated females, having the ability to induce cuticular plasticization, were digested with both trypsin and chymotrypsin. The proteases ($100\mu\text{g}/\mu\text{l}$ in 0.1 M Tris-HCl buffer pH 8.0) were added to a final concentration of $2.0\mu\text{g}/\mu\text{l}$. The mixtures were incubated at 37°C for 30 minutes.

Inflation experiments. Degree of induction of cuticular plasticization caused by injection of haemolymph, organ extracts or biogenic amines was examined with "inflation experiments" (Okura *et al.*, 1996). A glass micropipet was made using a micropipet puller (PB-7, Narishige scientific instrument lab.); the tip of the pipet was broken to $\sim 100\mu\text{m}$ in external diameter. The pipet was filled with the following substances from the tip to the base (Fig. 1a): (1) a candidate for induction of cuticular plasticization, that is haemolymph, organ extract or biogenic amine; (2) paraffin oil partition; (3) physiological saline supplied from the irrigator. The pipet was then inserted into the haemocoel of the tick through the scutum using a micromanipulator

Fig.1. Apparatus for inflation experiments. A schematic diagram showing glass micropipet used in the experiment (a). General view of the apparatus (b). Asterisk indicates a tick inflated. GM, glass micropipet; MH, micropipet holder; MM, micromanipulator; TT, teflon tube connected with irrigator. Scale bar indicates 5 cm.

(MM-188, Narishige scientific instrument lab.) visualized through a stereomicroscope (Figs. 1 b and 2). Solutions in the pipet were infused at 150 mmHg for 48 hours at 25°C. The physiological saline and paraffin oil without a candidate plasticization inducer were infused for 48 hours as a control.

The candidate compounds injected were as follows: 10 μ l of haemolymph or organ extracts; 10 μ l of protease digested haemolymph or extracts; 5 μ l of octopamine, noradrenaline, dopamine or serotonin in physiological saline having concentrations of 2×10^{-8} , 2×10^{-7} , 2×10^{-6} , 2×10^{-5} , 2×10^{-4} , 2×10^{-3} or 2×10^{-2} M. Kaufman and Phillips (1973) showed that haemolymph volume remains at a constant 23% of body weight throughout the feeding period in the ixodid tick, *Dermacentor andersoni*. On the basis of these data, the haemolymph volume of recipient ticks in the present inflation experiments, who had an average body weight of 23 mg, was estimated to be $\sim 5 \mu$ l. So that, concentrations of biogenic amine in the haemolymph just after injection were $\sim 1/2$ of the concentration of injected amines.

Idiosomal length, width, and depth (the length between dorsal and ventral surfaces) were measured with a 0.01 mm precision caliper at onset of injection immediately followed by inflation and after 48 hours of inflation. In accordance with Yano *et al.* (1989), the body volume of ticks was estimated using the stereometry of a spheroid ($\pi/6 \times \text{length} \times \text{width} \times \text{depth}$). The relative body volume was also calculated: relative body volume = the body volume after 48 hours of inflation divided by the volume at the onset of inflation. The relative body volume was taken as a measure of the degree of induction of cuticular plasticization. Significant differences between means of the relative body volume or the degree of induced plasticization were analyzed by Mann-Whitney U test.

RESULTS

When cuticular plasticization did not occur in the recipient tick, the ticks slightly expanded or gained a small relative body volume (Figs. 2 a and b). Whereas, the ticks

Fig. 2. Ticks in inflation experiments. At the onset of the experiment (a). At 48 hours of inflation without induction in cuticular plasticization (physiological saline control) (b). At 48 hours of inflation with induction in cuticular plasticization (octopamine 2×10^{-4} M) (c). Scale bar indicates 5 mm.

receiving induction of cuticular plasticization fully expanded or gained a large relative body volume (Figs. 2a and c).

Effects of various organ extracts and haemolymph on the induction of cuticular plasticization (or an increase in relative body volume) in the partially fed virgin females are shown in Table 1. Synganglion extracts from non-copulated females and haemolymph from copulated females induced significantly large plasticization of the cuticle.

The active components of the synganglion extract and the haemolymph retained the ability to induce cuticular plasticization after protease digestion (Table 2).

Effects of biogenic amines injected into the partially fed virgin females on the induction of cuticular plasticization are shown in Fig. 3. Both octopamine and noradrenaline induced cuticular plasticization at low ($\geq 10^{-6}$ M) concentrations. Whereas,

Table 1. Effects of haemolymph and organ extracts injected into haemocoel of 5-day fed virgin females on induction of cuticular plasticization. Degree of induction is indicated by relative body volume (RBV) (mean $\pm$ S.E.) after 48 hours of inflation.

Injected component	n	RBV (-fold)
Non-copulated female		
Haemolymph	3	6.13 $\pm$ 0.58
Synganglion extract	3	13.47 $\pm$ 1.07*
Salivary gland extract	3	6.10 $\pm$ 2.19
Midgut extract	3	6.23 $\pm$ 1.46
Trachea extract	3	6.13 $\pm$ 1.39
Ovary extract	3	5.60 $\pm$ 1.28
Copulated female		
Haemolymph	3	13.23 $\pm$ 1.32*
Synganglion extract	3	6.57 $\pm$ 1.04
Salivary gland extract	3	7.03 $\pm$ 1.94
Midgut extract	3	6.30 $\pm$ 1.78
Trachea extract	3	5.90 $\pm$ 1.65
Ovary extract	3	6.50 $\pm$ 0.46
Physiological saline	5	6.28 $\pm$ 1.45

* Significantly different from physiological saline control at $P < 0.05$.

Table 2. Effects of protease digested synganglion extract and haemolymph on the induction of cuticular plasticization. Degree of induction is indicated by relative body volume (RBV) (mean $\pm$ S.E.) after 48 hours of inflation.

Injected component	n	RBV (-fold)
Digested extract of synganglion from non-copulated females	3	11.90 $\pm$ 1.81*
Digested haemolymph from copulated females	3	12.77 $\pm$ 1.40*
Physiological saline	5	6.28 $\pm$ 1.45

* Significantly different from physiological saline control at $P < 0.05$.

dopamine at a considerably high ($\geq 10^{-4}$ M) concentration induced plasticization. However, serotonin did not induce cuticular plasticization at the present doses. All amines used in the present experiments were not lethal to ticks at the present doses.

DISCUSSION

Synganglion extracts from non-copulated females and haemolymph from copulated females induced cuticular plasticization. In addition, protease digestion did not prevent these components from inducing plasticization. These results imply that a cuticular plasticization inducing factor which is neither a peptide nor a protein is released from the synganglion into the haemolymph after copulation.

One of possibilities is that a candidate for the cuticular plasticization inducing factor having the resistance to protease is a sort of biogenic amines. In this connection, catecholamines that may function as not only neurotransmitters in the central nervous

Fig. 3. Abilities to induce cuticular plasticization of four biogenic amines injected into the haemocoel of virgin ticks; dose-response curve. Degree of induction is indicated by relative body volume after 48 hours of inflation. Asterisks indicate significant difference from physiological saline control (0 M) at $P < 0.05$. Solid circle and vertical bar indicate mean and S.E., respectively. Numbers in parentheses are sample numbers.

system (CNS) but also as neurohormones outside the CNS are histochemically demonstrated in a tick, *Boophilus microplus* (Binnington and Stone, 1977; Binnington and Obenchain, 1982). Biogenic amines, octopamine, noradrenaline and dopamine induced the cuticular plasticization in the present experiments. These facts are not contradictory to the possibility mentioned above.

At doses exceeding 10^{-7} M in the haemolymph, octopamine and noradrenaline induced cuticular plasticization in the present experiments. The concentrations just above the threshold of the induction in the tick are comparable to physiological concentrations of the biogenic amines in the haemolymph of the locust (Goosey and Candy, 1980) and the male cricket (Nagao *et al.*, 1991). In the locust, the haemolymph octopamine level increases to 173 nM (1.73×10^{-7} M) during flight. In the cricket, octopamine at $>10^{-7}$ M and serotonin at $>10^{-6}$ M modify the interval time of mating behavior. Therefore, the effective concentration of injected amine in a female tick seems to be similar to the physiological level.

Actions of octopamine and noradrenaline injected into the female tick in the present experiments seem to be explained in two different ways: (1) the injected amines penetrate into the CNS and modify the conditions of the neuroendocrine system, resulting in the induction of cuticular plasticization; (2) the injected amines directly change the physiological state of the integument, resulting in the induction. The perineurium of synganglion consisting of cortical glial cells which have septate or other types of junctions possesses the blood-brain barrier (Sonenshine, 1991). It is unlikely that biogenic amines with physiological concentration permeate the barrier. Thus, the lowness in the concentrations of injected octopamine and noradrenaline acting in the tick appears to support the latter explanation.

Cuticular acidification is a direct cause of cuticular plasticization in the bug (Reynolds, 1975) and the tick (Okura *et al.*, 1997). A novel component of the soft integument, "acidophilic epidermal cells" in the tick is a candidate for the cuticular acidifier (Okura *et al.*, 1997). Interactions between these cells and the cuticular plasticization inducing factor will be interesting questions.

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摘 要

フタトゲチマダニ雌成虫における総神経節抽出液、血リンパまたは生理活性アミン注射によるクチクラ柔軟化誘導 大倉信彦・毛利孝之・白石哲（九州大学農学部動物学教室）・安藤光一（九州産業大学国際文化学部生物学研究室）

交尾刺激によって誘導されるクチクラ柔軟化の神経内分泌的な調節機構を調べるために、

フタトゲチマダニ部分吸血雌成虫にクチクラ柔軟化を人為的に誘導することを試みた。未交尾雌成虫の総神経節抽出液または交尾雌成虫の血リンパを未交尾雌成虫の血体腔にそれぞれ注射後、生理食塩水を48時間注入加圧すると、著しい体積増加すなわちクチクラ柔軟化が起こった。これら抽出液と血リンパの蛋白分解酵素処理は、クチクラ柔軟化誘導能を阻害しなかった。血リンパ中に注射された生理活性アミン、オクトパミンとノルアドレナリンもまた比較的低濃度 ($\geq 10^{-6}$ M) でクチクラ柔軟化を誘導した。

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