The Journal of Experimental Biology 213, 000-000

doi:10.1242/jeb.044412

Cuticular plasticization in the tick, *Amblyomma hebraeum* (Acari: Ixodidae): possible roles of monoamines and cuticular pH

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Accepted 4 May 2010

SUMMARY

The degree of plasticization of the alloscutal cuticle of a 'hard' (ixodid) tick, *Amblyomma hebraeum*, and a 'soft' (argasid) tick, *Ornithodoros moubata*, was assessed throughout the blood-feeding period. Cuticle viscosity was calculated from rate of creep of cuticle under constant load using a Maxwell model. Feeding-related plasticization (i.e. increased rate of extension under a constant load) occurred in *A. hebraeum* but not in *O. moubata*. Maxwell viscosity of unfed *A. hebraeum* cuticle was relatively high (720 GPa s) but was significantly lower in feeding ticks. Small partially fed ticks displayed a viscosity of ~108 GPa s. Still, lower values (42 GPa s) were observed in the largest of the engorged ticks. Following cessation of feeding, there was a significant but limited reversal in viscosity back to ~100 GPa s. The water content of cuticle of unfed *A. hebraeum* (23.4% of wet mass) rose sharply after the onset of feeding and reached a plateau value of 34.0% at a fed/unfed weight ratio of 3 and beyond. Ixodid ticks lay down new endocuticle during the feeding period. The observed increase in cuticle hydration suggests that both old and new cuticles are hydrated during feeding. Monoamines may play an important role in controlling cuticle viscosity. Dopamine (DA) injected into partially fed *A. hebraeum* caused plasticization. 5-Hydroxytryptamine (serotonin, 5-HT), which induces plasticization in the blood-sucking insect *Rhodnius prolixus*, had no statistically significant effect on tick cuticle. Octopamine (OA) and tyramine both caused cuticle stiffening (i.e. opposed plasticization). This suggests a possible inhibitory effect but co-injection of OA with DA did not reduce DA-induced plasticization. The mechanism leading to plasticization of tick cuticle may involve a change in cuticular pH. The viscosity of tick cuticle loops was highest at pH 8.0 (389 GPa s) and fell precipitously in the acidic range to a low value of 2.2 GPa s at pH 5.5–5.7. A cuticular pH of ~6.5 would account for the lowest viscosity observed under physiological conditions (42.4 GPa s for large, day 0, engorged ticks). The V-ATPase inhibitor, concanamycin A, was a potent inhibitor of DA-induced plasticization. These results are consistent with a model in which DA acts to cause plasticization through transport of H^+ ions into the cuticle. Measurement of cuticular ion (Na^+, K^+, Ca^{2+}, Mg^{2+}) content did not suggest that plasticization is caused by any of these ions. Taken together, our results suggest that the mechanism of cuticular plasticization in feeding *A. hebraeum* is related to hydration, and involves the transport of H^+ ions into the sub-cuticular space by cells in the hypodermis. Feeding-induced plasticization was not observed in the rapid feeding tick, *O. moubata*.

Key words: cuticular plasticization, ticks, *Amblyomma hebraeum*, *Ornithodoros moubata*, dopamine, octopamine, 5-hydroxytryptamine, tyramine, concanamycin A.

INTRODUCTION

The integument of arthropods consists of a layer of epidermal cells and its overlying cuticle. At the time of ecdysis, the newly deposited cuticle becomes more compliant (i.e. it is more readily deformed in response to an applied force), a process called 'plasticization'. Plasticization is assumed to be an adaptation to reduce the metabolic energy required to expand the cuticle or to increase the speed with which it can be expanded (reviewed by Ewer and Reynolds, 2002).

Vertebrate blood, because of its high water content, is a relatively dilute source of nutrients. Consequently, blood feeders must expand their body wall integument substantially in order to accommodate a huge blood meal. Thus, the 5th stage larva of the blood-sucking reduviid bug, *Rhodnius prolixus*, imbibes about 10–15 times its own unfed mass in host blood within ~10 min; during this time the endocuticle is plasticized (Bennet-Clark, 1962). Maddrell showed that this plasticization is locally controlled by nerves that terminate in the abdominal dorsal and ventral cuticular plates (Maddrell, 1966). Reynolds (Reynolds, 1975a) stretched excised loops of *R. prolixus* abdominal integument to measure cuticle mechanical properties and showed (Reynolds, 1974) that plasticization can be induced pharmacologically by the monoamine, 5-hydroxytryptamine (5-HT), both *in vivo* and *in vitro*. The mechanism of plasticization involves a chain of events (including increased hydration of the endocuticle resulting from acidification) that ultimately weakens secondary molecular interactions among cuticular proteins (Reynolds, 1975b). Plasticization also occurs during feeding in another blood-sucking reduviid, *Triatoma infestans* (Melcón et al., 2005). Another study suggests that mere physical contact of the proboscis with a warm surface but without the intake of a blood meal may be sufficient to stimulate plasticization in both *R. prolixus* and *T. infestans* (Ianowski et al., 1998).

In female ticks of the family Ixodidae (informally known as 'hard ticks') feeding occurs in two phases: (1) during the initial slow phase of feeding (~7–8 days), the tick is continuously attached the host, increasing its mass ~10-fold; and (2) during the subsequent rapid phase of feeding (~1 day), the tick increases its mass a further 10-fold (Kaufman and Lomas, 1996; Kaufman, 2007). In most species, copulation occurs while on the host. In both blood-sucking insects

and ixodid ticks, the epicuticle and part of the outer endocuticle is folded into surface lamellae; these lamellae flatten considerably during engorgement (Bennet-Clark, 1963; Hackman, 1975; Hackman and Filshie, 1982; Hackman and Goldberg, 1987).

The first experimental study of cuticular plasticization in an ixodid tick (*Haemaphysalis longicornis*) was presented by Okura et al. (Okura et al., 1996). They reported that a significant increase in body cuticle extensibility occurs following copulation. Cuticle loops were stiff at pH 7 and 8 but became progressively more extensible at pH 6, 5 and 4 (Okura et al., 1997a). An extract of the synganglion [a structure comprising the complete central nervous system (CNS) of ticks] from virgin females stimulated cuticular plasticization when injected into partially fed virgin females, as did an extract of hemolymph from copulated females (Okura et al., 1997b). Octopamine (OA; at 1 µmol l^{-1}), noradrenaline (at 1 µmol l^{-1}) and dopamine (DA; at 1 mmol l^{-1}) also stimulated plasticization when injected into virgin females; 5-HT had no plasticizing effect up to 10 mmol l^{-1}. The latter authors proposed that copulatory stimuli result in a release into the hemolymph from the synganglion of a factor – perhaps a biogenic amine such as OA, noradrenaline and/or DA – that triggers plasticization (Okura et al., 1997b).

In this study, we investigate several aspects of feeding-related cuticular plasticization in the African ixodid tick, *Amblyomma hebraeum* Koch. Ticks of the family Argasidae are informally known as 'soft ticks' because their cuticle, even in the unfed state, is pliable. We also tested for cuticular plasticization in the African argasid tick, *Ornithodoros moubata*, but we could detect no evidence for feeding-induced plasticization in this species.

MATERIALS AND METHODS

Ticks: *A. hebraeum*

The adult *A. hebraeum* ticks used in this study came from a laboratory colony established at the University of Alberta, Canada. Some batches had moulted to adults in April 2007 and others in October 2007. The experiments were carried out between November 2007 and July 2008, beginning with the April batches and progressing through to the October batches. (If kept at high relative humidity, unfed ticks can survive for a year or more without feeding.) Unfed masses of females used in this project spanned the range of 15–45 mg. Because of this relatively wide mass range, partially fed ticks at any given mass would not necessarily be at a similar physiological stage of the feeding cycle. For this reason, we plotted loop stretch as a function of fed/unfed weight ratio (henceforth simply called 'weight ratio') of each partially fed and fully engorged tick, in preference to the absolute weight. To measure the weight ratio, weighed unfed ticks were marked individually, prior to feeding, by gluing (cyanoacrylate glue) a knot of fine, colored silk thread to one of the eight legs. Ticks were fed on the shaved backs of New Zealand white rabbits (Latin name, Taxonomic authority?) to which was fastened a cloth-covered foam rubber arena using a tissue adhesive (Kalmar adhesive, Steamboat Springs, CO, USA). Ticks were removed at various stages of feeding depending on the particular experiment.

Ticks: *O. moubata*

The *O. moubata* ticks were nymphs (various stages) coming from a colony at the NERC Centre for Ecology and Hydrology (CEH), Oxford, UK, and provided through the courtesy of Dr Miles Nunn. The nymphs were confined to the backs of rabbits within a cloth-covered foam rubber arena fixed using Kalmar adhesive. In some cases guinea pigs ((Latin name, Taxonomic authority?) served as hosts, and for these feeds, ticks were confined to the shaved backs in small, plastic, gauze-floored capsules held in place by loosely wrapped gauze bandage. *Ornithodoros moubata* ticks were allowed to remain on the host animal for up to 2 h to engorge fully. Although the nymphs provided to us were a mixed batch of unknown stages, for our experiments we weight-matched pairs as closely as possible, and fed one of each pair on a host.

All animal handling procedures of this study were carried out according to protocols approved by the British Home Office under Animals (Scientific Procedures) Act 1986, Project License # 30/2473. The experiment carried out at the University of Alberta (see Fig. 12) was conducted in accordance with the guidelines of the Canadian Council on Animal Care and with approval from the Animal Care and Use Committee for Biosciences.

Preparation of cuticle loops

In female ixodid ticks, the body cuticle is composed of two basic types: the scutum and the alloscutum. The scutum is a small plate of sclerotized cuticle situated on the anterior dorsum just behind the mouthpart-bearing capitulum. The alloscutum, as the name implies, is the reminder of the body cuticle, the only sclerotized portions being small islands of cuticular plates associated with the gonopore, the spiracles and the anus. It is only the non-sclerotized, alloscutal cuticle that expands enormously during the feeding period and from which cuticle loops were cut (Fig. 1).

Prior to all experiments, ticks were briefly rinsed under a stream of tap water and wiped dry with soft tissue paper. Cuticle loops were cut from the posterior alloscutum using a 'loop cutter'

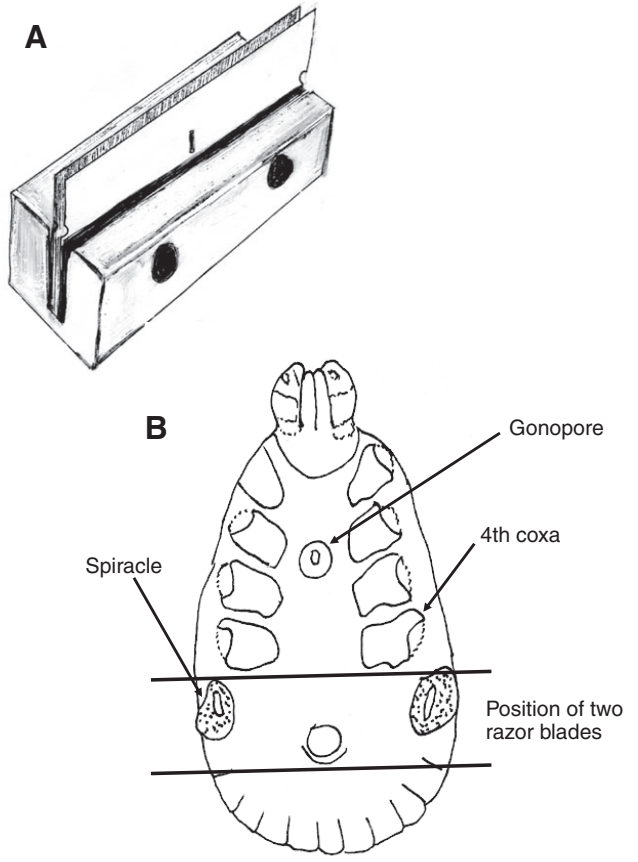

Fig. 1. (A) Diagram of the loop cutter (two single-sided razor blades held in a clamping device), and (B) the ventral surface of an unfed ixodid tick to show the approximate level at which the cuticle loops were cut (two parallel lines) in relation to various morphological features.

consisting of a clamping device that held two single-sided razor blades at a distance that could be adjusted by inserting shims (Fig. 1A). Ticks were laid dorsal-side down on a dissection platform made of Sylgard 184 silicon encapsulant (Dow-Corning, Town, State, Country), and the loop cutter aligned just behind the 4th coxae (Fig. 1B). The loop cutter was pressed down firmly until it cut completely through a cross-section of the body from the ventral to dorsal side, the loop appearing between the two fixed blades. The loops usually included the two spiracles and the anal pore. In most cases, the loop turned out to be somewhat wider on the ventral than the dorsal side. For unfed and partially fed ticks, the blades were set apart at ~1.25 mm. For engorged or near-engorged ticks, the approximate separation was set at 1.75 mm. It was necessary to use slightly wider loops for engorged *A. hebraeum* and *O. moubata* ticks, because in early trials the loops tended to break under load during the creep tests, perhaps because of a greater disparity in width between the dorsal and ventral sides in engorged ticks compared with unfed and small partially fed ticks.

For experiments testing rate of creep (RoC) throughout a normal feeding cycle, and for those testing pharmacological induction of plasticization, the cut loops were immediately transferred to disposable Petri dishes containing a simple isosmotic saline [1.2% NaCl buffered with 10 mmol l^{-1} morpholinopropanesulfonic acid (MOPS); pH=7.0–7.1]. Much of the soft tissue contained within the loop was removed with fine forceps and fine scissors before mounting the loop on the device for measuring RoC (Fig. 2). However, the hypodermis was not scraped away, and the ends of large tracheal branches emanating from the spiracles, and various muscle insertions, were left intact. The preparation of cuticle loops usually required only a minute or so. For measuring the effect of pH on cuticle plasticization, the loops were prepared differently, as described under 'pH tests' below.

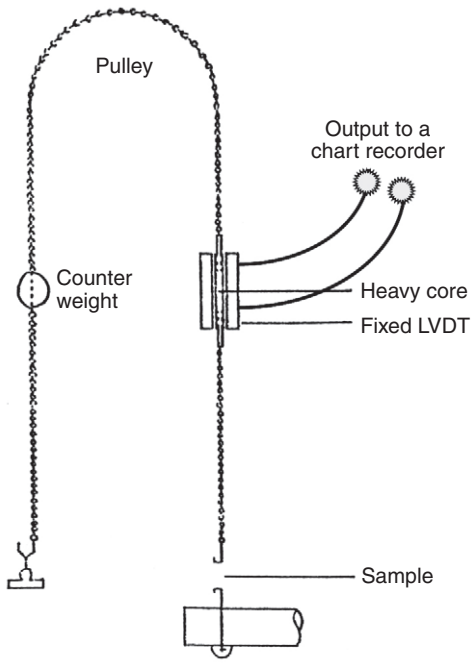

Fig. 2. Experimental setup for measuring rate of creep (RoC) in cuticle loops of *Amblyomma hebraeum* and *Ornithodoros moubata*. The loops were cut from alloscutal cuticle as described in the Materials and methods and suspended between the two hooks shown in the diagram. The lower hook was fixed; the upper hook could move as the cuticle stretched under the imposed load (70 g for *A. hebraeum* and 40 g for *O. moubata*). Stretching of the cuticle results in movement of the heavy core relative to the fixed linear variable differential transformer (LVDT). The electrical output caused by movement of the heavy core was relayed to a chart recorder.

Creep tests

RoC was measured using the mounting frame and linear variable differential transformer (LVDT) described by Reynolds (Reynolds, 1975a), and shown here in modified form in Fig. 2. Output from the LVDT was sent to a Rikadenki R21 chart recorder (Town, State, Country). The appropriate load to impose on the cuticle loops was determined in a series of preliminary experiments. For *A. hebraeum*, the imposed load ultimately chosen for all experiments was 70 g, and for *O. moubata* the imposed load was 40 g. RoC was measured over a period of 10–25 min (most frequently 12 min). Chart values at 30 s, 1 min and thereafter at 1-minute intervals, were transcribed to a Microsoft Excel® spreadsheet (Microsoft Corporation, Redmond, WA, USA) for further analysis.

Measurement of loop dimensions

Tick body size, and hence cuticle loop size, increased enormously throughout the feeding cycle (Fig. 3). It has also long been known that cuticle thickness increases substantially throughout the slow phase of engorgement because a significant amount of endocuticle is laid down at this time (Lees, 1952). Then, during the last day of feeding, the cuticle thins out again because of the rapid rate of expansion that occurs at this time (Lees, 1952) (see also Fig. 6). We measured thickness of the cuticle at the cut face of the carcass just anterior to where the loop had been cut. The carcass was mounted on a piece of modeling clay on the stage of a dissecting microscope, so that the cut faces of the dorsum and ventrum were horizontally displayed. Thickness of each cut face was measured using a calibrated ocular micrometer under high-power magnification. Four

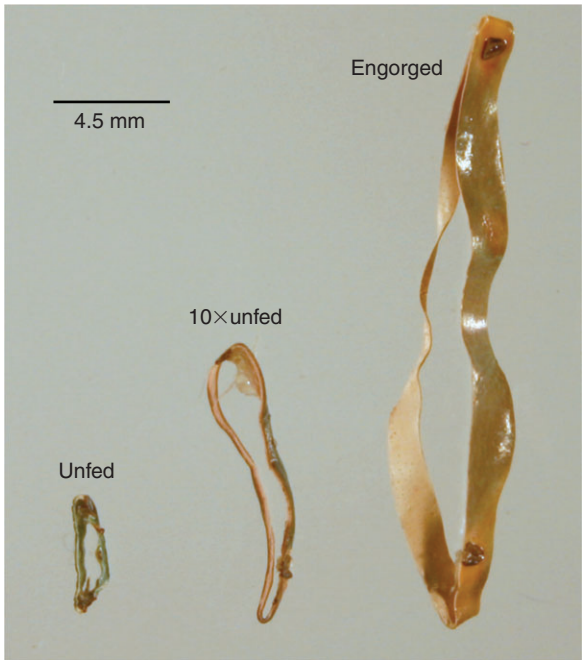

Fig. 3. Photographs of cuticle loops taken from *Amblyomma hebraeum* at three stages of feeding: unfed, end of slow phase of engorgement (~10× unfed mass), and engorged (~80× unfed mass).

to 10 replicate measurements were averaged for the dorsum and ventrum independently, but for calculating cross-sectional area of each loop, we recorded a single mean value of all replicate measurements. We measured total thickness and, in some experiments, we made independent measurements of the cuticular lamellae and outer and inner endocuticle (see Fig. 6). Length of the cuticle loops was measured under low-power magnification after recording RoC (i.e. post-stretch length).

Calibration and setup of the LVDT

The output of the LVDT deviated somewhat from linearity over the range of displacements measured in our experiments. The extent of this deviation was dependent on the starting position of the LVDT's heavy core relative to the fixed LVDT. Therefore, for all creep tests, the top of the heavy core was aligned initially to the top of the fixed LVDT, and displacement values were corrected by an experimentally determined correction factor.

Analysis of creep data

To analyze the stretch results for differing values of loop length, width and thickness, a classical engineering analysis of the stiffness of the loops was performed. Stretch was converted to engineering strain, dL/L_0, where dL is the stretch and L_0 is the initial loop length. L_0 was calculated by subtracting the observed stretch at the end of the creep test from the post-stretch loop length. The force on the loop was converted to engineering stress (units Pa): the force per unit of initial cross-sectional area. At large values of stretch (strain values >0.1), engineering stress and strain can deviate from true stress and strain [for a discussion, see Vogel (Vogel, 2003) and Vincent (Vincent, 1990)]. In this study, strain values were usually less than 0.1. Engineering stress and strain were used in all calculations. This approach enabled a direct measurement of cuticle stiffness and viscosity, as opposed to indirect measurements such as rate of increase in body volume under pressure (Maddrell, 1966; Okura et al., 1996; Ianowski et al., 1998). Viscosity as a measure of plasticization of cuticle is related to the rate of stretch of cuticle, $(dL/L_0)/dt$. The rate of increase in body volume under pressure is proportional to $L^2\times(dL/L_0)/dt$, and hence is not a valid measurement of viscous extensibility of cuticle, unless the specimens in the study are all of similar size.

Arthropod cuticle is composed of chitin fibrils in a complex protein matrix, and cuticle deformation under load is time-dependent (Vincent, 1990; Vincent and Wegst, 2004). Stretch data for each tick were normalized by calculating strain/stress as a function of time. Fig. 4 shows a typical trace for a partially fed female *A. hebraeum*.

Time-dependent stretch in cuticle can be modeled as a viscoelastic material undergoing both plastic (permanent) and viscoelastic (recoverable) deformation. Various models have been developed to characterize time-dependent stretch; for the RoC pattern observed in this study, a Maxwell model gave the best fit to the observed pattern of stretch [for a full discussion of modeling time-dependent stretch, see Vogel (Vogel, 2003) and Vincent (Vincent, 1990)]. The Maxwell model treats stretch as an instantaneous deformation characterized by a modulus of elasticity (units Pa) followed by a period of continuous extension characterized by a viscosity (units Pa s^{-1}). When full stretch data from the exact moment of imposition of load is available, a Maxwell model analysis can be used to estimate both initial modulus and viscosity. In this study, however, the experimental apparatus did not permit the recording of stretch at the instant of imposition of load, and hence we used the Maxwell analysis to calculate only a viscosity value for the cuticle loops. A linear best fit was calculated for the straighter portion of the stress/strain curve for each tick (e.g. Fig. 4). The inverse of the slope of strain/stress as a function of time gives the viscosity value, referred to in this study as the 'Maxwell viscosity'. Values observed in this study ranged from 5 to 1100 GPa s.

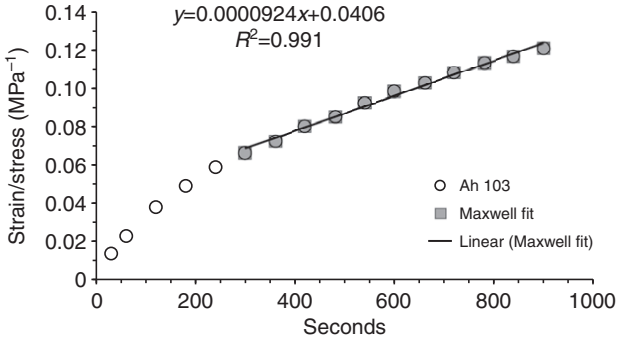

Fig. 4. Loop distension under constant 70 g load normalized to strain/stress, for a normal partially fed *Amblyomma hebraeum* female. The Maxwell viscosity value is the inverse of the slope of the fitted curve. This tick weighed 384 mg, the weight ratio being 17.4.

The limit for meaningful detection of RoC for our LVDT apparatus was in the order of 0.07 mm over 12 min, equivalent for an unfed tick to a strain of about 0.015, and a strain/stress value less than 0.007 MPa^{-1}. For statistical calculation purposes, ticks measuring below this threshold were treated as having a minimal viscosity of 1100 GPa s, i.e. the highest viscosity value measured in this study.

For a number of reasons, we did not have sufficient measurement data on the loops of *O. moubata* to carry out a proper Maxwell analysis. For these experiments comparing unfed and fed specimens, we report the RoC values directly (μm min^{-1} mm^{-1} loop length) on weight-matched specimens.

Water and ion content of the cuticle

Two series of tests were done on cuticular water content (University of Bath and University of Alberta), and one on ion content (University of Bath). In the first series (University of Bath), after taking the cuticle loops, there remained anterior and posterior parts of the tick carcass. For determining water and ion content of the alloscutal cuticle, these pieces were transferred to a disposable Petri dish containing light mineral oil (Sigma Chemical Co., St Louis, MO, USA). The posterior portion was half of a flattened, hollow ellipsoid, open at the straight edge, thus resembling a half pita bread (pocket bread); the joined dorsal and ventral surfaces of this piece of cuticle were separated by cutting along the lateral margin of the carcass with fine scissors. All of the soft tissue adhering to the internal dorsal and ventral surfaces was scraped away using the blunt side of a razor-blade scalpel. The dorsal alloscutum of the anterior carcass was likewise cut away using fine scissors but excluding the sclerotized scutal cuticle. As with the posterior portion, the internal surface of the anterior portion was scraped clean of adhering soft tissue using the blunt side of a razor-blade scalpel. These three pieces of cuticle from each tick were further cleansed of soft tissue and adhering mineral oil (supplier details, Town, State, Country) by wiping the internal and external surfaces with soft tissue paper. The cuticle pieces from each tick were then placed in a pre-weighed porcelain vessel (~13–15 g, weighed to the nearest 0.1 mg on an electronic balance), and the wet mass of the cuticle recorded. The vessels were covered with loose-fitting porcelain lids to protect the

samples from contaminating dust, and then they were dried in a 55°C oven. Several timed weighings over 24 h demonstrated that the cuticle loops reached a constant mass within 2 h, so subsequently this was the minimal drying time used.

After recording dry mass (to the nearest 0.1 mg) of the pieces of cuticle, the porcelain vessels were placed in a small laboratory furnace, and the temperature was raised from 250°C to 550°C in six steps (50°C each) in order to ash the cuticle samples; the cuticle samples were held at each temperature for ~20–40 min. Each ashed sample was then dissolved in 0.8 ml of 1 mol l^{-1} nitric acid. The dissolved ash was transferred to a polystyrene 15 ml conical-bottomed tube using a pipettor, and the porcelain vessels were washed twice with 3.6 ml of deionized (Milli-Q, Town, State, Country) water, the two washings being added to the conical-bottomed tube, so that the final concentration of nitric acid was 0.1 mol l^{-1}.

Standard solutions for each ion [sodium (0–5 p.p.m.), potassium (0–10 p.p.m.), calcium (0–5 p.p.m.) and magnesium (0–1 p.p.m.)] were prepared in 0.1 mol l^{-1} nitric acid. Sodium and potassium concentrations were measured by flame emission spectrophotometry (emission peaks at 589 nm and 766 nm, respectively), and calcium and magnesium concentrations were measured by flame absorption spectrophotometry (absorption peaks at 423 nm and 285 nm, respectively), all using a Varian AA 275 Atomic Absorption Spectrophotometer (Town, State, Country). Ion content of the cuticle samples is expressed as µmol mg^{-1} dry mass of cuticle.

In the second series of tests (University of Alberta), we measured water content on excised dorsal cuticle of ticks from which a loop had not been removed. A miniature razor-blade scalpel was used to cut around the lateral margin of the body mid-way between the dorsum and ventrum. On both sides of the anterior end, we sliced around the scutum/allosctutum margin. The severed dorsum was then removed by peeling it posteriorly. All soft tissue attached to the cuticle was removed by scraping it with the blunt edge of the razor-blade scalpel under light mineral oil (Fisher Scientific, Pittsburgh, PA, USA). After blotting the sample with tissue paper, each cuticle sample was transferred to a small, pre-weighed (nearest 0.01 mg) porcelain vessel, and wet mass was recorded on a microbalance to the nearest 0.01 mg. The sample was then dried as described above and the dry mass was then recorded to the nearest 0.01 mg. Water content of the cuticle is expressed as a percentage of wet mass.

Drug tests

The general procedure for testing the effects of drugs on RoC was to dissolve the drug of interest in a suitable vehicle at the desired concentration so that when 10 µl was injected into the hemocoel per 100 mg of body mass, the drug dose was 1 mmol kg^{-1} body mass. Hence, the concentration injected was 10 mmol l^{-1}. Injections were performed using a microliter syringe (Hamilton Company, Reno, NV, USA) fitted with a fine needle. The needle was advanced into the hemocoel *via* the camero-stomal fold (the articulation between the mouthpart bearing capitulum and the scutum), to just beyond the bevel. Injecting at this site almost never results in puncturing the gut. Cuticle loops were taken and RoC measured in the usual way within 30–60 min of injecting the drug of interest. Because we had initially hypothesized that a major plasticization event would occur during the rapid phase of engorgement, for all drug tests we chose ticks from the slow phase of engorgement. Most ticks used for drug tests were at a weight ratio of 8–11, although a few were at a weight ratio as low as 5–8, and a few at a ratio as high as 11–15. However, we felt justified in pooling the data for all ticks in this range because there was no statistically significant difference in Maxwell viscosity in normal ticks over this weight ratio range (Fig. 7).

For water-soluble drugs, the diluent was the MOPS-buffered saline described above. For some drugs (e.g. concanamycin A and butaclamol), the diluent was dimethylsulfoxide (DMSO) and, once dissolved, the drug was diluted in saline to the desired concentration for injection. Controls were injected with the equivalent concentration of DMSO. DMSO up to 12% in MOPS-buffered saline appeared not to have an adverse effect on the ticks and not to induce plasticization, although much higher concentrations did (see results for butaclamol).

For experiments in which a drug was being tested as an inhibitor of DA-induced plasticization, the putative inhibitor was injected first in a volume of 5 µl per 100 mg of body mass. Thirty minutes later, DA was injected in a volume of 10 µl per 100 mg of body mass. Controls were injected first with 5 µl per 100 mg of body mass of the appropriate vehicle, followed 30 min later by 10 µl per 100 mg of body mass of DA. RoC was measured 30–40 min following injection of DA [ixodid ticks easily tolerate injected fluid loads of 15% body mass and beyond (Kaufman et al., 1980)].

pH tests

For experiments testing the effect of pH on RoC, the loops were cut as described above and placed under light mineral oil (Sigma Chemical Co.) instead of MOPS-buffered saline so as to minimize disturbance of the endogenous pH of the cuticle. All soft tissue (muscle, tracheae, hypodermis) was then scraped away from the loop using the blunt side of a small razor-blade scalpel. These loops, free of soft tissue, were blotted with tissue paper to remove the mineral oil, and were then incubated in the desired buffer solution for 30–40 min before being mounted for the creep test. The pH levels tested were: 5.5, 5.7, 6.0, 6.5, 6.8, 7.0, 7.5 and 8.0.

The buffers (20 mmol l^{-1}) were as follows. Morpholinoethansulfonic acid (MES buffer; Sigma Chemical Co.) was used for pH range 5.5–6.5; Hepes buffer (Sigma Chemical Co.) was used for pH range 6.7–8.0. In an attempt to minimize the redistribution of endogenous salts from the cuticle during incubation, the buffer diluent was a solution of Na^+, K^+, Ca^{2+} and Mg^{2++} at the same concentrations measured in the cuticle, and calculated for the mean water content of cuticle (34% of cuticle wet mass). Thus, the diluent for all the buffers was a solution comprising 13.1 mmol l^{-1} NaCl, 4.9 mmol l^{-1} KCl, 1.0 mmol l^{-1} $CaCl_2$ and 0.36 mmol l^{-1} $MgCl_2$. This calculation was based on the assumption that all the ions in the cuticle are associated with free water and not bound to the cuticular macromolecules. While this assumption may not be completely valid, we consider it unlikely that any deviation from free solution behavior would materially affect the conclusions.

Statistics

Unless otherwise stated, statistical significance was determined, using Microsoft Excel® software (Microsoft Corporation), by paired *t*-tests, or by one-tailed or two-tailed *t*-tests, assuming non-equal variances.

RESULTS

Mechanical behavior of cuticle loops

When subjected to a maintained load, excised loops of *A. hebraeum* alloscutal cuticle displayed 'creep', i.e. they increased in length in a time-dependent way (Fig. 4). In this respect *A. hebraeum* cuticle behaves in a similar way to the abdominal cuticle of *R. prolixus* (Reynolds, 1975a).

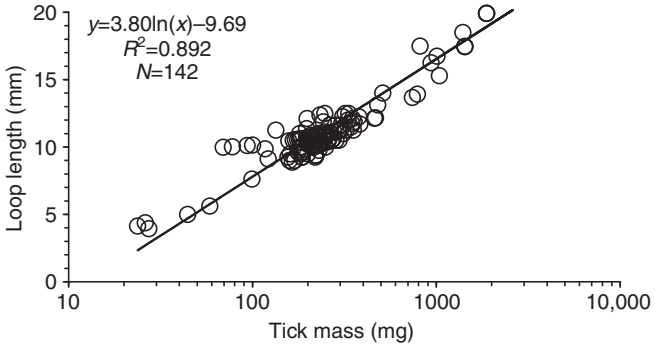

Fig. 5. Scatter plot of loop length as a function of tick mass (log scale) in *Amblyomma hebraeum*. Loop length varied linearly as a logarithmic function of tick mass over the full range of feeding.

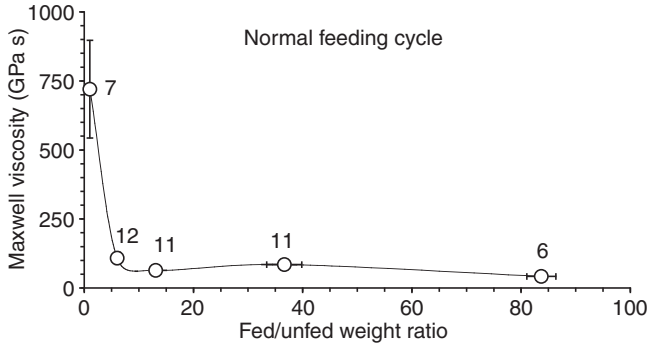

Fig. 7. Plasticization of the cuticle throughout the feeding cycle in *Amblyomma hebraeum*. Ticks were pooled into the following weight ratio groups: 1.0 (unfed; *N*=7), 2–10 (*N*=12), 10–20 (*N*=11), 20–60 (*N*=11) and >60 (*N*=6). Means, standard error of the mean (s.e.m.) and *N* are shown wherever they exceed the dimension of the symbol. NB: a finite value for Maxwell viscosity could not be calculated for three of the seven unfed ticks shown, because there was no significant movement recorded by the linear variable differential transformer (LVDT) over 12 min. However, we can state that the minimal value for these loops had to be 1100 GPa s, so to calculate the mean for unfed ticks, we assigned this minimal value to those three ticks. Hence, the mean value shown for unfed ticks (720 GPa s) is also a minimal value.

Loop dimensions throughout a normal feeding cycle

The size of a loop varied enormously with progression of feeding as seen in Fig. 3. Loop length varied linearly with the logarithm of tick mass (Fig. 5). The thickness of the cuticle also varied with progression of feeding but not in a straightforward way. Fig. 6 shows that for *A. hebraeum*, there is a progressive increase in cuticle thickness through the slow phase of engorgement (up to a weight ratio of ~13). For some of the ticks we also measured, independently, the thickness of the external pigmented layer comprising the epicuticular lamellae, the outer endocuticle and the inner endocuticle. For all of these measurements, there was a significant thinning in the large and fully engorged ticks (weight ratio >10; Fig. 6).

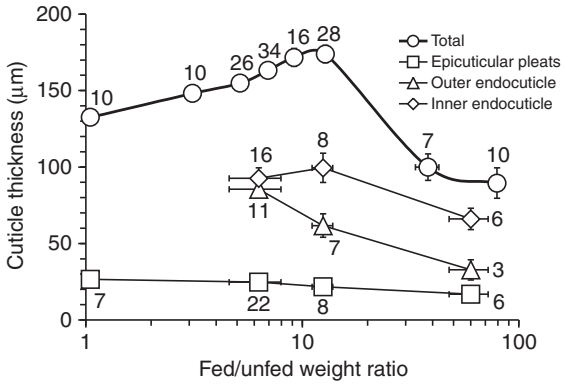

Fig. 6. Cuticle thickness in *Amblyomma hebraeum* as a function of weight ratio. Mean values are shown over the full range of feeding. Standard errors of the means (s.e.m.) are shown wherever they exceed the dimension of the symbol. There was a progressive thickening of the total cuticle during the slow phase of engorgement (up to ~13× the unfed mass) from 133±5.0 µm (*N*=10) for unfed ticks to 174±6.0 µm (*N*=28) for ticks at the end of the slow phase of engorgement (mean weight ratio=13). The differences between each of the seven means and the unfed group were highly significant (one-tailed *t*-test, equal variances) with the respective *P*-values being 0.0200, 0.0009, 0.0001, 0.0001, 0.0001 for the thickening of the cuticle during the slow phase of engorgement, and 0.0014 and 0.0005 for the thinning that occurs during the rapid phase. The outermost pigmented layer of the cuticle is pleated, so the thickness recorded for these 'epicuticular pleats' very much exceeds that of the epicuticle *per se*. The apparent thickness of the epicuticular pleats in the large and engorged group (16.9±4.2 µm; *N*=6) was significantly thinner (*P*=0.04) than the equivalent measurement in the unfed specimens (26.6±2.3 µm *N*=7). The outer endocuticle also thinned significantly from the small partially fed stage (85.4±4.1 µm *N*=11) to the largest ticks (32.7±6.5 µm *N*=3; *P*=0.002). Likewise for the inner endocuticle, there was a significant thinning from the small partially fed stage (92.7±6.7 µm *N*=16) to the largest ticks (66.0±7.0 µm *N*=6; *P*=0.008). Numbers?

Viscosity of cuticle through the course of a normal feeding cycle

Maxwell viscosity of cuticle loops from unfed female *A. hebraeum* was high (≥720 GPa s; Fig. 7) compared with values for both partially fed and engorged ticks. The decrease in Maxwell viscosity between unfed ticks and the 2–10 weight ratio group (108 GPa s) was statistically significant (*P*=0.0133). The viscosity for unfed loops was also significantly higher than that for all the other groups: for the 10–20 (64 GPa s), 20–60 (85 GPa s) and >60 (42 GPa s) weight ratio groups, *P*=0.0100, *P*=0.0114 and *P*=0.0087, respectively. Among the partially fed and engorged ticks, the Maxwell viscosity for the 2–10 weight ratio group was significantly higher than only the >60 weight ratio group (*P*=0.0139).

Engorged females post-engorgement

In *R. prolixus*, the normal plasticization induced by feeding is reversed within 6 h post-engorgement (Maddrell, 1966). We therefore asked whether the significant reduction in tick cuticle viscosity pertaining to the >60 weight ratio group would likewise reverse following engorgement. Fig. 8 demonstrates that such a reversal does occur within the first day post-engorgement. Day 0 engorged ticks (i.e. those that had detached from the host 0–24 h previously) had a mean viscosity of 42 GPa s, and this reversed to 102 GPa s by day 1 post-engorgement, with no further statistically significant change thereafter. The range of mean viscosity for engorged ticks, days 1–4 post-engorgement (90–123 GPa s) was within the range characteristic of normal partially fed ticks of weight ratio group 2–60 (64–108 GPa s). However, the Maxwell viscosity of post-engorgement alloscutal cuticle did not revert to anything approaching the high values characteristic of unfed ticks (≥720 GPa s).

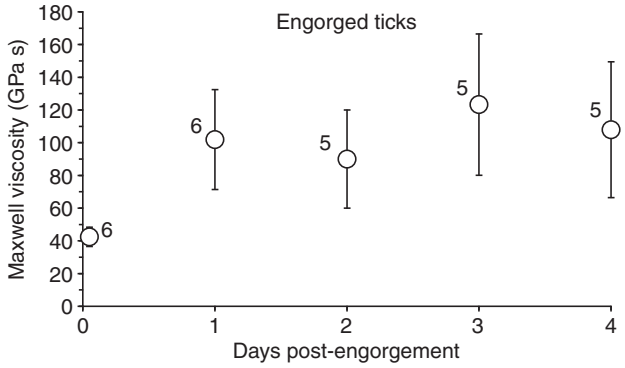

Fig. 8. Partial reversal of cuticle plasticization in *Amblyomma hebraeum* following engorgement. Reversal was complete within the first day post-engorgement. Means, standard error of the mean (s.e.m.) and *N* are shown for each day. *P*-values (one-tailed *t*-test, unequal variances) for each day compared with day 0 were 0.055, 0.095, 0.067 and 0.095 for days 1–4, respectively. The difference between day 0 (42.4±5.9 GPa s; *N*=6) and the pooled engorged ticks, days 1–4 post-engorgement (106±17 GPa s; *N*=21) was highly significant (*P*=0.0009).

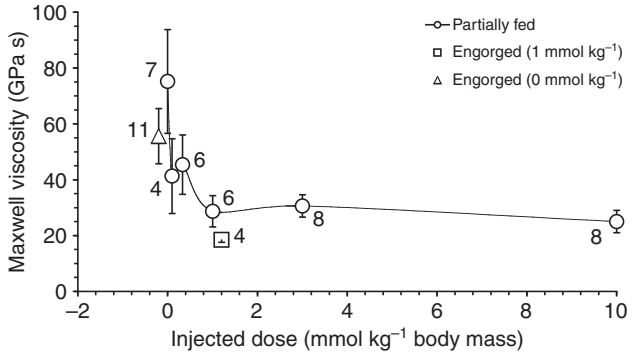

Fig. 9. Dose–response curve for the effect of dopamine (DA) on Maxwell viscosity in partially fed and engorged *Amblyomma hebraeum*. Means, standard error of the mean (s.e.m.) and *N* are shown for each concentration. Differences between control *vs* the other doses (*P*-values from one-tailed *t*-tests, unequal variances, in parentheses) were as follows: 0.1 mmol (0.1722). 0.33 mmol (0.1956), 1.0 mmol (0.0472), 3 mmol (0.0268) and 10 mmol (0.0176). *P*-value for the difference between 1.0 mmol and 10 mmol was 0.3012. *P*-value for the difference between normal engorged and engorged receiving 1.0 mmol was 0.0037.

Male ticks

Male ticks imbibe a very small blood meal while on the host. During this study the mass of a small sample of unfed males was 24.3±3.5 mg (*N*=5), and that of a small sample of fed males was 34.7±2.0 mg (*N*=6), for an 'engorged' weight ratio of 1.43. The dorsal cuticle of males is sclerotized. Although the ventral cuticle is pliable, so presumably not sclerotized, and does bulge out slightly in fed specimens, it does not appear to stretch during a blood meal.

We tested five loops from unfed males and five from fed males, under the same conditions as we did for females but none of these 10 loops stretched significantly, so the minimal viscosity for both groups was 1100 GPa s. This high viscosity is not due to the possession of a thicker cuticle; the thickness of adult male dorsal cuticle was 77.5±3.9 µm (*N*=6) and that of male ventral cuticle was 76.5±4.3 µm (*N*=6). By comparison, the thickness of cuticle from unfed females in this study was 133±5 µm (*N*=10; Fig. 6).

Drug effects on Maxwell viscosity

Dopamine

Table 1 summarizes the effects of several drugs on Maxwell viscosity following injection into small partially fed ticks. DA was the only drug to stimulate plasticization compared with control (28.7 *vs* 75.2 GPa s; *P*=0.047). Fig. 9 shows the dose–response curve for DA. We also tested DA on engorged females (Fig. 9). The decrease in viscosity between control engorged ticks (55.6 GPa s) and engorged ticks injected with 1 mmol DA kg^{-1} body mass (18.5 GPa s) was statistically significant (*P*=0.037).

5-HT and OA

The apparent increase in viscosity caused by injecting 1 mmol 5-HT kg^{-1} body mass (114 GPa s) compared with control (75.2 GPa s) was not statistically significant. The increase in viscosity affected by 1 mmol OA kg^{-1} body mass (210 GPa s) compared with control was highly significant (*P*=0.0005). Neither OA nor 5-HT were able to significantly reverse the effect of co-injected DA (*P*=0.072 and 0.445, respectively), although the *P*-value of the former was close to significant (Table 1).

Tyramine

The increase in Maxwell viscosity caused by injecting tyramine at 1 mmol kg^{-1} body mass (146 GPa s) compared with the control was statistically significant (*P*=0.048) (Table 1).

Butaclamol

Butaclamol is a potent inhibitor of the DA-receptor that mediates salivary fluid secretion in *A. hebraeum* (Kaufman and Wong, 1983). Because DA also induced cuticular plasticization in partially fed ticks (Table 1 and Fig. 9), we tested butaclamol *in vivo* as a putative inhibitor of DA on the cuticle. At the high concentration required for injection, however, we were unable to keep butaclamol dissolved following dissolution in DMSO and subsequent dilution to 50% DMSO in MOPS-buffered saline. We nevertheless injected this butaclamol suspension, followed 30 min later with DA (1 mmol kg^{-1} body mass). Controls were injected with (1) saline alone, (2) 50% DMSO in saline, and (3) 50% DMSO followed by

Table 1. Effect of various drug treatments *in vivo* on cuticle plasticization in *Amblyomma hebraeum**

Treatment	Maxwell viscosity (GPa s) Mean	s.e.m.	*N*	*P* (cf. no drug)†
MOPS-buffered saline control	75.2	18.6	7	–
1 mmol OA‡ kg^{-1} body mass	210	6.1	6	0.0005
1 mmol 5-HT‡ kg^{-1} body mass	114	19.8	9	0.173
1 mmol tyramine kg^{-1} body mass	146	25.1	6	0.048
1 mmol DA‡ kg^{-1} body mass	28.7	5.6	6	0.047
				P (cf. 1 mmol DA)§
1 mmol DA + 1 mmol OA kg^{-1} body mass	42.1	6.4	7	0.072
1 mmol DA + 1 mmol 5-HT kg^{-1} body mass	51.9	27.6	6	0.445

*Cuticle loops were taken from partially fed ticks, fed/unfed weight ratio in the range of 5–12. Drugs were injected at the indicated doses as described in the Materials and methods.
†Two-tailed *t*-test, unequal variance.
‡OA=octopamine; DA=dopamine; 5-HT=5-hydroxytryptamine.
§One-tailed *t*-test, unequal variances.

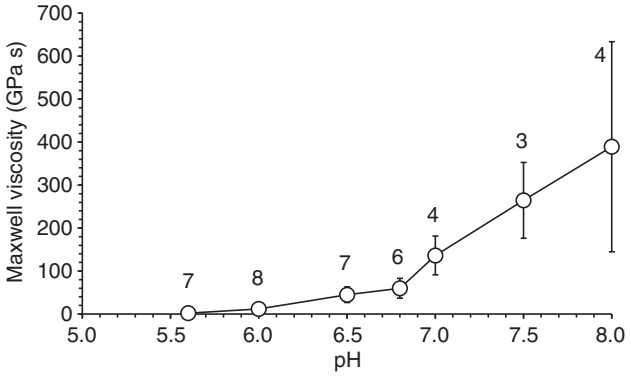

Fig. 10. The effect of pH on cuticle plasticization in *Amblyomma hebraeum*. There was a marked and progressive reduction in Maxwell viscosity from pH 8.0 (389 GPa s) to pH 5.6 (2.2 GPa s).

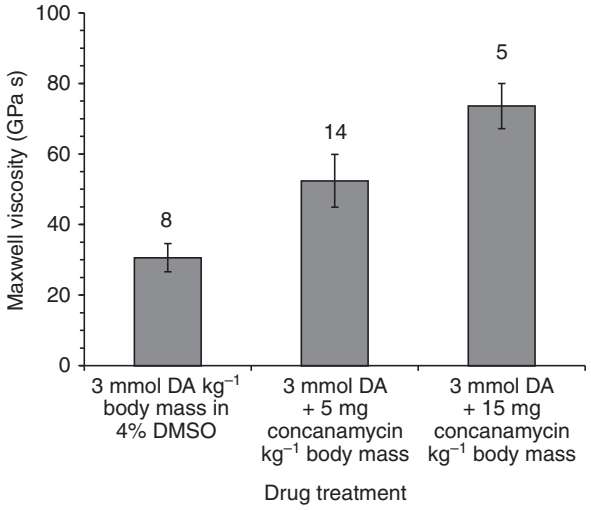

Fig. 11. Effect of the proton-transport inhibitor, concanamycin A, on dopamine (DA)-induced cuticle plasticization in *Amblyomma hebraeum*. Ticks were injected either with concanamycin A (5 or 15 mg kg^{-1} body mass) or 4% DMSO (control for the 5 mg dose) or 12% DMSO (control for 15 mg), followed 30 min later with 3 mmol DA kg^{-1} body mass. Means, standard error of the mean (s.e.m.) and *N* are shown for each treatment. The increase in Maxwell viscosity by 5 mg concanamycin A kg^{-1} body mass over the control was statistically significant (P=0.0189), and that by 15 mg concanamycin A kg^{-1} body mass was highly significant (P=0.00071). The difference between the two doses of concanamycin A was also significant (P=0.0491). DMSO, dimethylsulfoxide; DA, dopamine.

DA. 50% DMSO in saline itself caused a marked decrease in Maxwell viscosity (15.1±3.1 GPa s; N=4) compared with saline alone (75.2±18.6 GPa s; N=7; P=0.017). In the presence of 50% DMSO, 1 mmol l^{-1} DA kg^{-1} body mass (16.2±3.8 GPa s; N=7) caused no further decrease in Maxwell viscosity over DMSO/saline (P=0.101). The apparent difference between 1 mmol DA kg^{-1} body mass in saline (28.7±5.6 GPa s; N=6; Table 1) and 1 mmol DA kg^{-1} body mass in 50% DMSO (16.2±3.8 GPa s) was not statistically significant (P=0.101). Finally, butaclamol did not reverse the reduction in Maxwell viscosity caused by 50% DMSO (16.2±2.1 GPa s; N=9).

Effect of pH on cuticle plasticization

Maxwell viscosity fell progressively from pH 8.0 to 5.6 (Fig. 10). At pH 5.5–6.0, the cuticle loops stretched so much that some of them broke within 12 min under the 70 g load (these loops were not tallied in the dataset shown in Fig. 10).

As suggested for other systems, the mechanism of plasticization in *A. hebraeum* may involve the transport of H^+ ions by cells in the hypodermis into the sub-cuticular space. Thus, we tested whether concanamycin A, a well-known inhibitor of H^+-ion transport in many tissues (Dröse et al., 1993; Huss et al., 2002), might inhibit DA-induced plasticization. Fig. 11 shows that it did so. In this trial, Maxwell viscosity at 3 mmol l^{-1} DA kg^{-1} body mass was 30.6±4.0 GPa s (N=8) and viscosity in the presence of 5 mg concanamycin A kg^{-1} body mass increased to 52.4±7.5 GPa s (N=14) (P=0.019; one-tailed t-test, unequal variance). A further increase in viscosity (73.6±6.4 GPa s, N=5) was observed at 15 mg kg^{-1} body mass (P=0.0007). The difference between the 5 mg dose and the 15 mg dose was also statistically significant (P=0.049).

Water and ion content of the cuticle throughout the feeding cycle

Because a change in general water and/or ionic composition of the cuticle could conceivably influence cuticular plasticization (Vincent, 1990), we measured the water and ion content of cuticle throughout the feeding cycle and following the injection of drugs in partially fed females. The data are compiled in Table 2. The only statistically significant effects were that the higher dose of DA (10 mmol kg^{-1} body mass) appeared to increase cuticular water content by 10% whereas OA appeared to increase cuticular sodium content by 41%.

Table 3 shows the water and ion content of cuticle throughout feeding and the first 4 days post-engorgement. There was no statistically significant change in cuticular water content from the slow phase of feeding through the first 4 days post-engorgement. The Na^+ concentration did not decrease significantly from the day 0 value before day 3 post-engorgement. There was no statistically significant change in cuticular K^+ or Ca^{2+} or Mg^{2+} content from the slow phase of feeding through the first 4 days post-engorgement.

In order to better understand the potential impact of water content on plasticization over the entire feeding cycle, cuticular water content was measured from excised dorsal cuticle over a wide range of weight ratios. Results are shown in Fig. 12. Water content of cuticle

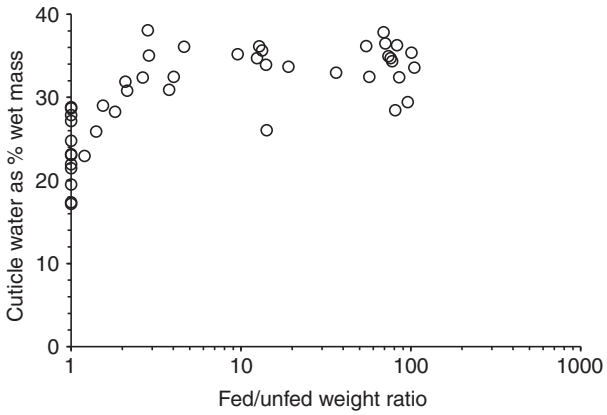

Fig. 12. Water content (as % wet mass) of cuticle during a normal feeding cycle of *Amblyomma hebraeum*. The water content observed at a fed/unfed weight ratio of >1–2.1 (28.1±1.3%; N=6) was significantly greater (P=0.011) than that of unfed ticks (23.4±1.2%; N=12).

Table 2. Effect of several drugs on water content and ion content of cuticle from partially fed ticks*

Water or ion	Drug and dose (mmol kg^{-1} body mass)‡	Water (% wet mass of cuticle) or ion (μmol g^{-1} dry mass cuticle) Mean	s.e.m.	*N*	*P* (cf. no drug)†
Water	No drug (control)	33.9	1.1	4	–
	5-HT (1.0)	33.3	1.2	3	0.694
	OA (1.0)	30.5	1.6	9	0.103
	DA (1.0)	32.7	0.7	7	0.381
	DA (10)	37.3	1.0	8	0.044
Sodium	No drug (control)	41.7	5.5	4	–
	5-HT (1.0)	73.4	10.1	3	0.066
	OA (1.0)	58.7	4.9	8	0.051
	DA (1.0)	44.7	2.4	7	0.642
	DA (10)	34.2	2.1	8	0.269
Potassium	No drug (control)	18.3	3.8	4	–
	5-HT (1.0)	17.5	1.2	3	0.851
	OA (1.0)	18.9	2.3	7	0.908
	DA (1.0)	14.3	2.2	7	0.403
	DA (10)	21.2	0.8	8	0.516
Calcium	No drug (control)	3.93	1.14	4	–
	5-HT (1.0)	5.04	0.77	3	0.460
	OA (1.0)	5.36	0.29	7	0.304
	DA (1.0)	4.36	0.49	7	0.793
	DA (10)	3.47	0.28	8	0.716
Magnesium	No drug (control)	1.41	0.49	4	–
	5-HT (1.0)	2.39	0.81	3	0.363
	OA (1.0)	2.33	0.34	7	0.176
	DA (1.0)	1.21	0.12	7	0.717
	DA (10)	1.27	0.12	8	0.798

*In each case the control group represents values for partially fed ticks of a normal feed.
†Two-tailed *t*-test, unequal variance.
‡OA=octopamine; DA=dopamine; 5-HT=5-hydroxytryptamine.

rose sharply at the onset of feeding and reached a plateau value of ~34% at a weight ratio of 3.

Cuticular plasticization in the argasid tick, *O. moubata*

Ticks of the family Argasidae are known as 'soft ticks' because their cuticle is noticeably pliable, even when unfed. So we asked whether feeding induces any degree of cuticular plasticization in this group of ticks. We matched fed ticks, for which the unfed masses were known, to a similar population of unfed ticks. The mean mass of the unfed ticks (69.2±6.5 mg; *N*=12) was not significantly different from the unfed masses of the fed ticks (74.9±6.7 mg; *N*=12) (*P*=0.562; paired *t*-test). As explained in the Materials and methods, we were not able to carry out a proper Maxwell analysis for this experiment, so we report only RoC. The RoC of cuticle from the fed ticks (6.57±0.81 μm min^{-1} mm^{-1} loop length; *N*=12) was not significantly greater than that of the unfed ticks (5.66±0.43 μm min^{-1} mm^{-1} loop length; *N*=12) (*P*=0.1675). Thus, feeding resulted in no statistically significant decrease in viscosity.

DISCUSSION

Here we report that the alloscutal cuticle of adult female *A. hebraeum* undergoes plasticization during feeding. We suppose this plasticization to be adaptive in facilitating the intake of a very large blood meal.

Our findings are consistent with the suggestion of Hackman (Hackman, 1975), Hackman and Goldberg (Hackman and Goldberg, 1987), and Andersen and Roepstorff (Andersen and Roepstorff, 2005) that, by analogy with *R. prolixus* (Reynolds, 1975b), the mechanical properties of tick cuticle might be modulated by relatively small changes in pH. The proteins present in the cuticle matrix in ticks and reduviids may well be different but the similar overall amino acid compositions of cuticle suggests that the pH dependence of their non-covalent molecular interactions might be similar.

The phenomenon of cuticle plasticization was first demonstrated by Cottrell in the blowfly, *Calliphora erythrocephala*, immediately following ecdysis (Cottrell, 1962). In the same year it was reported for *R. prolixus* during the early minutes of blood feeding (Bennet-Clark, 1962). Importantly, feeding-induced plasticization in *R. prolixus* occurs extremely rapidly, and is subsequently reversed within about 6 h of engorgement (Maddrell, 1966), suggesting that the process is unlikely to represent a significant change in the macromolecular content of the cuticle but a change in the extent of interactions between macromolecules.

Cuticle plasticization in *R. prolixus* is induced locally by a factor released from abdominal nerve endings in the integument (Maddrell,

Table 3. Water and ion content of cuticle samples

Feeding stage (p.e.=post-engorgement)	Water content (as % wet mass)	Micromoles per gram of dry cuticle (means ± s.e.m.; *N*)† Na^+	K^+	Ca^{2+}	Mg^{2+}
Ticks weight ratio <60*	**35.2**±2.5 (11)	**55.4**±6.3 (10)	**20.1**±2.3 (10)	**3.87**±0.51 (9)	**1.29**±0.19 (10)
Ticks weight ratio >60*					
Day 1 p.e.	**35.3**±2.3 (6)	**52.7**±8.5 (6)	**19.8**±1.3 (6)	**3.75**±0.19 (6)	**1.43**±0.15 (6)
Day 2 p.e.	**34.4**±1.6 (4)	**51.7**±5.4 (4)	**22.2**±2.3 (4)	**3.91**±0.27 (4)	**1.52**±0.14 (4)
Day 3 p.e.	**31.8**±0.9 (5)	**39.3**±0.9 (5), *P*=0.0157	**18.4**±1.4 (5)	**3.63**±0.37 (5)	**1.25**±0.06 (5)
Day 4 p.e.	**33.4**±0.8 (5)	**42.5**±1.6 (5), *P*=0.0378	**18.8**±1.0 (5)	**3.14**±0.29 (5)	**1.28**±0.12 (5)

*The mean fed/unfed weight ratio of the small partially fed and small engorged ticks in this dataset was 26±5 (11) and that of the ticks >60 weight ratio was 75±2 (20).
†Where a *P*-value is shown, it refers to a statistically significant difference compared with the respective <60 weight ratio value (one-tailed *t*-test, unequal variance). None of the other differences between ticks <60 weight ratio and any of the other groups, for water content or any of the four ions, were statistically significant (*P*>0.05). There were no statistically significant differences between the pooled days 1–4 values and the corresponding value for the <60 weight ratio value.

1966). It is probable that the neurotransmitter of these abdominal nerves is 5-HT. Indeed, 5-HT triggers plasticization when injected into unfed *R. prolixus*, and this action is blocked by 5-HT antagonists (Reynolds, 1974). Furthermore, 5-HT can cause plasticization even when the abdominal nerves are severed, and can cause cuticle loops to plasticize *in vitro*. 5-HT evidently acts *via* presumptive receptors on the epidermis, because cuticle loops stripped of the epidermal layer do not plasticize when exposed to 5-HT (Reynolds, 1974). It has also been shown that 5-HT is present in integumental nerve endings (Orchard et al., 1988), that 5-HT is released during feeding and causes elevated levels of cyclic AMP in epidermal cells (Barrett et al., 1993).

The mechanism of plasticization in *R. prolixus* is characterized by an increase in intracuticular hydration, believed to be the result of a transport of protons into the sub-cuticular space, because extensibility is markedly increased in cuticle loops exposed to buffers in a physiologically acidic range (~pH 6.0), and because plasticized cuticle appears to have a reduced pH (Reynolds, 1975b). Similarly, we propose that in *A. hebraeum* alloscutal cuticle, plasticization involves acidification associated with an increase in water content that occurs shortly after the onset of feeding. A complication in ixodid ticks is that (unlike *R. prolixus*) new endocuticle is deposited in abundance during the course of feeding (Lees, 1952) (Fig. 6). To what extent might this new endocuticle be inherently more hydrated and less viscous than the old endocuticle, independent of proton-induced hydration? We suggest that the increase in cuticular hydration is the result of a physiologically induced change in the entire cuticle. First, there is unlikely to be a water permeability barrier between the old and newly synthesized endocuticles. Moreover, by a weight ratio of 3, the cuticular water content has reached a plateau level of approximately 34% (Fig. 12). At this early stage of feeding, the cuticle has not yet increased in thickness very much [133±6 µm (N=8) for unfed ticks *vs* 148±5 µm (N=10) for ticks at a weight ratio=3, P=0.037; Fig. 6]. By a simple geometric analysis, the area of cuticle is approximately double ($3^{2/3}$) the pre-feeding value. At this stage, slightly less than half of the cuticle mass is newly formed. If the original cuticle of the unfed tick had kept its initial water content of 23.4% (Fig. 12), the tick could not have reached a plateau value of 34% water content so soon in the feeding period. We therefore conclude that the cuticle's mechanical properties are actively modified, probably by regulating pH.

Acidification as a means for disrupting secondary bonds among macromolecules seems to be a very widespread phenomenon. For example, in plants, cellulose serves both as a rigid skeletal support and as the means to withstand the particularly high turgor pressures generated within the plant cell: typically in the region of 0.5 MPa but up to 50 MPa in stomatal guard cells (McQueen-Mason et al., 2006). Expansins are a family of plant proteins that facilitate growth by means of a process of acid-induced cell wall extension (reviewed by McQueen-Mason et al., 2006). Similar to what occurs in *R. prolixus* (Reynolds, 1975b), the tick *H. longicornis* (Okura et al., 1997a) and now *A. hebraeum*, growing plant cells stretch little when placed under load following exposure to media of neutral or alkaline pH but at acidic pH, they expand much more rapidly under the same load (Rayle et al., 1970). The mechanism of this acid-induced 'plasticization' is probably the disruption of hydrogen bonds '… between cellulose microfibrils and the hemicellulose polymers that coat them in the cell wall' (McQueen-Mason et al., 2006).

Our measurements of cuticle dimensions during feeding indicate that the outer and inner endocuticles are appreciably thinned during feeding and that the epicuticular pleat layer is unfolded (Fig. 6). This is consistent with data from Dillinger and Kesel on another ixodid tick, *Ixodes ricinus* (Dillinger and Kesel, 2002). They found that the epicuticular pleats were 13.5 µm high and 2.7 µm wide in unfed specimens, 5–8 µm high and 14.8 µm wide in partially fed specimens, and about 4 µm high and 29.5 µm wide in engorged specimens. The epicuticle thickness remained the same in unfed and engorged ticks (~0.3 µm). So the process that occurs during feeding of both *A. hebraeum* and *I. ricinus* seems to be one of expansion of the inner layers of the endocuticle and unfolding of the outer layers.

Reynolds evaluated the potential effects of cuticle cation content on plasticization in *R. prolixus* (Reynolds, 1975b). Although altering the exogenous levels of some cations could cause cuticle plasticization, he concluded that the endogenous concentrations of these ions were insufficient to account for the extent of plasticization observed *in vivo*. Similarly, in *A. hebraeum*, we have found no evidence that altered levels of Na^+, K^+, Ca^{2+} or Mg^{2+} ions could account for plasticization of the body cuticle. However, we note that OA caused a 41% increase in Na^+ content of the cuticle (Table 2). Whether this increase has anything to do with OA's stiffening mechanism of the alloscutal cuticle (Table 1) remains to be tested.

Cuticular plasticization in ixodid ticks was first investigated by Okura et al., who monitored the subsequent increase in body volume of virgin and mated female *H. longicornis*, after subjecting the hemocoel to a constant pressure excess of 20 kPa for a very long 30–50 h (Okura et al., 1996). A similar 'inflation technique' had also been used in the original investigations on *R. prolixus* by Bennet-Clark (Bennet-Clark, 1962) and Maddrell (Maddrell, 1966), albeit over a much shorter duration. As noted earlier, however, rate of increase in volume of a tick confounds body size with a true measure of extensibility of cuticle. Time-dependent deformation of a material is proportional to $(dL/L_0)/dt$ whereas the rate of increase in volume of an object is proportional to $L^2\times(dL/L_0)/dt$. As a result, relating the rate of increase of volume to plasticization is valid only when the sample population is of relatively uniform size. If there is a wide range in size, as occurs during a normal feeding cycle of ticks, rate of volumetric increase will overstate the time-dependent deformation that occurs in larger specimens.

Okura et al. proposed that copulatory stimuli were central to the mechanism for cuticular plasticization (Okura et al., 1996). In further studies, they adopted a methodology similar to the method used in the present study of *A. hebraeum*: measuring the RoC *via* an LVDT. They also demonstrated a marked increase in cuticular extensibility at acidic pH in the physiological range, and a modest increase induced by copulation [RoC=0.174 mm min^{-1} for copulated females *vs* 0.134 mm min^{-1} for virgin females (Okura et al., 1997a)]. We have not yet tested *A. hebraeum* for an effect of mating status on cuticular plasticization.

Histology and ultrastructure of the cuticle of *H. longicornis* revealed a population of mitochondria-rich cells (adjacent to the large dermal gland cells) which appear similar to the H^+-secreting cells in the gills of euryhaline fishes. These 'acidophilic epidermal cells' increased in size and their 'tubulovesicular system' became more prominent following copulation, suggesting that they may be the cells responsible for acidifying the cuticle (Okura et al., 1997a). The acidophilic epidermal cells were found only within the epidermis underlying the non-sclerotized alloscutum, and not elsewhere. Okura et al. also claimed that the intracuticular pH fell significantly from 6.92 in virgin females to 6.43 in mated, rapidly feeding females (Okura et al., 1997a). However, they recognized some potential problems with their method that raise questions as to the accuracy of these numbers. Moreover, they restricted their attention to partially fed virgin and mated ticks of unspecified sizes, so unfortunately we have no information from their study on changes that may occur throughout a normal feeding cycle of *H. longicornis*.

In ixodid ticks, it is during the final 12–24 h or so of feeding ('rapid phase of engorgement') that 90% of the blood meal is imbibed (Kaufman, 2007). On launching this study we hypothesized that a major plasticization event might occur during the rapid phase of engorgement. But our results here tell a more complex story. While it is clear that Maxwell viscosity of alloscutal cuticle is lower in feeding ticks than in starved ticks, the major decrease in viscosity occurred between unfed (weight ratio of 1.0) and small partially fed ticks (weight ratio of 2–10). Thereafter, for most ticks, there was no significant further reduction through to full engorgement. Only those ticks that ultimately exceeded a weight ratio of ~60 appeared to have a yet lower viscosity (Fig. 7). Although the reduction in viscosity between small engorged ticks (weight ratio of 30–60) and large engorged ticks (weight ratio >60) was statistically significant (P=0.006), the biological significance of this observation and the mechanism responsible for the enhanced reduction of viscosity remain uncertain.

Unlike females, male *A. hebraeum* take in a very small blood meal (p. xxx) so it was perhaps not surprising that we could find no evidence for plasticization in males.

There are *a priori* reasons to suppose that the cuticle of female ixodid ticks might not plasticize as rapidly as that of the 5th larval stage of *R. prolixus*. The female of *A. hebraeum* ultimately achieves an engorged mass 10-fold greater than does the 5th stage larva of *R. prolixus*. But the rate of expansion is much higher in *R. prolixus* (a 10-fold increase within a few minutes) than in the tick during the rapid feeding phase (a 10-fold increase over a period of 12–24 h). However, a direct quantitative comparison between the degree of plasticization observed in *A. hebraeum* and that observed earlier for *R. prolixus* (Reynolds, 1974; Reynolds, 1975a; Reynolds, 1975b) is not possible because loop dimensions (length, width, thickness) were not recorded for the latter.

Injecting biogenic amines into partially fed and engorged female *A. hebraeum* affected Maxwell viscosity significantly: DA reducing it and OA and tyramine augmenting it (Table 1). The hypodermis of female ixodid ticks receives an extensive innervation (Binnington, 1981; Shoukrey and Sweatman, 1984), which is consistent with the hypothesis for the existence of a putative neural control pathway for cuticular plasticization. We do not yet know whether the antagonist effect of OA and tyramine reflects an endogenous inhibitory pathway or whether the effect is merely a pharmacological one – the drugs binding to the DA-receptor but not triggering the intracellular signaling cascade leading to plasticization.

The observation that cuticle loops, even from replete ticks, responded to exogenous DA (Fig. 9) implies that ticks cease to feed before they are obliged to do so by a limit to cuticle stretching. In large engorged *A. hebraeum* ticks, the epicuticular pleats are virtually flat (our unpublished observations). The observed effect of DA on engorged ticks suggests that the flattened epicuticle does not impose a barrier to a degree of expansion even beyond that which normally occurs.

Amblyomma hebraeum appears to respond differently to injected drugs than does *H. longicornis*, in which DA and OA were both reported to stimulate plasticization (Okura et al., 1997b) (using an inflation technique over 48 h). In *H. longicornis*, 5-HT had no significant effect (Okura et al., 1997b). We do not know at this point whether this is a genuine difference between the two species or only an apparent one due to the significant difference between the two studies in how plasticization is expressed and analyzed.

Dillinger and Kesel observed blue fluorescence in tick alloscutal cuticle, which they interpreted to indicate the presence of the elastic protein resilin, and speculated on the possible role of this protein in feeding-related cuticle extension (Dillinger and Kesel, 2002). As noted by Andersen and Roepstorff, however, such fluorescence is indicative of dityrosine cross-links rather than resilin *per se*, and therefore speculation on the possible significance of resilin in tick alloscutal cuticle is premature (Andersen and Roepstorff, 2005). Andersen and Roepstorff suggested that these covalent cross-links may account for the relative insolubility of tick (*I. ricinus*) cuticle proteins (Andersen and Roepstorff, 2005). One might expect this partially cross-linked cuticle to be appreciably stiffer than the abdominal cuticle in *R. prolixus*, which lacks covalent cross-links of this or any other kind. However, because the extent of cross-linking is modest in *I. ricinus* [Andersen and Roepstorff commented that the dityrosine content of tick cuticle is about one-tenth that of locust resilin (Andersen and Roepstorff, 2005)], one would expect tick alloscutal cuticle to display viscoelastic properties.

Okura et al. also tested the effects of tissue extracts on relative body volume using their inflation technique over 48 h (Okura et al., 1997b). Whereas injecting extracts of hemolymph, salivary gland, midgut, trachea and ovary from partially fed virgin females into partially fed virgin females resulted in no greater expansion than injected saline (all between 5.6 and 6.2-fold increase in body volume), an injected extract of synganglion from virgin females into virgin females had a significantly greater effect (an increase in relative body volume of almost 13.5-fold). Interestingly, a hemolymph extract from copulated females caused an increase in relative body volume of over 13-fold in recipient copulated females whereas a synganglion extract from such females had no greater effect than saline or the other tissue extracts (all between 6.3 and 7.0-fold). These positive effects of hemolymph and synganglion on copulated and non-copulated females, respectively, were not inhibited by protease digestion of the extracts, supporting their hypothesis that the endogenous mediators of plasticization in *H. longicornis* may be biogenic amines (Okura et al., 1997b).

Regarding *O. moubata*, this tick is a rapid feeder, completing engorgement within an hour. Moreover, the cuticle of unfed *O. moubata* is inherently quite pliable. One would not anticipate the necessity to plasticize the cuticle during feeding, and this was confirmed by our observations (p. xxx).

In conclusion, female *A. hebraeum* plasticizes its alloscutal cuticle early in the feeding period. The pharmacological data suggest that control of plasticization is by dopaminergic nerves innervating the alloscutal integument. The mechanism of plasticization is likely the disruption of hydrogen bonding between cuticular macromolecules resulting from acidification and hydration of the cuticle by a proton transport system in the hypodermis.

LIST OF ABBREVIATIONS

CNS	central nervous system
DA	dopamine
dL	stretch
DMSO	dimethylsulfoxide
dt	?
5-HT	5-hydroxytryptamine (serotonin)
Hepes	hydroxyethypiperazineethanesulfonic acid
L_0	initial loop length
LVDT	linear variable differential transformer
MES	morpholinoethansulfonic acid
MOPS	morpholinopropanesulfonic acid
N	sample size
OA	octopamine
p.p.m.	parts per million
RoC	rate of creep
s.e.m.	standard error of the mean

ACKNOWLEDGEMENTS

We are indebted to The Leverhulme Trust and the Alberta Heritage Foundation for Medical Research (AHFMR) for their most generous financial support of this project. This project could not have been carried out without the extensive support from Ms Louise Anderson and her staff of the Animal Facility, University of Bath. We are most grateful to the following for their respective contributions: Mr Steve Routley (Bath) provided much technical support in several areas, including fashioning of the device for holding together the razor blades for cutting cuticle loops, and making an elegant set of brass weights used in the creep tests. Ms Susan M. Rogers (St Albert, Alberta) produced the diagram of the loop cutter shown in Fig. 1. Mr Chris Davey (Bath) took the photograph of cuticle loops shown in Fig. 3. Mr Robert Stevens of the Chemistry Department (Bath) offered technical advice for ashing the cuticle samples for ion analysis and made available to us the high temperature furnace. Mr Alan Carver of the Chemistry Department (Bath) performed the ion analysis by flame spectrophotometry. Dr Miles Nunn of the NERC CEH Institute in Oxford provided nymphal specimens of *O. moubata*, and Dr DeMar Taylor, University of Tsukuba, Japan, provided us with capsules for feeding *O. moubata* on guinea pigs and detailed instructions on how to feed them. Professors Phillip Choi and Morris Flynn of the Faculty of Engineering, University of Alberta provided helpful advice on modeling tick cuticle as a viscoelastic material and the mathematical analysis of results. We also thank the anonymous reviewers for their valuable comments on the submitted manuscript.

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