RESEARCH ARTICLE

Mechanical properties of the cuticle of the tick *Amblyomma hebraeum* (Acari: Ixodidae)

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ABSTRACT

Female ticks of the family Ixodidae increase their mass up to 100-fold during the 7–10 day feeding period. We determined the material properties of the alloscutal cuticle of female *Amblyomma hebraeum* from the time of moulting through to full engorgement. The material properties of the cuticle were evaluated by a Kelvin–Voigt analysis of compliance determined from the stretch of loops of cuticle under stress. There was a 3-fold increase in cuticle dry mass during the first 3 weeks post-moult, during which the ductility and stiffness of the cuticle increased substantially. Under stress, the cuticle displayed time-dependent stretch, with a plastic (non-recoverable) and viscoelastic (recoverable) component. Plastic deformation was reasonably constant in the range 10–15% over a wide range of induced stress above ∼0.6 MPa. The plastic component of tick alloscutal cuticle was about 5–10 times higher than that of unsclerotized insect cuticle. Tick cuticle is far more ductile than unsclerotized insect cuticle. Material properties of the cuticle did not change significantly as a function of cuticular water content over the normal range throughout the feeding cycle (13–37% wet mass). Injected dopamine (DA) reduced one measure of the viscosity of the cuticle by 38%. Plastic deformability of the cuticle was reduced by 70% after an *in vitro* stretch, but restored in fully engorged ticks, and in *in vitro* stretched loops by treatment with DA and reduced pH. Thinning of the cuticle by half during the rapid phase of engorgement requires plastic deformation (irreversible strain) in two orthogonal dimensions in excess of 40%. Treatment with DA increased plastic deformation and enabled extensibility (strain at the point of rupture) above 40%.

KEY WORDS: Cuticular plasticization, Viscoelastic, Plastic deformation, Dopamine, Young's modulus, Extensibility, *Amblyomma hebraeum*, Kelvin–Voigt

INTRODUCTION

In ixodid ticks, the feeding period comprises three phases: a preparatory phase (about 1 day), during which the tick establishes the feeding lesion, a slow phase (∼7 days), during which body mass increases about 10-fold, and a rapid phase (∼12–24 h), during which there is a further 10-fold increase in mass, and the replete tick detaches from the host (Apanaskevich and Oliver, 2014). The enormous size increase during engorgement in ixodid ticks pertains only to the female. The male feeds much more modestly, increasing its mass by only ∼50%. Unless otherwise stated, in this article 'tick' refers exclusively to the female. *Amblyomma hebraeum* grows

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endocuticle during the slow phase and well into the rapid phase of engorgement (Flynn and Kaufman, 2011; Kaufman, 2014). Endocuticle thickens substantially during the slow phase (from 130 to 175 µm; Kaufman et al., 2010), when the rate of cuticle deposition exceeds the demand for increased surface area. The opposite occurs during the rapid phase. Notwithstanding the continued synthesis of endocuticle, the rapid expansion of the tick is accompanied by a marked thinning of the cuticle to 90 µm (Kaufman et al., 2010).

The fifth stage larva of the blood-sucking hemipteran *Rhodnius prolixus* increases its mass ∼10-fold during a feeding period of only ∼15 min (Maddrell, 1964, 1966). The cuticle stretches more easily during feeding than before; this phenomenon has been termed 'plasticization' (Bennet-Clark, 1962; Maddrell, 1966). Cuticle plasticization in *Rhodnius* is under neural control (Maddrell, 1966), the neurotransmitter probably being 5-hydroxytryptamine (5-HT; Reynolds, 1974). 5-HT triggers plasticization probably by stimulating the hypodermis to transport H^+ ions into the subcuticular space, causing a concomitant increase in cuticular hydration (Reynolds, 1975). Dopamine (DA) appears to play an equivalent role in cuticle plasticization in *A. hebraeum* (Kaufman et al., 2010).

In terrestrial arthropods, at least four variables influence the properties of the cuticle: (1) the relative amount of chitin fibrils and protein matrix, (2) the protein composition, (3) the pH/water content of the matrix and (4) the degree of cross-linking of the matrix protein. Variations in these parameters lead to the wide range of properties observed in the exoskeleton between, for example, flying insects and ticks. Here, we explored the mechanical properties of the tick cuticle at various stages of feeding by analyzing the time-dependent stretch under load, and compared it with cuticle from three species of flying insects. In ixodid ticks, some regions of the cuticle are fully sclerotized (mouthparts, legs and the dorsal 'scutum'). The so-called alloscutum (roughly equivalent to the abdomen in insects) is the only cuticular region that expands during feeding, although it does contain small islands of sclerotized (non-expandable) cuticle as well (spiracular, anal and gonopore plates). Unless otherwise stated, in this paper, 'cuticle' refers to alloscutal cuticle.

Many engineering materials show 'elastic deformation' under load: stretch occurs virtually instantaneously (at the speed of sound), with recovery to the original length after removal of the load. As with many biological materials and synthetic polymers, stretch in ixodid tick cuticle is time dependent, or 'viscoelastic' (Vogel, 2003; Vincent, 1990). A viscoelastic material is either a viscoelastic liquid or solid. Viscoelastic liquids stretch indefinitely under load, eventually breaking, and are characterized solely by viscosity, a measure of the rate of stretch. Viscoelastic solids have an asymptotic limiting value of stretch at a given stress. Such solids are characterized by, in addition to viscosity, the ultimate extent of stretch, measured by compliance (fractional distension per unit stress).

Previous papers on the stretch of insect and tick cuticle have introduced the concept of plasticization, a term referring to both an increased rate of stretch (Maddrell, 1966; Okura et al., 1996) and an increased extent of stretch (Reynolds, 1975). In this paper, we use a more rigorous terminology for categorizing the observed time-dependent stretch behaviour, consistent with characterization of polymeric and fibre composite materials (Ferry, 1980). The component of stretch that is recovered when the load is removed is 'viscoelastic', whereas the component that does not recover, causing permanent deformation, is 'plastic'. We show here that, under an imposed stress, tick and insect cuticle display both plastic and viscoelastic deformation. What some earlier authors termed 'plasticity', referring to rate of stretch, will be referred to here as 'viscosity'. Note that both plastic and viscoelastic deformation absorb energy during stretch (force multiplied by distance), but the ultimate strength and stiffness of the material derives only from the viscoelastic component, as the plastic component is not recoverable.

Multiple processes are involved in the stretch of a polymeric material; for proteins, this would include, for example, disentanglement of side groups and uncoiling of the protein backbone, all aided by vibrational and rotational motion within the molecules (Ferry, 1980; Vincent, 1990). Viscoelastic materials can be further characterized by their spectrum: the time scales over which stretch occurs. We used the technique of Tschoegl (1989) to analyse the spectrum of cuticular stretch; this method models the observed time-dependent stretch as an initial immediate stretch and a series of exponential functions at distinct time scales with unique individual parameters for viscosity and modulus.

In this paper, we focused on three key material issues. (1) What is the ultimate stiffness of the cuticle over the life cycle of the tick? This is the key measure of the ability of the cuticle to resist deformation under sustained load. (2) What is the ductility of the cuticle: how much energy can it absorb by stretching? This is a key measure of the ability of the cuticle to resist breaking under short-term loads as might occur if the host of a tick grooms itself by rubbing against an object. Ductility in fibre composite and polymeric materials arises in large part from plastic (non-recoverable) deformation. (3) By what mechanism does the tick cuticle thin by 50% during the rapid phase of engorgement?

RESULTS
Amblyomma hebraeum females grow cuticle after moulting and before feeding

After moulting, *A. hebraeum* synthesizes additional endocuticle (Fig. 1). Wet mass increased from 42.0 ± 1.6 µg mg^{-1} body mass (M_b) ($N=7$) on the day of moulting to 88.7 ± 3.6 µg mg^{-1} M_b ($N=6$) by day 21. There was no further change in mass from days 21 to 148 (84.3 ± 2.0 µg mg^{-1} M_b, $N=30$; Fig. 1A). Dry mass increased from 21.8 ± 1.7 µg mg^{-1} M_b ($N=7$) to 61.0 ± 4.5 µg mg^{-1} M_b ($N=6$) by day 21. There was no change in mass from days 21 to 148 (57.9 ± 2.3 µg mg^{-1} M_b, $N=29$) (Fig. 1B).

The cuticle has viscoelastic and plastic deformability over a wide range of stress; Young's modulus increases post-moult

Fig. 2 shows the typical pattern of engineering strain (ES) as a function of time, in this case for the cuticle of an unfed tick. Both stretch and recoil approach an asymptotic limiting value characteristic of a viscoelastic solid, providing evidence of bonding between protein molecules in the matrix. Fig. 3 shows the viscoelastic component of engineering strain (ES$_v$) as a function of stress for newly moulted ticks up to the maximum stress
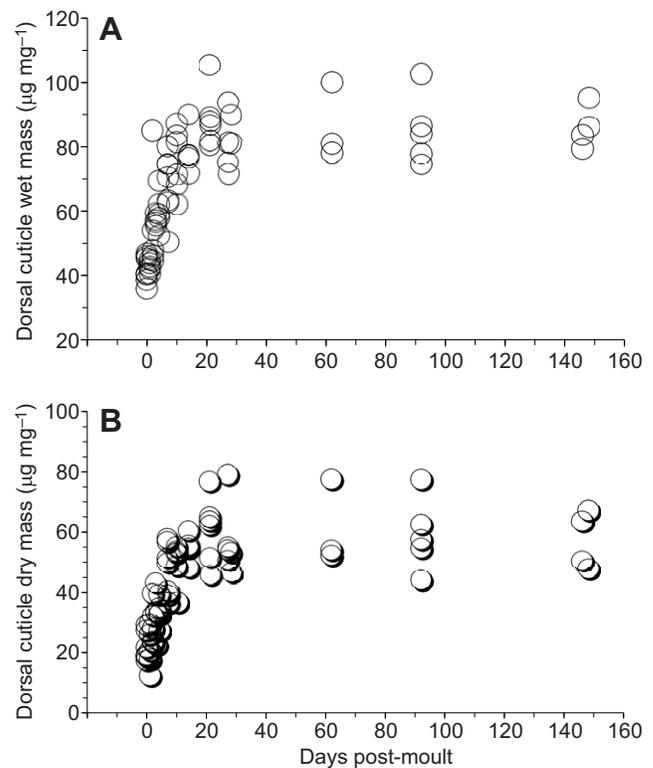

Fig. 1. Wet and dry mass of the dorsal cuticle for unfed ticks as a function of days post-moulting. Mean wet mass doubled (A), and mean dry mass almost tripled (B) over the first 3 weeks post-moult. There was no further significant increase in either measure between 21 and 148 days post-moult.

achievable before break (~0.5 MPa), and for unfed ticks at 18+ days post-moult. As noted above, stiffness under sustained load arises from this component. Stress/ES$_v$ (the inverse of the slope in Fig. 3) can be considered, in traditional engineering terms, the ultimate Young's modulus available to the tick: 18 MPa for newly moulted ticks and 27 MPa for ticks 18+ days post-moult. The value for partially fed ticks in the same stress range as unfed ticks 18+ days post-moult was 33 MPa. The difference in Young's modulus between ticks 18+ days post-moult and newly moulted ticks was
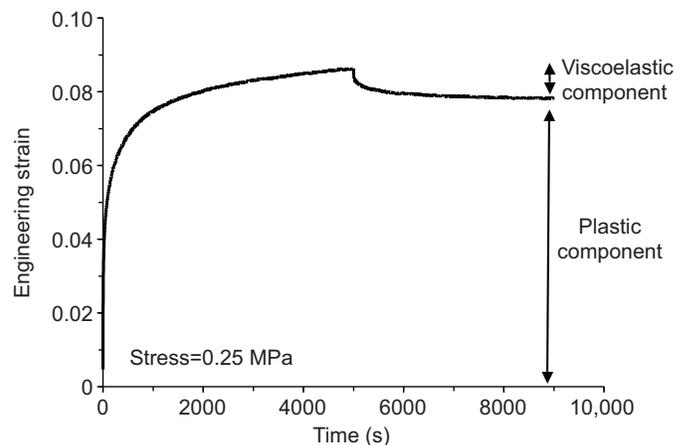
Fig. 2. Stretch and recoil of cuticle from an unfed tick. Here, 5 g (0.159 MPa) was added at time 0 to the cuticle loop, which had been set up 18 s earlier with 2.8 g (0.089 MPa) for tautness. At ~5000 s, the 5 g was removed.
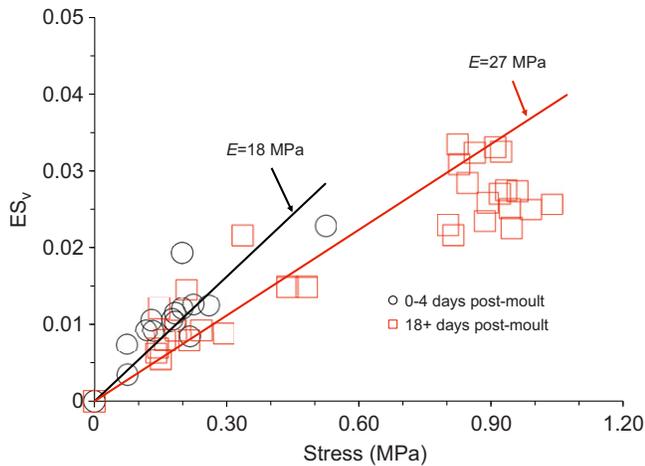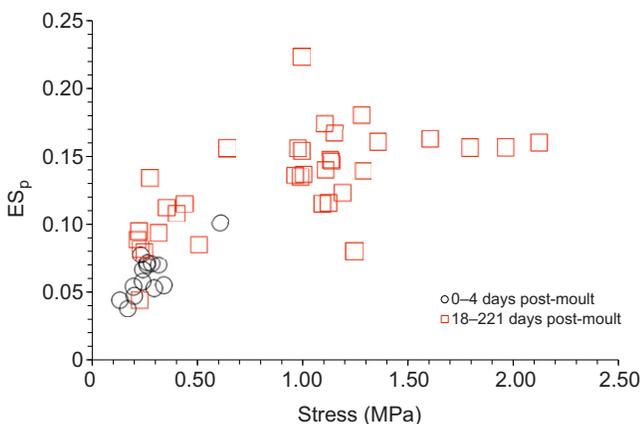
Fig. 3. Viscoelastic component of engineering strain (ES_v), for unfed ticks, as a function of applied stress to a maximum of 1.2 MPa. Data are from individual creep tests. Young's modulus (*E*) is calculated from the slope through the intercept. For ticks 0–4 days post-moult, $y=0.0541x$, and for ticks more than 18 days post-moult, $y=0.0372x$.

highly significant ($P<0.0001$); the difference between ticks 18+ days post-moult and partially fed ticks was not ($P=0.13$).

Fig. 4 shows the plastic component of engineering strain (ES_p) as a function of stress for the newly moulted ticks and those 18+ days post-moult. ES_p for the 18+ day group reached a plateau value of 0.150±0.007 ($N=29$) at a stress level in the range 0.6–2.1 MPa. For the newly moulted ticks, a plateau level was not achieved: the cuticle loops broke at stress levels above ~0.5 MPa. At a stress of 1 MPa, ES_p for the 18+ day group was much higher (~0.15) than ES_v (~0.03, 1 MPa÷Young's modulus). The general pattern of stretch and recovery was also observed in partially fed and engorged ticks.

For a sample of six partially fed ticks, 18±0.6% of the total stretch at 5000 s was reached by 3 s, and 35±1% by 60 s. But ES_p broke through the plateau at stress levels above ~2.5 MPa. Fig. 5 and Table 1 show ES_p as a function of stress for various stages of the feeding cycle and treatments. DA had a minor impact on ES_p at stress levels below 2.5 MPa, relative to its substantial impact above 2.5 MPa. Note that thinning of the cuticle by half, observed in the rapid phase of engorgement (Kaufman et al., 2010), requires an ES_p above 0.4 in two orthogonal directions. Feeding reduced ES_p available at stress levels

Fig. 4. Plastic component of engineering strain (ES_p) for unfed ticks as a function of applied stress. Data are from individual creep tests. ES_p is a linear function of stress up to about 0.6 MPa; thereafter, ES_p is independent of applied stress up to a stress in excess of 2.2 MPa.
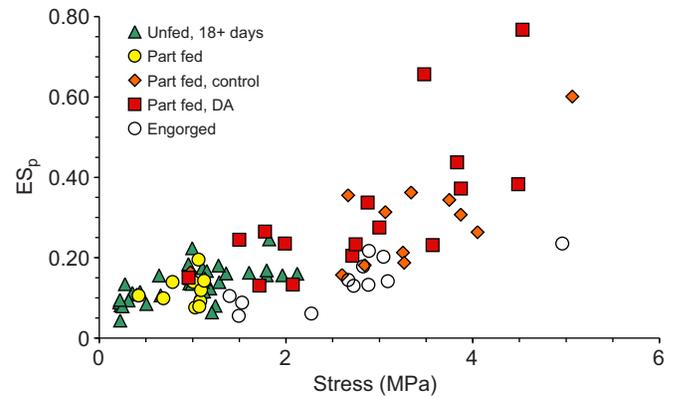
Fig. 5. ES_p as a function of stress plotted for various stages of the feeding cycle, and for ticks treated with dopamine. Data are from individual creep tests. In general, ES_p increases above ~2.5 MPa; dopamine (DA, 1 mol l^{-1}) appears to substantially enhance ES_p above the level for the tissue culture (TC) medium 199, but only at stresses above ~2.5 MPa. Note that thinning of the cuticle (assumed to be isochoric) requires an $ES_p>0.4$ in two orthogonal directions during the rapid phase of engorgement.

below 2.5 MPa from 0.15 to 0.08 by the time of full engorgement (Table 1). In the latter stress range, less than 30% of the total predicted asymptotic compliance occurred in step 1 for unfed ticks (27.9±1.1%, $N=29$), versus 47.4±2.7% ($N=9$) and 45.0±3.9% ($N=4$) for partially fed and engorged ticks, respectively.

The water content of cuticle does not impact measured material properties

Water constitutes about 23% of the wet mass of cuticle from unfed ticks, and increases to about 35% in partially fed ticks, where it plateaus at a fed:unfed mass ratio of 3:1 and beyond (Kaufman et al., 2010). We plotted (data not shown) the following material properties as a function of cuticular water content: ES_p, stress/ES_v, the modulus for steps 1–3 of the Kelvin–Voigt (KV) analysis, characteristic times for steps 1–3 and viscosity for steps 1–3. None of these material properties varied significantly as a function of water content; the R^2-values were all in the range 0.000–0.09, and the P-values were all well over 0.05.

Plastic deformability is 'used up' in a stress-induced stretch; treatment of stretched cuticle with DA and low pH restores ES_p

ES_p on the second and third stretch–recoil cycles was only 28% and 14%, respectively, of that of the first cycle (Table 2). In contrast, a

Table 1. ES_p values for various treatments and stress ranges

Treatment	Stress range (MPa)	ES_p
Unfed (18–221 days post-moult)	0.6–2.1	0.150±0.007 (29)
Partially fed	0.6–1.1	0.120±0.013 (9)*
Engorged	1.4–2.3	0.077±0.012 (4)**
	2.6–5	0.177±0.014 (9)
Partially fed, TC medium 199	2.6–4.1	0.269±0.025 (10)***
Partially fed, 1 mmol l^{-1} DA	1.0–2.1	0.193±0.025 (6)
	2.7–4.5	0.424±0.064 (11)

ES_p (plastic component of engineering strain) data are means±s.e.m. with N values in parentheses. DA, dopamine.
*Difference between partially fed, 0.6–1.1 MPa stress and partially fed plus 1 mmol l^{-1} DA, 1.0–2.1 MPa stress was significant ($P=0.014$).
**Difference between engorged, <2.5 MPa stress and engorged, >2.6 MPa stress was highly significant ($P=0.001$).
***Difference between partially fed plus tissue culture (TC) medium 199 and 1 mmol l^{-1} DA in the range 2.6–4.5 MPa stress was significant ($P=0.041$).

Table 2. ES_p over three successive stretch–recoil cycles for a population of unfed and partially fed ticks

	Cycle 1	Cycle 2	Cycle 3
Mean±s.e.m.	0.144±0.023	0.041±0.009	0.020±0.009
N	6	6	2

The stress range was 0.65–1.8 MPa. The fall in ES_p during the second cycle was highly significant ($P=0.002$). The difference in ES_p between the second and third cycles was not significant ($P=0.252$).

second stretch showed no significant change in ES_v/stress; so plasticity is lost in an *in vitro* stretch, but not stiffness.

Ticks that have engorged have obviously stretched and thinned their cuticle significantly; hence, normal engorgement might be considered to cause an initial stretch equivalent to that imposed by an *in vitro* cycle. Fig. 5 suggests (and subsequent work in preparation confirms) that during engorgement the tick generates sufficient internal pressure to create a stress in the cuticle in excess of 2.5 MPa. ES_p for engorged ticks in the stress range 2.6–5 MPa was 0.177±0.014 ($N=9$) (Table 1); hence, some process during or immediately following normal engorgement restores ES_p. Cuticle from engorged ticks was subjected to a first stretch–recoil cycle. The cuticle was then incubated overnight, either in a solution of 1 mmol l^{-1} DA at pH 6.5 or in tissue culture (TC) medium 199 at pH 7.2. Notwithstanding the small sample size, cuticle treated with DA at reduced pH appeared not to suffer a reduction of ES_p during the second cycle: ES_p during the second cycle was 0.167±0.046 ($N=2$) compared with 0.137±0.004 ($N=2$) during the first cycle. In contrast, cuticle treated with TC medium 199 at a pH of 7.2 appeared to suffer a reduction of ES_p: 0.052±0.007 ($N=3$) compared with 0.142±0.051 ($N=3$) during the first cycle.

DA increases cuticle extensibility and increases plastic deformability of cuticle

Data from experiments in which the cuticle loop broke allowed a calculation of extensibility, the ES at the point of break. Measured extensibility for unfed newly moulted ticks (less than 4 days post-moult) was 0.37±0.01 ($N=4$), and for partially fed ticks (fed:unfed mass ratio ranging from 4:1 to 20:1) it was 0.33±0.007 ($N=3$). However, for DA-treated ticks, extensibility was 0.51±0.027 ($N=2$). In the stress range 2.6–4.5 MPa, DA increased the average ES_p (Table 1): 0.424±0.064 ($N=11$) versus 0.269±0.025 ($N=10$) for the control group treated with TC medium 199 only. DA increased the maximum realized value of ES_p to 0.77 (Fig. 5).

DA reduced the viscosity of the cuticle, confirming an earlier finding (Kaufman et al., 2010): step 1 viscosity for DA-treated ticks in the stress range 2.5–4.5 MPa was 66±9 GPa s ($N=11$) versus 106±13 GPa s ($N=10$) for the control group treated with TC medium 199 only ($P=0.0146$, one-tailed). A similar drop in viscosity was also noted for step 2 viscosity: 22±2 ($N=11$) for DA-treated ticks versus 29±3 ($N=10$) for TC medium 199-treated ticks ($P=0.0405$, one-tailed), and the Maxwell viscosity (the inverse of the slope of the curve of compliance versus time) measured at 1/3 of the value of tau 1 was: 85±10 ($N=11$) versus 136±18 ($N=10$) ($P=0.011$, one-tailed). The step 1 modulus for DA-treated ticks was 25±4 MPa ($N=11$) versus 16±2 MPa ($N=10$) for the control group ($P=0.022$, one-tailed).

Tick cuticle has material properties significantly different from those of insect cuticle

Fig. 6 shows the values of ES_p, the overall Young's modulus and step 1 viscosity for ticks and freshly moulted insects. The
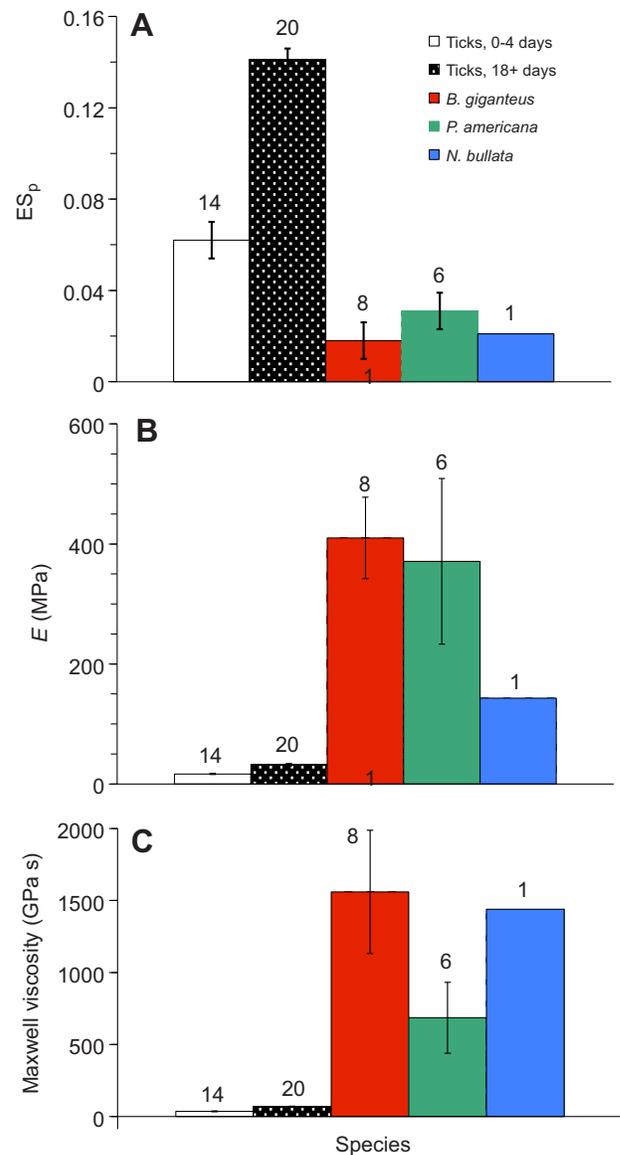
Fig. 6. Mechanical properties of newly moulted unfed ticks, unfed ticks 18+ days post-moult and three insects prior to sclerotization. The two cockroaches (*Periplaneta americana* and *Blaberus giganteus*) were taken on the day of ecdysis, and the fleshfly (*Neobellieria bullata*) was dissected out of the puparium just before ecdysis to the adult. (A) ES_p, (B) Young's modulus and (C) Maxwell viscosity at 1/3 of the value of tau 1 (the inverse of the slope of compliance versus time). The differences between the ticks and the insects were substantial for all measures.

unsclerotized insect cuticle is much stiffer and less ductile than the tick cuticle. Using the mean values of ES_p from Fig. 6, the estimated work of extension from plastic deformation as stress was applied up to 0.6 MPa was 42 kJ m^{-3} for the unfed tick at 18+ days, versus 5, 9 and 6 kJ m^{-3} for the three insects.

DISCUSSION
Female *A. hebraeum* cuticle is synthesized and stored at two points in the life cycle

A critical issue for a female ixodid tick is to have sufficient cuticle to contain the ~100-fold increase in body volume at the end of feeding. As noted above, females synthesize sufficient endocuticle during the feeding period to thicken their cuticle by the end of the slow phase of engorgement, and cuticle growth continues well into

the rapid phase of engorgement (Kaufman et al., 2010; Flynn and Kaufman, 2011). In addition to cuticle growth during feeding, females synthesize endocuticle during the first 3 weeks or so following moulting (Fig. 1). During the same period, chemical changes within the cuticle increase both its stiffness and its capability to sustain plastic deformation. Young's modulus increases by 50% by 18 days post-moult, and the mature cuticle is able to sustain stress up to 5 MPa versus only 0.5 MPa for the newly moulted cuticle. These changes are perhaps due to the formation of additional bonds between protein molecules in the matrix surrounding the chitin fibrils (mild sclerotization) and/or the synthesis of distinct proteins during cuticle growth post-moult. As mass ratio increases, the reduction of ES_p available at stress levels below 2.5 MPa (Table 1) also suggests that cuticle composition may be changing as the tick grows significant cuticle during feeding (Flynn and Kaufman, 2011).

Tick cuticle has two functions, energy absorption and stiffness; both properties are significantly different from that of unsclerotized insect cuticle

One striking feature of stretching alloscutal cuticle under load is the significant component of plastic deformation, ES_p. For example, in a partially fed tick, ES_p (~0.12; Table 1) is typically 4 times the value of ES_v (~0.03, 1/Young's modulus) at a stress of 1 MPa. The ES_p component provides ductility to the tick: the ability to absorb energy under deformation without rupture. One-sixth of the deformation occurs in only 3 s and one-third within 60 s. For the tick, the estimated work of extension in the stress range 0.0–0.6 MPa (42 kJ m^{-3}) was 4–8 times higher than the estimated values for the unsclerotized insect cuticle (5–9 kJ m^{-3}). Post-sclerotization, the insect cuticle has even lower plastic deformability and capacity for work of extension. The 27 MPa stiffness of cuticle from unfed ticks 18+ days post-moult in the stress range 0.0–1.1 MPa, was substantially lower than that calculated for the insects in our study, and lower still than values in the range 5–20 GPa for sclerotized insect cuticle (Vincent and Wegst, 2004). Insect cuticle, even when unsclerotized, is stiff but has low ductility.

The differences in cuticular properties between ticks and insects arise in part from a difference in the relative amount of chitin and protein matrix (Kaufman, 2014). Chitin comprises 3–4% of the cuticle in *Boophilus microplus* (Hackman, 1975; Hackman and Goldberg, 1987), and 12% in that of unfed female *Hyalomma dromedarii*, falling to 4% in the fully engorged female (Bassal and Hefnawy, 1972). Values for non-blood-sucking insects are in the range 25–60% (Gillott, 2005). The higher chitin content gives higher stiffness to the insect exoskeleton [chitin fibrils have a Young's modulus of 80–150 GPa (Vincent, 1990); the lower stiffness of the cuticle arises from the matrix, not the chitin]. The higher fibre content contributes to the 10- to 20-fold higher viscosity of the unsclerotized insect cuticle relative to that of the tick. The stiffness of the insect cuticle is further enhanced by post-eclosion sclerotization of the protein matrix. The differences in exoskeletal material properties provide a clear advantage to both the tick and the insects. The tick does not require a rigid exoskeleton against which to operate flight muscles, and is at risk of bursting from both short-term and longer term forces applied by the host animal; a short-term force might arise from the host rubbing against a tree to relieve irritation from ticks feeding on it, a longer term force from the animal lying down. The tick can utilize its total deformation to absorb energy under stress, particularly because so much of the plastic deformation is realized over a short time scale. The insect is less likely to experience crushing stress, but needs

rigidity of the skeletal frame to utilize its wing muscles; ductility in a flying insect would impair energy-efficient flight.

Multiple processes are involved in the stretch and recoil of cuticle; material properties are not affected by cuticular water content

Modelling stretch of the cuticle as a spring and four KV elements indicates that deformation of the cuticle involves more than one process at the molecular level, a phenomenon typical of polymeric materials (Ferry, 1980; Vincent, 1990). The characteristic time constant, tau, derived from the modelling of stretch as a series of exponential processes, represents the ratio of viscosity to modulus for each 'step' in the stretch. The various molecular components contributing to stretch, such as disentanglement of side chains and straightening of the protein backbone, are associated with different resistances to deformation. Note, however, that in the tick there was no measurable impact of cuticular water content on ES_p, ES_v/stress or any other calculated material property. This contrasts with previous reports on insects which indicated that increased hydration is a significant factor in accounting for increased compliance of the cuticle (Reynolds, 1975; Hackman and Goldberg, 1987; Vincent, 1990). Although we did observe a substantial increase in cuticular water content from an average of 23% to 35% as the feeding cycle progressed (Kaufman et al., 2010), this increase seems not to correlate to the mechanical properties of the cuticle. As noted above, additional cuticle is being synthesized until well into the rapid phase of engorgement (Flynn and Kaufman, 2011) and a change in protein composition and the ratio of protein matrix to chitin would be other factors affecting material properties of the cuticle.

Cuticle must accommodate rapid growth; DA impacts the material properties of cuticle during and after the rapid phase of engorgement

Over a wide range of stress, ES_p in unfed ticks (18+ days post-moult) and in partially fed ticks reached a plateau value in the 0.10–0.15 range. As noted above, this ductility can absorb energy when an external stress is applied to the tick. However, the cuticle of the partially fed tick must achieve a higher permanent deformation during the rapid phase of engorgement. The 50% thinning of alloscutal cuticle during the rapid phase results from the expansion of body volume; the stress causing this expansion arises from a very large increase in internal hydrostatic pressure (W.R.K., S. Kaufman and P.C.F., in preparation). Assuming a Poisson's ratio at or near 0.5 (Flynn and Kaufman, 2011), i.e. the cuticle is isochoric, this degree of thinning requires an ES_p of more than 0.4 to be achieved in each orthogonal direction of the cuticle. At stress levels in the range 2.6–4.5 MPa, ES_p for ticks in the control group often exceeded 0.15, reaching the maximum observed value in our experiments of 0.36 (Fig. 5).

DA triggers fluid secretion from the salivary gland of ixodid ticks (Kaufman, 2010). We speculated that DA might play a role in easing the stretch of the cuticle during the rapid phase of engorgement and restoring ductility (ES_p) after an *in vivo* stretch. That DA might play such a role was suggested by our earlier demonstration that DA reduces the Maxwell viscosity of cuticle during stretch (Kaufman et al., 2010). Studies of other arthropod cuticles are relevant here as well.

Hackman and Goldberg (1987) reviewed the amino acid composition of the cuticle of seven arthropods, three of which are blood feeders, and thus subject to rapid expansion: the insect *R. prolixus*, the ixodid tick *Rhipicephalus* (*Boophilus*) *microplus* and the argasid tick *Argas robertsi*. They made several observations and hypotheses. (1) They are all characterized by low chitin content

and predominantly basic and hydrophobic proteins that have a low number of amino acids containing bulky side chains. (2) Prior to expansion, the cuticle is maintained at a pH value within the range of the isoelectric point of the major proteins, giving the strongest bonding between proteins. (3) During rapid stretching, bonds linking the proteins are broken; a reduction in pH is the most rapid way to alter the inter-protein bonding. (4) The breaking of bonds can be reversed by a return of pH to a value in the range of the isoelectric points of the major proteins, restoring the mechanical properties characteristic of unfed specimens.

Reynolds (1975) postulated that plasticization of the cuticle of *R. prolixus*, known to be triggered by the neurotransmitter 5-HT, is due to mild acidification of the cuticle. We postulated a similar mechanism for the reduction in Maxwell viscosity triggered by DA (Kaufman et al., 2010). Here, we observed that DA causes a significant reduction in both the viscosity and the modulus (stiffness associated with that step) calculated by the KV analytical approach for the longest two stretch elements. Furthermore, treatment of stretched cuticle with DA at a pH of 6.5 did restore ES_p that was otherwise lost during the first stretch of cuticle. If acidification of the cuticle induced by DA occurs during the rapid phase of engorgement, this might account for why engorged tick cuticle retains ES_p but *in vitro* stretched cuticle does not. DA increased ES_p in the range 2.6–4.5 MPa, and the maximum ES_p value observed was 0.77 (Fig. 5), a degree of deformation that is well in excess of the extensibility at breakage of cuticle not treated with DA. The critical role of DA might be to reduce the threshold stress for ES_p to exceed 0.4; this would reduce the internal hydrostatic pressure required to thin the cuticle during the rapid phase of engorgement. Data from the limited number of cuticle loops that broke during KV analysis also suggest that DA enables ES_p above the required level of 0.4, as also observed from Fig. 5. Contrast this to extensibility values of 0.01 to 0.02 reported for sclerotized insect cuticle (Vogel, 2003). It is clear from Fig. 5 that in the absence of DA, stress levels above 2.5 MPa induce a higher ES_p in both partially fed and engorged ticks, but the effect is far greater for partially fed ticks. Hence, the DA treatment may be supplementing a process already initiated in the partially fed ticks.

Conclusions

In summary, our study of the tick *A. hebraeum* revealed the following. (1) An unfed female *A. hebraeum* doubles its cuticle wet mass and triples its dry mass in the first 20 days post-moult. (2) Over that time period, the cuticle increases in stiffness (Young's modulus) by 50% and increases the extent to which it can deform without breaking. (3) The cuticle displays time-dependent plastic (permanent, ES_p) and viscoelastic (recoverable, ES_v) deformation under stress. Plastic deformation absorbs energy during stretch but does not contribute to ultimate cuticular stiffness. (4) ES_p reaches a plateau value in the stress range 0.6–2.5 MPa; above 2.5 MPa, ES_p increases. (5) Partial feeding and engorgement reduce the plateau value of ES_p relative to unfed ticks 18+ days post-moult; but for all stages of feeding the tick has substantial ES_p, giving the cuticle ductility. (6) Relative to tick cuticle, unsclerotized cuticle from three species of insect has much higher stiffness and viscosity and lower ductility. (7) An *in vitro* stretch of cuticle removes 3/4 of its ductility in a subsequent stretch, but an engorged tick that has undergone stretch during feeding (causing expansion of the cuticle) retains its ductility. (8) ES_p increases in partially fed ticks in a control group treated with TC medium 199 and subjected to a stress greater than 2.5 MPa; the observed ES_p is below the level of 0.4 that would be required to accommodate the thinning of the cuticle by half

observed during the rapid phase of engorgement. (9) Extensibility (ES at the point of breakage) is less than 0.4 for cuticle loops not treated with DA. (10) Injection of partially fed ticks with DA in TC medium 199 increases ES_p, on average above 0.4, increases extensibility and reduces the viscosity of the cuticle. Incubating loops (already stretched) *in vitro* in DA and reduced pH restores ES_p.

MATERIALS AND METHODS
The ticks and insects

The *Amblyomma hebraeum* Koch 1844 ticks used in this study came from a laboratory colony. Ticks were maintained in darkness at 26°C at a relative humidity exceeding 95%, then fed on laboratory rabbits (Kaufman and Phillips, 1973). A cloth-covered foam arena (~12×8×2.5 cm) was glued to the shaven back of a rabbit with a latex adhesive. The next day, male ticks (equal in number to the anticipated number of female ticks to be used) were added to this 'backpack' and allowed to attach and feed for 1 day. Over the following days, small batches of unfed females were introduced to the backpack, ensuring that females at the desired feeding stage would not exceed the number that could be experimented with on any one day. The use of rabbits was approved by the Biosciences Animal Policy and Welfare Committee, University of Alberta, which functions under guidelines established by the Canadian Council on Animal Care. Before feeding, each tick was weighed and identified by a piece of coloured silk thread glued to a leg. Ticks were removed at various stages of feeding; we recorded the fed:unfed mass ratio rather than the absolute mass.

We used three species of insect: two cockroaches, *Periplaneta americana* (Linnaeus 1758) and *Blaberus giganteus* (Linnaeus 1758), and the fleshfly *Neobellieria bullata* (Parker 1916). The insects were reared according to standard procedures adopted by the Entomology section of the Department of Biological Sciences.

Measuring the growth of endocuticle

Ticks were collected at various stages post-moult or post-detachment from the host, and the dorsal alloscutal cuticle was cut out using a razor-blade scalpel. The cuticle was cut all the way around the lateral margin of the tick, and then the cut followed the contour between the scutum and alloscutum as shown in Fig. 7. The dissected cuticle was submerged in Hank's saline and all the soft tissue on the inner surface was scraped away gently using the blunt surface of the scalpel so as not to scrape away endocuticle. The piece of cuticle was then blotted with tissue paper, and the wet mass recorded on a 5-place electronic balance. The sample was dried in an oven at 150°C for

Fig. 7. An unfed female tick. The pathway of the cut used to sample the dorsal alloscutum is indicated by the white dashed curve. The contour of the cut follows along the lateral demarcation between the dorsal and ventral alloscutal surfaces, and then follows the V-shaped dorsal margin separating the scutum from the alloscutum. The same contour shown here on an unfed female is easily recognized in ticks at all stages of engorgement.

about 60–90 min, and the dry mass was recorded. Although this method measures endocuticle growth of only the dorsal alloscutum, it is reasonable to suppose that the remainder of the alloscutum grows by the same percentage.

Preparing the cuticle for stretch analysis

We characterize cuticular mechanical properties by a compliance (creep) test under fixed stress. Cuticle loops of ticks were prepared as described by Kaufman et al. (2010). These loops were taken from the alloscutal region, but always contained the anal plate, a small island of sclerotized cuticle. Loops taken from unfed and very small partially fed ticks occasionally also included the spiracular plate on either or both sides; in such cases, the total area of sclerotized cuticle would still have been a small (though unmeasured) percentage of the alloscutal cuticle. Length, width and thickness of the cuticle loops were measured under a dissecting microscope, both before and after recording stretch and recoil data. A diagram of the mounting frame apparatus is shown in Fig. 8. To prevent desiccation of the cuticle, all specimens were submerged in a mineral oil bath during stretch and recoil.

When we attempted to mount loops of insect cuticle on the hooks shown in Fig. 8, the loops invariably broke at low stress at the pleural membrane joining the tergite and sternite. Hence, for the insects, the hooks shown in Fig. 8 were replaced with small, flat-faced clips with fine ridges, one of them fixed to the curved arm and one attached to the linear variable differential transformer (LVDT). The specimens (usually the pronotum, occasionally a more distal tergite) were dissected out and the internal surface gently scraped with the back of a microscalpel to remove the adhering muscle insertions; the sample was then trimmed with fine scissors at the anterior and posterior edges to produce an approximately rectangular strip. Width and thickness of the sample were recorded. The lateral ends were then gripped by the clips, which were always wider than the specimen, and the cuticle was subjected to the desired stress. Only the free length of the cuticle between the noses of the two clips was measured for the analysis of compliance; no slippage of the specimen within the clips was observed.

Conducting the stress tests

Stretch data were collected by a LVDT (Macro Sensors, Pennsauken, NJ, USA); two models were used (DC 750 125 010, DC 750 050 010) depending on the expected amount of cuticle distension. Three improvements were made to the earlier apparatus described in Kaufman et al. (2010): (1) greater rigidity in the apparatus by additional cross-bracing and stiffer hooks, (2) a more accurate and linear LVDT and (3) improved data collection through an analog to digital converter (National Instruments, model USB-6008). Apparatus stretch was measured before each experiment by applying the experimental weight with the clips or hooks attached (without cuticle); measured cuticle stretch and recoil were subsequently corrected to remove the contribution of apparatus stretch and recoil. In most cases, apparatus stretch was not more than ~10% of the loop stretch. All tests were conducted at room temperature (approximately 21–23°C).

Drug trials

Ticks were injected with a solution of DA in modified TC medium 199 (see below) so as to achieve the desired dose (1 mmol kg^{-1} M_b; 5 µl 100 mg^{-1} M_b). Controls were injected with the same volume of TC medium 199 alone. After a 30–45 min delay, the cuticle loop was dissected out as described by Kaufman et al. (2010) and subjected to the appropriate stress test. Powdered TC medium 199 made with Hank's salts (Sigma Chemical Company) was dissolved in 1 l milliQ (Millipore) water along with 2.09 g of the buffer morpholinopropanesulphonic acid (MOPS) plus 2.1 g NaCl; the pH was adjusted to 7.2.

Kaufman et al. (2010) demonstrated that when cuticle loops were exposed to the pH range 8.0–5.5, Maxwell viscosity decreased progressively. Injecting ticks with DA (1 mmol kg^{-1} M_b) likewise reduced Maxwell
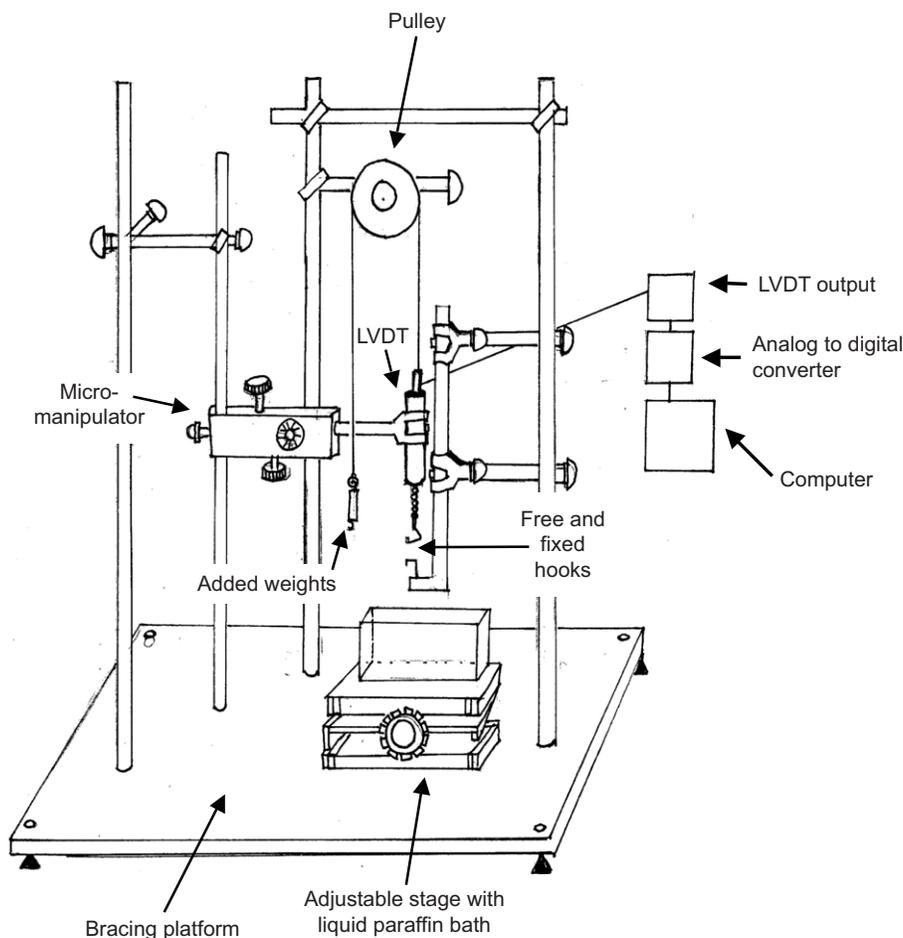
Fig. 8. Mounting frame and instrumentation for fixed stress testing of compliance. Apparatus stretch, which is small relative to loop stretch, was first measured by loading weights with the two hooks connected; this was subtracted from the subsequent measured loop stretch. The loop was then placed across the hooks and the mineral oil bath was raised to cover the loop to prevent loss of moisture during the stretch. LVDT, linear variable differential transformer.
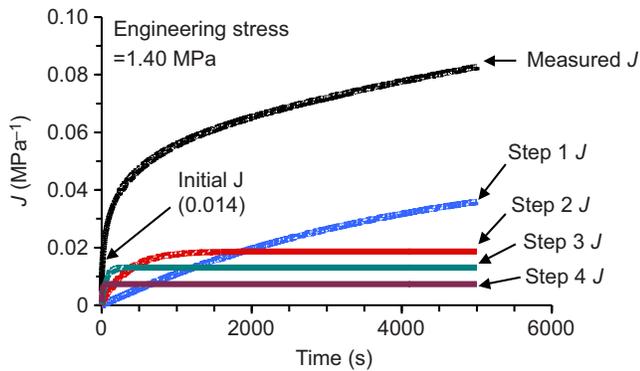
Fig. 9. A Kelvin–Voigt analysis of compliance from stretch of the cuticle from an engorged tick. The analysis models total compliance (J, strain per unit stress) as four exponential stretch elements and an instantaneous initial stretch.

viscosity. The fact that the V-ATPase inhibitor concanamycin A inhibited the effect of DA (Kaufman et al., 2010) suggested that the effect of DA is mediated by the transport of H^+ ions into the cuticle. Here, we found that an initial stretch–recoil cycle significantly reduces ES_p during a second cycle. So, we tested whether ES_p can be restored by incubating cuticle loops in a weakly acidic medium containing DA prior to the second stretch–recoil cycle. Following an initial stretch–recoil cycle, the cuticle loop was incubated in TC medium 199 containing 1 mmol l^{-1} DA, with pH adjusted to 6.5; the incubating loop was held overnight in a refrigerator. The following morning, the loop was re-mounted for the second cycle of a stress test. Controls were treated identically except that the overnight incubation was in TC medium 199 alone, pH 7.2.

Normalization of force and stretch

ES was calculated by dividing the increase in length by the original length of the loop (dL/L_0). The imposed force was converted to 'engineering stress' (N m^{-2} or Pa) by dividing the force by the initial cross-sectional area of the loop. The typical range of imposed stress was 0.1–5 MPa. True strain (dL/L) and true stress (force per unit cross-sectional area adjusted for the reduction in area as stretch occurs) differ from engineering strain and stress (Vogel, 2003; Vincent, 1990). Most of our experiments resulted in ES values of less than 0.2; up to this value, the difference between engineering strain/stress and true strain/stress is less than 10%, a difference that is modest relative to other sources of variation in this work. Strain per unit stress is called compliance; compliance as a function of time enables comparison of experiments over a wide range of loop sizes and imposed stress.

Analysis of compliance

In the range of stresses applied in our experiments, the cuticle behaved like a viscoelastic solid. A KV model (a viscous dashpot coupled in parallel with a spring) yields a first order exponential asymptotic approach to a limit. We used the approach of Tschoegl (1989) to model observed compliance, by assuming a series of KV elements with different time constants. Long-term compliance (step 1) was modelled as an exponential approach by best fit in the time range 1500–3500 s for stretch (400–1500 s for recoil); the stretch due to this first model KV element was then subtracted from the original compliance curve. This process was repeated three times at successively lower time ranges, as follows. Step 2: 200–1000 s for stretch, 100–250 s for recoil. Step 3: 20–120 s for stretch, 10–25 s for recoil. Step 4: 2–10 s for stretch, 2–6 s for recoil. These time ranges were identified by trial and error; typical R^2 values were 0.99 or higher for the first step, but usually above 0.9 for subsequent stretches. Remaining compliance after four steps was assumed to be instantaneous. Fig. 9 shows the compliance arising from different time ranges superimposed on a sample run. In effect, cuticle stretch was modelled as a single spring (instantaneous stretch) and four KV elements for which a modulus (stress per unit strain) and viscosity (and hence a characteristic time:viscosity divided by modulus) can be calculated. This model will work if the time scale (tau) of the elements is sufficiently

different from each other, because in an exponential approach to an asymptote, at t=3 tau, 95% of the ultimate stretch attributable to that KV element has occurred. The method identifies that different processes are occurring within the protein matrix; for example, Ferry (1980) suggests short distance events in polymeric plastics (kinks), medium (curls) and long (convolutions). The values of tau are a spectrum identifying the time range at which components of the stretch occur (Ferry, 1980). Sample spreadsheets and a more detailed methodology used in the analysis of stretch and recoil are filed at www.ualberta.ca/~pcflynn.

In practice, when the weight was added to the apparatus by hand, the starting point of stretch was accurate to only about 1 s. Given this uncertainty, we combined the compliance modelled by the fourth KV element with the calculated initial stretch component and treated the two as occurring almost instantaneously. Note also that stretch continued to occur after our typical upper range of measurement (3500–5000 s). Stretch observed in two longer runs showed that the stretch observed in our range was ~70% of that observed in 20 h, and ~60% of that observed in 60 h. One might model very long-term cuticle stretch by additional KV elements representing very slow extension or disentanglement of macromolecules, but the functional benefit of such long-term stretch for the tick seems dubious. The effective ultimate compliance in stretch or recoil was predicted from the asymptotic values of the initial stretch plus the four time-dependent components of stretch in the analysis.

Real polymeric materials exhibit stretch behaviour that is more complex than a model of KV elements; for example, the material does not return to its original length when the load is removed. A key insight of this study arises from separating the strain due to the plastic and viscoelastic components of deformation. In a stretch and recoil experiment, the asymptotic value of the recoil strain is the viscoelastic component (ES_v), and the asymptotic value of the stretch minus the asymptotic value of the recoil strain is the plastic component (ES_p).

Deforming materials store energy (force multiplied by distance); a key measure of this is work of extension (Vogel, 2003). A rigorous measure of this is the integral of a stress–strain curve from a progressive increase in stress. We approximated this value at a strain of 0.6 MPa using the assumption that plastic deformation is linear in the range 0–0.6 MPa, the point at which its maximum value was assumed to be reached. The resulting approximate values were used to illustrate the substantial difference in work of extension between tick cuticle and that of unsclerotized insects.

Statistics

Data are reported as means±s.e.m. (N). Statistical significance was calculated (Microsoft Excel software) using the specific test indicated; P-values are from a two-tailed t-test unless otherwise noted.

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