

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

Peter C. Flynn<sup>1</sup> and W. Reuben Kaufman<sup>2</sup>

<sup>1</sup> Prof. Emeritus, Department of Mechanical Engineering, University of Alberta, Edmonton,  
Alberta, Canada T6G 2G8

email: peter.flynn@ualberta.ca

<sup>2</sup> Prof. Emeritus, Department of Biological Sciences, University of Alberta, Edmonton,  
Alberta, Canada T6G 2E9

email: rkaufman@ualberta.ca

Summary Statement: Female *Amblyomma hebraeum* grow cuticle post moult prior to feeding; the cuticle stiffens. Mechanical analysis suggests that dopamine but not water content enables stretch and thinning of cuticle in the rapid phase of engorgement.

## Abstract

Female ticks of the family Ixodidae increase their weight 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 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 weight during the first 3 weeks post moult, during which the ductility and stiffness of the cuticle increased substantially. Under stress, cuticle displayed time-dependent stretch, with a plastic (non-recoverable) and viscoelastic (recoverable) component. Plastic deformation was reasonably constant in the range of 10 to 15% over a wide range of induced stress above ~0.6 MPa. The plastic component of tick alloscutal cuticle was about five to ten times higher than that of unsclerotized insect cuticle. Tick cuticle is also 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 weight). Injected dopamine (DA) reduced one measure of the viscosity of cuticle by 38%. Plastic deformability of 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 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 weight increases about 10-fold, and a rapid phase (~12 - 24 h), during which there is a further 10-fold increase in weight, and the replete tick detaches from the host (Apanaskevich and Oliver Jr., 2014)\*. *A. hebraeum* grows endocuticle during the slow and well into the rapid phase of engorgement (Flynn and Kaufman, 2011). Endocuticle thickens substantially during the slow phase (from 130 to 175  $\mu\text{m}$ ; 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 cuticle to 90  $\mu\text{m}$  (Kaufman et al., 2010).

The 5<sup>th</sup> stage larva of the blood-sucking hemipteran, *Rhodnius prolixus*, increases its weight ~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<sup>+</sup>-ions into the subcuticular space, causing a concomitant increased 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: (a) the relative amount of chitin fibrils and protein matrix, (b) the protein composition, (c) pH/water content of the matrix, and (d) 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 explore the mechanical

---

\* The enormous size increase during engorgement in ixodid ticks pertains only to the female. The male feeds much more modestly, increasing its weight by only ~50%. Unless otherwise stated, in this article “tick” refers exclusively to the female.

properties of the tick cuticle<sup>†</sup> at various stages of feeding by analyzing the time-dependent stretch under load, and compare it to cuticle from three species of flying insects.

Many engineering materials show ‘elastic deformation’ under load: stretch occurs virtually instantaneously (at the speed of sound), with recovery to the original length after removing 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 times distance), but the ultimate strength and stiffness of the material derives only from the viscoelastic component, since 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 use the technique of Tschoegl (1989) to analyze

---

<sup>†</sup> 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.

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 focus 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

### *Female A. hebraeum grows cuticle after moulting and before feeding*

After moulting, *A. hebraeum* synthesizes additional endocuticle (Fig. 1). Wet weight increased from  $42.0 \pm 1.6 \mu\text{g.mg body weight (bw)}^{-1}$  (7) on the day of moulting to  $88.7 \pm 3.6 \mu\text{g.mg bw}^{-1}$  (6) by day 21. There was no further change in weight from days 21 through 148 ( $84.3 \pm 2.0 \mu\text{g.mg bw}^{-1}$  (30; Fig. 1a). Dry weight increased from  $21.8 \pm 1.7 \mu\text{g.mg bw}^{-1}$  (7) to  $61.0 \pm 4.5 \mu\text{g.mg bw}^{-1}$  (6) by day 21. There was no change in weight from days 21 through 148 ( $57.9 \pm 2.3 \mu\text{g.mg bw}^{-1}$  (29) (Fig. 1b).

*The cuticle has viscoelastic deformability, proportional to stress, and plastic deformability, independent of stress, over a wide range of stress; the capacity for plastic deformability and the stiffness (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, evidence of bonding between protein molecules in the matrix. Fig. 3 shows the viscoelastic component of engineering strain (ESv) as a function of stress for newly moulted ticks up to the maximum stress achievable before break ( $\sim 0.5$  MPa), and for unfed ticks, 18+ days post moult. As noted above, stiffness under sustained load arises from this component. Stress/ESv (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 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 (ESp) as a function of stress for the newly moulted ticks and those 18+ days post moult. ESp for the 18+ group reached a plateau value of  $0.150 \pm 0.007$  (29) at a stress level in the range of 0.6 -2.1 MPa. For the newly moulted ticks, a plateau level was not achieved: the loops broke at stress levels above ~0.5 MPa. At a stress of 1 MPa, ESp for the 18+ group was much higher (~0.15) than ESv (~0.03,  $1 \text{ MPa} \div \text{Young's modulus}$ ). The general pattern of stretch and recovery was also observed in partially fed and engorged ticks.

For a sample of six partially fed ticks,  $18 \pm 0.6\%$  of the total stretch at 5000 s was reached by 3 s, and  $35 \pm 1\%$  by 60 s. But ESp broke through the plateau at stress levels above ~2.5 MPa. Fig. 5 and Table 1 show ESp as a function of stress for various stages of the feeding cycle and treatments. DA had a minor impact on ESp 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 ESp above 0.4 in two orthogonal directions. Feeding reduces ESp available at stress levels 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 occurs in Step 1 for unfed ticks ( $27.9\% \pm 1.1$  (29)), vs.  $47.4\% \pm 2.7$  (9) and  $45.0 \pm 3.9$  (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 weight of cuticle from unfed ticks, and increases to about 35% at a fed-unfed weight ratio of 5-14 (Kaufman et al., 2010). We plotted (data not shown) the following material properties as a function of cuticular water content: ESp, stress/ESv, the modulus for steps 1-3 of the 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 of 0.000 to 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 in DA and low pH restores ES<sub>p</sub>*

ES<sub>p</sub> on the 2<sup>nd</sup> and 3<sup>rd</sup> stretch/recoil cycles was only 28% and 14%, respectively, of that of the first cycle (Table 2). In contrast, a second stretch showed no significant change in ES<sub>v</sub>/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<sub>p</sub> for engorged ticks in the stress range 2.6 to 5 MPa was  $0.177 \pm 0.014$  (9) (Table 1); hence, some process during or immediately following normal engorgement restores ES<sub>p</sub>. Cuticle from engorged ticks was subjected to a first stretch/recoil cycle. The cuticle was then incubated overnight, either in a solution of 1 mM DA at pH 6.5, or in TC 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<sub>p</sub> during the 2<sup>nd</sup> cycle: ES<sub>p</sub> during the 2<sup>nd</sup> cycle was  $0.167 \pm 0.046$  (2), compared to  $0.137 \pm 0.004$  (2) during the 1<sup>st</sup> cycle. In contrast, cuticle treated with TC 199 at a pH of 7.2 appeared to suffer a reduction of ES<sub>p</sub>:  $0.052 \pm 0.007$  (3) compared to  $0.142 \pm 0.051$  (3) during the 1<sup>st</sup> cycle.

*DA increases cuticle extensibility and increases plastic deformability of cuticle*

Data from experiments in which the loop broke allowed a calculation of extensibility, the ES at point of break. Measured extensibility for unfed newly moulted ticks (less than four days post moult) was  $0.37 \pm 0.01$  (4), and for partially fed ticks (fed-to-unfed weight ratio = 4 - 20)  $0.33 \pm .007$  (3). However, for DA-treated ticks extensibility was  $0.51 \pm .027$  (2). In the stress range 2.6 to 4.5 MPa, DA increased the average ES<sub>p</sub> (Table 1):  $0.424 \pm 0.064$  (11) vs.  $0.269 \pm 0.025$  (10) for the control group treated with TC 199 only. DA increased the maximum realized value of ES<sub>p</sub> 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 to 4.5 MPa was  $66 \text{ GPa s} \pm 9$  (11) vs.  $106 \text{ GPa s} \pm 13$  (10) for the control group treated with TC 199 only ( $p = 0.0146$ , one-tailed). A similar drop in viscosity was also noted for step 2 viscosity:  $22 \pm 2$  (11) for DA-treated vs  $29 \pm 3$  (10) for TC 199-treated ( $p = 0.0405$ , one-tailed), and the Maxwell

viscosity (the inverse of the slope of the curve of compliance vs. time) measured at 1/3 of the value of tau 1:  $85 \pm 10$  (11) vs.  $136 \pm 18$  (10) ( $p = 0.011$ , one-tailed). The step 1 modulus for DA treated ticks was  $25 \text{ MPa} \pm 4$  (11) vs.  $16 \text{ MPa} \pm 2$  (10) for the control group ( $p = 0.022$ , one-tailed).

#### *Tick cuticle has material properties significantly different from that of insect cuticle*

Fig. 6 shows the values of ESp, the overall modulus and Step 1 viscosity for ticks and freshly moulted insects. The unsclerotized insect cuticle is much stiffer and less ductile than the tick cuticle. Using the mean values of ESp from Fig. 6, the estimated work of extension from plastic deformation as stress is applied up to 0.6 MPa was  $42 \text{ kJm}^{-3}$  for the unfed tick 18+ days, vs. 5, 9 and  $6 \text{ kJm}^{-3}$  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 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 vs. 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 weight ratio increases, the reduction of ESp 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<sub>p</sub>. For example, in a partially fed tick ES<sub>p</sub> (~0.12, Table 1) is typically 4 times the value of ES<sub>v</sub> (~0.03, 1/Young's modulus) at a stress of 1 MPa. The ES<sub>p</sub> 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 kJm<sup>-3</sup>) was 4 – 8 times higher than the estimated values for the unsclerotized insect cuticle (5 – 9 kJm<sup>-3</sup>). 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 still further lower than values in the range of 5 to 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. 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 of 25 to 60% (Gillott, 2005). The higher chitin content gives higher stiffness to the insect exoskeleton (chitin fibrils have a Young's modulus of 80 to 150 GPa (Vincent, 1990); the lower stiffness of cuticle arises from the matrix, not the chitin). The higher fiber content contributes to the 10 to 20 fold higher viscosity of the unsclerotized insect cuticle relative to the tick. The stiffness of 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 force applied by the host animal, both short term and longer-term forces; a short-term force might arise from the host rubbing against a tree to relieve irritation from ticks feeding on it, the 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 at 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*

Modeling the stretch of cuticle as a spring and four KV elements indicates that deformation of cuticle involves more than one process at the molecular level, a phenomenon typical of polymeric materials (Ferry, 1980; Vincent, 1990). The characteristic time constants,  $\tau$ , 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 ESp, ESv/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, ESp in unfed ticks (18+ days post moult) and in partially fed ticks reaches a plateau value in the 0.10 to 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 expansion of body volume; the stress causing this expansion arises from a very large increase in internal hydrostatic pressure (Kaufman et al., in preparation). Assuming a Poisson’s ratio of at or near 0.5 (Flynn and Kaufman, 2011), i.e. the cuticle is isochoric, this degree of

thinning requires achieving an ESp of more than 0.4 in each orthogonal direction of the cuticle. At stress levels in the range of 2.6 to 4.5 MPa, ESp 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 cuticle during the rapid phase of engorgement and restoring ductility (ESp) 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 cuticle 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, *Boophilus (Rhipicephalus) microplus*, and the argasid tick, *Argas robertsi*. They made several observations and hypotheses:

- 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;
- 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;
- During rapid stretching, bonds linking the proteins are broken; a reduction in pH is the most rapid way to alter the inter-protein bonding;
- 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 viscosities and the modulus (stiffness associated with that step) calculated by the Kelvin-Voigt analytical approach for the longest two stretch elements. Furthermore, treatment of stretched cuticle with DA at a pH of 6.5 did restore ESp that is 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 ESp but in-vitro

stretched cuticle does not. DA increased ESp in the range of 2.6 to 4.5 MPa, and the maximum ESp 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 ESp 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 loops that broke during KV analysis also suggest that DA enables ESp 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 ESp 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

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 cuticle increases in stiffness (modulus) by 50% and increases the extent it can deform without breaking.
3. Cuticle displays time-dependent plastic (permanent, ESp) and viscoelastic (recoverable, ESv) deformation under stress. Plastic deformation absorbs energy during stretch but does not contribute to ultimate cuticular stiffness.
4. ESp reaches a plateau value in the stress range 0.6 to 2.5 MPa; above 2.5 MPa ESp increases.
5. Partial feeding and engorgement reduce the plateau value of ESp relative to unfed ticks 18+ days post moult; but for all stages of feeding the tick has substantial ESp, 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 which has undergone stretch during feeding (causing expansion of the cuticle) retains its ductility.

8. ESp increases in partially fed ticks in a control group treated with TC 199 and subjected to a stress over 2.5 MPa; the observed ESp is below the level of 0.4 that would be required to accommodate the thinning of 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 199 increases ESp, on average above 0.4, increases extensibility, and reduces the viscosity of cuticle. Incubating loops (already stretched) *in vitro* in DA and reduced pH restores ESp.

## Materials and Methods

### *The ticks and insects*

The ticks used in this study came from a laboratory colony. Ticks were maintained in darkness at 26°C at a RH exceeding 95%, then fed on laboratory rabbits Kaufman & Philips (1973). A cloth-covered foam arena (~12 cm X 8 cm X 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 numbers that could be experimented with on any 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-to-unfed weight ratio rather than the absolute weight.

We used three species of insect, two cockroaches (*Periplaneta americana*, *Blaberus giganteus*) and the fleshfly, *Neobellaria bullata*. 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 weight recorded on a 5-place electronic balance. The sample was dried in an oven at 150<sup>0</sup>C for about 60-90 min, and the dry weight 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 included also 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 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 was collected by a linear variable differential transformer (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): (a) greater rigidity in the apparatus by additional cross bracing and stiffer hooks, (b) a more accurate and linear LVDT and (c) 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 was 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 a modified TC 199 so as to achieve the desired dose (1 mmol per kg bw; 5 µl per 100 mg bw). Controls were injected with the same volume of TC 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 litre 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 to 5.5, Maxwell viscosity decreased progressively. Injecting ticks with DA (1 mmol per kg bw) likewise reduced Maxwell 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<sup>+</sup>-ions into the cuticle. Here we show that an initial stretch/recoil cycle significantly reduces ESp during a second cycle. So we tested whether ESp 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 mM 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 199 alone, pH = 7.2.

### *Normalization of Force and Stretch*

‘Engineering strain’ (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’ (Newtons per square meter, or Pascals) by dividing the force by the initial cross-sectional area of the loop. The typical range of imposed stress was 0.1 to 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, cuticle behaves like a viscoelastic solid. A Kelvin-Voigt (KV) model (a viscous dashpot coupled in parallel with a spring) models 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 modeled as an exponential approach by best fit in the time range of 1500 to 3500 s for stretch (400 to 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 typically 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 modeled 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 are sufficiently different from each other, because in 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/~pcflynns](http://www.ualberta.ca/~pcflynns).

In practice, when the weight was added to the apparatus by hand, the starting point of stretch was accurate to only about 1 second. Given this uncertainty, we combine the compliance modeled by the fourth KV element with the calculated initial stretch component and treat the two as occurring almost instantaneously. Note also that stretch continues to occur after our typical upper range of measurement (3500 to 5000 s). Stretch observed in two longer runs showed that the stretch observed in our range was ~70% of that observed in 20 hours, and ~60% of that observed in 60 hours. 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<sub>v</sub>), and the asymptotic value of the stretch minus the asymptotic value of the recoil strain is the plastic component (ES<sub>p</sub>).

Deforming materials store energy (force times 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 of 0 to 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 mean  $\pm$  SEM (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.

### **Acknowledgments**

This research was generously supported from a Discovery Grant to WRK from the Natural Sciences and Engineering Research Council (NSERC) of Canada. Julian Vincent, Department of Mechanical Engineering, University of Bath, and Phillip Choi and Michael Williams, Department of Chemical and Materials Engineering, University of Alberta, advised us on the analysis of viscoelastic materials. David Nobes and Amanda Kuchon helped set up the data gathering system for the compliance testing. Morris Flynn assisted in understanding the mathematical analysis of stretch. Adam Tomaszewski, Alex Smith and Shahid Ullah assisted in creating the apparatus and performing experimental runs. Jens Roland conducted the statistical analysis for the non-effect of cuticular water content on various mechanical properties. Susan Rogers produced the diagram in Fig. 8.

## References

- Apanaskevich, D.A. and Oliver, Jr., J.H. (2014) Life cycles and natural history of ticks, In: Sonenshine, D.E. and Roe, R. M. (Eds.) *Biology of Ticks* (2<sup>nd</sup> edition), Volume 1, Oxford University Press, Oxford, New York, etc, pp. 59 – 73.
- Bassal, T.T.M. and Hefnawy, T. (1972). Biochemical and physiological studies of certain ticks (Ixodoidea). The effect of unfed female weight on feeding and oviposition of *Hyalomma (G) dromedarii* Koch (Ixodidae). *J. Parasitol.* 58, 984-988.
- Dillinger, S. G. C., and Kessel, A. B., (2002). Changes in the structure of the cuticle of *Ixodes ricinus* L. 1758 (Acari, Ixodidae) during feeding. *Arth. Struct. & Develop.* **31**, 95-101.
- Ferry, J. D. (1980). *Viscoelastic Properties of Polymers*, New York: John Wiley & Sons.
- Flynn, P.C. and Kaufman, W.R. (2011). Female ixodid ticks grow endocuticle during the rapid phase of engorgement. *Exp. Appl. Acarol.* **53**,167-178.
- Gillott, Cedric (2005). *Entomology* (3<sup>rd</sup> Edition), Springer, Dordrecht, The Netherlands.
- Hackman, R. H. (1975). Expanding abdominal cuticle in *Rhodnius* and *Boophilus*. *J. Insect Physiol.*, **21**, 1613-1623.
- Hackman, R.H. (1982). Structure and function in tick cuticle. *Ann. Rev. Entomol.* **27**, 75-95.
- Hackman, R.H. and Goldberg, M. (1987). Comparative study of some expanding arthropod cuticles: the relation between composition, structure and function. *J. Insect Physiol.* **33** 1, 39-50.
- Kaufman, W.R. (2010) Ticks: physiological aspects with implications for pathogen transmission *Ticks and Tick-Borne Diseases*. Ticks and Tick-borne Diseases 1, 11–22.
- Kaufman, W.R. (2014) Integument and Ecdysis. Chapter 5 in *Biology of Ticks*, 2<sup>nd</sup> Edition, Volume 1. Daniel E. Sonenshine and Michael Roe (Eds). Oxford University Press, Oxford, New York, pp. 99 – 121.
- Kaufman, W.R., Flynn, P.C., and Reynolds, S.E. (2010). Cuticular plasticization in the tick, *Amblyomma hebraeum* (Acari: Ixodidae): possible roles of monoamines and cuticular pH. *J. Exp. Biol.* 213, 2820-2831

- Maddrell, S.H.P. (1964). Excretion in the blood-sucking bug, *Rhodnius prolixus* Stal II. The normal course of diuresis and the effect of temperature. *J. Exp. Biol* **41**, 163-176.
- Maddrell, S.H.P. (1966). Nervous control of the mechanical properties of the abdominal wall at feeding in *Rhodnius*. *J. Exp. Biol* **44**, 59-68.
- Reynolds, S.E. (1974). Pharmacological induction of plasticization in the abdominal cuticle of *Rhodnius*. *J. Exp. Biol* **61**, 706-718.
- Reynolds, S.E. (1975). The mechanism of plasticization of the abdominal cuticle in *Rhodnius*. *J. Exp. Biol* **62**, 81-98.
- Tschoegl, N. W. (1989). *The Phenomenological Theory of Linear Viscoelastic Behavior*, Berlin: Springer-Verlag.
- Vincent, J. F. V. (1990). *Structural Biomechanics Rev. ed.*, Princeton, NJ: Princeton Univ. Press.
- Vogel, S. (2003). *Comparative Biomechanics: life's physical world*, Princeton, NJ: Princeton Univ. Press.

## Figures

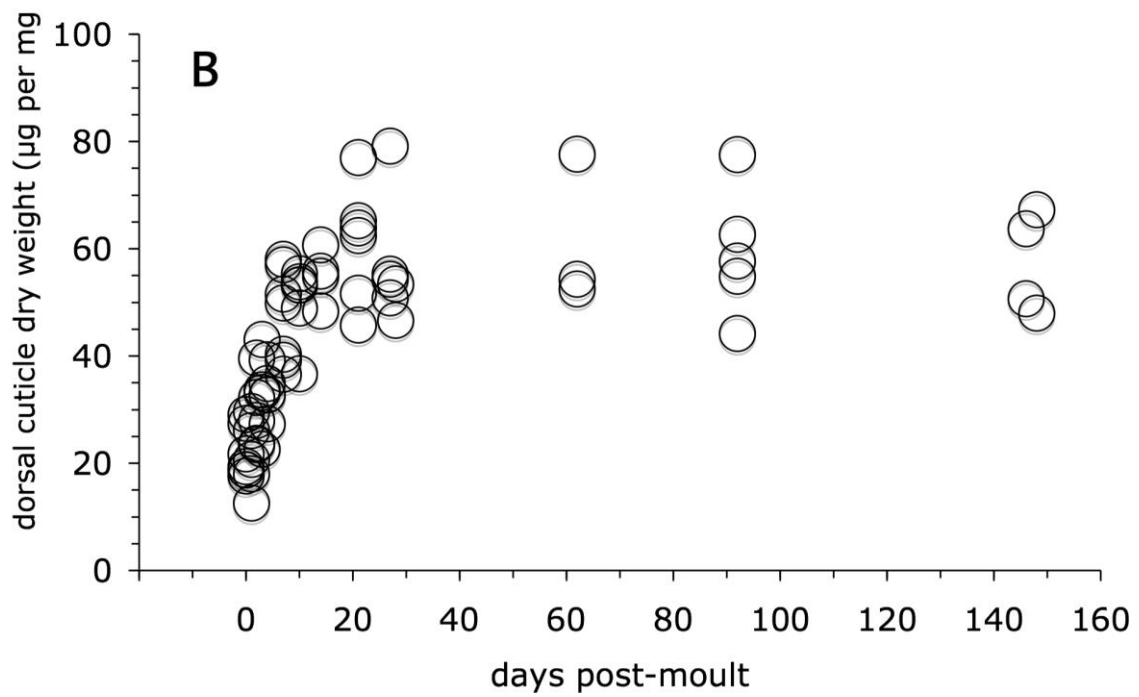
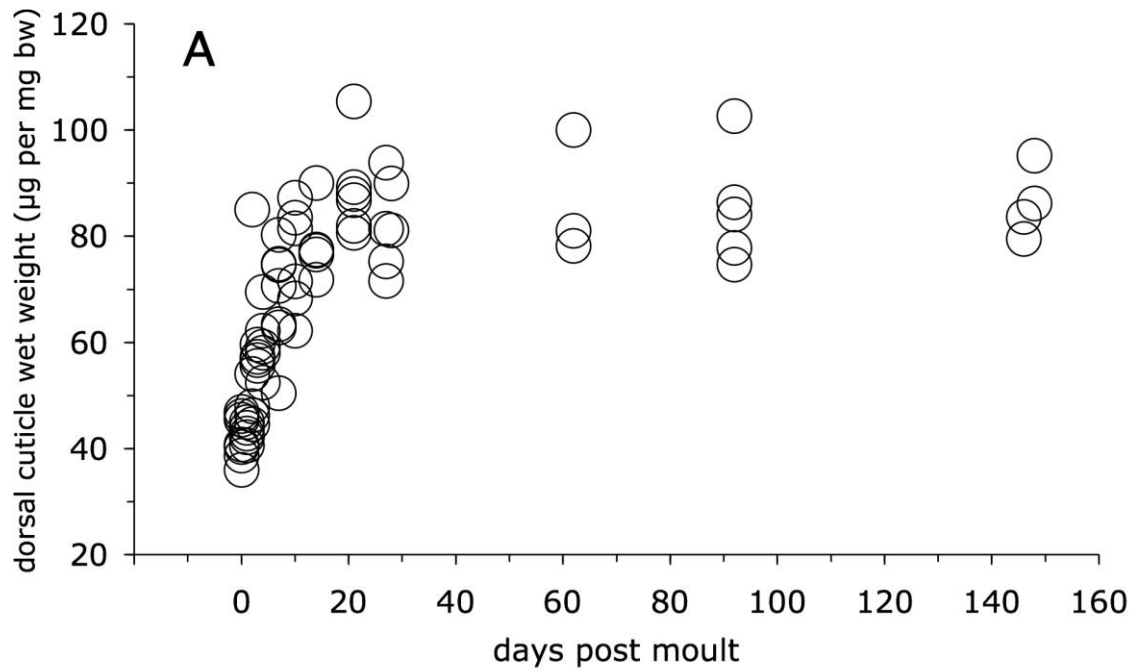


Fig 1. **Wet weight (A) and dry weight (B) of the dorsal cuticle for unfed ticks as a function of days post moulting.** Mean wet weight doubled, and mean dry weight almost tripled over the first 3 weeks post moult. There was no further significant increase in either measure between 21 and 150 days post moult.

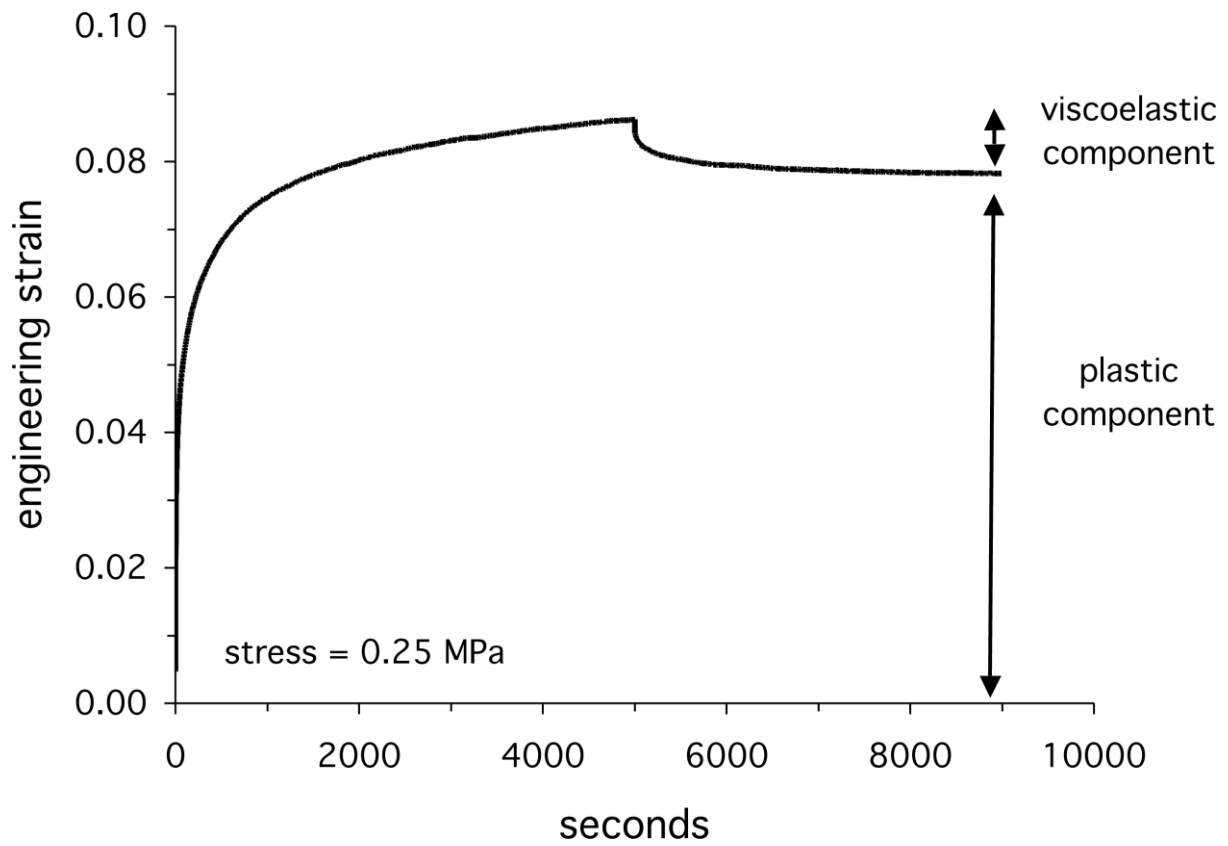


Fig. 2. **Stretch and recoil of an unfed tick.** 5 g (0.159 MPa) was added at time 0 to the loop which had been set up 18 seconds earlier with 2.8 g (0.089 MPa) for tautness. At ~5000 s, the 5 g was removed.

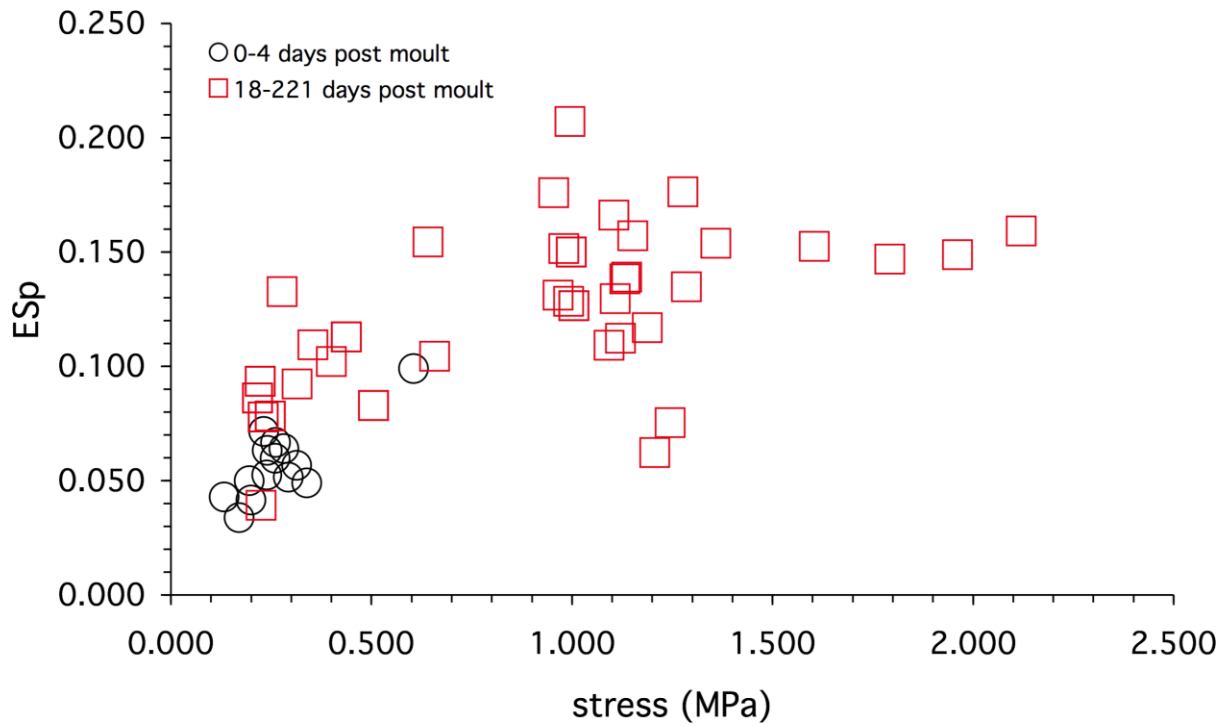


Fig. 3. **Viscoelastic component of engineering strain (ESv), for unfed ticks, as a function of applied stress to a maximum of 1.2 MPa.** Data are from individual creep tests. Young's Modulus is calculated from the slope through the intercept. For ticks 0-4 days post-moult,  $Y = 0.0541X$ , and for ticks more than 18 days post-moult,  $Y = 0.0372X$ .



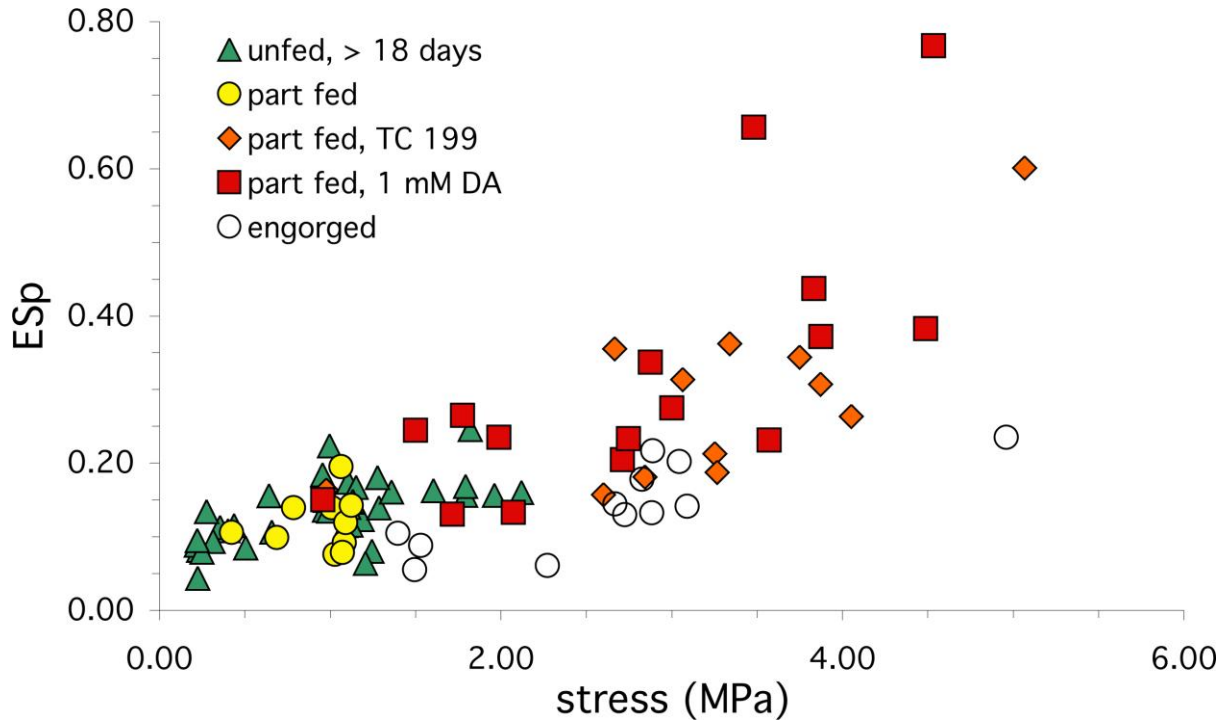


Fig 4. **Plastic component of engineering strain (ESp) for unfed ticks as a function of applied stress.** Data are from individual creep tests. ESp is a linear function of stress up to about 0.6 MPa; thereafter, ESp is independent of applied stress up to a stress of in excess of 2.2 MPa

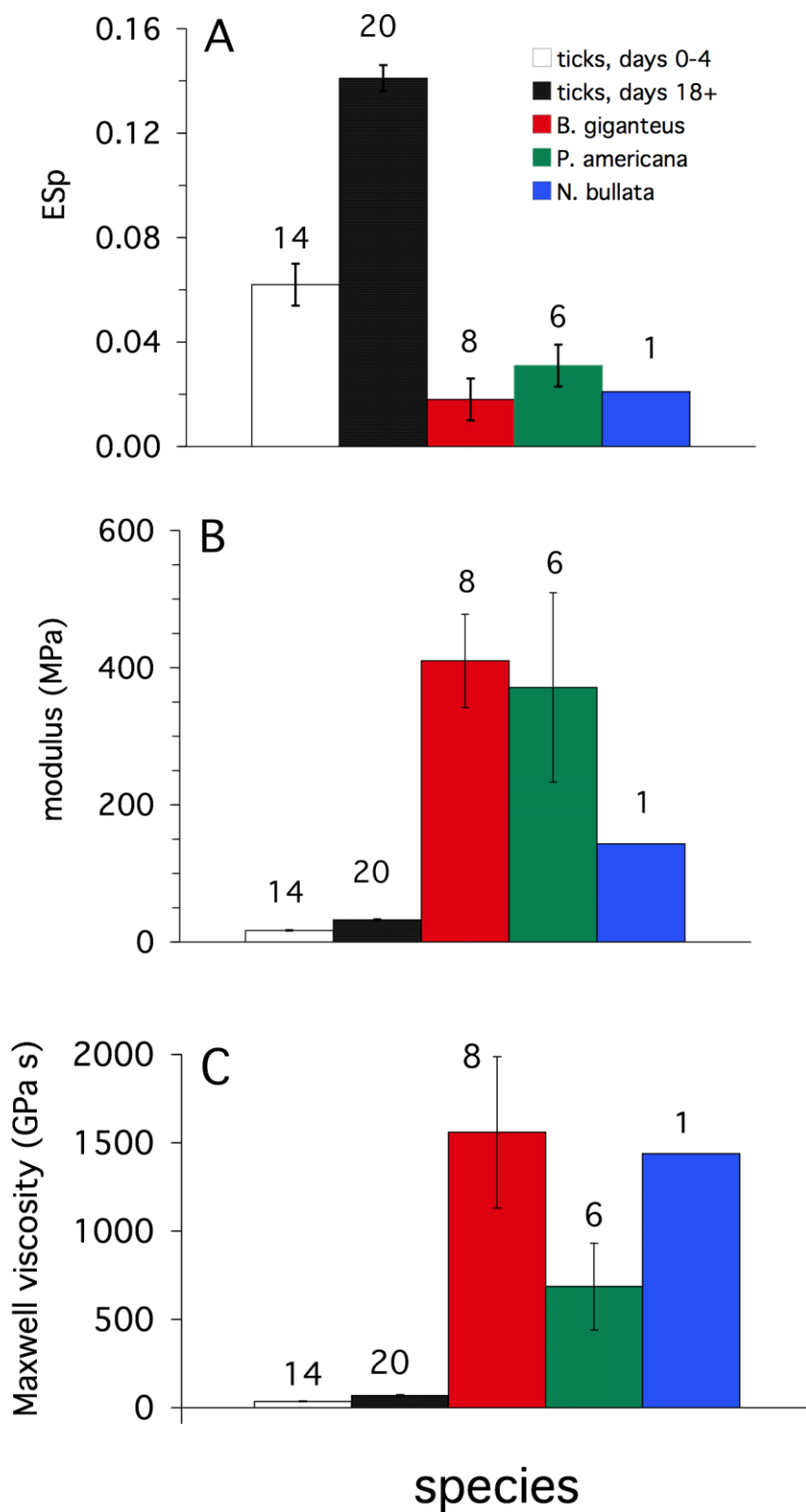


Fig. 5. ES<sub>p</sub> as a function of stress plotted for various stages of the feeding cycle, and for ticks treated with DA or with the TC 199 vehicle. Data are from individual creep

tests. In general, ESp increases above ~2.5 MPa; DA appears to substantially enhance ESp above the level for the TC 199 vehicle, but only at stresses above ~2.5 MPa. Note that thinning of the cuticle (assumed to be isochoric) requires an ESp >0.4 in two orthogonal directions during the rapid phase of engorgement.



Fig. 6. **Mechanical properties of newly moulted unfed ticks (days 0 - 4 post-moult), unfed ticks 18+ days post-moult, and three insects prior to sclerotization.** The two cockroaches (*A. americana* and *B. giganteus*) were taken on the day of ecdysis, and the fleshfly (*N. bullata*) was dissected out of the puparium just before ecdysis to the adult. (A) ESp, (B) Young's modulus, (C) Maxwell viscosity at  $1/3 \tau_1$  (the inverse of the slope of compliance vs. time). The differences between the ticks and the insects were substantial for all measures.

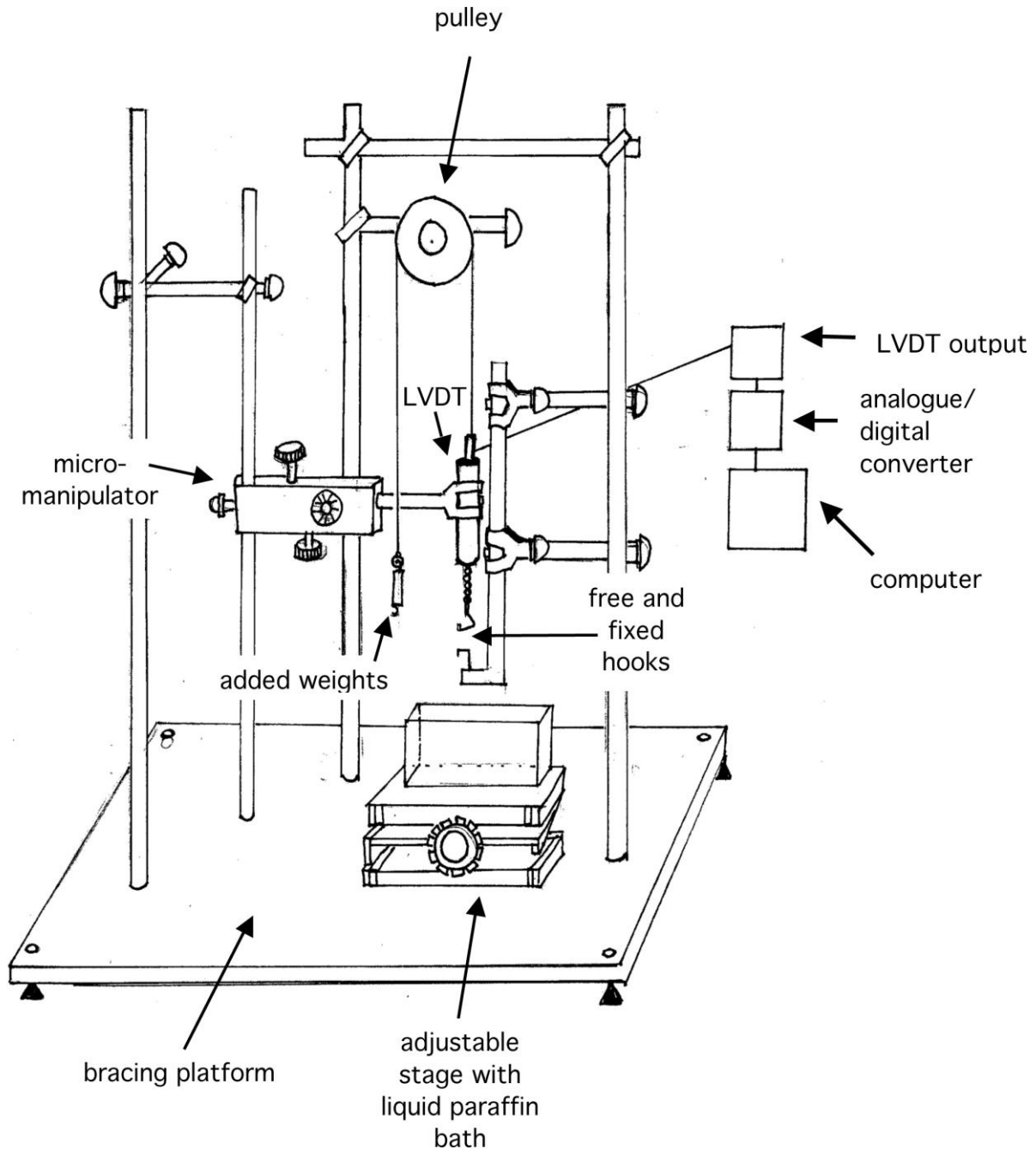


Fig. 7. **The pathway of the cut used to sample the dorsal alloscutum is indicated by the white dashed curve superimposed on an unfed female tick.** The contour of the cut follows along the lateral demarcation between the dorsal and ventral alloscutal surfaces, and then follows the V-shaped dorsal margin 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.

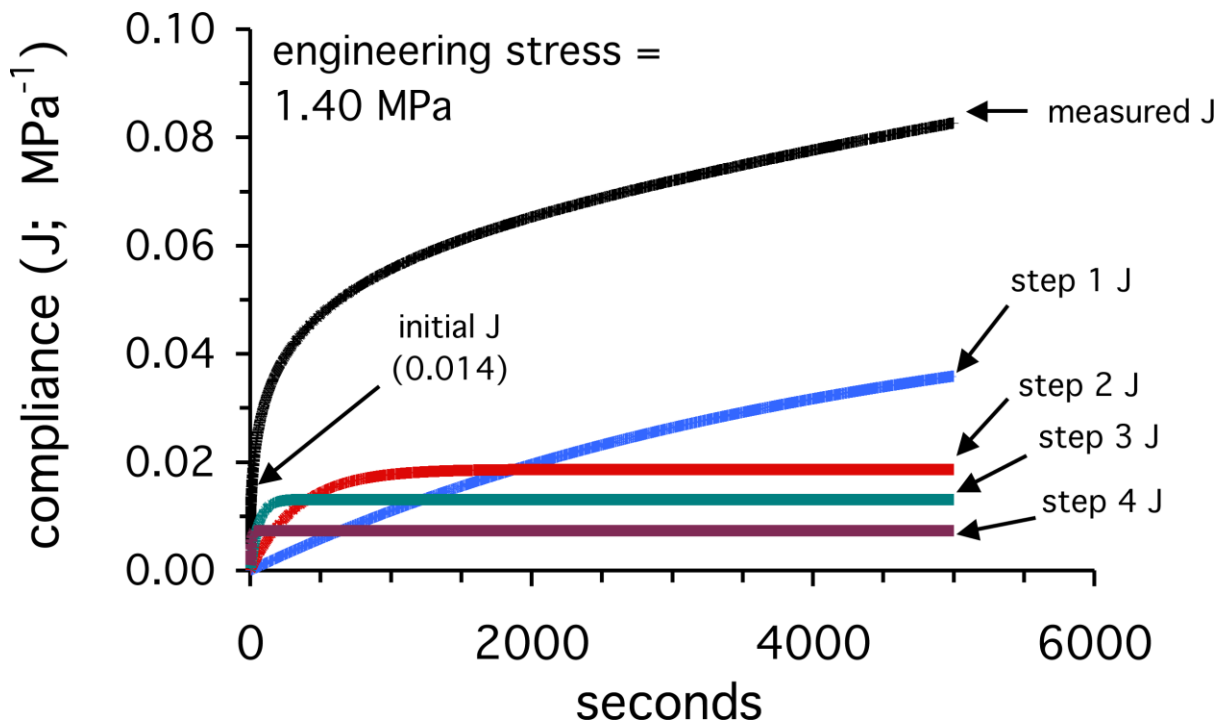


Fig. 8. **Mounting frame and instrumentation for fixed stress testing of compliance.** Apparatus stretch, small relative to loop stretch, is first measured by loading weights with the two hooks connected; this is subtracted from the subsequent measured loop stretch. The loop is then placed across the hooks and the mineral oil bath is raised to cover the loop to prevent loss of moisture during the stretch.

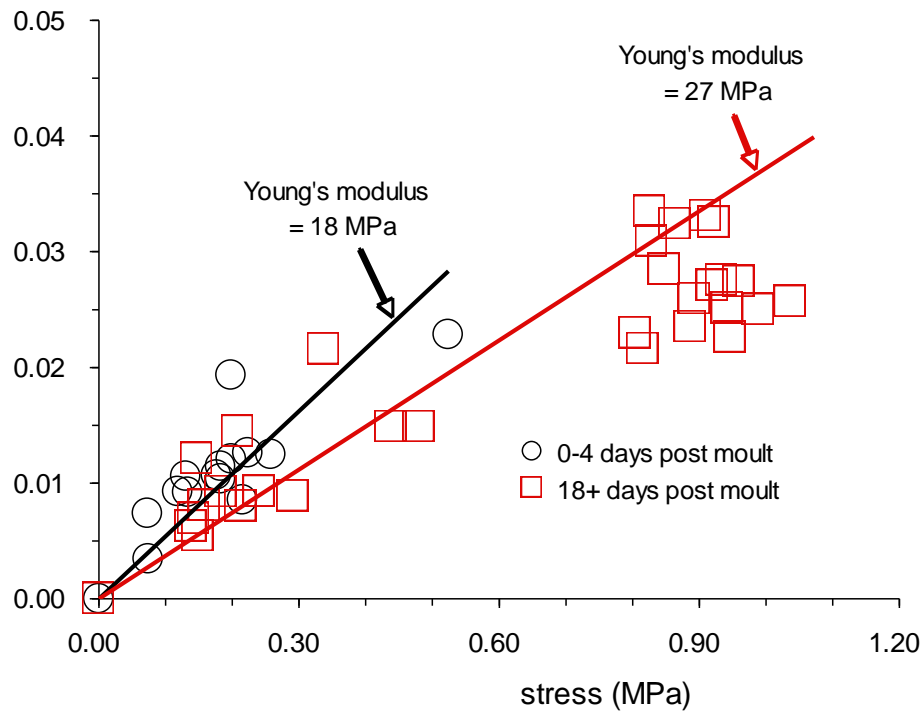


Fig. 9. **A Kelvin-Voigt analysis of compliance from stretch of tick cuticle from an engorged tick.** The analysis models total compliance (strain per unit stress, units  $\text{MPa}^{-1}$ ) as four exponential stretch elements and an instantaneous initial stretch.

## Tables

Table 1. ES<sub>p</sub> values for various treatments and stress ranges.

Treatment	Stress range, MPa	ES <sub>p</sub> +/- SEM (n)
Unfed (18-221 d post-moult)	0.6 to 2.1	0.150 ± 0.007 (29)
Partially fed	0.6 to 1.1	0.120 ± 0.013 (9)*
Engorged	1.4 to 2.3	0.077 ± 0.012 (4)**
Engorged	2.6 to 5	0.177 ± 0.014 (9)
Partially fed, TC 199	2.6 to 4.1	0.269 ± 0.025 (10)***
Partially fed, 1 mM DA	1.0 to 2.1	0.193 ± 0.025 (6)
Partially fed, 1 mM DA	2.7 to 4.5	0.424 ± 0.064 (11)

\* Difference between partially fed 0.6 to 1.1 MPa and partially fed 1 mM DA, 1.0 to 2.1 MPa was significant (p = 0.014).

\*\* Difference between engorged < 2.5 MPa stress and engorged >2.6 MPa was highly significant (p = 0.001).

\*\*\* Difference between partially fed TC 199 and 1mM DA in the range 2.6 to 4.5 MPa was significant (p = 0.041).



Table 2. ES<sub>p</sub> over three successive stretch/recoil cycles for a population of unfed and partially fed ticks.  
The stress range was 0.65 – 1.8 MPa.

	ES <sub>p</sub>		
	Cycle 1	Cycle 2	Cycle 3
Mean	0.144	0.041	0.020
SEM	0.023	0.009	0.009
N	6	6	2

The fall in ES<sub>p</sub> during the 2<sup>nd</sup> cycle was highly significant ( $p = 0.002$ ).  
The difference in ES<sub>p</sub> between the 2<sup>nd</sup> and 3<sup>rd</sup> cycles was not significant ( $p = 0.252$ ).