

RESEARCH ARTICLE

Age-related responses to injury and repair in insect cuticle

Maeve O'Neill*, Diego DeLandro and David Taylor

ABSTRACT

We evaluated the ability of female adult desert locusts (*Schistocerca gregaria*) to repair injuries to their exoskeletons and restore mechanical strength over the course of their natural life. We discovered that younger insects are more capable of repairing injuries, displaying no significant decreases in failure strength, stiffness or bending moment to failure after 3 weeks of repair. Older insects, in contrast, were only capable of repairing to ~70% of their original strength. Both older and younger insects carry out targeted deposition to repair injuries. We also examined different mechanisms of failure, and we discovered that the cuticle of older insects is more susceptible to crack growth due to a large decrease in fracture toughness with age, making them more sensitive to scalpel cuts and punctures. The biological mechanisms that drive these changes are still under investigation.

KEY WORDS: Repair, Arthropod, Biomechanics

INTRODUCTION

The mechanical properties of mammalian tissue such as bone and cartilage, along with how they repair themselves have been well studied (Frost, 1994; Cowin, 1986; Woo et al., 1981), and are understood to a significant extent. In contrast, the mechanical properties of cuticle remain poorly studied, leaving a lot of scope for further investigation. Cuticle is one of the most abundant biological materials. It makes up the skeleton of all arthropods and boasts a wide range of properties and functions even within a single insect. Cuticle is composed of chitin fibres embedded in a protein matrix (Wigglesworth, 1951; Vincent and Wegst, 2004). It is a hierarchical material composed of nanofibrils of chitin crystallites (Vincent and Wegst, 2004). These are coated with proteins, creating chitin-protein fibrils (Fabritious et al., 2011). These further assemble into large fibres and orient themselves in a 'Bouligand structure' (Bouligand, 1972), resembling plywood. The outermost layer is a waxy epicuticle (Wigglesworth, 1951), similar in function to the stratum corneum found in mammals, both of which serve as protection from desiccation. The layer below it, the procuticle, is acellular and is the main determinant of material properties of the cuticle. The mechanical properties of the cuticle are strongly affected by the extent of sclerotization (Anderson, 2010), the process that occurs after ecdysis in which the soft cuticle required for the insect to swell and grow in size becomes more rigid (Chapman, 1969). A couple of days after moulting, the proteins distributed throughout the cuticle matrix cross-link, stiffening the cuticle considerably (Chapman, 1969). The extent of this process

will depend on the final function of the cuticle; for example, the abdominal cuticle of *Rhodnius*, which needs to expand quite dramatically, will remain relatively soft with little cross-linking (60 MPa) (Reynolds, 1975), in contrast to the tibial flexor apodeme of the locust, where the stiffness has been measured to be 20 GPa (parallel to chitin orientation) (Vincent and Wegst, 2004). The tibia of the locust lies somewhere between these two (Dirks and Taylor, 2012).

Recently, there has been a growing interest in cuticle, to obtain a better understanding of the biomechanics of insects and other arthropods, and also for biomimetic applications. However, most previous studies only examined cuticle in the first few days of adult life (Vincent and Wegst, 2004; Hepburn and Joffe, 1974; Weis-Fogh and Jensen, 1962). The natural lifespan of insects is highly species dependent and that of locusts is estimated to be 4–5 months from eclosion for females (Schmidt and Albutz, 1994). Considering the well-known changes in properties observed in mammalian tissue, such as bone, with age, it is reasonable to consider whether cuticle experiences changes in its properties over the course of the insect's life. However, there have been very few studies investigating this question. Previous research has found that the adhesive pads of insects lose their flexibility and become less compliant as they age, reducing the ability of the insect to adhere (Ritzmann and Ridgel, 2005). Additionally, a reduction in the locomotive performance of insects as they age has also been observed (Zhou et al., 2015). To the best of our knowledge, there has only been one systematic study, carried out by Parle and Taylor (2017), directly examining the changes in mechanical properties in older insects. They found the mechanical properties of the cuticle vary as the insect ages. They discovered that Young's modulus and failure strength for older insects is much greater than that for younger insects, although the mechanism governing these changes remains unidentified. The present work serves to expand on this, and examine how response to injuries, and ability to repair them, may vary as insects age.

Parle et al. (2016) showed that insects are able not only to close wounds to prevent infection, which was previously known (Dushay, 2009; Hoffmann et al., 1999; Li et al., 2002), but also to restore mechanical strength. Scalpel cuts were found to have been repaired by targeted deposition, creating an internal patch – this mechanism is distinctly different from that used to repair mammalian bones (Taylor et al., 2007).

However, the study by Parle et al. (2016) was limited because it only examined the repair capabilities of insects at one age – 30 days – and following one type of injury – a scalpel cut – a neat precise injury. The fitness for survival of an insect is strongly related to its ability to withstand injury. Exoskeletal injuries may affect important locomotory functions, so an insect's survival will be dependent on the degree to which an injury reduces the strength of a body part such as the hind tibiae, and on its ability to repair the damage in a reasonable time. This is particularly relevant for long-lived insects, such as locusts, which can take several months through numerous moults to reach sexual maturity.

The aims of this study were to answer the following questions: how does injury affect the strength and viability of the exoskeleton

Trinity Centre for Bioengineering, Trinity College Dublin, College Green, Dublin 2, Ireland.

*Author for correspondence (oneilm10@tcd.ie)

 M.O., 0000-0003-0708-1125

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in insects of different ages?; is the ability to repair injury affected by age?; and how do insects respond to different types of injuries?

MATERIALS AND METHODS

We acquired female desert locusts (*Schistocerca gregaria* Forsskål 1775) as fifth instar nymphs. They were raised on a controlled 12 h:12 h day:night schedule with corresponding temperatures of 32:20°C, and fed fresh greens *ad libitum*. As the insects underwent their final moult into adulthood, they were separated based on the date of their final moult so that the exact age of the adult insects could be known. For all experiments, the hind tibiae were removed just below the femorotibial joint under sedation by cooling. Samples were tested fresh, within 15 min of excision, to prevent desiccation effects, which alter mechanical properties (Dirks and Taylor, 2012).

Subjects were tested at different ages (i.e. different times since the final moult), referred to as either ‘young’ or ‘old’ depending on whether they were tested before or after 3 weeks of age, because previous work showed important changes in cuticle at this time point, due to the sclerotization that occurs post-moult in adult locusts (Parle and Taylor, 2017).

We examined two different injuries in this investigation, both on the dorsal side of the hind tibia: a scalpel injury and a puncture. For the first type of injury, a transverse cut was made by drawing a scalpel blade across the dorsal side of the hind tibia of a sedated animal at either 1 or 12 weeks of age, and then left to repair for a subsequent 3 weeks. We used a specially designed cutting rig (Parle et al., 2016) to ensure the cut was always of the same size (average length $L=675\ \mu\text{m}$, ~23% of the total circumference); such injuries were regarded as severe but not serious enough for the animal to lose the limb immediately. A repaired injury can be seen in Fig. 1B.

For the second type of injury, we made a hole in the hind tibia of a sedated animal at either 2 or 8 weeks of age using acupuncture needles, chosen for their ease of availability and because they are provided pre-sterilized. We acquired stainless steel MAC acupuncture needles of three sizes, 0.3 mm and 0.22 mm diameter (TCM Supply, Dublin, Ireland) and 0.12 mm diameter (The Acupuncture Supply Co., Monfin, Enniscorthy, Ireland). We pushed the needle into the

dorsal side of the hind tibia, between the spines, ensuring not to pierce the ventral side of the hind tibia. A repaired needle injury and a depiction of the procedure can be seen in Fig. 1A,C. Tibia were placed inside the same rig used for the scalpel injuries, ensuring that the injury was always in approximately the same location for all the animals, approximately 5 mm from the tibiofemoral joint.

We tested some subjects immediately after injury to measure the effect of the cut or puncture on mechanical properties. Other subjects were kept alive to allow time for repair, in individual 20×10×10 cm tubs. This was the best compromise to prevent fighting amongst individuals that could lead to loss of the limb, yet still allowing full mobility and movement. They were raised in the same conditions as above for the desired length of time. Control animals were raised together in larger tanks.

We kept all individual tubs stacked and alongside other tubs to ensure the animals could see and smell each other to prevent them turning into the solitary form. The locations were rotated frequently to ensure all animals spent most of their time surrounded by others. No animals showed any indications of becoming solitary, interacting normally with other animals if reintroduced into communal tanks. The number of insects used in each testing procedure and the time allowed for recovery are outlined in Table 1.

The injured and intact hind tibia of each insect were tested in immediate succession to allow for paired comparison. To account for the possibility of the intact limb becoming stronger to compensate for the injured one, several age-matched controls were tested as well. For insects of all ages, no significant difference was found between the intact legs of injured insects and samples taken from the control insects, so they were treated as a single group in the analysis.

Test procedure

The test procedure was carried out using a protocol previously developed by Taylor and Dirk (2012) and Dirks et al. (2013). Immediately after removal, legs were set into a block containing a well of quick-drying polymer cement (see Fig. 2B), to prevent dehydration. We performed a cantilever bending test by applying a position-controlled displacement near the distal end of the sample, until failure,

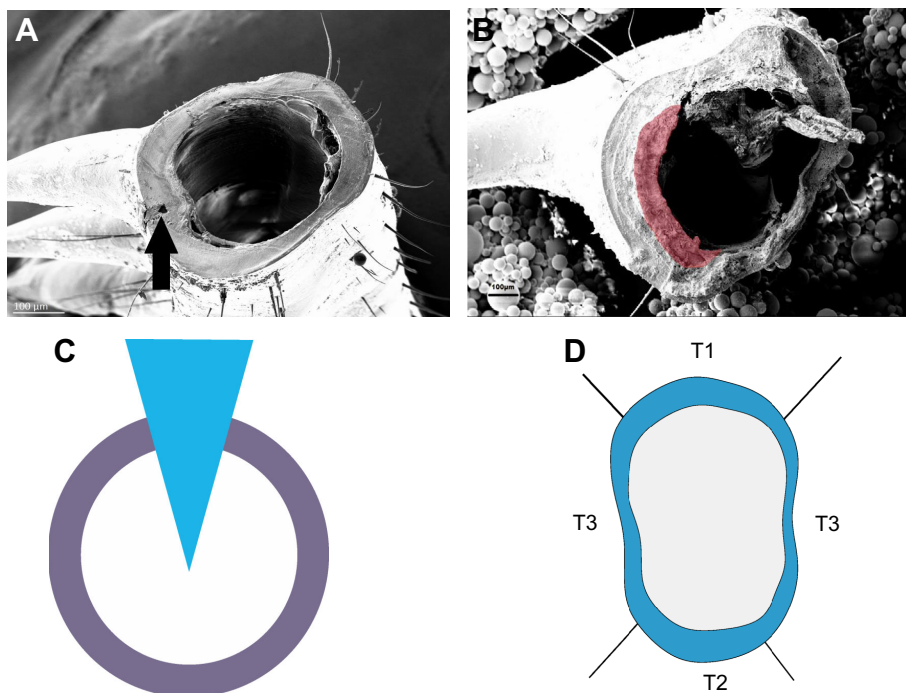


Fig. 1. Injury sites on the hind tibia. (A) Scanning electron microscopy (SEM) image of a recovered puncture injury. The arrow indicates the injury, still present as a small hole in the cuticle. A thick layer of repaired cuticle is evident behind the injury. (B) A repaired scalpel injury from a sample injured at 12 weeks and allowed to repair for 3 further weeks. The red shading indicates the fresh cuticle laid down underneath the scalpel injury, evident as a fracture surface, whereas the scalpel injury can be seen as a smoother surface. (C) A depiction of the needle entering the hind tibia. It pierces the dorsal side fully (including the epidermal layer), without coming into contact with the ventral side. (D) Schematic drawing of an insect's hind tibia. T1, T2 and T3 indicate the dorsal, ventral and medial/lateral sides, respectively, where cuticle thickness was measured.

Table 1. Experimental time line

Injury	Young	Old
Scalpel	1 week	12 weeks
3 weeks repair	35	23
Age-matched controls	10	6
Puncture (initial test)	2 weeks	8 weeks
Large (0.3 mm diameter)	10	12
Medium (0.22 mm diameter)	–	3
Small (0.12 mm diameter)	–	4
Puncture (repair)	2 weeks	8 weeks
Large (0.3 mm diameter) 2 weeks	–	4
Large (0.3 mm diameter) 3 weeks	–	15
Medium (0.22 mm diameter)	–	5
Age-matched controls	–	6

Data indicate the number of insects, not the number of samples tested. Except for controls, in each case one hind tibia was injured and the other left intact for comparison purposes. For controls, both hind tibia were tested as uninjured samples.

using a Zwick machine (5 N load cell, Zwick/Roell Z005, Ulm, Germany). Displacement increased at a rate of 5 mm min⁻¹. Failure was characterized by a sudden drop in the load. The distance between the applied force and fixed end was recorded, before placing the samples in a 3.7% solution of formalin to preserve them. Samples were placed in ethanol after 48 h for further storage.

We prepared samples for scanning electron microscopy (SEM) by sectioning the samples before mounting and sputter coating them with an Au:Pd target. For those that broke in two or fractured, we examined their fracture surfaces. Those that did not separate, we examined as transverse sections cut with a scalpel. The dimensions of the tibia were taken from these images. Any fresh cuticle or cracks were also evident. We recorded average values for the diameter and thickness using ImageJ Fiji software (Schindelin et al., 2012). Following previous models (Dirks and Taylor, 2012), the hind tibiae were assumed to be hollow cylindrical tubes, with circular cross-sections, and thus nominal bending stresses could be calculated from the forces applied and the displacements using the following standard equations for nominal stress (Eqn 1) and nominal strain (Eqn 2):

$$\sigma = \frac{FLr}{I}, \quad (1)$$

$$\varepsilon = \frac{3dr}{L^2}, \quad (2)$$

$$I = \frac{(r^4 - (r-t)^4)\pi}{4}, \quad (3)$$

where F is the maximum force, L is the distance from the fixed end of the sample to the applied load, r is the radius of the sample, I is the second moment of area of the sample and d is the maximum displacement of the sample. We then plotted stress–strain curves, as in Fig. 2A. Young's modulus can be calculated from the initial slope where the samples act as a Hookean material with a straight line slope. The failure strength of the material was taken as the largest stress experienced by the cuticle before failure. All mechanical properties reported are for the combined material of the cuticle, not individual layers. For the uninjured control samples, the value we obtained in this way for Young's modulus gives an indication of the average value for the cuticle. When an injury is present, this parameter will tend to be reduced as a result of the increased flexibility of the sample, and is no longer a true estimate of the material's modulus; however, it is presented here to give a sense of the changing stiffness of the leg as a whole.

Fracture toughness is a parameter which measures how easily a crack grows in a material and thus its susceptibility to brittle fracture. We calculated the fracture toughness of the cuticle as in Eqn 4:

$$K_c = F_b l_b Q \frac{r}{J} \sqrt{\pi a}, \quad (4)$$

where K_c is the fracture toughness, F_b is the maximum force before failure, l_b is the bending distance between the notch and the applied load, r and I are as before, crack length is $2a$ and Q is a constant that depends on the type of loading and also upon the geometry of the sample and the notch. Approximating our sample as a hollow cylinder of constant radius and thickness (Parle et al., 2015), values of Q are attainable (Takahashi, 2002). This approach will give an accurate estimate of K_c for the samples with scalpel cuts, and a reasonable indication for those containing punctures. The value obtained for K_c after repair will not be a true measure of the toughness of the material, but provides an indication of how effective the repair has been.

We measured the thickness of the cuticle using the SEM images at three locations, denoted T1, T2 and T3. These can be observed in Fig. 1D: T1 is the dorsal side, where the injury was made, T2 is the ventral side and T3 is an average of the medial and lateral sides. We pooled the lateral and medial sides as they displayed no difference. The aim of these measurements was to investigate whether additional cuticle was deposited during the repair period and, if so, whether it was being deposited preferentially near the injury site.

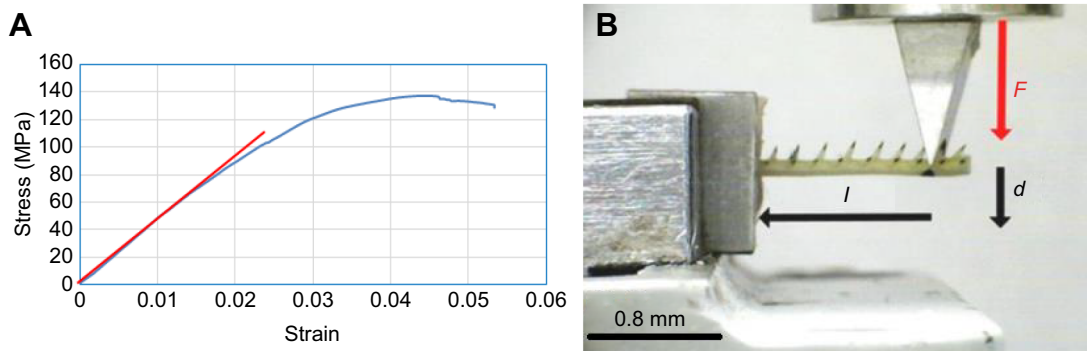


Fig. 2. A typical stress–strain curve and the mechanical testing rig used in the experiments. (A) A typical (nominal) stress–strain curve; the red line is a straight line fit to the early part of the curve, to obtain Young's modulus. F , maximum force; l , distance from the fixed end of the sample to the applied load; d , maximum displacement of the sample. (B) The set-up for the mechanical testing: the leg is implanted at its proximal end and the positioning of the pointer can be observed.

Statistical analysis

Statistical analysis (ANOVA, *t*-tests and *post hoc* Fisher's LSD) was carried out using Excel 2013 and Minitab 2018. We tested all data for normality by the Ryan–Joiner test. We found normal distributions for all datasets except one: the young insects receiving puncture injuries. In this case, we found no significant difference anyway. All values are reported as means±s.d. unless otherwise stated. We performed all tests for a 95% confidence level unless otherwise stated.

RESULTS

Our group has previously reported on the change in mechanical properties of adult locusts with age (Parle and Taylor, 2017). We found that strength and Young's modulus of the hind tibia increased sharply during the first 21 days, after which these properties

remained almost unchanged up to an age of 63 days. Fig. 3A presents a re-analysis of the data from Parle and Taylor (2017). By finding the mean of the data at each time point and constructing best-fit lines, we found that the data showed a rapid increase (increasing from ~50 MPa to ~150 MPa) in strength over the first 3 weeks, with little change thereafter. We constructed a scatter band around these lines representing 1 s.d. of the data from the mean. This exercise is useful for the present paper because it provides a description of normal behaviour against which the effects of injury and repair can be compared.

Fig. 3B shows failure strength results from the present study, reproducing the scatter band from Fig. 3A for comparison. Some additional data from previous work (Dirks and Taylor, 2012; Parle and Taylor, 2017) are also included in this figure and will be discussed below.

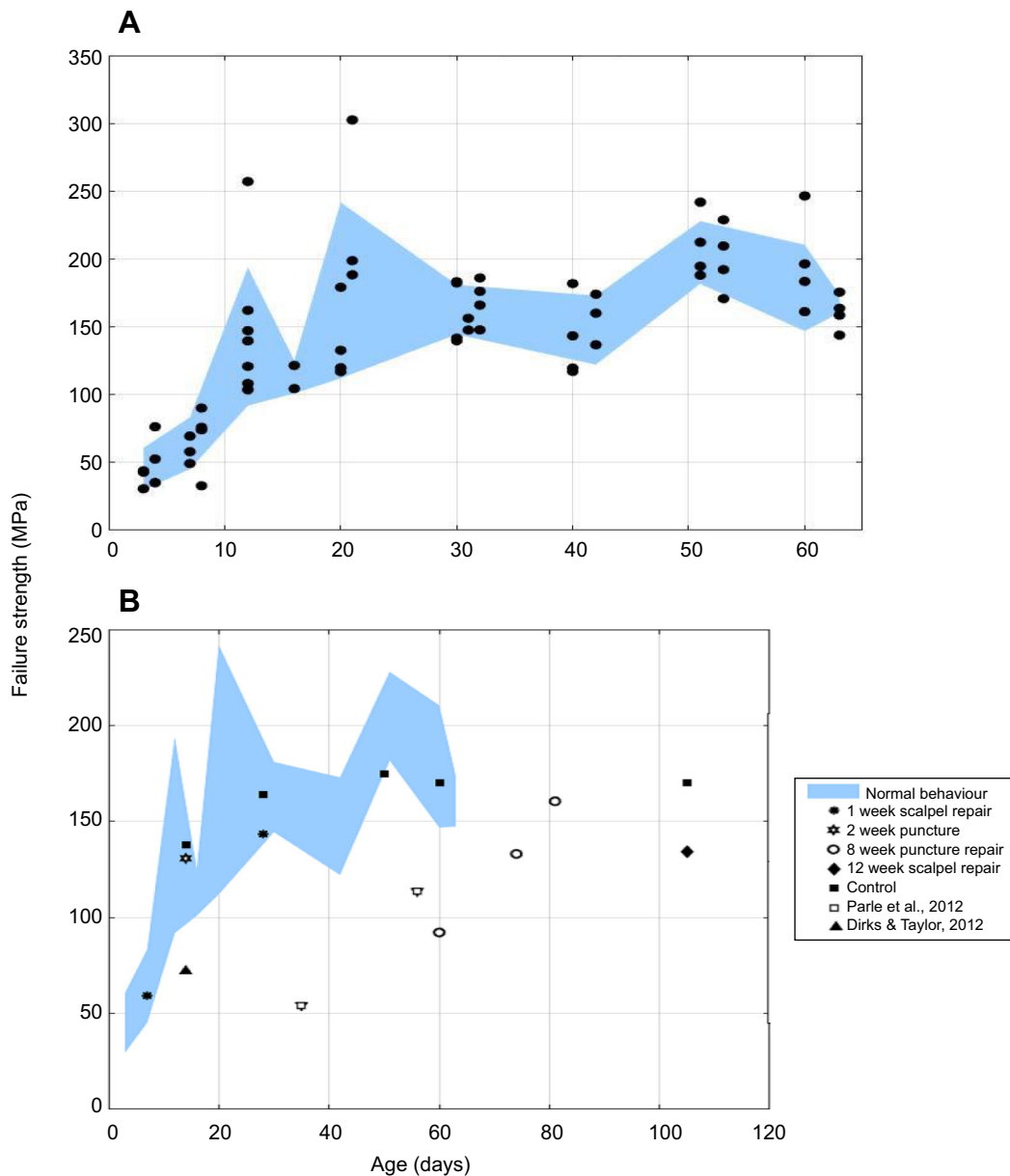


Fig. 3. Experimental data showing cuticle strength as a function of age, from the present study and previous work. (A) Re-analysis of data from Parle and Taylor (2017). The blue scatter band covers ± 1 s.d. of the data. (B) Changes in mechanical properties with age, injury and repair. The highlighted area represents previous findings.

Table 2. Results for insects that received scalpel cuts across the dorsal side of the hind tibia at either 1 week (young) or 12 weeks (old) and were then left to repair for 3 weeks

Scalpel injury	Young			Old		
	σ (MPa)	E (GPa)	M (N mm)	σ (MPa)	E (GPa)	M (N mm)
Control	158.86±47.32	5.38±2.57	8.85±2.38	171.55±27.08	3.38±1.06	9.28±1.50
Injured	142.29±30.49	3.64±2.02	9.55±1.99	137.26±35.62	2.739±1.49	7.82±2.01

Values for measured failure strength (σ), Young's modulus (E) and bending moment to failure (M) are compared with those for uninjured control samples. Data are means±s.d.

Response to scalpel injury

There was a slight decrease in strength after repair for the young insects, but this was not significant ($P=0.11$) (Table 2). The bending moment to failure also indicated that full repair had occurred, with no statistically significant difference between the injured and uninjured (control) groups ($P=0.33$). The decrease in Young's modulus was statistically significant ($P=0.04$), showing that the limbs were more flexible after repair.

By contrast, the repaired hind tibia of the old insects (those injured at 12 weeks) had significantly lower values of failure strength, bending moment to failure and Young's modulus (Table 2). The failure strength for the older insects was on average ~70% that of controls of the same age. We observed similar decreases for Young's modulus and bending moment to failure. Paired t -tests showed that the decrease for failure strength, Young's modulus and bending moment to failure were all statistically significant ($P<0.05$ for all 3).

Injuries seem to affect older insects more than their younger counterparts (Parle et al., 2016). We observed a similar trend for young insects injured with a scalpel 7 days after their final moult; their failure strength of 59.6 MPa is comparable to values in the literature for intact cuticle of insects of a similar age (Taylor and Dirk, 2012).

Response to needle puncture injury

It is evident from Table 3 that the needle injuries had no significant effect on younger insects, those injured 2 weeks after their final moult, even without any time to repair. We found that failure strength, Young's modulus and bending moment were almost the same for injured and uninjured (control) hind tibiae. Paired t -tests showed no significant difference between the samples ($P>0.05$).

However, we found older insects suffered a significant reduction in their failure strength after injury, down to ~53% of their original strength, when tested immediately after injury. When allowed to repair the cuticle, significant recovery was observed after 2 weeks. At this point, the cuticle had been restored to ~76% of its intact failure strength. After 3 weeks, the repair was relatively complete, with the failure strength being ~92% that of the controls. The bending moment at failure showed a similar restoration, as did Young's modulus. Paired t -tests showed a slight decrease in the failure strength between uninjured cuticle and cuticle that had repaired itself for 2 weeks ($P<0.05$). However, after 3 weeks, the two groups were not statistically different ($P>0.05$).

Microscopy

Fig. 4B,D shows that the damage done to the older cuticle is evidently more severe than just the hole made by the needle. We can see in Fig. 4B that the injury is quite long, with a crack close to 500 μm in length running away from the injury in both directions, along the length of the leg. Fig. 4D is a close-up of a similar injury; the cracking of the cuticle away from the initial injury is evident, with the injury being almost triangular in shape. However, the older insects are able to repair such injuries by the deposition of new cuticle under the injury, as in Fig. 4A,C, where new cuticle has been marked in red.

Fracture toughness

Fracture toughness of cuticle for insects injured at 14 days has previously been reported as 4.12 MPa $\text{m}^{1/2}$ (Dirks and Taylor, 2012). Insects receiving a scalpel injury at 1 week, followed by 3 weeks of repair displayed an apparent fracture toughness of 8.48 MPa $\text{m}^{1/2}$. Older insects (injured at 12 weeks) that repaired for the same length of time were only able to restore their fracture toughness to 6.09 MPa $\text{m}^{1/2}$.

We found a value of 3.98 MPa $\text{m}^{1/2}$ for insects receiving a puncture injury at ~2 weeks. However, similar to the results from the scalpel injuries, we found that older insects (8 weeks) receiving the same injury had a fracture toughness of just 2.44 MPa $\text{m}^{1/2}$. These data are presented in Fig. 5. For the younger insects, as a result of repair, very few of the samples fractured, allowing the repaired wound to be visible. Consequently, we were only able to measure the fracture toughness of two samples, so statistical comparison with other groups was not performed.

Cuticle deposition

For the young insects, ANOVA comparing the control and repaired cuticle groups indicated that the thickness was not the same across the two groups ($P<0.05$, $F=6.15$). We investigated this further using paired t -tests, which revealed a significant difference in cuticle thickness on the T1 side between the two groups ($P<0.05$). T2 thickness was also greater in the repaired group; however, this difference was not significant ($P>0.05$, $t=-1.474$). These data are presented Fig. 6B.

ANOVA was also carried out in the same way for the older samples, once again indicating a significant difference in the thickness between groups ($P<0.05$). Paired t -tests revealed a

Table 3. Results for insects that received puncture injuries on the dorsal side of the hind tibia at either 2 weeks (young) or 8 weeks (old)

Puncture injury	Young			Old		
	σ (MPa)	E (GPa)	M (N mm)	σ (MPa)	E (GPa)	M (N mm)
Controls	137.9±42	3.3±1.4	6.7±2.0	160.8±34.4	4.4±2.0	7.4±1.4
Injured (0 weeks repair)	130.8±26	3.12±1.5	6.4±1.3	92.2±41	1.9±1.1	4.1±1.8
Injured (2 weeks repair)	–	–	–	133.1±44.2	2.9±1.8	7.1±1.1
Injured (3 weeks repair)	–	–	–	160.5±24.7	5.2±2.5	7.8±1.2

Some insects were tested immediately with no time to repair and the remainder were left to repair for 2 or 3 weeks. Values for measured failure strength (σ), Young's modulus (E) and bending moment to failure (M) are compared with those for the uninjured control samples. All values are means±s.d.

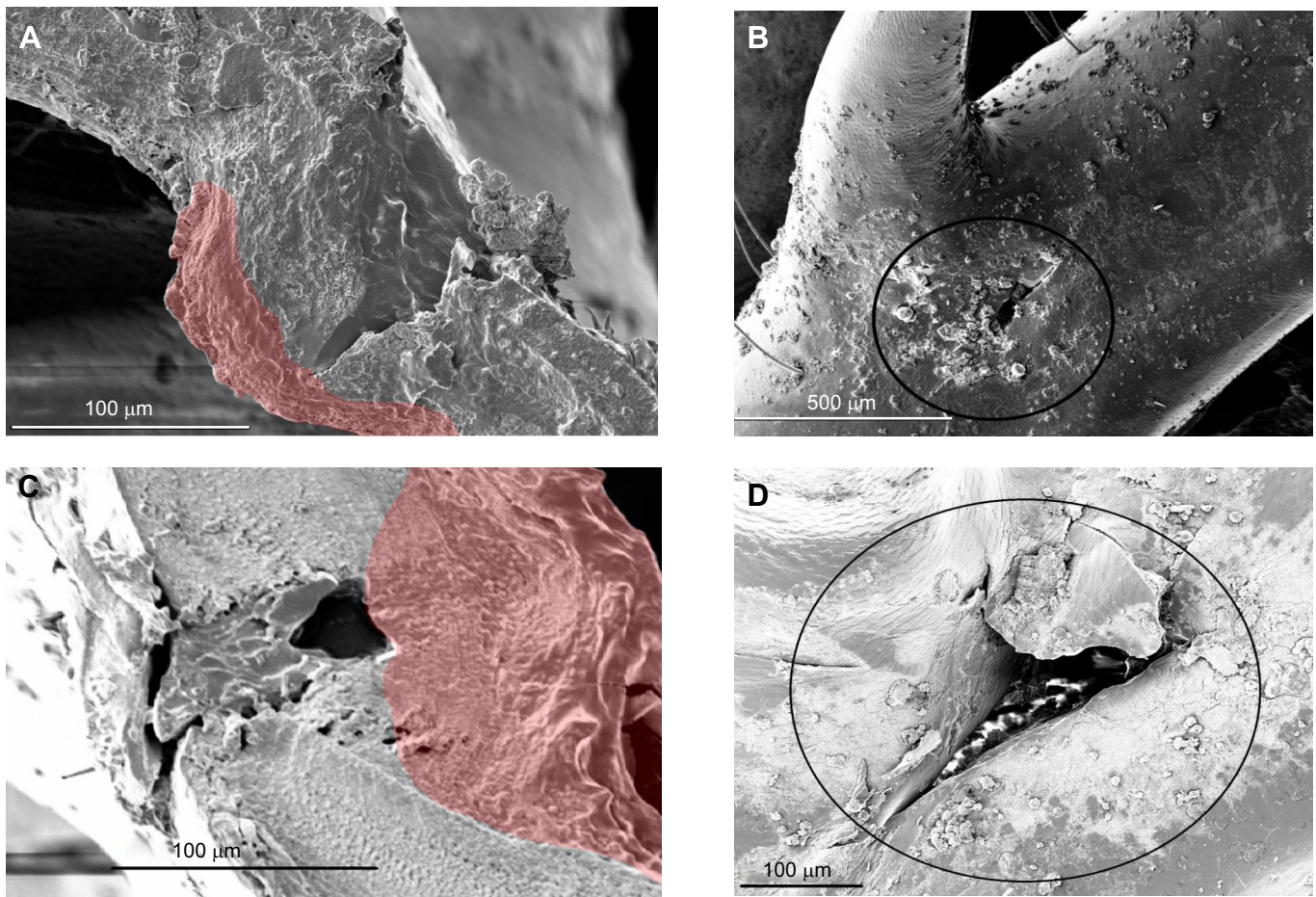


Fig. 4. SEM analysis of puncture injuries made at 8 weeks. (A) Close-up of a cross-section of a repaired puncture injury (2 weeks of repair time). Fresh cuticle has been shaded red for emphasis. (B) SEM image of a puncture injury, looking at the dorsal side of the hind tibia. The injury was made at 8 weeks and the image taken after testing, i.e. with no time for repair. The injury is marked with a circle and is evident as a dark hole with extensive cracking extending from it. (C) SEM image of a repaired puncture injury, from an insect injured at 8 weeks and left to repair for 3 subsequent weeks (a close-up of the SEM photo depicted in Fig. 1A). Fresh cuticle deposited after the injury is shaded red. (D) Close-up of a needle injury on a horizontal sample, with the same view as in B. This image was also taken without any repair time. The injury is indicated with a circle. The presence of cracks spreading out from the initial injury can be seen clearly.

significant increase in the thickness between the T1 sides in the repaired and control groups ($P < 0.05$, $t = -3.18$). We also observed an increase in the thickness of T2 between the groups, and here it

was significant ($P < 0.05$, $t = -3.16$). These data are presented in Fig. 6C. We found no differences in the thickness of the T3 side for either group ($P > 0.05$, $t = -1.2$ and $t = -1.48$ for young and old, respectively).

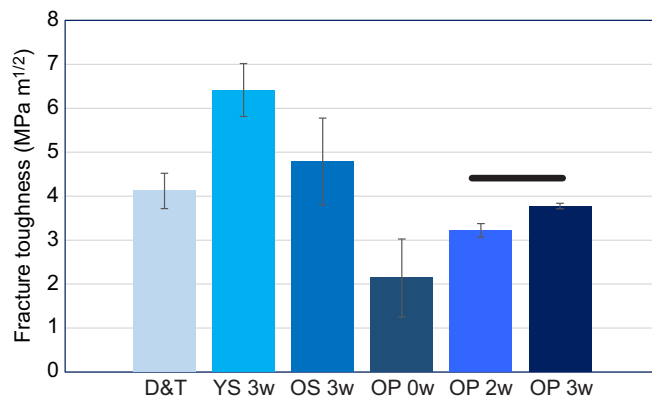


Fig. 5. Fracture toughness of cuticle tested at different ages. The value for 14 days is taken from Dirks and Taylor (2012) (D&T). YS, young scalpel; OS, old scalpel; OP, old puncture; W, weeks. Values are means \pm s.d. The line indicates the samples that are not statistically different from each other.

DISCUSSION

We have shown for the first time that young and old insects have different responses to injury. A remarkable ability to withstand damage was demonstrated by young insects: the strength of the hind tibiae was not significantly reduced even after severe injury such as a cut over 23% of the leg's diameter; almost complete restoration of strength was observed in the younger insects, achieved via cuticle deposition. By contrast, a major loss of strength is experienced by the older insects, even after relatively mild injuries. The response of these older insects was repair via targeted deposition, but they were unable to fully restore strength. The failure strengths reported here should be more than sufficient for normal activities, such as jumping, for which the applied stress has been estimated to be 42 MPa (Bennet-Clark, 1975; Taylor and Dirk, 2012). Complete repair of the exoskeleton was not performed by either group ('scarring' was still evident at the wound site on the hind tibia) but the repairs appear to be sufficient for their continued survival.

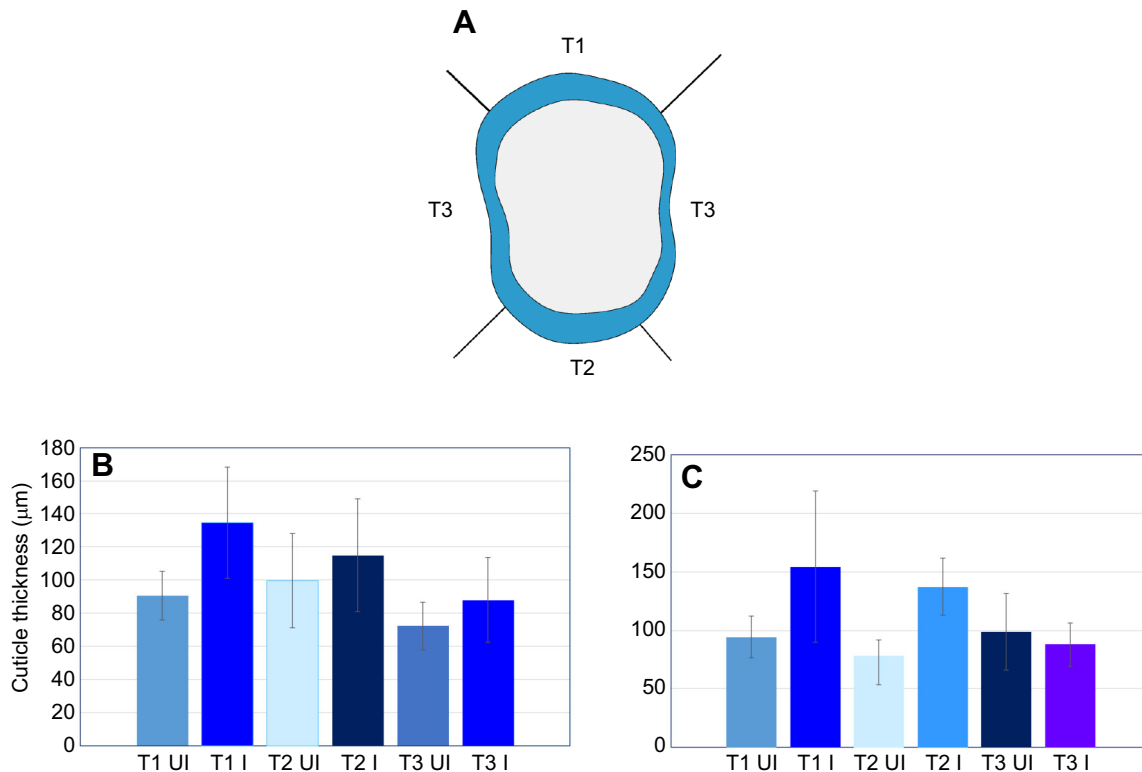


Fig. 6. Thickness values and locations for young and old insects. (A) The relevant sides of the hind tibia to aid understanding. (B) Cuticle thickness for insects that were injured at 1 week (i.e. younger insects) and left to repair for 3 further weeks. (C) Cuticle thickness for insects that were injured at 12 weeks (i.e. older insects) and left to repair for 3 further weeks. T1, T2 and T3 indicate the dorsal, ventral and medial/lateral sides, respectively; I, injured; UI, uninjured.

The repair process reported by Parle et al. (2016) – targeted deposition – occurred for both age groups. This is particularly interesting as the young insects are already in a rapid growth phase, depositing $1.8 \mu\text{m}$ of cuticle per day (Parle and Taylor, 2017), yet an even greater rate of deposition was possible. This high rate of deposition may be why the younger insects repair injuries to a greater extent than their older counterparts.

The puncture injuries had little effect on the younger insects, which is perhaps not surprising because the holes created were relatively small and rounded, compared with the scalpel cuts. More surprising was the strong effect of the same puncture wounds on the older insects. The explanation for this appears to be that, owing to the lower toughness of the cuticle, cracks formed around the puncture wounds, turning them from relatively mild injuries to more severe ones, similar in their effects to cuts of the same size. These kinds of puncture wounds are important because they might be inflicted by bites from predators. Additionally, as restoration of the continuity of the epidermal layer is necessary for repair (Locke, 1966; Lai-Fook, 1968), their ability to repair the injury is surprising, as the needle displaces tissue upon entry, leaving a wedge-shaped hole (Fig. 4C), which could disrupt the healing process. But this is possibly dependent upon the size of the injury, and it may simply be the small size of the needle relative to the tibia that allows repair to occur.

An explanation for the varying effect of injury on strength lies in the relative values of fracture toughness and failure strength of the cuticle as it ages. When receiving a puncture injury, younger insects were found to have a fracture toughness of $\sim 4 \text{ MPa m}^{1/2}$ whereas older insects (8 weeks) had a lower fracture toughness of $2.44 \text{ MPa m}^{1/2}$. Using Eqn 4, the critical crack length (the length for which the failure stress is equal to the strength of the cuticle) can be estimated. A re-analysis of the results of Dirks and Taylor (2012) yields a critical

crack length of 1.2 mm, which is approximately the same as the diameter of the leg. However, the critical crack length for older insects (those at 8 weeks that received a puncture injury) was just 0.36 mm, which is significantly smaller than the diameter of the tibia ($\sim 1 \text{ mm}$).

Combined with the lower fracture toughness, this means that small injuries could prove to be catastrophic at scales much lower than those of their younger counterparts. Thus, the declining tolerance to injury can be seen to result from the tendency of the cuticle to become stronger but less tough over time. These findings are in agreement with those of some previous workers who noted that insect cuticle gets stiffer and less flexible as the insects age (Ritzmann and Ridgel, 2005; Zhou et al., 2015).

Toughness values calculated after repair showed significant increases. After 3 weeks of repair, the fracture toughness of the older insects (12 weeks) had increased to $6.09 \text{ MPa m}^{1/2}$. In contrast, the fracture toughness of the younger insects (injured at 7 days) was increased to $8.48 \text{ MPa m}^{1/2}$ after 3 weeks of repair. These changes do not reflect an increase in the toughness of the material itself, but rather the effect of the repair process, which has made it more difficult for failure to occur by crack growth at the injury site (Vincent and Wegst, 2004; Vincent, 1990).

Consequently, the targeted deposition of new material observed here serves two functions: it both plugs the wound and prevents infection, as well as increasing the fracture toughness of the hind tibia, as reflected in the outlined results. Though it is known that the thickness to radius ratio of the hind tibia is optimized for resisting both transverse fracture and buckling (Dirks and Taylor, 2012), there may be a trade off occurring with this increased thickness. Little difference in thickness was observed between the young and old uninjured samples in all three directions of the cuticle. It was found that the increase in thickness across the T1 (dorsal) side of the

cuticle was comparable in both groups, though there was far more scatter in the older group. That they are of similar thickness by the end of the repair period is to be expected: cuticle is being rapidly deposited by the younger insects immediately after moulting to reach the optimum thickness to radius ratio. But the reason for the older insects being less capable of mechanical repair must still be elucidated. These differences can probably be attributed to the ageing process; though carrying out similar functions, a seemingly poorer performance is exhibited by the older insects. This could be due to the fact that the younger insects are still in their rapid growth phase at the time of injury, with cuticle being deposited immediately after growth so that an optimum radius to thickness ratio (Dirks and Taylor, 2012) can be reached and enabling sclerotization of some of the newly deposited cuticle, conferring additional strength. The endocuticle in older insects may also be getting less flexible as time passes; this would align with the reduced fracture toughness observed as the insects age. Further histological studies into changes in the composition of the exocuticle and endocuticle with age would be an interesting path to pursue, and may reveal parallels with osteoporosis and other age-related declines observed in mammalian bones (Rizzoli and Ammann, 2003; Marumo and Saito, 2010).

It is not clear why these dramatic changes occur in this species of locust at the age of 3 weeks, but it could be linked to animals becoming redundant in this species once they have passed sexual maturity. The phenomenon of ageing, and in particular the changes in the mechanical properties of structural materials, is very widespread in nature, and the effects of ageing can have a large impact upon survival. Up to now, the study of ageing has been lacking in insects: the present study has provided some new results in an area which merits further investigation.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.T.; Methodology: M.O.; Formal analysis: M.O., D.D.; Investigation: M.O., D.D.; Resources: D.T.; Data curation: M.O., D.D.; Writing - original draft: M.O.; Writing - review & editing: M.O., D.T.; Visualization: D.T.; Supervision: D.T.; Project administration: D.T.

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