

# Conflicting Requirements for Transparency and Mechanical Stability in the Compound Eyes of Desert Locusts

Chuchu Li,\* Hamed Rajabi, and Stanislav N. Gorb

Compound eyes of insects should be both thin and transparent to allow light to pass through, and at the same time mechanically stable to serve as exoskeleton. These conflicting requirements make the corneal cuticle an interesting example for studying cuticle biomechanics as well as for designing composite materials that seek similar properties. Here, scanning electron microscopy, confocal laser scanning microscopy, and nanoindentation are combined to investigate the microstructure, material composition, and material properties of the corneal cuticle of desert locust *Schistocerca gregaria*. The results suggest that a fully helicoidal architecture and large proportion of resilin in the corneal cuticle are likely to be adaptations for light transmission. Even though the corneal cuticle is resilin-rich, its elastic modulus is at least three times higher than that previously reported for other resilin-rich cuticles. This is likely due to the specific microstructure of the corneal cuticle with densely packed layers. This study presents one of a series of studies, in which multidisciplinary approaches are used to understand the link between the structure, material, property, and function in insect cuticle.

## 1. Introduction

Compound eyes of insects have previously attracted great interest, because of their optic properties and high potential for biomimetic research and potential technical applications.<sup>[1]</sup> Compared with human eyes, compound eyes have poor image resolution, but possess a large view angle and ability to detect fast movement objects and, in some cases, the polarization

of light.<sup>[2]</sup> Previous studies have focused mainly on the internal structures of the compound eyes, as they are key to decode the distinguished visual performance of insects.<sup>[3–8]</sup>

Some literature data are present on the ultrastructure, material composition, and sclerotization of the corneal cuticle and their influence on insect vision,<sup>[9–12]</sup> however, no study has ever established a quantitative link between the structural and biochemical factors and the biomechanical properties of the corneal cuticle. This is especially important, because as a part of insect exoskeleton, eyes should not only possess good optic properties, but also be able to resist mechanical stresses. For instance, the compound eyes should be able to prevent insect head from damage, maintain the mechanical stability between the ommatidia, and support the internal nervous system.<sup>[13]</sup> Currently, using the

existing data, we can hardly explain the mechanisms behind the mechanical stability of corneal cuticle, especially knowing that the elasticity modulus of resilin-rich cuticle (1–60 MPa) is too low to allow for the observed stability.<sup>[14]</sup>

Here, to fill this gap in the literature, we studied the microstructure, sclerotization, and mechanical properties of locust corneal cuticle. The desert locust *Schistocerca gregaria* was chosen, because it is one of the most established species in studies of cuticle biomechanics.<sup>[15–20]</sup> We used scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) for the structural and material analyses, respectively, and nanoindentation for measuring cuticle stiffness and hardness. Our results enabled us to shed light on the complex relationship between the structure, material, and properties of the corneal cuticle and their influence on the mechanical function of insect eyes.


## 2. Results

### 2.1. Microstructure and Material Composition of Eyes

Transverse fractures on the minor axis of both deep-frozen and air-dried eyes were visualized by SEM. The results revealed that the corneal cuticle consists of the typical layers of epi-, exo-, and endocuticle (Figure 1a,b). The epicuticle is very thin and unstructured, whereas the exocuticle has dense sublayers which are arranged helicoidally, similar to that observed in

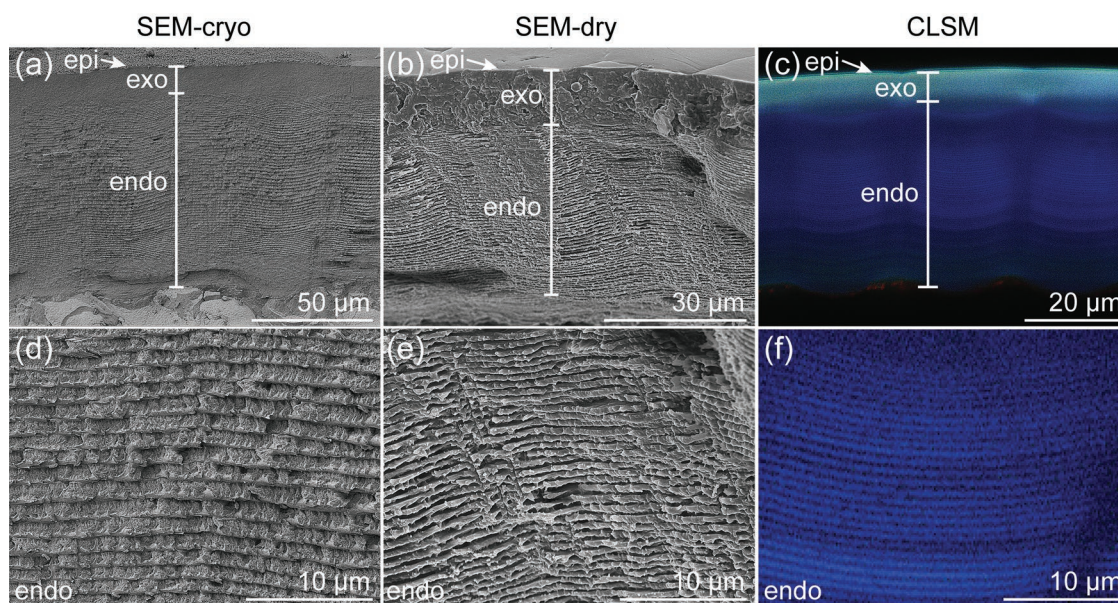
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**Figure 1.** Microstructure and material composition of the eyes. a,b,d,e) Scanning electron microscopy (SEM) images of the lateral section of the a,d) frozen (Cryo-SEM,  $n = 3$ ) and b,e) air-dried (regular SEM,  $n = 3$ ) eyes showing the epi-, exo-, and endocuticle layers. c,f) CLSM images of the lateral section of the fresh eye ( $n = 3$ ) showing different sclerotization levels of the exo- and endocuticle. epi: epicuticle, exo: exocuticle, endo: endocuticle.

many other cuticles of arthropods.<sup>[21]</sup> The endocuticle consists of less dense sublayers in comparison to the exocuticle, but has a similar helicoidal architecture (Figure 1d). The total thickness of the eye cuticle was  $86.0 \pm 4.3 \mu\text{m}$ . The thickness of epi-, exo-, and endocuticle was  $0.60 \pm 0.07$ ,  $13.0 \pm 1.8$ , and  $75.0 \pm 2.4 \mu\text{m}$ , respectively.

The sclerotization level of a cuticle specimen can be characterized based on the intensity of the blue, green, and red autofluorescence in the CLSM image of that specimen.<sup>[10,22]</sup> The exocuticle of the eye cuticle appeared in peacock green, i.e., a mixture of the blue and green colors, whereas the endocuticle appeared in dark blue (Figure 1c,f). This indicates the stronger sclerotization of the exocuticle in comparison to the endocuticle. The alternating light and dark stripes of blue color in Figure 1f result from the helicoidal architecture of the cuticle, in which layers change direction subsequently.<sup>[23]</sup>

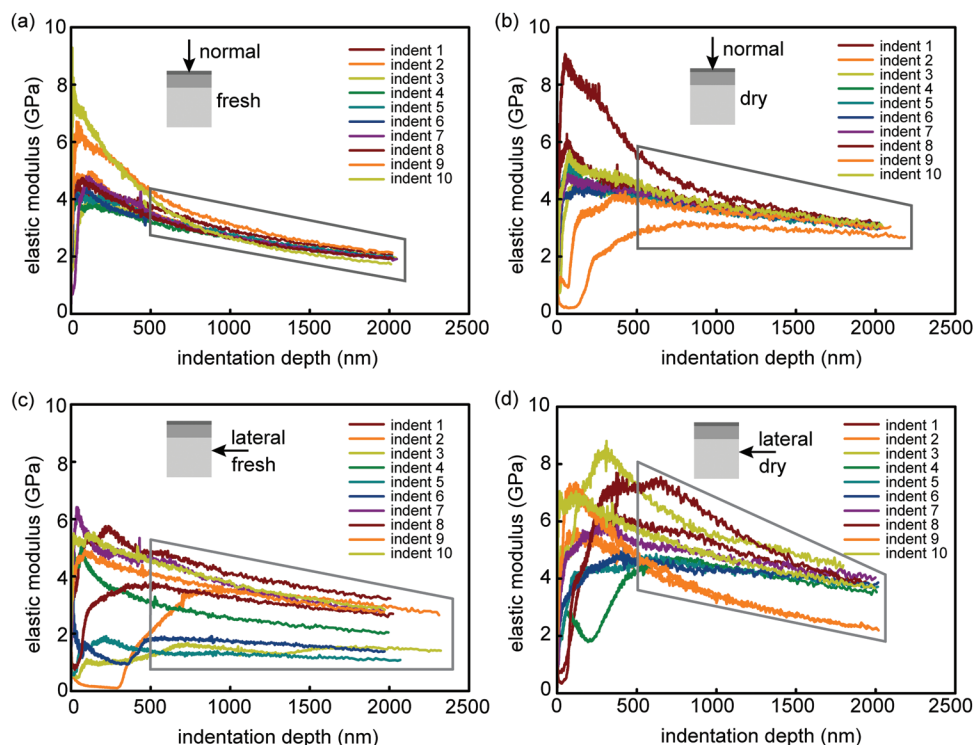
## 2.2. Elastic Modulus of Compound Eyes

We have measured the elastic moduli of fresh and dry corneal cuticles in the normal and lateral directions, using the continuous stiffness measurement (CSM) technique which allows the measurement of depth-dependent elastic modulus. The elastic moduli obtained from indentations in  $\approx 500 \text{ nm}$  from the surface were affected by the surface roughness (Figure 2). As shown in Figure 2, the elastic moduli of fresh and dry corneal cuticle measured in the normal and lateral directions are depending on the indentation depth (Figure 2, gray boxes). In this study, the elastic modulus of each specimen is calculated by averaging the elastic moduli obtained in indentation depths of 500–2000 nm. As shown in Figure 3a, the elasticity moduli of the fresh corneal cuticle in the normal and lateral

directions were  $1.6 \pm 0.5$  and  $2.2 \pm 0.8 \text{ GPa}$ , whereas the elasticity moduli of the dry corneal cuticle in the normal and lateral directions were  $3.7 \pm 0.2$  and  $2.4 \pm 0.3 \text{ GPa}$ . Such degree of variation in mechanical property values is expected in biological samples. Significant differences were found in the elastic modulus between fresh and dry cuticles, in normal direction (Holm–Sidak two-way analysis of variance (ANOVA) test,  $p < 0.001$ ), but not in lateral direction (Figure 3a). Furthermore, significant differences were also found in the elasticity moduli between the normal and lateral directions in dry cuticles (Holm–Sidak two-way ANOVA test,  $p < 0.001$ ), but not in fresh cuticles (Figure 3a). This means that the fresh cuticle possesses mechanical isotropy and the dry cuticle is mechanically anisotropic in elastic modulus.

## 2.3. Hardness of Corneal Cuticle

We also measured the hardness of fresh and dry corneal cuticles in the normal and lateral directions. The hardness in all measurements (i.e., fresh and dry cuticles in the normal and lateral directions) was independent from indentation depth (Figure 4). Similar to the elastic modulus, the hardness of each specimen is calculated by averaging hardness measured from the depth of 500–2000 nm. As shown in Figure 3b, the hardness of the fresh corneal cuticle in the normal and lateral directions was  $0.10 \pm 0.04$  and  $0.12 \pm 0.03 \text{ GPa}$ , respectively, whereas the hardness of the dry corneal cuticle in the normal and lateral directions was significantly higher than the corresponding fresh cuticles (Holm–Sidak two-way ANOVA test,  $p < 0.001$ ) and equal to  $0.24 \pm 0.03$  and  $0.24 \pm 0.04 \text{ GPa}$ , respectively. No significant differences were found in the hardness between directions, in both fresh and dry cuticles.

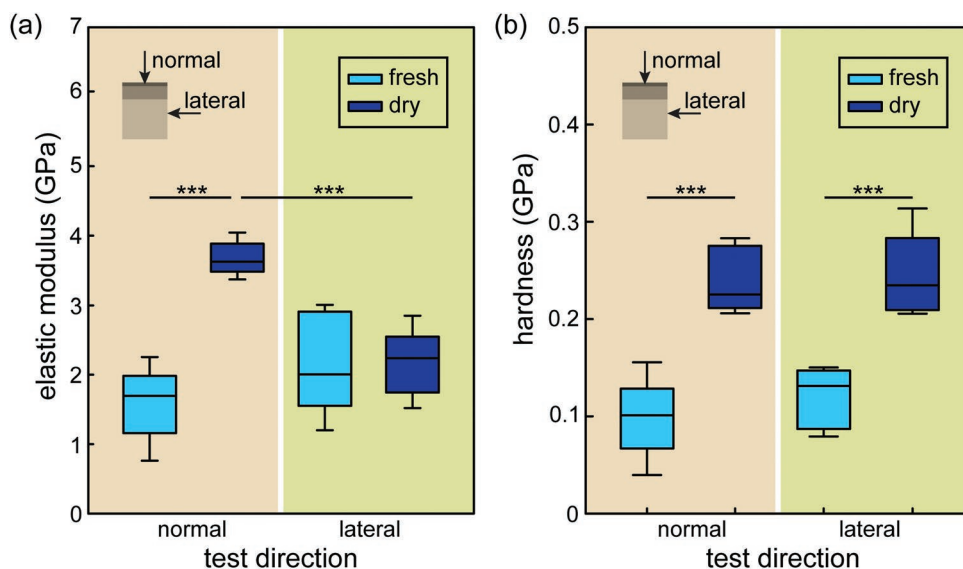


**Figure 2.** Elastic modulus against indentation depth obtained from CSM technique. a,b) Elastic modulus of a) fresh and b) dry corneal cuticle measured from normal direction. c,d) Elastic modulus of c) fresh and d) dry corneal cuticle measured from lateral direction. Indents were performed on ten indentation sites on each specimen.

### 3. Discussion

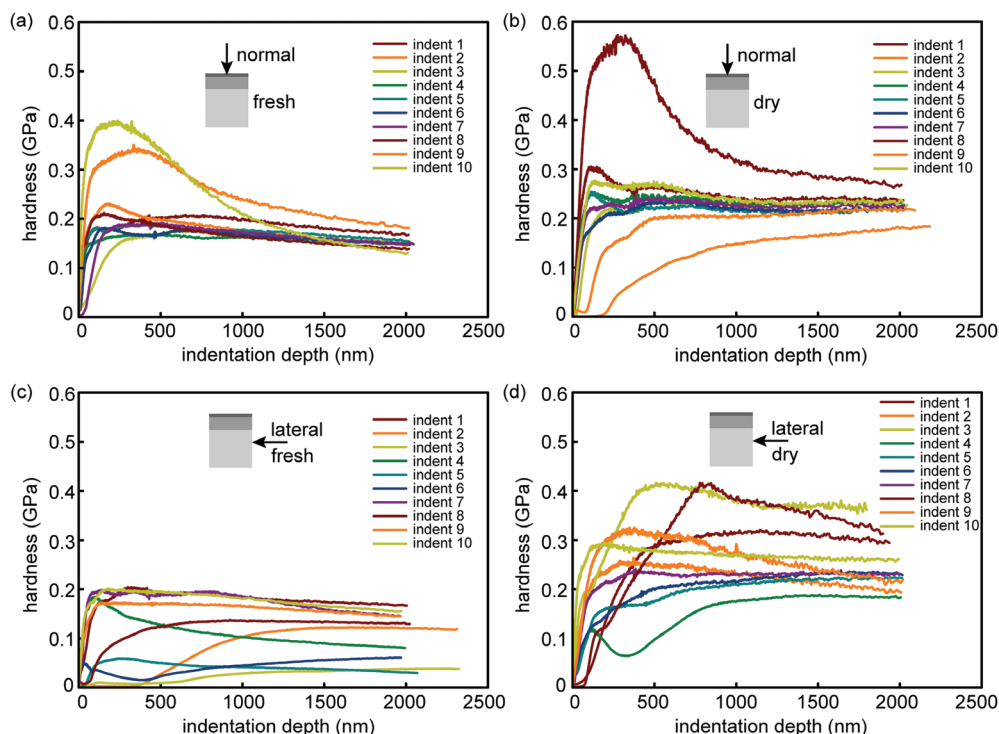
The compound eye lens of insects, including locusts, is made entirely of cuticle.<sup>[21]</sup> As a part of insect exoskeleton, the corneal

cuticle to some extent is similar to the cuticle of other body parts and should be mechanically stable, to protect the fragile inner eye structures. Considering that the compound eyes



**Figure 3.** a) Elastic moduli and b) hardness of the corneal cuticle in different directions and testing conditions. Indentation in the normal direction demonstrates mainly the properties of the exocuticle, and in the lateral direction results in the estimation of endocuticle properties. Statistical analyses were performed by Holm–Sidak two-way ANOVA test. Sample size: a) elastic modulus of fresh eyes in the normal direction ( $n = 50$ ) and in the lateral direction ( $n = 50$ ), elastic modulus of dry eyes in the normal direction ( $n = 50$ ) and in the lateral direction ( $n = 50$ ), b) hardness of fresh eyes in the normal direction ( $n = 50$ ) and in the lateral direction ( $n = 50$ ), hardness of dry eyes in the normal direction ( $n = 50$ ) and in the lateral direction ( $n = 50$ ), \*\*\*:  $P < 0.001$ .





**Figure 4.** Hardness against indentation depth obtained from CSM technique. a,b) Hardness of a) fresh and b) dry corneal cuticle measured from normal direction. c,d) Hardness of c) fresh and d) dry corneal cuticle measured from lateral direction. Indents were performed on ten indentation sites on each specimen.

protrude from the locust head capsule, they should also resist mechanical damage caused by striking and scraping. These requirements call for a cuticle that is thick and sclerotized. On the other hand, being a part of the visual system, the corneal cuticle should enable the specialized function of eyes by transmitting light to the light-sensitive cells. This requires a thin, transparent cuticle and explains the presence of large proportion of resilin in the corneal cuticle (Figure 1c,f), which is known for its transparency and amorphousness.<sup>[24]</sup> Our results comply with previous reports describing large proportions of resilin in the corneal cuticle of other insects, such as dragonfly *Mesogomphus lineatus*, Cixiidae (*Oecleus* sp.), green lacewing *Chrysoperla carnea*, ant (*Monomorium pharaonis*) worker, and beetle of the genus *Circocerus*.<sup>[10,25–27]</sup>

Besides its transparency, resilin is rather soft and in its pure form has an elastic modulus of about  $\approx 1$  MPa.<sup>[14,28]</sup> When mixed with other cuticle components, resilin composites can reach an elastic modulus of 700 MPa.<sup>[16,29]</sup> Hence, it is unlikely that a resilin-rich cuticle as that found in the ultra-thin eyes can provide them with the required mechanical stability. Surprisingly, our measurements showed that the elastic modulus of the corneal cuticle, despite being enriched with resilin, is at least three times greater than that reported in the literature for the resilin-rich cuticle.<sup>[16]</sup> The stronger elastic modulus of the corneal cuticle, reported here, is likely due to the specialized microstructure of its cuticle. As seen in the microscopic images (Figure 1), the corneal cuticle is made of densely packed sublayers with a density of  $\approx 1.6$  layers per  $\mu\text{m}$ , in contrast to the endocuticle of the tibial cuticle that has

only  $\approx 0.4$  layers per  $\mu\text{m}$ .<sup>[19]</sup> Generally, one can expect that a more sclerotized cuticle has a higher stiffness than a less sclerotized cuticle at similar microstructure. This is valid, e.g., for the exocuticle of the tibial and corneal cuticles of desert locusts, which are similar in the microstructure with dense helicoidally oriented layers.<sup>[19]</sup> Hence, the exocuticle of the tibial cuticle is stiffer than the corneal cuticle ( $\approx 3$  GPa vs  $\approx 1.6$  GPa), as it is more sclerotized.<sup>[19]</sup> In contrast, the endocuticle of the corneal cuticle is less sclerotized than that of the tibial cuticle, yet it has a higher elastic modulus ( $\approx 2.2$  GPa vs  $\approx 1.2$  GPa), as the two are microstructurally different. In brief, dense sublayers of corneal cuticle contribute to its elastic modulus.

Another interesting observation here is the fully helicoidal architecture of the locust corneal cuticle in its both thickest parts, the exo- and endocuticle. The occurrence of a helicoid architecture is common for the exocuticle and has been observed in many insect species.<sup>[21,30,31]</sup> The endocuticle of locust exoskeleton, however, usually consists of alternately helicoidal and unidirectional layers, which is known as “locust-style” cuticle.<sup>[32]</sup> This was first described for rubber-like cuticle of locusts,<sup>[33]</sup> and then their wing veins and legs.<sup>[19,20,23,34]</sup> We suggest that the helicoidal structure of corneal endocuticle is an adaptation to provide eyes with isotropic optical properties. De Vries and Kuiper have reported that,<sup>[35]</sup> when the dioptric apparatus of insect eyes is observed through a rotating polaroid, no change in the intensity of the transmitted light can be detected. If the corneal cuticle of locusts had a “locust-style” structure, the anisotropic property of unidirectional layers

would have resulted in an anisotropic light transmission. The helicoidal structure with very small pitch angles, by contrast, having isotropic properties can be ideal for the vision function. Our data on mechanical isotropy of the fresh corneal cuticle support this hypothesis. Interestingly, dry material loses isotropic mechanical properties (Figure 3a) and presumably also the optical properties.

The observed significant difference in the elasticity modulus between fresh and dry corneal cuticle in the normal direction is not surprising, as it is known that water content can change mechanical properties of insect cuticle.<sup>[14,16,36]</sup> In general, water loss increases the elastic modulus of cuticle, and the increased stiffness is positively correlated with the water content of the cuticle.<sup>[37]</sup> The water content of native cuticle varies from 15%, in hard cuticles, to 75% in soft cuticles.<sup>[14]</sup> Thus, soft cuticles usually experience a higher change in the elastic modulus after drying out, when compared with stiff cuticles. For example, the elastic modulus of the stiff exocuticle of locust sternal plate increases by a factor of 2.4 after drying out, whereas that of the soft endocuticle increases by a factor of 7.4.<sup>[16,38]</sup>

However, it is interesting to see that the elastic modulus of the corneal cuticle in the lateral direction did not significantly change by desiccation (Figure 3a). This can be explained by the change in the cuticle microstructure after desiccation. We have seen that when desiccation takes place, the layered structure of the corneal endocuticle becomes more pronounced in the microscopic images (Figure 1e). This is because some layers shrank more than others leading to the appearance of low-density layers (where fibers run perpendicular to the indentation direction) in the cuticle. Under lateral loading, the fibers running parallel to the indentation direction, would buckle easily due to having lesser support from the neighboring low-density layers. We assume that this might reduce the stiffness of the dried cuticle and counterbalance the influence of usual stiffness increase due to the desiccation.

Unlike the elastic modulus, the hardness of corneal cuticle significantly increased after desiccation (Figure 3b). This is not a surprise because, as we mentioned above, water content can change mechanical properties of insect cuticle. What surprised us here, is that the relationship between the elastic modulus and hardness is different between fresh and dry corneal cuticles (Figure 3). It is widely accepted that hardness  $H$  is proportional to the elastic modulus  $E$ , and in biological materials their relationship follows:  $H \approx 0.05E$ .<sup>[39]</sup> Our results ( $H \approx 0.06E$ ) are in agreement with the literature data,<sup>[39]</sup> except for the dry corneal cuticle tested in the lateral direction ( $H \approx 0.1E$ ). This suggests disproportional changes in the mechanical properties of cuticle by desiccation.

## 4. Conclusions

In this study, we attempted to explore the complicated link between the microstructure, material composition, mechanical properties, and two distinct functions (vision and mechanical stability) of the corneal cuticle of desert locusts. Based on our results, we can draw the following conclusions:

- i) Although the corneal cuticle of desert locust is resilin-rich, its elastic modulus reaches 2 GPa, which is three times higher

than that previously reported data for resilin-rich cuticle. This is likely the result of the densely packed helicoidally arranged chitin sublayers of the corneal cuticle.

- ii) Combination of the large resilin proportion and dense helicoidal architecture enables the corneal cuticle, to satisfy conflicting requirements for both functions of vision and mechanical stability.
- iii) Desiccation may not always increase the elastic modulus of cuticle in the lateral direction, as its effect can be counterbalanced by other factors, such as changes in the microstructure.

Our study provides a step toward understanding the complex properties of insect cuticle.

## 5. Experimental Section

**Ethics:** All procedures in this study comply with ethical guidelines at Kiel University.

**Animals:** Desert locusts *S. gregaria* originated from a pet shop in Kiel, Germany, and raised under controlled room temperature (25–30 °C) and humidity (30–40%). They were kept under a natural day/night cycle (12 h/12 h) and fed with fresh vegetables. In all experiments, only adult locust individuals that were aged more than 3 weeks after post molt were involved. Prior to experiments, locusts were euthanized with CO<sub>2</sub>.

**Measurement of the Elastic Modulus through Nanoindentation:** Compound eyes were dissected from the freshly killed adult locusts. Mechanical measurements were performed on the surfaces (normal direction) and transverse sections (lateral direction) of eyes. For measuring along the normal direction, the eyes were glued (5925 Ergo, Kisling AG, Wetzikon, Switzerland) on the columnar aluminum holder with a diameter of 12 mm (standard sample holders), equipped with an additional columnar aluminum block with a diameter of 1 mm on the top. For measuring along the lateral direction,  $\approx 2.0 \times 1.5 \times 0.1$  mm<sup>3</sup> pieces of the eye cuticle were separated from the middle of the eyes using a razor blade and glued on the standard sample holders. The whole process of dissection and fixation was finished within 2 min. To preserve moisture, the specimens were surrounded by pieces of wet cotton which were covered by the parafilm film (BEMIS Packaging Deutschland GmbH, Rheinbach, Germany).<sup>[19]</sup> Prior to testing, only the transverse sections of specimens were polished with sandpaper with a grain diameter of 0.3  $\mu$ m (ITW Test & Measurement GmbH, Esslingen, Germany), while the surfaces of compound eyes were smooth enough for the measurements.

Indentations were performed using a SA2 Nanoindenter (MTS Nano Instruments, Oak Ridge, Tenn., USA) equipped with a Berkovich diamond tip. The elasticity moduli and hardness of compound eyes were measured using the CSM technique.<sup>[40]</sup> The left eyes were used to perform normal direction measurements, while the right eyes were used for lateral direction measurements. For both directions, we have measured the elastic moduli and hardness of eyes under fresh and dry conditions. In total, 20 specimens selected from ten individuals were measured. Nanoindentations were performed on ten indentation sites on each specimen. The distance between adjacent indentation sites on the surface of each specimen was set to be >30  $\mu$ m, to avoid the interference between consecutive measurements. In all indentations, maximum indentation depth, strain rate, harmonic displacement, and harmonic frequency were set to be 2  $\mu$ m, 0.05 s<sup>-1</sup>, 1.0 nm, and 75 Hz. Poisson's ratio of specimens was assumed to be 0.3. Allowable drift rate was set to be 0.1 nm s<sup>-1</sup> to minimize the effect of material creep.

**Microstructure Characterization using SEM:** For SEM, a Hitachi S-4800 (Hitachi High-Tech., Tokyo, Japan) with the cryo-preparation chamber was used. For preparation, some eye samples were air dried, and others deep-frozen at -140° in the cryo-preparation chamber of the microscope. Each dry eye was transversely fractured and mounted on a metal holder with carbon Leit-tabs (Plano GmbH, Wetzlar, Germany)

and Leit-C conductive carbon cement (Neubauer, Munster, Germany) for the analysis at room temperature. Fresh eyes for Cryo-SEM were attached to the metal holders using Tissue-Tek O.C.T. compound (Sakura Finetek Europe B.V., Zoeterwoude, Netherlands) and fractured in the cryo-chamber after freezing. Dry specimens were sputter-coated with gold-palladium (6–10 nm thickness) using a Leica EM SCD 500 High-Vacuum Sputter Coater (Leica Microsystems GmbH, Wetzlar, Germany). Deep-frozen specimens were sputter-coated with gold-palladium (6–10 nm thickness) within the internal sputter coater built in the above-mentioned Gatan cryo-preparation chamber. They were visualized at an accelerating voltage of 3–5 kV. Deep-frozen specimens were visualized at –120 °C cryo-stage within the SEM. In total, six air dry eyes and six deep-frozen eyes from six individuals were measured.

**Material Composition Characterization through CLSM:** Freshly dissected eyes were first frozen at –70 °C for 1–2 min, and then pieces  $\approx 1.0 \times 0.3 \text{ mm}^2$  of eyes were made using a sharp razor blade. The specimens were washed with 75% ethanol and then immersed in glycerin ( $\geq 99.5\%$ , Carl Roth GmbH & Co. KG, Karlsruhe, Germany). After fixing the specimens on their transverse sections in glycerin between a glass slide and a cover slip (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for  $\approx 2 \text{ h}$ , these were visualized with a CLSM (Zeiss LSM 700, Carl Zeiss Microscopy, Jena, Germany). The CLSM was equipped with four lasers (laser wavelengths: 405, 488, 555, 639 nm). Four emission filters transmitting 420–480,  $\geq 490$ ,  $\geq 560$ , and  $\geq 640 \text{ nm}$  were used to visualize the autofluorescences of the eye cuticle under the laser light of different wavelengths. In total, nine transverse sections from three individuals were scanned.

**Statistical Analysis:** No statistical methods were used to predetermine sample size. All the statistical tests in the present study were two-tailed and performed in Sigmaplot v.12.5 (Systat Software GmbH, Erkrath, Germany). Assumptions of normality and homogeneity of variances were tested by Shapiro–Wilk test and Brown–Forsythe test before further analysis. The elastic moduli ( $n = 200$ ) and hardness ( $n = 200$ ) obtained from nanoindentations were compared between the fresh and dry eyes in both normal and lateral directions, and between both directions by ANOVA (two-way ANOVA) followed with Holm–Sidak method for multiple comparison with the testing level  $p < 0.001$ . All values shown in the manuscript were mean  $\pm$  s.d.

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## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

Conceptualization: S.N.G. and H.R.; Formal Analysis: C.L.; Funding Acquisition: C.L. and S.N.G.; Investigation: C.L.; Methodology: C.L., H.R., and S.N.G.; Resources: S.N.G.; Supervision: H.R. and S.N.G.; Validation: C.L.; Visualization: C.L.; Writing—Original Draft Preparation: C.L. and H.R.; Writing—Review and Editing: C.L., H.R., and S.N.G.

## Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

## Keywords

cornea, cuticle, insect eyes, mechanical properties of insect cuticle, microstructures, resilin

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