Arthropod Structure & Development xxx (2016) 1–9

Contents lists available at ScienceDirect

Arthropod Structure & Development

journal homepage: www.elsevier.com/locate/asd

# Effect of sample treatment on biomechanical properties of insect cuticle

Benjamin Aberle [a], Raouf Jemmali [b], Jan-Henning Dirks [a, *]

[a] Dept. New Materials and Biosystems, Max-Planck-Institute for Intelligent Systems, Heisenbergstr. 3, 70563 Stuttgart, Germany
[b] Institute of Structures and Design, German Aerospace Center (DLR), Pfaffenwaldring 38–40, 70569 Stuttgart, Germany

ARTICLE INFO

*Article history:*
Received 12 November 2015
Received in revised form
13 July 2016
Accepted 1 August 2016
Available online xxx

*Keywords:*
Insect cuticle
Biomechanics
Sample treatment
Dynamic mechanical analysis

ABSTRACT

Experimental limitations often prevent to perform biomechanical measurements on fresh arthropod cuticle samples. Hence, in many cases short- or long-term storage of samples is required. So far, it is not known whether any of the standard lab-techniques commonly used to fix or store insect cuticle samples in any way affects the biomechanical properties of the respective samples.

In this paper we systematically address this question for the first time, with a focus on practical, easily accessible and common lab-methods including storage in water, ethanol, glutaraldehyde, freezing and desiccation. We performed a comprehensive and sensitive non-destructive Dynamic Mechanical Analysis (DMA) on locust hind leg tibiae using a three-point-bending setup. Our results show that from all tested treatments, freezing samples at −20 °C was the best option to maintain the original values for Young's modulus and damping properties of insect cuticle. In addition, our results indicate that the damping properties of locust hind legs might be mechanically optimized in respect to the jumping and kicking direction.



## 1. Introduction

Although arthropod cuticle is one of the most common biological composite materials, yet very little is known about its basic biomechanical properties. Only recently, with the availability of more sensitive mechanical testing equipment and visualisation tools, the interest in biomechanical characterisation of cuticle samples has risen again.

Besides the usually small size and complex morphology of the samples, a major challenge in characterizing the biomechanical properties of samples made from cuticle is their notable sensitivity to hydration. Several previous studies have shown that only small changes in water content can strongly affect the static and dynamic biomechanical properties of cuticle (Klocke and Schmitz, 2011; Dirks and Taylor, 2012). With decreasing hydration, Young's modulus and strength of cuticle increase, whilst fracture toughness decreases. As a consequence, biologically relevant mechanical characterisation either need to be performed on a "fresh" sample basically immediately after removal from the animal, or samples need to be kept in a humid environment before and during the mechanical measurements.

Given that it is not always possible or practical to perform mechanical measurements immediately after removal of the sample, often a short- or long-term sample treatment or storage is required. So far, it is not known whether any of the standard lab-techniques commonly used to fix or store insect cuticle samples in any way affects the biomechanical properties of cuticle.

In this paper we systematically investigate this question for the first time. Based on our previous experience, we have selected representative methods typically used for treatment of cuticle samples during "every day lab use": water, ethanol, glutaraldehyde, freezing and desiccation (Hepburn and Chandler, 1980; Locke and Huie, 1980).

To examine even small changes and possible damage of the samples, we chose non-destructible Dynamic Mechanical Analysis (DMA). DMA is one of the most sensitive experimental approaches to characterise complex composite materials such as cuticle (Chandra et al., 1999). In contrast to typical bending experiments to measure maximum strength or fracture toughness, measurements of Young's modulus and damping of samples via DMA are possible without damaging the sample, thus allowing for an even more

* Corresponding author. Present address: Biomimetics-Innovation-Centre, Hochschule Bremen – City University of Applied Sciences, Neustadtswall 30, 28199 Bremen, Germany.
*E-mail address:* jan-henning.dirks@hs-bremen.de (J.-H. Dirks).

http://dx.doi.org/10.1016/j.asd.2016.08.001
1467-8039/

sensitive paired (before vs. after) statistical comparison. Given the non-uniform distribution of the endocuticle within the cross-section of the locust tibia, and to test how the treatments might differently affect endo- and exocuticle, we also performed the DMA tests in the dorsal-ventral and medial-lateral direction of the tibia.

## 2. Materials & methods

### 2.1. Sample preparation and treatment

*Schistocerca gregaria* (desert locust) were bought as 5th instar and kept in a lab colony with controlled day-night cycle (12 h/12 h at 30°C/21 °C) and fresh food *ad libitum*. For all experiments, the hind tibiae from adult mature (older than 3 weeks) locusts were cut off directly below the femur-tibia joint. Tarsal segments were cut off within the distal 1–2 mm of the tibia, leaving the tibia samples in the form of hollow tubes, open at the proximal and distal ends. Dorsal spines were carefully clipped from the tibia to facilitate the mechanical tests and improve the penetration of the histological treatment. The preparation of a sample typically took less than 3 min.

To improve the sensitivity of our experiments, paired mechanical tests on samples were performed before and after the respective treatments. Each sample was tested in a fresh state immediately after removal from the insect. Samples were then always separately stored in 1 ml Eppendorf tubes and subject to the respective treatment for 48 h or three weeks in the dark:

**Water**: Millipore-filtered water at room temperature. **Ethanol**: 1 ml 70% Ethanol at room temperature. **Glutaraldehyde**: 1 ml 2.5% aqueous Glutaraldehyde-solution (Sigma Aldrich, Germany, G6257) at room temperature. **Freezing**: Storage in a standard lab freezer at −20 °C for 48 h without medium. Frozen samples were thawed for 15 min in Millipore water to room temperature. In contrast to the other treatments, where chemical reactions within the cuticle could take place over time, freezing is a purely physical effect. Since any effect should thus most likely occur through the process of freezing and/or unfreezing (Karlsson and Toner, 1996), it was not necessary to repeat the measurements after three weeks. Previous studies on the effect of freezing substitution on moth antennae have shown that occurring damage was due to primary freezing damage during the cooling process (Steinbrecht, 1982). **Desiccation**: Previous studies have shown that locust hind leg tibia completely desiccate at room temperature within approximately 3 h (Dirks and Taylor, 2012). Samples were dried at room temperature and were mechanically characterised again in air. To quantify the water loss, samples were weight before and after desiccation.

### 2.2. Dynamic Mechanical Analysis (DMA)

All DMA measurements were performed using a tensile testing machine (22N loadcell, Bose Electroforce Series III). For repeated DMA measurements (before vs. after treatment) we chose a non-destructive three-point-bending test with a length between the sample-supports of 10 mm. To prevent desiccation of the samples, all measurements (if not stated otherwise) were performed with samples fully submerged in Millipore-filtered water at room temperature. Measurements were performed at seven different frequencies (1, 2.15, 4.65, 10, 21.5, 46.5 and 100 Hz) within 15 min. To test whether the measurement range stayed within the linear elastic region of the samples, which is a necessary requirement for DMA analysis, the measurements were performed at three different mean deflection levels (0.15, 0.2 and 0.25 mm). In addition this routine was executed for every sample in two directions, dorsal/ventral (d/v) and medial/lateral (m/l) in random order. The two deflection directions refer to a leg as positioned in a locust sitting in resting position.

The Young's modulus E of the samples was calculated from the DMA measurements using a custom made Matlab script with the force *F*, the deflection *d*, the spacing between the supports *S* (10 mm, see Eq. (1) and Supplemental Figure 1A) and the respective moment of inertia (I).

$$E = \frac{\left(\frac{F}{d}\right) \cdot S^3}{48I} \quad (1)$$

As a measurement of the mechanical damping we here report the dissipation factor (DF), which is equal to the tangents of the phase shift δ of the actuating movement and the samples' mechanical response (tan δ, see Supplemental Figure 1B). A higher DF means stronger mechanical damping. The dynamic damping properties of the cuticle samples were calculated using a standard DMA software (BOSE, WinTest 7.0).

### 2.3. MicroCT measurements

To distinguish between bending stiffness (EI) of a structure and the Young's modulus (E) as a material property, it is important to acquire detailed morphological data of the tested samples. MicroCT measurements have become a very powerful tool for entomological studies on insect morphology and biomechanics (Friedrich and Beutel, 2008; Metscher, 2009; Goyens et al., 2014; Iwan et al., 2015). This method allows a fast, comprehensive and destruction free measurement of the sample geometry at high resolution, which is a requirement for reliable biomechanical analysis.

To calculate the moment of inertia from the microCT scans, fresh tibia samples were fixed in custom designed Teflon (PTFE) holders and scanned in 70% ethanol using a high resolution microCT setup (Phoenix X-ray, 80 kV, 180 μA, 2.9 μm voxel size, see Fig. 1A–C). Data for morphological characterisation was post-processed using custom made Matlab Scripts. Maximal and minimal moment of inertia ($I_{max}$ and $I_{min}$) as well as maximum and minimum cuticle thickness were calculated using Fiji (Bonej plugin (Doube et al., 2010), see Fig. 1B). Measurements were taken for each sample at three different spine-free locations along the axis of the tibia.

### 2.4. Statistics

We defined the relative effect of treatment for all measurements as the difference between measured values divided by the respective reference value. Statistical analysis was performed using "R" (version 3.2.0). If not stated otherwise, paired t-tests were used to test for significant differences between the groups. The normal distribution of the differences of paired values was tested using the Shapiro-Wilk-test. If not stated otherwise, the requirements for statistical tests were all fulfilled. Box and whiskers plots show the median, the 50% quartiles and the 95% quartiles. Reported p-values are abbreviated to three different significance levels (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.01$).

## 3. Results

The average moments of inertia calculated from the microCT scans were 0.027 ± 0.004 $mm^4$ for $I_{max}$ (dorsal-ventral direction) and 0.015 ± 0.002 $mm^4$ for $I_{min}$ (medial-lateral direction, 15 slices from five tibiae from different locusts). The maximum thickness of the cuticle was 133.7 ± 4.9 μm and the minimum thickness 104.3 ± 4.8 μm.

Please cite this article in press as: Aberle, B., et al., Effect of sample treatment on biomechanical properties of insect cuticle, Arthropod Structure & Development (2016), http://dx.doi.org/10.1016/j.asd.2016.08.001