Effect of mechanical stress on insect exoskeleton

Karen Stamm
Submitted for the degree of Doctor of natural sciences
Supervisor: Prof. Dr. Jan-Henning Dirks
Supervisor: Prof. Dr. Marko Rohlfs
Date of defense: 27.09.2022
Cross-section of locust (*Locusta migratoria*) hindleg tibia under polarization light microscopy. The cross section turned during mounting into a Möbius loop.
Acknowledgments

I would like to express my gratitude towards my PhD supervisor Prof. Dr. Jan-Henning Dirks, who not only supervised the PhD project but also encouraged and supported my professional and personal development. His expertise and guidance through this fascinating and complex research area has challenged and motivated me during my studies and was invaluable over the last years.

I thank Prof. Dr. Marko Rohlfs for the fruitful discussions and his open ear for all conceivable questions and stumbling blocks and thus contributed to the progress and success of my work.

Many thanks to all former and present members of the Biological Structures and Biomimetics Workgoup:
Dr. Brian Daniel Saltin, Christoph Bruns, Raman.
Sharing knowledge, experience and science memes was a fundamental support during the last years.

I am very thankful to the Biomimetics Innovation Center in Bremen for providing an inspiring academic and social atmosphere. I would especially like to thank Martina Schnaars and Pauline Berger for their help and guidance.

I thank Maren Rheders and Klaudia Brix (Jacobs University Bremen), who kindly granted us access to their cryostat facilities and assisted with measurements.
The Institute for Biophysics of Dorothea Brüggemann (University of Bremen) kindly allowed me to perform polarization light microscopy.

I am very grateful for the financial support by the deutsche Forschungsgemeinschaft (Projectnumber: 395713517) and the Promotionsabschlusstipendium of the Hochschule Bremen during the last 3.5 years.

I am deeply indebted to Marc Rodemer. His support, unconditional love and faith in me kept me going forward, even in difficult times. I would like to thank my family and friends for their understanding, support and love since the beginning of my studies, which finally lead to this thesis.

From the bottom of my heart thank you!
Summary

Insects possess one of the most successful forms of “outer body protection” - the exoskeleton. Insect exoskeleton is mainly build from cuticle, a complex, hierarchical composite material, which shows a huge variety of properties. Its variety of properties seems to be linked to insect body part function. On a daily basis an insect body experiences stress on several levels (e.g. walking, jumping, predators, ...) and has to withstand the acting forces. However, its reactions to long-term applied mechanical load are not fully understood. In this dissertation, four studies have been conducted to investigate the effect of mechanical stress on insect exoskeleton.

Biomechanical properties of the insect exoskeleton have been increasingly investigated in the last centuries, using the most sophisticated scientific methods available at that time. Investigations revealed a biological material, which is considered to be one of the most complex and diverse biological materials worldwide. In spite of that, it is unclear if and how the utilized measurement techniques and sample treatments influence the measured properties. As the first study of this dissertation, a systematic literature review aims to provide an overview of measurement techniques and their advantages and disadvantages to define cuticle properties. Results suggest that there is no “prototypical cuticle” and subsequently investigated body part, sample condition and utilized measurement method should selected carefully to determine cuticle properties. To calculate between species comparable biomechanical properties, like Young’s modulus and bending strength, detailed knowledge of the sample morphology is required. More precisely, two cuticle main components, exo- and endocuticle morphology, influence the biomechanical properties and thus detailed knowledge about cuticle composition is required.

In the second study of this dissertation, a novel, destruction free staining method was developed to measure thickness of exo- and endocuticle directly from µCT scans. Semi-automated measurements of stained tibia cross-sections showed exocuticle and endocuticle variation of thickness distribution along the hindleg for the first time in a 3D environment. This will help us to understand the role of exo- and endocuticle within insect exoskeleton and subsequently its possible influence on biomechanical properties.

If and how can biomechanical properties of the insect exoskeleton change under long-term mechanical stress? To explore the effects of increased mechanical load on insect exoskeleton, in the third study of this dissertation a custom build centrifuge was utilized to increase mechanical load up to 8g. After their final mold, locust were kept under hyper gravity for two weeks. Afterwards, established morpholog-
ical and biomechanical tests were performed. Results showed a primarily increase of survival rate at 3g, which then decreases with increasing mechanical stress. Both, survival rate and body mass gain, were slightly increased again, when an “1 hour lunch break” was introduced three times a week. Further, the biomechanical properties (e.g., Young’s modulus and bending strength) also showed an increase at 3g. Cuticle properties of higher mechanical loads than 3g were comparatively similar to the 1g control group. A possible explanation might be that locust are used to short-term mechanical loads of up to 3g during mating. The male sits on top of the female and thus increases the mechanical load to up to 3g. It can be concluded that in a reasonable “physiological range” (3g) the exoskeleton does react to the mechanical load, but insects are physiologically not able to deal with higher mechanical loads. It is remarkable that the exocuticle thickness is similar in all treatments, while the endocuticle thickness is reduced with increased mechanical load, but still the Young’s modulus and bending strength properties of the 1g control group were met. That indicates that exocuticle might play a primary role in stress distribution during cantilever bending and other types of mechanical stress testing. In summary, the insect exoskeleton does react to increased mechanical load.

So far it was shown, that insect cuticle reacts to mechanical stress. But it remains unclear if its a specific response towards mechanical stress or a more general stress response of the exoskeleton. Another type of stress can be changes in the circadian clock of locust. This can be achieved by 24 hours of daylight. Neville (1965b) has already shown that this not only stresses the animals but also changes the endocuticle chitin fiber orientation. In a normal 12h day:night cycle fibers are oriented in changing pattern of lamellate and non-lamellate layers, whereas during 24h daylight only non-lamellate cuticle is build. In the fourth study, measurements of 24h daylight stressed locust showed a slightly increased survival rate and body mass gain. The thickness of exo- and endocuticle is comparable to the 1g 12h day:night cycle control group. The Young’s modulus and bending strength increased and values are comparable to the 3g stressed animals, this might indicate that the exoskeleton have a general stress response.

In summary, the insect exoskeleton is able to react to long-term mechanical stress as well as to other stress factors. Survival rate and body mass gain are significantly influenced by mechanical stress as well as the biomechanical properties (e.g. Young’s modulus and bending strength). Thickness distribution of the endocuticle changes when mechanical load is increased, while exocuticle thickness is comparable between treatments. But, the exocuticle seems to play a main role when it comes to biomechanical testing of cuticle properties. In summary, this study pro-
vides the first comprehensive investigation of cuticle under increased mechanical load. The measured properties (e.g. Young’s modulus, bending strength, exo- and endocuticle thickness) of mechanically stressed insect exoskeleton indicate that it reacts towards increased mechanical load.
Zusammenfassung


Ob und wie können sich die biomechanischen Eigenschaften des Insekten-Exoskeletts unter mechanischer Langzeitbelastung verändern? Um die Auswirkungen einer erhöhten mechanischen Belastung auf das Exoskelett von Insekten zu untersuchen, wurde eine speziell angefertigte Zentrifuge verwendet, um eine Hypergravitation von bis zu 8g zu erzeugen. Nach der letzten Häutung wurden die adulten Heuschrecken...
Publications

Some ideas and figures have appeared previously in the following publications:


Contents

List of Figures XVIII
List of Tables XIX
List of Equations XX

1 Introduction 1
   1.1 Insect exoskeleton composition .............................. 3
   1.2 Daily challenges of insect exoskeleton ........................ 4
   1.3 Reactions of biological materials to mechanical stress .......... 5
       1.3.1 Utah paradigm ............................................. 5
   1.4 Reactions of insect cuticle to mechanical stress ................. 8
   1.5 Aims and overview of the conducted studies ....................... 10

2 Biomechanics of insect cuticle – an interdisciplinary experimen-
tal challenge 13

3 Semi-automated differentiation of insect exo- and endocuticle
   in X-ray microtomography 15

4 Insect cuticle reacts to mechanical stress 17
   4.1 Abstract ...................................................... 18
   4.2 Introduction .................................................. 18
   4.3 Material & Methods ........................................... 20
       4.3.1 Animal keeping and treatments ............................ 20
       4.3.2 Cantilever bending ......................................... 20
       4.3.3 Geometric analysis .......................................... 22
       4.3.4 Statistics .................................................. 22
   4.4 Results ....................................................... 22
       4.4.1 Survival rate and body mass change ....................... 22
6.4 Comparison of mechanically stressed, light induced stressed and non stressed cuticle .......................................................... 57
6.4.1 Fiber alignment ............................................................... 58
6.5 Future studies ..................................................................... 61
6.5.1 Reaction of insect muscles to higher mechanical load .... 61
6.5.2 Locomotion .................................................................... 62
6.6 Concluding summary ........................................................... 63

7 References .................................................................................. 65

A Appendix ...................................................................................... 79
A.1 Centrifuge properties ............................................................ 80
A.1.1 Basic principles of centrifugal forces ............................... 80
A.2 Young’s modulus and bending strength ............................... 83
# List of Figures

1.1 Utah paradigm of several biological materials .......................... 10
4.1 Experimental set up ................................................. 21
4.2 Survival rate and body mass of mechanically stressed locust .... 24
4.3 Cuticle thickness distribution on locust hindleg tibia ............. 26
4.4 Biomechanical properties of mechanically stressed cuticle ...... 28
5.1 Light spectrum of LED stripe ......................................... 40
5.2 Survival rate and body mass of mechanical and light induced stressed locust ......................................................... 41
5.3 Biomechanical properties of mechanical and light induced stressed cuticle ......................................................... 43
5.4 Cuticle thickness distribution of mechanical and light induced stressed locust hindleg tibia ........................................ 44
6.1 Light polarization microscopy of mechanically and light induced stressed cuticle ......................................................... 60
A.1 Technical drawing and properties of custom build centrifuge ... 82
List of Tables

4.1 Geometrical features of mechanically stressed locust hindleg tibia from µCT scans ........................................ 25

5.1 Light intensity of used LED stripes ........................................ 40

A.1 Applied and corrected g forces per treatment ........................ 81
A.2 Statistics of survival rate per treatment ............................... 84
A.3 Statistics of locust body mass gain per treatment .................. 85
A.4 Statistics of Young’s modulus per treatment ....................... 86
A.5 Statistics of bending strength per treatment - dorsal ............ 87
A.6 Statistics of bending strength per treatment - ventral .......... 88
A.7 Statistics of endocuticle thickness per treatment ................ 89
List of equations

A.1 Centrifugal force .................................................. 80
A.2 Young’s modulus ................................................... 83
A.3 Bending strength ................................................... 83
Chapter 1

Introduction
For over 3000 years, humans took inspiration from nature. This was the beginning of the so called field of “biomimetics”: the study of biological mechanisms and functions and its application to engineering, design, chemistry, electronics and many more (Vincent et al. 2006; White and Vernerey 2018). One of the earliest and quite famous researcher for biomimetics was Leonardo da Vinci. For his flight research, Leonardo da Vinci studied bats, birds and dragonflies (Leishman 2000). Especially, the interest in insects like dragonflies or locust as biomimetic role models increased over time and scientific research outstretched from their -remarkably- flight performance to its non the less remarkable outer protection - the exoskeleton. It is one of the most successful forms of “outer body protection”, build of one of the most common biological composite material worldwide: cuticle (White and Vernerey 2018). Focusing only on arthropod cuticle of adult insects, already a huge variety of structure, function, properties and material composition can be observed.

The high diversity within insect’s exoskeleton seems often to be linked to the function of the investigated body part. Many parts are specialized for various purposes:
The compound eyes of insects, which arose along with the first arthropods in early Cambrian, show a repetitive architecture build from transparent cuticle (Nilsson and Kelber 2007; Waloszek 2003), whereas cuticle composition of the mouth part can be completely different. Insect mandibles used for cutting plant material have highly sophisticated surface structures and some are even reinforced with zinc and manganese (Quicke et al. 1998). For instance, leaf-cutter ants show size-specific modifications to increase bite force. The bite force is increased through an increase in muscle volume, resulting in specialized filamentattachment sides build by cuticle. Apart from the muscular investment, the apodeme, which is also build from cuticle, needs to withstand acting muscle force without failure (Püffel et al. 2021). Failure can not only occur during biting but also during locomotion. In both cases this can have severe influences on the insect and thus some “prevent mechanisms” in the exoskeleton can be found. In insect wing for example, a crack can occur in the wing membrane. But this crack is stopped by the cross veins in the wings (Dirks and Taylor 2012b).
During walking on any surface, insects use an alternating tripod gait (Cavagna et al. 1977, 1964). But special movements, like jumping, require specialized mechanisms to generate these rapid movements. Locust, who jump using their hindlegs, are using a “catapult mechanism” in which energy is stored and rapidly released. These catapult mechanisms are used to overcome the limitations of direct muscular
contraction (Gronenberg 1996; Patek et al. 2011), resulting in jumps, where locust body accelerate $20 - 30\, ms$ to a take-off velocity of $3\, ms^{-1}$ by extension of their hindlegs (Bennet-Clark 1975; Brown 1967). However, in order to stick to a surface, different attachment systems have been developed and highly investigated in the past years: Several cuticle micro-structures have been described, like smooth and hairy pats, which can have a gradient of stiffness within the cuticle microstructure or produce two-phasic fluids for attachment (Dirks 2014; Dirks et al. 2010; Dirks and Federle 2011; Federle and Labonte 2019; Gorb 2001; Gorb 2005; Labonte and Federle 2013; Scherge and Gorb 2001).

Generally, the thickness, arrangement and composition of cuticle layers determine its properties. The same cuticle, which is covering the eyes, can be very soft, thin and flexible in joints, can also be thicker, sclerotized and hard for the mandibles of an insect (Chandran et al. 2016; Müller et al. 2008; Vincent and Wegst 2004).

1.1 Insect exoskeleton composition

The structure and composition of arthropod exoskeleton enables the insect to successfully inhabits many ecosystems, which is certainly due to the highly versatile material properties of cuticle regarding its stiffness, strength, toughness and fatigue resistance (Dirks et al. 2013; Dirks and Taylor 2012a; Stamm et al. 2021; Vincent and Wegst 2004). Cuticle is a hierarchically organized structure secreted by epidermal cells. Generally, cuticle is a broad term that can incorporate any variety of tough but flexible, non-mineral, outer coverings of an organism. Also, it can be found in organismal parts providing protection. Typically, two relatively distinct layers can be found in insect cuticle: A thin outer layer, namely the “Envelope”, and a thicker “Procuticle” layer (Locke 2001). The envelope consists of proteins, lipids, and waxes. Underlying the envelope is the epicuticle, which is built out of proteins without chitin (Schwarz and Moussian 2007). However, the Procuticle is much thicker and contains chitin and proteins, which are secreted early during the molting cycle. Another property of the procuticle is “tanning” (or “sclerotization”). It describes the hardening and darkening of insect cuticle and is generally caused by cross-linking of proteins. The cross-linking of proteins is not fully understood, yet. Generally, it is hypothesized that quinone-tanning during insect cuticle sclerotization proposes oxidation of N-acylcatecholamines by phenoloxidase to quinones and quinone methides, which
serve as electrophilic cross-linking agents to form covalent cross-links between cuticular proteins. Further, insect cuticular proteins are oxidatively conjugated with catechols and then become cross-linked, forming oligomers and subsequently polymers (Suderman et al. 2006; Vincent and Hillerton 1979).

This causes the procuticle to form two relatively distinct layers: (1) The denser and more sclerotized “Exocuticle” which is a hardened outer layer containing chitin filaments, lipids and phenols. (2) The inner “Endocuticle”, which remains tough and flexible. It is usually the thickest part of cuticle and consists of fibrous chitin and (cross-linked) proteins building a “sandwich”- or laminated-like pattern (Harrison et al., 2012). Chitin fibers are arranged in daily growing layers which are visible under polarized light microscopy (Neville 1965a,b, 1967, 1983). Within the first 2-3 weeks post-mold endocuticle builds chitin crystallites organized in non-lamellae layers at day and lamellae layers at night. Wavelength, temperature and light intensity are key factors for the chitin lamellogenesis. Keeping insects (locust) under 24h daylight can uncouple these mechanisms and resulting in an all non-lamellate endocuticle (Neville 1965a). The crystalline structures of chitin within the cuticle and the effect of the hierarchical arrangement of chitin fibers within a complex protein matrix plays an important role in determining the overall properties of cuticle, which is reviewed in detail by Vincent and Wegst (2004). Beneath these layers the epidermis, as a single layer of cells connected to the endocuticle via pore canals, and the basement membrane are present in insect exoskeletons.

The internal structure of the exoskeleton has bumps and ridges functioning as muscle attachments and providing support. As stated by Andersen (2010) cuticular mechanical properties are influenced by dehydration and crosslinking during sclerotization. Nevertheless, the term cuticle is generally used to describe the whole exoskeleton or mainly the exo- and endocuticle. In this study the term “cuticle” is used to describes the whole insect exoskeleton if not stated otherwise.

1.2 Daily challenges of insect exoskeleton

Despite the complex composition of cuticle, one of the main exoskeleton functions is protection. This protection includes to withstand higher mechanical loads. For example, the locust jump is completed within 20-30 ms. During that time the hind leg tibia remains straight and the leg is extended up to 180 degree (Bayley et al. 2012). The peak loads during jumping are approximately twenty times the forces of gravity (Bennet-Clark 1975). These forces are only distributed over the
two jumping legs, while forces during walking are distributed to six legs. In some cases, when the tarsus slipped on the surfaces, buckling of tibia up to 28° could be observed (Bayley et al. 2012). This could lead to landing on only one hind leg. Even then, the kinematic energy is absorbed in the single leg and no failure or damage occurs in the leg, indicating an “overbuild” in the locust hindleg (Katz and Gosline 1992). Another form of higher mechanical load acting on the exoskeleton is its protection against predators. Insects can show behavioral or structural defense mechanisms. In both the cuticle needs to be adapted to withstand the acting forces to hide, escape or withstand bite-forces of a predator (Rivera et al. 2020; Simpson et al. 1986). The cockroach *B. craniifer* burrows itself into soft substrates by using its head and pronotum to hide before or after an (unsuccessful) predator attack (Simpson et al. 1986). Mechanically measured bite forces of lizards compared to numerical simulations of required force to fail weevil cuticle, have shown that the force needed to damage cuticle is just above the bite force (Wang et al. 2019).

In summary, the insect exoskeleton must withstand high mechanical loads on a daily basis. But how can this material withstand these various loads so successful?

## 1.3 Reactions of biological materials to mechanical stress

Insect cuticle as a biological material experiences various types of stress on a daily basis as any biological material given its functionality to a living organism. For example without its stiff cuticle, the weevil would be more often consumed by lizard predators (Wang et al. 2019). From a material point of view, stress in form of strain, tension, compression and increased mechanical load can be a difficult challenge for any material. What kind of mechanisms can we find in biological materials to withstand higher mechanical loads? Some materials like wood and bone are already well understood in their composition and their reactions towards higher mechanical loads. Their reactions are described in the “Utah paradigm” model by Frost (2001).

### 1.3.1 Utah paradigm

The Utah paradigm combines results and hypotheses from multiply disciplines, takes information on tissue-level and anatomical features into account and is origi-
Introduction

Finally developed from “Wolff’s Law”, which firstly stated that mechanical influences can affect bone architecture (Burr and Martin 1992; Frost 2000; Frost 1992; Jee and Frost 1992; Schönau 1996; Takahashi 1995, 1999; Wolff 1892, Figure 1.1). When a material is stretched, shortened, twisted and/or bent, the mechanical load always causes strain in form of compression, tension or shear resulting in a deformation or shape change of the structure or material. For example, a bone fracture can occur on different length scales. Bone micro-damage is a microscopic physical damage in a structural material due to material fatigue (Martin et al., 1989). Skeletons can detect and repair this damage to a certain level and thus only need enough strength to resist strains below a level, which could cause greater damage. This level is the micro-damage threshold. It defines the strain range above which micro-damage begins to accumulate and can not be repaired anymore. In engineering only the increase of structural material can prevent micro-damage. A similar mechanism can be found in bone, when the so called “modeling threshold” is reached. It is the genetically determined minimum effective strain range for mechanically controlled bone modeling. When this threshold is exceeded, modeling is activated and when the strain stays below the modeling threshold, modeling is deactivated. The modeling itself describes a biological process that produces functional material for skeletal organs (Jee 1989). Usually it adds to the mechanical usage of bone to prevent damage by creating a strong bone, where daily strain peaks do not exceed the modeling threshold. When the regularly experienced mechanical load changes, remodeling can take places. To activate the remodeling process, the remodeling threshold, which is the genetically-determined minimum effective strain range, must be reached. It controls the switching of so called “basic multicellular units” (BMU), which are responsible for remodeling, e.g. conservation or disuse of material. During remodeling small packages of BMU are active on the bone (Jee 1989). Disuse of material requires a reduced regular strain below the remodeling threshold. It defines the relationship between material strength and the size of its usual peak strains. This can also be expressed as the strength-safety factor. It states how much stronger a bone is than needed to carry usual occurring higher loads (Frost 2001).

Reactions of wood to mechanical stress

Wood from trees provides structural support, anchoring and nutrients transport. Tree trunk cross-sections reveal heartwood, ray cells, sapwood, cambium and bark (from inside to outside). The cellular structure consist of fibers (single cells) embedded in middle lamellae. The cells can be further separated into the primary and secondary cell wall, a watery layer and the lumen. Molecular components
of the secondary cell wall are cellulose microfibrills with lignin and hemicellulose. The microfibrills are build from cellulose chains (Sorieul et al. 2016). When wood experiences increased mechanical load it forms so called “reaction wood”, which formation is regulated by an interplay of hormones. Generally reaction wood has modified anatomical and chemical properties and is formed in response to a non-optimal stem orientation (e.g. prevailing wind, slope, asymmetric crown shape) (Du and Yamamoto 2007).

In gymnosperms, reaction wood is referred to as “compression wood”. It develops on the lower side of leaning stems and branches. The tissue is comparatively under compression at this location and cell walls are thicker, containing more lignin but less cellulose. Auxin and ethylene are the regulating plant hormones in the formation of compression wood (Du and Yamamoto 2007).

In angiosperms, on the other hand so called “tension wood” is build, which is formed on the upper side of the leaning. It is mainly regulated by auxin and gibberellin. A thick, inner cell-wall layer that consists of highly crystalline cellulose with microfibrils, which are almost oriented longitudinal and parallel to the fiber axis, is typical for tension wood (Du and Yamamoto 2007).

Generally, reaction wood helps to restore growth, especially to incline vertical stems to move above its surroundings (Burgert and Fratzl 2009; Donaldson and Singh 2016; Du and Yamamoto 2007; Gardiner et al. 2014).

Reactions of bone to mechanical stress

To understand the above briefly described reactions of bone to mechanical stress, it is important to know its hierarchical organization. Bones, which are important to organisms structural support and blood cell formation, are build from osteons. The osteon itself is a composite of collagen fibres, build from tropocollagen, and hydroxyapatite crystals.

Within bones basic multicellular units (BMU, Jee 1989) resorb and remodel equal amounts of bone to “conserve” the already existing bone and repair micro-damage. They are informed by osteocytes, which detect the presence of micro-damage and cracks (Burr, 2000). When mechanical loads deform or strain the bone and exceed the modeling threshold, which is individual for each bone, the bone modeling starts to increases strength and reduces strain. If an increase or reduction of bone material is necessary, it is regulated by strain-dependent signals from the bone itself compared to “the standard threshold”. During this remodeling of bone material the BMUs are active. BMUs use osteoblasts and osteoclasts to remodel the bone. Osteoblast synthesize bone and usually function in groups of connected cells. Osteoclast on the other hand are breaking down the bone tissue. In different part of
the same bone and even at the same time osteoblast and osteoclast can increase or disuse bone material to respond to the same stimulus (Chen et al., 1995; Yeh et al., 1995). In the so called “disuse-mode”, less bone is build by the BMU due to decreased loads acting on the bone and thus the remodelling threshold. Usually bone next to the marrow is disused. This property is a main difference to plants. Plants do not possess the ability to disuse already build material. Additionally, muscles influences the bone and add to its load and strain capacity. Thus, the muscle force acts in the bone, which experiences strains and the mechanism of modeling and remodeling is activated. Also hormones and vitamins can influence modeling and remodeling but they do not replace mechanical control over the bone, which causes bone adaptations from present and past loads to prevent future injuries (Frost 2001).

1.4 Reactions of insect cuticle to mechanical stress

Even though cuticle is the second most common biological material worldwide, the knowledge of its reaction to mechanical stress is less studied for short-term applied loads and abundant for long-term mechanical loads. According to Hepburn and Joffe (1974) the normalized cuticular stiffness is maintained across instars, which suggest that cuticular material properties are not able to change. Further, they suggested that the ratio of stiffness to mass is constant for tanned cuticle across instars due to the load experienced in a jump compared to body mass across the instars. After the moulting within each instar, endocuticle is added and so far it is unclear if the added material alters the leg diameter and thereafter tibia mechanical properties and the second moment of area (Katz and Gosline 1992; Neville 1975). Contradictory, Bayley et al (2021) observed temporarily changes in cuticle mechanical properties of locust hindleg during repeated bending, which recover within 24 hours. Similar mechanical “adaptation” could be observed in living and dead tibia (Parle et al. 2017; Parle and Taylor 2017) and are likely due to the thixotropic material properties. These properties are achieved by changes in the fiber orientation, a process which is common for fiber-composite materials (Harris 2003). Within the insect tibia, chitin fibers are primarily aligned parallel to the tibia axis (Neville 1965a). By applying a load, the fibers might get slightly off-axis and/ or misaligned fibers reorient themself within the viscoelastic matrix to resist the applied load (Parle et al. 2017). Thus, cuticle itself possesses passive adaptation possibilities (e.g. become stiffer due to applied load). Further, Parle et al. (2017) suggested that the two different growth rates in cuticle can allow some adaptations within the exoskeleton towards mechanical loads. During the
first 21 days post-molt, insect cuticle is deposited quite rapidly. This marks the first growth rate phase. The second growth rate phase takes place after the 21 days, where cuticle deposition is slowed down (Parle et al. 2016). So far it remains unclear if and how the cuticle growth rates influence the exoskeletal biomechanical properties and if the cuticle deposition is influenced by external factors such as increased mechanical load (Parle et al. 2017). Further, Parle and colleagues found indications of a signaling system between epidermis, which deposits the cuticle, and the cuticle itself, which could also create a purposeful reaction of cuticle towards its environment. This could be important for wound healing and deposition of new cuticle. Also, it has been suggested that insect exoskeleton is less mineralized compared to bone and may enable exoskeletal modulations in ways that are fundamentally different from vertebrates. Presumably morphological changes in skeletal support structures can increase the mechanical stiffness and subsequently the amount of load-bearing material (Katz and Gosline 1992). Parle et al. (2017) has shown that the geometry and stiffness of locusts tibia can vary significantly between species and even between legs from the same individual. These variations suggest that during an emergency behavior each leg is build to operate closely to its individual structural limit. Thus insect tibia can withstand everyday loads within its safety factor, which is similar to structure and safety factor relationship in bone or trees. In the field of engineering, the safety factor defines the ratio of a structure’s failure strength to the maximum allowable stress that it is expected to withstand during its use (Biewener 1993). The safety factor of locust hindleg is estimated to be no more than 1.2 (Bennet-Clark, 1975). Taylor (2011) showed that, without self-repair mechanisms, mammal bones have a safety factor of less than 1, which means structural failure is certain, and illustrates how important it is for a biological material to be able to react to mechanical load.

Overall the insect exoskeleton is still different to wood or bone and thus it might be possible that the material properties can be modulated to meet mechanical demands differently to the known mechanisms in vertebrates (Katz and Gosline 1992). However, so far it remains unknowns if and how cuticle reacts to long-term higher mechanical load.
1.5 Aims and overview of the conducted studies

Within the past 400 million years, insects evolved to one of the largest animal kingdoms with a considerably variety of different morphological adaptations, which allow them to inhabit almost every ecosystems on earth. Their success is partly due to the versatile exoskeleton, which bears fascinating biomechanical properties in respect to stiffness, strength, toughness and resistance to fatigue (Dirks et al. 2013; Dirks and Taylor 2012a; Vincent and Wegst 2004). During insect movement, the exoskeleton experience short-term higher mechanical loads leading to highly adapted material properties. However, how insect exoskeleton reacts to long-term higher mechanical stress is so far unknown. Considering the Utah paradigm of
Frost (2001), possible reactions of cuticle to applied higher mechanical load could be material “disuse”, “adaptation” or “overload”. Katz and Gosline (1992) suggested that the insect exoskeleton, which also functions as an integument and is not as mineralized as bone, can react to mechanical loads in fundamentally different ways than vertebrates. The aim of this thesis is to investigate possible reactions of insect cuticle under long-term increased mechanical load.

H0: Insect cuticle, as a biological material, reacts to long-term applied higher mechanical load.
To address this hypothesis, four studies were conducted:

How to measure insect cuticle biomechanical properties?
The insect exoskeleton build from cuticle shows a huge variety of forms, functions and properties. First, a general overview of known exoskeleton biomechanical properties is given to address this highly diverse material in a proper way. In contrast to the strongly investigated properties of insect cuticle, there is very limited coherent data on cuticle mechanical properties available. Previous studies have used a wide range of mechanical tests and sample treatments, insect species and body parts as well as incoherent terminology. The research has lead to a review of previous studies to examine a proper way to study cuticle properties for my aim to investigate possible cuticle reactions to long-term applied higher mechanical load (Chapter 2).

How can cuticle morphology be visualized in high detail?
Katz and Gosline (1992) pointed out that measurement of external dimensions alone do not provide sufficient information to determine material mechanical behavior. X-ray micro-tomography (µCT) is a tool to investigate destruction free 3D structures. Nowadays this method is widely used for morphological and developmental studies of insects and other organisms (Bäumler and Büsse 2019; Lösel et al. 2020; Parsons et al. 2008a). When it comes to µCT investigations, one major problem is low contrast of the specimen, which -so far- does not allow to distinguish between different types of cuticle and soft tissue (Betz et al. 2007; Beutel et al. 2008; Friedrich and Beutel 2008; Hörnschemeyer et al. 2002; Metscher 2009; Mizutani et al. 2008, 2007; Ribi et al. 2008). In this study, a novel staining protocol for X-ray micro-tomography, which combines histological procedures and semi-automated analysis algorithms, shows exocuticle and endocuticle directly in
the 3D environment of a µCT scan for the first time (Chapter 3).

If and how does insect cuticle react to long term applied mechanical load?

Contrary to highly researched biomaterials, like wood or bone, less is known about insect exoskeleton possible reactions towards long-term higher mechanical load. To investigate possible cuticle reactions, insects were raised in hyper gravity using a custom build centrifuge. After final molt, locust (*Locusta migratoria*) were placed in 3g, 5g or 8g hyper gravity for two weeks. Survival rate, body mass, biomechanical properties (Young’s modulus and bending strength) and thickness of exo- and endocuticle were measured and compared to a 1g control group. Further an “one hour lunch break” was introduced for 3g, 5g and 8g treatment, where three times a week the centrifuge was stopped for an hour. Results of this study show first evidence of insect exoskeleton reaction towards increased mechanical load (Chapter 4).

**Insect exoskeleton under mechanical and light induces stress**

Additionally to the mechanically stressed locust, insects were stressed with constant daylight at 1g to test for “general” exoskeleton stress reactions. Survival rate, body mass, biomechanical properties and cuticle thickness of light induced stressed cuticle were measured and compared to mechanically stressed cuticle. In summary, the performed treatments and tests give a first overview of insect exoskeleton properties under light induced and mechanical induced stress (Chapter 5).
Chapter 2

Biomechanics of insect cuticle – an interdisciplinary experimental challenge
Abstract

The cuticle exoskeleton plays a key role in facilitating the evolutionary success of insects. Since the mid of the last century many different biomechanical properties of exoskeletons have been investigated, always utilizing the most sophisticated scientific methods available at the time. So far, information on the biomechanical properties of cuticle seems to be as diverse as the methods used to measure them. As a consequence, insect cuticle is often considered to exhibit the most complex and diverse biomechanical properties of any biological material. However, it remains unclear which role the respective measurement methods and sample treatments used in previous studies play in supporting this claim. This review provides a broad overview of examination techniques used to study biomechanical properties of insect exoskeletons and discusses their respective advantages and disadvantages in describing the properties of a complex material such as cuticle. Our meta-analysis of the present data confirms significant effects of the respective measurement methods, sample treatments and body parts on the obtained mechanical properties. Based on our findings we highlight research gaps and point out important factors which should be taken into account in future studies on insect cuticle.
Chapter 3

Semi-automated differentiation of insect exo- and endocuticle in X-ray microtomography
Abstract

One of the most versatile and complex biological composite materials, the insect exoskeleton shows a huge range in biomechanical properties. The cuticle exoskeleton can be differentiated into two main histologically different layers with distinct properties: the outer, more sclerotized exocuticle and inner, softer endocuticle. For most biomechanical research questions, it is of great importance to be able to selectively characterize geometrical features of these layers. However, most conventional preparation methods (cross-sections, histological staining, SEM) require complex and destructive sample preparation, which provides only two-dimensional information. Here, we present a novel, simple staining method using X-ray microtomography to distinguish between exo- and endocuticle in a 3D environment without sample destruction. We illustrate the power of our method using locust (Locusta migratoria) hindleg tibia, a well characterized biomechanical sample. Our method allows an easy and direct measurement of exo- and endocuticle and their respective geometric features. Applying our method will help to understand the biomechanical role of exo- and endocuticle within an insect exoskeleton and will allow us to understand its composition and morphological features in more detail.
Chapter 4

Insect cuticle reacts to mechanical stress
4.1 Abstract

A typical feature of many biological materials is their ability to adapt to mechanical load. Bone remodeling for example is stimulated by deformation whilst trees can react to increased load by formation of compression or tension wood. This ability allows for efficient investment in building material, as it is only deposited when biomechanically needed. However, it is still not known whether cuticle exoskeletons of insects, one of the most common biological materials, also show the ability to remodel under increased mechanical load. For the first time, we show direct experimental evidence that increased mechanical load affects the biomechanical properties of an insect exoskeleton. Locusts were raised for several weeks in a custom-designed centrifuge at various levels of increased gravitational load. Comprehensive biomechanical measurements and high-resolution X-ray microtomography show that up to 3g load, the Young’s modulus and bending strength of the tibiae increased. Higher gravitational loads however decreased the survival rate, body mass and endocuticle thickness. Our results clearly show that cuticle exoskeletons are capable of direct reactions to mechanical load, which has so far only been known for bone endoskeletons and plants. These findings are not only a starting point for fundamental questions regarding the proximate mechanisms behind this ability of cuticle exoskeletons. But also add important context to the discussion on general factors in the evolution of adaptive biological materials.

4.2 Introduction

For an organism it is crucial to adapt and react to its environment. Any environment includes several stress factors, e.g. resources, mating, predators. These factors influences and determine larval growth and the timing of adult emergence in holometabolous insects (Abrams et al. 1996; Gotthard 2008). Growth, more precisely adaptive growth, can be observed on different length scales. “Wolff’s law” and its expansion by the “mechanostat” model describes three strategies of bone, muscles and wood to react to mechanical stress (Frost 2001; Wolff 1986). Generally biological materials can react in form of material remodeling, disuse, adaptation or overload. For bone this means during a regularly experienced mechanical load
Insect cuticle reacts to mechanical stress

it maintains its material and repairs micro damage to prevent failure. When the regularly experienced mechanical load changes, remodeling can take place. If the bone is experiencing higher mechanical stress, it increases its overall material and thus performs “overloading”, or it reduces material when less stress is experienced (“disuse”). During these processes, small packages of “basic multicellular units” (BMU) are active on the bone to perform the required adaptations (Jee 1989). For plants it is known that they can increase their material and build so called “reaction wood” (“overload”), but are not able to reduce their material (Donaldson and Singh 2016).

In contrast to these intensely investigated materials, properties of the second most common material worldwide, insect cuticle, remain so far unknown. Generally, the insect cuticle exoskeleton is known to show a huge range of biomechanical properties (Stamm et al. 2021), which are determined by the chitin fiber orientation, cross-linking and hydration of the protein matrix, embedded metals and minerals, amount of exo- and endocuticle and structure shape (Andersen et al. 1996; Fontaine et al. 1991; Parle et al. 2017). The chitin fibers are hierarchical arranged in the protein matrix and can be deposited in daily growth bands with alternating fiber orientation (Neville 1965b, 1983; Vincent and Wegst 2004). The exoskeletons of insects experience a variety of mechanical stress on a daily basis. Besides changing body position causes pressure changes through the body, which are controlled by principles of fluid mechanics (Full and Tullis 1990; Hicks and Badeer 1992; Lillywhite 1987; Lillywhite and Donald 1994; Pilowsky and Goodchild 2002; Seymour et al. 1993). Previous studies have indeed shown that cuticle is able to temporarily react to localized mechanical load, presumably due to thixotropic material properties (Bayley et al. 2012). Additionally, insect exoskeleton material properties may be modulated differently in order to meet mechanical demands of increasing body size (Katz and Gosline 1992). During cyclic loading tests, Parle et al. (2017) for example could observe that the Young’s modulus of a locust hind leg was affected by short term cyclic loading, which could be a temporary passive reaction of the cuticle. Thus, it seems likely that during their ontogenesis, insects might be able to alter the properties of their cuticle exoskeleton as a reaction to changes in mechanical stress. However, there is still no experimental evidence whether insect exoskeletons possess this ability when subject to long term mechanical loading. To answer this fundamental question, we designed an experimental setup in which adult locusts were subject to continuous mechanical stress for several weeks. We then performed a comprehensive biomechanical and morphological characterization of the hind leg tibia as a representative body part.
Insect cuticle reacts to mechanical stress

4.3 Material & Methods

4.3.1 Animal keeping and treatments

Locust (Locusta migratoria, Linneaus 1758) were bought at fifth instar and kept in a controlled 12h day: night cycle till final molt. Insects were fed with fresh plant and dried cereals material ad libitum. To apply continuous levels of mechanical stress over a long period of time, freshly molted locusts were raised in a custom build centrifuge setup (with identical environmental conditions) under 3 g, 5 g and 8 g load (see section A.1, Table A.1). To test for a possible effect of reduced feeding due to increased stress levels, we performed additional centrifuge experiments with 1-hour “lunch break” mid-day. To differentiate between complete physiological stress on the entire organism (including diffusion, etc.) and selective mechanical load on the exoskeleton, we also equipped locusts with backpacks on their pronotum with equivalent mass to the increased mechanical stress experienced in the centrifuge. The backpacks were attached as close to the center of gravity as possible (Pekin and Chichkov 2008). For all locust, pre-treatment and post-treatment weight and survival during treatment was documented. Sample size of all experiments is documented in each plot.

4.3.2 Cantilever bending

Two weeks after their final moult, the locust hindleg tibia were cut off directly at the femur–tibia joint and tarsal segments were cut off as close as possible from the tibia and biomechanically characterized using established protocols (Dirks and Taylor 2012a). Samples were either tested immediately or temporarily frozen at −20 C, which has no significant effect on the biomechanical properties of hind leg cuticle (Aberle et al. 2017). The proximal 5 mm of the tibiae were immediately embedded into a customized sample holder filled with fast curing dental cement (Simplex ACR308, Kemdent, Swindon, UK). Using a tensile testing machine (22N loadcell, Bose Electroforce Series III) samples were cantilever-bent at a distance of 7-11 mm from fixation point at a constant speed of 15mm * min $-1$ in dorsal-ventral plane till failure (Figure 4.1). As the tibiae are histologically not symmetrically, with different ratios of exo- and endocuticle on the dorsal and ventral side (Stamm and Dirks 2022), we performed deflection in both the dorsal-ventral and the ventral-dorsal direction. Spines on the dorsal side were removed.
Insect cuticle reacts to mechanical stress with a razor blade to allow a precise contact between sample and actuator. Duration of the experiments never exceeded 20 minutes. Young’s modulus ($E$) of the tibia was calculated within the linear elastic region of the force deflection curve, bending strength ($S$) was calculated by using the maximum force supported by the sample before failure.

Figure 4.1: Experimental set up. (a) Photo of spinning centrifuge by Marcus Meyer photography©. Red, dashed box highlights locust in set up. (b) Close up of locust (L. migratoria) by Marcus Meyer photography ©. Red arrow points to hindleg tibia. (c) Hindleg tibia and its orientation along the locust body. (d) schematic measurement set up of one point cantilever bending, where $l_{fix}$ is the distance between the sample holder and actuator, $d$ the deformed distance, when a load ($F$) is applied with constant speed. (e) Typical measurement curve with displacement (d in mm) on X-axis and load in N on the Y-axis. Red highlighted part indicates the linear regression line used for Young’s modulus calculation and blue square indicates the highest measured force before structural failure occurs, which is used for bending strength calculation.
4.3.3 Geometric analysis

Sample geometric features, such as minor and major second moment of area ($I_{\text{max}}$, $I_{\text{min}}$), tube wall thickness and radius ($r$), were taken from µCT measurements (Table 4.1). Following an established µCT protocol, tibia were prepared for XRM scans (Xradia 520 Versa, Zeiss, 80kV) (Stamm and Dirks 2022). Using the BoneJ Plugin (Doube et al. 2010) for ImageJ (V 1.52p, Wayne Rasband, National Institutes of Health, USA) the second moment of area around the major and minor axis ($I_{\text{max}}, I_{\text{min}}$), the max. and min. radius ($R_1, R_2$) and subsequently the mean radius ($R_{\text{mean}}$) of the tibia cross-sections were calculated. The exo- and endocuticle thickness was measured at eight positions along the cross-section.

4.3.4 Statistics

All statistical tests were performed using R Studio software (RCoreTeam 2021). Values which were $\leq 1.5 \times IQR$ were classified as outlies. Treatment effects were investigated using analyses of variance. If test assumptions (homogeneity of variance and normal distribution) were not met, non-parametric Kruskal-Wallis and subsequent Dunn-test with Benjamini-Hochberg adjustment for post-hoc pairwise comparisons were used.

4.4 Results

4.4.1 Survival rate and body mass change

With increasing mechanical stress the survival rate significantly decreased (Kruskal-Wallis test, $\chi^2(9) = 322.03, p > 0.001$; Figure 4.2). The 1g control group showed a survival rate of 76% after two weeks. The 3g group within the centrifuge showed the overall highest survival rate with 81% without lunch break and 77% with a lunch break. During 5g treatment the survival rate decreased to 51% without a lunch break. Introducing a lunch break at 5g however did not change the survival rate (51%). At 8g mechanical stress only 7% of locust survived without a lunch break, but 27% survived with 1 hour lunch break. Survival rate of locusts equipped with backpacks was dramatically reduced in comparison to the centrifuge experiments. After two weeks only 4% of locusts with 3g centrifuge backpacks and 17% of locusts with 5g centrifuge backpacks survived. 8g centrifuge backpacks were tested as well but due to no movement of the animals during the first 4 hours of trial this group was not considered any further. Due to the high mortality rate and observed continuous locomotion problems (e.g. falling to either side,
turning over), all backpack groups were excluded from any further analysis. In all treatment groups except the 8g centrifuge treatment, the animals increased their weight over the two weeks. The mass in the centrifuge 8g animals decreased over time. This is in the agreement with the observation that the centrifuge animals consumed less of the provided food and showed the highest mortality rate. The 1g control group showed the highest increase of body mass over time. In the 5g centrifuge treatment animals with a lunch break had a slightly higher body mass compared to the 5g animals without the lunch break. Overall the body mass and survival rate lie in good agreement with each other, both showing an impact of the higher mechanical stress on the locust.
Figure 4.2: Survival rate and body mass of mechanically stressed locust. (a) *L. migratoria* survival in percent during 2 weeks after final mold. Survival rate of the 1g control (beige) compared to higher mechanical stress of 3g (dark green), 3g with lunch break (light green), 5g (dark blue) 5g with lunch break (light blue), 8g (dark purple) and 8g with lunch break (light purple) centrifugal forces. For increased mechanical load without lunch break the survival rate preliminary increases at 3g centrifugal forces and then rapidly decreases with higher mechanical stress. The lunch break treatments show a “stabilization” of survival rate at 3g and 5g, while the survival rate at 8g strongly increases. (b) Locust body mass (g) changes in different treatment groups during 2 weeks after final mold. The body mass strongly decreases and at 8g the locust even loses mass, when 1g control (beige) is compared to higher mechanical stress of 3g (dark green), 5g (dark blue) and 8g (dark purple) centrifugal forces. The lunch break on 3g (light green), 5g (light blue) and 8g (light purple) increases the body mass at 5g and 8g centrifugal forces.
4.4.2 Morphological analysis

To test whether the centrifuge induced mechanical stress affects the composition of the tibial cuticle, we analyzed the thickness of the exo- and endocuticle (Figure 4.3). Even though the Kruskal-Wallis test showed a significant effect of the treatment on the exocuticle thickness ($\chi^2(7) = 14.672, p < 0.05$), the Dunn Test verified that this is a false significant result and significance values were found to be above the threshold of $p = 0.05$ for the exocuticle thickness of the tested treatments. This was expected, as the exocuticle was deposited before the animals were placed into the centrifuge. The exocuticle of the tested samples had a mean thickness of 2-25 $\mu$m, depending on the location along the circumference of the leg (Neville 1965b; Stamm and Dirks 2022). Analysis of the endocuticle thickness however showed that the introduction of mechanical stress decreased the thickness of endocuticle (Figure 4.3) (Kruskal-Wallis test $\chi^2(7) = 247.378, p < 0.005$). The thickness of endocuticle of the 1g control group was significantly higher than the endocuticle thickness in all treatments, except the 3g centrifuge treatment.

Table 4.1: Geometrical features of mechanically stressed locust hindleg tibia from µCT scans: Max., min. and mean radius ($R_1, R_2, R_{mean}$), second moment of area around the major and minor axis ($I_{max}, I_{min}$) and max. and mean 2d thickness distribution of tibia cross-sections per treatment ($n = 10$ per treatment). Values for the 1g control group were taken from the previous chapter (Chapter 3). All values are mean ± s.d. Treatment group abbreviations: 1g control (1g), 24h daylight (24h), 3g centrifuge with 1 hour lunch break (3g 1h), 3g centrifuge (3g), 5g centrifuge with 1 hour lunch break (5g 1h), 5g centrifuge (5g), 8g centrifuge with 1 hour lunch break (8g 1h) and 8g centrifuge (8g).

<table>
<thead>
<tr>
<th></th>
<th>$R_1$(mm)</th>
<th>$R_2$(mm)</th>
<th>$R_{mean}$(mm)</th>
<th>$I_{min}$ (mm)</th>
<th>$I_{max}$ (mm)</th>
<th>max. 2D thickness (mm)</th>
<th>mean 2D thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1g</td>
<td>0.452±0.151</td>
<td>0.542±0.142</td>
<td>0.498±0.147</td>
<td>0.027±0.073</td>
<td>0.034±0.074</td>
<td>0.146±0.259</td>
<td>0.105±0.027</td>
</tr>
<tr>
<td>24h</td>
<td>0.450±0.016</td>
<td>0.530±0.055</td>
<td>0.490±0.036</td>
<td>0.018±0.003</td>
<td>0.028±0.007</td>
<td>0.126±0.018</td>
<td>0.095±0.019</td>
</tr>
<tr>
<td>3g</td>
<td>0.444±0.022</td>
<td>0.522±0.044</td>
<td>0.483±0.033</td>
<td>0.017±0.003</td>
<td>0.026±0.006</td>
<td>0.122±0.011</td>
<td>0.09±0.019</td>
</tr>
<tr>
<td>3g 1h</td>
<td>0.452±0.024</td>
<td>0.546±0.031</td>
<td>0.499±0.027</td>
<td>0.014±0.003</td>
<td>0.022±0.004</td>
<td>0.092±0.020</td>
<td>0.066±0.014</td>
</tr>
<tr>
<td>5g</td>
<td>0.452±0.026</td>
<td>0.542±0.039</td>
<td>0.497±0.032</td>
<td>0.014±0.003</td>
<td>0.022±0.005</td>
<td>0.088±0.013</td>
<td>0.064±0.014</td>
</tr>
<tr>
<td>5g 1h</td>
<td>0.493±0.129</td>
<td>0.588±0.163</td>
<td>0.541±0.146</td>
<td>0.026±0.046</td>
<td>0.038±0.071</td>
<td>0.075±0.025</td>
<td>0.053±0.011</td>
</tr>
<tr>
<td>8g</td>
<td>0.442±0.024</td>
<td>0.535±0.032</td>
<td>0.488±0.028</td>
<td>0.013±0.003</td>
<td>0.020±0.005</td>
<td>0.086±0.018</td>
<td>0.061±0.014</td>
</tr>
</tbody>
</table>
Figure 4.3: Cuticle thickness distribution on locust hindleg tibia. (a-d) *L. migratoria* hindleg tibia cross-section stained and µCT scanned from different mechanical loads: 1g (a), 3g (b), 5g (c), 8g (d). (e) Exocuticle (orange) and endocuticle (blue) thickness were measured at eight different positions (0-315°) along the leg cross-section, where 0° refers to the ventral side. The 1g control compared to 3g, 5g and 8g mechanically stressed cuticle shows a decrease in endocuticle but not exocuticle. This effect can be observed in the lunch break treatments as well (3g, 5g, 8g, dotted barplots).
4.4.3 Biomechanical properties

Young’s modulus

For the Young’s modulus, no significant differences between dorsal and ventral measurement direction were found (Mann-Whitney-U test, $W = 15522, p > 0.05$). Thus, for further analysis, results of both deflection sides were pooled. Our results show that raising insects in the centrifuge had a significant influence on the measured Young’s modulus (Kruskal-Wallis test, $\chi^2(7) = 46.589, p < 0.05$). The control group showed a Young’s modulus of $3.4 \pm 2.4$ GPa ($\text{median} \pm \text{sd}$), which is in good agreement with previously measured Young’s moduli for locust hindleg tibia (Aberle et al. 2017; Dirks and Taylor 2012a). Increased mechanical load of 3g resulted in an increased Young’s modulus ($5.7 \pm 2.9$ GPa). Higher mechanical stresses of 5g and 8g had a Young’s modulus of $4.0 \pm 2.7$ GPa and $1.8 \pm 0.6$ GPa, respectively. Introducing the lunch break treatments caused a ‘stabilization’ of the Young’s modulus and is comparable between all lunch break treatment groups.

Bending strength

Bending strength results show significant differences between the dorsal and ventral measurement direction and treatment groups (Mann-Whitney-U test, $W = 25785, p < 0.05$; Kruskal-Wallis test, $\chi^2(7) = 75.376, p < 0.05$). In general, the 1g control group had a bending strength of $41.7 \pm 11.5$ MPa on the dorsal side and $16.60 \pm 10.1$ MPa on the ventral side ($\text{median} \pm \text{sd}$). The 3g centrifuge had a higher strength with $67.6 \pm 17.4$ MPa when deflected from dorsal and $32.0 \pm 13$ MPa when deflected from the ventral side. Compared to these results, the treatments with higher mechanical stress, 5g and 8g with and without lunch break, the bending strength decreases on both sides.
Figure 4.4: Biomechanical properties of mechanically stressed cuticle. (a) Young’s modulus of dorsal and ventral *L. migratoria* hindleg tibia. The 1g control (beige) compared to higher mechanical stress of 3g (dark green), 5g (dark blue) and 8g (dark purple) centrifugal forces show a preliminary increase at 3g and a continuous decrease of Young’s modulus with higher mechanical load. Introducing the lunch break on 3g (light green), 5g (light blue) and 8g (light purple) centrifugal forces caused a comparatively similar Young’s modulus across all tested higher mechanical stressed groups (Table A.4). (b) Bending strength of mechanically stressed *L. migratoria*. Dorsal (orange outline) and ventral (black outline) measurements are shown in separated boxplots for each treatment. The 1g control (beige) compared to higher mechanical stress of 3g (dark green), 5g (dark blue) and 8g (dark purple) centrifugal forces shows an increase at 3g and a continuous decrease in bending strength with higher mechanical loads. Introducing the lunch break on 3g (light green), 5g (light blue) and 8g (light purple) centrifugal forces caused a comparatively similar bending strength across all tested higher mechanical stress groups. But the decreasing trend of bending strength with higher mechanical load can still be observed (Table A.5, Table A.6).
4.5 Discussion

In this study the effects of increased mechanical stress on insect exoskeletons were systematically investigated for the first time. Our study presents comprehensive data of the survival rate and body mass as well as biomechanical properties, like Young’s modulus and bending strength, and geometric properties of locust hindleg tibia from insects subjected to long term mechanical stress.

4.5.1 Survival rate and body mass change

Typically, maturing locusts increase their body weight over a period of seven days by 53.8% (Phipps 1950). In our experiments, the results of the 1g control group support these findings, as these insects showed the highest gain of body mass. Our results, however, also clearly show that with higher mechanical stress, the body mass and the survival rate of the locust significantly decreased (Figure 4.1). Tauber et al. (1986) stated that environmental stress can induce quiescence behavior, which ultimately leads to starvation. To check for and minimize any possible effects of hunger or starvation in our study, we therefore performed experiments with a “1 hour lunch break” at mid-day. Indeed, locust from the 5g and 8g treatment with a break increased their mass compared to the same treatment without break. This may be due to reduced stress and/or the possibility to move (easier) to the food source. Surprisingly, the presence of a lunch break further decreased the body mass of 3g centrifuge animals. Additionally, locusts are used to this particular level of external load due to the mating process, where the female carries the male around and thus experiences short-term external load of up to 3g (assuming a general mass of 1.5g per locust). Le Bourg tested drosophila under increased mechanical load and suggested that mild hypergravity could lead to increased body mass, metabolism and life span in insects. This partly contradicts with our results, where the increase of mechanical load decreases body mass and life span within the tested two weeks post-mold. However, Le Bourg also found that hypergravity of 3g is the limit between mild and strong stress for drosophila. This finding might also be true for locust regarding the observed differences in body mass and survival rate between 3g and 5g mechanical load.

To “artificially” apply equal mechanical stress on the insect exoskeleton, weighted backpacks, which are mounted approximately to locust center of gravity, could be utilized. This is a fast and straightforward method, however it lacks the possibility to distribute stress equally on the locust body. Another possibility could be a large centrifuge, where the animals are kept over time.
Geometric analysis

The observed differences in cuticle thickness may be strongly influenced by the observed survival rate and body mass reduction and thus should be viewed carefully. However, the exoskeleton thickness has a huge influence on the material properties (e.g. Young’s modulus and bending strength). Generally, a trend within the thickness of the cuticle is visible. For the exocuticle, no significant differences between measurements were found. On the other hand the endocuticle, which is assumed to be built within two weeks after final mold (Neville 1963a,b, 1983), did show some significant differences between treatments and reduces with increased mechanical stress (Figure 4.3). The 3g centrifuge group showed no significant differences in their endocuticle thickness compared to the control group. The endocuticle itself experienced short-term 3g forces during mating of the animals. Speaking from an evolutionary point of view, this particular mechanical stress is already well known within the insect exoskeleton and thus might explain the endocuticle thickness results for 3g. Contra dictionary, Parle et al. (2017) pointed out that the relative amount of exo- and endocuticle and the structural shape itself also plays an important role and stated that cuticle shape can contribute to rigidity. But in our study, reduction of endocuticle thickness under higher mechanical load did not affect all treatments. The material properties are similar between all treatments, except in the 3g treatment. However, changes in endocuticle thickness automatically result in changes of the moment of area, which plays a fundamental role in the measured biomechanical properties (Table 4.1, Figure 4.4). Considering this, the primary increase of Young’s modulus and bending strength at 3g as well as the comparable range of the other increased load treatments are remarkable and indicate an adaptation reaction within the cuticle.

4.5.2 Biomechanical properties of stressed cuticle

Our results clearly show that raising insects under increased mechanical load significantly affected the biomechanical properties of the cuticle exoskeleton. Whilst the Young’s moduli and bending strength of the 1g control treatment are in good agreement with previously reported values for locust hindleg tibia (Dirks and Taylor 2012; Li et al. 2020; Parle and Taylor 2017; Stamm et al. 2021), a mechanical load of up to 3g significantly increased the Young’s modulus of the tested tibiae. Higher mechanical stresses of 5g and 8g however decreased the Young’s modulus again. For the 5g and 8g stress-level, the presence of a short-time break increased the respective Young’s modulus of the leg cuticle. The physiological reasons for these changes are still unclear. A mechanical load of 3g is common during mating
Insect cuticle reacts to mechanical stress

and thus cuticle might already have some kind of acclimatization processes for this particular stress level. In addition, as suggested by Le Bourg (2015), slight hyper gravity might a still undetermined positive general influence on the insect, which might lead to the changed cuticle properties reported in our study. Our results of insects raised with a lunch break further support this hypothesis, where for each stress level insects with a higher body mass and a higher survival rate also showed a higher respective Young’s modulus (Figure 4.4). A very similar pattern can be found for the bending strength of the tibia cuticle. In general, the dorsal and ventral side of the tibia showed a significantly different bending strength. This might be due to the different thickness distribution of exo- and endocuticle in dorsal-ventral plane as well as the methodological required removal of spines on the dorsal side. However, the pattern follows the effect observed for the Young’s modulus. The bending strength of the 3g centrifuge treatment increased, but with higher loads the bending strength decreased again. This is also true for the lunch breaks, which always notably increased the respective bending strength of each treatment stress level.

4.5.3 Overall Discussion

In summary, our results clearly show that the cuticle found in the hind leg tibia of locusts reacts to higher mechanical stress. This is the first time that this kind of reaction is experimentally shown for insect exoskeleton. Our study further shows that the biomechanical properties, Young’s modulus and bending strength, primarily increase at 3g and decrease with higher mechanical forces, respectively. The lunch break experiment confirmed that the observed effects in the centrifuge treatments are not a result of starvation effects, however, closely linked to the experienced mechanical stress. Histological characterisation of exo- and endocuticle show an increase of the endocuticle layer thickness to 3g with lunchbreak. With higher mechanical load the endocuticle thickness decreased again. The exocuticle thickness did not change over the tested treatments. This was expected, as the exocuticle was deposited before the animals were placed into the centrifuge. Prior studies have shown that locust can experience accelerations of 15 g during jumping (Bennet-Clark 1975; Dettner and Peters 2011). Thus, locust body and hindleg tibia might be already adapted towards short-term “bursts” of higher mechanical load but not towards a constant load. This lies in good agreement with our measured changes in Young’s modulus and bending strength (Figure 4.4). Considering the body mass reduction of the 8g animals, the lunch break had a remarkable effect. Previous studies have shown that food quantity and resting period after
feeding are positively correlated with the time spent without feeding. Thus, it is reasonable to assume that locust in the lunch break groups show stress-correlated but not starving-correlated responses (Dkhili et al. 2019). This aligns well with the observed weight gain and subsequently increased survival rate. The animals are still for about 98 % of their time in the centrifuge with the lunch break and thus these observations might be more reliable due to the increased survival rate and body mass. To further investigate possible long-term mechanically stressed cuticle it would be sensible to keep several instar molds or generations in the centrifuge. A main interference to test this hypothesis using our set up would be that freshly molted, soft animals would physically not be able to withstand the centrifugal forces. Preliminary experiments have shown that 73.59 % subadult locust die at a 5g centrifuge treatment, while at 1g 40.18 % of subadult die during three weeks of trial. Thus, it is not reasonable to test for long-term, even generational insect adaptations within the centrifuge.

4.6 Conclusion

For the first time, the insect exoskeleton cuticle material (e.g. Young’s modulus, bending strength) and structural properties (thickness of exo-, and endocuticle) were investigated under higher mechanical load and showed significant influences, respectively. Nevertheless, these results lead to more, fascinating questions: Are there behavioral changes in the insects? Can muscles or joints adapt to higher mechanical load? Prior studies have noted that the thin walled morphology of locust hindleg tibia achieve large volumes, which are only limited by buckling (Currey 1980; Katz and Gosline 1992). Considering the observed changes in exoskeletal thickness, now there might be more space for larger muscles etc., which needs to be tested in a large, randomized-controlled trial. Also, it remains unknown which role gravity sensors are playing in the conducted study. As they are linked to physiological responses, they could be linked to the control of cardiorespiratory functions (Harrison et al. 2020) and influence the development of cuticle in general. Another option might be to add splints or small prosthetics to specific body parts to increase mechanical stress locally to provide an insight into specific body part adaptation possibilities. Future research might explore various insect species like leaf cutter ants, who are famous for their ability to carry load twice as large as their own body weight. These insects might already have developed mechanisms to react to higher mechanical stress on a short-term time scale, but how would they react to high mechanical load on a long-term time scale? Overall, findings of this study may help to understand more about the reaction of cuticle to mechanical...
stress and thus the properties of the second most common biomaterial worldwide.

4.7 Outlook

For the first time, insect exoskeleton possible reactions towards higher mechanical load were investigated. Results indicate a reaction of the exoskeleton at 3g. Mechanisms, which trigger this reaction, are so far unknown. Also it remains unclear if the observed different properties are specific to mechanical load as a stress factor. To test if cuticle reacts in a specific or in a more “general” way towards mechanical stress, cuticle, which is stressed by another factor -light-, will be compared against the mechanically stressed cuticle.
Insect cuticle reacts to mechanical stress
Chapter 5

Mechanical and light induced stressed cuticle
5.1 Abstract

In order to survive, every organism must react to variations of environmental conditions. These changes can be due to biotic (e.g., symbionts, parasites) or abiotic (e.g., light, mechanical stress) factors. An easy to control and quite well understood abiotic factor in insects is light. During 24h daylight exposure for at least two days, the circadian clock of locust and its chitin lamellogensis are uncoupled. Here, insects experience another form of stress and exoskeleton composition changes. Neville (1965a) has shown that only non-lamellate cuticle is build under 24h daylight. Results of Chapter 4 show that the exoskeleton also reacts to mechanical stress in the form of increased gravitational forces of 3g. But are the properties of light and mechanically stressed insect exoskeletons similar or are there differences?

Locust (\textit{L. migratoria}) were raised in a controlled 12h day:night cycle at 1g and 3g as well as in a light induced stress environment with 24h daylight exposure at 1g. Results show that light induced stressed locust have an increased survival rate and body mass compared to the control group. Biomechanical and morphological properties of light induced stressed and mechanically stressed cuticle indicate that the Young’s modulus and bending strength are comparatively similar, as well as the thickness of exocuticle and endocuticle. Even though the mechanisms behind the reaction of the 3g stressed cuticle are so far unclear, both abiotic stress factors have shown that cuticle is able to react towards stress. In summary, the results suggest that cuticle can have a “general stress response” and might react similar to other biotic and abiotic stress factors.

5.2 Introduction

Environmental conditions are defined by biotic factors, like parasites and predators, and abiotic factors, like mechanical stress, humidity and light, among others. Both factors can induce stress and can significantly influence insects (Khaliq et al. 2014). These factors can not only affect insects behavior but also disturb physiological mechanism, like their activity pattern, ovulation, development, survival rate and immune response (Hori et al. 2014; Khaliq et al. 2014; Overgaard and Sørensen 2008; Saunders 2002). The daily cycle of light and dark is a major abiotic environmental factor and plays an important role during insect exoskeleton development.

Generally, the exoskeleton is build out of three layers: A waxy, outer “epicuticle”, an exocuticle, and a more softer inner endocuticle. Neville (1965b) has shown
that endocuticle chitin lamellogenesis is controlled by the circadian clock, which is coupled to daily environmental changes (temperature, light). Assuming a 12h day:night cycle, during cuticle development (first 2-3 weeks after molting) endocuticle builds a layer of chitin crystallites, which is organized into several lamellae at night, whereas at day the layer is non-lamellate, while containing the same relative quantity of chitin. This mechanism can be uncoupled when locust are kept under constant light during cuticle deposition, resulting in a non-lamellate endocuticle. Light intensity and wavelength are key factors to uncouple the chitin lamellogenesis (Neville 1965a). A more recent study from Hori et al. (2014) has shown that different wavelength can have lethal effects on *Drosophila melanogaster* pupae. Depending on the utilized wavelength, puppa mortality rate increased or decreased.

In summary, light cycle or spectrum changes can cause stress in the insects and changes in the cuticle composition. Considering results of the previous chapter (Chapter 4), it was shown that another abiotic stress factor, mechanical load, stresses the animals and changes cuticle composition as well. When locust are exposed to 3g for two weeks (12h day:night cycle), the biomechanical properties, e.g., Young’s modulus and bending strength, increase. So far, the biomechanical properties of 24h daylight stressed cuticle are unknown. However, both abiotic stress factors do influence the insect and its exoskeletal composition. Thus, it is unclear if the observed changes in mechanical and or light induced stressed cuticle are unique. Does 24h daylight stressed cuticle differ in its biomechanical properties? Or is there a “general” reaction of cuticle towards all types of abiotic stresses (light, mechanical load)?

In this study, results from the previous study (Chapter 4) will be compared to morphological and biomechanical properties of 24h daylight stressed cuticle, in order to gain more knowledge about cuticle reactions towards abiotic stress factors.

### 5.3 Material & Methods

#### 5.3.1 Induced light spectra and intensity

To ensure unintentional changes during chitin lamellogenesis, all light sources must provide the same light intensity and spectra. Consequently, only LED from the same manufacture were employed (Lucky Reptile Mini Light Strip LED set, Artikel-Nr. RK0914). In order to control the LED’s efficiency, the light spectrum of LED stripes was measured using a spectrometer (Ocean Optics, LD Didactic USB Compact-Spectrometer (USB2000), resolution: 1.3 nm) in a controlled, dark
environment. The LED stripes were mounted onto a custom designed holder with marks for thirty-two measurement positions every 0.5 cm. LED were measured at each position. To compare the light spectra of the LED stripes, an overall mean spectrum was calculated and each position spectra were adjusted to the local maximum.

Further, light intensity in insect cages was measured using a luxmeter (Lunasix 3, Firma Gossen) at three different position around the insect cages.

5.3.2 Animal keeping

Locust (*Locusta migratoria*, Linnaeus 1758) were bought at fifth instar and after final moult kept for two weeks in either 12h day:night cycle at 1g or 3g using a costum build centrifuge, as well as in a 24h daylight cycle at 1g. Data for 12h day:night at 1g and 3g were taken from the previous chapter (Chapter 4). Animals were fed three times a week (fresh plants, dried cereals) *ad libitum*. Biomechanical measurements were performed on left and right tibia, directly cut off below the femur-tibia joint and before the tarsal segments. Sample size of all experiments is documented in each plot.

Cantilever bending

Sample biomechanical properties were characterized using established protocols (Dirks and Taylor 2012a). Cut off tibia were either tested immediately or temporarily frozen at -20°C, which has no significant effect on the biomechanical properties (Aberle et al. 2017).

For cantilever bending, samples were embedded into a customized sample holder filled with fast curing dental cement (Simplex ACR308, Kemdent, Swindon, UK). Using a tensile testing machine (22N loadcell, Bose Electroforce Series III) equipped with a custom build actuator, samples were bent at a distance of 7-11 mm from fixation point at a constant speed of 15mm * min⁻¹. Samples were tested in dorsal-ventral plane till failure. Ventral side corresponds to the side where the actuator got in contact with the sample and subsequently the sample was bend from ventral to dorsal side. Spines on the dorsal side were removed with a razor blade to allow a precise contact between sample and actuator. Duration of the experiments never exceeded 20 minutes. Finally, Young’s modulus ($E$) and bending strength ($S$) were calculated (Equation A.2, Equation A.3).
Morphological analysis
Locust hindleg tibia were prepared for XRM scans (Xradia 520 Versa, Zeiss) following an established staining protocol (Stamm and Dirks 2022). Cuticle thickness, second moment of area around the major and minor axis ($I_{\text{max}}$, $I_{\text{min}}$), the max. and min. radius ($R_1$, $R_2$), and subsequently the mean radius ($R_{\text{mean}}$) of the tibia cross-sections were calculated.

5.3.3 Statistics
All statistical tests were performed as described in Chapter 4 using R Studio software (RCoreTeam 2021) and data of all treatments.

5.4 Results
5.4.1 Induced light spectra and intensity
Light of the LED strip added up to 275.57 lux, which is highly above the minimal intensity (107.64 lux, Neville 1967) needed to uncouple the chitin lamellogenesis within the first two days of treatment. Whereas measurements next to the insect cage or next to the blinds reached lower intensities (Table 5.1). Thus, LED stripe light did not influence the chitin lamellogenesis out of its utilized insect cage.
Spectrometer measurements confirm a steady light spectra of the used LED strips (Figure 5.1) for at least 150 days without significant changes over time ($\chi^2(5) = 0, p = 1$; Kruskal-Wallis test).
Figure 5.1: Light spectrum of LED stripe measured for 150 days. Shown is the intensity (%) for measured wavelength (nm) after 0 (red), 30 (olive), 60 (green), 90 (light blue), 120 (blue), 150 (pink) days of non-stop usage. There are no significant differences in the changes of LED light spectrum ($\chi^2(5) = 0, p = 1$; Kruskal-Wallis test).

Table 5.1: Light intensity of used LED stripes in insect cages at three different measurement positions. Each insect cage was equipped with two LED stripes. The measured mean value of 273.57 lux for one LED stripe is highly above the needed value to uncouple the chitin lamellogenesis within the first two days post-mold (107.64 lux, Neville 1967). The light intensity measured next to the blinds, which separates the 24h treatment and 12h treatment, is neglectable and does not influence the chitin lamellogenesis.

<table>
<thead>
<tr>
<th>Lux ($lm/m^2$)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>273.57</td>
<td>directly under LED stripe</td>
</tr>
<tr>
<td>32.5</td>
<td>next to insect cage</td>
</tr>
<tr>
<td>4.73</td>
<td>next to blinds</td>
</tr>
</tbody>
</table>
5.4.2 Survival rate and body mass change

Survival rate of both, 3g centrifuge and 24h daylight treatment, increased slightly compared to the 1g 12h day:night cycle control group. However, the treatment does not significantly influence the survival rate (Table A.2). Even though, locust body mass of 3g treatment decreases compared to 1g control and 24h daylight treatment, no significant differences could be found between treatment and body mass (Table A.3, Figure 5.2). Concluding that mechanical and light induced stress do not significantly influence the vital parameters.

![Figure 5.2: Survival rate and body mass of mechanical and light induced stressed locust. (a) Shown is the survival rate in percent for three different treatments. The 1g control (beige), the 3g centrifuge (green) and the 24h daylight treatment (orange). For both, the 3g and 24h daylight treatment, the survival rate slightly increases. (b) Body mass gain (g) over two weeks of treatment at 1g (beige), 3g (green) and 24h daylight (orange). The 3g treatment gains comparatively less body mass than the 1g and 24h daylight treatment.](image)

5.4.3 Biomechanical properties

Young’s modulus and bending strength

Measurements of the Young’s modulus were not significantly influenced by measurement direction (Mann-Whitney-U test, \( W = 15522, p > 0.05 \)), and thus dorsal and ventral side were grouped together for further analysis. An increase of Young’s modulus’ median in the 3g treatment as well as the 24h daylight treatment compared to the control group could be measured (Figure 5.3). For the control group, a Young’s modulus of 3.4 ± 2.5 GPa (median ± sd), for the 3g treatment 5.7 ± 2.9 GPa and for the 24h daylight treatment a 7.2 ± 2.2 GPa was measured. Thus, the 24h daylight treatment showed the highest measured value for Young’s modulus so far. Differences between Young’s modulus and treatment were not significant.
A similar pattern can be observed for the bending strength. Here, the measurement side had a significant effect on the bending strength (Mann-Whitney-U test, $W = 25785, p < 0.05$). Thus, results are presented for dorsal and ventral side (Figure 5.3). In general, the dorsal side had a higher bending strength than the ventral side within each treatment. The 24h daylight treatment had a bending strength of $74.9 \pm 15.8$ MPa (median ± sd) on the dorsal and $47.0 \pm 13.3$ MPa on the ventral side. For the 3g treatment, a bending strength of $67.3 \pm 20.4$ MPa was measured at the dorsal side and $32.0 \pm 13.0$ MPa at the ventral side. The 1g control group has a bending strength of $41.7 \pm 11.5$ MPa on the dorsal and $16.6 \pm 10.1$ MPa on the ventral side. For both measurement directions, an increase in median bending strength from 1g control to 3g centrifuge to 24h daylight treatment can be observed. The highest bending strength was measured in the 24h treatment, even though the differences between bending strength and treatment are not significant (Table A.5, Table A.6).
Figure 5.3: Biomechanical properties of mechanical and light induced stressed cuticle. (a) Young’s modulus of 1g control (beige) and 3g treatment (green) with a 12h day:night cycle and 24h daylight treatment at 1g (orange). Their are no significant differences between the measurement direction and the Young’s moduli and treatment (Kruskal-Wallis test, $\chi^2(1) = 0.127, p > 0.5$, Table A.4). However, an increase in Young’s modulus for the 3g treatment as well as the 24h daylight treatment is visible, when compared to the 1g control group. (b) Bending strength of the 1g control treatment (beige), the 3g treatment (green) and the 24h daylight treatment (orange). Here, significant differences between measurement direction were found (Kruskal-Wallis test, $\chi^2(1) = 79.955, p < 0.001$) and thus dorsal (orange outline) and ventral (black outline) results are shown separately. Generally, dorsal measurements resulted in a higher measured bending strength. But for both measurement directions, an increase of bending strength in 3g centrifuge and 24h daylight treatment can be observed compared to the 1g control treatment. Both, Young’s modulus and bending strength, are slightly influenced by treatment and increase compared to the control group.

5.4.4 Morphological analysis

Thickness measurements of exo- and endocuticle along the hindleg tibia cross-sections showed no significant differences between treatments (Table A.7). The exocuticle thickness varies as previously described along the cross-section, but its thickness at each position is similar in all treatments (Chapter 4). Here, the same differences in endocuticle thickness distribution can be observed. It varies along the tibia cross-sections, but there seems to be no differences in between the tested treatments (Figure 5.4).
Figure 5.4: Cuticle thickness distribution of mechanical and light induced stressed locust hindleg tibia. Treatments from left to right are, in a 12h day:night cycle, 1g control (1g), 3g centrifuge (3g) and, in a 24h daylight cycle, 1g (24h, striped barplot). Thickness in µm of exocuticle (orange) and endocuticle (blue) was measured at eight different positions (0-315°) along tibia cross-section from stained µCT scans. Even though endocuticle of 24h daylight treatment is build differently (Neville 1967), results show no significant differences of cuticle thickness distribution along the leg between treatment groups.
5.5 Discussion

5.5.1 Induced light spectra, survival rate and body mass gain

Light irradiation can influence the survival rate as well as the chitin lamellogenesis in insects (Hori et al. 2014; Neville 1965a). Considering the high survival rate of all tested treatments, the utilized LED had a for insects beneficial light spectra. Unintentional changes in chitin lamellogenesis can be excluded due to no significant changes of light spectra and intensity over time, as measured at six time points with an interval of thirty days.

As suggested by Khaliq et al. (2014), biotic and abiotic factors can significantly influence the insects and their population dynamics. Our results support these hypothesis. The survival rate of the mechanically and light induced stressed locust increased slightly compared to the control treatment. Further, the body mass gain over the 2 weeks of treatment has shown that the 3g centrifuge treatment has the lowest body mass gain. 1g control and 24h daylight treatment have a similar body mass gain. As mentioned in Chapter 4, the reduced body mass in the 3g centrifuge treatment might be due to the increased mechanical stress as well as the increased energy consumption during movement at 3g.

5.5.2 Morphological analysis

No significant differences between the exo- and endocuticle thickness could be found between the tested treatments (Table A.7). Considering the reduced body mass of the 3g centrifuge treatment, it is an interesting finding that the cuticle thickness distribution seems not to be influenced by the reduced body mass. Further, the thickness distribution and increased biomechanical properties of mechanically and light induced stressed cuticle indicate that their might be a general response of cuticle towards abiotic stress.

Our results support the theory of exocuticle as the main stress distributor during mechanical testing (e.g., cantilever bending). Changes in the endocuticle chitin lamellogenesis due to 24h daylight treatment, which are not happening at the 3g centrifuge treatment, did not result in significant differences between these two treatments even though the biomechanical properties increase compared to the control group. Also, both types of stresses do not influence the endocuticle thickness.
5.5.3 Biomechanical properties of mechanically and light induced stressed insect cuticle

Slight differences between the 1g, 3g and 24h daylight treatment could be observed, even though there are below the significance threshold. For both, Young’s modulus and bending strength, the 24h daylight treatment had the highest measured values (Figure 5.3). Considering the changes in chitin lamellogenesis due to the light treatment, it is remarkable that the biomechanical properties increase. Regarding the possible stabilization of the three-dimensional chitin framework by lamellae, the biomechanical properties of endocuticle might not be measurable with cantilever bending but with torsion test or, as mentioned above, the exocuticle plays a major role in mechanical stress distribution.

5.5.4 Overall discussion

Even though there are no significant differences between the 3g and 24h daylight treatment, slight differences can be found. Excluding the reduced body mass of the 3g treatment, both treatments, 3g and 24h daylight, had a slightly increased survival rate as well as an increase of biomechanical properties (e.g., Young’s modulus and bending strength) compared to the control group (Figure 5.2, Figure 5.3). Regarding the thickness distribution of exo- and endocuticle along the tibia, no significant differences can be found in both cuticle layers. This results indicate that there could be a general stress response of the cuticle, which is not depended on the body mass gain. The difference in endocuticle regading 3g with a 12h day:night rythm and the 24h daylight treatment seems not to influence these parameters. This might be further evidence for the exocuticle role in mechanical stress distribution during cantilever bending test.

In summary, so far it is possible that cuticle does have a “general” stress response and thus the measured values for 3g and 24h daylight are similar increased compared to the 1g control group. Our hypothesis that cuticle biomechanical properties change due to a stress reaction of the exocuticle, could be verified in the current sample as well. Thus, not only mechanical stress but also light induced stress seem to influence the exocuticle. To verify this hypothesis, more tests would be needed in form of a randomized-controlled 2x2 intervention study, including 1g and 3g at 24h daylight and a 12h day:night cycle. Do the biomechanical properties change? Are they comparable to 3g and 24h daylight stressed cuticle or are they different? How does the composition of 3g 24h daylight cuticle change?
5.6 Conclusion

Different abiotic stress types (e.g., mechanical load and light induced stress) influence insect exoskeleton properties. Due to changes in microstructure of the light induced stressed cuticle, which are not present in mechanically stressed cuticle, both treatments are difficult to compare. Both seem to have similar biomechanical properties in response to the stress factor, but so far it is not possible to identify the mechanisms behind these stress reactions. It is remarkable that the body mass seems not to influence the cuticle biomechanical properties, when responding to stress.

Our results illustrate how important it is to have as detailed information as possible about the environmental conditions under which an insect has grown. These conditions can influence insect development, exoskeleton biomechanical properties and presumably much more.

5.7 Outlook

It was unclear if the observed differences in 3g stressed cuticle are a specific or general response of the insect exoskeleton towards mechanical load as a stress factor. Stressing locust with 24h daylight has shown that also light induced stressed cuticle has different biomechanical properties compared to 1g 12h day:night cycle control group. These findings indicate that there could be a “general” reaction of insect exoskeleton towards stress. This observation as well as all other findings of the conducted studies will be discussed in a broader context in the next chapter (Chapter 6).
Chapter 6

Overall discussion
Within the world of biological materials, the insect exoskeleton is unmatched in its property range and task performance. However, compared to other materials like bone or wood, less is known of cuticle’s possible reactions to increased mechanical load. In this thesis, four studies were conducted to investigate the effect of long-term higher mechanical stress on the insect exoskeleton. The H0 hypothesis, that insect cuticle, as a biological material, reacts to long-term applied higher mechanical load, was approached via a literature review and experimental studies.

The first study sought to determine insect exoskeleton biomechanical properties and the ideal method to investigate such a complex material. There are plenty of measurement techniques with their own advantages and disadvantages. In respect to the first research question, it was found that sample treatment and utilized method influence the results. Further, depending on knowledge about sample geometry and utilized method, results have to be differentiated between structural and material properties. However, for all results, it is important to put them into a functional biological context. So far, there is a huge variety of measured properties, which are difficult to compare or not comparable at all. In summary, the review has shown how important it is to differentiate between material and structural value, measurement technique, sample treatment and geometry in order to interpret the cuticle properties (Chapter 2).

When insights of the sample geometry are needed, μCT scans can provide the desired information in high detail. X-ray micro-tomography is able to deliver the required information in high resolution without sample destruction, but usually lacks the differentiation of exoskeleton layers (e.g. exo- and endocuticle). Thus, a novel staining method was established to visualize exocuticle and endocuticle directly in μCT scans (Chapter 3).

The third study in this project combined the gathered information from the literature review and the novel staining method to investigate the effect of long-term higher mechanical load on insect exoskeleton. Insects were raised under higher mechanical load of up to 8g for two weeks. Using cantilever bending and μCT scans, the biomechanical properties of cuticle under higher mechanical load were determined (Young’s modulus, bending strength, thickness of exo- and endocuticle, Chapter 4).

To ensure that the results of mechanically stressed cuticle are a result of the mechanical stress and not a “general” stress response, another group was introduced. It was shown that light can stress insects as well. Thus, insects were stressed with 24h daylight at 1g and results were compared to the mechanically stressed cuticle (Chapter 5).

All conducted studies aim to answer the hypothesis, that insect cuticle, as a bio-
logical material, reacts to long-term applied higher mechanical load.

### 6.1 How to measure insect cuticle biomechanical properties?

The literature review pointed out that the insect exoskeleton is a highly diverse and complex material, which fulfills a huge variety of functions (Chapter 2). Even though research interest in insect exoskeleton increased over the past decades, the utilized methods and investigated body parts differ between studies. Thus, it is a challenge to summarize the properties of cuticle and subsequently the exoskeleton. Further, the review has illustrated how sample treatment and measurement technique influence the results. Also depending on the method, knowledge of sample geometry is important to get structural or material property values. For example, when nanoindentation is performed the sample geometry does not need to be characterized due to the known shape of the indentor. In cantilever bending, on the other hand, the sample geometry must be known in high detail to calculate material values (section A.2).

For cantilever bending on fresh and frozen samples, it was shown that data have a good reliability and can be compared to other data, when the sample geometry is known. Knowledge about sample geometry can be collected via µCT scans as shown in Chapter 3. In the experimental studies of this thesis, measurements were performed using cantilever bending and µCT scans. Even though the cuticle is not characterized on a nano scale, which would be the case using nanoindentation, material information about the exoskeleton on locust hindleg tibia were collected. Results of the 1g control group have shown, that the data are in good agreement with the previously published studies (Aberle et al. 2017; Dirks and Taylor 2012a). Thus, results can be compared to other studies, who have tested the same body part using the same measurement technique, adding to the information about insect exoskeleton biomechanical properties in a comparable way.

Even though the literature review summarizes results of the past decades and contains about 279 cuticle property values, it is by all means not a complete list of all measured insect cuticle properties. But overall results of the literature review showed that in order to generate comparable data, it is ideal to use fresh cuticle with a known geometry and measuring its properties with classical engineering tests (e.g., cantilever bending). Detailed geometric characterization of a specific body part from any biological organism is a difficult challenge, but necessary to report meaningful biomechanical properties. Thus, either structural or material
values are reported - depending on the complexity of the investigated structure. Here, the nowadays more accessible X-ray micro-tomography can come in handy to visualize various body parts destruction free and highly detailed in a 3D environment.

6.2 How can we visualize cuticle morphology in high detail?

µCT scans are gaining more popularity as a method to investigate morphological and developmental stages of organisms in a highly detailed 3D environment (Bäumler and Büss 2019; Lösel et al. 2020; Parsons et al. 2008b). It is also a more frequently utilized tool to investigate insect exoskeleton. Often insect exoskeleton lack high contrast in µCT investigations and thus it is difficult to differentiate between soft tissue and cuticle layers. Only staining can provide these information of interest within a µCT scan (Betz et al. 2007; Beutel et al. 2008; Friedrich and Beutel 2008; Hörnschemeyer et al. 2002; Metscher 2009; Mizutani et al. 2008, 2007; Ribi et al. 2008).

Via an iodine-methanol staining the exocuticle and endocuticle were visualized directly in µCT scans. The staining caused different grey values for each layer, which can be separated in five post-processing steps. Afterwards, sample geometry can be measured in high detail (e.g. second moment of area ($I_{min}$ and $I_{max}$), mean radius, thickness of exocuticle and endocuticle at eight different positions). Resulting in detailed knowledge of exo- and endocuticle thickness distribution along locust hindleg tibia.

It was shown that the staining worked reliable on locust hindleg tibia. There was no over-staining of the samples and exocuticle was significantly different from endocuticle in the µCT scans. Thickness could be observed in a 3D environment or in 2D cross-sections of the sample. Results of cross-sections could be used for precise calculations of the second moment of area for Young’s modulus and bending strength calculations (section A.2). However, the presented staining methods needs to be evaluated with other specimen and/or body areas. Further, not all cuticle layers can be observed. The thin envelope can not be visualized at the used scan resolution, because its thickness (ca. 4µm) is slightly larger than one pixel (Schwarz and Moussian 2007). Also information about the chitin lamellogenesis within the endocuticle are so far not visible by µCT scans. But µCT scans still provide the highest resolution to measure sample geometric features.

In summary, µCT scans of locust raised under control conditions (1g), under in-
creased mechanical load (up to 8g) and under light induced stress (24h daylight) were performed and the second moment of area, thickness distribution and other geometrical features were measured (Chapter 3, Chapter 5). Resulting in first information of insect exoskeleton properties under long-term increased mechanical load and light induced stress.

6.3 If and how does insect cuticle react to long term applied mechanical load?

The “Utah paradigm” of Frost (2001) is a combination of results and hypothesis from multiple disciplines to describe the mechanical influence on bone architecture. Generally, three reactions towards increased and reduced mechanical load can be distinguished: “disuse”, “adaption” and “overload”. This is well studied for vertebrates and plants. Plants can only add material but can not “disuse” material. However, less is known about the second most common biological material worldwide: insect cuticle (Chapter 1).

In this study, insect exoskeleton was investigated under long-term increased mechanical load, for the first time. A higher mechanical load was achieved by raising the locust in a custom build centrifuge creating an even distribution of mechanical load over the whole body. Three increased mechanical loads (3g, 5g, 8g) were tested and analysed using cantilever bending and µCT scans of stained samples. Results indicate that cuticle does react to an increased load of 3g, but not to any higher load (Chapter 4).

The data has shown, that with a long-term increased mechanical load the survival rate is reduced, and therefore it is not sensible to increase the mechanical stress to more than 8g, where less than 10% of locust survive the treatment for two weeks. Further, the Young’s modulus and bending strength have shown that cuticle seems to react to higher mechanical load between 1g to 3g. Higher mechanical load seems to stress the insects strongly and they do not seem to be able to adapt to this stress. This effect could be reduced by the introduction of the 1h “lunch break” group. The decreasing trend in survival rate and body was was still measured, but compared to the same treatment without the “lunch break”, survival rate and body mass increased. This can be evidence that with longer or more regularly breaks (cyclic loading), the exoskeleton might react different to the mechanical stress. Thus, not only the 3g treatment but also treatments, which experience cyclic loading of increased mechanical load, might show different
Overall discussion

The insect exoskeleton displays a huge variety of forms and functions, making insects a highly diverse group. Hence, some insect species may have developed different strategies for solving skeletal scaling problems and subsequently mechanical stress (Katz and Gosline 1992). For example, leaf cutter ants are well known for their possibility to withstand short-term higher mechanical loads and might show different reactions to long-term higher mechanical load (Püffel et al. 2021). Also when a weevil (Pachyrhynchus sarcitis kotoensis) gets caught by its predator (lizard), it experiences short-term increased mechanical load. But the exoskeleton mechanical properties are just above the bite force stress and thus the weevil gets a chance to escape and survive (Wang 2000). As another example of cyclic loading, the fruit fly (Drosophila viridis) uses a combination of jumping and flapping during take-off. Wings experience only about 5%–10% of the force during take-off jumping while the remaining force is attributed to the legs (Chen and Sun 2014). Switching the perspective from insects to vertebrates, Vij and Mao (2006) have performed cyclic loading experiments on Sprague-Dawley rats. They have shown that cyclic forces can be a potent stimuli for modulating postnatal structural development in rats. Presumably the osteogenesis and osteoclastogenesis are stimulated by this type of loading and may be used in future clinical adaptations. Among others, these findings illustrate the importance of cyclic loading and its impact on a living organism. Thus, cyclic loading experiments and (generally) tests with other species might provide more insights into cuticle reactions under mechanical stress.

6.3.1 Influences of long-term and short-term mechanical load

The effect of mechanical stress on insect exoskeleton could be observed in centrifuge treatment with and without the “lunch break”. In summary, survival rate increased at 3g and afterwards decreased with increasing mechanical stress. The body mass reduced with increasing mechanical stress over two weeks of treatment. Both, survival rate and body mass, could be slightly increased with the introduction of an 1 hour “lunch break”.

The short break of one hour, where fresh food was directly provided, increased the survival rate and body mass significantly. Comparing duration of the lunch break to the time insect spend under higher mechanical stress, the effect of the break was surprisingly “strong”. Nevertheless, locust spend more than 90% under higher mechanical load condition and it was out of scope of the study to find a
possible ratio or balance between increased and reduced higher mechanical load to “create optimal treatment conditions” to observe changes in cuticle properties within a reasonable survival rate. Thus, effects of the “lunch break” are difficult to determine. Experiments with an 1 hour break three times a week, where no food is provided till the centrifuge is turned on again, can help to understand effect of the “lunch break”. Preliminary data of the experiment mentioned above have shown have shown differences in survival rate and body mass gain at 3g and 5g centrifuge treatment with a 1 hour break, where no food is available. At 3g with 1 hour break, the relative survival rate was at 83% and for 5g treatment at 36%. The survival rate of 5g centrifuge with 1h break is noticeably lower compared to the survival rate with and without a lunch break at 5g, while 3g centrifuge treatment with 1 hour break is similar to the 3g centrifuge treatment without any break. Comparing the body mass gain, locust in the 3g treatment with 1h break gained 0.455 ± 0.075 g (mean ± sd.), while locust at 5g with 1h break gained 0.381 ± 0.04 g. Here, the 3g and 5g centrifuge with 1h break gained even more mass than the treatments with and without the “lunch break”. However, results of survival rate and body mass indicate that locust experience some form of stress. Here, opposite statements can be found in literature regarding locust behavior under increased stress: Tauber et al. (1986) stated that stress can induce quiescence behavior, which ultimately leads to starvation, while Ellis and Hoyle (1954) reported that starvation leads to higher movement in locust. More information about (movement) behavior, food consumption and locomotion (e.g. energy expansion) during the treatments are needed to understand the effect of the “lunch break”, “break” and “no break” treatments on the effects of mechanical stress in insect exo- and endocuticle.

6.3.2 Influence of exocuticle and endocuticle on biomechanical properties

As summarized in previous studies, cuticle properties are strongly influenced by sample geometry (e.g. exo- and endocuticle thickness), the amount and orientation of chitin fibers, cuticle constitution (e.g., hardening), degree of cross-linking, hydration within the protein matrix and protein presence itself (e.g., resilin)(Andersen et al. 1996; Dirks and Taylor 2012a; Vincent and Wegst 2004). In the conducted studies, sample geometry, more precisely exo- and endocuticle thickness, were measured of mechanically and light induced stressed cuticle (Chapter 4,Chapter 5). There were no significant differences in exocuticle thickness between all treatment groups (Chapter 4). But with increased mechanical load the thickness of endocu-
ticle reduced. This could also be observed in the lunch break treatments, where the endocuticle thickness is comparatively higher to the treatments without lunch break (Figure 4.3). However, there were no significant differences in exo- and endocuticle thickness between mechanically (3g) and light induced (24h daylight) stressed cuticle. Regarding the higher biomechanical properties of 3g centrifuge and 24 daylight stressed cuticle, these findings indicate that there might be a general reaction of cuticle towards stress.

Parle et al. (2017) pointed out that the relative amount of exo- and endocuticle, as well as the structural shape play an important role and cuticle shape itself can contribute to rigidity. This differs from the findings presented here. Even though the endocuticle thickness is reduced under higher mechanical load, the material properties (e.g., Young’s modulus, bending strength) are similar in all treatments, except in the 3g treatment. Here, the exoskeletal properties are increased compared to the control treatment. Regarding the similar amount of exocuticle, while endocuticle thickness is reduced, result indicate a strong influence of exocuticle in stress distribution and subsequently in measured biomechanical properties. The exo- and endocuticle thickness directly influences the second moment of area, which is used to calculate the material properties (section A.2). But our results indicate that sample geometry created by exocuticle influences the properties more than endocuticle thickness. This hypothesis is supported by the study of Katz and Gosline (1992). They showed no decrement in flexural stiffness within any instar that could be correlated with apolysis. Even though, one or two days before molting in each instar the second moment of area is decreased, due to enzymatic digestion of endocuticle during apolysis (Queathem 1991; Zacharuk 1976).

Another influencing factor on endocuticle thickness can be the internal structures and muscles within the exoskeleton. The weight of muscles and circulatory fluid may not represent the same penalty in vertebrates (acting on their marrow or fat) as in exoskeletal animals. But Currey (1980) suggested, that a thin-walled morphology, which achieves large volume and will only be limited by buckling, can be a locust strategy to maximize internal volumes. Considering our findings of reduced endocuticle resulting in thin-walled tibia, locust muscles, tendons, among others, might be able to increase their material mass within the exoskeleton. Thus, locust muscles or tendons might have different properties when exposed to long-term increased mechanical load.

Further, Parle et al. (2017) suggested that two different cuticle growth rates are
present, which might process the ability to influence cuticle properties, and can be tweaked or altered by load changed during insects' adult lifetime. The underlying principles are so far unclear. Nonetheless, these growth rates might alter within locust of the centrifuge treatment and thus produce differences in cuticle properties or endocuticle thickness. Here, long time experiments of insects raised in the centrifuge as described in Chapter 4 might provide new insights into cuticle growth and development. Within the first 21 days post-mold the cuticle is supposed to be deposited quickly and afterwards cuticle deposition is slowed down. Thus, daily measurements of cuticle thickness in correlation with the time needed to deposit the new cuticle, could show the time it takes for cuticle to react to increased mechanical load. Thickness of cuticle layers can be determined by µCT scans or cross-sections investigated using SEM or polarization light microscopy. The latter two methods would also provide information about the chitin lamellogenesis.

6.4 Comparison of mechanically stressed, light induced stressed and non stressed cuticle

Increased mechanical load acts as a stress factor on the insect regarding survival rate and body mass. Further at 3g, biomechanical properties change compared to 1g cuticle properties (Chapter 4). But so far it is unclear if mechanical stress causes specific changes in insect cuticle or if it is a more “general” stress response of the exoskeleton. Thus, another well understood abiotic stress factor was needed to test against mechanically stressed cuticle. Light and dark cycle, light intensity and wavelength are abiotic factors, which stress insects and can easily be manipulated (2014). The influences of light on insect cuticle was studied in high detail by Neville (Neville 1965a,b, 1967). Therefore, 24 hours daylight stressed locust cuticle (raised at 1g) was compared to mechanically stressed cuticle (3g) and 1g “control” cuticle (12h day:night cycle). Survival rate, body mass and thickness of exocuticle and endocuticle are similar between the 1g treatment and 24h daylight treatment. Measurement results of biomechanical properties increase compared to the control treatment. Both, 24h daylight and 3g centrifuge treatment, increase Young’s modulus and bending strength in a similar range. These results indicate that, even though cuticle experiencing another type of stress, the exoskeleton could react in a similar way (Chapter 5). But are there any differences between mechanically and light stressed cuticle?
6.4.1 Fiber alignment

In locust leg, chitin lamellogenesis and subsequently the fiber alignment is regulated by the circadian clock (Neville 1965a,b). So far it is not fully understood if and how the fiber alignment in insects can change and/or influences the material properties. Neville (1965a) has shown that inhibition of chitin lamellogenesis lowers the structural stability of the chitin framework of locust cuticle. Further, the chitin lamellogenesis cannot be inhibited in all body parts, like wing hinge and compound eye, as well as in the innermost layer of endocuticle and in the exocuticle as a whole. Neville hypothesized that this may be due to its strengthening function for the exoskeleton.

In material sciences it is a common practice to reinforce materials using fibers. For example, to prevent poor behavior under tensile load and sudden failure, reinforcing steel bars or other kinds of fibers, like minerals, glass, polymer or carbon, are introduced. Especially in 3D printing, the introduction of fibers has lead to high flexural strength in samples (Fischer et al. 2019; Hambach and Volkmer 2017). However, fiber reinforcement does not necessarily improves tensile strength. Fibers might be below a “critical length”, or the matrix and fiber bond is “weak” and can not support a full transition of load between materials, resulting in no increase in material tensile strength (Banthia and Sappakittipakorn 2007; Brückner et al. 2006; Larisa et al. 2017; Li et al. 2001; Lochner and Peter 2020). But nonetheless fibers can transmit stresses, which ultimately leads to an overall increase in the material stress–strain response (Rutzen et al. 2021). For example, the addition of stiff and strong carbon fibers in an extrusion-based alignment process of a cementitious composite has lead to an increased flexural strength of 100 N/mm\(^2\) (Hambach et al. 2016). However, due to the fiber alignment, measurement technique and direction should be chosen carefully. Measuring along the aligned fiber direction results in higher tensile and compressive strength than measurements in other directions (Ma et al. 2020). More precisely, in systematic fiber alignment, Rutzen et al. (2021) has shown variations with respect to the direction of applied tensile load, that fibers enhance the flexural strength of test specimens as long as their alignment angle does not deviate by more than 20° from the direction of the acting tensile stress. Thus, fiber alignment and utilized measurement technique play a huge role in measured material properties.

The importance of fiber alignment within insect exoskeleton has also been investigated in previous studies. Dirks et al. (2013) have performed fatigue test on locust hind leg tibia and hind wing. The leg could withstand a higher material strength of up to 30% than the wings. Wing membrane seems not to be reinforced by chitin fibers, but wing cross-veins are acting as crack-stoppers and structure reinforce-
ment, which could increase fatigue resistance as well (Smith et al. 2000). The tibia on the other hand has chitin fibers, arranged in a complex layered pattern, where fibers lying mostly parallel to the longitudinal axis of the leg, which increase their fatigue resistance, as well as axial bending (experienced during jumping) and results in a good crack resistance by parallel loading to grain direction (Dirks et al. 2013; Dirks and Taylor 2012a; Neville 1965a, Chapter 5).

Results of the biomechanical test from the mechanically and light induced stressed cuticle lie in good agreement with these findings. Investigating mechanical and light stressed cuticle under polarization light microscopy, it becomes clear that the endocuticle fiber alignment is not influenced by the mechanical stress. In a 12h day:night cycle, lamellate and non-lamellate layers differ according to the circadian clock, which gets uncoupled when locust are raised under 24h daylight. During 24h daylight, only non-lamellate cuticle is build (Neville 1965a). In the 8g centrifuge treatment (12h day:night cycle), the endocuticle thickness is reduced and less layers are visible in the thin endocuticle of 8g (Figure 6.1). However, the Young’s modulus and bending strength are similar compared to the control group. Thus, a certain material strength is presumably achieved by the exocuticle, regardless of endocuticle composition.
Figure 6.1: Light polarization microscopy of mechanically and light induced stressed cuticle from locust (*L. migratoria*) hindleg tibia. Cross-sections were cut with a cryo-microtome (12 µm) and visualized using polarization light microscopy as described in Chapter 3 and by Neville (1983). (a) High magnification cross-section of 24h daylight treatment tibia cross-section showing exocuticle (orange) and endocuticle (blue). In continuous light non-lamellate endocuticle is build and thus no layers in endocuticle can be observed. Image was adapted from Neville (1965b). (b) Tibia exo- and endocuticle of 1g, 12h day:night cycle treatment in polarization light microscopy. In the endocuticle the different growth layers, as described by Neville (1983), are clearly visible. (c) Cross-section of 8g centrifuge treatment (12h day:night cycle). Even though the endocuticle is significantly slimmer compared to other treatments, the different growth layers are still clearly visible. Scale bar: 100 µm.

Comparing the 24h daylight stressed and 3g mechanically stressed cuticle, they showed similar biomechanical properties, which are higher compared to the 1g control group (Chapter 4). For the 24h daylight stressed cuticle, the fiber alignment might play a role to achieve the different properties. But the differences in endocuticle thickness and fiber alignment of the mechanically stressed cuticle indicate that the exocuticle plays a major role during the performed biomechanical tests (subsection 6.3.2). Exocuticle contribution towards stress distribution within the 24h treatment can not be excluded or clearly separated from endocuticle influence in stress distribution. Thus, it could be possible that the exocuticle has a general response towards stress and biomechanical properties of both, mechanically and light stressed cuticle, increase similar. More detailed investigations regarding exocuticle composition and role in biomechanical/cantilever tests are needed to answer this question. Here, numerical simulations might be a convenient
approach. Cuticle layers and thickness can be altered comparatively easily and influences of each parameter can be investigated separately.

6.5 Future studies

The conducted studies have shown that insect exoskeleton reacts to mechanical stress. But this opens up many more questions:
How is this mechanisms regulated? Is there a “stress threshold”? Are there other reactions in insect body? What if an insect experiences increased mechanical load for longer than two weeks? What happens in the exoskeleton during cyclic loading? And do muscles and subsequently the locomotion changes during long-term increased mechanical load?

6.5.1 Reaction of insect muscles to higher mechanical load

Regarding the Utah paradigm, it seems likely that another reaction to higher mechanical load is the increase of muscle mass. It could be beneficial for locomotion under increased mechanical load to increase muscle mass. But the majority of insects operate close to the maximum volume occupancy and for locust it is assumed that the design in locust legs already maximizes the internal volume (Katz and Gosline 1992). Theoretically, the internal volume can be completely filled with apodeme. But in insects, individual muscle fibers can attach using a single, thin, filament-like process of the apodeme. This type of “filament attachment” can be beneficial due to significant material reduction, resulting in an energy saving and “light weight” construction, which optimizes area utilization (Püffel et al. 2021). Across species the direct and filament-like attachment is different and assumed to display functional specialization (Gronenberg et al. 1997; Paul and Gronenberg n.d.; Paul 2001). However, the apodeme itself needs to have a sufficient cross-section to withstand acting muscle forces without failure (Püffel et al. 2021).

Apart from apodeme attachment, the exoskeleton has only two options in order to increase muscle volume. First, the muscles can be rearranged and increased to make a “more efficient” use of the existing space/ volume. Second, more space is created by building larger structures to increase internal volume (Püffel et al. 2021). Changes in insect muscles to increase force are well studied in leaf cutter ants. Ant workers show substantial, size-specific modifications to internal and external anatomy in order to increase bite force. Püffel et al. (2021) suggested that the bite force is not only altered by size but also by shape differences to avoid
disproportional larger heads (which would limit other functions) or in general more heavier or larger ants (which would also be limiting their task performance), resulting in a forced up-scaling and reinforcement of mandibular cutting force. Changing the perspective from biting to flying, it was shown, that insects are capable of changing their flight muscle mass (Marden 2000; Robertson 1998). This can lead to a “better” flight performance and consequently to successful mating (Marden 1989). In both examples, the exoskeleton must provide enough “space” to increase muscle mass. This can be achieved by providing sufficient attachment sides, which allow the muscle an attachment without fiber shorting.

Taken together, the complex interactions of filament length, apodeme displacement, pennation angle, muscle contraction and average force, are a whole other field of investigations and out of scope of the presented study. Nonetheless it may be worth investigating it in future research. Here, µCT scans may provide first insights. Insects could be raised under increased mechanical load at 3g as reported in Chapter 4 and afterwards stained samples could be scanned in µCT, to visualize muscles and attachment sides in high detail. Here, it would be important to track insects during treatment to exclude behavior differences, which could lead to a different use of muscles. Furthermore, muscle strength could be measured and compared to muscle volume and density and to attachment side type and geometry within the exoskeleton. Results may lead to new design ideas in light weight constructions.

6.5.2 Locomotion

The possible reactions of muscles discussed above can have an influence on the locomotion as well. Insects usually use an alternating tripod gait. Cavagna et al. (1977, 1964) stated that there is an interplay of energy due to gravity and horizontal kinetic energy as the center of mass undergoes repeated accelerations and declarations with each step.

Considering the daily body position changes of an insect during locomotion (e.g., walking on a wall, ceiling, ...), the hemolymph system, which provides water, ions, nutrients and hormones, should be adapted to slight shifts in gravity (Chapman et al. 2013; Harrison et al. 2020, 2012). However, changes in body orientation and thus fluid distribution can change the center of gravity within an organism and require neuromuscular adjustments to maintain balance and coordinated movement (Chapman et al. 2013). Harrison et al. (2020) found that locust
actively resist gravity-driven shifts in hemolymph. If and how the hemolymph distribution is changed within locust of the centrifuge treatment remains so far unknown. Possible changes in the distribution can partly explain the obtained results with regard to the survival rate and body mass gain. With higher increased mechanical load, the circulation and distribution of nutrients, among others, might be interrupted, resulting in a high mortality rate. Also movement under increased mechanical load may cost comparatively more energy. If and how insects move during centrifuge treatment needs further investigations, for example by video tracking insects. However, increased energy cost of movement or no movement or no food consumption could all lead to the observed reduction of body mass. Contradictory, Le Bourg (2015) suggested that mild hyper gravity could lead to increased body mass, metabolism and life span in insects. He also found that hyper gravity of 3g is the limit between mild and strong stress for drosophila. This finding lies in good agreement with our results. The 3g centrifuge group showed, as the only centrifuge treatment group, an increase in survival rate and biomechanical properties, indicating that also for locust “mild hyper gravity stress” of 3g might be beneficial for insect life span as suggested by Le Bourg (2015). Considering the reduced body mass and survival rate of 3g centrifuge with 1h lunch break compared to the 3g centrifuge treatment without lunch break, switching between hyper gravity and “normal” gravity seems to be stressful for the animals. Here, future research might look into the (hormonal) stress levels and other metabolic mechanisms, which might result in the observed differences in survival rate and body mass at increased mechanical load.

6.6 Concluding summary

The aim of this thesis was to investigate possible reactions of insect exoskeleton towards higher mechanical load. The conducted studies have shown that:

- Insect exoskeleton cuticle is a highly complex and versatile material, which properties are influenced by sample treatment and utilized measurement technique. Thus, meaningful property-function conclusions on cuticle can only be drawn when detailed knowledge about sample treatment, biological function and material and structural properties are measured in a comprehensive way (Chapter 2).

- A destruction free, semi-automated differentiation of exo- and endocuticle within a 3D environment is possible by µCT scans. The thickness varia-
tion along locust hind leg tibia cross-section can be analysed in high detail, allowing more precise calculations of material properties (Chapter 3).

- Raising locust in hyper gravity of up to 8g influences the survival rate and body mass gain. At 3g, the survival rate increases compared to 1g. With higher mechanical load than 3g, the survival rate and body mass decrease. At 8g, the locust even lose weight during two weeks of treatment (Chapter 4).

- The introduction of an “1 hour lunch break” three times a week during treatment has increased survival rate and body mass at the tested g force compared to the treatment at the same g force without the lunch break (Chapter 4).

- Young’s modulus and bending strength increase at 3g compared to 1g treatment. But at higher loads than 3g, the biomechanical properties stay in a similar range compared to the 1g control group (Chapter 4).

- Thickness of exocuticle is similar between all treatments, while the endocuticle thickness is reduced with higher mechanical load (Chapter 4).

- From distinct properties of exoskeleton main layers, exo- and endocuticle, mainly the exocuticle seems to influence the biomechanical properties (Young’s modulus, bending strength) (Chapter 4).

- Mechanically or light induced stressed cuticle show an increase in their biomechanical properties. The insect exoskeleton might have a more “general” response towards stress (Chapter 5).

In summary, the conducted studies provide a first insight into fundamental biomechanical and evolutionary properties of stressed insect cuticle and the working hypothesis H0: Insect cuticle, as a biological material, reacts to long-term applied higher mechanical load, can be confirmed. Findings of this thesis add important information to the evolution and stress response of the second most common biological material worldwide.
Chapter 7

References
References


References


References

*Cinca* 30.4, pp. 495–506. ISSN: 0567-7718, 1614-3116. DOI: 10.1007/s10409-014-0059-0.


Ma, Y., Q. Wu, L. Duanmu, S. Wu, Q. Liu, B. Li, and X. Zhou (2020). “Bioinspired composites reinforced with ordered steel fibers produced via a magnetically assisted 3D printing process”. In: Journal of Materials Science 55.32,


References

Failure”. In: *Physiological Entomology* 23.1, pp. 75–80. ISSN: 1365-3032. DOI: 10.1046/j.1365-3032.1998.2310075.x.


Chapter A

Appendix
A.1 Centrifuge properties

To apply equal mechanical stress on the insect exoskeleton, a custom build centrifuge (diameter: 2 meters) was used. By adjusting the motor frequency (n, turns per minute) and distance of the cages from the center (r, radius), different g forces (a, centrifugal force) could be applied due to physical principle:

A.1.1 Basic principles of centrifugal forces

When a body rotates along a stiff axis, the point P inside the body follows a circular path along a radial line (r) from the center (O). The turn direction can be determined using the right hand screw rule. The direction of thumb and fingers determine if it is a clockwise or counter clockwise rotation. In our custom build centrifuge, the rotation is in clockwise direction. However, angular position, change, speed, and centrifugal acceleration are the main components to describe centrifugal forces. The angular position (r) is defined by the angle θ between a fixed line and r. Regarding $d\theta$ as a vector with norm $d\theta$ as degree and knowing the radian and turns (1 turn equals $2\pi$ radius) the angular change can be determined. Angular speed ($\omega$) describes the time depended change of angle position $d\theta$, while the time change of angular speed defines the angular acceleration (a). Given a constant angular acceleration, angular speed and position are functions of time. To calculate the speed of point $P (\nu)$, r remains constant and the polar coordinates ($\nu$) are needed. Further, the centrifugal acceleration is the acceleration of point $P$ represented by normal and tangential coordinates. Tangential components can have different effects on $\nu$ depended on increase, constant or decrease of the speed. The normal component of acceleration is the time depended change of speed direction and has an effect direction of the circuit center (O). This so called centripetal force acts towards the center of rotation, while centrifugal force acts away from the center of rotation. Considering the acting forces, centrifugal acceleration and position vector ($\nu$), slight corrections of the g factor are necessary (Table A.1) (Hibbeler 2012).

$$a = 4\pi^2rn^2 \tag{A.1}$$
Table A.1: Applied and corrected g forces per treatment. Motor frequency ($M$), radius ($r$), rotation speed ($n$), acceleration ($a$), were used to calculate the acting g-force ($g$). During centrifuge test the angle of insect cage was measured ($\alpha$) and corrected g-forces ($g_{corr}$) calculated. Within the thesis following abbreviation (Abrev.) were used to refer to the acting g-forces. Notice that there are only slight differences between the calculated and corrected g-force.

<table>
<thead>
<tr>
<th>$M$ [Hz]</th>
<th>$r$ [m]</th>
<th>$n$ [U/s]</th>
<th>$a$ [m/s²]</th>
<th>$g$</th>
<th>$\alpha$ [°]</th>
<th>$g_{corr}$</th>
<th>Abrev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>140.4</td>
<td>0.59</td>
<td>1.133</td>
<td>29.887</td>
<td>3.047</td>
<td>13.863</td>
<td>3.213</td>
<td>3g</td>
</tr>
<tr>
<td>140.4</td>
<td>0.97</td>
<td>1.133</td>
<td>49.137</td>
<td>5.001</td>
<td>6.269</td>
<td>5.483</td>
<td>5g</td>
</tr>
<tr>
<td>180.1</td>
<td>0.97</td>
<td>1.443</td>
<td>79.694</td>
<td>8.124</td>
<td>6.269</td>
<td>8.892</td>
<td>8g</td>
</tr>
</tbody>
</table>
Figure A.1: Technical drawing and properties of custom build centrifuge. (a) Side view of the centrifuge with two insect cages (technical drawing, adapted from Dirks unpublished). Rotation orientation along the center (dashed blue line) is indicated by the red arrow. Insect cage, ball bearing, frequency motor and abrasive wheel for power transmission are labeled in brown. Insect cages can be mounted at different distances (dashed dark blue line, \( r \)) to the rotational center. Acting forces are the position vector (\( \vec{g} \), blue) and the centrifugal acceleration (\( \vec{a} \), blue). Together they build an angle (\( \vec{g} + \vec{a} \), blue, \( \alpha, \beta \): red). (c) Centrifuge properties, g force (left Y-axis) and turns per minute (right Y-axis), are determined by the motor frequency in Hz (X-axis). Measured turns per minute are shown in brown and calculated g force is shown in blue.
A.2 Young’s modulus and bending strength

The Young’s modulus or elastic modulus \((E)\) is a measure of material tensile or compressive stiffness. It displays the relationship between tensile/compressive stress and axial strain in the linear elasticity region of an uniaxial deformation curve (Euler 1980). Within the linear elastic region a material deformation is reversible. Meaning after load application the material returns to its original shape. Young’s modulus is calculated using the deflection \((d)\) of a sample with one fixed end and measuring the load \((F)\) at an applied distance \((l_{\text{fix}})\) from the fixation point (Equation A.2, Figure 4.1). Further, sample geometry, the second moment of area \((I)\) is needed to calculate the Young’s modulus.

\[
E = \frac{Fl_{\text{fix}}^3}{3dI} \quad (A.2)
\]

Between the Young’s modulus and other material properties, like bending strength, often complex interaction can be observed. Thus, reporting Young’s modulus alone is only very small part of the overall material properties. Another property, the flexural or bending strength \((S)\), defines the stress in a material before failure. It represents the maximal stress a material can experience (Ashby and Johnson 2013) before it yields. The bending strength is calculated with the maximum force before failure \((F)\) of a sample, the distance from the fixation point \((l_{\text{fix}})\), the samples mean radius \((r)\) and the second moment of area \((I)\) (Equation A.3, Figure 4.1).

\[
S = \frac{Fl_{\text{fix}}^3}{Ir} \quad (A.3)
\]

Notice the influence of the second moment of area in both equations. Especially, for biological samples, where sample geometry is often rather complex and not equivalent to “standard” geometries, detailed knowledge of sample geometry is necessary to get as close as possible to a “real material” value in biological samples.
Table A.2: Statistics of survival rate per treatment. Using R studio software, a Dunn test with Benjamini-Hochberg adjustment was performed to check for significance’s between the different treatment groups and the survival rate. Notice that the 24h daylight and 3g centrifuge treatment with and without lunch break are not significantly different to the 1g control treatment. But the survival rate of higher mechanical stresses than 3g (5g and 8g centrifuge treatment with and without lunch break) are significantly different to the 1g control group. Significant differences are marked by * and highlighted in gray. Treatment groups abbreviations: 1g control (1g), 24h daylight (24h), 3g centrifuge with 1 hour lunch break (3g 1h), 3g centrifuge (3g), 5g centrifuge with 1 hour lunch break (5g 1h), 5g centrifuge (5g), 8g centrifuge with 1 hour lunch break (8g 1h) and 8g centrifuge (8g).

<table>
<thead>
<tr>
<th></th>
<th>1g</th>
<th>24h</th>
<th>3g 1h</th>
<th>3g</th>
<th>5g 1h</th>
<th>5g</th>
<th>8g 1h</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>−1.10811</td>
<td>0.172</td>
<td></td>
<td>0.046282</td>
<td>0.888259</td>
<td>0.4815</td>
<td>0.2277</td>
</tr>
<tr>
<td>3g 1h</td>
<td>0.046282</td>
<td>0.888259</td>
<td>0.4815</td>
<td>0.2277</td>
<td></td>
<td>0.046282</td>
<td>0.888259</td>
</tr>
<tr>
<td>3g</td>
<td>−0.968236</td>
<td>−0.133026</td>
<td>−0.856596</td>
<td>0.2081</td>
<td>0.4572</td>
<td>0.2266</td>
<td></td>
</tr>
<tr>
<td>5g 1h</td>
<td>4.395178</td>
<td>4.626816</td>
<td>3.0426769</td>
<td>3.622344</td>
<td>0.0000*</td>
<td>0.0000*</td>
<td>0.0016*</td>
</tr>
<tr>
<td>5g</td>
<td>4.377351</td>
<td>4.665313</td>
<td>3.207502</td>
<td>3.76114</td>
<td>0.447026</td>
<td>0.0000*</td>
<td>0.0000*</td>
</tr>
<tr>
<td>8g 1h</td>
<td>11.30802</td>
<td>10.04925</td>
<td>7.639384</td>
<td>7.567949</td>
<td>6.316841</td>
<td>5.140009</td>
<td>0.0000*</td>
</tr>
<tr>
<td>8g</td>
<td>13.59469</td>
<td>12.24313</td>
<td>9.799067</td>
<td>9.50658</td>
<td>9.11216</td>
<td>7.84429</td>
<td>3.66232</td>
</tr>
</tbody>
</table>

Significant differences are marked by * and highlighted in gray.
Table A.3: Statistics of locust body mass gain over two weeks of treatment. Using R studio software, a Dunn test with Benjamini-Hochberg adjustment was performed to check for significance’s between the different treatment groups and the body mass. Notice that the body mass gain of all lunch break groups is significantly different to the 1g control group. The 24h daylight and 3g centrifuge treatment are not significantly different to the 1g control treatment. Significant differences are marked by * and highlighted in gray. Treatment groups abbreviations: 1g control (1g), 24h daylight (24h), 3g centrifuge with 1 hour lunch break (3g 1h), 3g centrifuge (3g), 5g centrifuge with 1 hour lunch break (5g 1h), 5g centrifuge (5g), 8g centrifuge with 1 hour lunch break (8g 1h) and 8g centrifuge (8g).

<table>
<thead>
<tr>
<th></th>
<th>1g</th>
<th>24h</th>
<th>3g 1h</th>
<th>3g</th>
<th>5g 1h</th>
<th>5g</th>
<th>8g 1h</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>0.988847</td>
<td>0.2793</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3g 1h</td>
<td>2.505424</td>
<td>1.507185</td>
<td>0.0162*</td>
<td>0.1348</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3g</td>
<td>0.491334</td>
<td>-0.222107</td>
<td>-1.444303</td>
<td>0.4124</td>
<td>0.4416</td>
<td>0.1454</td>
<td></td>
</tr>
<tr>
<td>5g 1h</td>
<td>3592824</td>
<td>2.168308</td>
<td>0.310406</td>
<td>1.83883</td>
<td>0.0006*</td>
<td>0.0339</td>
<td>0.4254</td>
</tr>
<tr>
<td>5g</td>
<td>4.407708</td>
<td>3.093482</td>
<td>1.305897</td>
<td>2.698178</td>
<td>1.201352</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8g 1h</td>
<td>11.01724</td>
<td>9.027972</td>
<td>6.636853</td>
<td>7.522991</td>
<td>7.428961</td>
<td>5.704272</td>
<td></td>
</tr>
<tr>
<td>8g</td>
<td>5.50053</td>
<td>4.930229</td>
<td>3.964446</td>
<td>4.737527</td>
<td>3.981096</td>
<td>3.301225</td>
<td>0.201763</td>
</tr>
</tbody>
</table>
Table A.4: Statistics of Young’s modulus per treatment. Using R studio software, a Dunn test with Benjamini-Hochberg adjustment was performed to check for significance’s between the different treatment groups and the Young’s modulus. Notice that the Young’s modulus of the 24h daylight, the 3g centrifuge treatment with and without lunch break and even the 5g centrifuge treatment with lunch break, is significantly different to the 1g control treatment. Significant differences are marked by * and highlighted in gray. Treatment groups abbreviations: 1g control (1g), 24h daylight (24h), 3g centrifuge with 1 hour lunch break (3g 1h), 3g centrifuge (3g), 5g centrifuge with 1 hour lunch break (5g 1h), 5g centrifuge (5g).

<table>
<thead>
<tr>
<th></th>
<th>1g</th>
<th>24h</th>
<th>3g 1h</th>
<th>3g</th>
<th>5g 1h</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>-4.667355</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0000*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3g 1h</td>
<td>-3.803</td>
<td>0.80661</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0007*</td>
<td>0.2799</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3g</td>
<td>-3.800382</td>
<td>0.391742</td>
<td>-0.336714</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0005*</td>
<td>0.3744</td>
<td>0.3818</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5g 1h</td>
<td>-3.7115</td>
<td>1.349543</td>
<td>0.455008</td>
<td>0.770163</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.006*</td>
<td>0.1378</td>
<td>0.3635</td>
<td>0.2808</td>
<td></td>
</tr>
<tr>
<td>5g</td>
<td>-0.719699</td>
<td>4.177387</td>
<td>3.274573</td>
<td>3.306669</td>
<td>3.141757</td>
</tr>
<tr>
<td></td>
<td>0.2871</td>
<td>0.0002*</td>
<td>0.0021*</td>
<td>0.0022*</td>
<td>0.0029*</td>
</tr>
</tbody>
</table>
Table A.5: Statistics of bending strength per treatment on the dorsal side. Using R studio software, a Dunn test with Benjamini-Hochberg adjustment was performed to check for significant differences between treatment groups and bending strength. Notice that the bending strength of the 24h daylight and 3g centrifuge treatment with and without lunch break is significantly different to the 1g control treatment. Significant differences are marked by * and highlighted in gray. Treatment groups abbreviations: 1g control (1g), 24h daylight (24h), 3g centrifuge with 1 hour lunch break (3g 1h), 3g centrifuge (3g), 5g centrifuge with 1 hour lunch break (5g 1h), 5g centrifuge (5g).

<table>
<thead>
<tr>
<th></th>
<th>1g</th>
<th>24h</th>
<th>3g 1h</th>
<th>3g</th>
<th>5g 1h</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>-5.82377</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3g 1h</td>
<td>-4.388625</td>
<td>2.165188</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3g</td>
<td>-4.225435</td>
<td>1.966752</td>
<td>-0.118195</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5g 1h</td>
<td>-1.145895</td>
<td>5.18472</td>
<td>3.572868</td>
<td>3.438616</td>
<td></td>
</tr>
<tr>
<td>5g</td>
<td>-0.50409</td>
<td>5.57209</td>
<td>4.065023</td>
<td>3.907543</td>
<td>0.65542</td>
</tr>
<tr>
<td></td>
<td>0.3583</td>
<td></td>
<td></td>
<td></td>
<td>0.3164</td>
</tr>
</tbody>
</table>

Notice that the bending strength of the 24h daylight and 3g centrifuge treatment with and without lunch break is significantly different to the 1g control treatment. Significant differences are marked by * and highlighted in gray. Treatment groups abbreviations: 1g control (1g), 24h daylight (24h), 3g centrifuge with 1 hour lunch break (3g 1h), 3g centrifuge (3g), 5g centrifuge with 1 hour lunch break (5g 1h), 5g centrifuge (5g).
Appendix

Table A.6: Statistics of bending strength per treatment on the ventral side. Using R studio software, a Dunn test with Benjamini-Hochberg adjustment was performed to check for significant differences between treatment groups and the bending strength. Notice that the bending strength of the 24h daylight and 3g centrifuge treatment with and without lunch break is significantly different to the 1g control treatment. But there are no significant differences between the 24h daylight and 3g centrifuge treatment with and without a lunch break. Significant differences are marked by * and highlighted in gray. Treatment groups abbreviations: 1g control (1g), 24h daylight (24h), 3g centrifuge with 1 hour lunch break (3g 1h), 3g centrifuge (3g), 5g centrifuge with 1 hour lunch break (5g 1h) and 5g centrifuge (5g).

<table>
<thead>
<tr>
<th></th>
<th>1g</th>
<th>24h</th>
<th>3g 1h</th>
<th>3g</th>
<th>5g 1h</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>-4.513386</td>
<td>0.0000*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3g 1h</td>
<td>-3.482609</td>
<td>0.75663</td>
<td>0.0006*</td>
<td>0.2621</td>
<td></td>
</tr>
<tr>
<td>3g</td>
<td>-3.413807</td>
<td>0.65826</td>
<td>-0.057061</td>
<td>0.0007*</td>
<td>0.2820</td>
</tr>
<tr>
<td>5g 1h</td>
<td>1.779183</td>
<td>6.457369</td>
<td>5.291486</td>
<td>5.163871</td>
<td></td>
</tr>
<tr>
<td>5g</td>
<td>-0.042471</td>
<td>4.845627</td>
<td>3.679384</td>
<td>3.58955</td>
<td>-1.927877</td>
</tr>
<tr>
<td></td>
<td>0.4831</td>
<td>0.0000*</td>
<td>0.0004*</td>
<td>0.0004*</td>
<td>0.0435</td>
</tr>
</tbody>
</table>
Table A.7: Statistics of endocuticle thickness per treatment. Using R studio software, a Dunn test with Benjamini-Hochberg adjustment was performed to check for significance’s between the different treatment groups and the endocuticle thickness. Notice that the endocuticle thickness of all treatments, except the 24h daylight and 3g centrifuge with and without lunch break, are different to the 1g control group. Significant differences are marked by * and highlighted in gray. Treatment groups abbreviations: 1g control (1g), 24h daylight (24h), 3g centrifuge with 1 hour lunch break (3g 1h), 3g centrifuge (3g), 5g centrifuge with 1 hour lunch break (5g 1h), 5g centrifuge (5g), 8g centrifuge with 1 hour lunch break (8g 1h) and 8g centrifuge (8g).

<table>
<thead>
<tr>
<th></th>
<th>1g</th>
<th>24h</th>
<th>3g 1h</th>
<th>3g</th>
<th>5g 1h</th>
<th>5g</th>
<th>8g 1h</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>−1.499924</td>
<td>0.0780</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3g 1h</td>
<td>2.866759</td>
<td>4.266286</td>
<td>0.0029*</td>
<td>0.0000*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3g</td>
<td>−1.477232</td>
<td>0.02217</td>
<td>−4.244116</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5g 1h</td>
<td>4.9549</td>
<td>6.306418</td>
<td>2.040131</td>
<td>6.284248</td>
<td>0.0000*</td>
<td>0.0000*</td>
<td>0.0263</td>
</tr>
<tr>
<td>5g</td>
<td>5.308471</td>
<td>6.65186</td>
<td>2.385573</td>
<td>6.629689</td>
<td>0.345441</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8g 1h</td>
<td>6.700926</td>
<td>8.0123</td>
<td>3.746013</td>
<td>7.990129</td>
<td>1.705881</td>
<td>1.360439</td>
<td></td>
</tr>
<tr>
<td>8g</td>
<td>11.52751</td>
<td>12.72791</td>
<td>8.461627</td>
<td>12.70574</td>
<td>6.421495</td>
<td>6.076054</td>
<td>4.715614</td>
</tr>
</tbody>
</table>
Declaration

Declaration on the contribution of the candidate to a multi-author article/ manuscript which is included as a chapter in the submitted doctoral thesis.

Chapter: 2. Biomechanics of insect cuticle - an interdisciplinary experimental challenge

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):
Experimental concept and design: ca. 75 %
Experimental work and/or acquisition of (experimental) data: ca. 80 %
Data analysis and interpretation: ca. 75 %
Preparation of Figures and Tables: ca. 95 %
Drafting of the manuscript: ca. 75 %

Chapter: 3. Semi-automated differentiation of insect exo- and endocuticle in X-ray microtomography

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):
Experimental concept and design: ca. 100 %
Experimental work and/or acquisition of (experimental) data: ca. 100 %
Data analysis and interpretation: ca. 80 %
Preparation of Figures and Tables: ca. 90 %
Drafting of the manuscript: ca. 90 %

Chapter: 4. Insect cuticle reacts to mechanical stress

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):
Experimental concept and design: ca. 75 %
Experimental work and/or acquisition of (experimental) data: ca. 100 %
Data analysis and interpretation: ca. 75 %
Preparation of Figures and Tables: ca. 80 %
Drafting of the manuscript: ca. 75 %

Date:
Signature:
Erklärung zur elektronischen Version und zur Überprüfung einer Dissertation

Ort, Datum: ............................................................


Ich bin mit der Überprüfung meiner Dissertation gemäß §6 Abs. 2, Punkt 5 mit qualifizierter Software im Rahmen der Untersuchung von Plagiatsvorwürfen einverstanden.

............................................................

Unterschrift
Versicherung an Eides Statt

Ort, Datum: ..............................................................

Versicherung an Eides Statt


Die Strafbarkeit einer falschen eidesstattlichen Versicherung ist mir bekannt, namentlich die Strafandrohung gemäß § 156 StGB bis zu drei Jahre Freiheitsstrafe oder Geldstrafe bei vorsätzlicher Begehung der Tat bzw. gemäß § 161 Abs. 1 StGB bis zu einem Jahr Freiheitsstrafe oder Geldstrafe bei fahrlässiger Begehung.

..............................................................

Ort, Datum Unterschrift