Insect exoskeletons react to hypergravity

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Abstract

A typical feature of biological materials is their ability to adapt to mechanical load. However, it is not known whether the cuticle exoskeleton, one of the most common biological structures, also shares this trait. Here, we show direct experimental evidence that prolonged exposure to hypergravity conditions affects the morphology and biomechanics of an insect exoskeleton. Locusts were raised for several weeks in a custom-designed centrifuge at various levels of hypergravity. Biomechanical measurements and X-ray microtomography show that up to 3g load the Young's modulus of the tibiae increased by about 67%. Higher gravitational loads however decreased the survival rate, body mass and endocuticle thickness. These results directly show that cuticle exoskeletons can react to hypergravity. This ability has so far only been known for bone endoskeletons and plants. Our findings thus add important context to the discussion on general ultimate factors in the evolution of adaptive biological materials and skeletal systems.

Keywords: Insect cuticle, Utah paradigm, Exoskeleton, Biomaterials

1 Introduction

A common feature of many biological materials, such as wood or bone, is their ability to react to external mechanical stresses [1]. Adaptive growth or remodelling on an individual level can happen on various different length scales.

This ability helps to improve the performance of the structure or material and to optimise the use of "costly" biomaterial, which can be an important evolutionary advantage for plants, vertebrates and other organisms.

The reaction of wood to mechanical stress is relatively well studied, which might be a result of the wood-industry's

commercial interest [2]. Prolonged exposure to increased mechanical load leads to the formation of "reaction wood" in the form of "compression wood" in gymnosperms and "tension wood" in angiosperms. These types of wood help the tree to restore vertical growth or incline vertical stems to move towards the top of the canopy. How these two types of wood sense mechanical load on a cellular level however (e.g. tuning of growth stress level) is not yet fully understood and still debated [3, 4].

The ability to adapt the structure of bone endoskeletons is usually considered to be more complex. Whilst trees can only adapt by *adding* new material, bone also possesses the ability to *remove* material when needed [5]. This remodelling is commonly described using the "mechanostat" theory or "Utah paradigm". This model, first published by H. M. Frost in the 1960s and later refined several times, expands the older "Wolff's law" by including anatomical features, muscle force as well as control mechanisms on the tissue level [6]. Depending on the mechanical stress (or strain, which is mechanically linked to the stress) a bone can be in one of three remodelling states: "disuse", "adapted" or "overload". In both the "disuse" and the "adapted" phase, normal remodelling replaces bone material and repairs microcracks. Within the "adapted" phase, the bone experiences a level of mechanical stress that is sufficient to maintain the current bone mass. In the "disuse" category however, the bone experiences very little stress, thus over time the overall bone mass is reduced. In the "overload" phase, the mechanical stress on the bone is sufficient to trigger the modelling of additional bone material.

So far, this mechanostat model is only applied to endoskeletons from amphibians, birds, reptiles and mammals, which are only approximately 20% of the world's animal species [6]. Surprisingly, it is not known whether the mechanostat model is valid for arthropod cuticle exoskeletons, which represent the remaining 80% of animal species.

On the materials level, once deposited, cuticle exoskeletons seem to have only very limited "adaptive" self-healing abilities, besides simple wound clogging mechanisms to prevent desiccation or encapsulation processes of pathogens [7]. Looking at adaptations of the cuticle as a material, experiments by Bayley et al. have only recently indicated that the mechanical properties of locust hind legs can temporarily change. Legs subject to repeated bending are able to store less mechanical energy compared to their first loading, however the legs mechanically fully recover within 24h [8]. This observation speaks against any long-term adaptation of the cuticle's structure, at least in this type of load case. The reported mechanical "adaptation" instead is thought to be due to thixotropic material properties (time-dependent shear thinning). However, despite such short-term "passive" adaptations, it is not known whether insect exoskeletons can actually also biologically change depending on the mechanical stresses they experience.

The main objective of this study is thus to answer a key fundamental question regarding arthropod exoskeletons: Do insect exoskeletons have the ability to adapt to increased mechanical stress?

To answer this question, we raised *Locusta migratoria* locusts, a commonly used biomechanical model organism, in a custom made centrifuge setup under

hypergravity conditions (figure 1 A). We then performed established biomechanical and morphological tests on the locust hind leg tibiae, as a representative body part of the insect exoskeleton with already well known morphology and biomechanical properties.

2 Methods

Animal keeping and exposure to hypergravity

Locusta migratoria (Linneaus 1758) were bought at fifth instar and kept in a controlled 12h day/12h night cycle until final molt. Insects were fed with fresh plant material *ad libitum*. For all locust pre-treatment and post-treatment weight and survival during treatment was documented.

To apply continuous levels of hypergravity over a long period of time, freshly molted adult locusts were raised with identical environmental conditions in a custom build centrifuge setup under 3 g, 5 g and 8 g continuous hypergravity load (figure 1 A and suppl. figure S1). The adults were raised in the setup for two weeks until the exoskeleton was fully developed [10]. In our experiments we tried to cover a wide range of mechanical loads, starting with a physiological plausible load of 3 g, which is in the range of stresses adult female locusts are subjected to during mating with males attached to their backs. Loads of 5g and 8g are likely to exceed typical loads experienced during normal activity, however were used to test possible extreme regions of mechanical stress.

During these experiments the centrifuge was only stopped every three days for max. 15min for animal care-taking. To test for a possible effect of reduced feeding due to increased stress levels, we performed additional centrifuge experiments with an 1h daily "lunch break" at mid-day, during which the centrifuge was stopped. A few minutes after the centrifuge had come to a complete halt the insects started to feed.

To differentiate between physiological stress on the entire organism (including diffusion, etc.) induced by the centrifuge and purely mechanical load on the exoskeleton we also performed additional experiments where locusts were equipped with "backpacks" on their pronotum (suppl. figure S2). For each insect a custom-made backpack was designed according to its respective body mass and an equivalent mass to the hypergravity experienced in the centrifuge (3g, 5g and 8g). The backpacks were made from tungsten putty (Korda Dark Matter, Korda Europe Bv, Kerkrade, The Netherlands) and attached as close to the centre of mass as possible using small amounts of epoxy glue [9, 11]. To prevent the locusts' legs from sticking to the putty, the backpacks were additionally coated with FIMO Gloss Varnish (Staedtler, Nürnberg, Germany).

Mechanical testing

Two weeks after their final moult the locust hind leg tibia were cut off directly at the femur-tibia joint. Tarsal segments were removed as close as possible from the tibia (figure 1 B). Samples were either tested immediately or temporarily frozen at -20°C, which has no significant effect on the biomechanical properties of hind leg cuticle [12]. 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
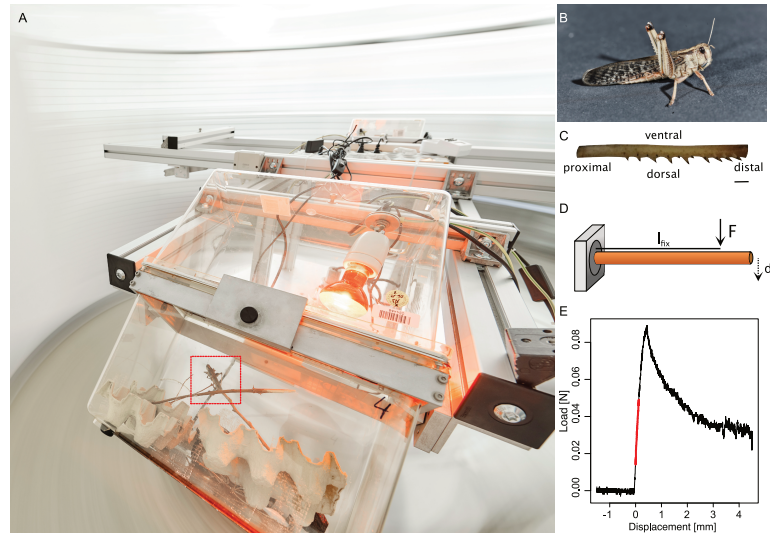
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Fig. 1 Experimental setup to increase mechanical stress on insect exoskeletons. (A) Photo of custom made centrifuge showing the standardised insect cages with individual heating and LED lighting. The red, dashed box highlights a locust in the setup. The centrifuge had a diameter of 2m and allowed to apply continuous hypergravity conditions up to 10g to the insects. (B) The locust *Locusta migratoria* is a typical model organism for biomechanical studies on insect cuticle. The cylindrical, thin-walled tibiae of the hind legs allow to apply standard engineering methods for biomechanical tests. (C) The hind leg tibia of *L. migratoria* showing the nomenclature used throughout the manuscript. (D) Schematic illustration of the cantilever bending experiments, where *l* is the distance between the sample holder and actuator, *d* the deflected distance, when a load *F* is applied with constant speed. (E) Typical force-displacement curve of a cantilever bending test on a hind leg tibia. The red highlighted part indicates the linear elastic region used for Young's modulus calculation. Figures A and B with kind permission from Marcus Meyer Photography.

load cell, TA Electroforce Series III) samples were cantilever-bent at a distance of 7-11 mm from fixation point at a constant speed of 15 mm*min^{-1} in dorsal-ventral plane until failure (figure 1 C and [13]). To test for possible effects of different ratios of exo- and endocuticle on the dorsal and ventral side of the tibiae [14], we performed deflection in both the dorsal-ventral and the ventral-dorsal direction. However, no significant differences between dorsal and ventral measurement direction were found (Kruskal-Wallis-test, $\chi^2(1)=0.127$, p>0.5). Thus for further analysis results of both deflection sides were pooled. 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 min. Young's modulus (E) of the tibia was calculated within the linear elastic region of the force deflection curve (figure 1 D, [13]).

Geometrical analysis

Samples were stained for X-ray microtomography (microCT) following an established protocol for cuticle samples [15]. The tibiae were cut off and directly placed in 100% EtOH (CAS: 64-17-5, CarlRoth) for 30 min. Samples were then transferred into a fresh solution of 1% iodine (CAS: 7553-56-2, SigmaAldrich)

dissolved in 100% methanol (CAS: 67-56-1, Carl Roth) for 48h at 8°C. For scans the samples were mounted with small amounts of dental wax (WN-92189, Henry Schein) in an Eppendorf tube filled with 100% methanol (CAS: 6756-1, Carl Roth). CT scans were performed using an X-Radia 520 Versa (Carl Zeiss Microscopy, Jena, Germany) at 80 kV, 7 W and a resolution of 3.33 µm voxel size. Using the BoneJ Plugin for ImageJ (V 1.52p, Wayne Rasband, National Institutes of Health, USA) the second moment of area around the major and minor axis (I_max, I_min, figure 3 A), the mean radius of the tibia cross-sections was measured and calculated for each treatment [13, 16]. To calculate the mean thickness of the exo-and endocuticle, the thickness was measured at eight different angles around the tibia perimeter (figure 3 B, [14]).

Statistics

All statistical tests were performed using R Studio software (RStudio, 2020). Values which were outside of 1.5* interquartile range (IQR) were counted as outliers. If homogeneity of variance and normal distribution criteria were not met, non-parametric Kruskal-Wallis and subsequent Dunn for post-hoc pairwise comparisons were used.

3 Results

To estimate the general effect of long-term exposure of locusts to hypergravity, we first measured the survival rate and changes in body mass at various levels of stress from 1g to 8g. We then performed detailed morphological and biomechanical analyses on the hind leg tibiae of the insects to study the effects on the cuticle exoskeleton.

Survival rate and body mass change

Our results show that prolonged exposure to hypergravity at levels between 1g and 8g significantly affected the survival rate of the insects (Kruskal-Wallis-test, χ^2 (9)=322.03, p<0.001; figure 2 A). Locusts raised at identical conditions at 1g (reference group) showed a survival rate of 76% after two weeks in the centrifuge. The 3g group showed the overall highest survival rate with 81% without lunch break. With 5g treatment the survival rate of the locusts significantly decreased to 51%. At 8g continuous hypergravity only 7% of locust survived.

To check for and minimize any possible effects of starvation in the centrifuge treatment, we performed experiments with a daily "1-hour lunch break" at mid-day. For 3g insects, this break non-significantly decreased the survival rate to 77%. For 5g insects the lunch break also did not significantly affect the survival rate (51%). Only for 8g animals we observed a significant improvement from 7% to to 27% survival with a lunch break.

Continuous application of hypergravity also significantly affected the body mass of the locusts. (Kruskal-Wallis-test, χ^2 (9)=142.92, p<0.001; figure 2 B). When placed into the centrifugal setup the initial mean body mass of the freshly moulted locusts was 0.864 ± 0.143 g. The 1g and 3g animals without lunch break showed no significant difference in their body mass gain. The 5g insects gained significantly less body mass than the 1g and 3g animals. The body mass change of the 8g insects was significantly lower than all other groups, with the animals even losing weight during maturation. This is in good agreement with the observation that 8g animals consumed less of

the provided food and showed the highest mortality rate.

Interestingly, the effect of the lunch break on the body mass was depending on the level of hypergravity. Whilst after two weeks 3g animals with lunch break showed a body mass significantly lower than 3g insects without the break, the lunch break did not significantly affect the body mass gain of 5g centrifuge animals. In 8g animals the presence of a lunch break also lead to an increased body mass.

Our results show that the survival rate of locusts equipped with backpacks was drastically reduced in comparison to the centrifuge experiments. After two weeks only 4% of locusts with 3g backpacks and 17% of locusts with 5g 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.

Morphological Analysis

To test whether the mechanical stress induced by the centrifuge affected the composition of the tibial cuticle, we analysed the thickness of the exo- and endocuticle using semi-automated microCT techniques (figure 3 and [15]). Even though a Kruskal-Wallis-Test showed a significant effect of the treatment on the exocuticle thickness (χ^2 (7)=14.672, p<0.05), a post-hoc Dunn Test showed no significant differences between 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 to 25 µm depending on the location along the circumference of the leg [15]. Growing under hypergravity conditions however affected the mean thickness of the endocuticle (Kruskal-Wallis-test $\chi^2(7)$=247.378, p<0.001). Post-hoc tests showed that the endocuticle of the 1g control group and the 3g group was not significantly different from each other - however significantly thicker than the endocuticle of all other treatments (figure 3 B).

The presence of the lunch break significantly affected the thickness of only the endocuticle ($\chi^2(3)$=77.498, p<0.01), significantly decreasing the endocuticle thickness of the 3g insects (p<0.001), however not significantly affecting the endocuticle thickness of the 5g insects (p=0.634).

Biomechanical properties

Our results show that raising insects at hypergravity had a significant effect on the Young's modulus of the hind leg tibia cuticle (figure 4 A and summary in table 1). The control group at 1g showed a Young's modulus of 3.4±2.4 GPa (median ± sd), which is in good agreement with previously measured Young's moduli for locust hind leg tibia [12, 13]. Hypergravity of 3g resulted in a significantly increased Young's modulus of 5.7±2.9 GPa. Insects subject to hypergravitational load of 5g however showed a reduced Young's modulus of 4.0±2.7 GPa, which was not significantly different to the 1g condition. Values measured from the very few insects surviving 8g hypergravity were not included due to the very low survival rate and respective sample size.

Introducing the lunch break treatments caused a "stabilization" of the
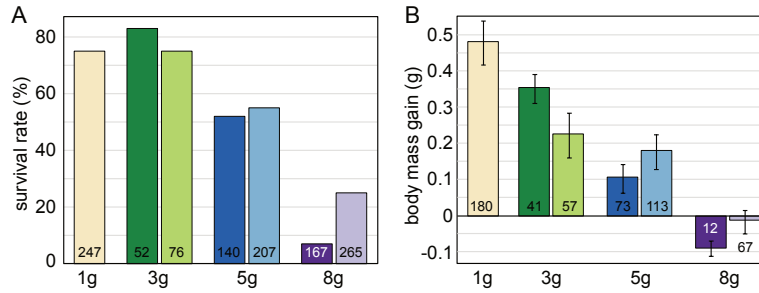
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Fig. 2 Survival rate and body mass of *L. migratoria* after two weeks under hypergravity conditions. A) After two weeks at up to a centrifugal force of 3 g the survival rate of locusts was not significantly reduced. Only stress levels of 5g and 8g reduced the survival rate significantly. Adding a lunch break (light colours) did not significantly affect the survival rate at 3g and 5g however significantly increased the survival of insects at 8g. B) After two weeks insects subject to increased mechanical stress of 5g gained significantly less body mass than insects at 1g and 3g conditions. At 8g centrifugal forces the locust even lost body mass in comparison to their initial weight. Whilst for 3g and 5g insects the presence of a lunch break (light colours) did not significantly affected the body mass gain, the break increased the respective body mass gain for 8g centrifugal forces. Bars show mean±SD.

Young's modulus. For insects raised at 3g hypergravity, the lunch break did not significantly affect the Young's modulus. At 5g hypergravity, the lunch break increased the Young's modulus to a level not significantly different to the 3g without lunch break insects. Insects at 8g hypergravity with a lunch break showed a Young's modulus significantly different to the control group and the 5g group without lunch break.

4 Discussion

We here show the effects of hypergravity on the morphology and the biomechanics of an insect exoskeleton. Our study presents comprehensive data of the survival rate and body mass, as well as geometric properties and Young's modulus of the locust hind leg cuticle.

Hypergravity decreased survival rate and affected body mass

The general effect of hypo- and hypergravity on the physiology of insects has been the focus of few studies on Drosophila [17–19]. Le Bourg kept adult Drosophila for several weeks under increased mechanical load in a centrifuge setup. His results show that mild hypergravity (< 3g) can lead to increased body mass, metabolism and life span in insects [17, 20]. Later studies even show that limited exposure to mild hypergravitational stress can help to cope with thermal stresses [21].

Our results show that *L. migratoria* subject to prolonged hypergravity showed a significantly reduced survival rate (figure 2 A). These results are not directly comparable with "longevity" from literature on Drosophila, as adult locusts usually live up to several months. However, our results confirm the general trend shown for Drosophila, which indicated that hypergravitational stress can affect homeostasis and metabolic rate in insects [22].

Our results further show that living under hypergravity conditions affected the body mass of locusts (figure 2 B). This could be an effect of starvation
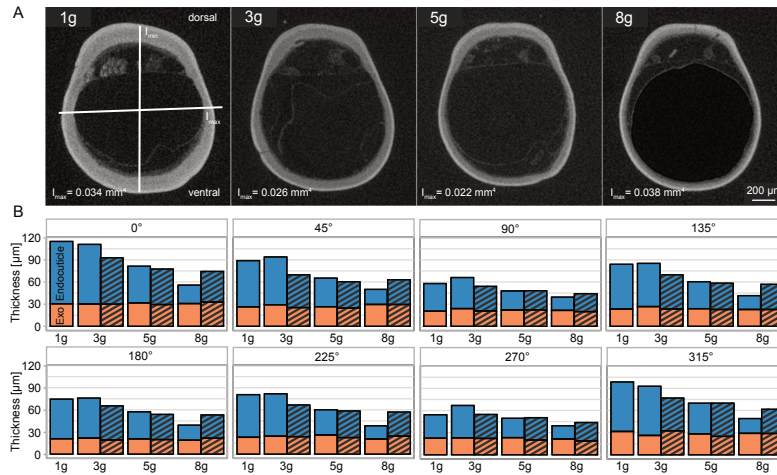
Fig. 3 Effect of long term hypergravity cuticle thickness in the locust hind leg tibia of *Locusta migratoria*. A) High resolution microCT scans of cross sections through the mid-tibial region showing the main axes for L*max* and L*min*. The exo- and endocuticle thickness B) Exocuticle (orange) and endocuticle (blue) thickness were measured at eight different positions (0-315°) along the leg cross-section, where 0° refers to the ventral side). This effect was also observed in the lunch break treatments (3g, 5g, 8g, hatched barplots). The exocuticle thickness (deposited before the animals were placed into the centrifuge) remained unchanged, whilst at hypergravity load of 5g and 8g the thickness of the endocuticle significantly decreased.
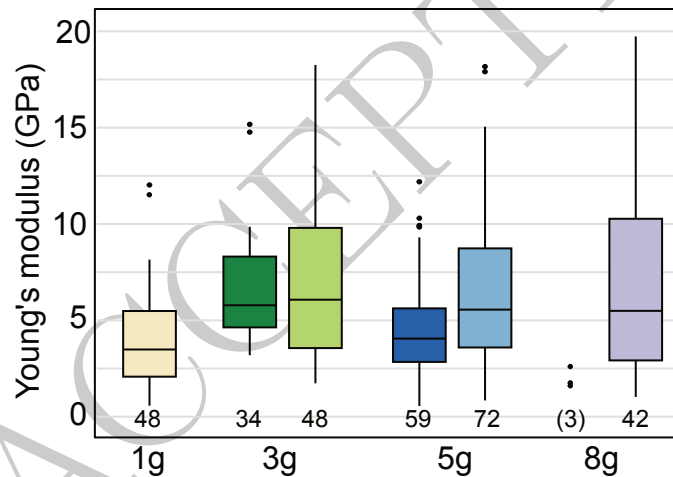

Fig. 4 Effect of hypergravity on biomechanical properties of locust hind leg cuticle. A) Hypergravity of 3g significantly increased the Young's modulus from 3.4±2.4 GPa to 5.7±2.9 GPa. Cuticle from insects subject to hypergravity of 5g however had a lower Young's modulus, which was not significantly different to the 1g control conditions. A lunch break had no significant effect at 3g, however significantly increased the Young's modulus for 5g conditions. Due to the very low survival rate at 8g without lunch break (3 data points), only values of insects with 8g plus lunch break were included in the analysis.

Table 1 Overview of statistical post-hoc comparison of Young's modulus between groups with different hypergravity conditions and lunch breaks (lb). Following a Kruskal-Wallis-test ($\chi^2(5)$=31.284, p<0.001) a Dunn test with Benjamini-Hochberg adjustment was performed for groupwise comparison. Significant differences (p<0.05) are shaded.

	1g	3g	3g lb	5g	5g lb
3g	<0.01				
3g lb	<0.01	n.s.			
5g	n.s.	<0.01	<0.01		
5g lb	<0.01	n.s.	n.s.	<0.01	
8g lb	<0.01	n.s.	n.s.	<0.01	n.s.

due to reduced feeding and/or increased metabolism. Tauber at al. (1986) indeed stated that in insects stress can induce quiescence behavior, which ultimately leads to starvation [23]. To test for the effect of starvation, we introduced a "lunch break", allowing the insects to move around at 1g conditions and feed. Control centrifuge experiments with 1h breaks without food showed no observable improvement of body mass and survival rates. However, even with the lunch break, none of the insects under hypergravitational conditions were able to match the body mass gain of the control experiments. Longer lunch breaks might allow the insects to gain additional body mass, however longer breaks would also decrease the time the insects spend under hypergravitational load. Our 1h lunch break thus seems to have been a good compromise, allowing the insects to feed whilst still keeping the exposure time to hypergravity as high as possible.

Interestingly, the presence of the break lead to an even bigger loss of body mass of 3g centrifuge animals (figure 2 B). The reason for this effect remains unclear. Previous studies however have shown that food quantity and resting period after feeding are positively correlated with the time spent without feeding. Thus, it seems likely that the responses of locust in the lunch break groups are also stress correlated but not starving correlated [24]. To further investigate the observed interaction of hypergravity, body mass and survival rate, it would therefore be very beneficial to collect detailed data on metabolic activity, locust behavior and locomotion patterns within the centrifuge setup in future studies.

Hypergravity affected cuticle thickness and Young's modulus

Our results show that raising locusts under hypergravity conditions significantly affected the morphology and biomechanical properties of the cuticle exoskeleton in the tibia. This is the first time that this kind of reaction is experimentally shown for exoskeletons of insects.

The detailed microCT measurements show that hypergravity affected the overall thickness of the tibia's cuticle. For the exocuticle no significant effect of the prolonged exposure to hypergravity was found in our centrifuge experiment. This is not surprising, as the exocuticle was deposited before the animals were placed into the centrifuge. The endocuticle however was deposited during the two weeks the insects were in the centrifuge [10, 25, 26].

Changes in cuticle thickness result in changes of the tibia's moment of area, which again is an important factor in the measurement of the Young's modulus [13]. Hence, one could argue that the observed differences of the Young's modulus are due to changes of the cuticle thickness and possible effects of starvation or reduced body mass alone. However, in our experiments we measured the moment of inertia for all hypergravity conditions and thus corrected for any morphological changes. The reported Young's modulus is therefore a material constant and not a structural "flexural stiffness" of a leg with reduced overall cuticular thickness [14]. In addition, the endocuticle of the 3g centrifuge group was not significantly different to the endocuticle of the control group - yet the Young's modulus was significantly higher than the control group. At least up to hypergravity conditions of 3g our results thus strongly imply different material properties of endocuticle deposited during exposure to hypergravity. Future studies analysing the detailed histological composition and ultrastrucrure of the stressed endocuticle (chitin-protein content, cross linking of the protein matrix, fibre orientation, etc.) and micromechanical tests using nanoindentation techniques are required to further investigate this difference.

Higher gravitational load of 5g and 8g led to a Young's modulus significantly lower than after exposure to 3g conditions. The physiological principles for these changes remain unclear. It seems likely that at these conditions physiological stress (metabolic changes and/or starvation effects) dominates the deposition of the cuticle.

Exoskeletons and the Utah Paradigm

Our results provide first experimental data on the reaction of insect cuticle to hypergravity, leaving many open physiological and biomechanical follow-up questions. However, our results do show that locusts subject to hypergravity seem to be able to deposit cuticle with different mechanical properties during their ontogenesis. This indicates that the ability of a biological material to adapt to mechanical load, previously only known for endoskeletons and higher plants, apparently is also present in insects and maybe even all arthropods. This ability thus might be a fundamental concept of almost all skeletal forms and - taking into account the diverse evolutionary pathways of exo- and endoskeletons and plants - could be a widely distributed convergent evolutionary trait of all load bearing skeletal biomaterials.
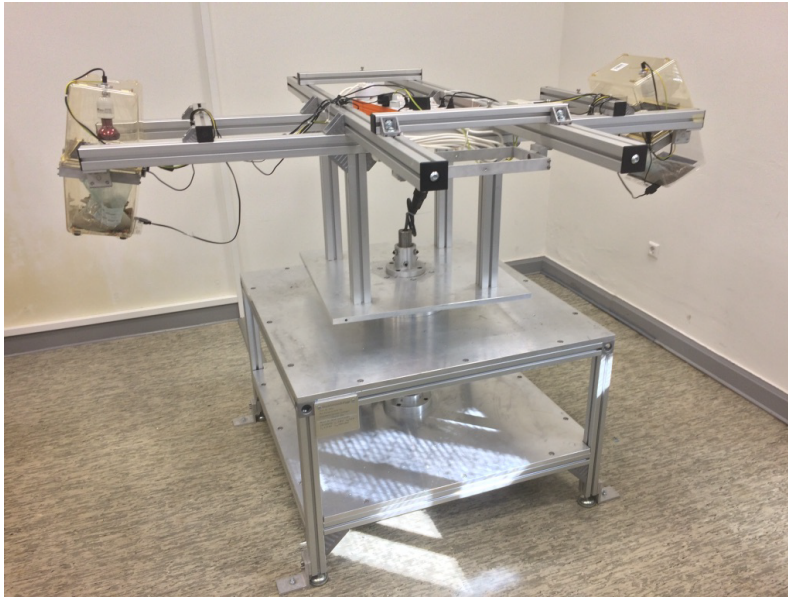
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5 Supplementary Material

Fig. S1 Photography of the custom designed insect centrifuge setup. Insect cages were fixed in a rotating centrifuge (diameter 2m) and allowed insects to live in a controlled hypergravity environment with individual controlled heating and daylight. Changing the rotational speed allowed to vary gravitational acceleration during otherwise identical experimental conditions.

Fig. S2 Close up of *L. migratoria* locusts with a custom designed "backpack", which was used in addition to the centrifuge experiments to experimentally uncouple the role of hypergravity (centrifuge) from purely mechanical stress (backpack). The backpacks were attached as closely to the centre of gravity, however still restricted the movement of the insects. Data from these experiments was thus not included in the analysis. Image adapted from [9].

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