Exoskeletal microstructure and tensile behavior of the acorn weevil rostrum

M. Andrew Jansen,[1, *] Jason Williams,[2] Nikhilesh Chawla,[2] and Nico M. Franz[1]

[1]*School of Life Sciences, Arizona State University, Tempe, AZ 85287, USA*

[2]*School for Engineering of Matter, Energy, and Transport,*
Arizona State University, Tempe, AZ 85287, USA

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INTRODUCTION

The exoskeleton of Coleoptera (beetles) is a hierarchically-structured fibrous composite typified by variously arranged α-chitin (N-acetylglucosamine) nanofibrils embedded in a heterogeneous protein matrix. Although α-chitin is brittle and strongly anisotropic, beetle cuticle is simultaneously rigid and tough due to its uniquely layered microstructure. Beetle cuticle is divided into two structurally and mechanically distinct regions, the (outer) exocuticle and (inner) endocuticle. The exocuticle is characterized by a transversely-isotropic helicoidal structure that is comparatively rigid (though brittle) and has been the subject of many prior studies. By contrast, the endocuticle of beetles is comprised of large, aligned bundles (macrofibers) of chitin that are strongly anisotropic. Arranged in unidirectional laminae, the macrofibers contribute to the toughness of the exoskeleton by inhibiting crack formation and propagation between successive plies [10–12].

In general, impact-prone areas and exaggerated structures, such as horns and legs exhibit cuticle organization that resists deformation and fracture. Acorn weevils in the genus *Curculio* are typified by an elongate structure of the head, called the rostrum (snout), which instead exhibits unusual distal flexibility. The rostrum is a hollow, strongly curved (over 90° in some species), cylindrical, exoskeletal extension of the otherwise nearly-spherical head, which bears at its apex the terminal chewing mouthparts. Despite being composed of the same material as other rigid body parts, the snout can be repeatedly bent without evident damage. This structure is used by the female to feed and excavate sites for egg-laying (oviposition); the latter process causes significant, apparently elastic, deformation of the rostrum. By maintaining constant tension on the snout and rotating around the bore-hole, females are able to flex the rostrum into a near-perfectly straight configuration and thereby produce a linear channel into the fruit.

While this behavior has been observed in many species of *Curculio*, it was unclear how the rostrum of female acorn weevils can withstand the repeated, often extreme bending incurred during the process of egg-chamber excavation. In this study we characterize the composite profile of the rostral cuticle to account for the observed flexibility of the snout. We show that the relative layer thicknesses and fiber orientation angles of the exocuticle and endocuticle of the rostrum are strongly differentiated from the head capsule and other body parts. Because recent studies have shown that the yield strength of the weevil rostrum exoskeleton is lower in tension than compression, we perform a comparative analysis of the ultimate tensile strength of the rostrum across species and snout morphotypes; we also report the results of displacement-controlled load cycling of the snout in a species with strongly curved morphology. We relate an observed increase in the volume fraction of endocuticle in the rostrum to higher tensile strength at the rostral apex in all tested species, and find that a strongly curved rostrum can be flexed repeatedly without harm to the structure.

We additionally describe the fracture mechanics of the snout, as pertains to both cuticle composite structure and tensile behavior, and consider how modification of the cuticle may reduce the risk of rostral fracture during oviposition. Based on our findings we posit that the composite profile of the rostral apex enables the snout to be flexed until straight while remaining within the elastic limits of the material, mitigating the risk of structural damage, and without evident alteration of the mechanical properties of the individual components of the cuticle across the structure and between species. Thus, the flexibility and tensile strength of the rostrum appear to be derived *exclusively* from modification of the composite architecture of the exoskeleton. To our knowledge, this is the first time that a modified composite profile has been reported as a means of enhancing structural elasticity in the insect exoskeleton.

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* corresponding author, email: majanse1@asu.edu

I. MICROSTRUCTURE OF THE *CURCULIO* ROSTRUM

Insect cuticle is made up of chitin and numerous uncharacterized proteins, which effectively act as a matrix into which the chitin is embedded [3–5]. The arrangement of the embedded chitin fibers is primarily responsible for the mechanical behavior of the cuticle, although this is modulated by the degree of tanning and water retention. [1, 3]. Accordingly, cuticle is often considered as a fiber-reinforced composite material; however, in beetles, the composite arrangement of the chitin fibers varies by cuticle region.

Insect cuticle is divisible into three regions (Figs. INSERT-REFS-HERE), including (1) endocuticle, which is the most compliant and innermost region; and (3) exocuticle, which is the stiffest, hardest, and outermost region. Between these lies (2) mesocuticle, which is similar in microstructure to exocuticle, but less sclerotized (tanned), usually acting as a thin transition zone [1–3].

In general, these regions are confluent, and not necessarily sharply defined; for instance, mesocuticle is not always evident, and is sometimes considered part of the exocuticle. In beetles, both exocuticle and mesocuticle (when present) are laminate, and have numerous laminae of unidirectional chitin fibers, each layer a single fiber thick (2-4nm), embedded in a proteinaceous matrix. These layers are stacked at a more or less constant angle to each other in a helicoidal arrangement, referred to as the Bouligand structure [6–8]. This microstructure allows exocuticle and mesocuticle to exhibit transverse isotropy, or complete isotropy in some cases, despite the strong anisotropy of α-chitin.

Conversely, the endocuticle of Coleoptera appears to take advantage of this anisotropy to improve fracture resistance of the exoskeleton. Beetles in particular have a highly modified endocuticle comprised of large (5-20μm OD) unidirectional bundles of chitin, called macrofibers. The macrofibers are strongly anisotropic, with an elastic modulus of 8-9GPa along their longitudinal axis, but only 1-2 GPa along the transverse plane. Macrofibers are aligned in layers, as in Figs INSERT-FIGS-HERE [11, 12]. Typically, endocuticle contains several layers of macrofibers, with the adjacent layers forming pairs. The layers *within* each pair are pseudo-orthogonal (i.e., angled approx. 90° to each other, see Figs. INSERT-REFS-HERE), while the stacking angle *between* pairs is typically acute, although other configurations have been observed [10, 11]. It is thought that this arrangement of the macrofibers contributes to the toughness of beetle exoskeletons by inhibiting crack formation and propagation between successive plies [10–12].

We have found that in *Curculio*, the head capsule (which is similar to the rest of the body) fits the general profile of endocuticle, with an angle of approximately 30° between successive pseudo-orthogonal plies. Additionally, in the head capsule, the thickness of the exocuticle in cross section is nearly equal to that of the endocuticle; compared to the snout, this part of the head is fairly rigid.

By contrast, the region beyond the scrobe (antennal channel) is quite flexible, even in fully desiccated specimens. Serial thin sectioning of the snout has demonstrated that the cuticle in this region has a different composite structure (see Fig INSERT-REFS-HERE) than the head capsule, namely:

1. The exocuticle is reduced to a thin shell, with the endocuticle thickened to offset this reduction.

2. The endocuticular macrofibers exhibit no rotation between successive pseudo-orthogonal plies, which are all oriented at approx. ±45° to the longitudinal axis of the snout.

We believe that each of these modifications contributes to the observed flexibility of the snout, but in different ways. Because the exocuticle is generally more rigid (and brittle) than the endocuticle due to its transverse isotropy and microstructure, a reduction in the relative thickness of this region would likely have the effect of increasing the flexibility of the resulting composite. Additionally, aligning the layers of endocuticle as a 45 deg cross ply would likely reduce the bending moment of the structure. The combined effect of these modifications is that the apex of the rostrum is able to bend until completely straight without fracture.

Correspondingly, the portion of the snout between the head capsule and apex of the scrobe exhibits a gradual transition in composite profile along an anterior-posterior gradient. In previous work we identified these modifications to the composite structure of the cuticle, but only within a single species, *C. longinasus* Chittenden, 1927 [? ?]. This composite profile has now been uncovered in the rostral apex of six additional, phylogenetically disparate, species (listed below), indicating that this is likely a genus-wide trait.

Notably, we could find no evidence of resilin, as indicated by florescent microscopy, anywhere in the cuticle of the head (including the rostrum). We also observed no difference in total cuticle thickness between the head capsule and rostral apex, only differences in relative layer thicknesses. Below we demonstrate that that the endocuticle does not vary in tensile strength across the rostrum, making it unlikely that differences in sclerotization or chitin composition within the cuticle are responsible for the mechanical behavior of the rostrum. The available evidence therefore suggests that the relative flexibility of the snout is solely derived from the composite profile of its cuticle.

II. FORCE-CONTROLLED LOADING TO FRACTURE

To better characterize the mechanical behavior of the snout as an integrated whole, we performed tensile test-

ing on the snouts of six species in the genus *Curculio*, representing a mixture of closely and distantly related taxa. Although the heads were rehydrated by immersion in de-ionized water for 24 hours, we observed comparatively brittle fracture (but see below), in contrast to GIVE EXAMPLES HERE. The tensile behavior for the cuticle of these weevils is a result of its microstructure, which lacks pores, etc (give reasons). THIS IS ALSO A GOOD SPOT TO SIGNPOST WHAT I FOUND IN GENERAL.

We observed that the maximum force sustained at the site of the break was strongly correlated with the cross-sectional area of the endocuticle, and not the exocuticle. Consequently, there was a negative correlation between utlimate tensile strenth of the specimen and the ratio of exocuticle to endocuticle cross-sectional area at the site of fracture, indicating that as the proportion of exocuticle increased, tensile strength decreased. In other words, UTS is strongly correlated with the cross-sectional area of the endocuticle across species. These associations were found to be statistically significant and independent of species membership, rostrum length, and location on the snout.

These data have three important implications:

1. There is very little variation in the gross elastic behavior of the cuticle across the genus, in agreement with our current understanding of cuticle mechanobiology. (singh 2016, jansen 2016)

2. The endocuticle contributes more to the tensile strength of the rostrum than the exocuticle, possibly because the endocuticle is organized in large bundles of aligned, anisotropic fibers.

3. Thus, in addition to making the cuticle more flexible, the altered apical composite profile makes it less likely to break, suggesting a possible means by which the system evolved (via negative selection of breakage, maybe say this in conclusions).

Finally, we observed that the elastic modulus is higher in specimens with a longer snout. This observation was initially quite puzzling, as we had expected that a longer (and typically more strongly curved [insert ecomorph paper ref here]) would need to be more flexible to avoid fracture during oviposition. Based on preliminary confocal microscopy data, we speculate that this phenomenon may be the result of a longer scrobe/transition/gradient from the basal profile to the apical profile in longer rostra. A decrease in exocuticle thickness along a longer portion of the base might reinforce the snout against buckling; however Young's modulus of the rostrum would be comparatively higher in these species because of the greater volume fraction of exocuticle. [OR SAY MORE CONCISELY THAT: based on prelim CLSM data we believe that the snout is reinforced against bucking with thicker exocuticle in the base of these species; as a result, the higher volume fraction of exocuticle in the snout increases young's modulus for such species]

III. LOAD CYCLING OF *CURCULIO CARYAE*

The shearing motion and accommodation of strain led us to question how the cuticle might accommodate repeated strain, as is seen in the living organism. We therefore performed fatigue testing on a female curculio longinasus, which exhibits the most extreme degree of bending in the sis species examined.

400K cycles, complete elongation, rehydrated, coated in grease to prevent loss of moisture and stiffening of the specimen. We observed visco-elastic behavior in the specimen, as indicated by histeresis in the stress-strain relationship during each cycle. Fmax decreased logarithmically with cycle number, etc., and the specimen appeared to have deformed plastically during the test. We initially believed that this indicated damage to the specimen; however, after cleaning the specimen in a 24 hour wash with ethanol and water, we observed that the specimen returned to its original shape. The specimen did not show any evidence of fractures or shear cusps anywhere in the surface of the exocuticle, and, furthermore, the tensile strength of the specimen was consistent with other members of its species. Given this surprising result, it appears that the specimen was undamaged by the testing. We therefore believe that under normal conditions in life, repeated bending of the snout does exceed the yield/plastic limit of the cuticle, and the bending strain is purely elastic/visco-elastic.

We cannot fully account for these results, but we speculate that the microstructure of the endocuticle is resposible for what we observed. The endocuticle is made of aligned α-chitin nanofibrils whose crystaline structure is enforced by hydrogen bonds between individual chitin chains along their length. The viscoelasticity of the cuticle is thought, in part, to come from slippage between these chains as the hydrogen bonds break and reform in response to shearing between the chitin molecules. We believe that repeated strain may have caused such slippage in the endocuticle of the rostral apex during the fatigue test, but without sufficient time for the material to *completely* relax after deformation, the specimen would slowly accumulate strain and consequently deform visco-elastically/visco-plastically. After 24 hours soaking in ethanol and water, the hydrogen bonds would relax sufficiently to allow the specimen to return to its original configuration, dissipating the accumulated strain without any damage to the specimen.

IV. FRACTOGRAPHY OF TEST SPECIMENS

Examination of the fracture surfaces and adjacent cuticle of tensile testing specimens revealed that although fracture was comparatively brittle, the fracture mechanics of the exocuticle and endocuticle differed according to their microstructure, in agreement with previous studies (name some).

In cross-section, the exocuticle consistently presented

a smooth, nearly continuous fracture surface, indicative of relatively brittle fracture, likely due to the microarchitecture and resultant transverse isotropy of the Bouligand structure (explain this). Endocuticle, on the other hand exhibited severe delamination, ply-splitting, and fiber pulling, consistent with viscoelastic/plastic behavior shown in previous studies and congruent with theoretical consideration of the microstructure of this material (name something here). These patterns indicate that the endocuticle is probably less brittle than the exocuticle, most likely due to the alignment of the α-chitin fibers in each macrofiber.

In addition, the exocuticle typically appears to fracture before the endocuticle, with shear-cusp formation evident over unbroken endocuticle. We note, however, that the exocuticle of weevils/beetles is anchored to the endocuticle by cross-linking fibers in a transition zone described by Kamp et al. (see refs.). The presence of shear-cusps therefore indicates that the fibers of the endocuticle are shearing past each other within each ply along the radial/normal plane (type II shearing???). Furthermore, given the delamination observed between plies, the layers of endocuticle are liekly shearing past each other (type III shearing) along the transverse plane. We therefore infer that, under tension, the endocuticle tends to deform visco-elastically and plastically along the longitudinal axes of the macrofibers, while the overlying exocuticle exhibits brittle fracture due to shearing between the stretching endocuticle fibers to which it is anchored.

Additionally, the fracture surfaces show a characteristic failure mode, based on the pattern of fiber dislocation in the plies of the endocuticle. (copy description from DATA PRESENTATION, for figure 5B).

From this pattern we hypothesize that the exocuticle-rich gular sutures are the most likely site for the initiation of void nucleation and failure of the integrated rostral cuticle in cross section. Structural failure would take place as cracks propagate through the endocuticle from these sutures, which penetrate the entire thickness of the laminate. We speculate that this could be the reason why the cross-sectional profile of *C. caryae* is flattened ventrally. Flattening this region may reduce tensile-strain across the gular sutures when the snout is bent dorsally, thus reducing the risk of fracture in the elongate, strongly-curved rostrum in this species.

V.   CONCLUSIONS

VI.   METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

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AUTHOR CONTRIBUTIONS

Andrew Jansen: Conducted sectioning and staining, microscopy and imaging, tensile and fatigue testing, statistical analysis, and participated in manuscript preparation.

Jason Williams: Conducted tensile and fatigue testing, participated in manuscript preparation.

Nikhilesh Chawla: Facilitated microscopy, tensile and fatigue testing, and participated in manuscript preparation.

Nico Franz: Facilitated specimen acquisition and imaging, participated in manuscript preparation.

ADDITIONAL INFORMATION

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

METHODS

Histological sectioning

Tensile and fatigue testing

Specimen imaging and microscopy

Statistical analysis

General approach To explore the relationships between the composite structure and mechanical properties of the cuticle, we fit phylogenetic linear mixed-effects models to the data using maximum likelihood estimation. In order to control for phylogenetic non-independence in the data, we included the species of each specimen as a random effect in all models. We also allowed for correlation in the error term of the models, as specified by a variance-covariance matrix generated from a Brownian motion model of trait evolution along the phylogeny. Response variables and covariates were natural-log transformed, as needed, to ensure model residuals were normally distributed and homoscedastic. In all models, we tested whether the inclusion of phylogenetic correlation in the model error produced significantly better model fit, using a likelihood-ratio test and R^2_σ-difference test between the fully-specified model and a model lacking the phylogenetic effect.

Hypothesis testing The following three hypotheses were tested using PGLMMs fitted using ML estimation:

1. The maximum sustained tensile force is proportional to the cross-sectional area of the endocuticle, and *not* that of the exocuticle.

2. The ultimate tensile strength of the samples is inversely proportional to the ratio of exocuticle to endocuticle at the location of fracture.

3. Young's modulus of the samples is proportional to the length of the snout.

We fitted a fully-specified model with the cross-sectional area of endocuticle and exocuticle at the site of fracture as fixed effects, including an interaction term, and with maximum tensile force sustained prior to fracture as a response variable. This model was then compared to models with only cross-sectional area of either endocuticle or exocuticle as the sole fixed effect in the model. We then tested the first hypothesis by using likelihood-ratio tests and $R^2_{\beta*}$-difference tests between each of the three models.

The hypothesis that

Model selection and fitting

Estimating phylogenetic signal

467 **Code availability**

468 **Data availability**

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469 **REFERENCES**

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