A ZINC-HISTIDINE, IRON PHOSPHATE, AND ZINC HYDROXIDE BIO-COMPOSITE IN SCORPION CUTTING AND PUNCTURING TOOLS

We propose to study a structural biomaterial that we recently discovered in a species of scorpion. The material differs from previously known biomaterials in several ways. First, it appears to be a composite of the two types of inorganic-enhanced materials used by organisms to construct hard and damage resistant tools. The first of the two material types, biomineralized materials, employ mainly Ca-, Fe-, and Si-based biominerals, like those in calcified teeth and bones or the magnetite teeth of certain mollusks. These materials contain two distinct phases, the organic matrix and biomineral inclusions that are usually larger than 100 nm in scale. We call the second type of materials Heavy Element Biomaterials (HEBs). They are distinguished by the predominance of organic material and the lack of separate organic and inorganic phases, down to a molecular scale. The homogeneity of HEBs may allow for sharper tools in force-limited small organisms. The jaws, leg claws, stings and other "tools" of a large fraction of arthropods, some worms and members of other phyla, employ these HEBs, which contain concentrations of heavy elements (e.g. Zn, Mn, Br, I and Cu), usually between 5% and 25% of dry mass, an atom for every couple of amino acid residues or so.

This newly-identified material appears to be a nano-scale composite of both material types, an iron biomineral, and a Zn-HEB. In addition, the nano-scale Fe-biomineral inclusions are smaller than inclusions in any other known biomineralized structural tissue. And third, the material appears to contain a second biomineral, a Zn-hydroxide, on an even smaller, sub-nanometer scale. This would be the first example of a Zn-biomineral in a structural role. These small biomineral inclusions are embedded in an HEB matrix that occupies roughly half of the material. This remarkable multi-scale, triple-component composite promises to extend our understanding of both biominerals and HEBs. It is also likely to have unique properties of its own that may inform development of man-made materials for small tools.

We discovered this material during the no-cost extension of our just-ended grant (NSF BMAT 1408933) for studying HEBs. We examined the scorpion specimen in hopes of establishing the Atom Probe Tomography (APT) resolution for the ant-tooth HEB that we were studying (APT resolution can vary with sample type). The problem was that the HEB was homogeneous and provided no resolution cues. In hopes of finding resolution indicators in a similar material, we examined a scorpion that we had previously observed (using a micron-resolution proton microprobe: Schofield, 2001) to contain Fe and Zn in the cheliceral tynes (homologous to the fangs and teeth of spiders). We were surprised to find that the material contained both a Zn-HEB a Zn-biomineral and an iron-phosphorus biomineral in an extremely small scale composite.

Tool materials in small organisms are good candidates for evolution of self-healing mechanisms because damage may often be fatal for smaller animals that are reliant on sharp tools to overcome force limitations. A wolf with dull teeth can use more force to puncture its prey, but a leafcutter ant with dulled mandibles cannot cut leaves (Schofield *et al.*, 2011).

One interesting possibility is that labile Zn-histidine bonds absorb energy by breaking and reforming in a manner similar to the self-healing mechanism proposed for mussel byssus threads (Schmitt *et al*., 2015). The idea would be that the ubiquitous histidine in the Zn-HEB would, at the HEB-mineral interface, bind to some of the Zn in the Zn-hydroxide biomineral, strengthening the connection and reducing the possibility that the composite would fracture at the interface. If high forces nevertheless break the bonds at the interface, energy would be absorbed and no longer available for fracture elsewhere. Later, the labile Zn-histidine bonds could reconnect, healing the incipient crack before further use of the tool enlarges the crack. If the Zn-hydroxide is mobile, it might even fill in wider cracks that open up between components of the composite. The Zn-hydroxide would be a ready reservoir of Zn for re-establishment of the high density of Zn-histidine bonds in the HEB.

Histidine-metal complexes have already been employed in polymer design, including for self-healing (Zechel et. al., 2019). The study of this new composite may suggest similar schemes that can be used to improve hard composites. For example, the self-healing performance of a hard composite that employs Zn-histidine complexes might be improved by having a ready source of zinc for healing, such as in the widely-distributed Zn-hydroxide in the scorpion composite.

The uniquely small size of the biomineral inclusions in this composite likely allows for sculpting of smaller scale features such as the sharp tip of the scorpion's cheliceral tyne. In general, small organisms do not use biominerals in sharp tools, possibly because of a crystal size limit that we may understand better by studying this exception. The small size of the inclusions may also reduce the likelihood of fracture at the boundaries between phases.

As humans continue to build ever smaller machines, we may learn from the materials that have evolved in small organisms to meet the distinctive challenges associated with mechanical interactions on a small scale, and with the organism's absolute reliance on sharp tools to overcome force limitations. A greater understanding of these biomaterials may lead to transformative approaches to man-made materials, such as new self-healing techniques and a better understanding of control of crystal size in human biomaterials like teeth and bones.

We propose to study the composition and mechanical properties of this material using APT, TEM, SEM, and nanoindentation. In addition, we will use a puncture tester along with scanning electron microscopy movies to study the chipping and plastic behavior of the material during puncture or cutting and to look for self-healing properties. We will compare this behavior to that of human-manufactured blades or tips. In addition, we will use time series of developing scorpions to compare the development of the composite material to our previous studies of the development of Fe-biominerals and Zn-HEBs.

BACKGROUND AND RESULTS FROM RECENT NSF SUPPORT

We begin by reviewing the components of the composite, HEBs and biominerals. Then we discuss their roles in crabs that employ both, although not in a composite. Finally, we focus in greater depth on our recent NSF-supported study of HEB properties, before discussing the proposed research.

HEAVY ELEMENT BIOMATERIALS (HEBs)

The recognition that the fracture-prone tools of many small organisms contain unique materials has developed slowly over the last few decades. The first indication of distinct materials came from Bryan and Gibbs who found Zn in the jaws of nereid marine worms and Cu in the jaws of glycerid marine worms and suggested that they might improve the mechanical properties of the jaws (Bryan and Gibbs 1979; Gibbs and Bryan 1980a, b, c). Subsequently, Hillerton and Vincent (1982) and Hillerton et al. (1984) detected Zn and Mn near the cutting edge of the mandibles of many insects (Hillerton *et al*., 1982).

Our group stumbled into the field after developing high-energy ion microscopy (Schofield *et al*, 1988; Schofield, 1990; Schofield & Lefevre, 1992, 1993), and observing Zn-rich mandibular teeth in an ant captured while crossing the lab floor. High-energy ion microscopy (Figure 1A, B, G and H) enabled rapid surveys of whole structures and concentration measurements on a micron scale which revealed the extremely high metal concentrations. We found that different regions of a single animal could be enriched with different metals, such as Zn in the fangs and Mn in the marginal teeth of spiders (Figure 1F) and we found that these enriched biological materials were employed in structures other than mouthparts, such as leg claws (Figure 1B) and stings (Figure 1C; Schofield & Lefevre, 1989, Schofield *et al*., 1989, Schofield, 2001). The HEBs are used for cutting, piercing, digging, grasping, clamping and traction. We did not find HEBs in structures such as joints that might benefit from hardening or abrasion resistance but do not directly contact the environment. The enriched structures were often sharp and susceptible to fracture, abrasion and impact. We have since engaged in studying the properties, biochemistry and behavioral corre-

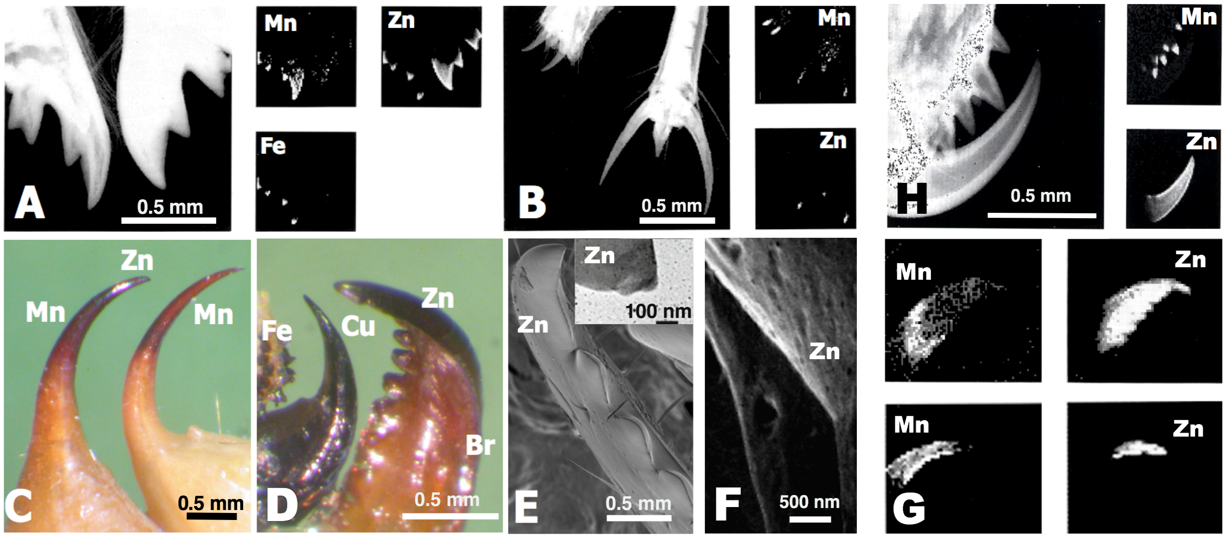


Figure 1. Images of HEB-containing tools. **A**: cheliceral tynes (mouth claws) of the scorpions *Centruroides exilicauda* (left, where we found the composite) and *Paruroctonus mesaensis* (right). **B**: leg claws of the scorpions *Centruroides exilicauda* (left) and *Vaejovis confusus* (right). **C**: Stings of the scorpions *Vaejovis confusus* (left) and *Centruroides exilicauda* (right). **D**: tools of marine worms, paragnaths of *Nereis vexillosa* (left), jaw of *Glycera convoluta* (middle), jaw of *Nereis vexillosa* (right). **E,** mandible of the ant *Atta cephalotes*, showing the 50 nm radius cutting edge (inset cross-section). **F.** Cutting edge of scorpion, *Centruroides exilicauda,* pedipalp teeth; edge radius is less than100 nm. **G.** Common motif of Zn at the tip and Mn in the shaft; Top row: marine worm *N. Brandti*, Bottom row, scorpion sting, *V. confususs*. **H.** Fangs and marginal teeth of the spider *Araneus diadematus*.

lates associated with these materials.

Mechanical Properties of HEBs

Several early investigations found a higher indentation hardness for Zn-enriched structures than for surrounding regions (Hillerton and Vincent, 1982, Hillerton, Reynolds & Vincent, 1982, Schofield, 1990, Edwards *et al*., 1993, McClements *et al*., 1993). However, similar levels of hardness had also been found in certain Zn-free regions (Hillerton, Reynolds & Vincent, 1982) and, more recently in other non-HEB cuticle (Cribb et al., 2010). To strengthen the correlation between Zn-enrichment and hardness, we took advantage of our observation that Zn is incorporated very late in cuticle development (Schofield, Nesson & Richardson, 2003). We measured the hardness of the mandibular teeth of adult ants, using nanoindentation, during the period when Zn concentration increases in early adult life (Schofield, Nesson & Richardson, 2002; NSF IBN 9817206). We found that the hardness increases nearly threefold as the Zn is incorporated, from about 0.3 to 0.9 GPa.

Broomell et al. (2006) found that removal of zinc, using the chelating agent EDTA, from internal regions of the jaws of a nereid worm reduced hardness from about 0.7 to about 0.2 GPa. Furthermore, when the depleted regions were soaked in $ZnCl_2$, $MnCl_2$ or $CuCl_2$, the hardness was substantially restored (Broomell, Zok & Waite, 2008).

Hardness and modulus of elasticity were, prior to our recent grants, the only mechanical properties that had been measured for HEBs.

Composition of HEBs

The first composition hypotheses were presented by Hillerton and Vincent (1982) who suggested that Zn in the mandibles of insects might increase the number of secondary bonds in the cuticle and thereby increase the density and fracture toughness of these structures. Amino acid analysis and sequencing of the dominant protein from the tips of Zn-enriched worm jaws, indicated that the fraction of histidine residues could be as high as about 27%, which has led to the suggestion that Zn cross-links proteins as $Zn(His)_3$ Cl (Bromell *et al*., 2006, Birkedal *et al*., 2006, Broomell *et al*., 2008). However, if Zn were bound in

$Zn(His)_3$ Cl, the maximum concentration would be about 5% Zn. In contrast, we have measured concentrations of about 18% in nereids and this analysis does not consider the difficulty of orienting every histidine so that it could participate in a Zn-mediated cross-link at the proper angle.

For NSF IOS 0422234, we tested the hypothesis that Zn was present in nano-scale biomineral inclusions using X-ray absorption spectroscopy (XAS) and other techniques. Our XAS results did not support this hypothesis; we found no Zn – Zn scattering signal though we would expect to find such a signal even for amorphous biominerals (Tao *et al*., 2007). These results suggest that either the Zn atoms in HEBs are further apart than about 0.6 nm, or else that the Zn-Zn distance is highly variable. In addition, we found no signs of order in X-ray and electron diffraction experiments and no distinguishable biomineral phases were evident at a TEM resolution of about 5 nm.

EXAFS did reveal the similarity of the chemical environment of Zn across many organisms from multiple phyla (Figure 2). For each of the organisms examined, EXAFS indicated that Zn is 4-coordinate, bound to O and/or N, and, for arthropod specimens, the data suggests that only one in four of these binding sites could be an imidazole, while two imidazoles could be possible in the worm jaws. EXAFS data were also consistent with binding to the oxygens and/or nitrogens of chitosan (Braier & Jishi, 2000, Okuyama *et al*., 1997, Schlick, 1986), or catechols (unpublished analysis). EXAFS data are not consistent with Cl binding as proposed by Broomell *et al*. (2006, 2008). The EXAFS spectra are similar to spectra for Zn in concentrated aqueous hydroxide (Pandya *et. al*., 1995).

BIOMINERALIZED TOOLS

Biomineralized tissues in animal tools require less of an introduction than HEBs because they are more widely studied, if not more widely employed. Biominerals likely played a central role in enabling the great diversity of organisms that developed in the Cambrian and after. Nevertheless, the mechanisms by which they are deposited are still being studied. Because of the Fe-biomineral in the new composite, we will focus on the main examples of iron-biomineral enriched tools, the teeth of mollusks.

Michael Nesson, who will be one of the senior personnel, first described the process of iron biomineralization in the teeth of chitons (Nesson & Lowenstam, 1985). The iron comes from membrane –bound storage vesicles filled with ferritin protein molecules which contain a core of ferrrihydrite (a weakly diffracting ferric hydroxide phosphate). The protein is digested, the iron core is released to the cytoplasm in a solubilized form (presumably Fe^{2+}) and is then pumped through bundles of microvilli into the organic tooth matrix. Alteration of pH and redox potential initiates precipitation of ferrihydrite on the organic framework of the matrix. As additional iron (presumably as Fe^{2+}) is transported into the matrix, the amorphous ferrihydrite is transformed into micron-scale magnetite crystals (Fe_3O_4) and the teeth reach their final, black form.

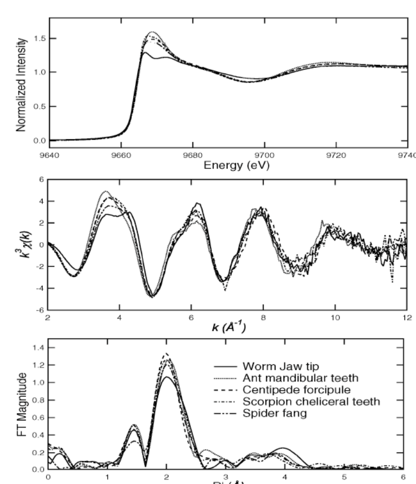


Figure 2. Comparison of Zn XAS data for a worm, an ant, a centipede, a scorpion and a spider. In all cases the chemical environment appears similar.

Recent APT work on chiton teeth (Gordon & Joester, 2011) showed that the organic filaments that make up the walls of the loosely-formed matrix compartments can, at least in the magnetite state, contain high concentrations of either sodium, or of magnesium. Possible roles for these elements are as nucleation sites or as sites for binding residual anions. We will search for similar features in the scorpion material.

An important point about the mature teeth of chitons, is that the magnetite crystals are micron-scale, and too large to produce the structures with sharp points and edges like many of those made from HEBs, including the tips of the tynes of scorpions. One possibility is that the biomineral formation process in the tynes is similar to that in the chiton, but halts at the amorphous phase. Alternatively, the tyne material may be made up of very small crystals.

HEBs AND BIOMINERALIZATION IN SEPARATE REGIONS OF CRAB CLAWS

Most crab claws are highly calcified and crustacean cuticle has become a model system for invertebrate biomineralization. We discovered that certain crabs employ Br-HEBs at the tips of their calcified claws (not a nano-scale composite of the two materials like the material we propose to study here). This gave us an opportunity to compare the advantages of biomineralized and HEB materials in a single organism (NSF IOS 0422234). We compared the two materials by measuring hardness, modulus of elasticity, abrasion resistance, energy of fracture and dynamic mechanical properties. In addition to Atomic Force Microscopy (AFM) nanoindentation measurements, we built miniaturized versions of standard abrasion and fracture testing equipment that could be used on small samples for these comparisons.

Our results (Schofield *et al*., 2009). suggested that the main advantage of brominated cuticle over calcified cuticle was resistance to fracture (9 times more energy was required to fracture similar surface areas). Other things being equal, a claw tip made of brominated instead of calcified cuticle would require a 6 times greater deflection, and a 1.4 to 3 times greater force to produce the same area of fracture. Another important difference was that the largest structures observed in the Br HEB were 7 nm laminae, 1/1000 of the size of crystals in calcified cuticle.

Calcified cuticle appeared to be best for regions designed to apply large pressures, but that were not sensitive to fracture, while brominated cuticle would be optimal for sharp edges and tips that must keep their shape under pressure and not fracture. This was consistent with the observed crab behavior. The HEB regions were used as forceps while the calcified regions were used for crushing and were more often damaged by small fractures.

WEAR MATTERS

A second part of our NSF IOS 0422234 grant, was to investigate the degree to which average levels of wear influenced the performance of arthropod tools. We used leaf-cutter ants (*Atta cephalotes*) as a model and found the cost of wear to be greater even than we expected (Schofield *et al*., 2011). We measured the cutting rates of leaf-cutter ants in Panama and then collected the cutting ants and measured wear of their mandibles. We also developed an instrument to measure the force required to cut leaves using their dissected mandibles. From our measurements of cutting rate and cutting energy as a function of wear, we estimated that a hypothetical colony of leaf cutter ants with un-worn mandibles would have spent about half as much time and half as much energy cutting leaves outside the nest than our field colony did. Factors of two in energy can be expected to produce huge evolutionary pressures and we were not surprised to find that the ants with the most worn mandibles contributed to the colony by carrying leaves but not by cutting them.

We also discovered that *Atta* leaf cutter ant mandibles start out literally as sharp as a razor blade (50 nm blade radius), and that the force required to cut leaves is about what it would be for a new scalpel blade.

RESULTS FROM OUR MOST RECENT NSF GRANT

In our most recent grant (NSF BMAT 1408933, Biomaterials for Small Tools) we modified or developed new testing equipment for even smaller test pieces than for the crab results, so that we could measure previously un-measured properties of HEBs. In addition, we began using Atom Probe Tomography (APT) to investigate the nanoscale chemical structure. We made major advances in the understanding of HEBs: their mechanical properties, why they are employed mainly by small organisms, and their remarkable composition. The mechanical property measurements and the APT investigations are summarized in Schofield *et. al.*, 2020 (https://darkwing.uoregon.edu/~rmss/TheHomogeneousAlternToBiomin.pdf).

The main advantage of the zinc- and manganese-containing materials, relative to plain organic structural materials, appears to be that they are stiffer but, nevertheless, equally or more damage resistant, as indicated by their high hardness, abrasion resistance, damping, and energy of fracture. Their high hardness makes them especially resistant to permanent indentation and scratch damage that might reduce the efficiency of sharp "tools" and mating surfaces (such as in scissors and forceps). The main advantage of the zinc- and manganese-materials, relative to the tested biomineralized materials, is likely their homogeneity, which may enable sharper and more precisely shaped structures since HEBs do not have mineral inclusions. In addition, the homogeneity of HEBs relative to biomineralization, may reduce chipping damage like that found along razor blades at boundaries between grains of differing stiffness (Roscioli *et al*., 2020). Some of the HEB-containing tools that we examined take advantage of HEB homogeneity by starting out, after molting, with edge and tip radii of less than 100 nm (Fig.1D, E), 100 times smaller than a single Ca-mineral inclusion in crab cuticle. The material we propose to study here has biomineral inclusions on a scale of a few nanometers, and may aid in the understanding of why most structural biominerals are on a micron rather than a nanometer scale.

Mechanical properties

The results from our most recent grant for Zn- and Mn-HEBs, represent, by far, the most comprehensive series of measurements of the mechanical properties of HEBs. To avoid scale or other systematic differences, we also measured mechanical properties of a variety of engineering and biomineralized materials for direct comparison. Table 1 shows the results of our measurements. The investigations in the past have generally been on single species, and often, single individuals. The table is compiled from at least 1,500 hand-positioned measurements on about 150 fresh organisms from colonies in our lab, representing several distantly-related species. The results illustrate the dangers from drawing conclusions from a single species, much less single organisms, as the HEBs can outperform unenriched materials in some species but not others. It is important to recognize that the variability of complex biological materials is usually much greater than that of man-made materials. The most important universal differences that we found follow.

Most significant and consistent differences between HEBs and plain materials

High hardness and modulus of elasticity. Figures 3A (hardness) and 3B (modulus of elasticity) show that the Mn- and Zn-HEBs were harder and had a higher modulus of elasticity than the non-HEB materials in all cases. The lowest hardness and modulus of elasticity values for both the Zn- and Mn-HEBs were significantly higher than the highest value of the tested non-HEB material in any of the organisms, even across phyla (hardness: $p<2e-10$; modulus of elasticity: $p<0.004$). The measured hardness of most Zn- and even Mn-HEBs was similar to that of the measured materials fortified by calcium biominerals (in crab claws and salmon teeth – Table 1), indicating that biomineralization is not the only way to achieve high hardness. Indentation hardness is especially important in maintaining the shape of sharp blades, tips, or mating surfaces (such as forceps-like tools). This is because higher hardness indicates smaller residual plastic deformation of the surface, plastic deformations that can reduce the sharpness and precision of the tools. A high modulus of elasticity is also important for tools that cut, puncture and manipulate, as it reduces the temporary elastic blunting of blades and points, and the mismatch of mating surfaces, as forces are applied to the tool.

High loss tangent for hardness. Another notable feature of the examined Zn- and Mn- HEBs was the high values for absorption of vibration energy, the loss tangent, especially given how hard they were (Figure 3C). One way of avoiding fracture damage is to rapidly absorb energy from the interaction of the tool with the environment, that could otherwise break bonds and produce new fracture surfaces.

*High energy of fracture for hardness***.** HEBs also appear to have a valuable balance of hardness and energy of fracture – typically there is a trade-off between hardness and energy of fracture (e.g. glass is hard but fracture prone). Table 1 shows that the measured values for the Zn-rich regions of ants, scorpions and spiders are about equal in hardness to the calcified claw cuticle, and yet have a higher energy of fracture (for Ashby-style plots see: (https://darkwing.uoregon.edu/~rmss/TheHomogeneousAlternToBiomin.pdf).

Table 1. Results from mechanical testing.

Material	Hardness (Gpa) from image	Std. Dev.	Hardness (Gpa) Oliver-Pharr	Std. Dev.	Reduced Modulus of Elasticity (GPa) from image	Std. Dev.	Reduced Modulus of Elasticity (GPa) Oliver-Pharr	Std. Dev.	n	Loss tangent, 10 Hz	Std. Dev.	n	Energy of Fracture (J/m2)	Std. Dev.	n	Impact resistance, 20 um tip (J/m to fracture)	Std. Dev.	n	Wear Resistance (J/m3)	Std. Dev.	n
Ant, HEB-free region (Atta cephalotes)	0.37	0.05	0.38	0.05	5.29	1.10	5.50	1.10	99	0.041	0.016	24	3885	2514	8				4.34E+09	3.55E+09	27
Ant, Zn region	0.99	0.43	0.77	0.14	9.21	2.16	8.36	2.01	82	0.069	0.031	26	546	151	4				4.99E+09	2.91E+09	13
Spider fang, HEB-free region (A. diadematus)	0.52	0.14	0.51	0.07	6.48	0.92	6.52	0.81	55	0.035	0.023	26	697	700	8	3.65E+00	1.69E+00	10	2.38E+09	1.40E+09	17
Spider fang, Zn region	1.37	0.38	1.17	0.30	12.04	2.39	11.10	1.91	56	0.036	0.032	28	903	690	6	4.83E+00	1.52E+00	7	2.36E+09	1.21E+09	16
Spider marginal teeth, Mn region	1.01	0.28	0.81	0.09	9.64	1.53	8.74	0.92	45	0.029	0.009	21							4.03E+09	8.82E+08	7
Worm jaw, HEB-free region (A. brandti)	0.51	0.12	0.50	0.08	8.64	1.28	8.62	1.17	48	0.025	0.015	22	907	697	5	2.09E+01	5.57E+00	9	2.35E+09	1.33E+09	15
Worm jaw, Zn region	0.78	0.21	0.76	0.07	10.79	1.62	10.76	1.55	50	0.021	0.012	22	945	747	5	1.89E+01	4.86E+00	12	5.80E+09	3.13E+09	13
Worm paragnath Fe region	0.26	0.07	0.16	0.06	2.60	0.76	2.07	0.84	3	0.148	0.009	3									
Scorpion sting, HEB-free reg. (H. Arizonensis)	0.51	0.13	0.45	0.06	6.09	2.15	5.79	1.94	21	0.002	0.006	21	2981	1641	6	1.34E+01	3.32E+00	5	2.66E+09	7.72E+08	4
Scorpion sting, Zn region	1.02	0.35	0.68	0.15	11.95	2.34	9.94	1.71	27	0.054	0.029	24	548	661	2	1.14E+01	4.85E+00	6	5.70E+09	2.03E+09	5
Scorpion sting, Mn region	0.80	0.22	0.69	0.10	10.61	1.73	9.93	1.00	38	0.021	0.013	32	180	298	5	9.08E+00	5.60E+00	12	5.19E+09	3.20E+09	15
Salmon tooth (O. tshawytscha)	1.24	0.12	1.31	0.12	25.76	2.53	27.46	1.70	6	0.072	0.006	3	1706	751	9						
Chiton radula, Fe-biomineral (K. tunicata)	5.21	4.02	3.14	1.28	49.51	23.21	41.48	15.87	29	0.198	0.081	20	20	22	4				2.87E+10	1.3E+10	9
Chiton radula, Fe-biomineral (C. stelleri)	6.27	1.07	2.48	0.11	71.09	15.73	45.40	12.83	3	0.231	0.010	3	6	3	3						
Crab claw, Ca biomineral region (P. crassipes)	1.79	0.35	1.49	0.4	35	11	35	12	38	0.020	0.020	20	348	138	7				1.25E+09	3.13E+08	7
Crab claw, Br HEB region (P. crassipes)	0.6	0.08	0.49	12	7.6	2.2	7.6	2.7	45	0.130	0.070	19	2931	574	6				1.00E+09	2.00E+07	7
Cat claw (F. cattus)	0.35	0.02	0.31	0.03	5.3	0.3	5.5	0.5	6				13321	8810	9						
Tarantula fang, HEB-free region, (A. hentzi)	0.67	0.11	0.66	0.03	8.98	1.27	8.91	0.57	6												
Tarantula fang, Zn-region	1.41	0.28	1.27	0.14	16.01	2.89	15.25	2.36	4												
Nylon 6 (not dried)	0.14	0.01	0.2	0.01	2.5	0.16	3.1	0.14	3	0.100	0.010	3							3.23E+09	5.10E+08	37
PMMA acrylic	0.29	0.06	0.34	0.04	5.85	0.65	6.74	0.83	17	0.071	0.007	6	188.3	151.849	8						
Polycarbonate	0.22	0.07	0.26	0.01	3.43	0.89	3.80	0.49	19	0.016	0.003	10				2.69E+00	7.50E-01	5			
Fused silica or glass = *	11.99	4.78	9.80	0.93	75.06	15.98	64.68	12.90	73	-0.001	0.025	73	3.08	3.49	5	2.44E+00	1.14E+00	11			
Aluminum, single crystal	0.72	0.17	0.86	0.04	42.28	16.04	45.38	16.04	7	0.208	0.014	7									
Stainless steel, 430	4.09	0.56	6.90	0.32	116.36	11.98	151.43	12.30	7	0.224	0.013	7									

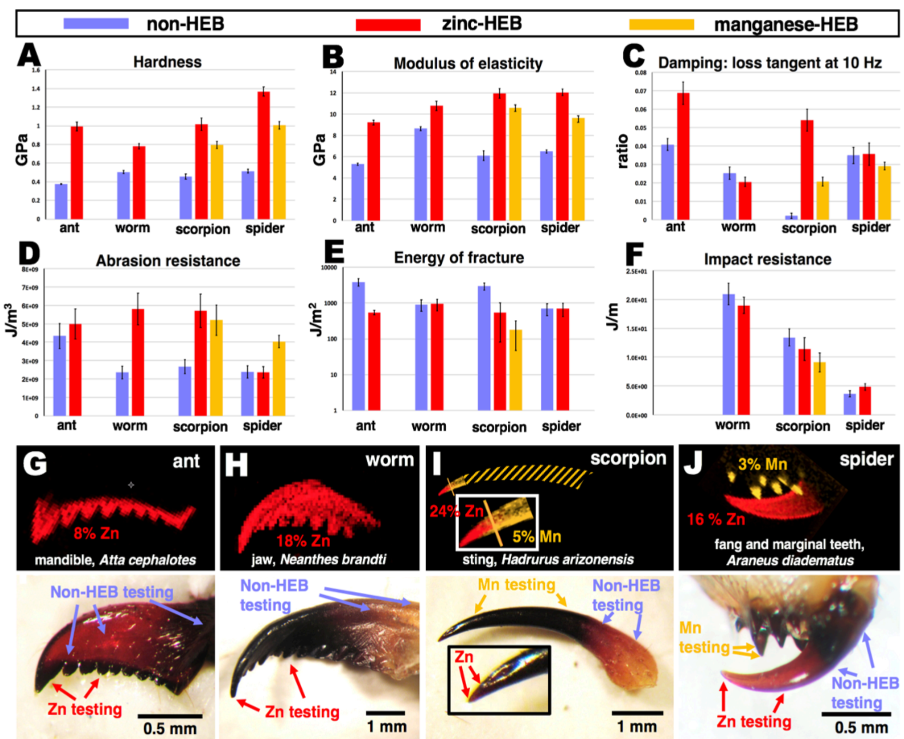


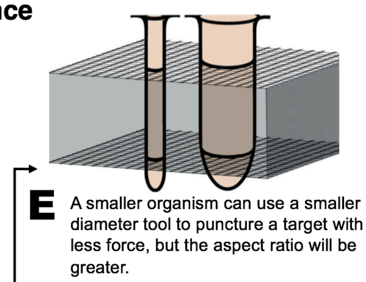
Figure 3. **A-F**. Results from mechanical testing of the main organisms. Zn-, Mn- and non-HEBs, are indicated by red, orange and blue coloring, respectively. Error bars show the standard deviation of the mean of measured values, standard deviations are given in Table 1. **G-H**: Images of the tested structures; top row, high-energy ion microprobe (Schofield, 2001) elemental maps of zinc (red) and, superimposed, manganese (orange). Bottom row, photographs of specimens of the indicated species. The element concentration values (percent of dry mass) are for the most enriched regions The orange hash-marked region of the scorpion sting in "I" indicates the extent of the Mn-HEB region in the photograph below it, but is not part of the microprobe image. Regions used in mechanical testing are indicated with arrows in the photographs. Because of the different scales of the tests, there may be differences in the material sampled.

Comparison to engineering materials tested in the same manner. The HEBs and even the plain cuticle, were harder and had a higher modulus of elasticity than the plastics we tested (Table 1). The HEBs had better balances of damping and energy of fracture for their hardness than the engineering materials tested. Most of the Zn-HEBs exceeded the Lake threshold of E*tan(delta) = 0.6 for highly damped engineering materials (Lakes, 2016), while none of the plain materials did.

Differences between Zn- and Mn-HEBs. Different HEBs are often used in different regions of tools. The examined Zn- and Mn-HEBs differed mainly in that the Mn-HEBs were intermediate in modulus of elasticity and hardness between Zn-HEBs and plain cuticle. This result may explain the observation that Mn-HEBs are often located between the plain materials and Zn-HEBs (see the similar motif in scorpion

A **Scaling rules for force, tool sharpness, and tool fracture resistance**

	Force require to produce a given stress		Tool diameter D, assuming equal stress and maximum force proportional to square of body length		Energy to fracture tool / Energy to puncture target	Energy stored in tool / energy to fracture tool	
	thick target	thin target	thick target	thin target		for lateral forces	for axial compression
punch	$F \propto D^2$	$F \propto D$	$D \propto L$	$D \propto L^2$	$\propto D$	$\propto 1/D^4$	$\propto 1/D^2$
blade	$F \propto D$	$F = C$	$D \propto L^2$	no change with L	= constant		
crushing surface		$F \propto D^2$		$D \propto L$	$\propto D$	N/A	N/A



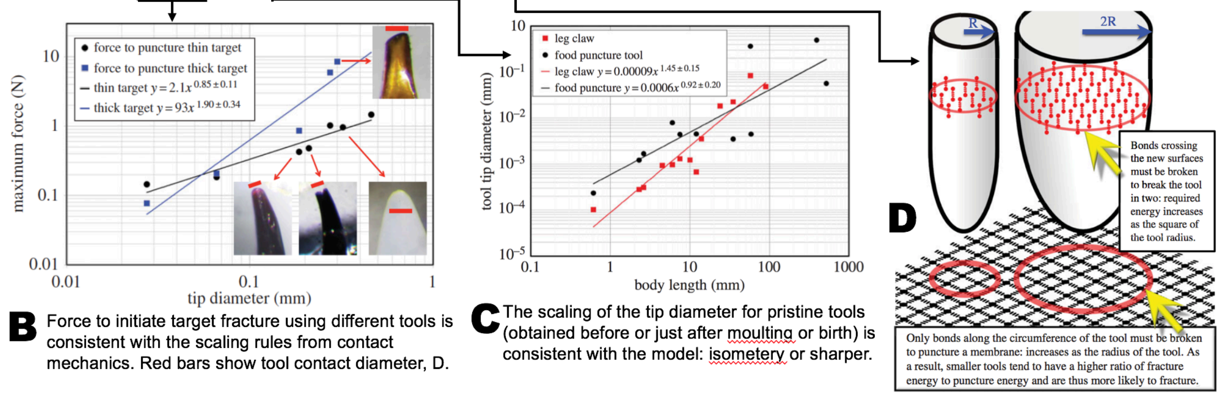


Figure 4. Scaling rules and consistency checks. **A.** Table of scaling rules derived using contact mechanics. **B.** Check of force rule for thin or thick puncture. **C.** Check of predicted tool sharpness as a function of body size. **D.** Cartoon explaining why there is less safety margin for fracture of sharper tools. **E.** Cartoon explaining why small organisms are likely to have high-aspect ratio tools that can store more energy as they bend, also reducing the fracture safety margin. Schofield *et. al.*, 2016.

stings and worm jaws of Figure 1G). Smaller steps in mechanical properties would reduce the tendency to concentrate stresses, that may lead to fracture at interfaces between materials.

The need for HEBs: tool tip radius scales isometrically or sharper with smaller body size, and sharper tools are more susceptible to fracture.

The hard, sharp, but fracture resistant structures that can be made with homogeneous HEBs, are likely important to small organisms because sharper tools can compensate for the force limitations associated with a smaller body. For the recent grant, we developed scaling rules that suggest that smaller animals do need to use sharper tools and sharper tools are more susceptible to fracture (Schofield *et al.,* 2016). Damage resistance is especially important because, when organisms rely on sharpness, blunting can be fatal.

The results of our investigations are summarized in Figure 4. The first two columns, derived from simple contact mechanics models, give the relation between the force required to reach failure stress levels, and the diameter of the tips or edges of tools such as teeth, claws and cutting blades. We made a machine to test puncture forces for small, real tools, and found that the required forces were well predicted by the scaling rule (Figure 4B). The scaling rule we developed for the sharpness of tools as a function of animal size, was based on the assumptions that maximum force goes as the square of body length and that animals of all sizes puncture the same materials (e.g. a wolf and a mosquito both have to puncture the same elk skin). We tested the assumptions and scaling rule using teeth and claws from organisms covering about 3 orders of magnitude in body length. The results (Figure 4C) show that the sharpness of pristine tools (from animals just before or after molting or birth) does indeed scale as body length or more rapidly with decreasing size. None of the organisms with pristine tool radii under a couple of microns, employed biominerals. Our results may explain why most arthropods use homogenous HEBs instead of biominerals.

We also developed scaling rules for the importance of fracture resistance as a function of tool sharpness, finding that fracture resistance is more important for sharper tools. In particular, for a simple model, we found that the ratio of the energy required to break a tool tip, to the energy required to puncture a given membrane, scales as the radius of the tip. That is, a larger tip has a greater safety margin - it requires more energy to fracture the tool tip relative to the energy required to puncture the membrane. This applies to man-made tools as well as animal tools. A simplified graphical explanation from the paper is shown in Figure 4D.

We further argued that, while sharper tools made it possible for smaller animals to puncture materials with less force, there would be a tendency for the tools to have a higher aspect ratio in order to reach to similar depths with a smaller diameter, illustrated in Figure 4E (and evident in Figure 1). The problem with higher aspect ratio tools, is that they tend to bend more and store more energy. The energy stored in bending can greatly increase the fracture risk for smaller-diameter tools of the same length as larger-diameter tools (last columns of Figure 4 A). Stiffening to reduce energy stored in bending may explain the use of Mn-HEBs, with high modulus of elasticity, in the shafts of many tools.

Composition: hypothesized zinc binding in ant teeth - a single histidine and water

APT fragments. We used the mandibular teeth of the leaf cutter ant, *Atta cephalotes*, as our representative Zn-HEB for APT studies. The use of APT on biological materials is relatively new and we are currently helping to address problems such as damage and analysis of complex fragments. We began by examining standards (such as zinc picolinate) and adjusting beam current densities and other parameters in both the FIB-SEM preparation of APT samples and in APT itself in order to minimize damage detected with SEM and to minimize differences in the elemental content obtained from APT of the standards and from their formulae (Wang *et. al.*, 2017).

About 67% of identified zinc-containing APT fragments consisted of $ZnNC^{+1}$ and $ZnN_2C_2H^{+1}$. These fragments could arise from a single histidine connected to the zinc atom. The other 33% of the fragments were isolated Zn ions. Our previous XAS data preclude Zn binding to C (Tao et al., 2007), so most of the zinc is apparently bound to nitrogen. This is consistent with the hypothesis that the zinc is mainly bound as individual atoms to nitrogen in an organic matrix, such as to the nitrogen in imidazole rings of histidine residues in proteins. However, the quantity of zinc in ant mandibular teeth is too high for each atom to be bound to four histidines (in the typical zinc binding motif: McCall et al., 2000, Karlin and Zhu, 1997). Instead, it may be that the dominant binding motif is to a single histidine and water.

Testing binding hypotheses using elemental analysis. The concentration of heavy elements in HEBs are so great that binding hypotheses can be tested using gross elemental analyses. We used energy dispersive X-ray spectroscopy in order to determine elemental ratios of Zn/N, Zn/O and N/C in the ant teeth. In order to improve the accuracy of the EDX technique we calibrated using three standards that were similar to the ant teeth in elemental composition. Amino acid analysis was used to estimate the elemental composition of the proteins in the ant teeth for developing composition models to test against the EDX elemental analysis.

The models were made up of varying ratios of components that included protein, chitin, water and the catechol NBAD, which is found in tanned cuticle (Suderman *et. al.*, 2006). The models were compared to the ant measurements using Markov Chain Monte Carlo and Bayesian parameter estimation methods. In order to obtain probability distribution functions for the actual elemental ratios in the ant teeth, we used the differences between formula and EDX-measured ratios for 5 standards (zinc picolinate, glycine zinc salt monohydrate, 8-hydroxyquinalone Zn, zinc acetate, and tris base). Information was combined from at least three measurements of each standard and 11 measurements of teeth from 5 different ant specimens.

The probability that the observed element ratios could be obtained from Zn binding to 4, 3, 2 or 1 histidine residues is, respectively, 0.01, 0.11, 0.37 and 0.37. Thus, the element concentrations are most consistent with Zn binding is to 1 or 2 histidines. If each Zn atom were bound to two histidines, 95% of all protein residues would have to be histidine, while histidine fractions of 28% have been measured (Broomell *et al.*, 2008). Therefore, our preferred model is to a single histidine residue and 3 waters.

The function of zinc in HEBs

Only specialized proteins like metallothioneins bind metals in such high concentrations – no previously identified structural proteins can bind so much metal over macroscopic volumes. A minor fraction of the zinc, but only a minor fraction, may bridge two or more histidine residues, or connect histidine residues to NBAD, reducing plasticiy with cross-links. But, considering cross-link densities in other materials, even if only a minor fraction of the zinc molecules were involved in cross-links, the cross-links may have a significant effect on the material. The zinc may also act to reduce plasticity and increase hardness by binding water and reducing its mobility and, as a result, reducing the water's ability to lubricate relative displacement of macromolecules. Finally, we speculate that the histidine-zinc-water binding motif may act as a damping pot, and, in the large numbers that are present, absorb significant energy from high frequency mechanical vibrations induced by tool use, energy that would otherwise be available for fracture. The argument that high-mass density may be useful, may help explain why heavy elements, like Mn, Zn, Br, and Cu are used in HEBs, and why their damping properties are so good.

PROPOSED RESEARCH

PRELIMINARY DATA

When we examined APT data for the cheliceral tyne of a buthid scorpion it was immediately clear that we were observing a new phenomenon. The APT data for the Zn-HEB in an ant, and TEM data from several other HEBs showed no structure on the scale of a few nanometers, but this new material had very distinct regions. The material appears to be composed of an Fe-biomineral, on a smaller scale than known biomineral inclusions, and a Zn-HEB, making it the first example of a biomineral-HEB composite. The techniques we have developed for studying biomineralization and HEBs, discussed above, position us perfectly for investigating this new material.

Figure 6 shows the scorpion source (Figure 6A), and details of the newly discovered composite. EDX data (Figure 6B) shows that iron and zinc are co-localized at the tip of the tynes, but the zinc-containing region is larger and extends proximal to the iron-containing region, suggesting a pure Zn-HEB region. The reconstruction from the APT data is shown with Fe and Zn fragments, and, as a representative of or-

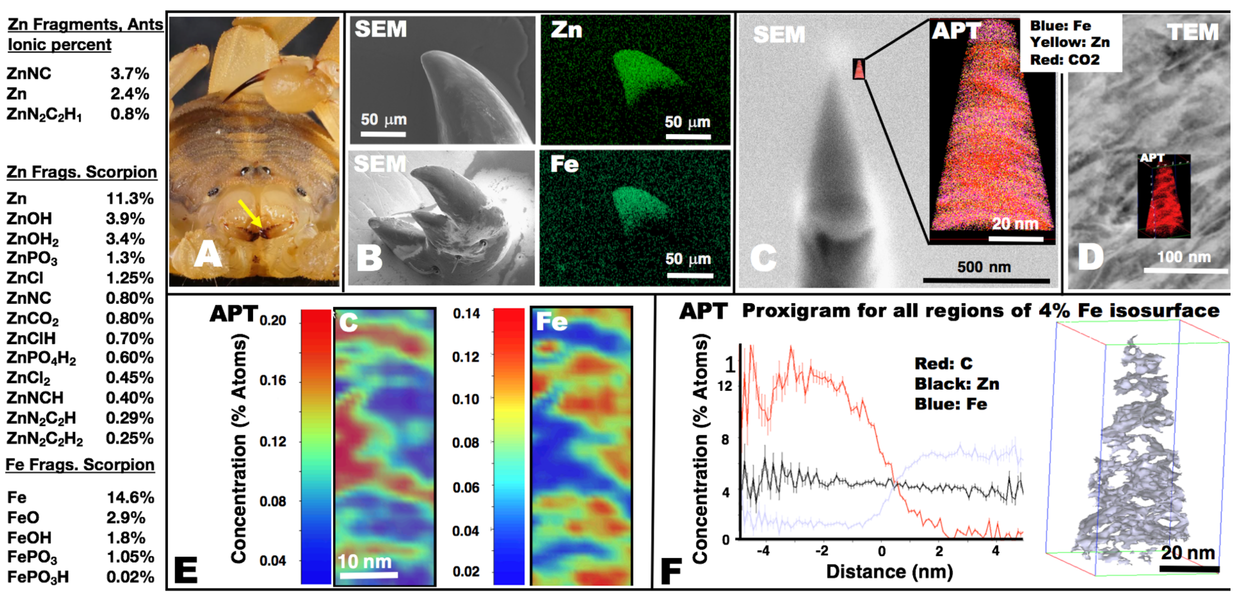


Figure 6. Preliminary data. **A.** Scorpion, *Centruroides exilicauda,* showing the chelicerae. **B.** Zn and Fe distributions in a cheliceral tyne. **C.** SEM of APT preparation of the Zn and Fe region, with the APT reconstruction shown on the same and larger scales. **D** TEM and APT images showing similarity in structure. **E.** 2-D distributions of C and Fe showing segregation. **F.** Proxigram and corresponding 4% Fe isosurface. There is very little C in the Fe-rich region, characteristic of biominerals.

ganic fragments, CO_2 fragments (Figure 6C). The APT reconstruction of the CO2-containing regions (red) is overlaid on a TEM image of this material, showing that the structure seen in the APT reconstruction of the carbon distribution is similar to the regions of low electron density in the TEM image, increasing confidence that the reconstruction is accurate (Figure 6D).

The APT 2-D concentration plots, (Figure 6E) and especially the Proxigram (Figure 6F), shows that there is little or no carbon in the iron-containing region, and little iron in the carbon-containing region. This separation into organic matrix and inorganic inclusion is characteristic of biominerals, but these inclusions are only a few nanometers in diameter (Figure 6E and 6F). Zinc, on the other hand, is co-localized with carbon on a scale smaller than the resolution of the image, estimated to be a nanometer or less. Interestingly, Figure 6F suggests that the zinc content is roughly the same in both the iron and the carbon regions. The list in Figure 6 compares the fragments from the iron and zinc region of the scorpion composite to those from an ant Zn-HEB and further demonstrates the differences between the HEB and this composite. The scorpion has $ZnN_xC_xH_x^{+1}$ fragments like the Zn-HEB, consistent with the presence of a Zn-HEB. But unlike the HEB, there are Fe- and P- containing fragments that are consistent with a biomineral. None of the Fe- or P-containing fragments also contain C. There are also $ZnOH_x$ and $ZnPO_xH_x$ fragments, suggesting the possibility of a second biomineral, and the first known Zn-based structural biomineral.

We hypothesize that the Zn-HEB in the scorpion differs from that in the ant in that, in addition to individual Zn atoms bound to histidine residues and water molecules, it contains zinc hydroxide and possibly zinc phosphates in sub-nanometer inclusions. We hypothesize that the zinc hydroxide is also present with the iron phosphates and iron hydroxides in the biomineral regions.

A movie showing a rotating APT reconstruction, that helps visualize the 3-D distribution of these regions is located here: https://www.youtube.com/watch?v=OmhxjnpedPY

PROPOSED ACTIVITIES

Investigations of a newly identified structural biomaterial, especially one as unique as the scorpion material, will likely lead to advances in both material and biological sciences. The proposed activities support the goals of the Biomaterials program as fundamental materials research in 1, biomaterials, and, 4, the processes through which nature produces biological materials. As the first investigation of a composite of biominerals and an HEB, which also appears to be the first example of a structural zinc biomineral and appears to have the smallest reported biomineral inclusions, this research is consistent with the goal of "discovery of new phenomena". This research will begin to answer the basic questions about a newly identified structural material, namely: **I)** its composition, **II)** its properties, with particular focus on properties or "tricks" useful to humans, but also, from a biological perspective, adaptive advantages, and**, III)** the developmental sequence, the steps by which it is made by the organism.

The buthid scorpion material appears to be a three-component, multi-scale composite: a Zn-HEB with nanometer-scale Fe-biomineral inclusions, and sub-nanometer scale Zn-hydroxide inclusions permeating both the Zn-HEB and the Fe-biomineral regions. Known iron phosphate and zinc hydroxide minerals, such as Vivianite and Wulfingite, are all quite soft. One possibility is that, as nano-scale inclusions, they are hard. Another is that the inclusions do not help harden the composite but play other roles. For example, we speculate that the Zn hydroxide might play a self-healing role. Invertebrate blades and tips are good candidates for self-healing innovations because tool damage in small organisms that overcome force limitations with sharp tools may be fatal.

The Zn-hydroxide might serve as a ready source of Zn for cross linking histidine residues after the material is damaged by high stress. If it is hydrated enough that it is mobile, it might fill in gaps that open up during high-force interactions. This might reduce the probability of a fracture initiating at stress induced cracks, especially if some of the in-filled Zn coordinates with histidine in the HEB walls of the gap.

Below we propose SEM during puncture measurements in order to investigate this possibility, altering the time between subsequent punctures in order to investigate such self-healing.

Our investigations will not only add to an understanding of the material properties associated with this phenomenon, but also will find wider application in the continuing development of APT for biological samples. We will continue to improve the methods we developed to gently prepare biological specimens and to identify the multitude of fragments associated with organic biological materials. We will also continue to develop the techniques we have pioneered to measure mechanical properties of micron-scale samples.

I) Composition and ultrastructure of the biomineral - HEB composite.

This is the core of the proposal, verification and testing of the composition hypothesis developed from the preliminary investigation. We will supplement APT with techniques that are sensitive to crystallinity and oxidation state of the iron. The investigation of the composition and ultrastructure involves multiple samples, enumerated below.

A. APT. We will continue to collaborate with PNNL using the gentle APT techniques that we have developed in order to examine the following or similar samples:
1) At least two confirmatory samples of the newly discovered composite in cheliceral tynes of *Centruroides exilicauda*.
2) A sample from the pedipalp teeth (the teeth on the large claws), from the same organism, in order to determine whether the scorpion uses the material in other similar "tools".
3) Standards of iron minerals and zinc hydroxide to make sure that the fragments observed from the scorpion are consistent with the hypothesized composition.
4) To investigate whether this phenomenon is wide spread, a sample from a distantly related organism with Zn- and Fe-containing tools (Schofield, 2001), a pseudoscorpion species such as *Halobisium occidentale* (which are about as related to scorpions as spiders are).
5) Two samples from organisms that might employ different biomineral – HEB composites, a sample from a glycerid worm jaw (which are known to contain the mineral atacamite, and a Cu-HEB), *Glycera convoluta*, and a scorpion that has such high concentrations of Zn in its pedipalps that it is likely to contain a Zn-mineral, e.g. *Vaejovis confusus*.

B. TEM with EDX and EELS. We will use TEM-associated techniques to validate APT results, search for crystallinity and to investigate the oxidation state of the iron.
1) Examine at least one scorpion APT needle using TEM before and after APT in order to estimate mass density, improve the reconstruction, and test the APT results.
2) Use TEM to validate structural features observed with APT, as needed.
3) Employ TEM electron diffraction techniques in order to test how amorphous the iron biomineral is and, possibly, to contribute to its identification.
4) EDX and EELS to investigate the composition of the scorpion material and to investigate the oxidation state of the iron.

C. SEM with EDX
1) Elemental analysis of multiple iron-containing standards in order to establish uncertainties for elemental analysis of the scorpion material. We have previously examined zinc-containing standards in order to establish the uncertainties for elemental analysis of HEBs.
2) Elemental analysis of multiple samples of the scorpion material to establish variance in the elemental analysis for modeling purposes.

II) Mechanical properties and the behavior of sharp edges and tips during puncture and cutting

A. Hardness, modulus of elasticity and loss tangent
Does the incorporation of the Fe-biomineral make the cuticle even harder or stiffer than in the regions of pure Zn-HEB? Could the nano-scale inclusions act to damp, not harden, the material, increasing the loss tangent? We will measure hardness, modulus of elasticity and loss tangent of the tips of the scorpion cheliceral tynes, the zinc-only regions, and the un-enriched regions for comparison. We will make similar measurements on zinc and iron containing pedipalp (main claw) teeth, as well as teeth from a vaejovid scorpion that contain only zinc for comparison. We will also test the "healing" hypothesis by repeatedly indenting at the same location, varying the force and repeat rate (up to 10 Hz with our equipment) in order to determine if there is a slow self-healing process, such as has been described in mussel byssus threads (Schmitt *et al*., 2015). If there is, the measured hardness value should be higher when sufficient time has elapsed between indents (scale of minutes for Zn-histidine bonds in byssus threads).

B) Behavior of sharp tips during puncture and comparison to human-engineered blades and tips
Different properties may be important in reducing damage in sharp tips and blades than in the bulk. For example, we have argued that the homogenaity of HEBs is one of the main advantages relative to biominerals because of more precise control of shape on a nano-scale and because there is a tendancy to fracture at interfaces. Roscioli *et*. *al*. (2020) have argued that regions of differing stiffness along the cutting edge of razor blades likely lead to fracture at the regional crystallite boundaries, and they developed an SEM technique to monitor the interaction of the blade and target down to a nanometer scale. We will adopt and modify this imaging technique in order to look for differences in the performance of the new composite, HEBs, and human-made blades or tips as they repeatedly puncture materials.

We will set up a vacuum compatible version of our puncture force tester (Schofield *et. al.*, 2016) inside the FIB-SEM instrument that we use for making APT target needles (Schofield has expertise in vacuum design). This FIB-SEM machine is equipped with electrical feedthroughs that can be used for the drive and force signals (see supplemental documents for facility director's approval). The optical stage will drive the scorpion cheliceral tyne or comparison blade or needle into cellophane sheeting and other membranes used in Schofield *et. al.*, 2016. However, for this investigation, we will puncture or cut repeatedly with the same blade or tip, because we are interested in the accumulating effect of chipping and plastic damage. We will quantify the damage by the rate of increase in force and energy required for subsequent punctures of a particular membrane. The SEM images will allow us to estimate the increasing radii with wear, and alert us to phenomena such as self-healing and recovery of the tip or edge radius. We will vary the puncture repeat rates, as with indentation, in order to investigate "healing". If there is "healing", the increase in required force with subsequent punctures should lessen when there is sufficient recovery time.

C) Behavioral observations to further understand advantages.
The ultimate test of a biological material is how behavior and, particularly, energy acquisition can be improved as a result of using that material. The diversity of scorpion biomaterials presents the possibility for behavioral comparisons: the chelicerae of buthid scorpions employ the composite, while others use only HEBs. We will compare the cutting and puncturing behavior of the different scorpions, testing for behavioral differences associated with the mechanical property differences that we find. For example, if the material is harder or has greater self-healing behavior, it may enable buthid scoprions to puncture and cut faster, instead of slowly searching for easy-to-puncture resilin between sclerites. We will make movies of the two types of scorpions feeding and establish timing from these movies. Differences in behavior may also point to other advantages of the composite material.

III) Compare the developmental course of the composite in the scorpion to that of Fe biominerals and HEBs.

An important technique for understanding the production of biological materials, are observations of the developmental course. One of us has played a role in elucidating the course of the iron biomineralization process in mollusks (Nesson & Lowenstam, 1985), and we have described the time course of the development of Zn-HEBs in ants and scorpions (Schofield, Nesson & Richardson, 2003), finding, for example, that Zn is deposited through nano-canals long after cuticle development. We will use these techniques to establish the time course of the development of the biomineral-HEB composite in scorpions.

We will obtain a developmental series of the scorpion *Centruroides exilicauda*, by monitoring litter mates as they transition between instars, freezing individuals at various points during development (as in Schofield, Nesson & Richardson, 2003). Litter mates develop and molt at very similar times, so observing litter mates that have been frozen in a time series is a reasonable approximation to observing development of a single individual.

We will use SEM and TEM with EDX in order to observe the time course of Fe, P, and Zn deposition, which, along with their spatial distributions, will allow us to test several hypotheses. One possibility is that the small-scale biominerals in the scorpion are produced through a process that is similar to the biomineralization process in mollusks but halts at an early stage. We will look for ferrihydrite or ferritin in cells, and initial deposition of ferrihydrite in the tyne cuticle prior to iron phosphate formation. Another possibility is that zinc hydroxide is a nucleation site for the Fe-biomineral.

BROADER IMPACTS

Humans may learn from the materials that have evolved in small organisms that, because they are limited to small forces, rely on tool sharpness for survival. These materials may have self-healing properties and other tricks to keep the tool sharp and thus prolong the life of the organism, tricks that could be useful for human made materials. While, so far, we know little about the properties of this new material, it is likely that there are distinct advantages that drove the evolution of such a complex material, employing 3 components and large quantities of two different heavy metals. For example, the zinc hydroxide permeating the material may make Zn easily available for re-establishing Zn-histidine bonds, as well as act as a filling and bonding agent for incipient cracks. These findings may lead to improved self-healing in human materials based on metal-histidine complexes.

Investigation of the extremely small biomineral inclusions in this material may help us explain why the inclusions are so large in human bones and teeth, and give us a better understanding of how to control mineral inclusion size and the resulting advantages. The possibility that the macromolecules in the matrix of the scorpion material are modified with individually bound metal atoms in order to make the matrix modulus of elasticity more like the mineral inclusions, improving damage resistance, may be of interest in design of human-made composites. If the "damping pot", and "water sequestration" hypotheses for the roles of the individually bound metal atoms are confirmed, the study of these materials could conceivably lead to transformative approaches to man-made materials.

For biologists, these studies will lead to better understandings of the role of material properties and the adaptive advantages associated with these specific materials. We will continue to interpret material property differences in biological terms to help biologists understand the importance of materials. For example, we argued in Schofield *et. al.* 2020, that for HEBs, the higher modulus of elasticity allowed tool contact regions to deform less, leading to a reduction in required force of 0.6 relative to normal materials for stiff targets in a simple model. Thus, the organism that employs the HEB could cut or puncture with 0.6 of the energy expenditure and 0.6 of the muscle mass, huge improvements from a biological perspective.

RESEARCH INFRASTRUCTURE

This research is particularly interdisciplinary, combining material science with organismal biology, biochemistry, and physics, at several institutions. Interdisciplinary collaborations tend to disseminate techniques and technology between fields. Research that highlights the importance of material properties in biology will tend to increase the interest and flow of ideas between material science and biology.

RESEARCH TRAINING

We will continue our research training with a focus on under-represented groups and non-traditional students. We will continue to partner with Lane Community College (LCC), in our area, which has significantly higher fractions of minority students than U of O, especially Hispanic and Native Americans/Alaskans as well as first generation students. During this most recent grant period, three LCC students worked in the lab through our partnership program with Lane Community College. All three of the students transferred to the U of O and continued to work in the lab – one of them, a non-traditional first generation student, has just graduated and started work towards a PhD in a prestigious program (David Lee). A letter is included in the supplemental documents section from the director of the LCC internship program expressing interest in continuing to send students to our lab.

In addition to the 3 undergraduate students who will be paid for by this grant, we usually have many undergraduate students who volunteer to work in our lab or work for research credit. This is one advantage of interdisciplinary research. The biology side of our work, and our colonies of ants and other organisms, are the initial draw, but students are introduced to, and often find interest in, the other disciplines of our research. In total, 90 undergraduate students gained research experience during the course of our most recent grant, and we expect similar numbers for the proposed grant period. We think that even lab training and meeting attendance for a single term give an improved understanding and appreciation of research. Finally, we will use the U of O student employment system in conjunction with a Cal-Tech SURF student rating system, that we have had experience with, that is designed to increase representation of under-represented groups without being unfair to others. In addition to introducing many undergraduate students to scientific research, we will train 1 graduate student.

Our students tell us that one of the reasons that working in our lab is popular on campus is that we have a reputation for close collaboration between senior personnel and undergrads. Although we will use senior students to train new undergraduates, each student will collaborate directly with senior personnel on their projects. Close mentorship improves training and results in more opportunities to make significant intellectual contributions to papers. Most of our papers have undergraduate co-authors and, during the previous grant period, another one of our first-generation student undergraduates, who started at LCC, served as lead author on a paper (Garrett *et. al.,* 2016). Our latest paper has 17 undergraduate co-authors and many more in the acknowledgements.

For all but one of the years of the previous grant, we participated in the summer Quark Net (http://quarknet.fnal.gov) continuing education program for high school science teachers. Teachers have said that the interdisciplinary nature of our research has helped them make connections for their students between physics and biology. We plan on continuing our Quark Net presentations and lectures for each year of the proposal period.

OUTREACH

Since the need for sharp, damage resistant tools to overcome the force limitations of small animals is such a simple and universal topic, it makes a good candidate for exciting students and the general public about material science and biology. About 20 million listeners heard radio interviews with the PI about our NSF IOS 0422234 research, and the results were featured in print publications with a readership of more than 50 million. Programs that interviewed us about behavioral correlates with wear (links here), included the BBC World Service, Voice of America, NPR's Science Friday, CBC Quirks and Quarks, BBC 5 Live Drive, and, most importantly according to some of the co-authors, our results were featured in a limerick on NPR's "Wait, Wait Don't Tell Me". Our research on mechanical properties of heavy-element materials was featured on more than 60 websites, and we were interviewed on the topic for the AAAS radio show. The biomaterial research was also featured in an NSF Highlight. We attribute our outreach successes to our ongoing relationship with the U of O communications office. We will continue to follow their suggestions and work with them to dramatically increase the reach of our outreach.