PROJECT DESCRIPTION

Biological accumulations of inorganic materials have shaped the course of evolution, characterizing such groups as molluscs and vertebrates. The calcium biominerals in the hard parts of these organisms and, to some degree, the iron and silicon biominerals employed in, for example, mollusk teeth and the skeletons of radiolarians, are thought to be responsible for major adaptive radiations in evolutionary history. The role played by a much less studied type of inorganic structural accumulation is as yet unknown. This alternative means of structural modification is characterized by heavy metals and halogens, Zn, Mn, Fe, Cu, Br and Cl, in lower concentrations than the Ca and Fe concentration in the shells, skeletons and teeth of molluscs and vertebrates, but, nonetheless, reaching 25% of dry mass. In our previous NSF funded project, we showed that the hardness of the mandibular teeth of leaf-cutting ants more than doubles as Zn is incorporated during early adult life. We suggested that observed differences in mandible employing behavior between young and older adults might, in part, be due to this Zn-correlated hardness change. In addition, we showed that the deposition time and sequence of Zn and associated elements in ants and scorpions were similar and suggested that this is evidence that heavy metal-halogen fortification evolved before these distantly related arthropods diverged.

Here we will propose to expand our investigations to include heavy metals and halogens other than Zn, mechanical properties other than hardness, and to begin to test hypotheses about the chemistry of these features and how they modify mechanical properties. First, we plan to use an isopod model to compare the developmental course and hardness of Br and Zn enriched features. We will also use isopods to search for similarities in cuticular canal systems associated with calcification and heavy metal-halogen fortification. Second, we will search for differences in mechanical properties other than hardness, such as resistance to wear, between Zn, Mn and Br enriched structures and between these features and biomineralized structures, particularly the calcified cuticle of crustaceans. Third, we plan to test a hypothesis that Zn-enrichment functions by binding or replacing water that softens cuticle under normal conditions. Fourth, we will use a battery of techniques to investigate the chemical environment of Mn, Fe, Cu, Zn and Br in these features. And fifth, we plan to tighten and expand the correlation between Zn enrichment and the behavior of ants.

BACKGROUND

Manganese, copper and zinc-enriched structures have not been identified until recently, probably because the metal bearing material is not visually distinct from surrounding tissue and suitable detection methods have not been available. Bryan and Gibbs (1979) were the first to note structural accumulations of these metals in animal material. Zinc was found in the jaws of nereids and Cu in the jaws of glycerids in concentrations that did not depend on the environmental levels (Bryan and Gibbs 1979; Gibbs and Bryan 1980a,b,c). Because of the functional nature of jaws, they suggested that these metals might improve their mechanical properties. Subsequently, Hillerton and Vincent (1982) and Hillerton et al. (1984) detected Zn and Mn near the cutting edge of the mandibles of herbivorous insects from several orders, after an initial finding that the cutting surface of dry locust

![Figure 1. STIM and PIXE images showing a cheliceral fang and marginal teeth from a garder spider, Araneus diadematus. The large image is a STIM (Scanning Transmission Ion Microscope) image in which lighter shades indicate greater projected mass. The four smaller images are PIXE (Proton Induced X-ray Emission) images of the same field as the STIM image showing the origin of Cl, Ca, Mn, and Zn X-rays. Lighter shades indicate greater numbers of detected X-rays. The fang is enriched with Zn and Cl. The tips of the marginal teeth contain Mn. Ca is enriched in the teeth, surrounding regions and the portion of the fang not enriched with Zn. Backgrounds have not been subtracted. Frame size: 1 mm x 1 mm. Source: Schofield. 2001.](image-url)
mandible was harder than surrounding cuticle (Hillerton et al., 1982). In each of the above cases, the metals were identified using wet chemical and electron microscopical techniques and quantified using wet chemical techniques. Because of the spatial imprecision of wet chemical techniques, these studies gave only hints of the extreme magnitudes of the metal concentrations in localized regions.

The development of high-energy ion microscopy has facilitated measurement of local metal concentrations and revealed the complex patterns of metal deposition in a diversity of organisms, including structures other than mouthparts.

High-energy ion microprobes employ a beam of protons or other ions that are accelerated by millions of volts and focused onto a specimen. Proton microprobes are analogous to electron microprobes but have two major advantages. First, the detection limits for most elements can be lower by 2 or 3 orders of magnitude (Legge et al., 1988). Second, the heavier protons penetrate much further than electrons; the probe at the University of Oregon can sample up to about a millimeter of dry, low density biological material while electron probes are limited to a few microns. Specimens can thus be surveyed with little or no sectioning (Schofield et al., 1988).

Two high-energy ion microprobe techniques have been used in the study of heavy metal-halogen biomaterials: STIM (Scanning Transmission Ion Microscopy; Lefevre et al., 1991) and PIXE (Proton Induced X-ray Emission; Johansson & Campbell, 1988). Used together these techniques allow simultaneous measurement of the concentrations of many elements in the specimen; the quantity of specific elements is given by PIXE, the total quantity of material by STIM (Schofield, 1990; Schofield & Lefevre, 1993). Images produced by these two techniques using a scanned beam of 4MeV protons are demonstrated in Figure 1.

The data plotted in Figure 1 can be used to calculate a concentration value for each of the 4096 (64x64) pixels, or picture elements, in the four small PIXE images. Near the tip of the spider fang, the measured Zn concentration reached 15(±3)% of dry mass. At this location the chloride concentration was found to be 3(±1)% In the marginal teeth, Mn concentrations reached 3.7(±0.7)% (Schofield, 1990). Each of these values is derived from a volume represented by a single pixel in Fig. 1. Since these volumes extend along the beam path through the specimen and may thus include unenriched material, the concentration values are lower limits to the peak local values. To measure concentrations in local volumes that may be completely internal, we developed PIXE-STIM tomography (Schofield & Lefevre 1992, 1993). Data is collected at a number of sample orientations, and the 3-dimensional distribution is reconstructed using a computer.

We have been using PIXE and STIM to survey a variety of organisms for...
structural metal accumulations. These findings are summarized in Table 1. While metal-rich structural features adorn a wide range of organisms in several phyla, it is difficult to predict which organisms will have them. For instance, these accumulations are not associated solely with herbivorous feeding behaviors as earlier investigators had suspected. In fact, heavy metals are commonly found in structures other than mouthparts. Even the phylogeny of the higher taxa can not be used to reliably predict the presence of heavy metal features. Within the order Hymenoptera, for example, metal accumulations have been found in the ant family, in jaws and tarsal claws, but not in the closely related wasp and bee families. Some more distantly related hymenopterans have them, others do not (Schofield, 1990). On the other hand, we have not found major differences in metal distributions among the examined species within any arthropod family.

**Metals In Scorpion Cuticle**

Of the organisms known to produce Mn, Cu, or Zn-rich structural materials (Table 1), the highest metal concentrations and the greatest variety of enriched structures have been found in scorpions. High concentrations of heavy metals were found in the teeth of the chelicera and pedipalps, the tarsal claws and the sting of every species for which these structures were examined (121 specimens from 15 species). In each of these structures, metals were localized in regions susceptible to abrasion and mechanical force by contact with the environment. Three metals with atomic weights between Ca and Br were found: Mn, Fe and Zn. Two very different enrichment patterns emerged: one characteristic of the family Buthidae (41 specimens, 4 species), the other characteristic of all other families. Figure 2 is a diagram of these two patterns; pattern A occurs in non-buthid scorpions, pattern B in buthids.

The buthid claws are distinct from the non-buthid claws and demonstrate that not all darkened cuticle contains heavy metals. The two lateral claws are enriched with Mn, in considerably greater quantities than in the non-buthid claws. However, the median claw is not enriched with heavy metals. The enriched lateral claws do not obviously differ from the median claw in color or darkness of tanning. In the scorpions in general, as well as in the other arthropods we have examined, we have not found metals in light cuticle, although darkly tanned cuticle is not a reliable indicator of metal presence. Even when metals are present, they often do not precisely overlap the darkly tanned regions.

The metal concentrations in heavy metal biomaterials have been measured for dozens of species in five phyla using STIM-PIXE, STIM-PIXE tomography, and electron microprobes (Schofield et al., 1988; Schofield and Lefèvre, 1989; Schofield et al., 1989; Schofield 1990; Schofield & Lefèvre, 1992; Schofield & Lefèvre 1993; Schofield 1995; Schofield, 2001). The measured concentrations of Mn and Zn were unusually high for elements that are not abundant in nature; the peak measured values of Zn (25% of dry mass) are among the highest reported in biological organisms (Underwood, 1977). In scorpions, peak concentrations of Fe, Mn, and Zn were calculated for 17 of the 121 specimens of cuticular structures examined with the proton microprobe (summarized in Schofield, 2001). Peak Zn values ranged between 3 and 25% of dry mass, Mn between 0.1 and 4% and Fe between 2 and 9%. Heavy metal concentrations measured in other body cuticle were generally on the order of 0.01% or less.

**Possible Molecular Forms**

Biological metals are known to be found in biominerall form such as the magnetite in the radular teeth of certain molluscs, and ionically bound in proteins. The concentrations of heavy metals in cuticular structures, particularly those in scorpions, are difficult to reconcile with either of these molecular forms. Organically Bound
The 25% Zn concentration in scorpion cuticle is too high to be easily accounted for by organic binding. Hillerton and Vincent (1982) presented such a hypothesis, suggesting 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. While the measured concentrations of Mn (up to 4% in spiders and scorpions) could be explained by such a hypothesis, the Zn concentrations are inordinately high. Estimations of the maximum possible Zn concentrations for two postulated binding mechanisms: binding to cuticular proteins or binding to catecholate ligands during cuticle sclerotization, both gave Zn concentrations under 8% of dry mass (Schofield, 2001).

Biomineral

The concentrations of heavy metals in cuticular "tools" are very low compared to cation concentrations in well known examples of biominalerized structures. This problem can be illustrated with a rough estimate of the volume fraction occupied by a biomineral (Schofield, 2001). The volume fraction of goethite (FeO(OH)) in the radular teeth of limpets is roughly 0.5. In contrast, if Zn is incorporated in a common Zn mineral such as ZnCO$_3$ or ZnO (Al, Si, P, and S were not associated with Zn), then the mineral would occupy only about 0.2 (ZnCO$_3$) or 0.07 (ZnO) of the volume in the richest accumulation. The Zn biomineral could not act like many previously studied structural biominerals, by filling the bulk to the extent that the mechanical properties of the tissue approached those of the pure mineral.

In any case, the characterization of the molecular form of this material may lead to the identification of novel modes of metal incorporation into biological material, whether the metals are incorporated in unusual biominerals, organic salts, organically bound, or in some combination of these.

Possible Function

All known Zn-enriched structures are associated by function: they are tools -- structures with which the organism manipulates its environment. The uses of these Zn-enriched structures include cutting, piercing, digging, grasping, clamping and traction. The performance and durability of each of the Zn-containing structures would seem to be enhanced by hardening and especially by increased resistance to wear. However, there are other structures that might benefit from mechanical enhancement; high concentrations of Zn have not been found in leg joints or in proximal mouthparts. But the enriched structures differ from the unenriched structures in that they routinely come in direct contact with environmental structures that may be much harder than ordinary cuticle.

RESULTS FROM PRIOR NSF SUPPORT

For NSF award IBN 9817206 ($180,000; 1999 - 2002), entitled "The Role of Heavy Metals in Biomaterials", we proposed three research projects. First, to select a species of ant as a model organism and, using MeV-ion microscopy, determine the developmental course of Zn features in the mandibles, as well as possibly related features throughout the whole organism. Second, to develop an Atomic Force Microscopy based technique to measure the hardness of these small features with a spatial resolution at least an order of magnitude smaller than previously possible. Using this technique we planned to search for a spatial and temporal correlation between Zn enrichment and hardness. Finally, we planned to ascertain whether Zn accumulates in sub-micron or larger features within the cuticle.

During the course of this funding, we prepared a chapter (Schofield, 2001), which included the early results of this project, and two papers (Schofield et al., 2001, Schofield et al., 2002). All results summarized in this section are detailed in these papers, also available (without figures) at: http://darkwing.uoregon.edu/~rmss

THE TIME COURSE OF Zn ACCUMULATION IN "TOOLS" AND WHOLE BODIES

An understanding of the time course of heavy metal-halogen accumulation during development of the organism is important in understanding the form, function and even the evolution of these features. We began by selecting Tapinoma sessile, the sugar ant, as our model organism because it was small enough that the Zn distribution throughout the entire body could be determined, without sectioning, using high-energy ion microscopy, and because colonies were fairly easy to collect and maintain. In order to compare the development of Zn enriched features in distantly related arthropods, we also obtained gravid scorpions,
We examined ant pupae of all stages as well as adults of different ages. To produce adults of known age, late stage pupae (darkened mandibular teeth and darkening abdomen cuticle) were isolated individually in 13 x 100 mm glass culture tubes. The tubes were horizontally held and about half filled with reverse-osmosis purified water held against the closed end of the tube by a glass–wool plug. The culture tubes were then sealed with a second glass–wool plug and incubated at 25 +/- 1 degrees centigrade. The pupae were inspected at intervals, and the time of eclosion (the last ecdysis: escape of the pharate adult from the cuticle of the pupa) was set to be at the center of the time span between the first observation that eclosion had begun and the prior inspection time. Eclosion was defined as having begun if the tarsal–tibial joints of at least 2 legs were observed to be at least two joint widths from their locations in the pupal posture.

First instars of the scorpions were isolated and monitored in a similar manner. The time of ecdysis (emergence from the old cuticle) of the second instar scorpions was set to be the time at which they first stretched upward through the split exuvium as they began to pull their legs out of the exuvium.

We found that zinc incorporation into the mandibles of the ant T. sessile took place after the cuticular structures had darkened in the pupa and predominantly after eclosion. The unfilled symbols plotted in Fig. 3 indicate that the zinc content of the mandibular teeth increases by a factor of about 5 during the interval between 1 and 100 hours after eclosion.

The curve plotted in Fig. 3 is an iterative fit to the mandible data based on a model in which zinc deposition begins abruptly and the deposition rate is proportional to the number of unfilled binding sites. This simplified model, which assumes that the concentration of zinc available for binding is constant even when the deposition rate is high, gives the content of bound zinc in the mandibles (Zn_{bound}) as:

\[ Zn_{\text{bound}} = Zn_{\text{max-bound}} \left(1 - e^{-(t-t_0)/\tau}\right) \]

where "Zn_{max-bound}" is the bound quantity of zinc when all sites are filled in both mandibles, t is the time elapsed since eclosion, "t_0" is the time at which binding began, and "\tau" is the time constant. An iterative algorithm was used to fit the data in Figure 3, giving values of 21 nanograms for "Zn_{max-bound}"; 41 hours for "\tau", and -10 hours for "t_0" (deposition began 10 hours before eclosion). A second non-overlapping data set consisting of mandible values for 10 different ants was also fit in order to assess the uncertainty in the above values (data is not shown). The fit for this second data set, considered less accurate because mandibles in images of whole ants were analyzed instead of the higher resolution images of isolated mandibles used above, gave the following values: Zn_{max-bound}: 14 ngm; \tau : 54 hours; t_0 : -22 hours. The fit for the primary data set gives an initial deposition rate of 0.51 ngm/hr declining thereafter, with a rate of 0.39 ngm/hr 1 hour after eclosion. At a rate of 0.39 ngm/hr, roughly 0.01
of the total body content of zinc must be relocated and bound in the mandibular teeth in one hour. The sudden onset of deposition late in cuticular development suggests either that an active zinc transport system turned on or that the cuticle matrix was modified to incorporate zinc just prior to eclosion, or both.

Accumulation of zinc in the teeth of the chelicera and pedipalp, tarsal claws and stings of the scorpions began even later in cuticular development, more than 40 hours after ecysis of the second instars (Figure 4).

The lateness of zinc deposition may necessitate the network of canals observed in metal-bearing exocuticle of the ants and scorpions. In TEM cross sections, the 10nm scale pores were smaller and more densely distributed than the pores in adjacent non-metal bearing cuticle and in other cuticular structures that were not metal fortified (Figure 5). The zinc may travel from the epidermis through this network of canals and into the sclerotized cuticle.

Cuticular implements enriched with zinc are often also enriched, to a lesser degree, with manganese, calcium and chlorine (see Figure 1 and Schofield, 2001). Within the structures, the spatial distribution of chlorine is tightly correlated with the zinc distribution while the highest concentrations of manganese and calcium are generally found in regions that have lower concentrations of zinc. The time course of deposition of these elements may offer clues to the biochemistry and reveal similarities between zinc fortification in different phyla.

Manganese accumulated before zinc in the examined cuticular implements (ants: mandibular teeth; scorpions: stings and tarsal claws). Early incorporation of manganese, relative to zinc, has also been reported in the mandibular teeth of the ant Atta sexdens (Grime et al., 1999, Schofield et al. 2002). In Tapinoma, manganese enrichment was observed in the mandibular teeth of eclosing individuals, though not in stage 3 pupae. Thus, in the ants and scorpions that we have examined, the cuticle began to darken, then manganese was deposited and finally, zinc. Calcium in the scorpions accumulated before zinc and at the same time as manganese. Distribution maps of calcium in

Figure 4. Zinc in cuticular implements of developing second instar scorpions, V. spinigeris. Three specimens from litter mates of different ages are shown in each image; clockwise from the top left in each image, approximately 40, 90 and 160 hours post-ecdysis. The first column shows pedipalp fingers, the second shows tarsal claws, and the third, chelicerae. The total zinc X-ray count (approximately proportional to content) for the distal 200 μm of the toothed regions of the pedipalps of the 40, 90 and 160 hour old specimens was 9.89 and 330 respectively; for the distal 50 μm of both lateral tarsal claws the count was 25, 38 and 214 respectively, and for the distal 50 μm of the right cheliceral finger the count was 18, 27 and 331 respectively. The frame edge lengths for the images of the pedipalps, tarsal claws, and chelicerae are, respectively, 1.1, 0.55 and 0.82 mm.

Figure 5. Higher pore density in a section of a zinc-enriched pedipalp tooth (or granule) then in a section of a zinc-free body armor tooth (carina granule). (A and B) Transmission electron micrographs (TEM) of unstained sections through the zinc-enriched exocuticular tip of a pedipalp tooth of an adult scorpion, V. spinigeris. In both images, the scalloped edge is the external surface of the tooth. (C) TEM image of an unstained section through a sclerotized, but zinc-free, tooth-like cuticular protuberance on a carina of the first metasomal segment of an adult V. spinigeris. The external surface is at the top. Exo, exocuticle; Endo, endocuticle; wd, wax ducts.
Tapinoma mandibles were also consistent with simultaneous deposition of calcium and manganese before zinc.

Our data were consistent with a hypothesis that chlorine and zinc accumulate at the same time. Chlorine is difficult to quantify in specimens that are as thick and as contoured as these cuticular structures because the low-energy chlorine X-rays are readily absorbed. However, the ratio of detected chlorine to sulfur X-rays (which are similarly attenuated) was at least ten times greater in the mandibular teeth of a 2-hour post-eclosion Tapinoma specimen than in the teeth of a stage P3 pupa. Thus the chlorine and zinc levels appeared to increase together, though we were unable to quantify this observation.

**The content and distribution of zinc in whole Tapinoma**

We used MeV-ion microscopy to determine the content and distribution of several elements in whole freeze-substituted *T. sessile* specimens, ranging from egg to adult, in order to answer questions about the availability, metabolism and storage of zinc and other elements. To our knowledge, this is the first developmental time series showing directly the distribution of elements in entire organisms.

In Fig. 3, the zinc content of whole ants during development is displayed along with the zinc content of the mandibular teeth. The zinc concentrations in the ants of Fig. 3 were at least an order of magnitude greater than typical environmental and food concentrations. (Holland *et al.* 1996; Schofield, unpublished data).

The high zinc content appeared to be regulated and roughly constant, not accumulative. Thus deposit excretion, which would be expected to produce a roughly linear increase in zinc content with time, is not supported by this ant data. Zinc is an essential component of more than 300 enzymes and transcription factors (Coleman, 1992). Yet the total zinc content in these molecules is probably a small fraction of the body zinc content and it is surprising that such large stores of zinc would be needed. It may be that much more zinc than necessary is accumulated because the zinc excretion rate does not exceed the accumulation rate until toxic levels are approached.

The zinc content of the mandibular teeth was small compared to the total body content and, considering the large variation in body content between individuals, it is not likely to have been a great additional energetic expense to acquire.

Fig. 6 shows the distributions of mass and zinc in the abdomen and head regions of a developmental series of *Tapinoma*. These are the ants whose total zinc contents are plotted in Fig. 3 as the colony 1 series. At all stages and ages shown in Fig. 6, zinc was distributed fairly uniformly throughout the
body with localized accumulations in the midgut region, the mandible region of the adults, and the infra
buccal pocket region of several specimens.

EVOLUTIONARY QUESTIONS
We argued that our results had bearing on two questions regarding the evolution of the heavy metal
-halogen system. First, the degree of relatedness of this system to biomineralization systems, particularly
calcification as manifest in crustaceans. Second, whether the multiple instances of heavy metal-halogen
fortification in distantly related phyla evolved independently, or whether the system predates the
divergence of these phyla.

Relation to calcification. Like zinc deposition in the ants and scorpions studied here, calcium deposition in
crustacean cuticle takes place mainly after ecdysis, late in cuticular development. Calcification may occur
after ecdysis so that the cuticle can expand to accommodate growth of the crustacean before the cuticle
becomes calcified. Whether or not there is an adaptive advantage associated with late zinc deposition, it is
possible that parts of systems needed for late modification of the cuticle, such as pore canals, are derived
from each other.

Both calcified crustacean cuticle and zinc fortified cuticle contain higher canal densities than
unenriched cuticle. Excess pore canals in calcified cuticle, relative to uncalcified cuticle, have been
observed in crustaceans (Compere and Goffinet, 1987a; Compere and Goffinet, 1987b), and similar
differences were observed in the ants and scorpions mentioned above. Further ultrastructural comparisons
between canals associated with calcification and canals associated with zinc fortification are needed to
investigate the possibility that the canals in the two systems are homologous.

In a wide variety of organisms, there is a zinc-calcium association: zinc often accumulates in small
quantities in calcified tissue, and structures that are predominantly zinc enriched are often also enriched
with calcium. Zinc accumulates preferentially in the regions of primate bone undergoing mineralization
(Gugenheim and Gaster, 1973), zinc has been found in 0.001 gm/gm of dry mass concentrations near the
tip of calcified hagfish teeth (Schofield, 1990), in concentrations approaching 0.10gm/gm of dry mass in
the calcified stylets of nemertians (Schofield, unpublished data), and zinc accumulates at about the same
time as calcium biominerals in the magnetite filled teeth of chitons and in the same region as calcium in
goethite filled teeth of limpets (Webb, et al. 1989). In structures that are predominantly zinc enriched,
calcium is often also enriched, usually in association with manganese (Schofield, 2001).

Notwithstanding the similarities in the time course of deposition, the excess canals, and the
associations between Zn and Ca in both systems, there are also major differences between the calcium and
zinc fortification systems that suggest that if parts of the two systems are related, they are not slight
modifications of each other.

First, the number density of zinc atoms in the ant cuticle was about 0.15 of the number density of
calcium atoms in typical crustacean cuticle. If the zinc were bound as a biomineral, the volume that the
mineral filled would be considerably smaller than the volume filled by CaCO$_3$ in crustacean cuticle - unless
the zinc were a much smaller fraction of the biomineral molecule than Ca is of CaCO$_3$. Elemental analysis
and EXAFS (Extended X-ray Absorption Fine Structure) analysis have eliminated most of the common
zinc minerals, except possibly an amorphous ZnO, as candidates; apparently, only zinc binding organic
salts could fill fractions of cuticle comparable to those filled by inorganic calcium salts in crustaceans
(Schofield, 2001).

A second difference lies in the type of cuticular features that are fortified. Calcium enrichment does
not necessarily substitute for heavy metal - halogen enrichment in cuticular "tools" of calcified arthropods.
For example, near the tips of the tarsal claws of the calcified millipedes, crabs and isopods that we
examined, calcium levels dropped and, instead, the predominant inorganic element was, in millipedes,
chlorine, and in crustaceans, bromine (data not shown).

In addition to differences in the types of cuticular features that are calcified or zinc fortified, there
appear to be differences in the enriched cuticular layers. Zinc fortification occurs primarily in the
exocuticular layer in the examined ants and scorpions (data not shown), while calcification in crustaceans is
found in the epicuticle, the exocuticle, and the endocuticle (Roer and Dillman, 1993).

It is preliminary to draw conclusions about any evolutionary relationship between calcification and
heavy metal - halogen fortification systems from the similarities and differences discussed above. The
lateness and time course of zinc incorporation, and the associations between calcium and zinc in both
calcification and zinc fortification systems suggest that parts of the systems are similar and that they may
be evolutionarily related. On the other hand, the differences between heavy metal - halogen and calcium
fortification, in particular the lower number densities of zinc, and the differences in enriched features and
icutular layers, suggest that the two systems may modify mechanical properties in very different ways and
that the biochemistry may be very different.

Origin. The detailed similarities in zinc fortification systems between distantly related phyla suggest to us
an early origin of heavy metal - halogen fortification. For example, we have previously noted that the
spatial distributions of zinc, chlorine, manganese and calcium accumulations in the jaws of a nereid worm
are similar to the spatial distributions of these elements in cuticular "tools" of scorpions (Schofield, 2001).
Our most recent work adds similarities in the time course of these accumulations in two distantly related
arthropods. In both the ant (a hexapod arthropod) and the scorpion (a chelicerate arthropod), zinc
fortification took place very late in cuticular development and zinc was deposited after calcium and
manganese. These developmental similarities further strengthen the argument that heavy metal - halogen
fortification arose before these phyla diverged.

We suggest three possible evolutionary precursors of heavy metal - halogen fortification, which are
not necessarily mutually exclusive: a biomineralization system such as calcification, an organic cross-
linking system such as that hypothesized for cuticle sclerotization, or a trace element transport and storage
system that might not be associated with mechanical properties. Of the first two suggested precursors, the
developmental course of the zinc system is more similar to the calcification system than it is to the
sclerotization system. The third precursor is suggested because the ability to obtain, transport, store and
regulate large quantities of zinc is well developed not only in the ants but also in fruit flies that do not have
zinc enriched cuticular structures (Schofield, et al. 1997). This system for handling quantities of zinc that,
in T. sessile, are much greater than those found in cuticular accumulations, may have predated heavy metal
-halogen fortification and may have required little modification for zinc procurement, transport to, and
perhaps even deposition in cuticular "tools".

CUTICLE HARDNESS CORRELATES WITH Zn ENRICHMENT

The function of each of the structures enriched with Zn would seem to be enhanced by increased
hardness relative to normal cuticle. Several investigations have found a higher indentation hardness for Zn-
enriched structures then for surrounding regions (Hillerton and Vincent, 1982, Hillerton et al., 1982,
Schofield, 1990, Edwards et al., 1993, McClements et al., 1993). This evidence has been inconclusive
because of differences, such as degree and timing of sclerotization, and artifacts associated with dessication
and curved structures.

The Chemistry Department and the Materials Science Institute of the Department of Physics at the
University of Oregon received an NSF Chemistry Research Instrumentation and Facilities Grant for multi-
user instrumentation for the purchase of a Digital Instruments AFM. We purchased, in conjunction with
another faculty member, an add-on force/displacement transducer (TriboScope, Hysitron Inc., Minneapolis,
Minnesota). The Hysitron 2-D transducer holds a polished diamond probe in place with vertically and
horizontally oriented capacitors which are used to sense the position of the probe and to impart vertical
forces for indenting and imaging the specimen. The small size of AFM-based indents makes it possible to
measure the hardness on the curved surfaces of fresh specimens. In addition, hardness measurements at a
defined location, before during and after Zn incorporation, circumvent the problems associated with
measurements on different regions.

The AFM hardness measuring technique was developed by Hysitron for indentation of large flat
surfaces, so we developed a technique for landing the probe with a 10 um precision and measuring
hardnesses on small, topologically complex structures. The specimens were floated in an epoxy composite
bed that we developed to meet the criterion that a glass shard, similar in size to and floated in the cement in
the same way as the mandibles, would yield hardness and modulus of elasticity values equivalent to those
obtained on a large piece of glass affixed directly to the specimen disk with an epoxy film.

We used this technique to measure the hardness of the mandibular teeth and off-tooth control
regions of the leaf cutter ant Atta sexdens. Like Tapinoma, Atta mandibular teeth contain very little Zn at
the time of eclosion. We tested the hardness of the teeth of eclosing adults and a range of body shades, which darken as the adults age.

After the hardness measurements, the relative Zn contents of the hardness-tested regions were determined using energy-dispersion X-ray analysis in a Scanning Electron Microscope (SEM). Figure 7 shows two extreme examples of the body-shade and Zn-content range, a mandible from a lightly shaded eclosing adult containing little Zn (on the left) and one from a fully-shaded adult with a full Zn complement (on the right). The zinc concentration in the hardness-tested mandibular tooth of the fully-shaded adult was about 200 times greater (16% of dry mass) than in the eclosing adult while there was no significant difference in the Zn-content of the off-tooth control region.

Figure 8 shows the average hardness values measured at the on-tooth and off-tooth locations as a function of the Zn X-ray count rates measured at the on-tooth locations. The data of Figure 8 show a correlation \( r = 0.92 \pm 0.02 \) between the indentation hardness of the mandibular teeth and Zn X-ray count rates (which were approximately proportional to the Zn concentration). The hardness of the tooth varies almost threefold with Zn count-rate while there is relatively little change in the hardness of the Zn-free off-tooth region.

In addition, Figure 8 shows that callow adults, defined by visibly lighter body-shading (at low magnification) than their darkest nest mates and presumed to be younger (Wilson, 1980), tended to yield lower hardness values and Zn-Xray count rates than fully-pigmented adults.

Because hardening occurred predominantly after eclosion, it took place after cuticle deposition and after pigmentation (preadecydial tanning) of the mandibular teeth. Pigmentation is thought to be generally cotemporal with sclerotization, the process by which typical arthropod cuticle is hardened (Andersen et al., 1996, Hopkins and Kramer, 1992). The apparent temporal lag between sclerotization and Zn-associated hardening suggests that there are two separate hardening processes. The fact that Zn-enrichment occurred after much of the cuticle maturation process was complete also makes it less likely that a separate process coincidentally hardened the cuticle as Zn was deposited.

In summary, the evidence for Zn-associated hardening is, first, the high degree of correlation between tooth hardness and Zn-Xray count-rate. Circles show hardness and count-rate values for the center of the anterior face of the fourth mandibular tooth (see Figure 1) for each of the 19 tested individuals. Squares show hardness values (but not count-rates) for the off-tooth control region; they are horizontally positioned to line up beneath the corresponding circles. White and grey symbol fills indicate callows, white signifies that they were not visibly darker than eclosing adults; black indicates full adults. Each point shows the average of three hardness measurement and one count-rate determination; the vertical and horizontal error bars indicate the 95% confidence interval, based on Students’ t-test (vertical) and X-ray counting statistics (horizontal). The displayed line fit yields an \( r \) of 0.91 \( \pm 0.02 \) (the standard deviation is for the \( r \) values of 1000 numerical simulations for which the position of the data points were varied randomly according to their uncertainty). Indents ranged from about 1 to 2 micrometers in depth.
and Zn content. Second, that the hardness difference between tooth and control regions increases with increasing Zn content. And third, the lateness of Zn incorporation and hardening relative to other cuticle maturation processes.

**A POSSIBLE BEHAVIORAL CORRELATE WITH Zn ENRICHMENT**

The nearly three-fold variation in the hardness values of the adult mandibles suggests the possibility of an associated differentiation in mandible-employing behaviors. Behavioral differences have been noted in *Atta sexdens* between callows and fully pigmented adults (Wilson, 1980). The callow adults of *Atta sexdens* (and of ants in general) are rarely seen outside of the nest and are instead over-represented in brood care (Wilson, 1980). Brood care seems less likely to involve mandibular contact with harder more abrasive materials than many of the tasks performed outside of the nest.

Perhaps more compelling is the reported difference in callow involvement in the processing of vegetation within the nest. The first stage of vegetation processing in the nest involves cutting with the mandibles while the second stage, chewing with the maxillae, does not. The fraction of the work force made up of callows is reported to be ten times less in the mandible-employing first stage than in the second stage (Wilson, 1980 and personal communication). Leaf-cutting can be extremely energy intensive (Roces & Lighton, 1995) and leaf toughness may limit harvesting (Nichols-Orians & Schultz, 1989). Harder mandibles would be energetically more efficient because the edges would deform less during cutting and they would also be less susceptible to dulling wear. We suggest that there is an adaptive advantage in postponing cutting until zinc enrichment brings an increased efficiency and a reduced risk of damage to the cutting edges.

**PROPOSED RESEARCH**

In our research so far, we have concentrated on Zn enrichment as a representative model of heavy metal - halogen enrichment. A major focus of the research proposed here is to begin to study the similarities and differences between Zn enrichment and enrichment with other elements. The crustaceans that we have examined are characterized by Br instead of Zn in their cuticular "tools" in concentrations that are sometimes as high as the Zn concentrations in other organisms. The mandibles of most of the millipedes that we have examined are enriched with Cl instead of Zn. In the tips of the stings of buthid scorpions, the marginal teeth of spiders and the mandibles of many beetles, Mn is the predominant metal, reaching concentrations greater than 5% of dry mass. The paragnaths of nereid worms contain Fe (7%) and the jaws of glycerid worms contain Cu (20%). These other cases are important to explore for several reasons. First, the presumption that these other elements alter mechanical properties, must be tested. Second, other elements may be associated with differing mechanical properties, a possibility suggested by their appearance in different regions of a single structure. A third justification is to develop a unifying evolutionary picture. We suspect that these features are all phylogenetically related and that heavy metal - halogen enrichment developed before the hypothesized adaptive-radiation during the Cambrian. A fourth reason is a search for a unifying biochemistry. Our initial attempt to create a unifying working hypothesis to account for the involvement of both heavy metals and halogens in cuticle strengthening follows.

We know that the sites of enrichment are, in general, strongly correlated with the occurrence of pre-ecdysial sclerotisation, which itself depends on a preferential involvement of N-á-alanyl-dopamine over N-acetyl-dopamine as the major sclerotizing catecholamine. Scattered reports of the occurrence of halogenated tyrosine derivatives in sclerotised structures include: the presence of low levels of 3-chloro-tyrosine in locust femur cuticle (Andersen, 1972), only after hardening (Andersen in Welinder, 1972); mono- and di- chloro- and bromo- and even 3-chloro-5-bromo- tyrosines in the cuticle of horseshoe crabs (Welinder, 1972) and in the operculum of a gastropod mollusk (Hunt & Breuer, 1971). Pryor (1962) suggested a functional role for halogenated tyrosine derivatives: that the electrophilic halogens might increase cross-link formation by generating a partial positive charge on the phenolic hydroxyl groups.

Hence, we suggest a hypothetical evolutionary sequence of cuticle strengthening strategies. The most primitive stage of hardening would be the N-acetyl-dopamine dependant sclerotisation, found in the general body cuticle. Then, N-á-alanyl-dopamine is transported to 'tool' cuticle, resulting in substantially harder final product after the sclerotisation process. Next, chlorine, bromine (and possibly iodine) would
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assume a role in the oxidative chemistry that underlies sclerotisation, resulting in the attachment of halides to the phenolic rings of protein side chains and, possibly, of the catecholamines, and produce an increase in the number of cross-links within the cuticular structures.

The ultimate stage of modification would involve the transport and deposition of heavy metals into the sclerotized cuticle. The metals might be involved in cross-linking or as a separate phase. The high concentrations of Zn in the extreme cases are difficult to reconcile with the estimated number of available cross-linking sites, unless one postulates the formation of cross-bridges containing multiple metal atoms (Schofield, 2001). A separate Zn-containing phase could harden and dessicate by filling space; our preliminary data limits the phase to amorphous nanoclusters.

Although we have no illusions about the likelihood of our validating the entire hypothesis, we will proceed to apply a number of techniques to investigate the chemical environment of the heavy metals and halogens within the cuticular structures in which they predominate.

I. AN ISOPOD MODEL OF Br ENRICHMENT AND CALCIFICATION

The horseshoe crabs, pycnogonid, priapulids and crustaceans (including terrestrial isopods) that we have examined contain Br, rather than Zn, in high concentrations in cuticular "tools". Most regions of isopod cuticle are calcified, but the tips of the tarsal claws and mandibles are not and are instead highly enriched with Br. We propose to use an isopod model to answer three questions. With these questions in mind we have established a laboratory colony of the isopod *Porcellio scaber*, and have begun preliminary studies.

1) At what stage is Br incorporated into the mandibles? We will use the timing techniques developed for the ants and scorpions above along with MeV-Ion microscopy in order to determine the time, relative to ecdysis, at which Br is incorporated into the mandibles. We have found that it is easy to isolate these isopods and to recognize signs of approaching ecdysis. We suspect that we will find that Br is incorporated late in cuticular development just as Zn and Cl in ants and scorpions. If this turns out to be the case, this developmental similarity will be a piece of evidence suggesting that the Zn and Br systems are phylogenetically related.

2) Is Br enriched cuticle harder than Br-free cuticle? We will use our AFM technique to compare the hardness of Br-rich and Br-free cuticle. If Br deposition occurs late in development, we will compare the hardness of cuticle before and after Br incorporation. We have tested a mandible of one specimen and found that the hardness was intermediate between Zn-enriched and Zn-free ant cuticle (0.53GPa).

3) What are the similarities and differences between canals in Br enriched, Zn enriched, and calcified cuticle? As discussed in our previous results section, similarities between calcification and heavy metal - halogen enrichment suggest the possibility of a phylogenetic relationship between the two systems. More densely distributed canals, relative to uneriched cuticle, have been noted in both calcified and Zn fortified cuticles. Similarities or differences between these networks of canals, such as origin, branching, and the cuticular layers in which branching take place, would have bearing on whether or not the two systems are homologous. Preliminary TEM examinations have revealed the presence of these dense pore systems in both calcified and Br enriched regions of isopod cuticle and we propose to continue these examinations in order to map out the two canal systems in isopods, and we plan to map out a third system in the Zn-fortified cuticle of scorpions.

The isopod model will also play an important role in the proposed research that follows.

II. BEYOND HARDNESS

While our results have demonstrated that Zn enriched cuticle is harder than unenriched cuticle, and our preliminary results have suggested that Mn and Br enriched cuticle are also harder than unenriched cuticle, the measured hardness values are lower than for biomineralized cuticle. For example, we tested the hardness of a calcified sclerite from an isopod and found it to be much harder than the mandibles (4.0 GPa). Biomineralized teeth are often harder than even the hardest Zn enriched cuticle (which reaches about 1 GPa): we measured the hardness of a magnetite containing chiton tooth, 5.0 GPa, the calcified tooth of a salmon, 0.80 GPa, and a human tooth, 5.0 GPa.

The presence of both calcification and Br enrichment in different regions of isopod cuticle affords the possibility of comparing the two types of cuticle modification. We hypothesize that Br enriched isopod...
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cuticle is more resistant to wear than calcified isopod cuticle. If this is the case, Br enrichment may be preferable to calcification in sharp or protruding structures even though calcified cuticle can be harder. **Resistance to wear.** In a preliminary investigation, we mounted an isopod mandible on a reciprocating shaft which plunged the mandible into moistened polishing grit. The white calcified cuticle at the base of the mandible wore away faster than the dark Br-rich cuticle at the tip. We propose to perfect and replicate this experiment, and to investigate wear resistance using the AFM.

A quantitative measurement of resistance to wear can be made using the Hysitron Triboscope. The diamond tip is held against the specimen with a selected force and then scanned repeatedly in a rectangular (e.g. 5 um x 5 um) pattern. The depth to which this rectangular area is worn away for a given force and number of scans can be compared to the depth worn at another region. A second somewhat related test is a scratch test. The diamond tip is pressed against the surface and the force necessary to slide the tip laterally is measured. The depth of the scratch gives an indication of resistance to scratching. We have demonstrated the feasibility of these tests on arthropod cuticle (scorpions) but we have not yet attempted measurements on isopods. We propose to compare calcified and Br enriched isopod cuticle and to compare these results to similar measurements made on unenriched cuticle. Our findings may suggest adaptive advantages other than hardness that are associated with the various forms of cuticle modification.

We also plan to make similar measurements on Mn enriched cuticle. Preliminary investigations have suggested that, over a range of atomic concentrations in two scorpion species, it takes only one Mn atom to harden the cuticle as much as two Zn atoms. We plan to complete this investigation, and to compare the wear resistance of Zn and Mn enriched scorpion cuticle.

### III. THE DESSICATION HYPOTHESIS

We suspect that the many forms of heavy metal - halogen fortification may be unified by similarities in how they modify the cuticle. One simple hypothesis is that they act by dessicating the cuticle. Protein structures that are impregnated with water are generally softer than when they are dry. Drying, along with protein cross-linking, may be a major contributor to the hardening that takes place during normal cuticle sclerotization (Hillerton & Vincent, 1979, Vincent & Hillerton, 1979, Andersen, 1981, Vincent & Ablett, 1987). Our preliminary results have also suggested the importance of dehydration in the Zn-associated hardening process. The following measurements were made on the mandibles of *Atta sexdens* in the manner of our prior results:

<table>
<thead>
<tr>
<th>SPECIMEN</th>
<th>TOOTH (GPa)</th>
<th>OFF-TOOTH (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>000630c: eclosing adult (no Zn), fresh (tested within 2h)</td>
<td>0.23</td>
<td>0.20</td>
</tr>
<tr>
<td>000630c: eclosing adult retested after 1.5 years (45 to 65% RH)</td>
<td>0.61</td>
<td>0.35</td>
</tr>
<tr>
<td>011225a: late stage pupal mandible heated 1h at 180 degrees C</td>
<td>0.88</td>
<td>0.45</td>
</tr>
<tr>
<td>010109b: fully Zn-enriched, fresh</td>
<td>0.89</td>
<td>0.27</td>
</tr>
<tr>
<td>010109b: fully Zn-enriched, retested after 1h at 180 degrees C</td>
<td>0.86</td>
<td>0.35</td>
</tr>
</tbody>
</table>

The first line in the above table shows the on-tooth and off-tooth hardness for a typical eclosing adult. The second line shows that the hardness of the tooth cuticle increased after 1.5 years in storage, but not to the level of hardness of a similarly Zn-free late-stage pupal mandibular tooth that was heated to 180 degrees C, shown in the third row. This suggests that the pupal mandibular tooth can be made harder by driving out water that remains in a dried specimen that is not heated. The fourth row shows that the hardness of a fresh mandibular tooth that is fully enriched with Zn is very close to the hardness of the heat treated Zn-free tooth. The final row shows that heat treatment of a Zn-enriched tooth did not further harden it. Thus Zn-hardening could be accounted for if the process removes, binds or otherwise mitigates the softening effect of the class of water molecules that were driven out by the heat treatment.

The hardness values in the Zn-free off-tooth regions of all specimens are consistent with the dessication hypothesis. The hardness value of the off-tooth region of the fully mature specimen did increase on heating even though the hardness of the Zn-enriched mandibular tooth did not. After heating, off-tooth hardness values were lower than the value of the heated Zn-free tooth, row 3; this suggests that there is a
hardening mechanism active in the mandibular teeth that is not associated with Zn-enrichment, presumably pre-ecdysial tanning.

We propose to test the dessication hypothesis by replicating these preliminary experiments and by using thermogravimetric analysis to compare the lability of water in Zn-enriched and unenriched mandibles.

**Thermogravimetric analysis (TGA).** The University of Oregon chemistry department has a TA Instruments Thermogravimetric Analyzer 2950 purchased under an NSF Chemistry Research Instrumentation and Facilities grant for multi-user instrumentation. The instrument will be available for our use at no cost. The basic technique is to raise the temperature in steps and allow the weight of the specimen to stabilize at each temperature before moving on to a higher temperature. Water that is more labile will be released at lower temperatures.

We plan to dissect the strips of mandibular teeth off of the mandibles of large *Atta sexdens*, and pool these strips for analysis. We estimate the mass of these strips to be about 10 ug (based on STIM measurements) and the Zn content to be about 0.4 ugs (from PIXE measurements). If we assume that one water molecule is replaced or bound up per Zn atom, the mass of water associated with Zn would be about 0.1 ug for one strip of mandibular teeth. The precision of the instrument is specified to be 0.1 ugs, so pooling at least 10 strips of mandibular teeth (from 5 or more ants) should give sufficient resolution.

We will first obtain mass-temperature curves for mandibular teeth that are not yet Zn-enriched. We will examine these curves for a mass-loss that may be associated with water that is not lost at room temperature but is lost at a higher temperature. We will test the hardness of mandibles that are heated to just below and just above this temperature feature in order to link this mass-loss feature with the hardness increase associated with heat treatment. Close correlation of hardness increases with water loss features would argue against the possibility that the hardness increase was due to heat induced-cross links. We will also obtain mass-temperature curves for Zn-enriched mandibular teeth.

**IV. THE CHEMICAL ENVIRONMENT OF Fe, Mn, Cu, Zn AND Br**

Whatever the role of water removal in the process of cuticle tool strengthening, we need to determine the exact chemistry of the various enriching elements in order to understand the mechanisms utilized by the epidermal tissues for cuticular tool modification. We propose to use a number of techniques to investigate the chemical environment of Mn, Fe, Zn, Cu and Br in cuticle in which they are the predominant heavy metals or halogens.

**Coordination.** Recently, our collaborator Paul Wyeth's group at Southampton have obtained EXAFS (Extended X-ray Absorption Fine Structure) analyses of Zn rich material form 3 insect and 1 nereid worm species. All analyses yielded similar results: O and/or N donors, coordination numbers between 3.9 and 4.5 and first shell radii between 1.96 and 1.99 angstroms (J. D. Fawke, J. G. McClements and P. Wyeth, personal communication). We have determined that this data is not consistent with Zn binding as ZnCO$_3$ but is consistent with ZnO or Zn(OH)$_2$ (Inorganic Crystal Structure Database, FIZ Karl Sruhe, Berlin, Germany). The EXAFS data is also consistent with binding in numerous organic salts (Cambridge Structural Database) and are typical for Zn binding by water, imidazole or carboxyl ligands (Bertini and Luchinat, 1994).

We propose to replicate this investigation using focussed beam EXAFS facilities, which may reveal details about second shell coordination, and to extend this investigation to Cl, Mn, Fe, Cu and Br enriched cuticle. We have begun this investigation by initiating a collaboration with Wolfgang Caliebe of Brookhaven National Laboratory (see attached letter). For a preliminary investigation, we have prepared specimens of nereid worm jaws which contain both Zn and Br. Using these preliminary results, we will prepare a proposal for extended investigations using the focussed beam at the Brookhaven facility. We have also contacted experts at the other high-energy synchrotron light sources in the United States, located at Stanford, Berkley, Argone and Cornell. These facilities do not charge for beam-time.

**Vibrational spectroscopy.** Performance of both Fourier-transform infrared (FTIR) and FT-Raman spectroscopy on unenriched and enriched cuticle can provide substantial information about the identities and the surrounding environments of the chemical bonds in the samples. Since FT-Raman spectroscopy
relies on low-energy laser excitation, possible fluorescence interference will be minimized. Our collaborator, Paul Wyeth, will analyze our specimens at an FTIR facility located at the University of Southampton, U.K.

Valence state methods. Two analytical methods can provide direct information about the valence state of sub-microgram quantities of elements. X-ray photoemission spectroscopy (XPS or ESCA) is a surface analysis technique that provides semi-quantitative elemental analysis information but also gives chemical state information from the distinctive chemical shifts of core level spectral peaks for different oxidation states. The XPS investigation will also be undertaken by Paul Wyeth. Electron energy loss spectroscopy is performed with an add-on accessory to a transmission electron microscope. We have begun collaborative efforts with Dr. Y.C. Wang (Senior Applications Engineer at FEI Company, Hillsboro, OR) on a Tecnai F20 Scanning Transmission electron microscope, equipped with a Gatan Enfina PEELS spectrometer. Capabilities include rapid ultra-high resolution elemental mapping and, for some elements, the ability to distinguish between different oxidation states. Initial studies with zinc-enriched cuticular tools have pointed out the need for suitable reference spectra for data interpretation.

Mass spectrometry. Preliminary experiments indicate the possibility of solubilizing certain of the enrichment elements by treatment of the cuticular structures with proteolytic enzymes. As well, there is the possibility of extracting a fraction of the catecholamines by mild treatments. The soluble fractions from such treatments will be analysed by suitable mass spectrometry methods. Zinc- chlorine- and bromine-containing fragments may be readily detectable due to the natural isotope pattern of these three elements. There is a well-equipped mass spectrometry Central Facility at Oregon State University.

V. BEHAVIORAL CORRELATES WITH Zn-ENRICHMENT

As discussed in the prior results section above, we have suggested that leaf-cutter ants might delay use of their mandibles until the mandibular teeth are fully Zn - enriched. This was based on observations of differences in leaf-processing behavior between light and darkly-colored adults (Wilson, 1980). The techniques that we have developed should allow us to tighten this behavioral correlation and to investigate other behavioral correlates in Atta and other ant species.

Behavior and Zn content. A simple approach to investigating behavioral correlates is to sample ants engaged in mandible-employing behavior and measure the Zn content of the mandibles. For example, callow Atta soldier castes do not engage in defense of the nest while older adults are highly aggressive (Wilson, 1980). We propose to test the hypothesis that aggressive soldiers have fully-enriched mandibles, while non-aggressive soldiers do not. We have observed that certain soldiers aggressively attack a foreign wooden dowel in the nest while others, generally lighter in shade, will not attack even if touched by the dowel. We propose to use this simple method to sort aggressive and non-aggressive soldiers (head size > 3.0 mm) and to compare the Zn content of their mandibles using high-energy ion microscopy.

Scratch Counting. In our AFM examinations we have noticed many more scratches on the mandibular teeth of adults that have a full complement of Zn than on those that do not. Of course one would expect that the number of scratches would increase with age. In order to determine if mandible-employing behavior suddenly begins and the rate of scratching suddenly increases when the mandibles reach full hardness, we need to be able to plot average numbers of scratches as a function of age. In preliminary experiments we have shown that we can mark individual recently eclosed Atta and track them as they age. We have also developed scratch counting techniques based on archaeological methods (King et al., 1999) and have counted scratches on about 20 specimens. We plan to continue these investigations on stable colonies of Atta and Camponotus. This novel use of the AFM has advantages over previously employed SEM techniques in that smaller scratches can be counted and their width and depth can be quantified.