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SHORT COMMUNICATION

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Tooth hardness increases with zinc-content in mandibles of young adult leaf-cutter ants

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Abstract A wide variety of arthropods and members of other phyla have elevated concentrations of Zn, Mn, other heavy metals and halogens in their jaws, leg claws, and other "tools" for interacting with the environment. While measured Zn concentrations reach 25% of dry mass in scorpion stings, concentrations are often lower than this and the enriched structures are not heavily biomineralized like vertebrate teeth and the radula of mollusks. For this reason, the degree to which the inorganic components of these structures modify their mechanical properties is in question. Here we address this problem by measuring hardness during the development of Zn accumulations in ant mandibles. We found that Zn is incorporated into the mandibular teeth of leaf-cutter ants during early adult life, reaching concentrations of about 16% of dry mass. We show that the hardness of the mandibular teeth increases nearly three-fold as the adults age and that hardness correlates with Zn content (r=0.91). We suggest that young adults rarely cut leaves partly because their mandibles are not yet rich in Zn. Zinc enrichment (along with enrichment by other heavy metals and halogens) may play an unrecognized role in the behavioral ecology and evolution of a wide variety of invertebrates.

Introduction

Concentrations of heavy metals and halogens exceeding 1% of dry mass have been found in the contact regions of mandibles, chelicerae, stings, pedipalps, forcipules, leg claws, and other environmentally interactive "tools" of a large fraction of examined arthropods, some annelids, and members of other phyla (Schofield 2001). The molecular form and biochemistry of these accumulations is not yet known. X-ray and electron diffractometry have not detected crystallinity in Zn-rich structures and the concentrations are often too high for the Zn atoms to be bound as individual ions to protein binding sites (Schofield 2001). Any clustering of Zn atoms is likely to be on a nanometer scale because we have not detected a separate electron-dense phase in transmission electron micrographs of Zn-enriched ant and scorpion cuticle, even though we were easily able to observe the 7 nm cores of ferritin molecules in transmission electron microscope images of several of the scorpion sections, and could confirm the ferritin identification using electron diffractometry (Schofield et al. 2003; M.H. Nesson and R.M.S. Schofield, unpublished data).

Behavioral correlates with Zn enrichment, such as an association with herbivory, have been suggested, but they have not withstood the expanding catalogue of enriched organisms and structures (Schofield 2001).

Zinc is incorporated late in the cuticular development of the arthropods that have been studied. Preliminary high-energy ion microprobe surveys found little Zn in the mandibles of pupal leaf-cutter ants, *Atta sexdens* (Grime et al. 1999), or in recently eclosed adults (R.M.S. Schofield and P. Wyeth, unpublished results). We have recently shown that Zn incorporation takes place late in the cuticular development of another ant species (*Tapinoma sessile*) and in a species of scorpion (*Vaejovis spinigeris*), and we have shown that the time course of deposition of Zn and associated elements, Mn, Cl and Ca, is similar in these distantly related groups. We have argued that these developmental similarities, along with the widespread phylogenetic distribution, suggest that this form of structural modification evolved before the insect and arachnid lines diverged (Schofield et al. 2003).

Several investigations have found a higher indentation hardness for Zn-enriched structures than for surrounding regions (Hillerton and Vincent 1982; Hillerton et al. 1982; Schofield 1990; Edwards et al. 1993; McClements et al.

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1993). This evidence has been inconclusive for three reasons: first, because the hardness differences could be attributed to different cuticle matrices in the Zn-rich and Zn-free regions or to different degrees of sclerotization in the pre-ecdysially tanned Zn-rich regions and the normally-tanned surrounding regions; second, because of artifacts associated with the large size of the indents and because many specimens were polished flat to avoid these artifacts; and third, also because of technique limitations, the specimens in previous studies were dry. We have found that two regions of ant mandible, initially equal in hardness, can differ in hardness by a factor of 2 after air-drying (R.M.S. Schofield and M.H. Nesson, unpublished data).

Here, instead of comparing Zn-rich to surrounding regions, we took advantage of late Zn incorporation and compared the hardness of the fourth mandibular tooth of individuals at various stages of Zn incorporation. We reduced artifacts associated with indentation size by employing an AFM (atomic force microscopy)-based nanoindentation technique that we modified for these small biological specimens. This technique produced indentations that were under 3 μm across, up to 100 times smaller than indentations in previous studies, and enabled us to test fresh specimens.

Materials and methods

Specimens

Adults representing the range of cuticular shades and with head widths between 1.4 and 2.4 mm were selected from three several-thousand-individual subcolonies that, on three occasions over the course of 1.5 years, were spooned with nest material from the center of a colony of *Atta sexdens rubipilosa* maintained at the University of Southampton, UK.

Callows were defined as those ants whose posterior head region visually matched swatches with a value of 4.5 or higher of the 7.5YR hue of the Munsell color notation system. Ants were compared with color swatches under a dissecting microscope illuminated by two 16.5-inch fluorescent lights (Sylvania F15T8/WW and F15T8/W). Marked, recently eclosed ants spent about 3 to 4 weeks in the callow phase.

Specimen preparation

Each right mandible was dissected off and floated in a bed of epoxy composite on an atomic force microscopy (AFM) specimen disk (TedPella, Redding, Calif.). The mandible was adjusted under a dissecting scope so that the anterior faces of the distal mandibular teeth were exposed parallel to the surface of the AFM specimen disk.

The epoxy composite and curing procedures were developed to minimize curing time and temperature, and 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. The epoxy composite was prepared by mixing about 0.4 g of 400-grit aluminum oxide powder (Buehler, Evanston, Ill.) with 0.075 ml each of 5-min-setting resin and hardener (Quick Set Epoxy; Loctite, Rocky Hill, Conn.). The mounted specimens were placed in an oven at 39°C for 20 min to cure the cement.

To reduce drying artifacts, the cut base of each mandible was rapidly buried in the epoxy composite, and all hardness measurements were completed within 2 h of dissection.

Hardness measurements

Hardness measurements were made using an AFM (NanoScope IIIa; Digital Instruments, Santa Barbara, Calif.) with an add-on force/displacement transducer (TriboScope; Hysitron, Minneapolis, Minn.). 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.

We used an inverted-pyramid shaped probe with cubic cornered facets (90° between the three faces) rather than the more conventional Berkovich pyramid which, because of its bluntness (the cross-sectional area increases with distance from the apex at about ten times the rate for the cubic tip), was more susceptible to interference from structures such as hair-like sensilla near the indentation region.

The diamond probe was positioned on the specimen using a 30× extra-short-focus monocular (M1030; Specwell Corporation, Tokyo, Japan) sighted down through a side window on the 2-D Hysitron transducer. Using the monocular and the hand positioning screws on the transducer, it was possible to position the probe to within about 10 μm of the desired location on the specimen. A single indentation sequence was used for all indents to minimize variation due to visco-elastic effects. The force on the indenting probe was ramped linearly from 0 to 2 mN in 1 s. This force was then maintained for 30 s and finally removed linearly over a period of 1 s.

The hardness (H) values reported here were calculated as $H=F/A$, where F is the force applied to the probe, calculated from the voltage applied to the probe-holding capacitor, and A is the projected area of the residual indentation obtained from the perimeter of the indentation, which was measured on an AFM image made by scanning the indenting probe itself immediately after indenting the specimen. We avoided the indirect technique of calculating indent area from the probe shape and its vertical displacement because the displacement of the probe was considerably larger than the depth of the indents in control regions of the most recently emerged adults, apparently because the entire face of the mandible deformed. However, indent area measurements yielded an accurate hardness value even for deformed structures because the applied force was nearly independent of probe displacement. To minimize inaccuracies in indent perimeter determination caused by the finite size of the imaging probe or other systematic errors, we calibrated our area measurements using a single crystal Al standard under identical imaging conditions as for the mandibles and with similar indent sizes. The calibration factor was set to give a hardness of 0.30 GPa for the Al standard.

High-energy ion microprobe elemental analysis

High-energy ion microscopy is more penetrating than scanning electron microscopy (SEM) and was used for surveying the entire volume of selected mandibles while SEM was used to sample a surface layer of approximately the same depth as the hardness indentations. The data displayed in Fig. 1 were obtained using two high-energy ion microscopy techniques: particle induced X-ray emission, to measure the quantity of specific elements in the sampled volume, and scanning transmission ion microscopy, to measure the total quantity of material in the sampled volume (Schofield 2001).

Scanning electron microscopy

Each specimen was Au-Pd sputter-coated and the indented region of the mandibular tooth was examined using a field-emission SEM (Amray 3300FE) equipped for energy-dispersive X-ray spectroscopy (IXRF Systems, Houston, Tex.). Each of the three batches of specimens was examined in a different SEM run. The Zn K-alpha X-ray count rates were multiplied by the fraction required to normalize the count rates for a standard (polyester resin, styrene, ZnO) examined immediately prior to or after the mandible specimen. The reported Zn X-ray count rates are thus proportional to Zn concentration in the sampled region (several micrometers in depth), in the approximation that the composition and topography of the mandibular tooth specimens were identical.

Results and discussion

The late incorporation of Zn into the mandibular teeth of *Atta sexdens* is demonstrated in Fig. 1, which shows mandibles from two individuals, a mandible from a light-colored eclosing adult containing little Zn (on the left) and one from a dark-colored adult with a full complement of Zn (on the right). The Zn concentration in the hardness-tested mandibular tooth of the fully shaded adult (16% of dry mass, measured using high-energy ion microscopy) was about 200 times greater than in the eclosing adult, while there was no significant difference in the Zn content of the off-tooth control region. This image also suggests that the Mn associated with Zn is mostly in place before the Zn begins to accumulate, consistent with our findings for another species of ant and a scorpion species (Schofield et al. 2003).

Specimens were hardness-tested on the central region of the fourth mandibular tooth and the off-tooth control region shown in Fig. 1. After hardness testing, the relative Zn contents of the mandibular teeth were determined by X-ray analysis using an SEM. Figure 2 shows a high correlation (r=0.91) between the hardness values of the teeth and the Zn K-alpha X-ray count rates (which were approximately proportional to the Zn concentration). Figure 2 also shows the hardness values of the corre-

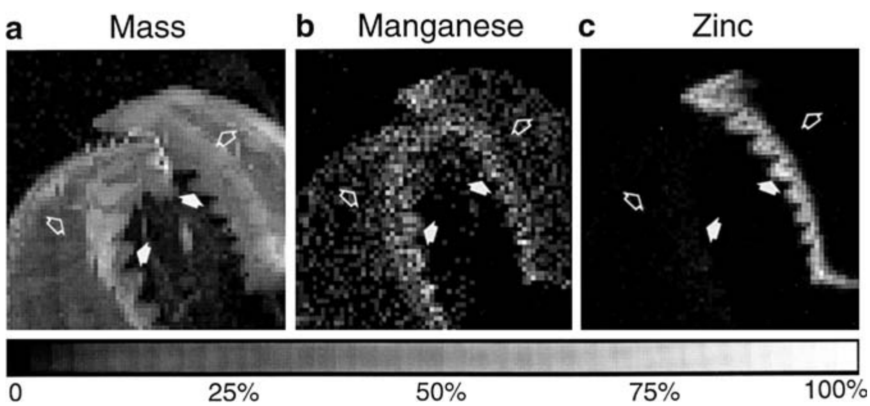

Fig. 1a–c Post-eclosion Zn enrichment in *Atta sexdens*. Each of the three high-energy ion microprobe images show two mandibles, one from an eclosing adult, *on the left*, and one from a fully-pigmented adult, *on the right*. The *white-filled arrows* point to the location of hardness measurements on the fourth mandibular tooth, the *open arrows* point to the off-tooth control region. **a** A plot of projected mass density, used to calculate concentrations from projected element densities. **b** A plot of Mn X-ray counts in 0.2 s. The Mn concentrations in the hardness-tested teeth: 0.0022 (±0.0004) and 0.0032 (±0.0006) of dry mass in, respectively, the eclosing and the fully pigmented adult (68% confidence intervals are given for all concentration values). **c** Zn X-ray counts in 0.2 s; the concentrations of Zn in the fourth teeth of the eclosing and fully-pigmented adults were 0.0008 (±0.0002) and 0.16 (±0.02) of dry mass, respectively. The grayscale maximum (*white*) is set at 0.020 g/cm^2 in **a**, 13 X-ray counts in **b** and 144 X-ray counts in **c**. The imaged area is 1×1 mm

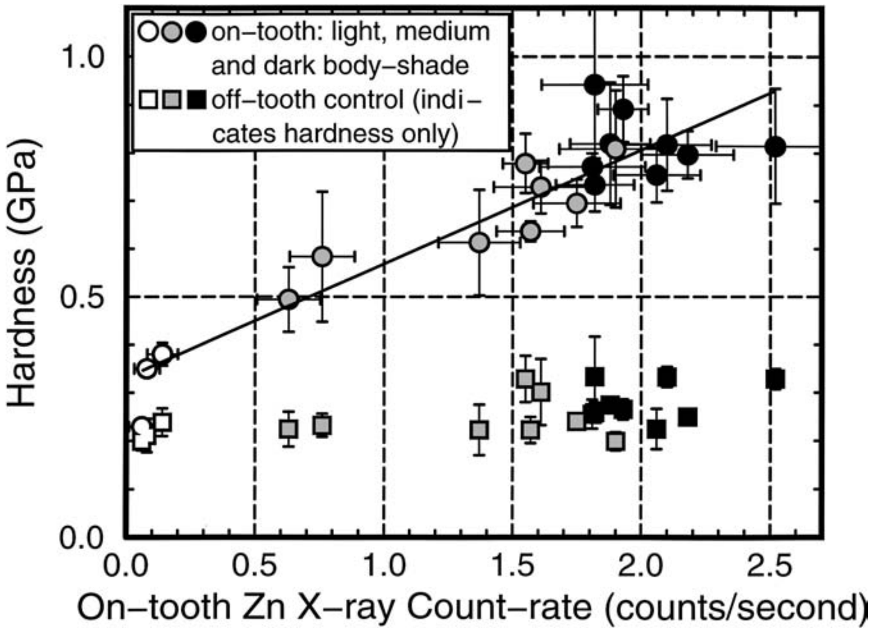

Fig. 2 Mandibular tooth hardness correlates with Zn X-ray count-rate. *Circles* show hardness and count-rate values for the center of the anterior face of the fourth mandibular tooth (see Fig. 1) for each of the 19 tested individuals. *Squares* show hardness values (but not count-rates) for the off-tooth control region; they are horizontally positioned to line up beneath the corresponding circles. *White and gray symbol fills* indicate callows (6), *white* signifies that they were not visibly darker than eclosing adults; *black* indicates full adults. *Each point* shows the average of three hardness measurements and one count-rate determination. The *error bars* indicate the 95% confidence interval for the mean; *vertical error bars* are based on Students' *t*-test for the three hardness measurements (t=4.3), *horizontal bars* are based on X-ray counting statistics. The displayed line fit yields an r value of 0.91±0.02 (the confidence interval is the standard deviation for the r values of 1,000 numerical simulations for which the position of the data points were varied randomly according to their uncertainty). Indents ranged from about 1 to 2 μm in depth

sponding off-tooth control regions. The hardness of the teeth varied with Zn count rate by more than a factor of 2, while there was relatively little change in the hardness of the Zn-free off-tooth region.

In addition, Fig. 2 shows that callow adults yielded lower hardness values and Zn X-ray count rates than darker adults. Although the specific ages of the individuals tested here were not known, we have shown, for a different ant species, that Zn accumulation began abruptly and that the rate of accumulation decreased as the adults aged (Schofield et al. 2003).

Because hardening occurred predominantly after eclosion, it took place after cuticle deposition and after pigmentation (pre-ecdysial tanning) of the mandibular teeth. Pigmentation is thought to be generally co-temporal 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 (teeth of eclosing individuals appear fully darkened) and Zn-associated hardening (which takes place during the 3 to 4-week-long callow adult phase) suggests that there are two separate hardening processes. The fact that Zn enrichment occurred after much of the cuticle maturation process was complete and the control region fully hardened also makes it unlikely that a separate process coincidentally hardened the cuticle as Zn was deposited.

In summary, the evidence for Zn-associated hardening is, first, that there was a high degree of correlation between tooth hardness and Zn content; second, that the hardness difference between tooth and control regions increased with increasing Zn content; and, third, that hardening and Zn incorporation both occurred later than other cuticle maturation processes.

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, which have been placed in separate temporal castes, and fully colored 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. The largest callows are relatively inactive until, as fully colored adults, they engage almost exclusively in mandible-employing defense.

Perhaps the most compelling evidence is the reported difference in callow involvement in the processing of vegetation within the nest. The first stage of processing involves cutting with the mandibles, while the second stage involves chewing with the maxillae. The proportion of callows in the cutting work force is reported to be ten times lower than the proportion in the chewing work force (Wilson 1980; E.O. Wilson, personal communication). The argument that mandible maturity plays an important role in behavioral differences is strengthened by this observation because, except for cutting, the tasks are similar and occur at similar locations in the nest. Leaf-cutting can be energy-intensive (Roces and Lighton 1995) and leaf toughness may limit harvesting ability (Nichols-Orians and Schultz 1989). Harder mandibles would be energetically more efficient because the cutting edges would deform less during cutting and because they would be less susceptible to dulling wear. We suggest that there is an adaptive advantage in postponing cutting until Zn enrichment provides more effective "tools". We further suggest that the advantages of Zn-associated hardening are significant enough to modify behavior in the wide variety of enriched invertebrates.

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