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Spectral cathodoluminescence analysis of hymenopteran mandibles with different levels of zinc enrichment in their teeth

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ABSTRACT

The inclusion of Zn in insect mandibles affects their hardness and is functional to their use during feeding or reproducing. However, little is known on the chemical/structural base of Zn enrichment. Here, we found that cathodoluminescence (CL) technique revealed two different types of CL spectra in the mandibles of Hymenoptera, depending on the Zn enrichment level assessed by Energy Dispersive X-ray Spectroscopy (EDS). Individuals having negligible traces to low % of Zn in their mandible teeth (≤3 wt%) presented a wide band of luminescence in the visible range which resembled those observed in the C–C structures of graphite. This spectrum is probably characteristic for un-enriched cuticle, since it did not differ from those obtained from the Zn-lacking inner part of mandibles. Individuals with moderate to high % of Zn in their mandible teeth (≥7 wt%), instead, presented additional CL peaks in the ultraviolet range. Comparisons with different minerals of Zn suggest that these peaks could be related with O–Zn–O bonds, with hydroxyl groups and with zinc-chlorine links (in agreement with Cl high levels detected by the EDS). Being a non-destructive technique, CL allows large comparative studies of the chemistry of metal-enriched insect cuticle even using unique specimens, such as those deposited in Natural History Museums.

1. Introduction

Cuticle of all insects, which includes most of the material of the exoskeleton, has a common fundamental structure and consists of a procuticle of up to few hundreds μm, comprising chitin filaments disposed within a protein matrix, and an upper non-chitinous epicuticle of less than 2 μm, composed of lipids and proteins (Neville, 1975; Andersen, 1979). Nevertheless, cuticle is extremely variable in terms of composition, thickness, stiffness, strength, elasticity and colour (Vincent, 2002; Vincent and Wegst, 2004), in part due to the different degrees of stabilization and structuring occurring during the sclerotization process, in which certain aromatic organic compounds are incorporated into the cuticle proteins. From the pioneer studies on the chemical base of sclerotization (see Pryor, 1940) to the more recent analyses on incorporation of phenolic compounds and chemistry of hydrocarbon profiles in the epicuticle (reviewed by Andersen, 2010; Blomquist and Bagnères, 2010), much effort was done to shed lights on insect cuticle structure and composition, and on their relationships with life-history traits.

From early studies in the 80's (Hillerton and Vincent, 1982; Hillerton et al., 1984) and especially in the last 20 years, many researchers have focused on a particular feature of the arthropod (and to a lesser extent non-arthropod) cuticle, i.e. the incorporation of transition metals such as Zn, Cu, Mn, Fe and of halogens in certain organs such as mandibles, ovipositors, and chelicera (e.g., Schofield and Lefevre, 1989; Quicke et al., 1998; Schofield et al., 2002; Lichtenegger et al., 2003; Polidori et al., 2013). Such arthropods seem to have benefits in possessing organs enriched with metals, because an enriched cuticle increases its hardness and wear resistance, and thereby allows a better and longer performance in cutting hard substrates, such as seeds, leafs or other modified plant

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tissues (e.g., galls), or wood, which are used as oviposition sites and/or food (Vincent, 2002; Lichtenegger et al., 2003; Schofield and Lefevre, 1989; Quicke et al., 1998; Fontaine et al., 1991; Birkedal et al., 2006; Morgan et al., 2003; Schofield et al., 2002, 2003; Cribb et al., 2008a; Hillerton and Vincent, 1982; Fawke et al., 1997; Kundanati and Gundiah, 2014). For example, in ants (Schofield et al., 2002), termites (Cribb et al., 2008b) and certain marine polychaetes (Lichtenegger et al., 2003), the hardness of the mandible teeth has been correlated with the content of Zn, and the experimental removing of Zn considerably decreases their hardness (Broomell et al., 2006). Halogens such as Cl are often co-located with Zn in these organisms (Lichtenegger et al., 2003; Schofield et al., 2003; Birkedal et al., 2006). Comparative studies also suggest that metal enrichment in the cuticle is beneficial to organisms with certain life-histories. Indeed, species which do not reproduce or feed in hard substrates tend to lack transition metals, while closely related species with prolonged activity of cutting or drilling in hard substrates during their life present such metals (e.g., Cribb et al., 2008b; Morgan et al., 2003; Polidori et al., 2013; Kundanati and Gundiah, 2014).

However, despite the many reports of metal enrichment in different insect organs, still little is known about its chemical/structural base. It has been observed that certain metals, such as Zn, showed no mineral formation within the cuticle (Broomell et al., 2006), and some authors have preliminarily proposed that the biochemical form of Zn may involve binding proteins (Lichtenegger et al., 2003; Birkedal et al., 2006; Broomell et al., 2006; Schofield, 2001). Nevertheless, other authors highlight that mineralization processes, as phosphating, silicification and carbonation are very common in biological systems, these phenomena having been observed in animals as different as Hemiptera (Garcia-Guinea et al., 2011), corals (Sanchez-Munoz et al., 2009), echinoderms (McClintock et al., 2011) and molluscs (Baronnet et al., 2008). Because it is believed that mineralization processes are responsible for major adaptive radiations in evolutionary history, it cannot be completely excluded that they are also linked with cuticle Zn enrichment.

The use of novel analytical techniques could help in a better understanding of metal enrichment in biological structures. For example, beyond bioluminescence, which was studied in a wide range of taxa (e.g., Lloyd, 1983; Viviani, 2002), the improved spatial resolution and efficiency of modern luminescence spectrometers coupled to electron microscopes could provide details of luminescent emission centres of insect cuticle. Cathodoluminescence technique (CL) is a non-destructive method in which spectral emission of light is produced by irradiating the surface of a solid by an electron beam. CL basic processes involve the excitation of an electron to a higher energy state followed by emission of a photon with energy λ ranges of ultraviolet (UV), visible or infrared (IR), when the electron returns to the lower energy state. The depth of penetration of the electrons in CL mode and therefore the depth of excitation depends on the energy of the electrons and is usually between 2 and 8 microns (Götze and Kempe, 2009), thus adequate to reach the procuticle of insects. However, as far as we know, CL analysis in insects to date was only applied to unveil the presence of ossification vesicles with calcium phosphate in the eyes of a hemipteran bug (Garcia-Guinea et al., 2011). In contrast, CL was largely used in the last decade to study structures of other organisms, such as shells in molluscs and thallus in algae (see Barbin, 2013).

In the present study we analyzed the relationship between the Zn enrichment in the mandibles of Hymenoptera, evaluated with electron microscopy and microanalysis of energy dispersive X-ray spectroscopy (EDS), and the spectra obtained through CL spectroscopy, to help unveiling how Zn incorporation may affect the cuticular structure. To reach such objectives we analysed CL spectra and chemical-elemental compositions of individuals with different Zn concentrations, together with Zn-bearing luminescent minerals for comparative purposes. With this mainly methodological study we aimed to provide new technical elements that can be used in future comparative ecological and evolutionary investigations.

2. Materials and methods

2.1. Selected minerals for the study

We selected four historical-international zinc minerals kept in the reserve of the Museo Nacional de Ciencias Naturales (CSIC) (Madrid, Spain) (MNCN) and commercial zinc compounds: hydrozincite ($Zn_5(CO_3)_2(OH)_6$), sphalerite (ZnS), zincite (ZnO), smithsonite ($ZnCO_3$), zinc chloride (ZnCl) and zinc sulfate ($ZnSO_4$). Details about the origin of the analyzed minerals are available in Table S1.

2.2. Selected biological sample for the study

We selected female individuals from a total of 11 species of Hymenoptera spanning 6 super-families and 10 families of bees and wasps (Table 1) for the study of mandibles. Biologically, these species span a wide range of life-histories, including parasitoid wasps, predatory wasps, gall-forming wasps, and both pollen-foraging and pollen-stealing (cuckoo) bees (Table 1). Because our aim was to evaluate the CL method as a tool to study Zn enrichment, and not to test differences between species, we used one individual per species, spanning a wide range of Zn concentrations based on previous published observations on related taxa (Quicke et al., 1998; Polidori et al., 2013). Zn content variability is known to be typically very low within species (Polidori et al., 2013); however, for two of the selected species (see Table 1), we also analyzed three females each and evaluated the degree of intra-specific variation in Zn% and CL spectrum. Differences were minimal (see Supporting Information, Fig. S1). In addition to mandible teeth, for three species (see Table 1) we also analyzed the inner part of mandibles, which is never enriched with Zn in Hymenoptera, to include a "control" CL spectrum to compare both mandible types, i.e. Zn-containing and Zn-lacking. Specimens preserved in 99% ethanol, belong to the collections of authors CP and JLNA (Table 1). Prior to the analyses, specimens were air-dried for 15–20 min, and then their mandibles were dissected under a light microscope and mounted on adhesive carbon pads attached to aluminium stubs for the subsequent analyses. Mandibles used in this study are deposited at MNCN.

2.3. Environmental scanning electron microscopy (ESEM) and energy dispersive X-ray spectroscopy (EDS)

The micromorphology, topography, and distribution of metals were determined using a Philips FEI INSPECT (Hillsboro, Oregon, USA), an environmental scanning electron microscope (ESEM) at the MNCN. This ESEM can work at low vacuum conditions, allowing analyzing samples without previous preparation, i.e. gold-coating or dehydrating. The ESEM resolution operating at low-vacuum was at 3.0 nm/30 kV (SE), 4.0 nm/30 kV (BSE) and <12 nm/3 kV (SE). The accelerating voltage was at 200 V–30 kV and the probe current up to continuously adjustable 2 μA. To obtain comparative analytical results, we always worked in low-vacuum mode with a backscattered electron detector (BSED) under vacuum conditions of 30 Pa, a high voltage of 20 kV, a suitable beam spot diameter for particular magnifications and to achieve good focus and astigmatism correction, and a working distance of approximately 10 mm to the detector. A picture of one mandible for each of the studies

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