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Strong phylogenetic constraint on transition metal incorporation in the mandibles of the hyper-diverse Hymenoptera (Insecta)

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Abstract

In several groups of insects, body structures related to feeding and oviposition are known to have a hardened cuticle by incorporation of transition metals. However, a functional link between metal enrichment and ecological pressures (i.e., adaptation) has been only rarely shown, opening the possibility that in some lineages, the evolutionary history may account for most of the observed variation (i.e., phylogenetic constraint). Here, we addressed this question in the hyper-diverse Hymenoptera (bees, wasps, ants, and sawflies), in which Zn and/or Mn have been found enriching the mandibles of a number of species. Across 87 species spanning most of the extant superfamilies, we found Zn enrichment to be widespread (57 species). Although lacking in the most primitive "Symphyta", our ancestral state reconstruction was not conclusive in determining whether Zn enrichment was a derived state for the complete order, but it was clearly the ancestral state for the Apocrita, where it was lost in few lineages, notably in Aculeata (where it was then reacquired at least three times). Mn, on the other hand, occurred very rarely in mandibles (10 species). Our comparative analysis revealed a strong phylogenetic effect explaining most Zn % and Mn % variation in mandibles. Additionally, species with herbivorous larvae were less prone to have Zn (but not Mn) than those with carnivorous larvae, although a causal effect of diet on this trait was unlikely. Furthermore, species emerging from concealed vs. unconcealed development sites have similar likelihood to have metal-enriched mandibles. Evolutionary history seems to constrain metal enrichment in hymenopteran mandibles, yet the few observed losses and regains of this trait during evolution claim for deeper investigations on the role of alternative, here untested, ecological pressures.

Keywords Insect cuticle · Hymenoptera · Transition metal · Mandible · Life history · Evolution

Introduction

Despite a common structure, the cuticle of arthropods is very variable in terms of chemical composition, thickness, and mechanical properties such as stiffness and elasticity (Vincent 2002; Vincent and Wegst 2004). One of the most variable traits associated with cuticle structure concerns the presence of transition metals, and more rarely alkaline earth

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metals (Hillerton and Vincent 1982; Fawke et al. 1997; Quicke et al. 2004; Schofield and Lefevre 1989; Polidori and Wurdack 2018). In most cases, these metals occur in mandibles and/or ovipositors, i.e., structures that are extensively used in a variety of activities, such as feeding, ovipositing, digging, and crawling as adults during emergence from the development site that incur great energetic costs and lead to progressive wear of these organs.

The most frequent transition metals found in insects are Zn and Mn (Schofield et al. 2002; Hillerton et al. 1984; Quicke et al. 1998; Morgan et al. 2003; Polidori et al. 2013). While the chemical form in which these metals are incorporated within the cuticle matrix is not fully understood, it seems that they showed no mineral formation within the cuticle (Broomell et al. 2006). Instead, it was suggested that binding to amino acid side chains of cuticular proteins, or binding to catecholate ligands, which accumulate during cuticle sclerotization, are involved (Schofield 2005). Species with high percentage of Zn in their mandibles presented cathodoluminescence peaks in the ultraviolet range which can be related with O–Zn–O bonds, with hydroxyl groups and with zinc-chlorine links (Jorge et al. 2017).

The enrichment of the cuticle with transition metals has been suggested to be an evolutionary adaptation in insects, given its verified relationship with increased hardness and wear resistance (Broomell et al. 2006; Cribb et al. 2008a). For instance, many insects which cut, drill, or chew hard substrates such as those of seeds, wood, leafs, or other modified plant tissues (e.g., galls) show Zn and/or Mn in their mandibles and/or ovipositors (Quicke et al. 1998; Fontaine et al. 1991; Morgan et al. 2003; Schofield et al. 2002; Cribb et al. 2008a; Hillerton and Vincent 1982; Kundanati and Gundiah 2014). The metals only occur on the parts of these organs involved in these activities (e.g., on the mandible tooth (Quicke et al. 1998; Polidori et al. 2013)), also suggesting an adaptive role. However, only few studies showed that metal enrichment evolved in responses to ecological pressures. For example, Cribb et al. (2008b) showed that the presence of Zn-enriched mandibles in certain species of dry wood termites appears to be related to the lack of free access to water to wet the wood. Morgan et al. (2003) showed that high concentrations of Zn and/or Mn occur in insect larval mandibles that pierce seeds, but not in those that attack previously damaged seeds. Ovipositors in species of wasps that lay eggs in hard parts of plants, such as wood, galls, and hard fig syconia, have more often high concentrations of transition metals, while those wasp species that oviposit in softer parts of plants, such as leaves or flowers, present no enriched or less enriched ovipositors (Polidori et al. 2013; Kundanati and Gundiah 2014).

The insect order Hymenoptera (sawflies, wasps, bees, and ants) is an extremely speciose group of insects that evolved a great diversity of life histories (Austin and Dowton 2000; Nieves-Aldrey and Sharkey 2014).

Within Hymenoptera, it is possible to find solitary as well as social species, species with carnivorous (parasitoids and predators) or herbivorous (defoliators, pollinators) larvae, and species with larvae developing in unconcealed (e.g., exposed hosts) or concealed (e.g., within a nest) sites. This makes this group of insects a good model to test the link between ecological pressures and morpho-physiological modifications.

Previous studies on Hymenoptera point towards a reasonably clear link between metal enrichment in the ovipositor and the oviposition substrate. For instance, despite the more complex trend found for the aculeus (i.e., the modified ovipositor) of Aculeata (Baumann et al. 2018), no metals were detected in the ovipositor across Hymenoptera in species that penetrate soft substrates or do not make holes at all (Quicke et al. 1998). This association was confirmed also within certain superfamilies (Polidori et al. 2013). On the other hand, the evolution of metal enrichment in mandibles is much less understood. Across 42 species spanning 18 superfamilies, Quicke et al. (1998) found Zn in the mandibles of almost all lineages. Explanations for the lack of Zn in few lineages were not conclusive, and Quicke et al. (1998), though not basing on a real correlational analysis, noted that the almost universal Zn incorporation in the mandibles contrasts with both the variation in larval diet (plant-leaf feeders, gall feeders, pollinivorous) and the strongly variable material that has to be cut or dug during emerging, digging, and/or foraging (wood, leafs, soil, insect cuticle). Things were similarly unclear with Mn enrichment, which was detected in few species spanning different life histories (Quicke et al. 1998).

Here, we used an expanded sample of species, families, and superfamilies (overall almost doubling the taxonomic spectrum analyzed by Quicke et al. (1998)) to investigate the effects of these two key ecological factors (adult emergence site, larval feeding resource) and of phylogenetic relationships on Zn and Mn enrichment variability (through a semi-quantitative evaluation) in Hymenoptera's mandibles. An effect of larval diet may suggest that variation in metal content in adult mandibles is a result of differences in metal uptake from types of tissues (e.g., animal or plant) by the larvae (Hillerton and Vincent 1982). In this case, physiological processes, but not necessarily adaptation, would explain variation. On the other hand, an effect of adult emerging site would suggest an adaptation of metal enrichment as a response to the ecological pressure of digging through the barrier of a concealed development site (Polidori et al. 2013). Alternatively, the strongest predictor of whether a species contains metals in its mandibles may be whether closely related species also show this trait, i.e., there is a strong phylogenetic constraint (Blomberg and Garland 2002).

Materials and methods

Sample, taxa, and life history traits

Females of 87 species of Hymenoptera spanning 64 families in 25 superfamilies were analyzed (Table 1). The sample covered > 80% of the 30 extant superfamilies and about 50% of 132 extant families (Aguiar et al. 2013; Peters et al. 2017). Individuals were identified to species when possible; otherwise, they were reported as higher taxon name with sp. The sample includes both newly analyzed taxa coming from the private collections of CP and JLN-A and from the collection of the Museo Nacional de Ciencias Naturales (CSIC) (Madrid, Spain) (MNCN) and data retrieved from previous works (Quicke et al. 1998; Polidori et al. 2013). To avoid biases in species representation among lineages, we analyzed a maximum of two species per family. The complete sample included species representing all the life histories known for the order (plant-feeders, gall-formers, ecto- and endo-parasitoids, inquilines, predators, pollinivorous, social and solitary species) and spans a wide range of substrates from which adults emerge (Table S1). Each species was then associated with two main traits: larval feeding resource (hereafter: LFR) (only plant (0) or animal (completely or partially) (1)), and larval development site (hereafter: LDS) (unconcealed (0) or concealed (2)) (which corresponds also to adult emergence site). A development site was considered concealed if the immature stages are located within a substrate, such as soil, wood, and plant galls. A site was defined as unconcealed if development takes place (including cocoon spinning) in an exposed environment, such as plant leaves or hosts not located in a substrate. LFR and LDS states were based on the available information about the biology of the studied taxa in the literature (Table S1).

Phylogeny and ancestral state reconstruction

The tree used for ancestral inferences was derived from the current knowledge about Hymenopteran phylogeny, yet the distances were discarded as evidence was combined from different sources. In particular, the phylogeny of Peters et al. (2017), based on 167 whole transcriptomes, was used as the backbone of the tree and covered all major groups. Within Chalcidoidea, taxa lacking in the transcriptome-based tree were added with first priority according to transcriptome-based phylogeny of Peters et al. (2018), then further missing taxa according to the 18S and 28S ribosomal gene phylogeny of Munro et al. (2011). Within the Proctotrupoidea, we followed Kozlov (1970). Ancestral states of Zn %, Mn %, and LFR and LDS ancestral states were reconstructed using Mesquite 3.6 (Maddison and Maddison 2018) with maximum likelihood reconstruction and as model Mk1. Reported likelihoods were illustrated in the trees as proportional likelihoods.

Scanning electron microscopy and energy-dispersive X-ray spectroscopy

Except for the few specimens coming from the Museum collection that were introduced in the scanning electron microscopy (SEM) without previous gold coating, individuals were dissected under light microscopy and the excised mandibles were gold-coated after mounting on adhesive carbon pads attached to aluminum stubs (Polidori et al. 2013; Jorge et al. 2017). One individual per species was analyzed, according to the very weak percentage variation previously recorded within species (i.e., percentage variation did not change the percentage rank of any individual of a species) (see Jorge et al. 2017).

The abundance of Zn and Mn was determined using a SEM Philips FEI INSPECT (Hillsboro, OR, USA) at the MNCN. We worked in a high-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 a working distance of about 10 mm to the detector. Pictures of the mandibles were taken in the SEM at appropriate magnification. The X-ray energy microanalysis (EDS) of the samples was conducted with an energy-dispersive X-ray spectrometer (INCA Energy 200 energy-dispersive system, Oxford Instruments) (Polidori et al. 2013; Jorge et al. 2017). For each specimen, we performed a point analysis, in which metal concentration was obtained at one point on the mandible tooth.

Data analysis

To check for significant metal enrichment in their mandibles, we performed a semi-quantitative analysis by using the maximum peak intensities obtained by a least-squares fitting routine that used standard peaks correlated to a spectrum of known compounds (Roomans 1988; Packwood 1991). After these intensities were determined, matrix corrections were applied (Pouchou and Pichoir 1991) to determine the concentration of each element by using approximated exponential curves and the $\varphi(\rho Z)$ model to describe the shape of the curves. We could thus obtain improved measurements of light elements in samples that are tilted in the direction of the incident electron beam (Pouchou and Pichoir 1991). Since the correction factors are dependent on the sample composition, we derived the concentrations using successive iterations, until achieving concentrations that are accurate to approximately 0.01% (Donovan and Tingle 1996). To calculate the statistical error in the concentration, the weight percentage of the sigma value was used to determine whether the element is below the detection limits of the sample analysis (Duncumb 1994). We applied a conservative condition: an element's weight percentage has be greater than three times the weight percentage of the sigma value resulting from the analysis (Polidori et al. 2013).

Table 1 List of the taxa analyzed in this study, together with their classification and the origin of the data. If data are original, the origin of sample is specified: MNCN = collection of the Museo de Ciencias Naturales de Madrid (CSIC), JLN-A = collection of José Luis Nieves- Aldrey, CP = collection of Carlo Polidori. Zn was ranked as 0 = < 0.1 wt%; 1 = 0.1–1.0 wt%; 2 = 1.0–5.0 wt%; 3 = 5.0–10.0 wt%; and 4 = > 10 wt%

Sub-order	Infra-order	Superfamily	Family	Species	Dara origin
Apocrita	Aculeata	Apoidea	Ampulicidae	*Dolichurus corniculus* (Spinola, 1808)	This study, MNCN
			Andrenidae	*Andrena agilissima* (Scopoli, 1770)	This study, CP
				Andrena limata Smith, 1853	This study, MNCN
			Apidae	*Apis mellifera* Linnaeus, 1678	This study, CP
				Xylocopa violacea Linnaeus, 1758	This study, CP
			Colletidae	*Colletes abeillei* Pérez, 1903	This study, MNCN
			"Crabronidae"	*Philanthus triangulum* (Fabricius, 1775)	This study, CP
				Stizus continuus (Klug, 1835)	This study, CP
			Halictidae	*Halictus albomaculatus* Lucas, 1849	This study, MNCN
				Sphecodes monilicornis (Kirby, 1802)	This study, CP
			Megachilidae	*Megachile apicalis* Spinola, 1808	This study, MNCN
				Rhodanthidium sticticum (Fabricius, 1787)	This study, CP
			Melittidae	*Dasypoda argentata* Panzer, 1809	This study, MNCN
				Melitta leporina (Panzer, 1799)	This study, MNCN
			Sphecidae	*Ammophila heydeni* Dahlbom, 1845	This study, MNCN
				Isodontia mexicana (Saussure, 1867)	This study, CP
		Chrysidoidea	Bethylidae	*Epyris niger* Westwood, 1832	Quicke et al. (1998)
				Pristocera depressa (Fabricius, 1805)	This study, MNCN
			Chrysididae	*Omalus* sp.	Quicke et al. (1998)
				Parnopes grandior (Pallas, 1771)	This study, MNCN
			Dryinidae	Dryinidae sp.	This study, JLN-A
		Formicoidea	Formicidae	*Atta* sp.	This study, CP
				Camponotus cruentatus (Latreille, 1802)	This study, MNCN
		Pompiloidea	Mutillidae	*Mutilla europea* Linnaeus, 1758	Quicke et al. (1998)
				Nemka viduata (Pallas, 1773)	This study, CP
			Pompilidae	*Anospilus orbitalis luctigerus* (Costa, 1887)	This study, MNCN
				Arachnospila (Melanospila) brevispinis Wahis, 1992	This study, MNCN
			Sapygidae	*Sapyga quinquepunctata* (Fabricius, 1781)	This study, MNCN
		Scolioidea	Scoliidae	*Scolia (Discolia) hirta hirta* (Schrank, 1781)	This study, MNCN
		Tiphioidea	Tiphiidae	*Meria tripunctata* (Rossi, 1790)	This study, MNCN
		Vespoidea	Vespidae	*Discoelius zonalis* (Panzer, 1801)	This study, CP
				Polistes dominula (Christ, 1791)	This study, CP
	"Terebrantia"	Ceraphronoidea	Ceraphronidae	*Ceraphron* sp.	This study, JLN-A
			Megaspilidae	Megaspilidae sp.	This study, JLN-A
		Chalcidoidea	Agaonidae	Agaonidae sp.	This study, JLN-A
				Elisabethiella stueckenbergi Grandi, 1955	Quicke et al. (1998)
			Aphelinidae	Aphelinidae sp.	This study, JLN-A
			Chalcididae	Chalcididae sp.	This study, JLN-A
			Encyrtidae	*Cerchysius subplanus* (Dalman, 1820)	Quicke et al. (1998)
			Eucharitidae	Eucharitidae sp.	This study, JLN-A
			Eulophidae	Eulophidae sp.	This study, JLN-A
			Eupelmidae	*Eupelmus spongipartus* Förster, 1860	Polidori et al. (2013)
			Eurytomidae	*Eurytoma* sp.	This study, JLN-A
				Sycophila biguttata (Swederus, 1795)	Quicke et al. (1998)
			Leucospidae	*Leucospis* sp.	Quicke et al. (1998)
			Mymaridae	Mymaridae sp.	This study, JLN-A

Table 1 (continued)

Sub-order	Infra-order	Superfamily	Family	Species	Dara origin
			Ormyridae	*Ormyrus nitidulus* (Fabricius, 1804)	Polidori et al. (2013)
				Ormyrus papaveris (Perris, 1840)	This study, JLN-A
			Perilampidae	Perilampidae sp.	This study, JLN-A
			Pteromalidae	*Philocaenus barbarus* Grandi, 1955	Quicke et al. (1998)
				Pteromalus bedeguaris (Thomson, 1878)	Polidori et al. (2013)
			Tetracampidae	Tetracampidae sp.	This study, JLN-A
			Torymidae	*Megastigmus stigmatizans* (Fabricius, 1798)	Polidori et al. (2013)
				Torymus sp.	This study, JLN-A
			Trichogrammatidae	*Trichogramma* sp.	This study, JLN-A
		Cynipoidea	Cynipidae	*Andricus kollari* (Hartig, 1843)	This study, JLN-A
				Ceroptres cerri Mayr, 1872	Polidori et al. (2013)
			Figitidae	*Neralsia* sp.	Polidori et al. (2013)
				Parnips nigripes (Barbotin, 1964)	Polidori et al. (2013)
			Ibaliidae	*Ibalia drewseni* Borries, 1891	Quicke et al. (1998)
				Ibalia rufipes Cresson, 1879	This study, JLN-A
		Diaprioidea	Diapriidae	*Trichopria verticillata* (Latreille, 1805)	Quicke et al. (1998)
			Monomachidae	*Monomachus* sp.	Quicke et al. (1998)
		Evanioidea	Aulacidae	*Pristaulacus* sp.	Quicke et al. (1998)
			Evaniidae	*Evania* sp.	Quicke et al. (1998)
			Gasteruptidae	*Gasteruption jaculator* (Linnaeus, 1758)	Quicke et al. (1998)
		Ichneumonoidea	Braconidae	*Archibracon servillei* Brullé, 1846	Quicke et al. (1998)
				Eubazus sp.	Quicke et al. (1998)
			Ichneumonidae	*Perithous* sp.	Quicke et al. (1998)
				Rhyssa persuasoria (Linnaeus, 1758)	Quicke et al. (1998)
		Megalyroidea	Megalyridae	*Megalyra fasciipennis* Westwood, 1832	Quicke et al. (1998)
		Platygastroidea	Scelionidae	Scelionidae sp.	Quicke et al. (1998)
		Proctotrupoidea	Heloridae	*Helorus* sp.	This study, JLN-A
			Pelecinidae	*Pelecinus polyturator* (Drury, 1773)	Quicke et al. (1998)
			Proctotrupidae	Proctotrupidae sp.	This study, JLN-A
			Roproniidae	*Ropronia* sp.	Quicke et al. (1998)
		Stephanoidea	Stephanidae	Stephanidae sp.	Quicke et al. (1998)
		Trigonaloidea	Trigonalidae	*Poecilogonalos costalis* (Cresson, 1867)	Quicke et al. (1998)
"Symphyta"		Cephoidea	Cephidae	*Calameuta filiformis* (Eversmann, 1847)	Quicke et al. (1998)
		Orussoidea	Orussidae	*Orussus sayii* Westwood, 1835	Quicke et al. (1998)
		Pamphilioidea	Pamphiliidae	*Cephalcia arvensis* Panzer, 1805	Quicke et al. (1998)
		Siricoidea	Siricidae	*Urocerus gigas* Linnaeus, 1758	Quicke et al. (1998)
		Tenthredinoidea	Argidae	Argidae sp.	This study, JLN-A
			Pergidae	*Phylacteophaga* sp.	Quicke et al. (1998)
			Tenthredinidae	Tenthredinidae sp.	This study, JLN-A
		Xiphydrioidea	Xiphydriidae	*Xiphydria* sp.	Quicke et al. (1998)
		Xyeloidea	Xyelidae	Xyelidae sp.	This study, JLN-A

Since the analytical method of metal detection is semi-quantitative, we ranked all the obtained percentage values. Zn was ranked as 0 = < 0.1 wt%; 1 = 0.1–1.0 wt%; 2 = 1.0– 5.0 wt%; 3 = 5.0–10.0 wt%; and 4 => 10 wt% (maximum value detected: 32%), while Mn was ranked as 0 = <0.1 wt% and 1 = 0.1–1.0 wt% (no species with more than 1 wt% of Mn

was found). This ranking procedure is the same used by Quicke et al. (1998). The data retrieved from Polidori et al. (2013) were obtained with exactly the same method as the new sample, given that they were analyzed in the same laboratory, with the same electron microscope and with the same software (see above and Polidori et al. (2013) for further details). Quicke et al. (1998) also used the energy-dispersive X-ray spectroscopy (EDS) analysis, and several observations suggest that the inclusion in the final dataset of some of the species analyzed in that study did not distort the results. First, for a species analyzed by us and by Quicke et al. (1998) (*Ormyrus nitidulus* (Fabricius, 1804)), Zn rank was similar (3 and 4). Second, within families for which one species came from Quicke et al. (1998) and the other came from our sample, the difference between Zn ranks were never larger than 1, which is also the maximum difference found between species that were both analyzed by us within families. Finally, all Vespidae analyzed by us and by Quicke et al. (1998) lacked Zn. The raw data of the EDS spectra for the newly used sample and the sample from Polidori et al. (2013) are available in the Supplementary file EDSdata.xlsx.

The results of Zn and Mn assessments were analyzed by an ANOVA in R (R Core Team 2014) to identify correlations between metal percentage ranks and LFR or LDS (function aov), with the discrete metal ranks treated as continuous variables and the two others as categorial. After that, we determined correlation of each trait with the species phylogeny using the R phytools package for Pagel's λ and Blomberg's K, 1000 simulations each (function phylosig) (Revell 2012). Since all traits were significantly correlated with phylogeny in both metrics, we repeated the above ANOVA with a phylogenetically corrected model using the R phytools package (function phylANOVA). We compared these results with the corresponding phylogenetic ANOVA as implemented in the R Geiger package, which yielded comparable results (data not shown).

Results

Zinc

The presence of Zn in the mandibles can be often predicted through the inspection of SEM pictures, since mandibles with Zn (particularly with ranks 3–4) show the cutting edge and the apex (i.e., the areas where the metal occurs) of a whitish color (Fig. 1a, c); such whitish area was not visible in specimens lacking Zn (Fig. 1e, g). The X-ray energy microanalysis returned spectra in which the presence (Fig. 1b, d) or the absence (Fig. 1f, h) of Zn is clearly visible by the presence or absence of peaks at both 1.2 (Zn_L), 8.8 KeV ($Zn_{K\alpha}$) and 9.8 KeV ($Zn_{K\beta}$). Chlorine (Cl), a halogen generally co-

occurring with Zn in insect mandibles (Jorge et al. 2017), is also well visible in these peaks (Fig. 1b, d).

Overall, Zn enrichment was widespread in hymenopteran mandibles, with 57 out of 87 species having from about 1 to 32 wt% of Zn detected with our method. Most of species possessing Zn fall into ranks 3–4 (42 out of 57). Low values of Zn % (rank 1) were only found in two species within the superfamily Chalcidoidea (Fig. 2). However, we found also a certain variability of Zn incorporation in the mandibles of Hymenoptera across lineages (Fig. 2).

Zn lacked in the three most primitive "Symphyta" superfamilies, while it was variably present in Apocrita (i.e., "Terebrantia" + Aculeata). In fact, while Zn is widespread in many lineages within "Terebrantia" (with only four exceptions), within Aculeata it is rarer, occurring in only 11 species out of 32 (Fig. 2). Although absent from the basal clades of the order, our ancestral state reconstruction was unable to define with high confidence that Zn enrichment in the mandibles did not represent the ancestral state for the Hymenoptera (Fig. 2). Our analysis instead showed that Zn in the mandibles was the ancestral state for Apocrita (node 1 in Fig. 2). Here, this trait was likely lost in Pelecinidae + Proctotrupidae (Proctotrupoidea) (node 2), in Agaonidae (Chalcidoidea) (node 3), and in most Aculeata. In the latter group, Zn enrichment was ancestral and occurs in the all members of the basal superfamily Chrysidoidea (node 4); Zn incorporation was then lost in the remaining clade but then again reacquired in Formicoidea (node 5), in Mutillidae + Sapygidae (Pompiloidea) (node 6), and in Ampulicidae (Apoidea) (node 7) (Fig. 2). Notably, all the other Apoidea (including all bees and apoid predatory wasps) and Vespoidea (including all the remaining predatory wasps) invariably lacked Zn.

Despite these losses and re-acquisition of Zn enrichment within certain clades, common ancestry resulted the factor accounting for most of the variance in the level of Zn, with the key life history traits having minimum or no effects. Concerning larval diet, species with carnivorous larvae were weakly but significantly more prone to have Zn than those with herbivorous larvae (which is the ancestral state in the order) (ANOVA $F = 20.95$, df = 1, before phylogenetic correction: $P < 0.001$, after phylogenetic correction: $P = 0.075$) (Fig. 3). Indeed, median rank value for Zn was 3 for species with carnivorous larvae ($n = 63$) and 0 for species with herbivorous larvae ($n = 24$) (Mann-Whitney $U = 369$, $P < 0.001$). Animal diet at larval stage was the ancestral state for the Apocrita in our reconstruction, being herbivory ancestral for the whole order (Fig. 3). However, species from entirely herbivorous clades (i.e., Cynipidae and several derived "Symphyta" families) had high percentage of Zn (ranks 3–4), similarly to species of closely related and entirely carnivorous clades (i.e., Figitidae and Ibaliidae within Cynipoidea, and Orussidae within "Symphyta," thus showing a large effect of phylogeny on this trait (Pagel's $\lambda = 0.99$, $P < 0.001$;

Fig. 1 SEM pictures of mandibles and representative spectra (on base-10 logarithmic scale) from X-ray energy-dispersive analysis of mandible teeth, generated with a primary electron beam energy of 20 keV. **a, b** *Camponotus cruentatus* (Formicidae); **c, d** *Ibalia rufipes* (Ibaliidae); **e, f** *Isodontia mexicana* (Sphecidae); **g, h** Argidae sp. Note that Zn occurs only in **a** and **b**, and Mn occurs only in **b**. Squares in the SEM pictures indicate the area of the mandibles where the EDS point analysis was done. Peaks for transition metals (Zn and Mn), for Cl (the halogen co-associated with Zn), and for the basic elements of the cuticle (C and O) are highlighted
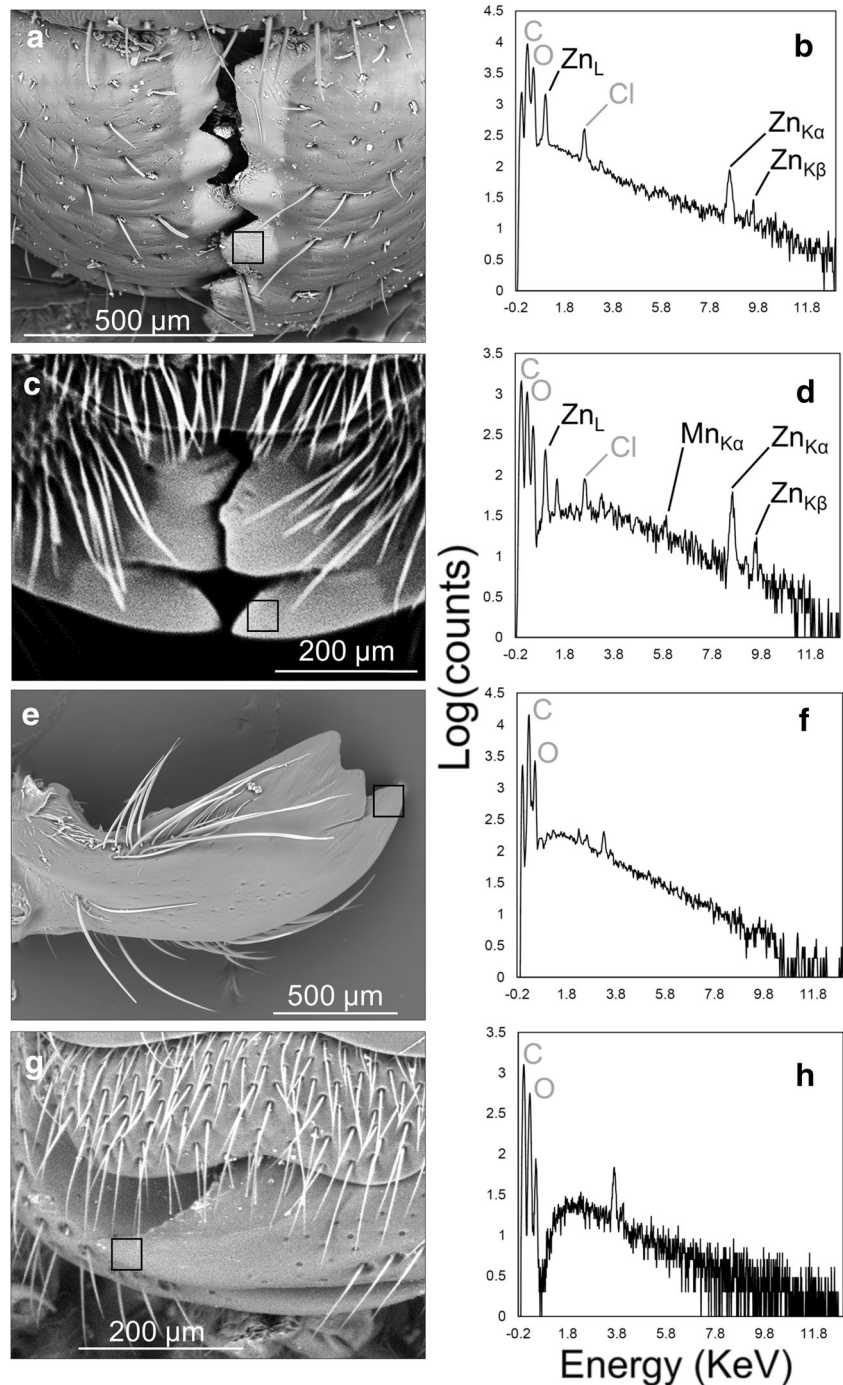
Blomberg's $K = 0.51$, $P < 0.001$). On the other hand, Zn enrichment did not depend on the type (unconcealed or concealed) of larval development site (ANOVA $F = 0.18$, df = 1, before $P = 0.67$ and after phylogenetic correction: $P = 0.85$) (Fig. 4). Indeed, median rank value for Zn was 2.5 for species with concealed larvae ($n = 64$) and 2 for species with unconcealed larvae ($n = 23$). Thus, for example, soil-nesting Apoidea (Aculeata) had no Zn, while parasitoid (i.e., non-nesting) Aculeata attacking soil-nesting Apoidea (and thus emerging from adults from the same substrate) often have

Zn (e.g., Chrysididae, Mutillidae). Another example is found within parasitoid Chalcidoidea, where both species attacking unconcealed (e.g., Trichogrammatidae, Mymaridae) and species attacking concealed (e.g., Torymidae, Eulophidae) hosts have medium-to-high percentage of Zn in their mandibles (ranks 2–4). Thus, again, phylogeny accounted for most of variance in Zn enrichment. Although the most primitive "Symphyta" just develop exposed on plant leaves, it was not clear from our analysis if developing on a concealed resource is ancestral or derived in the order (proportional likelihoods:

Fig. 2 Phylogenetic tree mapping the ranked Zn % for each taxon analyzed in this study. Zn was ranked as 0 = < 0.1 wt%; 1 = 0.1–1.0 wt%; 2 = 1.0–5.0 wt%; 3 = 5.0–10.0 wt%; and 4 = > 10 wt%. Node 1 (in violet) identifies Zn enrichment as ancestral state for Apocrita (proportional likelihoods for Zn %: 0 = 0.0015, 1 = 0.0015, 2 = 0.121, 3 = 0.9785, 4 = 0.0062). Nodes 2 (Proctotrupidae + Pelecinidae) and 3 (Agaonidae) (in pink) identify losses of Zn enrichment in non-Aculeata (proportional likelihoods for Zn %: node 2: 0 = 0.9390, 1 = 0.0036, 2 = 0.0483, 3 = 0.0052, 4 = 0.0037; node 3: 0 = 0.9454, 1 = 0.0038, 2 = 0.0389, 3 = 0.0050, 4 = 0.0068). Nodes 4 (Chrysidoidea), 5 (Formicidae), 6 (Mutillidae + Sapygidae), and 7 (Ampulicidae) (in violet) identify re-acquisitions of Zn enrichment within Aculeata (proportional likelihoods for Zn %: node 4: 0 = 0.0597, 1 = 0.0112, 2 = 0.3890, 3 = 0.5156, 4 = 0.0244; node 5: 0 = 0.2138, 1 = 0.0173, 2 = 0.0174, 3 = 0.2796, 4 = 0.4717; node 6: 0 = 0.3439, 1 = 0.0233, 2 = 0.1286, 3 = 0.1291, 4 = 0.3748; node 7: 4 = 1)
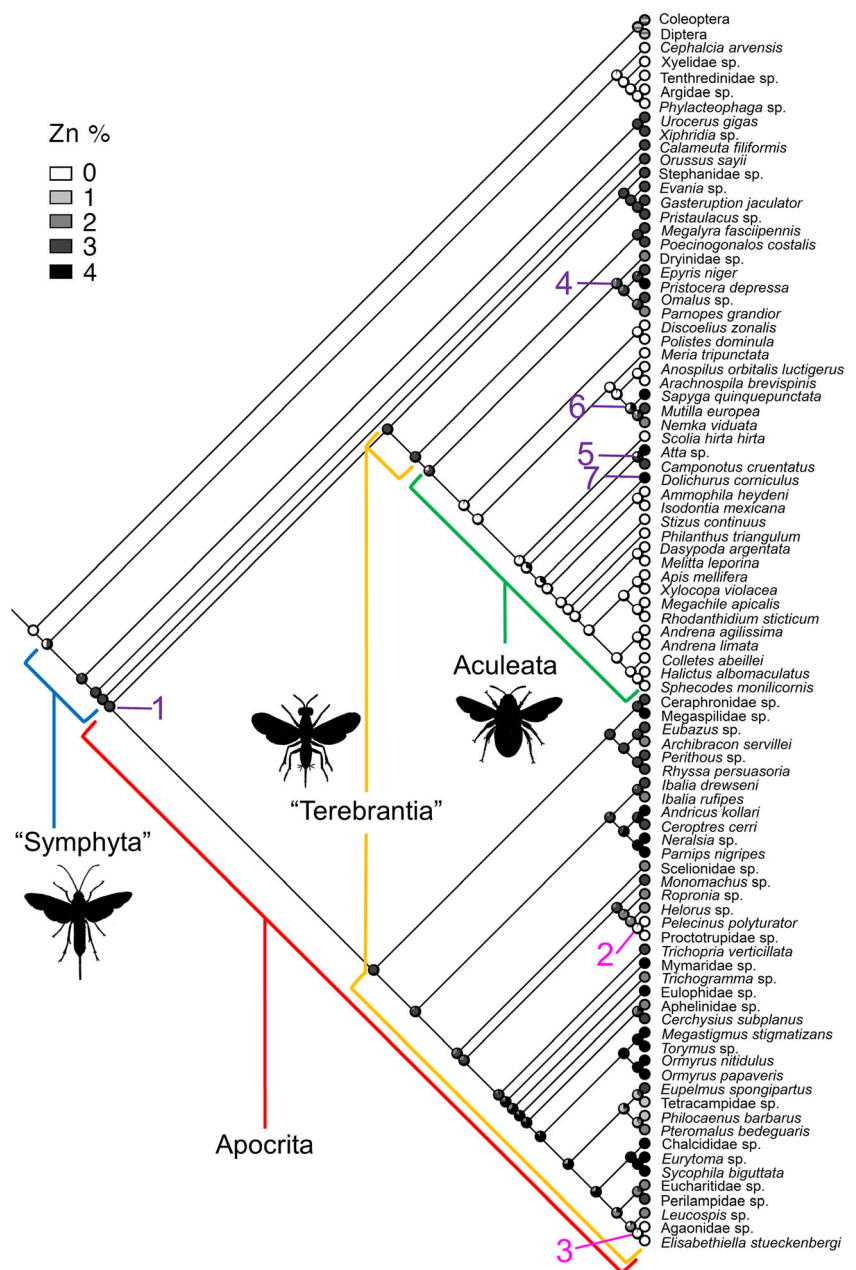
0 = 0.5026, 1 = 0.4974), while it was clearly the ancestral state for the Apocrita (proportional likelihoods: 0 = 0.0165, 1 = 0.9835) (Fig. 4).

Manganese

Mn was recorded in 10 species spanning three distantly related superfamilies (Chalcidoidea, Cynipoidea, and Formicoidea) (Fig. 5). In the spectra obtained from the X-ray energy micro-analysis, the presence of Mn was visible by a peak at 5.9 KeV ($Mn_{K\alpha}$) (Fig. 1d), which lacks in species with no Mn (Fig. 1b, f–h). Because no species with Mn were found in "Symphyta", Mn enrichment in mandibles clearly resulted as a derived state

for the order, and it evolved independently three times in the three superfamilies listed above (Fig. 5). Species with Mn-enriched mandibles spanned both herbivorous (e.g., Agaonidae) and carnivorous (e.g., Figitidae) larval diets, as well as both concealed (e.g., gall-parasitoids, ants) and unconcealed (e.g., parasitoids of exposed Diptera larvae) development sites (Figs. 3 and 4). No associations were found between Mn % and life history traits (LFR: ANOVA $F = 0.31$, df = 1, before $P = 0.58$ and after phylogenetic correction $P = 0.84$; LDS: ANOVA $F = 1.56$, df = 1, before $P = 0.22$ and after phylogenetic correction $P = 0.56$) and yet with phylogeny (Pagel's $\lambda = 0.99$, $P < 0.001$; Blomberg's $K = 0.40$, $P < 0.01$).

Fig. 3 Phylogenetic tree mapping the larval feeding resource (LFR) (0 = plant, 1 = animal (at least partially)) for each taxon analyzed in this study. The circle with a picture of a herbivorous larva (the bee *Andrena agilissima* (Andrenidae), feeding on a pollen ball) and of a carnivorous larva (the apoid wasp *Sceliphron spirifex* (Sphecidae), feeding on spiders) mark the ancestral state for Hymenoptera and for Apocrita, respectively. The histogram in the lower part of the figure shows the distribution of cases for a given rank of Zn, for species with either LFR type
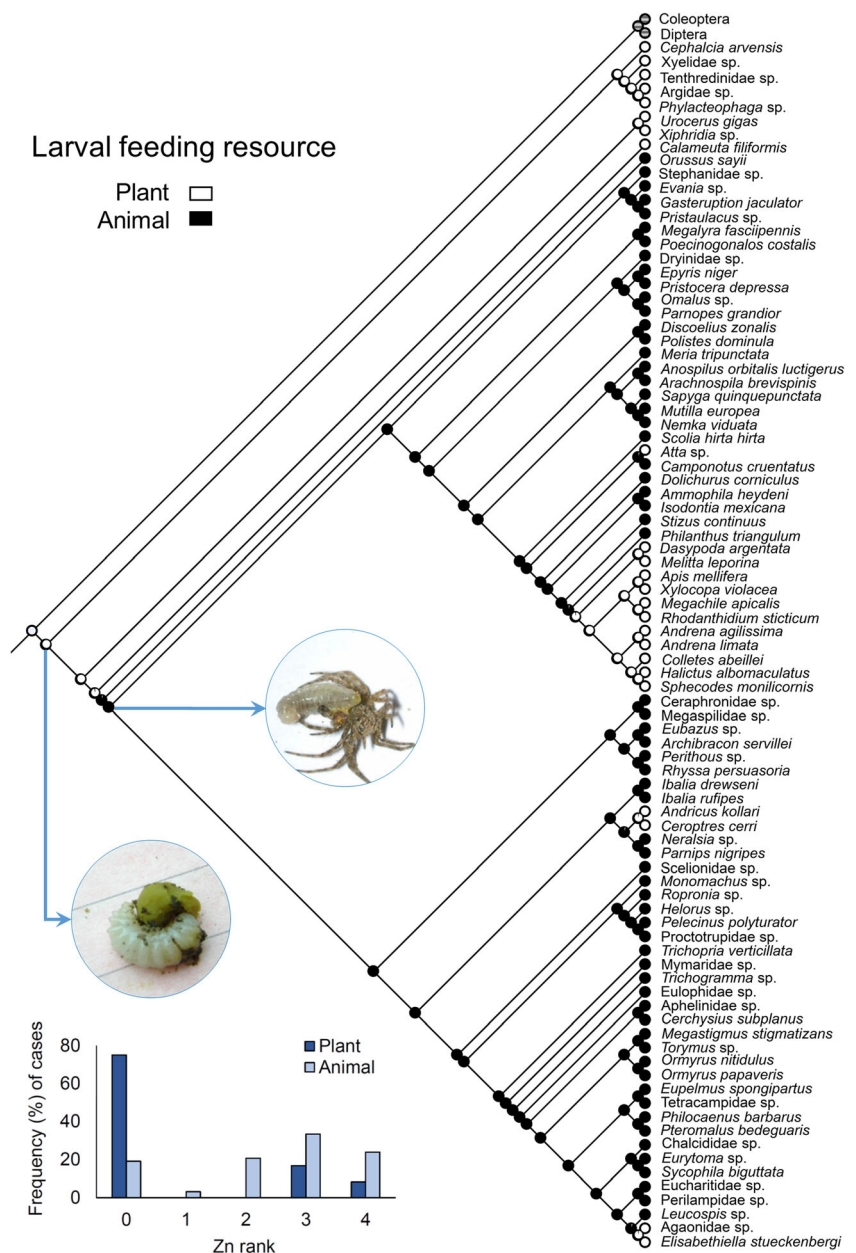

Discussion

It was previously shown that mandibles of Hymenoptera are often enriched with transition metals, with most of the studied superfamilies including most, if not all, species with Zn (and much more rarely Mn) in their mandible teeth (Quicke et al. 1998; Polidori et al. 2013; Jorge et al. 2017). However, few superfamilies do not have this trait in any or most of their members. In this study, we hypothesized that this variability may reflect either the larval diet, and thus amount of metals that can be uptaken by the insects during the development, or a functional response to ecological pressure related with mandible use, i.e., the necessity to cut or break hard emerging substrate after adult development.

Zinc

Until the present study, across Hymenoptera families, patterns of Zn enrichment remained unclear because many lineages were not studied by Quicke et al. (1998) and because a phylogeny-based quantitative analysis was not performed in that study. Quicke et al. (1998) showed that the cutting edge of the mandibles of many hymenopterans that chew hard substrates during emergence almost invariably contain high concentrations of Zn, and the occurrence itself of Zn was extremely widespread in the sample analyzed by these authors. In fact, they found Zn to lack only in two aculeate wasps, two "Symphyta", and only one member of "Terebrantia" (Proctotrupoidea). By looking at the superfamily-level

Fig. 4 Phylogenetic tree mapping the larval development site (LDS) (0 = unconcealed, 1 = concealed) for each taxon analyzed in this study. The circle with a picture of a species developing in a concealed site (the apoid wasp *Stizus continuus* (Crabronidae), emerging from its nest) marks the ancestral state for Apocrita, while the circle with a question mark indicate the unclear ancestral state for Hymenoptera. The histogram in the lower part of the figure show the distribution of cases for a given rank of Zn, for species with either LDS type
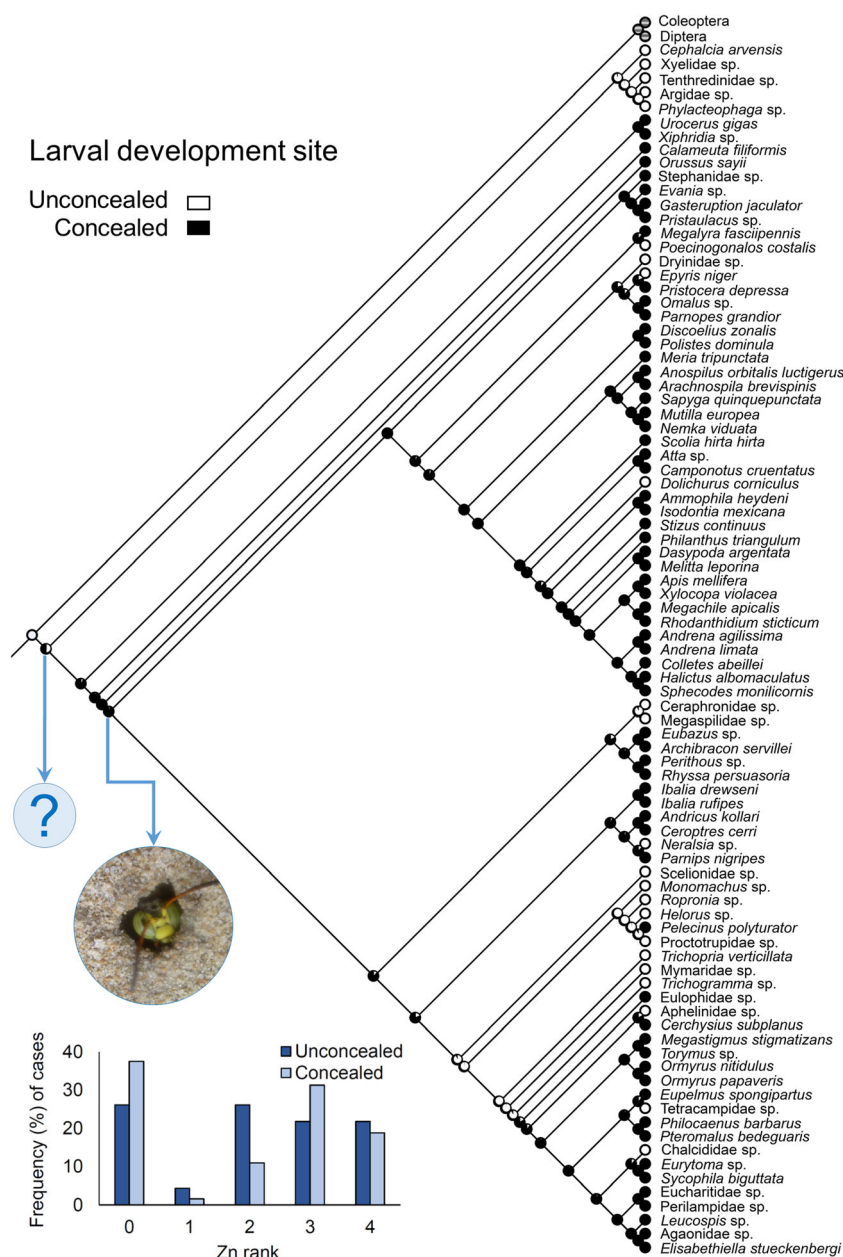

phylogeny of Hymenoptera known at the time of their study, Quicke et al. (1998) thus suggested that Zn enrichment appeared once in "Symphyta", when the so-called Unicalcarida (Peters et al. 2017) separated from the rest of the group and then was conserved, in Apocrita, across all groups with only two exceptions: Pelecinidae (Proctotrupoidea) and Vespidae (Vespoidea) (both considered reversals). We confirm that most Hymenoptera have Zn in their mandibles, though our enlarged sample spectrum showed that this trait is less widespread than suggested by previous studies on these insects. Quicke et al. (1998) suggested that the use of metal for cuticular hardening in mandibles is not ancestral to the order. Our ancestral state reconstruction was however not conclusive to confirm this possibility.

Zn enrichment and larval development site

Our adaptive hypothesis that species which have to dig or cut hard substrates during emergence are more prone to have metal-enriched mandibles finds its base on previous studies about variation in hymenopteran mandible morphology. For example, some parasitic wasps developing in wood have often very robust mandibles with chisel-like cutting teeth (Quicke 1997). Camargo et al. (2015) found morphological differences among mandibles of grass-cutting ant species (which have generally shorter and massive mandibles with two teeth) and leaf-cutting ant species (which have generally larger and more elongated mandibles with numerous teeth). In some social wasps (Vespidae: Epiponinae), strong torsion of mandibles

Fig. 5 Phylogenetic tree mapping the ranked Mn % for each taxon analyzed in this study. Mn was ranked as 0 = <0.1 wt%; 1 = 0.1–1.0 wt%

Mn %

□ 0
■ 1
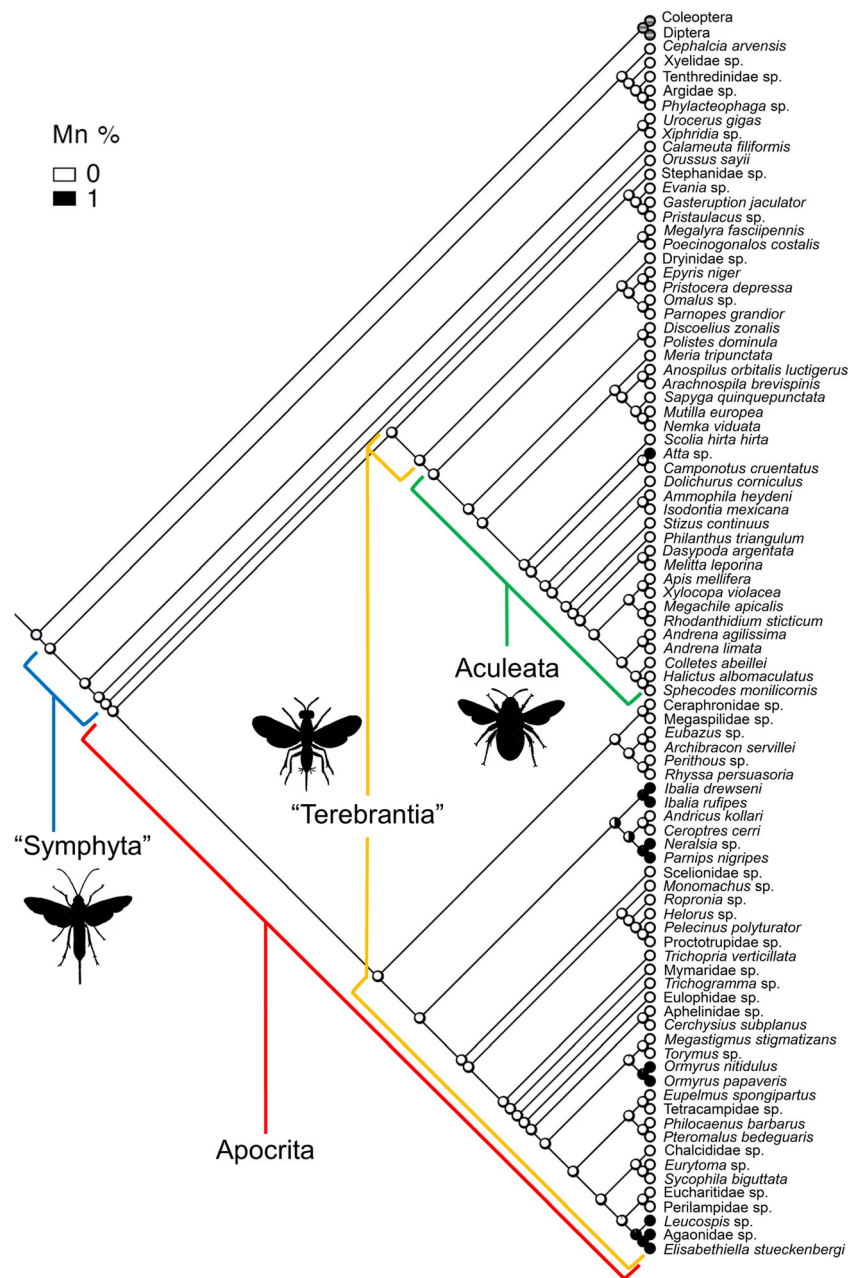
may be partially associated with the use of wood fibers for nest construction (Silveira and Santos 2011). Across *Osmia* bees (Apoidea: Megachilidae), Williams and Goodell (2000) found greater similarity among bees that use leaf material for nest construction than among the species which use mud for nest construction. Within apoid wasps (Apoidea: Sphecidae and "Crabronidae"), ground-nesting species have more robust and enlarged mandibles than species that colonize already excavated tunnels (Bohart and Menke 1976).

Furthermore, the hypothesis of ecology-driven metal incorporation in hymenopteran mandibles can also find its base on what observed in other insect lineages. For example, the presence of metals in mandibles of some herbivorous insects

reduces the abrasive wear that mandibles suffer through chewing hard, often silica-rich, plant materials (Hillerton and Vincent 1982; Hillerton et al. 1984; Edwards et al. 1993), while the ability to moisten wood predicts the presence of Zn in termite lineages (Cribb et al. 2008b). The adaptive role of Zn in mandibles is also suggested by the fact that metal enrichment occurred in localized areas, typically on the cutting edges or teeth of the mandibles (e.g., Polidori et al. 2013; Jorge et al. 2017). Despite these observations, however, a link between emerging substrate and Zn incorporation was not found in our study.

Despite Zn lacks in the three more basal groups of "Symphyta" and it appears first in the Siricoidea, i.e., in

correspondence of a transition to an endoxylous larval habit (thus somehow supporting the adaptive hypothesis, Quicke et al. (1998)), the occurrence of Zn is then invariably conserved across many lineages which include species with both concealed and unconcealed larvae. Our analysis failed to unambiguously determine if developing in an unconcealed site is ancestral in the order. Peters et al. (2017) stated that the inferred relationship between the most primitive "Symphyta" and Unicalcarida implies that the most recent common ancestor of Hymenoptera could have been ecto- or endo-phytophagous. Accordingly, it was not possible in our analysis to ascertain if Zn enrichment is ancestral or derived in the order. Adding new sample and new phylogenetic analysis may elucidate this point.

Interestingly, Zn seemed to be lost a first time in the family Agaonidae (fig wasps), the only family in Chalcidoidea lacking Zn in the mandibles. It is difficult to hypothesize why fig wasps do not have Zn; adults emerge from fig fruits, so from a relatively soft substrate (Cruaud et al. 2012), but there are many other groups of Chalcidoidea emerging from apparently soft tissues, such as unconcealed host pupae or larvae (Heraty et al. 2013), which have Zn in their mandibles. Zn seems also to be lost twice in Proctotrupoidea (in Pelecinidae and in Proctotrupidae). This is also difficult to explain, particularly for Pelecinidae, which are parasitoids of beetle larvae developing into soils (Johnson and Musetti 1999). A third loss of Zn occurred in the more derived Aculeata, with all soil-nesting bees and wasps lacking Zn, in clear contradiction with our hypothesis. Perhaps, soil is a substrate that does not require metal enrichment, and larger and more robust mandibles may be a sufficient adaptation to soil digging (Bohart and Menke 1976). A further evidence of no role of emerging substrate is clear when looking at the basal Aculeata (Chrysidoidea), which possess Zn despite including many species attacking nesting bees and wasps, and thus developing in the same sites (Kimsey and Bohart 1991).

Furthermore, Zn enrichment have been found in the mandibles of species of Formicidae which range widely in both nesting behavior and habitat (Hillerton and Vincent 1982; Edwards et al. 1993; Schofield 2001; Zeng 2016; Obando Brito et al. 2017; Khalife et al. 2018). Additionally, Polidori et al. (2013) previously found Zn in the mandibles of all species of Cynipidae and all species of Figitidae, despite the important variation in emerging site, which ranges from hard galls for gall-wasps and their inquiline wasps (Cynipidae) to simply the host body in case of parasitoids attacking unconcealed hosts (many Figitidae) (Nieves-Aldrey 2001; Ronquist 1999; Ronquist et al. 2015).

Within the derived Aculeata, interestingly, Zn was reacquired in Mutillidae, Sapygidae, Formicidae, and Ampulicidae, the latter (basal to all the other Apoidea) being the only apoid family to have this trait. Again, this pattern cannot be related with a change in developing substrate, since species from some of these lineages (Mutillidae, Sapygidae) develop into the nests of their bee and wasp hosts (Grandi 1962; Brothers et al. 2000), which lack Zn. Leaf-cutting behavior in some ants (*Atta*) was hypothesized to be associated with Zn enrichment (Schofield et al. 2002). However, also, ants which do not cut leaves (*Camponotus*) have Zn, while leaf-cutting bees (*Megachile*) and paper wasps (*Polistes*) (which also cut leaves to build the nests) do not (Hölldobler and Wilson 1990; Michener 2007; Turillazzi and West-Eberhard 1996).

Zn enrichment and larval food resource

Food is the major source of zinc intake, since drinking water usually makes a negligible contribution to total intake (Longnecker and Robson 1993). A major intake of Zn from animal tissues could thus at least partially explain the found positive, though weak, association between larval diet and Zn %. Indeed, by inspecting the literature, it appears that animal tissues seem to have, on average, higher Zn % than plant tissues (about 150 mg/kg vs. about 40 mg/kg, mean values calculated across species and across studies (Mwangi et al. 2018; Esenin and Ma 2000; Longnecker and Robson 1993; Konieczyński and Wesołowski 2007; Ansari et al. 2004; Irmak et al. 2008; Kabata-Pendias 2010). This would contrast with what proposed by Hillerton and Vincent (1982), who suggested that herbivory explains why Mn and Zn more likely occur in the mandibles of herbivorous insects.

However, despite animal larval diet could potentially increase Zn incorporation, it seems that phylogeny has still a major role in the observed variance. Further observations are in fact against an important role of diet on Zn level variation among herbivorous species. For example, bee-collected pollen have similar amount of Zn (about 40 mg/kg vs. about 44 mg/kg, mean values calculated across species and across studies) (Stanciu et al. 2011; Somerville and Nicol 2002; Kostić et al. 2015; Yang et al. 2013) than those recorded for plant leaves (see above), thus not explaining why bees have no Zn while other herbivorous have Zn in the mandibles. Additionally, the high levels of Zn found in gall-wasps did not seem to depend on higher percentage of Zn in gall tissues relative to normal tissues of the host plants, as apparently insect galls possess less Zn than normal tissues in the attacked plants (data available for Hymenoptera, Diptera, and Coleoptera) (Brewer et al. 1987; Bagatto and Shorthouse 1994; Bagatto et al. 1991; Florentine et al. 2005).

In addition, food-stealing (kleptoparasitic) chrysid wasps and their wasp hosts feed on the same animal resources at the larval stage, and only the former have Zn, while all Formicidae studied to date possess Zn in their mandibles, despite some have herbivorous larvae and some have carnivorous larvae (Hillerton and Vincent 1982; Edwards et al. 1993; Shoefield 2001; Zeng 2016; Obando Brito et al. 2017;

Khalife et al. 2018). The same occurs within Cynipoidea: all species of Cynipidae and all species of Figitidae studied in Polidori et al. (2013) have Zn in the mandibles, despite the former have herbivorous larvae and the latter have carnivorous larvae (Nieves-Aldrey 2001; Ronquist et al. 2015).

These observations may agree with the hypothesis that genetic and cellular regulation, rather than environmental Zn availability, is at the base of Zn incorporation (Degtyar et al. 2014). Given our results, such genetic and cellular regulation may be constrained by phylogeny.

Manganese

In the present study, Mn was rarely detected in hymenopteran mandibles, at low percentage and, except in fig wasps (Agaonidae), always co-occurring with Zn. These low values are not a product of the much higher Zn % in these organs, which hypothetically may hide Mn peaks in the spectral background noise. Indeed, Mn stays at similarly lower values even when it is present as the only metal in mandibles (Agaonidae) and in the ovipositor (Quicke et al. 1998; Polidori et al. 2013; Jorge et al. 2017). Mn was recorded in only 10 species spanning three distantly related superfamilies and including both herbivorous and carnivorous species at the larval stage, as well as species emerging both from concealed and unconcealed development sites. Thus, at this broad, order-level analysis, this trait seemed to have a strong phylogenetic component, since no association appeared with life history traits. However, previous studies on other insects showed that diet might directly affect Mn content in mandibles. For example, when the beetle *Rhyzopertha dominica* (Fabricius, 1792) is reared on wheat germ, it generally has no Mn in the mandibles, but if 300-ppm manganese is added to the diet, deposition of Mn in both their larval and adult mandibles increases (Morgan et al. 2003). Further studies should investigate experimentally how diet affects Mn content in hymenopteran mandibles.

Quicke et al. (1998) also found Mn to be very rare in mandibles (only 4 species), and our enlarged sample did not increase the number of lineages with Mn-enriched mandibles. Within families, previous studies showed that Mn did not occur universally, and, again, no apparent association with life history traits was found at this scale (Hillerton and Vincent 1982; Polidori et al. 2013). On the other hand, phylogeny seems to be responsible for the likelihood to have or lack Mn. In Hymenoptera, Mn seems to be more common in ovipositors (Quicke et al. 1998; Polidori et al. 2013). Cuticle incorporating Zn or Mn are thought to differ in their mechanical properties (Schofield and Lefevre 1989), so that the alternative occurrence of these two metals in different body parts (mandibles vs. ovipositors) or even in different parts of the same organ (e.g., upper valve vs. lower valve) may mirror different functional roles. For example, Agaonidae have Mn

in their mandibles. This may suggest that digging through fig fruit requires special mechanical properties that this metal, rather than Zn, provides to the mandibles. *Atta* ants also have Mn in their mandibles perhaps because cutting leaves requires such metal enrichment. However, other leaf-cutting species, such as *Megachile apicalis* Spinola, 1808, do not have Mn. There could be also a competition between the absorption of Zn and Mn that may affect their relative proportions. For example, Morgan et al. (2003) show that the majority of stored-product feeding insects deposited Mn in their mandibles when their diet was supplemented with Zn, suggesting that the absorption of one metal may influence the absorption of the other one. Hillerton and Vincent (1982) found that certain orders of insects, such as Orthoptera and Lepidoptera, have Zn-enriched mandibles, while Coleoptera has Mn-enriched mandibles, and ants have both metals. Interestingly, all of the species analyzed in that work are herbivorous, suggesting that the type of diet of each species should be inspected in deep detail to better understand its relationship with metal incorporation.

Additionally, the link between metal enrichment and cuticle hardness seems much clearer for Zn than for Mn in other insect lineages (Cribb et al. 2008a). It is clear that studies on Mn enrichment in hymenopteran mandibles require deeper investigations with larger within-families or even within-genera species sample and by taking into account detailed data on the behavioral ecology of the species.

Conclusions

Our results suggest that a phylogenetic constraint (sensu Blomberg and Garland 2002), i.e., the alternative hypothesis to adaptation by natural selection when attempting to explain interspecific variation, largely explains the occurrence and distribution of Zn and Mn enrichment in the mandibles of Hymenoptera. However, we identified a few cases of reversal from Zn presence to absence and vice-versa, which may suggest that further life history traits, here not investigated, may actually have also played a role in shaping mandible structure of Hymenoptera. While it is possible that habitats or diets with very different metal availability affect their incorporation in mandibles, at the broad level of our comparative analysis, phylogenetic constrains may still play a major role in its distribution across hymenopteran lineages. We argue that a limited effect of habitat or diet may be more visible within species, across individuals sampled in different environments or by experiments with modified diets. Furthermore, new studies on the different biomechanical properties achieved through the incorporation of Zn and Mn may shed light on the variable use of these metals across wasps and ants.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Aguiar, A. P., Deans, A. R., Engel, M. S., Forshage, M., Huber, J. T., Jennings J. T., Johnson, N. F., Lelej, A. S., Longino J. T., Lohrmann, V., Mikó, I., Ohl, M., Rasmussen, C., Taeger, A., & Yu, D. S. K. (2013). Order Hymenoptera. In: Z. Q. Zhang, (Ed.) Animal biodiversity: an outline of higher-level classification and survey of taxonomic richness (addenda 2013). Zootaxa, 3703 (1), 51–62.

Ansari, T. M., Ikram, N., Najam-ul-Haq, M., Fayyaz, I., Fayyaz, Q., Ghafoor, I., & Khalid, N. (2004). Essential trace metal (zinc, manganese, copper and iron) levels in plants of medicinal importance. Journal of Biological Sciences, 4, 95–99.

Austin, A. D., & Dowton, M. (Eds.). (2000). The Hymenoptera: evolution, biodiversity & biological control. Melbourne: CSIRO Publishing.

Bagatto, G., & Shorthouse, J. D. (1994). Seasonal acquisition of mineral nutrients by a chalcid gall on lowbush blueberry. Entomologia Experimentalis et Applicata, 73, 61–66.

Bagatto, G., Zmijowskyj, T. J., & Shorthouse, J. D. (1991). Galls induced by Diplolepis spinosa influence distribution of mineral nutrients in the shrub rose. HortScience, 26, 1283–1284.

Baumann, K., Vicenzi, E. P., Lam, T., Douglas, J., Arbuckle, K., Cribb, B., & Brady, S. G. (2018). Harden up: metal acquisition in the weaponized ovipositors of aculeate hymenoptera. Zoomorphology, 137, 389–406.

Blomberg, S. P., & Garland, T. (2002). Tempo and mode in evolution: phylogenetic inertia, adaptation and comparative methods. Journal of Evolutionary Biology, 15, 899–910.

Bohart, R., & Menke, A. (1976). Sphecid wasps of the world: a generic revision. Berkeley: University of California Press.

Brewer, J. W., Bishop, J. N., & Skuhravý, V. (1987). Levels of foliar chemicals in insect-induced galls (Dipt., Cecidomyiidae). Journal of Applied Entomology, 104, 504–510.

Broomell, C. C., Mattoni, M. A., Zok, F. W., & Waite, J. H. (2006). Critical role of zinc in hardening of Nereis jaws. Journal of Experimental Biology, 209, 3219–3225.

Brothers, D. J., Tschuch, G., & Burger, F. (2000). Associations of mutillid wasps (Hymenoptera, Mutillidae) with eusocial insects. Insectes Sociaux, 47, 201–211.

Camargo, R. S., Hastenreiter, I. N., Brugger, M., Forti, L. C., & Lopes, J. F. S. (2015). Relationship between mandible morphology and leaf preference in leaf-cutting ants (Hymenoptera: Formicidae). Revista Colombiana de Entomología, 41, 241–244.

Cribb, B. W., Stewart, A., Huang, H., Truss, R., Noller, B., Rasch, R., & Zalucki, M. P. (2008a). Insect mandibles-comparative mechanical properties and links with metal incorporation. Naturwissenschaften, 95, 17–23.

Cribb, B. W., Stewart, A., Huang, H., Truss, R., Noller, B., Rasch, R., & Zalucki, M. P. (2008b). Unique zinc mass in mandibles separates drywood termites from other groups of termites. Naturwissenschaften, 95, 433–441.

Cruaud, A., Cook, J. M., Yang, D., Genson, G., Jabbour-Zahab, R., Kjellberg, F., Pereira, R. A. S., Rnsted, N., Santos-Mattos, O., Savolainen, V., Ubaidillah, R., Van Noort, S., Yan-Qiong, P., & Raplus, J. Y. (2012). Fig-fig wasp mutualism: the fall of the strict cospeciation paradigm? In S. Patiny (Ed.), Evolution of plant-pollinator interactions (pp. 68–102). Cambridge: Cambridge University Press.

Degtyar, E., Harrington, M. J., Politi, Y., & Fratzl, P. (2014). The mechanical role of metal ions in biogenic protein-based materials. Angewandte Chemie, 53, 12026–12044.

Donovan, J. J., & Tingle, T. N. (1996). An improved mean atomic number background correction for quantitative microanalysis. Microscopy and Microanalysis, 2, 1–7.

Duncumb, P. (1994). Correction procedures in electron probe microanalysis of bulk samples. Microchimica Acta, 114, 3–20.

Edwards, A. J., Fawke, J. D., McClements, J. G., Smith, S. A., & Wyeth, P. (1993). Correlation of zinc distribution and enhanced hardness in the mandibular cuticle of the leaf-cutting ant Atta sexdens rubropilosa. Cell Biology International, 17, 697–698.

Esenin, A., & Ma, W. (2000). Heavy metals (Cd, Cu, Zn) in wood and wood-feeding insects and other invertebrates associated with decaying pine trees. Bulletin of Environmental Contamination and Toxicology, 64, 242–249.

Fawke, J. D., McClements, J. G., & Wyeth, P. (1997). Cuticular metals: quantification and mapping by complementary techniques. Cell Biology International, 21, 675–678.

Florentine, S. K., Raman, A., & Dhileepan, K. (2005). Effects of gall induction by Epiblema strenuana on gas exchange, nutrients, and energetics in Parthenium hysterophorus. BioControl, 50, 787–801.

Fontaine, A. R., Olsen, N., Ring, R. A., & Singla, C. L. (1991). Cuticular metal hardening of mouthparts and claws of some forest insects of British Columbia. Journal of the Entomological Society of British Columbia, 88, 45–55.

Grandi, G. (1962). Contributi alla conoscenza degli imenotteri Aculeati XXXI. Bollettino dell'Isituto di Entomolgia della Università di Bologna, 26, 55–102.

Heraty, J. M., Burks, R. A., Cruaud, A., Gibson, G. A. P., Liljeblad, J., Munro, J., Rasplus, J. Y., Delvare, G., Jansta, P., Gumovsky, A., Huber, J., Woolley, J. B., Krogmann, L., Heydon, S., Polaszek, A., Schmidt, S., Darling, D. C., Gates, M. W., Mottern, J., Murray, E., DalMolin, A., Triapitsyn, S., Baur, H., Pinto, J. D., van Noort, S., George, J., & Yoder, M. (2013). A phylogenetic analysis of the megadiverse Chalcidoidea (Hymenoptera). Cladistics, 29, 466–542.

Hillerton, J. E., & Vincent, J. F. V. (1982). The specific location of zinc in insect mandibles. Journal of Experimental Biology, 101, 333–336.

Hillerton, J. E., Robertson, B., & Vincent, J. F. V. (1984). The presence of zinc or manganese as the predominant metal in the mandibles of adult stored-product beetles. Journal of Stored Products Research, 20, 133–137.

Hölldobler, B., & Wilson, E. O. (1990). The ants. Cambridge: Harvard University Press.

Irmak, S., Surucu, A. K., & Aydin, S. (2008). Zinc contents of soils and plants in the Çukurova Region of Turkey. Asian Journal of Chemistry, 20, 3525–3536.

Johnson, N. F., & Musetti, L. (1999). Revision of the proctotrupoid genus Pelecinus Latreille (Hymenoptera: Pelecinidae). *Journal of Natural History, 33*, 1513–1543.

Jorge, A., Polidori, C., Garcia-Guinea, J., & Nieves-Aldrey, J. L. (2017). Spectral cathodoluminescence analysis of hymenopteran mandibles with different levels of zinc enrichment in their teeth. *Arthropod Structure & Development, 46*, 39–48.

Kabata-Pendias, A. (2010). *Trace elements in soils and plants* (4th ed.). Boca Raton: CRC Press/Taylor & Francis Group.

Khalife, A., Keller, R. A., Billen, J., Hita Garcia, F., Economo, E. P., & Peeters, C. (2018). Skeletomuscular adaptations of head and legs of *Melissotarsus* ants for tunnelling through living wood. *Frontiers in Zoology, 15*, 30. https://doi.org/10.1186/s12983-018-0277-6.

Kimsey, L. S., & Bohart, R. M. (1991). *The Chrysidid wasps of the world*. New York: Oxford University Press.

Konieczyński, P., & Wesołowski, M. (2007). Determination of zinc, iron, nitrogen and phosphorus in several botanical species of medicinal plants. *Polish Journal of Environmental Studies, 16*, 785–790.

Kostić, A. Ž., Pešić, M. B., Mosić, M. D., Dojčinović, B. P., Natić, M. M., & Trifković, J. Đ. (2015). Mineral content of bee pollen from Serbia. *Archives of Industrial Hygiene and Toxicology, 66*, 251–258.

Kozlov, M. (1970). Supergeneric groups of the Proctotrupoidea (Hymenoptera). *Entomological Review, 49*, 115–127.

Kundanati, L., & Gundiah, N. (2014). Biomechanics of substrate boring by fig wasps. *Journal of Experimental Biology, 217*, 1946–1954.

Longnecker, N. E., & Robson, A. D. (1993). Distribution and transport of zinc in plants. In A. D. Robson (Ed.), *Zinc in soils and plants*, *Developments in Plant and Soil Sciences* (Vol. 55, pp. 79–91). Dordrecht: Springer.

Maddison, W.P., & Maddison, D. R. (2018). Mesquite: a modular system for evolutionary analysis. Version 3.51. http://www.mesquiteproject.org.

Michener, C. D. (2007). *The bees of the world* (2nd ed.). Baltimore: John Hopkins University Press.

Morgan, T. D., Baker, P., Kramer, K. J., Basibuyuk, H. H., & Quicke, D. L. J. (2003). Metals in mandibles of stored products insects: do zinc and manganese enhance the ability of larvae to infest seeds? *Journal of Stored Products Research, 39*, 65–75.

Munro, J. B., Heraty, J. M., Burks, R. A., Hawks, D., Mottern, J., Cruaud, A., Rasplus, J.-Y., & Jansta, P. (2011). A molecular phylogeny of the Chalcidoidea (Hymenoptera). *PLoS One, 6*(11), e27023. https://doi.org/10.1371/journal.pone.0027023.

Mwangi, M. N., Dennis, G. A. B. O., Stouten, T., Veenenbos, M., Melse-Boonstra, A., Dicke, M., & van Loon, J. J. A. (2018). Insects as sources of iron and zinc in human nutrition. *Nutrition Research Reviews, 31*, 248–255.

Nieves-Aldrey, J. L. (2001). Hymenoptera, Cynipidae. In M. A. Ramos, J. Alba-Tercedor, X. Bellés-i-Ros, J. Gosálbez-i-Noguera, A. Guerra-Sierra, E. MacPherson-Mayol, F. Martín-Piera, J. Serrano-Marino, & J. Templado-González (Eds.), *Fauna Ibérica* (Vol. 16, pp. 1–636). Madrid: Museo Nacional de Ciencias Naturales, CSIC.

Nieves-Aldrey, J. L., & Sharkey, M. J. (2014). Hymenoptera: ants, bees, wasps and the majority of insect parasitoids. In P. Vargas & R. Zardoya (Eds.), *The tree of life* (pp. 395–408). Sunderland: Sinauer.

Obando Brito, T., Elzubaira, A., Sales Araújo, L., de Souza Camargo, S. A., Pereira Souza, J. L., & Almeida, L. H. (2017). Characterization of the mandible *Atta laevigata* and the bioinspiration for the development of a biomimetic surgical clamp. *Materials Research, 20*, 1525–1533.

Packwood, R. (1991). A comprehensive theory of electron probe micro-analysis. In K. Heinrich & D. Newbury (Eds.), *Electron probe quantitation* (pp. 83–103). New York: Plenum Press.

Peters, R. S., Krogmann, L., Mayer, C., Donath, A., Gunkel, S., Meusemann, K., Kozlov, A., Podsiadlowski, L., Petersen, M., Lanfear, R., Diez, P. A., Heraty, J., Kjer, K. M., Klopfstein, S., Meier, R., Polidori, C., Schmitt, T., Liu, S., Zhou, X., Wappler, T., Rust, J., Misof, B., & Niehuis, O. (2017). Evolutionary history of the Hymenoptera. *Current Biology, 27*, 1013–1018.

Peters, R. S., Niehuis, O., Gunkel, S., Bläser, M., Mayer, C., Podsiadlowski, L., Kozlov, A., Donath, A., van Noort, S., Liu, S., Zhou, X., Misof, B., Heraty, J., & Krogmann, L. (2018). Transcriptome sequence-based phylogeny of chalcidoid wasps (Hymenoptera: Chalcidoidea) reveals a history of rapid radiations, convergence, and evolutionary success. *Molecular Phylogenetics and Evolution, 120*, 286–296.

Pouchou, J. L., & Pichoir, F. (1991). Quantitative analysis of homoge-neous or stratified microvolumes applying the model "PAP". In K. Heinrich & D. Newbury (Eds.), *Electron probe quantitation* (pp. 31–76). New York: Plenum Press.

Polidori, C., & Wurdack, M. (2018). Mg-enriched ovipositors as a pos-sible adaptation to hard-skinned fruit oviposition in *Drosophila suzukii* and *D. subpulchrella*. *Arthropod-Plant Interactions*. https://doi.org/10.1007/s11829-018-9641-x.

Polidori, C., Jorge, A., & Nieves-Aldrey, J. L. (2013). Breaking up the wall: metal enrichment in ovipositors, but not in mandibles, co-varies with substrate hardness in gall-wasps and their associates. *PLoS One, 8*, 1–7. https://doi.org/10.1371/journal.pone.0070529.

Quicke, D. L. J. (1997). *Parasitic wasps*. London: Chapman & Hall.

Quicke, D. L. J., Palmer-Wilson, J., Burrough, A., & Broad, J. R. (2004). Discovery of calcium enrichment in cutting teeth of parasitic wasp ovipositors (Hymenoptera: Ichneumonoidea). *African Entomology: Journal of the Entomological Society of Southern Africa, 12*, 259–264.

Quicke, D. L. J., Wyeth, P., Fawke, J. D., Basibuyuk, H. H., & Vincent, J. F. V. (1998). Manganese and zinc in the ovipositors and mandibles of hymenopterous insects. *Zoological Journal of the Linnean Society, 124*, 387–396.

R Core Team. (2014). *R: A language and environment for statistical computing*. Vienna: R Foundation for statistical computing URL http://www.R-project.org/.

Revell, L. J. (2012). Phytools: an R package for phylogenetic compara-tive biology (and other things). *Methods in Ecology and Evolution, 3*, 217–223.

Roomans, G. M. (1988). Quantitative X-ray microanalysis of biological specimens. *Journal of Electron Microscopy Technique, 9*, 19–43.

Ronquist, F. (1999). Phylogeny, classification and evolution of the Cynipoidea. *Zoologica Scripta, 28*, 139–164.

Ronquist, F., Nieves-Aldrey, J. L., Buffington, M., Liu, Z., Liljeblad, J., & Nylander, J. A. A. (2015). Phylogeny, evolution, and classifica-tion of gall wasps: the plot thickens. *PLoS One, 10*(5), e0123301. https://doi.org/10.1371/journal.pone.0123301.

Schofield, R., & Lefevre, H. (1989). High concentrations of zinc in the fangs and manganese in the teeth of spiders. *Journal of Experimental Biology, 114*, 577–581.

Schofield, R. M. S. (2001). Metals in cuticular structures. In P. Brownell & G. Polis (Eds.), *Scorpion biology and research* (pp. 234–256). Oxford: Oxford University Press.

Schofield, R. M. S. (2005). Metal–halogen biomaterials. *American Entomologist, 51*, 45–47.

Schofield, R. M. S., Nesson, M. H., & Richardson, K. A. (2002). Tooth hardness increases with zinc content in mandibles of young adult leaf-cutter ants. *Naturwissenschaften, 89*, 579–583.

Silveira, O. T., & Santos Jr., J. N. A. (2011). Comparative morphology of the mandibles of female polistine social wasps (Hymenoptera, Vespidae, Polistinae). *Revista Brasileira de Entomologia, 55*, 479–500.

Somerville, D. C., & Nicol, H. I. (2002). Mineral content of honeybee-collected pollen from southern New South Wales. *Australian Journal of Experimental Agriculture, 42*, 1131–1136.

Stanciu, O. G., Mărghitas, L. A., Dezmirean, D. S., & Campos, M. G. (2011). A comparison between the mineral content of flower and honeybee collected pollen of selected plant origin (*Helianthus*

annuus L. and *Salix* sp.). *Romanian Biotechnological Letters, 16*, 6291–6296.

Turillazzi, S., & West-Eberhard, M. J. (Eds.). (1996). *Natural history and evolution of paper-wasps*. Oxford: Oxford University Press.

Vincent, J. F. V. (2002). Arthropod cuticle–a natural composite shell system. *Composites Part A: Applied Science and Manufacturing, 33*, 1311–1315.

Vincent, J. F. V., & Wegst, U. (2004). Design and mechanical properties of insect cuticle. *Arthropod Structure & Development, 33*, 187–199.

Williams, N. M., & Goodell, K. (2000). Association of mandible shape and nesting material in *Osmia* panzer (Hymenoptera: Megachilidae): a morphometric analysis. *Annals of the Entomological Society of America, 93*, 318–325.

Yang, K., Wu, D., Ye, X., Liu, D., Chen, J., & Sun, P. (2013). Characterization of chemical composition of bee pollen in China. *Journal of Agricultural and Food Chemistry, 61*, 708–718.

Zeng H (2016) Iron teeth. Available at: https://horacesnature.com/2016/02/23/we-found-zinc-in-a-hopeful-place/. Accessed 5 Dec 2018.