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A comparison between sexes, castes, and life-histories supports the adaptive role of metal enrichment in the mandibles of *Lasius* ants

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Abstract

Ant mandibles perform different functions (e.g. feeding, hunting, and nest building), and previous studies on workers of some species revealed that they are enriched in transition metals (Zn and Mn), which improve cuticle hardness. Whether this trait is adaptive in all ant species is still unclear. One way to test for such adaptation is to compare sexes and castes within species as well as species with different life-histories. Here, we evaluated the transition metal enrichment in workers, queens, and males of 12 species of the Holarctic genus *Lasius* Fabricius 1804. The genus is adequate to test for the evolution of this trait because of two reasons. First, males show small, often toothless mandibles and do not contribute to nest activities, hence leading to the prediction of lower metal-enrichment compared with workers and queens. Second, this genus includes both socially parasitic (of other *Lasius* species) and non–parasitic species, leading to the prediction that females of the former, because they engage in fights with the host, have higher metal-enrichment compared with females of the latter. Our Scanning Electron Microscopy/Energy Dispersive X-ray analysis provided evidence for an adaptive role of Zn and Mn in *Lasius* mandibles. At intra-specific-level, males had lower amount of Zn and Mn in their mandibles compared with workers and queens (which did not differ). Although parasitic and non-parasitic queens had similar metal amounts, parasitic workers showed higher Mn content and marginally higher Zn content than workers of non-parasitic species. Overall, our results strongly suggest that both colony activities and parasitic life-style promoted greater metal-enrichment in *Lasius* females. Given the huge biological diversity of ants, large comparative studies are needed to assess the generality of our findings.

Keywords: *Formicidae*, *social parasite*, *cuticle*, *zinc*, *manganese*, *Energy dispersive X-ray analysis*

1. Introduction

Arthropod exoskeleton has been widely studied and highly appreciated for its mechanical properties (Vincent & Wegst 2004; Dirks & Taylor 2012; Parle et al. 2016; Li et al. 2020). Despite chitin – the main cuticle component – is known for its properties of lightness and elasticity (Vincent & Wegst 2004), exoskeleton hardness is frequently connected with elemental enrichments, which has evolved in arthropods as improvement of cuticular performance (Cribb et al. 2008). While some crustaceans possess calcium carbonate within certain parts of their cuticle, some insects have evolved an alternative strategy to improve cuticle hardness and abrasion resistance: the inclusion of transition metals such as zinc (Zn) and manganese (Mn), likely as metal cross-link proteins (Schofield et al. 2021). Such transition metals have been found in several clades of insects, particularly in the mandibles and ovipositors (Hillerton & Vincent 1982; Broomell et al. 2008; Cribb et al. 2008; Lehnert et al. 2019), i.e. organs prone to be frequently used during the life to chew, cut, and drill hard objects. The presence of transition metals mandible cuticle is connected to a greater hardness and wear limitation, thus increasing their efficient use (Hillerton & Vincent 1982; Hillerton et al. 1984; Edwards et al. 1993; Schofield et al. 2002).

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In the hyper-diverse insect order Hymenoptera, Zn is the transition metal more commonly found in mandibles, while Mn has been seldom found in a few families often not phylogenetically related (Schofield 2001; Schofield et al. 2003; Jorge et al. 2017; Polidori et al. 2020). The presence of transition metals is also common in the ovipositor and sting of Hymenoptera (Quicke et al. 1998; Polidori et al. 2013; Baumann et al. 2018).

In the hymenopteran family Formicidae (Hymenoptera: Aculeata), metal enrichment in mandibles was previously detected in a limited number of species, with Zn always present (Hillerton & Vincent 1982; Edwards et al. 1993; Schofield et al. 2002; Larabee & Suarez 2010; Brito et al. 2017; Polidori et al. 2020; Chowdhury & Rastogi 2021) and was also studied in terms of its mechanical effects on the cuticle (Schofield et al. 2002). The relationship between force applied and Zn content was recently assessed, pointing out the importance of metal enrichment in improving mechanical properties of cuticule (Birkenfeld et al. 2024). Furthermore, it has been demonstrated, across several ant species, how cuticular enrichment, together with mandible shape and position/number of teeth, is linked with feeding habits (granivorous, predatory, necrophagous, nectivorous), territoriality, type of nesting (arboreal, psammophilous, lithophilous), and foraging methods (solitary or by recruitment) (Chowdhury & Rastogi 2021).

The evolution of incorporation of transition metals into the ants' mandibles has allowed the reduction of the force applied, as well as the energy consumed for their use (Schofield et al. 2021; Klunk et al. 2024; Birkenfeld et al. 2024). Ants seem, as all other studied Hymenoptera to date (Polidori et al. 2020), to have essentially evolved this strategy to increase cuticle hardness, with the only, striking exception of *Acromyrmex echinatior* Forel 1899, which have high-magnesium calcite crystals as an additional coating in the exoskeleton which improves its biomechanical properties (Li et al. 2020). Despite transition metals are apparently ubiquitous in the mandible teeth of ants, their adaptive role is still not fully clear. Indeed, metal enrichment could merely be a consequence of phylogenetic heritance (Polidori et al. 2020) and not necessarily an evolutionary response to ecological or behavioural pressures in all ants. While comparison between sub-castes within one species was performed (Birkenfeld et al. 2024), comparative analysis between males, queens, and workers within species, as well as between species with different life-histories, have been never carried out to date. For instance, no evaluation of metals in the mandibles of either queens or males of any ant species was performed to date, and only free-living, non-parasitic ant species were studied from this point of view to date.

Here, we tested for the possible adaptive role of mandibles' metal enrichment in the ant genus *Lasius* Fabricius 1804, a member of Formicinae with Holarctic distribution (Wilson 1955), through intra-specific and inter-specific comparisons. *Lasius* includes 115 known extant species occurring in a wide range of habitats including grasslands, woodlands, and meadows (Seifert 2018). Nests can be built in wood or in the soil. Queens and workers differ in body size, with an overall little marked caste dimorphism (Bolton 2018). *Lasius* mandibles possess from six to ten (queens and workers) or from zero to seven (males) teeth on their inner margin, according to the fact that only females are engaged in feeding, hunting, nest building, and all the other colony activities. Hence, the genus is adequate for intra-specific comparisons of mandible cuticular features. Within this genus, furthermore, both temporary socially parasitic species and autonomous, nest-founding species, occur (Seifert 2018). Temporary parasitism involves queens decapitating the host species' queens (frequently a non-parasitic species) and exploiting workers of the target nest in order to found their own colony (Buschinger 2009). Mandibles of parasitic workers of *Lasius* are known to possess a slightly different mandible shape compared with workers of non-parasitic species (Seifert 2018). Hence, this ant genus is also adequate for inter-specific comparisons of mandible chemical composition.

In particular, our predictions were the following two. First, males, not involved in colony activities, are predicted to have lower metal-enrichment in mandibles compared with workers and queens. Second, females (both castes) of socially parasitic species, because they engage in fights with the host, are expected to have higher metal-enrichment in mandibles compared with females (both castes) of the non-parasitic species.

2. Material and methods

2.1. *Sample origin and species synopsis*

Ninety-one individuals belonging to 12 species of *Lasius* and spanning queens, workers, and males were collected from different Italian localities in spring and summer from 2014 to 2021 (Table S1). Specimens were identified through dichotomous key provided in Seifert (2018), Seifert (2020) and Lebas and Galkowski

(2021). Because males are particularly difficult to identify morphologically, they were gathered together with females from nests. Nests were detected on sight, waiting for nuptial flights or otherwise searching under stones. Samples were preserved in ethanol 95% until their preparation for the microscopy investigations.

The 12 studied species encompass five different species-group and included both free-living, colony-founding species (here after non-parasitic species) as well as socially parasitic species (here after parasitic species) (Figure 1(a–b)). Males, workers, and queens were studied in all species, except for *Lasius brunneus*, for which males were not available.

2.2. *Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDS)*

Specimens were dissected under light microscopy in a way to gently separate the heads from the rest of the body. Mandibles were open up to achieve exposition. The heads were then mounted on adhesive carbon pads attached to aluminium stubs (Polidori et al. 2013; Jorge et al. 2017) and introduced in the scanning electron microscopy (SEM) without previous gold or platinum coating. From two to four individuals *per* species and caste were analysed (Tables S1-S2). Pictures of the specimens` heads were taken at convenient magnification to show their entire mandibles. For the Energy Dispersive X-ray Spectroscopy (EDS) analysis, we focused on the occurrence and abundance (weight percentage, wt%) of the transition metal zinc (Zn) and manganese (Mn). A point-analysis was performed on each specimen, i.e. the X-ray was applied to a point on the distal portion of one tooth of the inner side of the mandible (or, in case mandibles lacked inner teeth, on the mandible apical tooth). Some point-analyses were also carried out in the proximal part of the mandible, far from the teeth, to confirm that transition metals lack in such cuticle area as already reported in other hymenopterans (e.g. Polidori et al. 2013).

A JEOL JSM-IT500 LV Scanning Electron Microscope (JEOL Ltd., Tokyo, Japan), equipped with Backscattered (BSE) and Secondary Electrons (SE) detectors, coupled with energy dispersive X-ray spectrometry, was employed to perform the chemical micro-analysis at the Department of Earth Sciences "Arditio Desio" of the University of Milan (ESD-UniMI). The operating conditions were: vacuum mode, 20 kV accelerating voltage, 10 mm working distance. The ZAF correction was applied to the

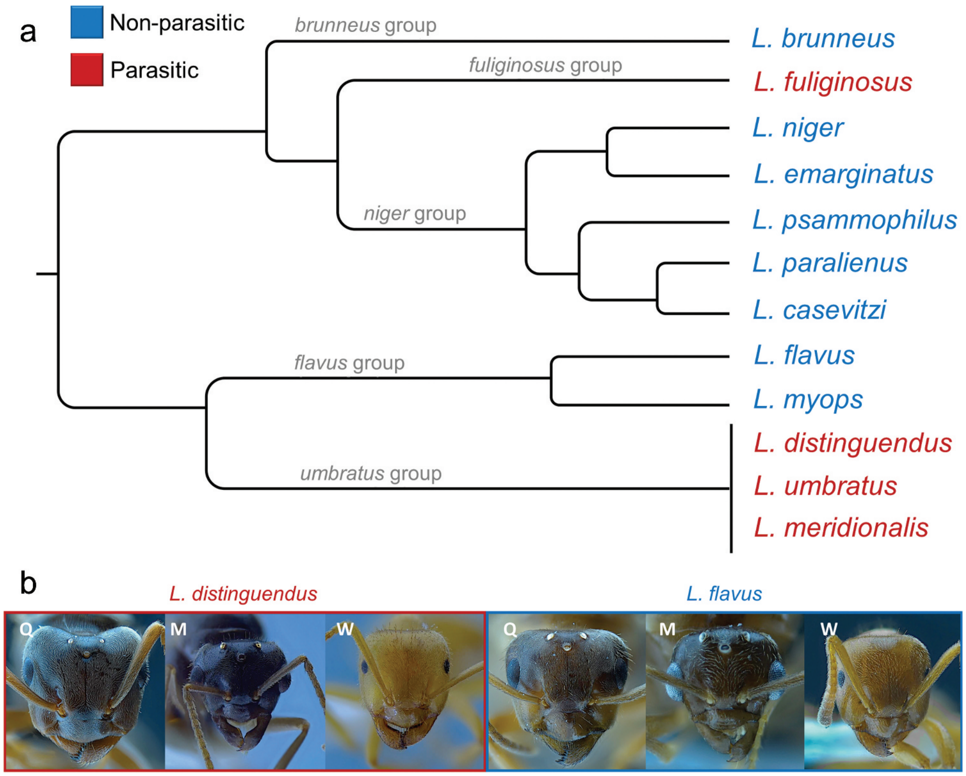

Figure 1. a, Phylogeny of the studied *Lasius* species, manually re-drawn based on Seifert (2020), Maruyama et al. (2008), Blatrix et al. (2020) and Boudinot et al. (2022). b, Pictures of the head of individuals (Q = queen, M = male, W = worker) from two of the studies species (one parasitic and one non-parasitic).

chemical data, as implemented in the JEOL suite of programs. The wt% of the sigma value (i.e. the error in the weight percent concentration at the one sigma level) was used to determine whether the element is below the detection limits of the sample analysis (Duncumb 1994). As in previous studies (Polidori et al. 2013, 2020; Jorge et al. 2017), we considered an element as actually occurring in the cuticle, its wt% had to be greater than three times the wt% of the sigma value (Polidori et al. 2013, 2020).

2.3. Statistical analysis

Since the used analytical method of metal detection is semi-quantitative, we ranked all the obtained wt% values. For Mn, we ranked values as follows: 0 = absent, 1 = 0.01–0.10%, 2 = 0.11–0.20%, 3 = 0.21–0.30%, 4 = 0.31–0.40%, 5 = 0.41–0.50%, 6 ≥ 0.51%. For Zn, we ranked values as follows: 0 = absent, 1 = 0.01–1.00%, 2 = 1.01–2.00%, 3 = 2.01–3.00%, 4 = 3.01–4.00%, 5 = 4.01–5.00%, 6 = 5.01–6.00%, 7 = 6.01–7.00%, 8 = 7.01–8.00%, 9 = 8.01–9.00%, 10 = 9.01–10.00%, 11 = ≥10.01%. All raw data of %wt recorded during the study can be found in Table S2.

Once ranked, we calculated the average values of wt% for Zn and Mn across individuals *per* each species (Table I). Then, we averaged the values *per* species across groups (caste, sex, parasitic or non-parasitic life-style) to apply the statistical analyses and test for our hypotheses. To evaluate the differences in elements' concentrations we first apply simple, univariate non-parametric statistics. To compare the medians of two groups, Mann–Whitney test was used, while the Kruskal–Wallis test was used for comparisons among more than two groups. In case of overall significance, the Kruskal–Wallis test was followed by Bonferroni paired comparisons. Then, we used Generalized Linear Models (GLMs) to evaluate

Table I. Mean values ± SE of the ranked % of Zn and Mn, *per* species and caste.

Species	Biology	Caste	Zn	Mn
Lasius brunneus (Latreille 1798)	Not parasite	Queen	3.0 ± 0.0	1.0 ± 0.0
		Worker	2.5 ± 0.5	0.5 ± 0.5
Lasius casevitzi Seifert & Galkowski 2016	Not parasite	Male	4.0 ± 0.6	0.0
		Queen	6.5 ± 4.5	3.5 ± 2.5
		Worker	6.3 ± 1.2	2.0 ± 1.0
Lasius distinguendus (Emery 1916)	Parasite	Male	2.5 ± 0.5	0.0
		Queen	4.0 ± 1.0	0.0
		Worker	5.0 ± 0.0	3.0 ± 1.0
Lasius emarginatus (Olivier 1792)	Not parasite	Male	0.0	0.0
		Queen	4.3 ± 1.9	1.3 ± 0.3
		Worker	4.7 ± 1.3	0.7 ± 0.7
Lasius flavus Fabricius 1782	Not parasite	Male	1.0 ± 0	0.0
		Queen	3.7 ± 0.7	0.0
		Worker	3.7 ± 1.2	1.0 ± 0.6
Lasius fuliginosus (Latreille 1798)	Parasite	Male	3.3 ± 0.3	0.0
		Queen	4.7 ± 1.7	1.0 ± 0.6
		Worker	11.0 ± 0.0	5.0 ± 0.6
Lasius meridionalis (Bondroit 1920)	Parasite	Male	4.0 ± 0.0	2.0 ± 2.0
		Queen	4.5 ± 0.5	2.0 ± 0.0
		Worker	6.0 ± 1.0	2.5 ± 0.5
Lasius myops Forel 1894	Not parasite	Male	2.5 ± 0.5	1.5 ± 1.5
		Queen	2.0 ± 0.0	1.0 ± 0.0
		Worker	6.5 ± 3.5	2.0 ± 2.0
Lasius niger L. 1758	Not parasite	Male	3.0 ± 0.6	0.0
		Queen	5.7 ± 2.2	2.0 ± 0.6
		Worker	3.7 ± 1.2	2.0 ± 0.6
Lasius paralienus Seifert 1992	Not parasite	Male	3.3 ± 0.7	0.3 ± 0.3
		Queen	5.0 ± 1.0	0.3 ± 0.3
		Worker	2.5 ± 0.5	0.5 ± 0.5
Lasius psammophilus Seifert 1992	Not parasite	Male	5.3 ± 2.0	0.0
		Queen	3.0 ± 0.6	1.0 ± 1.0
		Worker	3.7 ± 0.7	0.7 ± 0.7
Lasius umbratus (Nylander 1846)	Parasite	Male	1.5 ± 0.5	1.0 ± 1.0
		Queen	5.0 ± 2.1	2.0 ± 0.6
		Worker	5.7 ± 2.0	2.3 ± 1.5

which of the two explanatory variables (caste/sex and biology (parasitic or non-parasitic)) had greater effect on the variability of element concentrations. One model was performed for each of the two considered elements (Zn and Mn). All statistics were performed in R through the RStudio Software v 2022.02.2–485, R v 4.1.3 (R Core Team 2022) (for GLM, using the R package *lme4* (Bates et al. 2015)) and in PAST 3.04 (Paleontological Statistics Software Package) Hammer et al. (2001 (for the non-parametric univariate tests and to create the graphs). In text and tables, average values are reported ± standard error (SE).

3. Results

The SEM pictures revealed that *Lasius* males of the studied species possess much fewer (often none) and smaller teeth on the inner side of the mandibles, compared with workers and queens (Figure 2). The EDS analysis revealed that Zn and Mn largely occur in *Lasius* mandible teeth (Figure 2). When occurring, the presence of Zn and Mn was visible in the EDS spectra by the presence of peaks at 1.2 (Zn_L) and 8.8 KeV ($Zn_{K\alpha}$) and 5.9 KeV ($Mn_{K\alpha}$) (Figure 2). The metal-enriched mandible teeth appeared whiter than the rest of the cuticle in the SEM pictures (Figure 2). On the other hand, none of the EDS analyses performed in the proximal part of the mandibles gave spectra with Zn or Mn peaks (Fig. S1). Apart from the obvious high abundance of Carbon (C), Oxygen (O), and Nitrogen (N) (the main components of chitin), other elements were detected at small concentrations and/or were not associated with a relevant function in insects, and thus were not further considered here (Table S2). Chlorine (Cl) was recorded at often relatively high abundances (up to 1%, Table S2) because it is known to co-occur with Zn in metal-enriched insect cuticle (e.g. Jorge et al. 2017), being incorporated in the ant mandibles at the same time as Zn (i.e. after pre-ecdysial tanning) (Schofield et al. 2003) and being probably involved in the protein complex including Zn (Jorge et al. 2017; Schofield et al. 2021).

Important difference emerged between castes, sexes, and life-histories in the transition metals. From a general inspection of the raw concentrations obtained from the EDS analysis, males had mandibles with 0 wt% to 8 wt% of Zn (<3 wt% in most individuals) and from 0 wt% to 0.4% of Mn (with no Mn in most individuals) (Table S2). Workers and queens, on the other hand, possessed mandibles with teeth enriched with 1 wt% to almost 15 wt% of Zn, with most of the individuals having >3 wt%. Mn in females was rarely absent and was >0.5 wt% in several individuals (Table S2). Once ranked, these values showed statistically significant variations among the tested groups.

Males, workers, and queens differed in the ranked wt % of Zn (Kruskal–Wallis test: $\chi^2 = 8.04$, $N = 35$, $p = 0.017$), with significant paired differences between males and both workers (Bonferroni paired comparison: $p = 0.007$) and queens ($p = 0.033$), but no differences between workers and queens ($p = 0.55$) (Figure 3a, Table I). Males, workers, and queens also differed in the ranked wt% of Mn (Kruskal–Wallis test: $\chi^2 = 10.02$, $N = 35$, $p = 0.006$) (Figure 3b, Table I), with significant paired differences between males and both workers (Bonferroni paired comparison: $p = 0.002$) and queens ($p = 0.034$), again with no differences between workers and queens ($p = 0.28$). Since males had much lower concentrations of both Zn and Mn, the comparison between parasitic and non-parasitic species was carried out only for workers and queens. Parasitic queens and non-parasitic queens did not differ in the ranked wt% of both Zn (Mann–Whitney test: $U = 12.5$, $N = 12$, $p = 0.59$) and Mn (Mann–Whitney test: $U = 15$, $N = 12$, $p = 0.93$) (Table I). However, workers of parasitic species possessed a significantly higher wt% of Mn compared with those of non-parasitic species (Mann–Whitney test: $U = 0$, $N = 12$, $p = 0.008$) and a marginally significantly higher wt% of Zn (Mann–Whitney test: $U = 6$, $N = 12$, $p = 0.10$) compared with those of non-parasitic species (Figure 3(c–b), Table I).

The GLMs largely confirmed these results. The caste/sex affected the ranked wt% of both Zn (Adjusted $R^2 = 0.247$, $F = 4.718$, $df = 3, 31$, $p = 0.008$) and Mn (Adjusted $R^2 = 0.3022$, $F = 5.909$, $df = 3, 31$, $p = 0.003$), with males possessing the lowest values compared with females, and the species biology affected the ranked wt% of Mn and marginally of Zn, with parasitic species possessing higher values than non-parasitic species (Table II). The statistical parameters of the GLMs and the associated *P*-values seem to give more importance to the differences between females and males than to those between parasitic and non-parasitic species (Table II).

Across workers and queens, Zn ranked wt% was correlated with Mn ranked wt% (Pearson test: workers: $r = 0.89$, $N = 12$, $p < 0.001$; queens: $r = 0.60$, $N = 12$, $p = 0.038$). However, a correlation between Zn and Mn did not appear in males (Pearson test: $r = 0.09$, $N = 11$, $p = 0.79$),

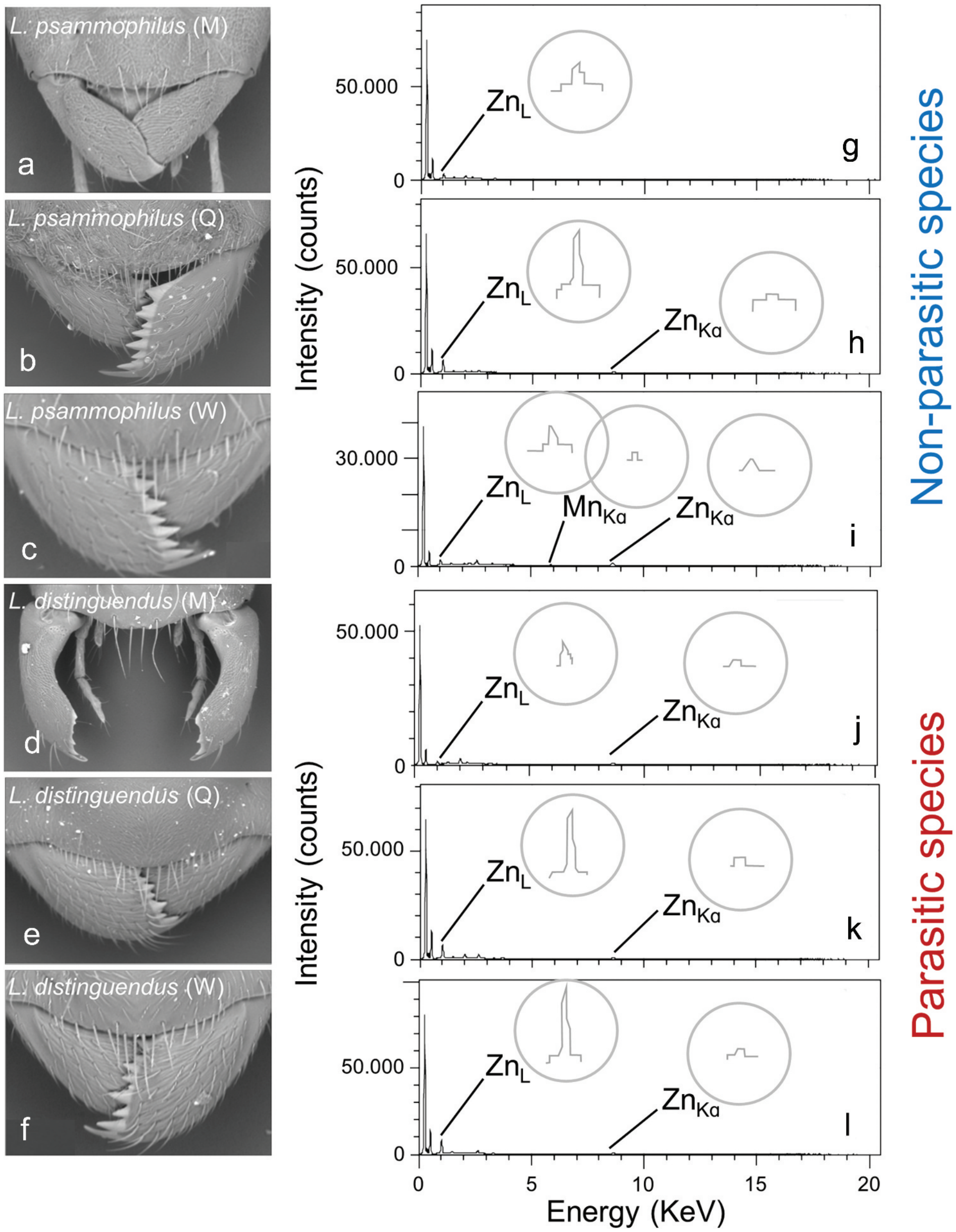

Figure 2. Examples of SEM images of male, queen and worker mandibles of *L. distinguendus* (parasite) (a–c) and *L. psammophilus* (not parasite) (d–f). g–l, the EDS spectra for the mandibles of the individuals shown in a–f. Zn and Mn peaks are indicated and drawn from zoomed images in grey circles.

since they often had no Mn even if Zn occurs in appreciable concentrations.

4. Discussion

Our study provided new information on the enrichment of mandible teeth by transition metals in ants, a group for which few species and only workers were previously investigated from this point of view. More in particular, workers of a total of 21 species were previously studied (Hillerton & Vincent 1982; Edwards et al. 1993; Schofield et al. 2002; Larabee & Suarez 2010; Brito et al. 2017; Polidori et al. 2020; Chowdhury & Rastogi 2021; Klunk et al. 2024) (Table S3) spanning only five out of the 16 extant subfamilies of ants. Furthermore, the leaf-cutter genus *Atta*, with five species studied, is over-represented in cuticular composition investigations (Table S3). In these studied ant species, workers have always Zn-enriched mandibles (roughly in a range of 3–16 wt%), with this metal often co-occurring with Mn (always <1 wt%).

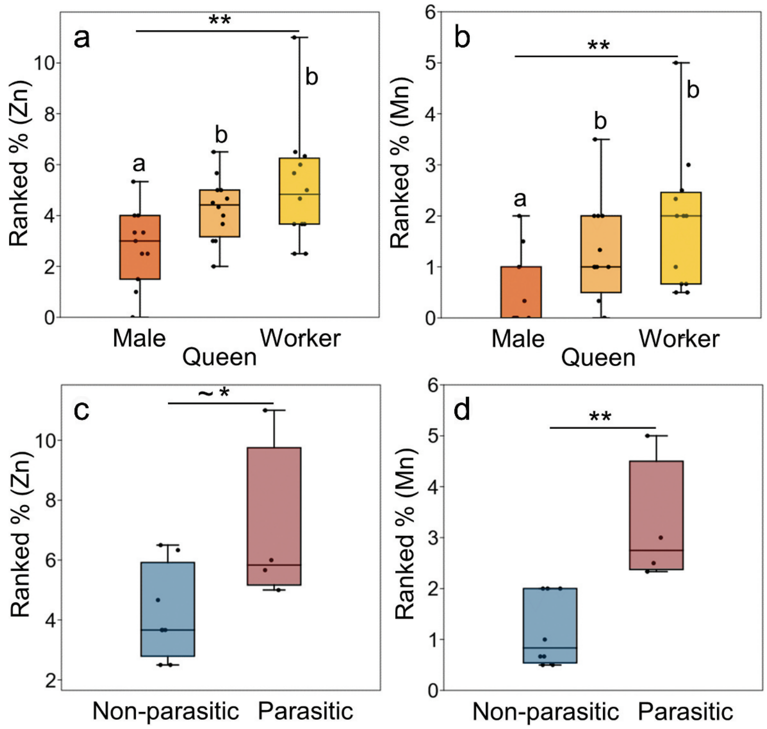

Figure 3. Box-and-whisker plots showing medians (horizontal lines within boxes), 1° and 3° quartile (horizontal lines closing the boxes), and maximum and minimum values (ends of the whiskers) for the ranked values of Zn (a, c) and Mn (b, d), calculated across species, in males (M), queens (Q) and workers (W) (a-b) and in workers of parasitic and non-parasitic species (c-d). * = [$0.1 \geq p \geq 0.01$], ** = $P < 0.01$.

Table II. Results of the General Linear Model (GLM) analysis for Zn, Mn, and Cl. Explanatory variables were caste (W = worker, Q = queen) and biology (*p* = parasitic). SE = Standard Error. In bold, *P*-values <0.05.

Element	Model and factors	Estimate	SE	*t*	*P*
Zn	Intercept	2.3769	0.5586	4.255	0.0002
	Caste (Q)	1.538	0.7108	2.164	**0.04**
	Caste (W)	2.3575	0.7108	3.317	**0.002**
	Biology (P)	1.0885	0.6065	1.795	0.08
Mn	Intercept	0.133	0.3215	0.415	0.68
	Caste (Q)	0.85	0.4091	2.078	0.05
	Caste (W)	1.4333	0.4091	3.503	**0.001**
	Biology (P)	0.8417	0.3491	2.411	**0.02**

Our study largely confirmed such general features for ants. Most importantly, our study is the first performed in a comparative framework within one single ant genus and that provided data on queens, workers, and males, as well as on socially parasitic species. This allowed us to evidence the adaptive role of metals in *Lasius*. A previous large comparative analysis of Zn and Mn enrichment in Hymenoptera mandibles, together with the above-cited studies on ants, revealed that phylogenetic inertia (Blomberg & Garland 2002) is the main responsible for metal-enrichment in the mandibles of ants as a group, which re-acquired Zn- and Mn-enrichment within Aculeata after having loss them at the ancestral node from which both Formicoidea and Apoidea arose (Polidori et al. 2020). However, at a smaller scale (i.e. within lineages) the possible evolutionary pressures driving the abundance of these metals were still not fully understood for ants. Nonetheless, such pressures were recognized, for other body parts (ovipositors), in other hymenopteran lineages. For example, within fig wasps (Agaonidae), species that lay eggs in hard fig syconia have more often high concentrations of transition metals in the ovipositor, and in gall wasps (Cynipidae) species that oviposit in hard plant tissues are more prone to have higher concentrations of metals (Polidori et al. 2013; Kundanati & Gundiah 2014). Even though mandibles evolution in ants still needed to be deepened, our results suggest that within the genus *Lasius*, behavioural specialization (caste/sex and parasitism) appeared from our study as a likely driver for the evolution of greater abundance of Zn and Mn in the mandibles.

Our first prediction was that males have lower metal-enrichment in mandibles compared with workers and queens. The limited use of the mandibles by males compared with queens and workers seems a reasonable and probable explanation for a reduction of transition metals in male mandibles' cuticle. This is in clear accordance with the striking morphological difference in mandibles between sexes in these, as well as other, ants (Wilson 1955; Dlussky & Pisarski 1971), with a notable reduction in mandible size and in teeth size and number in males. In males of the genus *Lasius*, both species with toothless mandibles and others with few ones have evolved (Kutter 1977; Seifert 1988). Despite the low autonomy of males and the relatively short lifespan (Hölldobler & Wilson 1990; Wilson 2020), an exceptional case of mandibles use has been reported in males of *Lasius meridionalis*, which are capable to move objects and feeding themselves independently (Collingwood 1979). Accordingly, males of this species had one of the highest value of Zn recorded here for *Lasius* males (though not Mn) and teeth on the inner side of the mandibles, emphasizing that there could be special, still unknown behaviors which might deserve further studies.

However, no differences emerged in either Zn or Mn between queens and workers. Workers live from a few months to a couple of years (Parker 2010) and make continuous use of their mandibles, requiring greater resistance to limit abrasion, while queens are much longer-lived, reaching 20–30 years (Keller 1998; Quque & Bles 2020) using the mandibles almost exclusively during the colony foundation phase. As the incorporation of transition metals is directly connected with mandibles' function efficiency, a positive relationship between hardness and the individual fitness was identified in insects (Laiolo et al. 2021). Therefore, we assume that queens with poorly enriched mandibles may have difficulties in the construction phase of claustrum and in its defense. Furthermore, given the longevity of queens, mandible hardness may have evolved to provide durability over time. As a consequence, it can be hypothesized that the similarity in the percentages of Zn and Mn between workers and queens may depend on a trade-off between longevity of individuals and use of mandibles. Our intra-specific comparison opens to a still unexplored question related with how, within a single species, metals concentrate more in the mandibles of a sex over the other. The larval diet seems to account for variation in hymenopteran mandibles' metal concentration (Polidori et al. 2020), but it is currently unknown if *Lasius* males and females are fed differently in the nest. Alternatively, evolution limited – because no more necessary – the biochemical pathway responsible for metal accumulation in males' mandible teeth, even in case of identical larval diet for both sexes.

Our second prediction was that females of socially parasitic species have higher metal-enrichment in mandibles compared with females of the non-parasitic species. We found a partial confirmation to this hypothesis, perhaps also because of the small sample of parasitic species. From one side, workers of parasitic species were significantly more enriched in Mn and marginally more enriched in Zn, than workers of non-parasitic species. This result can be explained by the behavior and interaction between host and parasite in young parasitized colonies: after the success of parasitic queen during colony foundation, workers of the two species coexist in the same nest for a long period, until the natural death of the host workers (Stukalyuk et al. 2021). While nests are mixed, the host species are able to recognize the workers of the parasitic one (Liu et al. 2000; Pulliainen et al. 2019; Schultner & Pulliainen 2020) and acts of aggression are reported in several ant genera (Buschinger 2009). Moreover, once increased sufficiently in number, parasitic workers kill the remains of the host ones (Hölldobler 1953). Consequently, workers of parasitic species may benefit from possessing stronger mandibles through higher metal-enrichment, as a response to their need to maintain control on the hosts.

On the other hand, while parasitic queens invade the host nests and decapitate the resident queen to establish as the new colony owner, we have found no significant difference between parasitic and non-parasitic queens in both Zn and Mn. An alternative hypothesis is that parasitic queens benefit from their larger body size and mandible shape, rather than from an increased concentration of metals, during host nest invasion. This hypothesis might find support by looking indeed at the difference in mandible inner (i.e. cutting) surface in parasitic *Lasius* species compared to the non-parasitic ones (Seifert 1988, 2018). In the former, the cutting surface seems visibly larger and the mandibles overall more robust. Micro-modification in shape may lead to a change in mandibular functioning in ants (Klunk et al. 2021), and further morpho-functional studies in parasitic and non-parasitic *Lasius* may highlight interesting difference in cutting performance.

Further considerations support an adaptive role linked with parasitism in *Lasius*. Indeed, the highest values of transition metals were measured in the workers of *Lasius fuliginosus* (Latreille 1798), the only known hyperparasite species in Europe (Seifert 2018). This species establishes a nest through social parasitism on another *Lasius* species which previously parasitized a nest of a non-parasitic species. Hence, *L. fuliginosus* may have evolved a higher mandible hardness than that – already greater than non-parasitic species – of its hosts. The high abundance of metals in the mandible of this species supports our hypothesis also for another reason. In fact, it is the only parasitic species analysed here belonging to a clade otherwise entirely composed of non-parasitic species (the *niger*-group) (Figure 1a). Similarly, the non-parasitic *Lasius flavus* and *L. myops*, both closely related with the completely parasitic *umbratus* group, have lower concentrations of metals than the species of the latter. At last, *Lasius brunneus*, a non-parasitic species, and the parasitic *L. fuliginosus*, both digging in wood (Seifert 2018), do not have similar concentrations of metals, thus further supporting the role of parasitism in shaping cuticular structure.

A larger species sample of both parasitic and non-parasitic *Lasius* species, analysed in a formally phylogenetically corrected framework, may further confirm our findings. In addition, many other ant genera have evolved social parasitism, showing a wide range of behaviors (Buschinger 2009; Rabeling 2020) and phenotypes (Hölldobler & Wilson 1990; Fischer et al. 2020) and the evaluation of the metal content in the mandibles of species from many ant lineages might support the generality of our results.

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Authors’ contributions

C.P. conceived the study, designed the methodology, collected the SEM/EDS data, performed the statistical analysis, and drafted the manuscript. E. N. carried out the field sampling, collected the SEM/EDS data, and drafted the manuscript.

Availability of data and material

Data analysed in this study are available in the Supplementary Table S2. The studied specimens are preserved at E.N. personal collection and at the University of Milan.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Supplementary material

Supplemental data for this article can be accessed online at https://doi.org/10.1080/24750263.2024.2355308

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