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Chemical deception among ant social parasites

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Abstract Deception is widespread throughout the animal kingdom and various deceptive strategies are exemplified by social parasites. These are species of ants, bees and wasps that have evolved to invade, survive and reproduce within a host colony of another social species. This is achieved principally by chemical deception that tricks the host workers into treating the invading parasite as their own kin. Achieving levels of acceptance into typically hostile host colonies requires an amazing level of deception as social insects have evolved complex species- and colony-specific recognition systems. This allows the detection of foreigners, both hetero- and con-specific. Therefore, social parasitic ants not only have to overcome the unique species recognition profiles that each ant species produces, but also the subtle variations in theses profiles which generate the colony-specific profiles. We present data on the level of chemical similarity between social parasites and their hosts in four different systems and then discuss these data in the wider context with previous studies, especially in respect to using multivariate statistical methods when looking for differences in these systems [*Current Zoology* 60 (1): 62–75, 2014].

Keywords Mimicry, Social parasites, Cuticular hydrocarbons, Multivariate statistics

Deception by mimicking the pattern of another species was first defined in 1862 by HW Bates. Since then, numerous examples of visual mimicry have been described. Until recently, there have been relatively few examples of chemical deception, despite chemical cues being the main form of communication in the majority of organisms (Bagnères and Lorenzi, 2010). An early example was that of the Bolas spider which mimics a female moth sex pheromone to help capture male moths (Stowe et al., 1987). Since then the advancement and availability of instruments that can detect small amounts of chemicals have started to reveal that chemical deception is widespread, especially among the social bees, wasps and ants (Dettner and Liepert, 1994). The best studied group are ants (Lenoir et al., 2001). Currently there the around 12,800 ant species described (Bolton, 2013) with approximately 230 that have evolved into social parasites (Agosti and Johnson, 2005). "Social parasitism" is the coexistence in the same nest of two social insect species one of which is dependent on the other (Hölldobler and Wilson, 1990), so social parasites parasitize complete societies. The level of social parasitism in ants is probably an underestimation since a detailed ant survey in Switzerland found almost one third of the 110 ant species were social parasites (Kutter, 1968). Parasites are often rare, have cryptic habits and live in low densities within a given habitat, making them very difficult to find, especially since many parasites occur only in localised areas that may only cover a few square meters. Within the Formicidae, four basic types of social parasitism have evolved (Hölldobler and Wilson, 1990; Buschinger, 2009): 1) xenobiosis where the parasite lives within the host colony but rears its own brood and is a potential precursor of inquilinism (Hölldobler and Wilson, 1990); 2) temporary parasitism**,** where a parasitic queen will invade a host colony, kill the resident queen and trick the host workers into rearing her worker brood in order to establish a new colony; 3) dulosis or 'slave-making' species, where the parasites invade the host colony, kill the queen and en-slave the host workers to work for them. They continually replenish slaves by invading other colonies and stealing the brood, so several batches of sexuals can be produced by a single colony; and finally 4) inquilinism which is permanent parasitism without slavery, but the parasite's brood which often only consists of sexuals, is raised by the host workers and is the most frequent type of social parasitism among ants. In some extreme cases, the parasite has lost the ability to produce its own workers and so relies directly on the host workers to rear the

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next generation of sexuals. This final type of association is predicted to be the most advanced and hence the level of deception to be most highly evolved.

Irrespective of their life-styles, all parasites face the same fundamental problem in their ability to invade and then remain within the host colony despite being vastly outnumbered by hostile host workers. As the interests of the parasites and hosts are diametrically opposed, evolutionary forces are strong in both directions, producing a deception-detection arms race. This is best exemplified by the highly complex chemical recognition system that ants and other social insects have evolved to ensure colony integrity and that all altruistic acts are directed only towards nest-mates that are typically kin (Blomquist and Bagnères, 2010). Any parasite that can successfully mimic this recognition system will become accepted as a 'nest-mate' reaping all the benefits afforded by the colony with none of the costs associated with colony establishment, foraging, or colony defence.

Over the past decade, a large amount of empirical data has indicated that the ant nest-mate recognition system is based around the hydrocarbons that are found on the cuticle of all insects (Blomquist and Bagnères, 2010). These cuticular hydrocarbons (CHC) are multifunctional since in addition to their role in chemical communication they also act as a barrier to transcuticular water movement and entry of pathogens. Most CHC profiles, consist of a carbon chain length up to C_{40}, which is either saturated (*n*-alkanes), possesses one or two double bonds (alkenes, and alkadienes), or has one, two or three methyl-groups attached to the molecule (mono-, di-, or tri-methyl-alkanes). The simple presence of a double bond or methyl group gives the molecule conformation (i.e. shape) that greatly increases its ability to be detected (Chaline et al., 2005; Dani et al., 2005). Thus, the strong role of chemical communication in ants has caused a high diversity of these types of CHCs to evolve and helps explain why over 1000 CHCs have been described from just 78 ant species (Martin and Drijfhout, 2009a). This allows each ant species to have evolved a unique species-specific CHC profile that remains stable across large geographical distances and timescales. Although we don't know the precise evolutionary history of the parasitic species it is a reasonable assumption that each parasitic species at one time possessed its own unique CHC profile, which it has had to change or adapt in order to invade the colony of another species, since there is no direct evidence for allopatric speciation among parasitic ants (Buschinger, 2009). It has long been assumed that a parasite will be a sister species of its host in accordance to Emery's rule (Emery, 1909). A further assumption is that sister species will also have similar CHC profiles so it is predicted that host-parasite CHC profiles will be similar between sister species and is lesslikely to occur when the parasite and host are more distantly related.

Within a species each colony possesses a unique CHC profile that allows discrimination between conspecific colonies. This is normally achieved by varying the ratios of compounds that form the species profile, or even the expression of different positional isomers of the same compound, that is when the carbon chain length remains the same but the position of the double bond or methyl group changes e.g. Z9-C_{25}alkene or Z7-C_{25}alkene (Martin et al., 2008b). Therefore, social parasites have to evade both the species and colony level recognition systems, which has evolved specifically to detect all 'non-nest-mates', irrespective of their relatedness. Amazingly some ant parasites can even exploit two or three hosts that have very different CHC profiles (Kaib et al., 1993; Johnson et al., 2001).

Despite these formidable barriers, social parasites have evolved a range of chemical deception strategies that allow them to persist in host colonies (Lenoir et al., 2001). These include chemical camouflage (obtaining the compounds directly from the host); chemical mimicry (actively producing the host compounds) and chemical insignificance where the parasite produces no or very low amounts of CHCs so it has an inert profile, which later may become similar to the host via mimicry or camouflage. Due to the complexity of ant CHC profiles, the analysis of differences between parasites and hosts has almost always been done using very sensitive multivariate statistical methods. Furthermore, social parasites are often very rare, so sample size can be an issue when using multivariate statistical methods (Martin and Drijfhout, 2009b; Mitteroecker and Bookstein, 2011).

This study investigates the CHC profiles of three Palaearctic social parasites (*Harpagoxenus sublaevis*, *Strongylognathus testaceus* and *Camponotus universitatis*) and their respective hosts (*Leptothorax acervorum*, *Tetramorium cf. impurum*, *T. semilaeve* and *Camponotus aethiops*). Despite belonging to very different genera, all three parasites have very similar lifestyles, being entirely dependent on their hosts and living in mixed colonies. *H. sublaevis* is a slave-maker, invading *Leptothorax* host colonies, killing the queen and enslaving the host workers. Since the parasite workers are not adapted to forage, killing the host queen means that the

parasite workers need to raid neighbouring host colonies to replenish the slaves. *Harpagoxenus sublaevis* co-exists with three species of *Leptothorax*; *L. acervorum*, *L. muscorum* and *L. gredleri*, although the former is the most commonly recorded host. Colonies can also consist of mixed *Leptothorax* spp. slaves (Bauer et al., 2010). The situation is a bit different in *S. testaceus*, which is a degenerate slave-maker of *Tetramorium caespitum* and *T. impurum* (although these *Tetramorium* species consist of a complex containing a number of undetermined cryptic species [Schlick Steiner et al., 2006]). The *Strongylognathus* queen does not kill the host queen, but instead pheromonally inhibits production of the host sexual brood. As the number of parasite workers rarely exceeds 1%, they rarely (or perhaps never) raid additional colonies for extra slaves (Sanetra and Gusten, 2001; Czechowski et al., 2002; Tinaut et al., 2005). Thus, this species may be considered an intermediate stage between slave-makers and inquilines. The parasite *C. universitatis* is an inquiline species that is a permanent parasite without slavery. It inhabits colonies of *C. aethiops* and *C. pilicornis* within Western Europe (Tinaut et al., 1992). Currently *C. universitatis* and *C. ruseni* (Karaman, 2012) are the only known *Camponotus* social parasites, despite this genus containing 1,097 species (Bolton, 2013). This study aims to compare the CHC profiles of these three parasites with their respective hosts. Since all the parasites are fully integrated into their host colonies, we expect CHC profiles of the parasites to be very similar to their hosts, possibly even down to the level of the colony.

1 Materials and Methods

1.1 Collection locations

Three colonies of *Harpagoxenus sublaevis* were found by breaking open dry pine twigs that contained *Leptothorax acervorum* workers and queens on the Hanko peninsular in Southern Finland during September 2012. Five additional colonies of non-parasitized *L. acervorum* were collected for chemical analysis. Roughly every one in 15 colonies of *L. acervorum* found was parasitized.

Strongylognathus testaceus were collected from two colonies of *Tetramorium c.f impurum* and a single colony of *T. semilaeve* in the Catalan Pyrenees, Spain during June 2011. Colonies were found under stones. The colony found with *T. semilaeve* contained *S. testaceus* sexuals (both males and females). Within Iberia, *S. testaceus* has only been recorded from *T. caespitum* and *T. impurum* (Tinaut et al., 2005), so this is also the first record of *S. testaceus* with *T. semilaeve*. Four colonies of non-parasitized *T. impurum* and seven *T. semilaeve* were also collected for analysis. Approximately one in 50 colonies of *Tetramorium* was parasitized.

Camponotus universitatis were also collected from the Catalan Pyrenees in June 2011 when four colonies were found with their respective hosts *Camponotus aethiops*. In addition, three colonies of unparasitized *C. aethiops* were collected from the same area. Parasitized colonies consisted of *C. universitatis* workers and female alates (young winged queens). All colonies of *Camponotus* were found in a small (10 m × 10 m) area on a dry slope that was stony and open with a sparse Mediterranean flora.

1.2 Chemical analysis

All ants were killed by freezing and then stored at -20°C until chemically analyzed. For each *Strongylognathus* and *Tetramorium* colony, one sample comprising a pool of 20 workers was used. Pools containing 10 individuals per colony were used for *H. sublaevis* and *L. acervorum*. Individuals of both *Camponotus* species were used as they are large enough to obtain enough extract from just one ant, allowing nine and 11 unparasitized and parasitized *C. aethiops* workers respectively to be analysed in addition to 13 *C. universitatis* individuals (six workers and seven female alates). Each sample was placed in a glass vial and immersed in 50 µl of high-performance liquid chromatography grade hexane that contained 1 mg 100 ml^{-1} of an internal standard (docosane, C_{20} alkane) for 10 min. Three individual workers of *C. aethiops* and *C. universitatis* from each colony were extracted in order to compare profiles within and between colonies. The ants were then removed and hexane evaporated to dryness. Prior to analysis, 30 µl of hexane was added to the vials. Samples were analyzed on an HP 6890 gas chromatograph (GC) connected to an HP 5973 MSD (quadrupole) mass spectrometer (MS: -70 eV, electron impact ionization). The GC was equipped with a ZB-5HT column (length, 30 m; ID, 0.32 mm; film thickness, 0.25 µm), and the oven temperature was programmed from 50°C to 110°C at 40°C min^{-1} and then from 110°C to 360°C at 20°C min^{-1}. Samples were injected in splitless mode, with helium as the carrier gas, at a constant flow rate of 1.0 ml min^{-1}. CHCs were characterized by using standard mass spectrum databases, diagnostic ions, and Kovats indices. Where compounds co-eluted resulting in a single peak that contained two or more compounds, the proportion of each compounds' characteristic ions was calculated using the ion extract function. The original

peak area was then divided into the relative proportions obtained from the ion extract function to obtain an estimate of the amount of each compound in the peak. If the abundance of any compound was consistently below 0.5% across all samples, it was excluded from the analysis.

1.3 Statistical analysis

Typical total ion chromatograms (TICs) are shown for each parasitized colony with the parasite TIC inverted to facilitate direct comparisons of the profiles. This allows species and colony differences to be clearly seen. A list of CHCs detected (mean percentage ± *SD*) from each group of ants is based on the proportion of each compound relative to all the compounds detected. However, for the *Camponotus* samples the di-methyl-alkanes were separated out from all other compounds and the percentage of each di-methyl-alkane calculated against only the other di-methyl-alkanes for each ant. These were then averaged across each colony, considering the host and parasite separately.

Comparisons between the absolute amounts of CHCs extracted from hosts compared to their parasitized and un-parasitized hosts were performed using non-parametric Kruskal-Wallis tests followed by post-hoc Mann-Whitney *U* tests if required, since the data were not normally distributed.

To investigate if the parasites and host workers had different profiles we subjected the *Camponotus* data to three different multivariate statistical methods. We also compare their performances as the analysis of multi-dimensional data can be complex and biased. Firstly, the commonly used Discriminant Function Analysis (DA) was employed. The data for each individual ant were placed into one of three groups (un-parasitized hosts, parasitized hosts and parasites). The percentage of each compound was calculated against all CHCs for that individual. As this generates a compositional data set the values are not independent and were transformed using the following standard method of Aitchinson (1986):

$$Zi,j = \mathrm{Ln}[Yi,j/g(Yj)]$$

Where Yi,j is the area of peak i for the individual j, $g(Yj)$ the geometric mean of the areas of all peaks for individual j, and Zi, j the transformed area of peak i for individual j. The transformed data values were then analysed using the Discriminant Function Analysis tool in SPSS version 20 (IBM Corp).

Secondly we used a non-metric Multidimensional Scaling (NMDS) method based on the Bray-Curtis dissimilarity distances using the untransformed data to assess overall ranking of pairwise dissimilarities. NMDS does not rely on the assumptions of normality of multi-dimensional data, thus having the advantage of omitting any distributional assumptions required for other methods (McCune and Grace, 2002) and pre-assumed hypothesis-driven groupings such as those used in discriminant analysis which can bias the final result. This was performed in R 3.0.2 (R core team, 2013) using the vegan package (Oksanen et al., 2013).

Finally we used the nest centroid discriminant analysis method (Seifert et al., 2013). This new approach is an unconventional, hypothesis-free application of linear discriminant analysis (LDA). Instead, each nest sample i.e. colony, composed of *n* individual ant workers is treated as a separate class. The DA computes the mean (centroid) for each sample (which is based on *n* individuals). This creates a multidimensional distance matrix between group centroids of nest samples and can be used as input data for additional clustering methods (Seifert et al., 2013). This nest centroid method can be applied to all cohesive systems providing repeats of definitely conspecific elements e.g., related nest mates of a single colony or leaves and flowers of the same plant (Seifert et al., 2013). The benefit is that no pre-imposed species hypotheses are placed upon the computation of the DA.

2 Results

As expected, each of the four host species had a highly distinctive and unique CHC profile (Fig.1). Three species (*T. cf. impurum, T. semilaeve* and *L. acervorum*) had CHC profiles that are very rich in alkene and alkadiene isomers (Table 1), whereas, *C. aethiops* was dominated by mono- and di-methyl-alkanes (Table 1). Despite these differences, in each case the parasites' CHC profile was indistinguishable from that of its hosts' profile (Fig. 1), even when the parasite (e.g. *S. testaceus*) is using more than one host species. This mimicry even extended to smaller colony-level differences (Fig.2). For example, the presence of 5,17-di-MeC_{31} was produced by only two out of the eight *L. acervorum* colonies, but its presence or absence was also mirrored in the parasites' profiles, as were subtle changes in the ratios of mono-and di-methyl-alkanes in different *Camponotus* colonies (Fig. 2). Focusing on di-methyl-alkane profiles of *Camponotus* revealed small but distinctive differences in their ratios that were consistent between nest-mates but different between colonies (Fig. 3). Again these small colony differences are matched by the parasites living in those colonies. In all cases the profiles of the un-parasitized colonies were similar to those that were parasitized (Fig. 1) indicating that it is the parasites that have adjusted their profile to

Table 1 A list of cuticular hydrocarbons and their proportions (mean percentage ± *SD*), detected from un-parasitized host (UPH), parasitized hosts (PH) and the parasites (P) for four different parasite-host species associations

		T. impurum		*S. testaceus*	*T. semilaeve*		*S. testaceus*	*L. acervorum**		*H. sublaevis*	*C. aethiops*		*C. universitatis*
		UPH = 4	PH = 2	P=2	UPH = 7	PH = 1	P=1	UPH=5	PH=3	P=3	UPH=9	PH=11	P=13
1	$C_{23:1}$	0.5±0.3	0.4±0.2	0.4±0.0									
2	C_{23}	7.5±2.1	8.3±1.7	8.3±3.2	5.6±2.2	2.9	6.8		0.2±0.3	2.5±2.9			
3	$9MeC_{23}$	0.6±0.2	1±0.6	1±0.5									
4	$5MeC_{23}$	0.4±0.1	0.4±0.0	0.5±0.2									
5	$3MeC_{23}$	**20.4±11.1**	**30.7±2.3**	**21.5±3.9**	0.3±0.2								
6	C_{24}	0.3±0.1	0.4	0.6±0.5	0.8±0.2	0.4	0.9						
7	$3,7diMeC_{23}$	0.2	0.4±0.1	0.3±0.1									
8	$10,12MeC_{24}$	0.2±0.1	0.3±0.1	0.5±0.4									
9	$2MeC_{24}$										0.6±0.2	0.6±0.2	0.6±0.1
10	$C_{25:1}$(2-isomers)	**41.6±20.9**	**21.8±4.3**	**18.9±16.5**	3.8±1.2	5.5							
11	C_{25}	6.2±3.6	6.0±0.6	6.8±3.7	14.4±4.0	14.5	13.7	0.1±0.1	1±0.7	3.8±3.8	0.9±0.3	0.8±0.2	1±0.3
12	$13MeC_{25}$										1.9±0.1	2±0.5	2.2±0.6
13	$11MeC_{25}$										0.9±0.1	1±0.3	1.2±0.3
14	$9MeC_{25}$	5±5	7.4±2.7	6.2±0.2	0.7±0.2				1.1±1.6	2.4±2.9	0.9±0.3	0.9±0.3	1.1±0.4
15	$7MeC_{25}$	1.8±0.6	3.3±1.2	3.8±1.4	1.6±1.0	1	1.5				2.6±0.6	2.2±1.2	2.3±1.3
16	$5MeC_{25}$	7.3±3.9	9.9±3.6	11.1±0.8	4.0±1.0		20.9		0.4±0.6	0.7±0.8	0.2±0.1	0.2±0.2	0.2±0.1
17	$3MeC_{25}$	2.5±2.7	2.8±0.6	4.7±2.2	3.5±1.0	2.3	2.3	0.1±0.1	1.1±1.3	2±2.3			
18	$9,13diMeC_{25}$										1.5±0.3	2.1±0.8	2.2±0.7
19	$7,11diMeC_{25}$										0.8±0.1	0.7±0.2	0.8±0.2
20	$5,11+5,9diMeC_{25}$										0.1	0.2±0.1	0.2±0.1
21	$5,17diMeC_{25}$	3.5±2.1	4.6±0.2	7.7±1.7							0.1±0.1	0.4±0.3	0.5±0.3
22	C_{26}				0.7±0.2	0.6	0.7				0.9±0.3	0.6±0.2	0.7±0.2
23	$3,11+3,13+3,7diMeC_{26}$										0.3±0.0	0.3±0.2	0.4±0.2
24	$10,12,14MeC_{26}$										2.1±0.4	1.8±0.3	2±0.3
25	$6,8MeC_{26}$										0.9±0.1	0.9±0.2	0.9±0.2
26	$2MeC_{26}$										8.4±1.0	7.5±0.8	7.4±0.7
27	$C_{27:2}$ (3 isomers)				2.8±1.2	0.3							
28	$C_{27:1}$				**20.5**±4.7	**28.7**	**16.5**						
29	$C_{27:1}$				**18.1**±2.3	**28.3**	**16.5**	1.3±0.7	2.4±1	1.1±0.1			

Continued Table 1

		T. impurum		*S. testaceus*	*T. semilaeve*		*S. testaceus*	*L. acervorum**		*H. sublaevis*	*C. aethiops*		*C. universitatis*
		UPH = 4	PH = 2	P=2	UPH = 7	PH = 1	P=1	UPH=5	PH=3	P=3	UPH=9	PH=11	P=13
30	C_{27}	0.5±0.2	0.7±0.3	2.1±1.5	3.5±1.1	3.5	17.9	1±0.3	1.8±1.1	3.2±3	4.3±1.1	3.4±0.6	3.9±1.5
31	$13MeC_{27}$										**9.1±0.7**	**8.7±0.9**	**9.1±0.8**
32	$11MeC_{27}$										6.1±1.3	5±1.2	5.4±1.0
33	$9MeC_{27}$	0.5±0.1	0.7±0.1	1.3±0.1	2.4±0.6	1.7	1	0.3±0.3	0.5±0.4	1±0.1	**9.5±1.2**	**9.9±1.1**	**9.9±1.5**
34	$7MeC_{27}$				2.9±0.8	1.9	1.7	0.1±0.0	0.3±0.3	0.4±0.4	5.3±1.7	4±0.8	4.5±1.2
35	$5MeC_{27}$				1.4±0.5	0.5	0.5	0.1±0.1	0.3±0.3	0.4±0.2	0.8±0.3	1.3±0.7	1.5±0.6
36	$3MeC_{27}$	0.4±0.2	0.8±0.2	2.8±1.7	2.0±0.7	2.7	6.1	3.5±1.4	4.1±0.8	3.2±1.5			
37	$5,17diMeC_{27}$	0.9±1.3	0.5±0.1	1.4±0.3									
38	$11,15diMeC_{27}$										**9.7±1.5**	**9.3±2.2**	**8.5±2.1**
39	$9,13diMeC_{27}$										**7.7±0.8**	**8.9±1.0**	**8.0±1.0**
40	$7,15diMeC_{27}$										2.6±0.3	2.9±0.4	3.1±0.4
41	$5,9+5,15diMeC_{27}$										0.8±0.1	1.1±0.3	1.2±0.3
42	$5,7,17triMeC_{27}$										0.5±0.1	0.6±0.1	0.6±0.1
43	C_{28}										0.9±0.4	0.5±0.1	0.6±0.2
44	$3,11+3,13+3,7diMeC_{28}$										2.2±0.4	1.6±0.9	1.5±0.8
45	$10,12,14MeC_{28}$										2.2±0.3	2.5±0.6	2.2±0.7
46	$7MeC_{28}$										0.6±0.2	0.7±0.2	0.6±0.2
47	$10,14+12,16diMeC_{28}$										1.4±0.3	1.6±0.4	1.3±0.4
48	$7,13diMeC_{28}$										0.4±0.1	0.5±0.1	0.5±0.2
49	$4,12+4,10diMeC_{28}$										0.2±0.0	0.3±0.2	0.3±0.1
50	$C_{29:2}$ (3 isomers)				8.9±4.6	1.8		3.8±0.9	4.3±1.0	3.0±0.4			
51	$C_{29:1}$				1.1±0.8	1.3		**30.7±4.2**	**30.3±1.9**	**25.8±5.8**			
52	C_{29}				0.2±0.1	1.1		1.1±0.2	1.6±0.5	1.6±1.1	1.0±0.4	0.9±0.3	1.8±1.2
53	$13MeC_{29}$										1.3±0.3	1.5±0.3	1.2±0.3
54	$11MeC_{29}$							0.2±0.1	0.4±0.2	0.8±0.1	2.4±0.9	3.1±1.3	2.3±1.1
55	$9MeC_{29}$				0.5±0.2			0.3±0.1	0.5±0.2	0.6±0.1	1.6±0.4	2.0±0.4	1.6±0.4
56	$7MeC_{29}$										2.5±0.5	2.8±0.3	2.6±0.3
57	$3MeC_{29}$							2.5±0.4	2.3±0.5	1.8±0.3			
58	$11,15diMeC_{29}$										0.9±0.2	1.0±0.6	0.9±0.4
59	$9,13diMeC_{29}$										0.6±0.2	0.7±0.2	0.4±0.1

Continued Table 1

		T. impurum		*S. testaceus*	*T. semilaeve*		*S. testaceus*	*L. acervorum**		*H. sublaevis*	*C. aethiops*		*C. universitatis*
		UPH = 4	PH = 2	P=2	UPH = 7	PH = 1	P=1	UPH=5	PH=3	P=3	UPH=9	PH=11	P=13
60	7,17diMeC$_{29}$										1.9±0.5	2.5±0.7	2.1±0.5
61	5,17dimeC$_{29}$										0.3±0.1	0.8±0.4	0.8±0.5
62	C$_{31:2}$ (4 isomers)							**8.9±2.2**	**7.0±1.1**	**6.4±2.5**			
63	C$_{31:1}$							**14.9±1.7**	**11.5±3.3**	**11.8±5.0**			
64	9,11,13MeC$_{31}$							0.9±0.3	1.5±0.2	2.1±0.2			
65	3MeC$_{31}$							0.8±0.3	0.4±0.2	0.4±0.3			
66	C$_{33:2}$ (4 isomers)							**14.0±4.8**	**10.3±2.9**	**8.5±3.3**			
67	C$_{33:1}$							1.8±0.4	1.6±0.4	1.2±0.5			
68	9,11,13MeC$_{33}$							0.9±0.3	1.6±0.2	1.7±0.3			
69	11,15+11,21diMeC$_{33}$							2.4±0.6	3.4±1.2	3.7±0.4			
70	C$_{35:2}$ (2 isomers)							4.5±1.2	3.4±0.8	2.8±1.4			
71	?MeC$_{35}$							0.2±0.0	0.5±0.1	0.5±0.1			
72	11,23diMeC$_{35}$							0.6±0.1	1.3±0.5	1.8±0.4			
73	C$_{37:2}$							1.2±0.3	1.1±0.4	0.9±0.5			
74	11,xdiMeC$_{37}$							0.3±0.2	0.6±0.3	0.8±0.1			
75	C$_{39:2}$							1.7±0.9	1.5±0.9	1.3±0.8			
76	?MeC$_{39}$							0.6±0.3	0.8±0.4	0.9±0.0			
77	?MeC$_{39}$							1.2±0.7	1.0±0.6	0.8±0.7			

* The CHC profile in two out of the eight L. *acervorum* colonies contained 5,17diMeC$_{31}$.

The value indicates the number of pooled samples of 20 workers (*T. impurum, T. semilaeve and S. testaceus*); 10 workers (*L. acervorum and H. sublaevis*) or individual ants (*C. aethiops* and *C. universitatis*).

The most abundance compounds in each group are shown in bold.

match that of their host and not *vice versa*. The profiles of the female *C. universitatis* alates (new queens) were the same as the *C. universitatis* workers so were not treated separately. There was no significant difference in the total abundance of ions (CHC amount) between the *H. sublaevis* parasite and *Leptothorax acervorum* irrespective of whether they were parasitized or not (H[2]=4.19, P=0.12). However, the amount of CHCs in the *Camponotus* parasites were significantly lower than that of their hosts (U =143, z= 4.17, P <.0001), but there was no significant difference between parasitized and non-parasitized *L. acervorum* workers (U= 32, z=1.36, P=0.08). There were insufficient samples to test the *Strongylognathus* parasites.

Despite the almost indistinguishable profiles between the various hosts and their parasites (Fig. 1), even down to the colony level (Fig. 3), we investigated the *Camponotus* data set using three multivariate statistical methods. Firstly the standard DA analysis indicated clear separation into the three groups i.e. un-parasitized host workers, parasitized host workers and their parasites (Fig. 4A). The DA was able to successfully classify 54.5% of colonies into the correct groups (cross validated) compared to the proportional by-chance accuracy rate of 34.1%. Non-parasitized *C. aethiops* colonies were classified with better accuracy (88.9%) compared to parasitized *C. aethiops* colonies (54.5%) and *C. universitatis* parasites (30.8%). Due to the low classification accuracy we cannot interpret the discriminant model with confidence. The MDS ordination did not

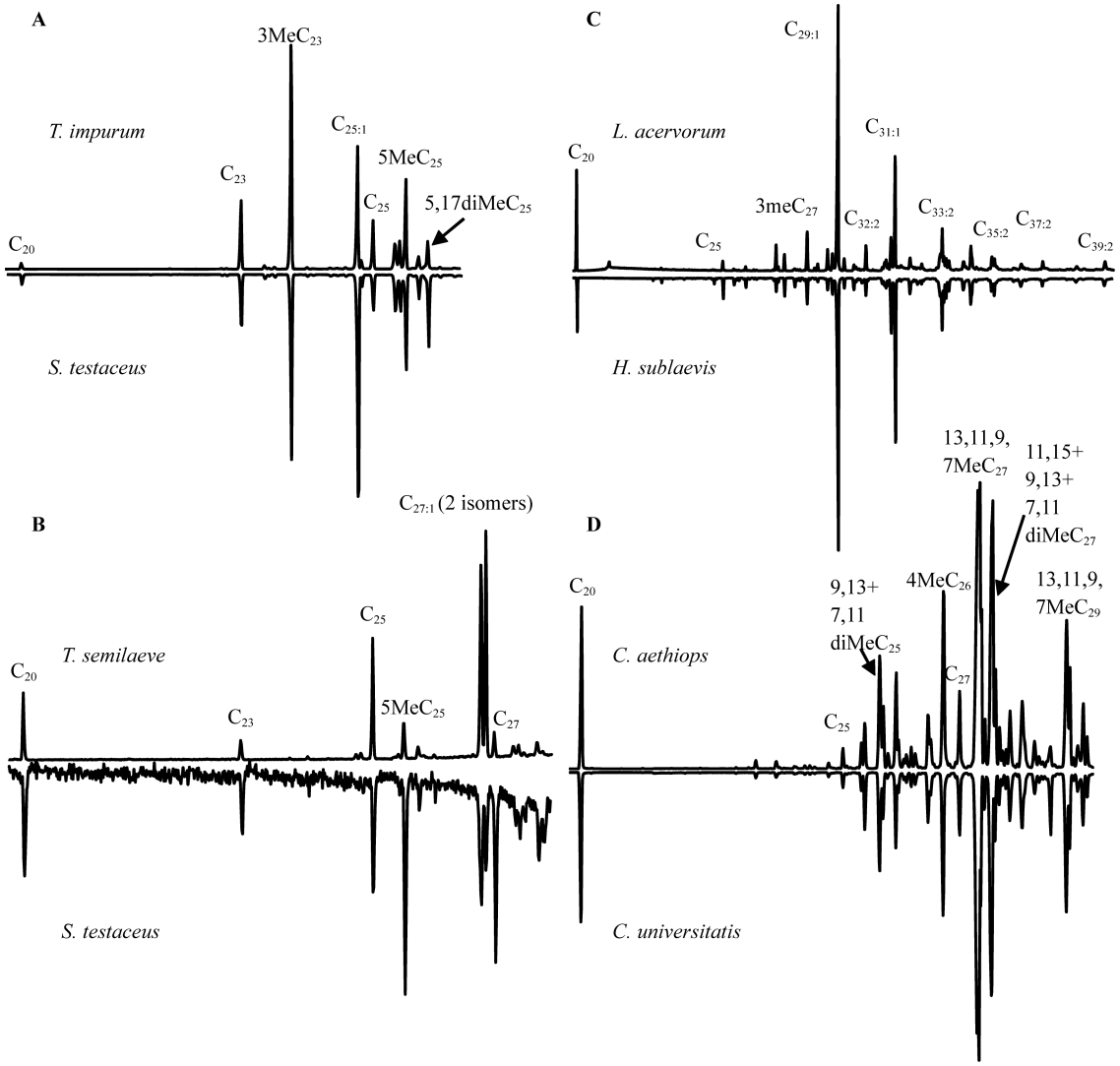

Fig. 1 Total ion chromatograms (TICs) of the four different ant species (upper profiles) and from the same colony their social parasites (inverted 'mirror' profiles)

A. *Tetramorium impurum* and *Strongylognathus testaceus*. **B**. *T. semilaeve* and *S. testaceus*, **C**. *Leptothorax acervorum* and *Harpagoxenus sublaevis*. **D**. *Camponotus aethiops* and *Camponotus universitatis*. The major peaks are labelled, C_{20} is the internal standard. All compounds are given in Table 1. Despite each host species having a very different profile in all cases the parasites mirror the hosts profile.

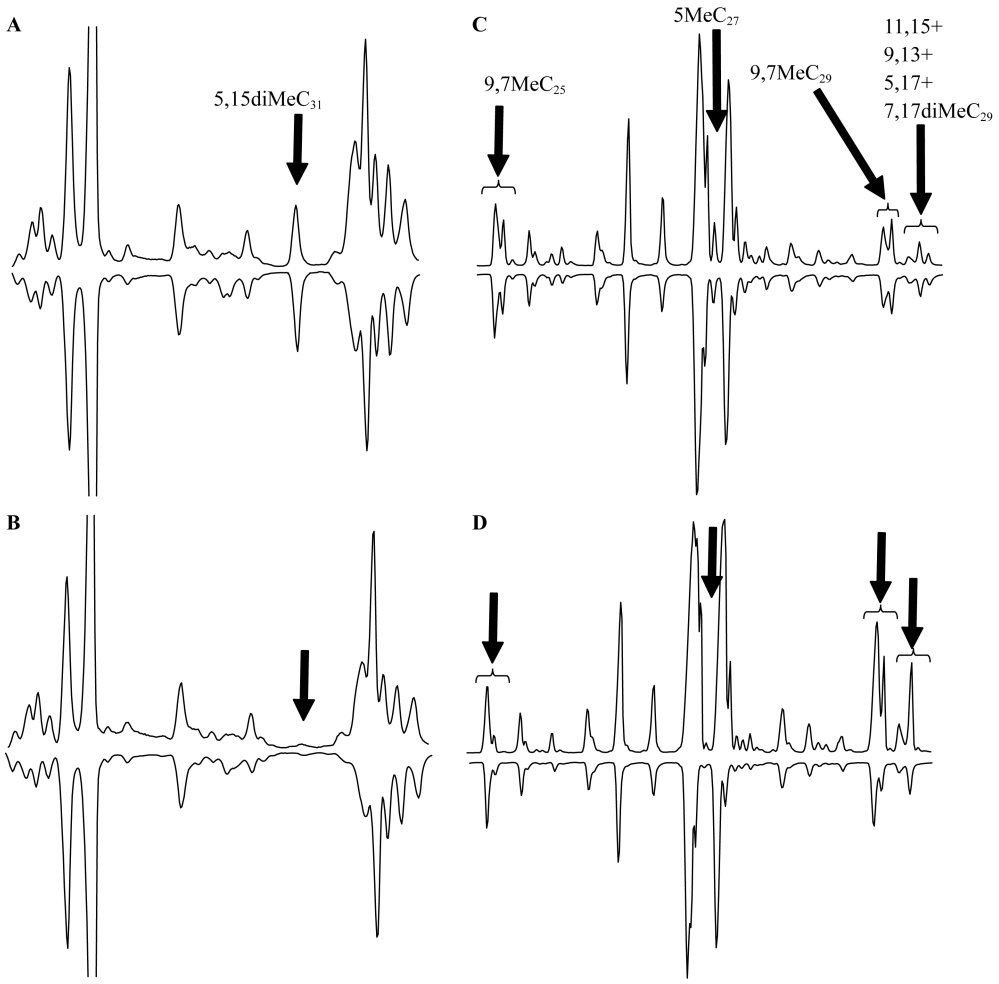

Fig. 2 Shows a part of the profile to indicates how small colony differences, indicated by arrows of ratios of certain compounds are mimicked by their parasites (invert profile) (A, B) *Leptothorax acervorum* and (C, D) *Camponotus aethiops*

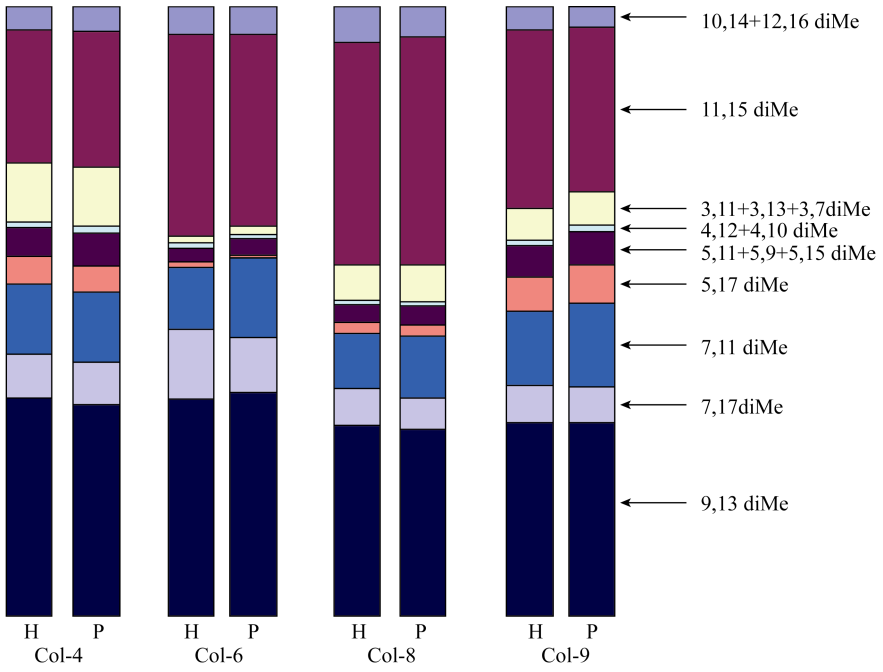

Fig. 3 Colony specific proportions of di-methyl-alkanes in four *Camponotus aethiops* colonies and how these are mirrored by the profiles of their parasites *C. universitatis* that inhabit these colonies

produce the distinctive groupings seen in the DA, and this is reflected in the relatively high STRESS value of 0.123 (Fig. 4B). This suggests that the data between non-parasitized hosts, parasitized hosts and parasites are extremely similar. However, some level of grouping can be observed where parasites display chemical profiles more similar to their respective host colonies than other non-host colonies (Fig.4B). The DA based on generated nest means (centroids) pair up the parasites and hosts from the same colony (Fig. 4C). However, the DA was only able to successfully classify 9.1% of colonies into the correct groups, compared to the proportional by-chance accuracy rate of 9.7%. Therefore, the criteria for classification accuracy is also not satisfied and we cannot interpret the model. The lack of successful classification may be due to the very small sample size (i.e. only three individuals from each nest were analysed) or the fact that all profiles are extremely similar in their di-methyl-alkane abundance.

3 Discussion

It is now becoming well established that each ant species has a unique species-specific CHC signature (Martin and Drijfhout, 2009a) that remains very stable even across large geographical areas (Guillem et al., 2012; Berville et al., 2013). Previous studies have analyzed the CHC profiles of *L. acervorum* (Kaib et al., 1993; Tentschert et al., 2002) and *C. aethiops* (Bos et al., 2012) from three different regions of Germany (*L. acervorum*) and Central Italy (*C. aethiops*). Not surprisingly, qualitatively their CHC profiles were almost the same as the samples collected from Finland (*L. acervorum*) and Spain (*C. aethiops)* used in this study. Some differences are present especially in the minor compounds, but this is often due to different detection limits and difficulties in compound identification. However, the main features of the CHC profile are always amazingly similar e.g. large amounts of $C_{29:1}$, $C_{31:2}$

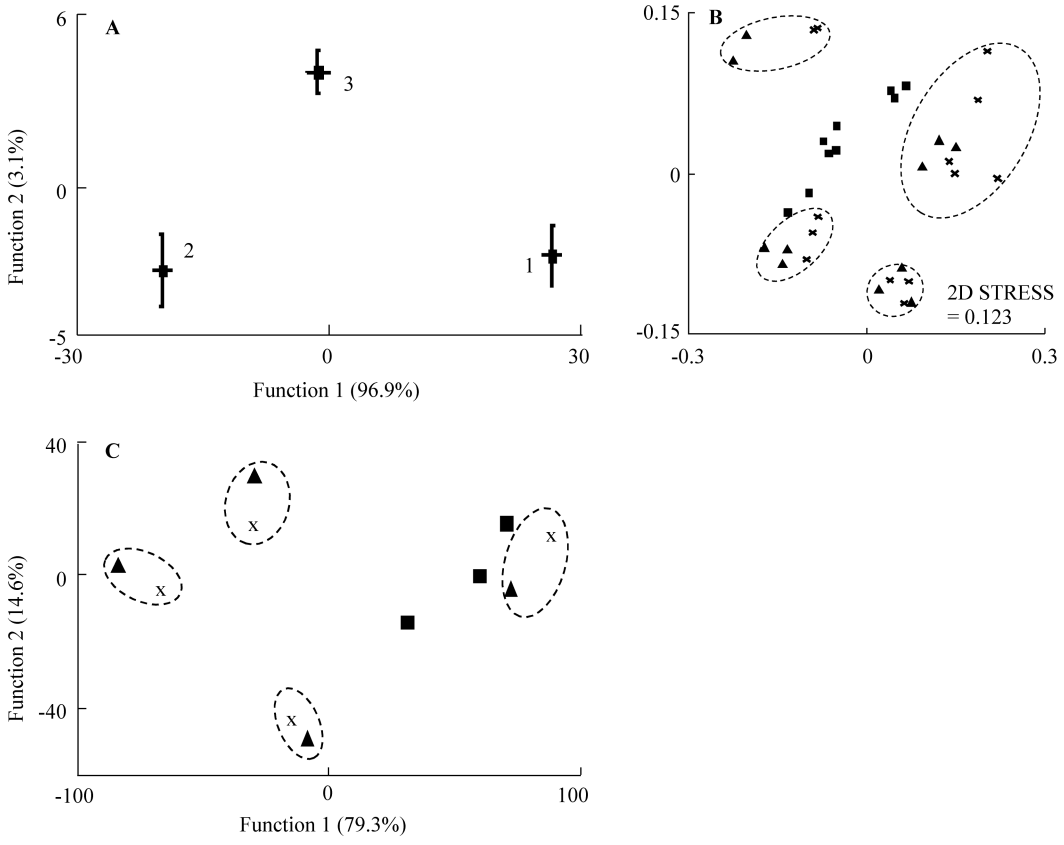

Fig. 4 The *Camponotus* di-methyl-alkane data analysed by A) Discriminant Function analysis. Group centroids ± *SD* of discriminant function analyses are shown. 1 = non-parasitized colonies of *C. aethiops*; 2 = parasitized colonies of *C. aethiops*; 3 = *C. universitatis* parasites. B) Non-metric multidimensional scaling (NMDS) ordination derived from the raw data. Squares = non-parasitized colonies of *C. aethiops*; triangles = parasitized colonies of *C. aethiops*; crosses = *C. universitatis* parasites. Colonies of host and equivalent parasite are enclosed in ellipses. C) Nest-centroid discriminant function analyses squares = non-parasitized colonies of *C. aethiops*; triangles = parasitized colonies of *C. aethiops*; crosses = *C. universitatis* parasites. Dashed lines indicate groupings of parasite with its original host colony.

and $C_{33:2}$ isomers in *L. acervorum* and a very large numbers of di-methyl-alkanes in *C. aethiops*.

Despite these unique species differences, all three parasitic species had CHC profiles that were indistinguishable from that of their host (Fig. 1 and 4B). The level of chemical mimicry even extended to the more subtle colony level differences (Fig. 2, 3 and 4C). This explains why these social parasites are fully integrated members of each colony and are treated as nest-mates. It also helps to explain why raiding parties of *Harpagoxenus* workers are frequently killed or driven off when trying to raid or invade new host *L. acervorum* colonies (Winter and Buschinger, 1986), since they are carrying their own host colony odour, which is likely to be different from that of the one they are raiding. This is why parasites continue to use a wide range of other chemical and morphological adaptations associated with their parasitic lifestyle. These include a thickened cuticle and production of appeasement or propaganda compounds (e.g. Allies et al., 1986; Lloyd et al., 1986; Ollett et al., 1987; D'Ettorre et al., 2000). These tactics allow the parasite time to make the necessary adjustments to its profile. Acquiring a host profile may be possible in just a few hours (R. Kather, pers. comm.).

The majority of ant social parasites have a very restricted (one to three) host range, with the exception of some xenobiotic species such as *Formicoxenus nitidulus* that inhabit colonies of several different wood-ant (*Formica*) species. However, unlike other social parasites, *F. nitidulus* uses a chemical deterrent rather than chemical deception to remain in the colony (Martin et al., 2007). In cases where the parasite has multiple host species they are always closely related (Emery, 1909; Hölldobler and Wilson, 1990). Therefore, it was assumed that they must have compatible communication systems (Buschiner, 2009). This is the case in *Protomognathus americanus*, whose two hosts *Temnothorax longispinosus* and *T. curvispinosus* are chemically almost identical (Brandt et al., 2005). However, other parasites are able to mimic very different host chemical profiles. For example, *H. sublaevis* is able to mimic the olfin (alkene and diene) rich profile of *L. acervorum* and the di-methyl-alkane rich profile of *L. muscorum* (Kaib et al., 1993; Bauer et al., 2010). Furthermore, when *H. sublaevis* nests contain both *L. acervorum* and *L. muscorum* slaves, the parasite's profile contains compounds of both species (Heinze et al., 1994; Brandt et al., 2005). In this study we found that *S. testaceus* can mimic both the 3-MeC_{23} and $C_{25:1}$ rich *T. impurum* and the $C_{27:1}$ rich *T. semilaeve* (Table 1). The phylogenetic relationships between the ants is not paralleled by their CHC profiles (Martin and Drijfhout, 2009a) so sister species can either have very similar or very different CHC profiles so the role of Emery's rule may have been over interpreted in the evolution of host-parasite relationships. Chemical mimicry occurs regardless of relatedness as demonstrated by the many Myrmecophiles that inhabit ant colonies despite often belonging to very different groups such as mites, butterfly larvae and adult beetles (Hölldobler and Wilson, 1990). Sanetra and Buschinger, (2000) studied the phylogeny of *Tetramorium* and some of its ant social parasites. They concluded that their results do not support Emery's rule in the strict sense since none of the parasites (*Stronglognathus*, *Anergates* or *Teleutomyrmex*) were sister species of their particular *Tetramorium* host species. The phylogenetic relationship between *C. universitatis* and *C. aethiops* currently remains unknown.

Johnson et al. (2001) found that prior to invasion, the CHC profile of the queen of the parasitic ant *Polyergus breviceps* was composed almost exclusively of *n*-alkanes, compounds known to be very difficult to detect relative to other CHCs (Chaline et al., 2005; Dani et al., 2005). Therefore, the parasite was effectively invisible and only after killing the host queen did it match the profile of its hosts *Formica gnava* or *F. occulta*, which contain 9 or 21 di-methyl-alkanes respectively (Johnson et al., 2001). This supports the earlier study of Yamaoka (1990), who found that the CHC profile of newly emerged *Polyergus samurai* workers consisted only of *n*-alkanes and within 10 days matched the profile of their host species; the alkene rich *F. japonica* or the alkadiene rich *Formica sp*. 5. It remains unknown whether all ant parasite queens use this chemical invisibility method to invade their hosts since if new queens matched their natal colonies prior to invading a new host colony, this would lead to the formation of gentes i.e. each gens (a sub-population) specializing in a particular host as occurs in avian cuckoos (Gibbs et al., 2000). However, *C. universitatis* new queens (alates) match their host's profile before leaving the nest to find new colonies to invade so chemical invisibility in this case is not been used.

The ability of social parasites to match their host profiles almost perfectly, seen in this and previous studies, suggests that they are acquiring their profiles from their hosts (i.e. chemical camouflage). Producing the wide range of host compounds needed (especially in the multi-host systems) requires the ability to biosynthesise many different compounds. This is supported by Ya-

maoka (1990), who found that after 14 days in isolation *Polyergus samurai* workers reverted back to a simple *n*-alkane profile. However, when *P. rufescens* was reared in isolation it produced the full range of host compounds, so in this case none were acquired from its host (Bonavita-Cougourdan et al., 2004). A similar phenomenon is seen in the parasitic bumblebee (*Psithyrus*) where the queens already produce their hosts' CHC profile prior to invading their host's colony (Martin et al., 2010). Further evidence for true chemical mimicry (synthesis) comes from the ability of *P. rufescens* to actively change its CHC profile when it is artificially switched between its two host species (Bonavita-Cougourdan et al., 1997). In addition, queens of the parasitic paper wasps *Polistes sulcifer* and *P. atrimandibularis* are able to alter synthetic pathways since the proportion of 9-, 15-di-methylC_{29} in *P. sulcifer* drops drastically after usurpation, while in *P. atrimandibularis* a whole suite of compounds disappears after colony takeover, only to reappear later in the colony cycle (Bagnères et al., 1996).

So it appears that social insects have evolved several mechanisms of chemical deception that allow them to invade and then blend into their host colonies. The high level of mimicry was expected in the inquiline *C. universitatis* as this form of parasitism is predicted to be the evolutionary end-point. However, the same level of mimicry was also found in the semi-inquiline *S. testaceus* and the slave making *H. sublaevis,* although it remains a mystery whether these parasites are able to synthesise a wide range of CHCs or become chemically invisible, acquiring their host's profile at a later stage.

Finally, using the same *Camponotus* data we subjected them to three different multivariate methods that produced two very different, apparently conflicting results (Fig. 4). The DA shows very clear groupings that are not present in the NMDS. These differences arise due the pre-assumed hypothesis-driven groupings used in discriminant analysis and a low sample to variable ratio (Mitteroecker and Bookstein, 2011). That is, Mitteroecker and Bookstein (2011) demonstrated that when the number of variables exceeds the number of samples, group affiliation is predicted exactly and the samples collapse to a single point per group in the canonical variates. So if the number of variables is sufficiently large, which is very common in CHC studies, the canonical variates separate the groups regardless of the actual group distributions. This is a statistical artefact without any scientific meaning (Mitteroecker and Bookstein, 2011). As individual compounds i.e. variables within a CHC profile are often highly correlated, which is common (Martin and Drijfhout, 2009b), this statistical grouping artefact can still occur even when the number of variables is less than the number of samples, because the last few eigenvalues of a covariance matrix often are negligibly small even when non-zero (Mitteroecker and Bookstein, 2011). Therefore, DA should be avoided as an analytical method where the sample to variable ratio is not at least 5–10 samples for each variable. NMDS has the advantage that no pre-assumed hypothesis-driven groupings are inferred (as used in a DA) with all the values treated as individual points. NMDS can be used when the data violate the assumptions of other multivariate methods and do not assume multivariate normality or linearity. Various distance measures can be applied, and although we use Bray-Curtis in this study, Euclidean distances produced a very similar ordination plot and associated stress value, with Bray-Curtis performing just a little better (i.e. slightly lower stress value). The use of nest centroids to create a DA also has the benefit of not imposing any pre-formed species hypotheses or forcing the data into pre-defined groupings which can bias the final results (as seen in Fig. 4A using the standard DA) as each colony is treated as a separate group. We recommend the use of NMDS for analysing large chemical data sets or the nest centroid DA method, providing repeatable samples (at least 5–10) can be analysed per colony. The latter method may be more suited to data that are very similar if colony level differences are to be investigated. Additionally, this method is advised for assessing cryptic species based on morphological characters (Seifert et al., 2013), but here we utilise it for analysing chemical data.

The hypothesis-free nest centroid discriminant analysis (Seifert et al., 2013), and to a lesser extent the NMDS, 'supports' the descriptive data the best i.e. that each colony has a different CHC profile, but those of parasites and their hosts have the closest match. However, it must be noted that our sample size was very low, and would benefit from the addition of further individuals. Much of the chemical ecology of social insects is based on the interpretation of multivariate statistical results, especially PCA and DA, and we often fall into the common trap of using the results to 'support' our ideas rather than for 'illumination'.

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