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RESEARCH ARTICLE

Acclimation in ants: Interference of communication and waterproofing through cuticular hydrocarbons in a multifunctional trait

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Abstract

1. Organismal traits may experience conflicting selection pressures if they fulfil different functions simultaneously. This can require trade-offs between functions or alternatively functional separation between elements of the trait.
2. An important multifunctional trait in insects is the cuticular hydrocarbon (CHC) layer. CHCs cover the body of nearly all insects, protect against desiccation and serve as a communication signal. In social insects like ants, they provide cues for nestmate recognition. To maintain their waterproofing function, insects have to adjust CHC composition to current temperatures. These changes might affect information content and interfere with communication, which would be especially detrimental in social insects.
3. Here, we studied how acclimation affects nestmate recognition in two sister species of the ant genus *Lasius*. Colony fragments were exposed to three climate regimes. We analysed behaviour towards same and differently acclimated conspecifics, and determined which CHCs were related to acclimatory changes, colony differences and inter-individual aggression.
4. Differential acclimation led to higher aggression and chemical distances among former nestmates. We identified small CHC subsets, which only differed among colonies or among acclimation treatments. Moreover, few compounds sufficed to explain inter-individual aggression, suggesting that ants do not use the entire CHC profile for nestmate recognition and that colony identity is encoded in a redundant way.
5. Across individual CHCs, their contribution to colony differences and to differences among acclimatory treatments was negatively correlated, indicating that there is some degree of functional separation. However, CHC classes could not be clearly assigned to one or another function, indicating that the role of each CHC is idiosyncratic and may differ among species. Acclimatory effects and colony differences were more independent from each other in *L. platythorax* than in *L. niger*, indicating that functional separation can differ even among sister species.

6. Our results show that CHC functions are more intertwined than previously assumed, suggesting that insects cannot optimise all functions independently. The main constraint might be the need to maintain a certain phase behaviour of the CHC layer, which depends on CHC composition and affects functionality. The need to separate functions might depend on species-specific ecological and life-history parameters.

KEYWORDS
acclimation, aggression, climate adaptation, cuticular hydrocarbons, Formicidae, nestmate recognition

1 | INTRODUCTION

An organism's traits are supposed to be selected for optimal functionality. However, this seemingly straightforward approach becomes hard to realise if a single trait fulfils multiple functions. For each function, different trait states may be optimal, and thus need to be traded off against each other. A commonly known trade-off are sexually selected traits, which often represent a handicap for its bearer—for example, male ornaments in birds are attractive to females, but enhance their predation risk (Zahavi, 1975). The conflicting selection pressures lead to a trade-off between natural and sexual selection. Multiple selection pressures arising from different functions also affect beak shapes in birds, which are important for foraging, singing and thermoregulation (Friedman et al., 2019). In plants, the bark of trees fulfils a wide range of functions, including mechanical stability, fire protection, transport of metabolites and in some cases even photosynthesis (Rosell, 2019). Conflicting requirements can either be solved by trade-offs between functions, or, preferably, by separation of the functions, for example, if they are fulfilled by different elements of that trait.

In insects, an important multifunctional trait is the cuticular hydrocarbon (CHC) layer. Being part of the integument, CHC layers protect against water loss (presumably the ancestral function), but also serve as communication signals (Blomquist & Ginzel, 2021). The information encoded in a CHC profile includes species membership, sex, health state and others (Sprenger & Menzel, 2020). CHCs are also under sexual selection in several insect species (Steiger & Stökl, 2014). Each of these functions may cause evolutionary or plastic changes that enhance functionality. For example, optimal waterproofing requires constant adjustment during an insect's lifetime (Hadley, 1994; Menzel et al., 2018; Sprenger et al., 2018). This is due to temperature changes causing CHCs to melt (Gibbs & Rajpurohit, 2010; Menzel et al., 2019), which can increase (undesirable) water permeability of the CHC layer (Rourke & Gibbs, 1999). However, it is hardly known how these changes affect information content.

In social insects like ants, CHCs encode much additional information (Dietemann et al., 2003; Sprenger & Menzel, 2020; van Zweden & d'Ettorre, 2010), which is vital to ensure colony functioning. In particular, the ability to recognise nestmates and distinguish them from non-nestmates is indispensable to maintain colony integrity. It allows them to show altruism in the form of care towards nestmates, while rejecting foreign individuals (Leonhardt et al., 2016). Upon encountering another individual, an ant compares the odour of its opponent to the neuronal template of the own colony odour, and only accepts the other one if they match. Aggression is not a discrete variable, but increases gradually upon mismatch with the template (Sturgis & Gordon, 2012; Tanner & Adler, 2009). However, it is still largely unknown which compounds are actually important for recognition, and whether the same set of recognition cues is used by multiple species (as hypothesised by Sturgis & Gordon, 2012).

Although CHC profiles are largely genetically determined (van Zweden & d'Ettorre, 2010), their quantitative composition is relatively plastic (Frizzi et al., 2015; Otte et al., 2018); for example, they can be influenced by nutrition (Otte et al., 2014). Insects can also adjust their CHC profile to the current temperature to maintain its waterproofing ability. These temperature-dependent effects are largely predictable, in the way that warm temperatures often trigger a higher production of compounds with high melting points (Sprenger et al., 2018). Waterproofing requirements vary with temperature due to CHC melting and changes in vapour pressure (Rourke & Gibbs, 1999; Sprenger et al., 2018). However, CHC changes might come along with a change in information content, and hence interfere with nestmate recognition. Currently, it is hardly known how this conflict is solved, and whether optimisation of waterproofing interferes with the information content of the CHC profile. Ideally, interference could be avoided completely if both functions were fulfilled by separate sets of hydrocarbons. Such a separation has repeatedly been hypothesised, assuming that nestmate information is only encoded in methyl-branched alkanes and alkenes, whereas *n*-alkanes carry little or no information, but are effective waterproofing agents (Guerrieri et al., 2009; van Zweden & d'Ettorre, 2010). Indeed, alkenes, but not *n*-alkanes carry nestmate information in the ant *Formica exsecta* (Martin et al., 2008). However, this species is unusual in that its CHC profile consists almost only of *n*-alkanes and alkenes. Many other ant

species only possess few *n*-alkanes (<5%), which seems too little to ensure waterproofing, such that a dichotomous separation of functions seems unlikely. Moreover, previous studies of other species showed that nearly all CHC classes can change in abundance during acclimation, suggesting that multiple CHC classes may be involved in waterproofing (Menzel et al., 2018; Sprenger et al., 2018). However, if the two functions are carried out by the same or by overlapping sets of CHCs, acclimation to different climates should result in changes in nestmate recognition ability.

In this study, we investigated how information content, such as the colony signal, is maintained during acclimatory changes in the sister species *Lasius niger* and *L. platythorax*. Given that acclimatory CHC changes are linked to increases in survival in these and other ant species (Baumgart et al., in revision; Menzel et al., 2018), it is plausible that those CHCs that increase during warm acclimation are those responsible for waterproofing. We used different temperature regimes to induce acclimatory CHC adjustment to different waterproofing requirements and investigated how these changes affected information content. Both *Lasius* species have complex profiles dominated by methyl-branched alkanes (>90%), with most compounds unique to each species. Their strong chemical differentiation despite close relatedness (Morrison & Witte, 2011) makes the species pair a good model system to analyse how information is encoded in similar species, and how functional separation differs between species. *Lasius niger* inhabits sun-exposed meadows, while *L. platythorax* nests in dead wood in forests or bogs (Seifert, 2007, FM pers. obs.). Due to the habitat differences, the two species may experience different degrees of environmental fluctuations. Hence, their recognition systems might require a different robustness to these fluctuations, and accordingly a varying degree of separation between the CHC functions.

Our research questions were first, whether acclimatory CHC changes affect their information content. If so, ants should exhibit aggression against nestmates from different acclimation regimes and a reduced ability to discriminate nestmates among differently acclimated workers. Second, we asked whether there is functional separation, that is, whether waterproofing and signalling colony membership is performed by different CHCs. If so, the importance of each CHC for colony differences and for acclimatory changes should be negatively correlated. Moreover, we predicted that colony-specific CHCs, but not acclimation-specific CHCs should be most important for aggression. Third, we tested whether CHC classes differ in their likelihood to perform one or the other function. If so, this might be linked to physical properties that differ among CHC classes, making them more suitable for one or the other function. Given that acclimatory CHC changes seem to be adaptive (Menzel et al., 2018), acclimation-specific CHCs should be those with high relevance for waterproofing, hence those with relatively high melting points. In contrast, CHCs important for colony separation and for interindividual aggression should be early-melting compounds, for example, multiply branched hydrocarbons, since these can encode more information and, being liquid and relatively less viscous, are easier to perceive by others and easier to transfer via trophallaxis.

2 | MATERIALS AND METHODS

2.1 | Study species

We studied *Lasius niger* Linnaeus, 1758 and *Lasius platythorax* Seifert, 1991, which was only recognised as a separate species from *L. niger* 30 years ago. They are morphologically similar, with both species being monodomous and monogynous. *Lasius niger* nests preferably in soil with little or no vegetation cover, that is, meadows, lawns or barren land. In contrast, *L. platythorax* nests in tree stumps or other dead wood in deciduous forests (Seifert, 2007; pers. observation). In our study sites, only one species was found in either habitat, although overlapping habitats have been reported earlier (Morrison & Witte, 2011).

2.2 | Collection and acclimation of ant colonies

Twelve colonies of each species were collected in May 2019. *Lasius niger* was collected on a field near Mainz (49°59′25.1″N 8°13′18.4″E). *Lasius platythorax* was collected in the Ober-Olmer Forest near Lerchenberg (49°57′46.4″N 8°10′56.7″E) (permit no. 42/553-254/287-19, issued by the Struktur- und Genehmigungsdirektion Süd on 20 May 2019). Each colony was split into six fragments: three smaller fragments, henceforth termed 'presented' nests, each contained at least 40 ants and at least 10 larvae and were housed in small plaster nests (10 cm × 10 cm × 5.5 cm). The other three fragments, henceforth termed 'reacting' nests, contained the remaining ants (minimum 80 ants, mostly several hundred ants per fragment), and were housed in larger boxes (23 × 17 × 9 cm, Licefa) in the original nest material (thus 72 nests per species). Except for one *Lasius platythorax* fragment (in the fluctuating treatment), they were all queenless. All fragments were fed twice a week with honey and crickets ad libitum. See Figure 1 for an overview of the experimental setup.

From each colony, one presented and one reacting fragment were put into one of three climate cabinets (Rumed E400, Rubarth Apparate GmbH). They were programmed to constant 20°C, constant 28°C or a fluctuating temperature regime, respectively, with a 12 hr:12 hr light:dark cycle. The fluctuating regime varied from 20°C at night to 28°C during the day, with 4 hr temperature ramps in between. Humidity was kept at >99% RH by regularly moistening nest material to saturation. Each fragment was acclimated for 3 weeks. Temperature and humidity in the nest boxes were recorded using data loggers (testo Mini) in one nest per climate cabinet.

2.3 | Behavioural assays

After 3 weeks of acclimation, aggression assays were conducted using live ants from the 'reacting' fragments and freeze-killed ants (frozen alive in air at −20°C) from the 'presented' fragments. To ensure the same behavioural caste for the presented ants, we chose

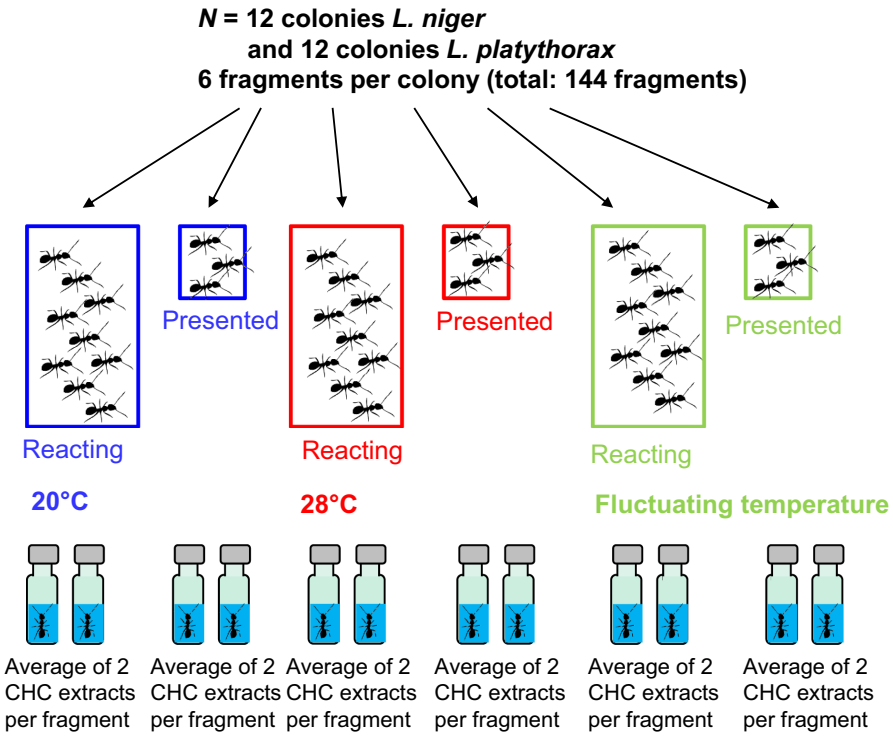

FIGURE 1 Experimental setup of our study, with the respective sample sizes

20 workers that stayed close to larvae (assumed to be nurses); due to low foraging activity in the presented nests, not enough foragers were available in these nests. For the live ants from reacting nests, we chose workers who were active outside the nest (assumed to be foragers, Pamminger et al., 2014). Foragers and nurses usually differ in CHC profile (Pamminger et al., 2014; Wagner et al., 1998) albeit this task-group signal is largely manifested in the *n*-alkane composition and thus may be independent from the colony signal (Sprenger & Menzel, 2020). However, in our experiment we only confronted living outside workers (likely foragers) with dead inside workers (likely nurses), so the task-group difference could not bias our results.

Workers from each reacting nest were confronted with dead ants from all three climate treatments, both with ants from the same original colony and from one (specific) other colony, that is, six combinations per reacting nest (Figure S1). This resulted in 432 combinations (12 colonies × 3 climate treatments of reacting ants × 2 colony origins [nestmate/non-nestmate] × 3 climate treatments of presented ants × 2 species). Each of these combinations was replicated three times on different days, resulting in a total of 1,296 planned behavioural assays per species. Due to low population sizes, there were not always enough ants to do three repetitions of each experiment. For 49 combinations in *L. niger* and 41 combinations in *L. platythorax*, only two repetitions per assay were carried out, resulting in a total *N* of 1,206 assays (599 assays in *L. niger*, 607 in *L. platythorax*). All assays were done in a climate chamber at 24°C (i.e. the average of the two constant temperatures) by one person (MW) in a blinded fashion.

In each assay, five foragers from a reacting nest were put into a 7 cm diameter arena with fluon-covered walls and a fresh paper sheet as ground. For each experiment at the same day, new foragers were taken from their nest, but at the end of the experiments for that day, they were returned to their nest. The ants were allowed to calm down for 1–2 min and then confronted with a dead ant, held by their legs with tweezers, for 3 min. During the experiments, we recorded and categorised all interactions into biting (incl. holding with locked mandibles), threatening (i.e. open mandibles), retreating (suddenly running away) and antennation. To give more weight to long-lasting interactions, they were recorded again if they lasted longer than 10 s. Bites into the tweezers were not considered. As a measure of aggression, we used the proportion of biting interactions (see below) compared to the total number of behavioural interactions. The behaviour of different individuals was not separated; for all further analyses, we used the averages over the two to three repetitions. No ethical approval was required for any of our experiments.

2.4 | Chemical analysis

For the chemical analysis, CHCs from workers from presented nests and reacting nests were analysed. Chemical distances were calculated between ants from reacting and presented fragments in order to compare chemical distances to aggression resulting from behavioural interactions. After 3 weeks of acclimation, two workers of each presented nest (presumably nurses) and two workers from each reacting nest (presumably foragers) were collected, freeze-killed in air at −20°C and individually extracted in hexane for 10 min (Figure 1).

The extracts were concentrated and 2 µl were injected into a gas chromatograph (GC 7890A, Agilent) coupled to a mass-selective detector (MSD 5975C, Agilent). The oven temperature started at 60°C for 2 min, then increased to 200°C at a rate of 60°C/min, and

then further increased to 320°C at a rate of 4°C/min, and then held for 10 min. Data were acquired with MSD ChemStation E.02.02 (Agilent). The peaks were integrated and aligned manually; identification was done based on retention indices and diagnostic ions.

The data were integrated by two different observers, using the software MSD Chem station (Agilent). Each observer integrated the data for all treatments for six colonies per species; all subsequent data processing, including alignment, CHC identification and all analyses, was done by the same person. For each sample, we determined the relative abundance per CHC peak. All CHCs with an average abundance above 0.1% were included. For all subsequent analyses, we used the average of the two samples per fragment, that is, for each CHC, we calculated the average relative abundance across the two samples. Thus, the total sample number was 2 species × 12 colonies × 3 acclimation treatments × 2 fragment types × 2 replicates per fragment = 288 samples. By averaging the two extracts per fragment, we obtained CHC datasets for 144 fragments (Figure 1). Due to problems with the GC–MS device, we could not obtain chemical data for one *L. niger* nest fragment and seven *L. platythorax* fragments, reducing the number of CHC datasets to 136.

2.5 | Statistical analysis: Behavioural assays

First, we analysed whether aggression towards nestmates and non-nestmates differed depending on whether presented and reacting ant belonged to the same colony and/or the same acclimation treatment. We created a linear mixed effects model (LMM) with the proportion of bites (average proportion over the two to three repetitions per assay) as dependent variable and colony-sameness (nestmate/non-nestmate), acclimation-sameness (same/different) and species as fixed factors, including all possible interactions. The colony ID of the reacting ants was included as random factor. Second, we analysed whether aggression differed between the three acclimation treatments of the reacting ants (Supplement S1; Figure S2). Third, we analysed the ants' ability to distinguish between nestmates and non-nestmates, calculated as the difference in aggression towards nestmates and non-nestmates (Supplement S1; Figure S3).

All statistics were done in R version 3.6. LMMs were created using the *lmer* function (package LME4, Bates et al., 2015). All models were evaluated using ANOVA; pair-wise comparisons were done using the *lsmeans* function (package LSMEANS, Lenth, 2016) including FDR correction.

2.6 | Statistical analysis: Chemical differentiation among treatments

First, we analysed how pair-wise chemical distances between ants differed between acclimation treatments and between nestmates and non-nestmates. We calculated the Bray–Curtis dissimilarity between each pair of reacting and presented ants in the same setup as in the behavioural assays ($n = 216$ per species), based on the average of the two chemical samples per fragment (Figure 1). These distance values were then analysed in the same manner as the aggression data above, that is, using an LMM with chemical distances as dependent variable and colony-sameness, acclimation-sameness and species as fixed factors and the colony ID of the reacting colony as random factor. The model was then analysed using the *Anova* command (package CAR).

Subsequently, we estimated effects of colony and acclimation treatment on the entire CHC profile using a PERMANOVA with 9999 permutations (command *adonis*, package VEGAN Oksanen et al., 2022), with colony ID and acclimation as predictors. The combination of observer (MW or LB) and nest type (large or small) was entered as strata. The data were visualised using NMDS ordinations based on Bray–Curtis distances. The PERMANOVA was first performed based on the entire CHC set, but also based on CHC subsets (see below). To compare the overall degree of acclimatory changes between the two species, we further determined the per cent change of each hydrocarbon from 20°C to 28°C. For each CHC, we calculated the absolute difference of the abundances at 20°C and at 28°C (averaged over all colonies), divided by the abundance at 20°C, and compared these values between the two species with a *t* test.

2.7 | Identifying which CHCs contribute to group separation

To analyse functional separation, we calculated for each hydrocarbon its importance for separation between colonies (*colonyMDA*), between acclimation treatments (*acclimationMDA*), and its importance for aggression (*IfA*) as described below. Then, we tested for correlations among these values using Spearman correlations. Furthermore, we analysed whether the three metrics differed among CHC classes using linear models, separately for each ant species and each of the three metrics.

First, we identified the importance of each hydrocarbon for separation between colonies and between acclimation treatments using random forests (package RANDOMFOREST, Liaw & Wiener, 2002). This method identifies CHCs that contribute most to the separation between groups that are given a priori. We used proportional abundance of each CHC as input data (adding up to 1 for each sample). Random forests were calculated once with colony and once with acclimation treatment as grouping variable. In both analyses, we used the 'mean decrease of accuracy' (MDA) for each CHC as a measure of importance for group separation. The MDA value is independent from compound abundance. It is high for a specific compound if this compound allows robust discrimination between groups, for example, if it occurs in one treatment but not in the other. Conversely, MDA is low if the compound is either uniformly present or even introduces noise, that is, varies randomly, but not according to the predefined groups. We used these MDA values to determine the importance of each CHC peak for colony differences or for acclimatory changes, respectively. These values are henceforth termed *colonyMDA* and *acclimationMDA*.

Next, we developed a method to find the CHCs with the highest importance for aggression (IfA). It relies on the Spearman correlation (rho) between aggression and chemical distance (henceforth 'aggression correlation') (n = 201 for *L. niger*, 175 for *L. platythorax*, each data point being a combination of inter-individual aggression and chemical distance). Aggression was defined as the mean biting proportion over all three repetitions of each pair of reacting and presented nest. For chemical distance, we used the Bray–Curtis dissimilarity between ants from reacting and presented fragments in the aggression assays, using the mean of the two chemical samples. If not all CHCs were used to calculate chemical distance, their proportions were re-standardised to 1. We then developed a bottom-up approach using random subsets of a CHC profile. We created 200 random sets of five CHCs each, and calculated the aggression correlation for each set. To estimate the importance for aggression for a single CHC, we added this focal hydrocarbon to each random sample (if it was not part of it already) and calculated the aggression correlation for the new set (now consisting of six CHCs). The mean difference of correlation for the random set with and without the focal CHC, that is, by how much the aggression correlation changed when adding this particular CHC, was used as the estimate of the IfA for this CHC. This method has the advantage that we could calculate a numerical estimate of IfA for each single hydrocarbon and relate this value to *colonyMDA* and *acclimationMDA*.

To further confirm that the random forests correctly identified hydrocarbons that were important for separation among colonies or acclimation treatments, we repeated the above-mentioned PERMANOVA based on two different CHC subsets instead of the entire profile: (a) all CHCs that had a positive *colonyMDA* but a negative or zero *acclimationMDA* (see below), that is, all CHCs that contributed to colony separation, but not for separation between acclimation treatments, and (b) vice versa all CHCs with a positive *acclimationMDA* but a negative or zero *colonyMDA*. These analyses allowed to check whether *colonyMDA* and *acclimationMDA* correctly reflect how well those CHCs explain the corresponding factors and thus also whether indeed some CHCs are primarily affected by one of those factors. The identities of the CHCs in each subset are denoted in Tables S3 and S4. As an alternative, top-down approach to determine the CHCs relevant for aggression, we developed a reductive algorithm to obtain a subset of CHCs that explained aggression best (see Supplement S2; Figure S4). The rationale behind this approach is that entire profiles may contain variation that is irrelevant for communication, thus concealing information. Reductive algorithms have proved useful in the past and allow to identify hydrocarbon sets (rather than single hydrocarbons) that explain aggression patterns best (Jongepier & Foitzik, 2015).

3 | RESULTS

3.1 | Behavioural assays and chemical distances

As expected, ants were more aggressive towards non-nestmates than towards nestmates, as measured by the biting proportion towards presented ants (LMM: colony-sameness: χ^2_1 = 83.36, $p < 0.0001$; Table S1). Ants were more aggressive against nestmates from another acclimation treatment (LMM: t = 2.67, df = 403, p = 0.0097). This effect was stronger in *L. platythorax* (t_{400} = 2.21, p = 0.028) compared to *L. niger* (t_{400} = 1.56, p = 0.12). Among non-nestmates, however, acclimation-sameness did not affect aggression (LMM: t_{403} = 0.087, p = 0.93; overall effect of acclimation-sameness: χ^2_1 = 3.32, p = 0.068; interaction acclimation-sameness x colony-sameness: χ^2_1 = 3.78, p = 0.052) (Figure 2a,c). The two species did not differ in overall aggression (LMM: species: χ^2_1 = 0.25, p = 0.62).

The aggressiveness of the reacting ants depended on the acclimation regime they experienced. In *L. niger*, they were most aggressive in the 28°C treatment, while in *L. platythorax*, aggression was highest in 20°C-acclimated ants (see Supplement S1, Figure S2). We also measured 'recognition ability' as the aggression difference between nestmates and non-nestmates. While *L. niger*'s ability to distinguish nestmates from non-nestmates was consistent across acclimation treatments, *L. platythorax* was poorer at distinguishing nestmates from non-nestmates when the acclimation regime was different, specifically when reacting ants were reared in a fluctuating temperature environment (see Supplement S1; Figure S3).

Overall chemical distances between the corresponding CHC profiles mirrored aggression between reacting and presented ants, being lowest for same-acclimated nestmates, higher for different-acclimated nestmates, and highest for non-nestmates (Figure 2b,d): as expected, the chemical distances were higher between non-nestmates than between nestmates (LMM: χ^2_1 = 31.0, $p < 0.0001$; Table S1). They were also higher for ants from different acclimation treatments (χ^2_1 = 9.8, p = 0.0018), although this effect was smaller than the colony effect. Interestingly, the overall distances were considerably higher in *L. niger* compared to *L. platythorax* (LMM: χ^2_1 = 5.5, p = 0.019).

3.2 | Composition of the chemical profiles

The profiles of *L. niger* and *L. platythorax* consisted of 52 and 47 CHC peaks respectively (Tables S3 and S4). In *L. niger*, dimethyl alkanes (54.04 ± 1.03% of the total CHC peak area) were the most abundant CHC class, followed by monomethyl alkanes (27.75 ± 0.53%); *n*-alkanes accounted for 5.94 ± 0.56% of the CHCs. In *L. platythorax*, the most abundant CHC class were also dimethyl alkanes (46.37 ± 0.76%), followed by trimethyl alkanes (34.31 ± 0.82%); *n*-alkanes accounted for 2.59 ± 0.35% of the CHCs only. In both species, many peaks consisted of multiple CHCs (usually from the same CHC class) that could not be separated due to similar retention times.

3.3 | Chemical differentiation between colonies and acclimation treatments

Concerning the entire CHC profile, chemical differences between colonies and between acclimation treatments were highly significant

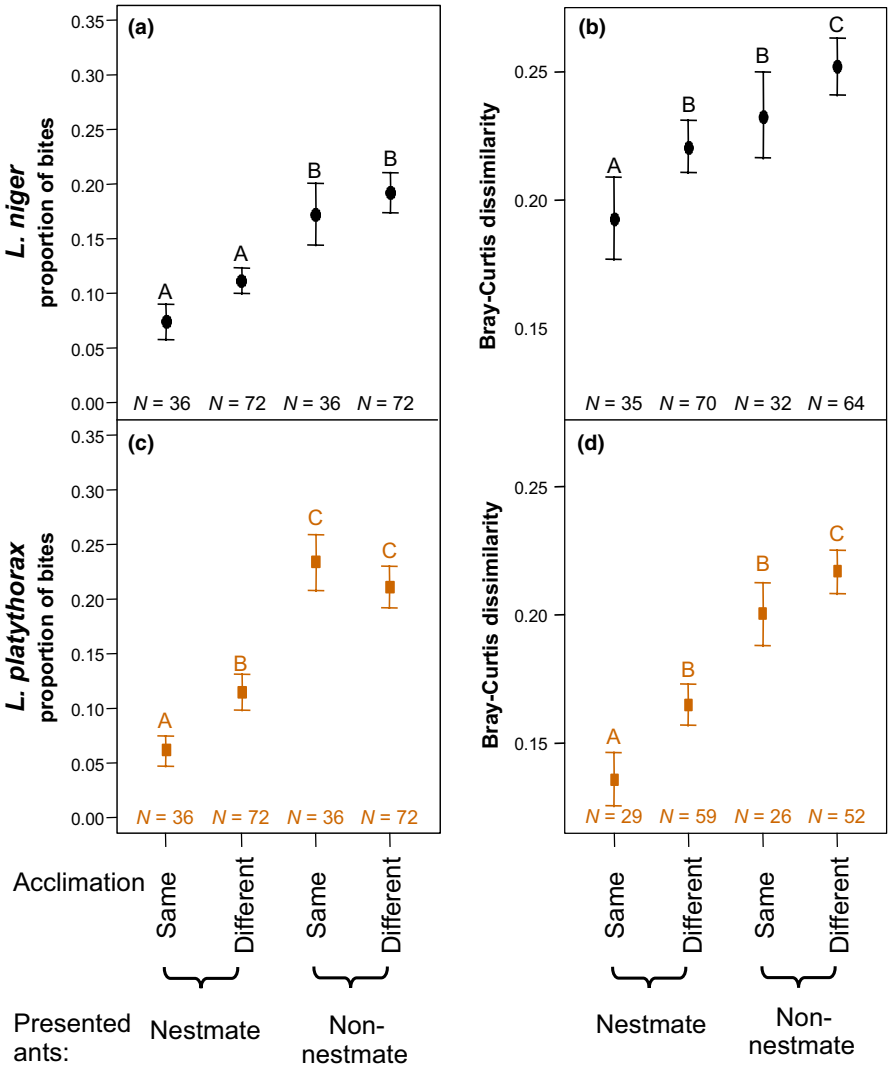

FIGURE 2 Behavioural interactions (a, c) and chemical distances (b, d). The graphs show proportion of bites (means ± *SE*) in *Lasius niger* (a) and *Lasius platythorax* (c) for confrontations of individuals from same and different acclimation, as well as from the same and different colonies. Chemical distances between the same groups are shown in (b) and (d). Plots with the same letters within the same panel are not significantly different

in both species. Notably though, their effect sizes were much lower in *L. niger* than in *L. platythorax* (Table S5). The interaction between colony and acclimation treatment was not significant in either species (both pseudo-$F < 0.95$, $p > 0.5$), indicating that the colony signal was relatively stable irrespective of acclimation treatment. Interestingly, the interaction explained less variation in *L. platythorax* than in *L. niger* ($R^2 = 0.085$ vs. 0.19), suggesting that the two sources of variation are more independent from each other in *L. platythorax*. By only including CHCs with positive *colonyMDA*, we could generate CHC sets that only differed among colonies but not among acclimation treatments. In analogy, we obtained CHC sets that differed among acclimation treatments but not among colonies. Such sets could be generated for both species; nevertheless, effect sizes and explained variances were much higher in *L. platythorax* than in *L. niger* (Table S5). This is reflected in NMDS ordinations. Here, overlap between acclimation treatments (Figure 3a) and between colonies (Figure 3b) was relatively high in *L. niger*. In contrast, acclimation treatments (Figure 3c) and colonies (Figure 3d) were separated much better in *L. platythorax* (note the variation along NMDS axis 1 vs. axis 2). Overall, acclimatory changes in relative abundance per CHC were lower for *L. niger* (relative change in proportion: 31.2 ± 3.2%, mean and *SE*) than for *L. platythorax* (57.4 ± 9.0%) between the 20°C and 28°C treatment (*t* test: $t_{57.9} = 2.74$, $p = 0.0081$).

3.4 | Which compounds are relevant for acclimatory differences, colony differences and aggression?

Functional separation among CHCs was significant, especially in *L. platythorax*: CHCs that were important for colony separation contributed little to separation between acclimation treatments, and vice versa. This is evidenced from the negative correlation of *colonyMDA* and *acclimationMDA* for both species (*L. niger*: Spearman's rho = −0.33; $p = 0.019$; *L. platythorax*: rho = −0.49; $p = 0.00061$) (Figure 4a). As expected, importance for aggression was not correlated with importance for acclimatory treatments in either species (rho = −0.18 and − 0.15; $p = 0.20$ and 0.32 for *L. niger* and *platythorax*). In contrast, importance for aggression was highly correlated with importance for colony differences in *L. platythorax* (Spearman's rho = 0.57; $p < 0.0001$). Unexpectedly, this was not true for *L. niger* (rho = 0.10; $p = 0.47$) (Figure 4b), suggesting that much of the

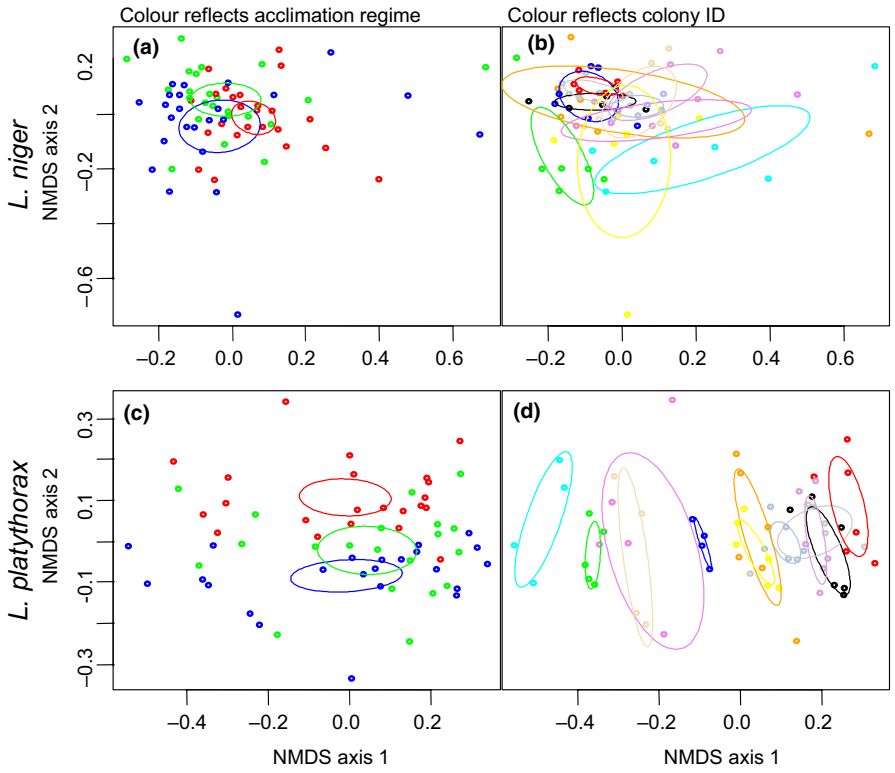

FIGURE 3 NMDS ordinations of CHC profiles (based on Bray–Curtis distances) of *Lasius niger* (a–b) and *Lasius platythorax* (c–d). Each dot represents the CHC profile of one colony fragment. The coloration in (a) and (c) reflects acclimation regime (red: 28°C, blue: 20°C, green: Fluctuating). In (b and d) the coloration reflects colony identity. Ellipses denote 95% confidence intervals around medoids of each acclimation treatment (a, c) or colony (b, d)

inter-colony variation was not relevant for aggression in this species. This notion is further confirmed by the fact that aggression and chemical distance (based on the entire CHC profile) were not correlated in *L. niger*, and the correlation was only revealed using a reductive algorithm that generated a subset of CHCs (Supplement S2, Figure S4). In *L. platythorax* in contrast, aggression and chemical distance of the entire profile were highly correlated.

The importance of CHCs for one or the other function varied across CHC classes in *L. platythorax*. Monomethyl alkanes were more important for acclimatory differences than the other CHC classes (LM: $\chi^2_4 = 7.02$, $p = 0.0002$) (Figure 5a), but less important for inter-colony differences (LM: $\chi^2_4 = 3.36$, $p = 0.018$) (Figure 5b). In contrast, no such differences were found in *L. niger* (LM: $\chi^2_5 < 2$, $p > 0.1$). However, as one would expect, dimethyl and trimethyl alkanes tended to be less important for acclimatory differences, but more important for colony differences, while the opposite was true for *n*-alkanes and monomethyl alkanes. Importance for aggression did not differ between substance classes for either species (*L. niger*: LM: $\chi^2_5 = 1.93$, $p = 0.11$; *L. platythorax*: LM: $\chi^2_4 = 0.5$, $p = 0.74$).

4 | DISCUSSION

4.1 | Does acclimation affect information content?

CHC profiles fulfil multiple functions at the same time. Hence, the optimisation of one might negatively affect another. In this study, we investigated whether the information content and acclimation to changing temperatures interfere on a behavioural level, and whether functions are separated within the CHC profile—by a separation between individual CHCs. Our assays showed that acclimation and nestmate recognition can indeed interfere with each other: acclimatory CHC changes increased aggression among former nestmates, especially in *L. platythorax*. Nevertheless, aggression towards non-nestmates was always higher than towards nestmates. Thus, although acclimation interferes with nestmate recognition, this does not compromise recognition ability. Notably, aggression against nestmates and non-nestmates also varied across acclimation regimes (Supplement S1, Figures S2 and S3), implying that the ability to recognise nestmates differs not only between species (Foitzik et al., 2007; Menzel et al., 2008), but also depending on the current climate. Our results match a previous study on *Cataglyphis* ants (Dahbi & Lenoir, 1998) where acclimation led to aggression between ants from hibernating and non-hibernating conditions.

4.2 | Is there functional separation across hydrocarbons?

Functions of the CHC layer can be decoupled best if they are fulfilled by different components of the profile. Here, we could indeed show some degree of functional decoupling, based on the negative correlation of importance for colony differences and for acclimation treatments: CHCs that differ among colonies differ less among acclimation regimes, and vice versa. In *L. platythorax* (albeit not *L. niger*), the CHCs that differed most among colonies were also those that mattered most for aggression, as one would expect.

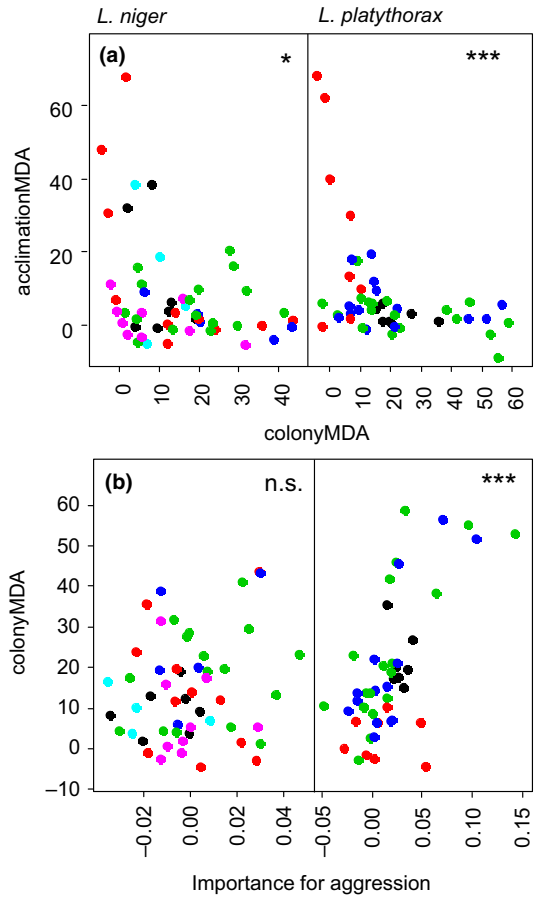

FIGURE 4 Importance of each CHC for differences between colonies, between acclimation treatments, and for inter-individual aggression. (a) Importance for differences between acclimation treatments (*acclimationMDA*) plotted against importance for colony differences (*colonyMDA*). (b) Importance for colony differences plotted against importance for aggression as determined from behavioural assays. Each dot is one hydrocarbon; the colours reflect CHC classes: Black (*n*-alkanes), red (monomethyl alkanes), green (dimethyl alkanes), blue (trimethyl alkanes), cyan (alkenes), magenta (unknown). $^{*}p < 0.05$, $^{***}p < 0.0001$, n.s. not significant according to Spearman correlation

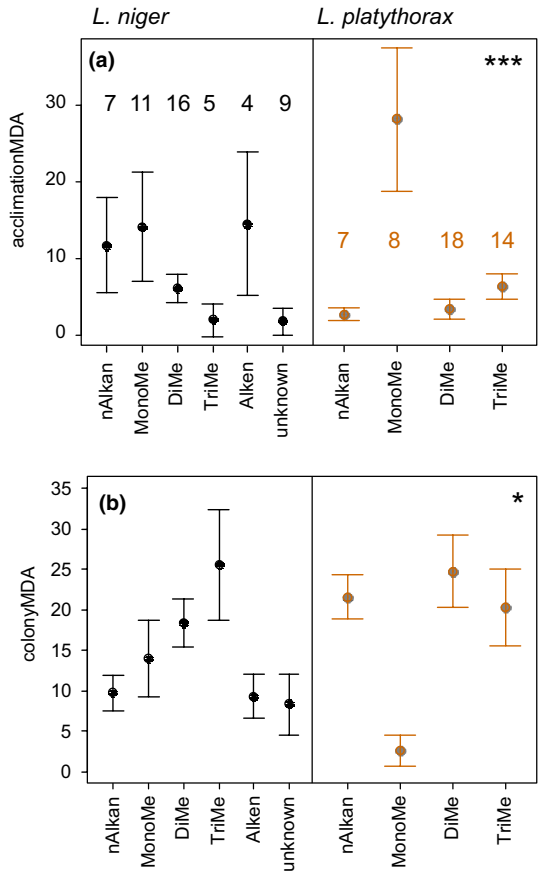

FIGURE 5 Functions across CHC classes. The plots show (a) importance of each CHC for differences between acclimation treatments (acclimationMDA) and (b) importance for colony differences, depending on CHC class. The plots show mean (±*SE*) importance per CHC class for *Lasius niger* (left) and *Lasius platythorax* (right). Numbers give the number of different CHCs in each CHC class. $^{*}p < 0.05$, $^{***}p < 0.0001$ according to linear models

An entirely different line of evidence for functional separation is that we could identify CHC subsets that differed only among colonies or only among acclimation treatments (Table S5). Similarly, the reductive algorithm identified subsets of few CHCs which sufficed to explain aggression patterns, indicating that many CHCs either are not important for recognition or redundant (Supplement S3, Figure S4). Nevertheless, the fact that acclimation still affected the nestmate recognition suggests that functional separation is not perfect, albeit high.

To our knowledge, a clear separation between two CHC functions was only found in the ant *Formica exsecta*, where two lines of information are separately encoded in the CHC profile (Martin & Drijfhout, 2009b). Here, (Z)-9-alkenes encoded colony identity, while *n*-alkanes differed between castes but did not affect aggression (Martin et al., 2008, 2012). This species is unusual in that *n*-alkanes and alkenes together account for >95% of its profile. This peculiar composition—solid *n*-alkanes and liquid alkenes, but hardly any intermediate compounds—may facilitate separation. In contrast, most other ants have more diverse CHC profiles, with methyl-branched alkanes being more abundant and more diverse (Martin & Drijfhout, 2009a; Menzel et al., 2017). Since all CHCs influence melting range and viscosity (Menzel et al., 2019), insects might not be able to perfectly separate acclimatory changes from changes in information content. This matches our finding that some hydrocarbons could not be clearly assigned to one or the other function, but differed between both colonies and acclimation treatments.

4.3 | Which hydrocarbons are responsible for waterproofing?

Depending on their melting points, different hydrocarbons may be more suitable for one or the other function. For waterproofing, it is hardly possible to empirically show the role of single CHCs. However, we have two lines of reasoning that those that changed

with acclimation are those important for waterproofing. First, in both species, CHCs that changed most with acclimation (highest *acclimationMDA*) are in relatively good accordance with predictions: Changes were high for the solid *n*-alkanes (albeit not in *L. platythorax* with its low abundance of *n*-alkanes) and the solid or highly viscous monomethyl alkanes, while lower for the less viscous dimethyl and trimethyl alkanes. Second and more importantly, previous studies showed that acclimatory CHC changes are linked to drought resistance (Menzel et al., 2018). Also, for the ants studied here, warm acclimation significantly increased drought survival in both species compared to cold acclimation, with ants from the fluctuating treatment in between (Baumgart et al., in press). However, waterproofing not only requires solid or highly viscous compounds like *n*-alkanes and monomethyl alkanes. Especially *n*-alkanes alone would aggregate too tightly (even shorter-chained ones) and thus be too brittle to reliable coat the insect body (Rourke & Gibbs, 1999). Hence, more liquid hydrocarbons are also necessary to ensure homogenous cuticle coating and an optimal CHC viscosity (Menzel et al., 2019). This suggests that a perfect functional separation may actually not be possible, even if insect species can separate the functions to different degrees, depending on their species-specific CHC composition.

Our results refute the idea that waterproofing is only performed by *n*-alkanes (D'Ettorre & Lenoir, 2010; Sturgis & Gordon, 2012; van Zweden & d'Ettorre, 2010), while methyl-branched or unsaturated hydrocarbons function exclusively as communication signals. This may seem plausible, since methyl-branched or unsaturated hydrocarbons can encode more information via number and position of methyl branches and unsaturations; and *n*-alkanes are indeed most effective waterproofing agents due to their high melting point (Gibbs, 1998; Gibbs & Rajpurohit, 2010) and are usually upregulated under warm conditions (Menzel et al., 2018; Sprenger et al., 2018; Wagner et al., 2001). However, our results clearly show that *n*-alkanes do not tell the entire story of acclimatory changes, especially in *L. platythorax* with their low *n*-alkane abundance. The most solid CHC class next to them is monomethyl alkanes, and these indeed changed most during acclimation.

4.4 | Which compounds are used for nestmate recognition?

Identifying which hydrocarbons actually carry the nestmate information is far from easy. Van Zweden and colleagues introduced the 'diagnostic power', a statistical approach to identify which CHCs differ most between colonies (van Zweden et al., 2009; van Zweden & d'Ettorre, 2010), but without relation to behavioural data. Other studies tried to identify which chemical differences correspond most to aggression via an iterative reductive algorithm (Jongepier & Foitzik, 2015; Martin et al., 2012), similar to what we used here. In our view, including behavioural data is preferable since any chemical difference between colonies does not necessarily mean that the ants use it for recognition. In our *L. niger* data for example, differences among colonies (*colonyMDA*) did not correlate with their importance for aggression in behavioural assays. Given that the complexity of CHC profiles prevents testing artificial mixtures, algorithmic solutions probably represent the best approach.

Our (and previous) approaches to identify the CHCs that carry information rest on the assumption that ants use relative proportion of CHCs for recognition. While this is plausible, it may be an oversimplification of the actual recognition process, as it is unknown so far how nestmate information is actually computed from CHC abundances in an ant's brain. For example, information might be encoded in the proportion of two CHCs relative to each other (as opposed to relative to the entire profile, as assumed here). This is an epistemological problem, since it is hardly possible to precisely determine how an ant perceives CHC profiles. However, we think that this assumption is reasonable at our current state of knowledge, especially given that our (and previous) models yielded meaningful results and explained aggression patterns.

Our results show that CHCs that consistently differ between colonies are not necessarily identical to those that elicit aggression. Importance for aggression and *colonyMDA* were correlated only for *L. platythorax*. This coincides with the strong correlation between aggression and chemical distance (Figure S4) in this species, as has been shown for other ant species as well (Jongepier & Foitzik, 2015; Lahav et al., 1999; Martin et al., 2012; Sprenger & Menzel, 2020). However, it was not true for *L. niger*. Beside the independence of *colonyMDA* and importance for aggression, aggression and overall chemical distance were not linked either (Figure 3; Figure S4). Nevertheless, even in *L. niger*, a reductive algorithm identified CHC subsets that strongly correlated with aggression. These 'aggression sets' consisted only of few compounds but predicted aggression better than the entire CHC profile. These results show that CHC profiles do encode nestmate information in both species. However, ants ignore most CHC variation and use only a small subset of compounds for recognition (Jongepier & Foitzik, 2015). Moreover, CHC profiles probably encode information in a redundant manner. This notion explains that, despite considerable overlap, the aggression sets from reductive algorithms do not contain only those CHCs with the highest 'importance for aggression' values (Tables S3 and S4). Such a redundant encoding would be highly useful in nature, where climatic conditions, but also other extrinsic factors like food (Liang & Silverman, 2000; Otte et al., 2014), may blur an individual's profile.

Many peaks of the GC analysis actually consisted of multiple hydrocarbons that could not be further separated chromatographically. Usually, they were structurally similar, for example, one peak could contain several 5,x-DiMeC33 alkanes or multiple internally branched monomethyl alkanes of the same chain length. This is a ubiquitous problem in the analysis of insect hydrocarbons, but was especially pronounced in our two species due to their high number of methyl-branched alkanes. Given this, it is notable that chemical distances were still accurate enough to explain aggression patterns.

The identity of recognition cues was species specific, and differed even among the sister species studied here. This important conclusion underlines that recognition systems may not be

comparable even among closely related species. Even CHC classes could not always be clearly assigned to aggression or colony separation. Dimethyl and trimethyl alkanes often had a high importance for colony separation (*colonyMDA*) or aggression, as one would expect given their low melting point and their high potential to encode information. Still, the patterns were far from predictable: *n*-alkanes were unexpectedly important for colony separation in *L. platythorax*, and in *L. niger*, CHC classes did not differ in importance despite trends in the expected direction. Thus, generalisations on the role of different CHC classes for one function or the other should be considered with care.

4.5 | Differences between species

The two sister species differed considerably concerning functional separation. This is shown by three lines of evidence: first, PERMANOVA effect sizes of colony and acclimation were smaller in *L. niger* than in *L. platythorax*, especially for the CHC subsets (Table S5). Second, *colonyMDA* and importance for aggression were correlated in *L. platythorax* but not in *L. niger*. Finally, the NMDS ordinations showed a weaker separation of the two factors for *L. niger* CHC profiles than for those of *L. platythorax* (Figure 3). At the same time, aggression of *L. niger* towards nestmates was less affected by their acclimation treatment than *L. platythorax* (Figure 2; Figure S3), which seems counter-intuitive at first sight. However, the results make sense considering that *L. niger* showed lower acclimatory responses overall. Beside the lower effect sizes of acclimation in the PERMANOVA analyses, per cent changes between 20°C and 28°C treatments were much higher in *L. platythorax* than in *L. niger*. The lower acclimatory changes also affected fitness: the acclimatory increase in drought survival was substantially lower in *L. niger* than in *L. platythorax* (Baumgart et al., in press).

We conclude that functional separation is lower in *L. niger*. Its lower acclimatory changes compared to *L. platythorax*, however, bring about that acclimation has little effect on nestmate recognition nevertheless. Two potential causations come to mind. The species may be constrained by lower functional separation, enforcing lower acclimatory variability to maintain nestmate recognition. Alternatively, *L. niger* might not need strong acclimatory changes if its colonies are on average exposed to lower temperature fluctuations than in *L. platythorax*, which is why it has not evolved stronger functional separation. So far, we cannot distinguish between these scenarios, which would require a deeper understanding of functional separation and acclimatory ability across species.

As monodomous species, both might not need a complete separation of CHCs carrying information and CHCs responsible for waterproofing. Since all individuals of a colony share the same nest, there might be little selection pressure for recognition cues to be robust to acclimation. In contrast, facultatively polydomous species (e.g. some *Formica* species) might require a stronger separation of the functions, especially if sub-nests are exposed to different microclimates despite spatial proximity, as is common in montane habitats. Thus, the ecology of a species might affect how CHC functions are separated from each other, even if a complete functional separation is probably impossible.

5 | CONCLUSIONS

The optimisation of a trait to multiple functions is a wide-spread problem in evolutionary ecology (Wainwright, 2007). Beside morphological structures (Rosell, 2019), it also concerns chemical mixtures like floral bouquets (Junker, 2016). Here, we studied how different CHC functions interfere with each other and whether different components can be assigned to one or another function. We expected low functional separation because of the relatively high phenotypic integration of CHC profiles compared to other chemical blends (Junker et al., 2017). To test this, we changed waterproofing requirements by exposing ants to different climates, and the resulting CHC changes indeed interfered with nestmate recognition. Notably though, a small subset of hydrocarbons was sufficient to predict aggression, suggesting that few compounds are sufficient for recognition and that information is encoded in a redundant way.

Possibly, a clear functional separation is not always possible due to physical constraints. To ensure waterproofing, solid phases are most efficient, but they must be embedded in more liquid phases so that a homogenous CHC coating is achieved and brittle fractures are avoided (Menzel et al., 2019). Here, species-specific CHC class composition may affect the potential for functional separation. A higher independence of information coding and acclimatory changes may be possible if the CHCs in a profile span a wider range from solid to liquid, low-viscosity compounds. Information encoded in early-melting, less viscous CHCs like alkenes or trimethyl alkanes may be less affected by acclimatory changes than information encoded in later-melting, more viscous CHCs like dimethyl or monomethyl alkanes (Gibbs, 2002). This is consistent with the fact that *L. platythorax* possesses more early-melting compounds (trimethyl alkanes), while *L. niger* is richer in later-melting monomethyl alkanes and n-alkanes, even if dimethyl alkanes are dominant in both species. In *Formica exsecta*, where functional separation of two information channels was shown (Martin & Drijfhout, 2009b), the CHC layer consists almost entirely of solid *n*-alkanes and very early-melting, liquid alkenes, which may enable relatively independent variation of the two classes. Our study exemplifies how trait optimisation for one function affects functionality in another. Especially when functional separation is not be possible, species are forced to prioritise one function over another. The severity of this evolutionary conflict may depend on species-specific, trait-dependent physical constraints on functional separation, but also from the species' ecology that defines the need for separation.

AUTHORS' CONTRIBUTIONS

F.M. conceived the study; M.W. and L.B. collected the data; M.W. and F.M. conducted the analyses and wrote the paper. All authors approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All raw data from this study are available via Menzel et al. (2022) (https://doi.org/10.5061/dryad.qz612jmj3).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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