How can population-level chemical diversity evolve through individual-based selection? An approach to resolve Crozier's paradox

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Introduction

Trait variation among conspecific individuals is ubiquitous, and has important effects on biotic communities and ecosystems (Bolnick et al., 2011; Schuman et al., 2016). Especially for communication, trait diversity concerning chemical or optical cues is the prerequisite for any form of recognition, be it in individualised groups (Gilfillan et al., 2016; Tibbetts & Dale, 2007) or in social insects, where individuals recognise group membership rather than individual entities. In eusocial insects in particular, recognition is crucial to maintain the integrity of the colony. Here, nestmate recognition ensures that behavioural altruism is directed only towards members of the own colony (Leonhardt et al., 2016; Vandermeer & Morel, 1998). In social insect colonies, like ants, bees or wasps, individuals discriminate between nestmates and non-nestmates, and decide whether or not an encountered individual is granted access to the nest and its resources. Nestmate recognition is mediated by cuticular hydrocarbons (CHCs), which form complex blends of up to 150 different compounds on a single individual (Sprenger & Menzel, 2020). There is strong selection to avoid that the nest is exploited by alien, unrelated individuals. Cheaters, on the other hand, often mimic others so that they are not recognised, i.e. they are selected to be as similar as possible to a potential 'victim' colony. Such chemical mimicry has frequently evolved in socially parasitic ants, but also in myrmecophilic insects such as staphylinid beetles or lycaenid butterflies (Guillem et al., 2014; Schlick-Steiner et al., 2004).

However, the evolution of recognition cues raises a theoretical problem concerning the direction of selection pressures: if there is high aggression against individuals from foreign colonies, then each colony should benefit from resembling the others (Crozier, 1986). Aggression between opponents often increases with increasing chemical distance (Kidokoro-Kobayashi et al., 2012; Wittke et al., 2022). Therefore, each individual should elicit less aggression if its chemical signature differs less from its opponent, and be selected to be as similar to the others as possible. However, this would result in a homogenous odour across the entire population of colonies – which would thwart all possibilities to actually perform nestmate recognition, since discrimination only works if groups differ from each other. This reasoning – that, in theory, there should be selection against being different from others - has been termed *Crozier's paradox*. But then, why does cue diversity not decline in natural populations, and how can diversity evolve at all? Crozier's paradox has the additional interesting twist of how individual-based selection (survival and fitness of individual colonies) can result in a population-level trait such as cue diversity across multiple colonies. Note that, in eusocial insects, selection acts on the colony level, since this is the reproductive unit.

A previous approach to solve Crozier's paradox used an agent-based model to show that cue diversity can be maintained if there is disassortative mating (Holman et al., 2013). Indeed, CHCs were shown to be sexually selected in solitary insects (Steiger et al., 2013; Steiger & Stökl, 2014). In social insects, CHCs mediate nestmate recognition, hence recognition cues may be under sexual selection as well. However, this idea rests on the assumption that insects mate disassortatively. This has been shown in solitary insects (Lihoreau & Rivault, 2009; Thomas & Simmons, 2011) but evidence for eusocial insects is scarce. Moreover, intra-sexual and inter-sexual selection in social insects seems to be weak, since the mating system in social

Hymenoptera leaves little room for female choice. Thus, variation in male reproductive success (number of matings) is far lower than in other organisms and males cannot monopolise multiple females due to once-in-a-lifetime partner selection (via mating and sperm storage) (Boomsma et al., 2005; Strassmann, 2001). Hence, it is unclear whether the disassortative mating required for previous models actually holds for many social insects.

A further, often-debated question concerns how exactly the recognition system determines the chemical distance of its opponent to an internal, neuronal template (which is the first step of the recognition process). Firstly, it might use the overall similarity, averaging chemical distance over all chemical compounds present. This has been suggested as the *Gestalt* model (Crozier & Dix, 1979; Vandermeer et al., 1998). Alternatively, the ant might only consider whether the opponent bears compounds that are not part of its internal template (*'undesired-absent'* or *U-absent)* or whether it bears all compounds that are part of the template (*'desired-present'* or *D-present*) (d'Ettorre & Lenoir, 2010). These models imply that the ant ignores any missing cues that are present in its template in the first case, or any additional cues not present in its template in the second one. Evidence supports *U-absent* rather than *D-present* (Nehring et al., 2016; van Zweden & d'Ettorre, 2010).

Here, we used an agent-based model to study how cue diversity can evolve despite Crozier's paradox, and compare the three recognition models mentioned above. Our model does not require disassortative mating. Instead, we simulate a population of social insect colonies that can invade and exploit each other. Each individual colony can forage on its own, but can also invade other colonies to exploit their resources; thus parasite and host are the same species, and each colony can adopt both roles simultaneously. Only the most successful colonies pass on their profiles to the next generation. Using this model, we studied how population-level cue diversity evolved over time. Furthermore, we assessed which recognition model gave the most realistic results, and how cue evolution was affected by different levels of signal production costs, selection strength and rate of cue evolution (evolvability).

Materials & Methods

The model

The model was developed in NetLogo version 6.2.2 (Wilensky, 1999) (also used e.g. in (Milles et al., 2020)). We simulated 50 ant colonies with 10 workers each. They could forage on 300 food sources. Each food source consisted of a single unit of food and each ant could carry exactly one unit of food. For this purpose, each colony had 10 ants that would find food at every time step (henceforth tick). At each tick, the ants select one after each other a random food source or foreign colony and try to pick up food. Note that the order in which ants act is independent of their colony membership and is randomized at each tick, thus simulating a simultaneous activity of all colonies. Due to the limited number of food sources, ants benefit from the additional possibility of stealing from other colonies. Any food source that was not collected in the previous time step is removed, (simulating other animals foraging and food perishing), and new food sources are generated, i.e. there would not be a "surplus" of food accumulating.

Metabolic costs of the ants are subtracted after each tick from the colony's "resource stock". We simulated a constant resource need plus a variable term that depended on the quantity of CHCs biosynthesised. The overall metabolic costs were calculated as 2 + *cost-factor* * *total cue abundance* / 1000. This formula was chosen so that costs contained a fixed factor but increased with the overall quantity of cues produced. *Total cue abundance* was the sum of all cues (see below). With this formula the overall amount of food available was enough for all colonies to survive with the *total cue abundance* they started with, so in theory it would be

possible for all colonies to find an equilibrium without the need to steal from others. The *cost-factor* was one of the variables we varied (default value: 40) to investigate varying costs of CHC biosysnthesis per unit CHCs, since the actual metabolic or fitness costs of biosynthesising a unit of CHCs are unknown. Colonies started with 25 units of food and died if they failed to collect enough food to have more than 0 units at the end of each tick. Each "generation" lasted 100 ticks, after which selection would take place and some colonies would die and be replaced.

Recognition signal

Each colony started with a random chemical profile, which served as recognition signal. In social insects, recognition is usually mediated by cuticular hydrocarbons (CHCs), which form complex mixtures that encode, among others, nestmate membership (Sprenger & Menzel, 2020; Sturgis & Gordon, 2012).

In our model, the signal consisted of 10 compounds (CHCs), each with an abundance of zero to infinite. The abundance of all compounds can vary independently from each other. In ant CHCs, it has been shown that not all CHCs vary independently (S. Martin & Drijfhout, 2009; S. J. Martin & Drijfhout, 2009). This may be due to physical, but also to biosynthetic constraints (F. Menzel et al., 2017). Hence, the 'compounds' of our model should not necessarily be seen as real CHC compounds, but rather as entities (e.g. groups of compounds) that vary independently from each other. For example, they might be viewed as homologous series of CHCs, such as the sum of 15-MeC25, 15-MeC27 and 15-MeC29, or as a principal component axis along which sets of hydrocarbons co-vary and which are by definition independent from each other. Initial abundance for each colony was generated by drawing from an exponential distribution with an expected value of 10.

In our model, each component had a colony-specific mean. To generate intra-colonial profile variation, we generated individual CHC profiles for each ant by drawing the abundance of each component from a normal distribution around the colony-specific mean (different for each component) with a standard deviation that was 10% of the evolvability (see below).

Colony invasion and the nestmate recognition process

Colonies could invade other colonies or search for food by themselves. If they invaded a colony, they tried to steal food. Every time an ant attempted to steal from another colony, a recognition process determined whether it was successful. Here, the chemical distance between the ant and its target colony was calculated as the chance of success. At the same time, ants also needed to recognise their nestmates. When bringing back food to their own colony, the returning ant's profile was compared to the colony profile the same way as when they entered another colony to steal. Only if they were considered a nestmate, the food was brought back and added to the colony stock. If not, the food was discarded but the ant could keep foraging in the next time step and re-try to enter its home colony.

For the recognition process, we considered three recognition models: *Desirable-present* (*D-present*), *undesirable-absent (U-absent)* and *overall similarity*. The models only affect the way the chemical distance between opponent and colony is calculated. *Overall similarity* is similar to the conjectured recognition in the *Gestalt* model (Crozier & Dix, 1979; Vandermeer & Morel, 1998) and is calculated as the Bray-Curtis distance between the profile of the encountered (thieving) ant and the colony profile of the colony it intends to enter. In contrast, the *D-present* model only pays attention to substances that occur in the template, but ignores any additional compounds. For this model, we calculated the Bray-Curtis distance, but considered components where the thieving ant had higher abundance than the target (i.e. the focal ant) to be

identical. The reasoning for this was that all components for which there is a higher abundance in the thieving ant would be considered as "present" by the targeted colony and thus not elicit aggression. The *U-absent* model, on the other hand, considered components with less abundance in the thieving ant than the target to be identical in both. This model focuses on compounds that are more abundant in the opponent than in the template, and hence are either novel to the focal ant, or only known for her in smaller abundances – they 'stick out' of the template (ref).

The calculated chemical distances ranged from 0 to 1 and were used as the likelihood that the intruder would be rejected. In case of rejection, it immediately returned to its home colony without food. If it was accepted, the target colony would lose one unit of food and the thieving ant attempt to return to its own colony. If it was later rejected at its home colony upon return, it would return later without the food. This assumption was made so that ants would not only have to recognise and reject non-nestmates, but also to recognise (and allow) nestmates.

Reproduction and selection

Each ant colony reproduced after 100 ticks (i.e. 1 generation). At the end of each generation, a defined percentage (default value: 40%) of the colonies died and was replaced by new colonies. If colonies died from starvation, they were replaced as well. The surviving colonies created offspring colonies, with the number of offspring colonies being proportional to their amount of collected food relative to the total amount of food available in all living colonies. The number of offspring colonies was adjusted so that each generation would start with the same total number of colonies.

Offspring colonies inherited their profiles from their parent colony, but with random variation. This variation was created for each component by drawing a number from a normal distribution with 'abundance in parent colony' as mean and *evolvability* as standard deviation (default value: 5). *Evolvability* reflected to the mutation rate in the loci underlying profile biosynthesis, and represented the evolutionary malleability (as opposed to its phylogenetic conservation) of a profile. Components were capped at a minimum of 0, i.e. they could not become negative, but without a maximum.

Experiments performed and data recorded

Our main question was how profile diversity evolves, and whether it is maintained over the course of time. Therefore, we performed a simulation with the model as described above, i.e. with a selection-percentage of 40%, production-cost parameter of 40 and standard deviation for profile evolution of 5. We varied the three recognition models (*overall similarity, D-present, U-absent*) and performed a control experiment in which the selection of colonies that reproduced or died at the end of a generation was done randomly, and not based on the amount of food collected. Each experiment ran for 2000 ticks (i.e. 20 generations) and was replicated 100 times. At the end of each simulation, we calculated population-level cue diversity as the average Bray-Curtis distance between all colonies. Secondly, we recorded the mean absolute cue abundance across all colonies. This was to assess how cue abundance evolves, and how their evolution is shaped by recognition models and profile costs.

To assess the robustness of our results and assess the effect of each individual parameter, we conducted simulations where we varied one of the main parameters and tested this for all three recognition models. The variables that were unchanged in the experiment were set to a default value in all experiments, as defined below. Namely, we varied:

- Selection strength. We simulated selection strength by varying the proportion of colonies dying after each generation as 10%, 40% (default) and 80%.

interaction: F_3 = 273.6, p < 0.0001). As in the main model, they were lowest for *U-absent*, followed by *overall similarity, D-present* and *control* (Fig. 2).

Costs of CHC biosynthesis

Higher profile costs often led to an increase of cue diversity (Fig. 3). However, this effect was weakest in *U-absent* (t = 6.0, p < 0.0001), moderate in *overall similarity* (t = 17.5, p < 0.0001), and high in *D-present* (t = 37.4, p < 0.0001). No effect of profile cost was found in the *control* assay (t = -0.4, p = 0.66) (recognition model: F_3 = 2651.8, p < 0.0001, profile cost: F_1 = 913.2, p < 0.0001, interaction: F_3 = 276.6, p < 0.0001).

As expected, higher profile costs led to a decrease in cue abundance. Again, this effect was weakest in *U-absent* (t = -3.8, p < 0.0001), moderate in *overall similarity* (t = -12.0, p < 0.0001), and high in *D-present* (t = -48.2, p < 0.0001). Thus, low profile costs led to very high cue quantities in the *D-present* model, but less so in the other two models. No effect of profile cost was found in the *control* assay (t = 1.2, p = 0.22) (recognition model: F_3 = 5569.2, p < 0.0001, profile cost: F_1 = 984.7, p < 0.0001, interaction: F_3 = 501.4, p < 0.0001). For all three levels of profile cost, cue abundances were highest in the control, followed by the *D-present* model (Fig. 3). Comparisons to the initial values revealed increases in cue diversity and decreases in cue abundance for all recognition models except for the control (Table S2).

Evolvability

Higher evolvability generally led to higher cue diversity (F_1 = 2353.3, p < 0.0001) (Fig. 4). However, the effect size differed between models (interaction: F_3 = 124.8, p < 0.0001; main effect of model: F_3 = 169.2, p < 0.0001). Effect sizes of evolvability were only similar for *overall similarity* and *U-absent*, but different for all other pairs of models. Interestingly though, cue diversity increased over time only for evolvability 5 and 20, but not for evolvability 1 (Table S2). For evolvability 1, cue diversity strongly decreased over time, with strongest decrease in *U-absent* and weakest in *D-present* (Table S2, Fig. S3).

Like cue diversity, cue abundance also increased with evovability (F_1= 8442.8, p < 0.0001). Again, effect sizes differed between models (interaction: F_3 = 4537.8, p < 0.0001; main effect of model: F_3 = 8490.4, p < 0.0001). Here, effect sizes differed significantly between each pair of models.

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Discussion

Crozier's paradox states that in theory, population-level diversity of recognition cues should erode quickly, because individuals are attacked less if they are similar to others, and hence benefit from being similar to the population average. However, this would render recognition impossible, which relies on inter-individual cue differences. Here, we investigated a potential solution to this scenario, and showed that cue diversity on population level can evolve based on individual-level selection. The main new angle of our approach compared to previous models (Holman et al., 2013; Rousset & Roze, 2007) is that cue perception is separated from the actual cue profile. Therefore, two individuals with different cue profiles need not necessarily consider each other as different. Cues and cue recognition mechanisms evolve independently, and the same cues can be interpreted differently by different individuals. Under this scenario, an individual may profit from being different from the population average, because it may be more efficient in recognising and fending off intruders.

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The separation of cue perception and the profile itself is realistic: cue production and cue perception, i.e. 'sender' and 'receiver' side, are separate pathways that evolve separately albeit they exert selection onto each other (Leonhardt et al., 2016). Cue perception involves the production of odorant receptors as well as the neuronal template to which the perceived profiles

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are compared. In social insects, odorant receptors diversified drastically, which underlines their importance for social interactions and for recognition (d'Ettorre et al., 2017; McKenzie et al., 2016; Mier et al., 2022; Pask et al., 2017). Furthermore, odorant receptor expression can evolve quickly and even change during an insect's lifetime, e.g. when they switch behavioural castes (Caminer et al., 2023). In ants for example, social parasites lost odorant receptors, exemplifying that odorant receptor suites can evolve rapidly (Jongepier et al., 2022).

Given that in *D-present* and *U-absent* not all CHCs are perceived, the perceived chemical distances between two individuals may be different, depending on the recognition model they use, their number of odorant receptors, but also depending on their own profile. Thus, an individuals A may regard individual B as different, but B may regard A as similar. This concept was not considered in previous models, but is implemented in our model through the separation of cue profile and cue perception.

Our model assumes a scenario of intraspecific parasitism. Colonies can forage on their own, but also raid food sources of other colonies, and thus are host and parasite simultaneously. Such a scenario has been shown e.g. in the neotropical *Ectatomma ruidum* (Formicidae: Ectatomminae), where intraspecific food robbing (cleptoparasitism) occurs and can reduce colony productivity (Jandt et al., 2015). Next to resources being thought of as food resources, it is also conceivable to think about them as workforce (larvae or pupae) that is being robbed – which would represent social parasitism. Indeed, intraspecific social parasitism is common in many ant species, especially as temporary social parasitism during colony foundation (Buschinger, 2009; Seifert, 2018). Also in established colonies, older and larger colonies often raid brood from younger colonies to enlarge their own size, which has been shown for Myrmicine and Ponerine ants (Paul et al., 2016; Stamps & Vinson, 1991). Social parasite species are often the closest relatives to their host species (Buschinger, 2009). This suggests that interspecific parasitism evolved from intraspecific parasitism, which further justifies the scenario we assumed. Parasitic interactions can shape the evolution of host diversity and host recognition systems. Parasitism can favour host diversification via exerting negative frequency-dependent selection (Hamilton, 1982; van Valen, 1973). This has also been shown for recognition-relevant traits, e.g. for cuckoos (Kilner & Langmore, 2011; Øien et al., 1995) and for ants where parasitised populations carry higher cue diversity than non-parasitised ones (Jongepier & Foitzik, 2016). This matches our results, where intraspecific parasitism led to higher cue diversity.

Our model is parsimonious in that it does not make any assumptions concerning disassortative mating or sexual selection. While one study showed that ants can mate disassortatively (i.e. they prefer non-nestmates) (Oppelt et al., 2008), further evidence for sexual selection is generally low in ants, and there seem to be few opportunities for males or females to actually choose between different mates (Heinze pers. Com.; (Boomsma et al., 2005).

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Evolutionary trajectory of diversity in the different recognition models

In our simulations, cue diversity increased over time. This was true for a range of values concerning evolvability, production costs and selection strength, thus showing that this result is robust to a wide variety of parameter combinations. Notably, *overall similarity* and *U-absent*