Asymmetry in olfactory generalization and the inclusion criterion in ants

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Animals constantly face the challenge of extracting important information out of their environment, and for many animals much of this information is chemical in nature. The ability to discriminate and generalize between chemical stimuli is extremely important and is commonly thought to depend mostly on the structural similarity between the different stimuli. However, we previously provided evidence that in the carpenter ant *Camponotus aethiops*, generalization not only depends on structural similarity, but also on the animal's previous training experience. When individual ants were conditioned to substance A, they generalized toward a mixture of A and B. However, when trained to substance B, they did not generalize toward this mixture, resulting in asymmetrical generalization. This asymmetry followed an inclusion criterion, where the ants consistently generalized from a molecule with a long carbon chain to molecules with a shorter chain, but not the other way around. Here I will review the evidence for the inclusion criterion, describe possible proximate mechanisms underlying this phenomenon as well as discuss its potential adaptive significance.

Communication is vital for all types of social interaction, and is achieved by the interplay of three separate components, previously described for recognition systems:[1] the expression, perception and action components. The expression component occurs when an organism produces a signal or cue (e.g., chemical, visual, acoustic). After expression, a receiver individual detects this product (perception component) and could then alter its behavior, based on the newly acquired information (action component).

Chemical communication is perhaps the most widespread means of communication in the animal world.[2] In insects, the importance of odour detection is highlighted by the fact that olfactory receptor genes are one of the largest families of genes, and underwent rapid evolutionary change.[3] In the olfactory modality of insects, the perception component can be divided into multiple steps.[4,5] First, odorant molecules are picked up by sensilla present on the antennae. These sensilla contain Odorant Binding Proteins (OBPs) and Chemosensory Proteins (CSPs[6,7]), both of which can bind specific odorant molecules and transport it through the lymph fluid to the Olfactory Receptors (ORs), located on the Olfactory Receptor Neurons (ORNs). These ORNs then transmit a signal to the antennal lobes, the first-order integration center of the insect central nervous system. The antennal lobe consists of many glomeruli, which are synaptic aggregations where ORNs are connected to local interneurons and projection neurons. These local interneurons modulate the response of other glomeruli through inhibition or stimulation. Afterwards, the projection neurons of the glomeruli transfer the modified, integrated information to the higher brain centers (mushroom bodies and lateral horn). These brain centers integrate all received information (olfactory, visual etc.), and regulate for example motor neurons, resulting in a behavioral response (action component).

A particularly fascinating area of study is that of olfactory generalization, which occurs when an individual treats two structurally different odorants as similar. Olfactory generalization can arise from either the perception or action component of communication. If it is due to the perception component, the animal can simply not distinguish between the two stimuli. If it arises from the action component, the animal actually does perceive a difference between the two stimuli, but treats them as being similar.

Asymmetry in Generalization and the Inclusion Criterion

We previously studied generalization of odorants in the carpenter ant *Camponotus aethiops*.[8,9] By using an associative learning protocol known as the Maxilla-Labium Extension Response (MaLER[10]), we conditioned individual ants by training them to for example substance A, and afterwards tested their response to a mixture of A and a novel substance B. Ants trained to substance A treated a mixture of A and B as similar, but surprisingly individuals trained to B did not generalize toward the mixture of A and B, indicating that generalization between stimuli is not only influenced by the similarity of molecular structure of the odorants involved, but also by the animal's previous experience.

While asymmetry in generalization itself has previously been found in many organisms, especially in the olfactory modality (e.g.[11-15]), most of these studies either used very few substances, or substances that greatly differed in their molecular structure or function (e.g., floral odour vs. alarm pheromone), making it impossible to draw conclusions about the molecular basis of the asymmetry.

In order to address this question, our experimental setup allowed us to describe how molecular structure systematically plays a role in predicting whether the ants would generalize or not. The ants consistently generalized from molecules with a long carbon chain to mixtures containing this trained substance and a molecule with a shorter chain. However, when trained to a molecule with a short chain, no generalization to a mixture containing this trained substance and a molecule with a longer chain was found. Additionally, we elucidated a hierarchy of stimuli regarding functional groups, where ants trained to alcohols generalized to aldehydes and ketones of similar or shorter length. When trained to aldehydes, ants would generalize to ketones of similar or shorter length, but not to alcohols. Lastly, when trained to ketones ants would not generalize to molecules with other functional groups at all. This asymmetry thus follows an inclusion criterion (a term adapted from Guerrieri et al.[16]), where substances with shorter chain lengths or certain functional groups are treated as if similar to the trained substance.

This raises the question about whether the inclusion criterion is a general phenomenon in the animal world, or whether it is exclusive to ants. Two pioneering studies by Smith and Menzel[11] and Guerrieri et al.[15] in honeybees allowed for extensive analysis of asymmetry in generalization with regards to molecular structure, as both studies used different volatile floral odours varying in either chain length or functional group. Although asymmetry in generalization was found in these studies, it could not systematically be predicted by molecular structure, thus not supporting the inclusion criterion. However, in these studies the test stimulus was always a single substance instead of a mixture of the conditioned stimulus and the test stimulus. In these conditions, ants also show no evidence of the inclusion criterion.[9] As generalization to a mixture after training to a single substance has, to my knowledge, not been studied in a systematic way in other animals, it is currently impossible to draw conclusions on how widespread the inclusion criterion is.

In ants, a similar phenomenon of inclusion appears to come into play in nestmate recognition, where non-nestmates (enemies) are aggressed only if their cuticular chemical profile contains additional substances in comparison to the profile of the discriminating individual, but are not recognized when they have less substances.[16,17] Indeed, some social parasites use this to their advantage, and are known to be chemically insignificant, having only a very small amount of substances on their cuticle, which ensures that they are not recognized by the host colony.[18] This implies that the inclusion criterion might be a general phenomenon, at least in ants.

Does it Arise from the Perception or Action Component?

Now that we established that, at least in the carpenter ant *C. aethiops*, asymmetry in generalisation follows an inclusion criterion, the question remains about whether it arises from the perception or action component of communication.

If inclusion arises at the perception component, the receiving individual does not notice the difference between the trained odorant and the substance or mixture that it generalizes toward. However, this does not necessarily mean the odour is not detected by the sensilla, as inclusion could potentially result from processes at different levels of the perception component.

The first level is that of the OBPs and CSPs in the sensilla. These sensory proteins are normally tuned to specific substances. Intuitively, one could predict that these proteins might also bind smaller substances due to the size of their binding pocket (while not binding larger molecules than the one they are tuned to). In turn, generalization toward a molecule smaller than the conditioned stimulus might be expected, as these proteins would bind to both to the trained substance, as well as smaller ones. However, evidence points in the other direction, where sensory proteins tuned to a certain substance bind, next to the molecule they are tuned to, larger molecules better than smaller ones.[19,20,but see 21] If larger molecules indeed activate proteins tuned to smaller molecules but not the other way around, a binary mixture containing a large and small molecule could activate the same sensory proteins as the large molecule alone, potentially explaining our results. Alternatively, not the binding pocket, but the chemical attributes of the odorants might cause asymmetry in generalization. Nearly all ligand interactions rely, to some extent, on hydrogen

bonding. For example, there is a difference in the capability of alcohols and aldehydes/ketones to form hydrogen bonds. The oxygen in all of these classes of molecules can build a hydrogen bond with the hydrogen atoms of the sensory protein, but only alcohols can also build a hydrogen bond with the protein's oxygen through the hydrogen in the alcohol's -OH group.[22] This might cause alcohols to bind better than aldehydes and ketones, possibly explaining the hierarchy of stimuli found in our study, where generalization occurred from alcohols to aldehydes and ketones, but not the other way around.

The second level is that of the olfactory receptors and the antennal lobe. Every odour elicits a unique pattern (much like a barcode) in the antennal lobe by activating a specific subset of glomeruli.[23,24] If higher chain length molecules elicit the same pattern as their lower chain length counterparts, but activate some additional glomeruli in the process, this could give rise to asymmetry in generalisation, as the activation of additional compounds seems to enable discrimination, whereas the deactivation of many glomeruli does not.[25] However, smaller molecules do not necessarily activate less glomeruli, at least in the honey bee.[24,26] Furthermore, mixtures are not always represented in the antennal lobe as the sum of their parts due to mixture interactions. Because of this, odour blends can acquire a unique quality, where sometimes not all information about the individual components is perceptually available anymore.[27] A map of the antennal lobe, including activation patterns of many different odorants and their mixtures would be required to test this hypothesis in ants. Unfortunately, due to the complex morphology of the ant antennal lobe, full mapping remains extremely challenging.[28,29]

Another potential cause for the inclusion criterion arising at the perception component could be inhibition due to overshadowing. Overshadowing occurs when an individual encounters a mixture, but only perceives a subset of that mixture.[30] For example, if ants trained to e.g., 2-hexanone are tested with a mixture of 2-hexanone and octanol, reaction might be inhibited if octanol overshadows 2-hexanone (i.e., the ant mainly perceives octanol, even though 2-hexanone is also present in the mixture). However, this seems to be an unlikely cause of our results, as we also conducted an overshadowing experiment by training ants to a mixture and afterwards testing their reaction to the individual components, which suggested that overshadowing is rare in *C. aethiops*.[9]

Instead of the perception component, the inclusion criterion could arise at the action component. This would imply that the animal receives all the information needed in order to discriminate between different substances, but follows certain decision rules resulting in asymmetrical generalisation. Our experiment showed that ants could perceive each tested substance,[9] making it likely that the action component is the cause for the inclusion criterion. However, as previous experience to a single substance can adjust processes in the perception component (through for example sensory adaptation[31]), more experimental data are needed in order to tease apart the two components from each other.

Conclusions

The question remains on how widespread the inclusion criterion is. More species should be studied in order to find out whether our study species (*Camponotus aethiops*) is unique with respect to the inclusion criterion or not. As learning paradigms are well established for many different animals,[12,32-35] a systematic investigation, including many substances of different chain-lengths and functional groups, of the reaction of individuals to a mixture of the conditioned stimulus and a novel odorant would elucidate whether the inclusion criterion is widespread or not. If the ant is indeed the exception and one of the only groups of animals that uses the inclusion criterion, this would suggest (but not prove) that it arises from the action component, as the olfactory pathway (which is part of the perception component) is well preserved between different groups of animals.[36]

Whether the inclusion criterion is adaptive or not is not clear. One potential benefit for ants could be during recognition of nestmates vs. non-nestmates. As their cuticular chemical profile consists of many different substances, the inclusion criterion could 'compact' this profile into something simpler to interpret by treating multiple different substances as similar. This hypothesis is supported by recent evidence in the Argentine ant (*Linipithema humile*).[37] Ants were exposed to a filter paper containing their own nestmate odour, to which they were not aggressive. When one of eight synthetic hydrocarbons (differing in branch position, chain length, or both) was added to this filter paper, the ants started to be aggressive. The ants displayed different levels of aggression depending on which hydrocarbon was added. The authors found functional homologs, where hydrocarbons differing in chain length (but not in the position of the functional group) received equal levels aggression, whereas this was not the case when comparing aggression levels against hydrocarbons differing in the position of the functional group. This could indicate that these functional homologs are 'compacted' into a single peak during the perception component.

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