

PERSPECTIVES

Critical thinking in the chemical ecology of mammalian communication: roadmap for future studies

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Summary

1. Sophisticated and sensitive technologies now allow separation, quantification and chemical characterization of numerous compounds that play roles in chemical communication, chemical defence and aggression, in interactions between conspecific or heterospecific individuals. In the particular subfield of mammalian chemical communication, these rapid technological advances, combined with a frequent lack of technical background, have led to important errors in both chemical characterization of molecules and interpretation of their roles as chemical mediators of communication.

2. The aim of this article is to highlight some of these methodological and analytical pitfalls and to provide a basis for better understanding of chemical mediation of communication in mammals. We compiled the recent literature treating molecules found in mammalian secretions and having putative roles in communication. A selection of 41 published studies dealing with 33 mammal species revealed reports of 857 different molecules. Based on the five main metabolic pathways responsible for the biosynthesis of most known secondary metabolites, we propose nine general biochemical rules that will help researchers to avoid errors of chemical characterization and to aid in interpreting the possible functional role of identified molecules as chemical mediators of mammal communication.

3. Following these nine rules, we show that published studies include reports of molecules that are incorrectly or ambiguously named, molecules of exclusively non-natural origin, molecules produced by other organisms but not directly by mammals, and molecules of biological origin and possibly produced by mammals. Only the last two of these classes could conceivably play roles as mediators of mammalian communication. We discuss the potential roles of these compounds as reported in the publications we reviewed.

4. Our recommendations concerning technical, analytical and statistical aspects of the identification of compounds and interpretation of their roles should help chemical ecologists ask the appropriate questions about the accuracy of their identifications of molecules, the biological relevance of molecules they do identify and the possible functional roles of these molecules in mammalian communication.

Key-words: chemical communication, chemical mediators, mammals

Introduction

Chemical ecology concerns chemically mediated interactions between organisms and their biotic environment, that is, other organisms of the same or different species. Chemi-

cal communication and chemical defence (and aggression) are two major areas of research in chemical ecology. The great potential to explain proximate mechanisms and ultimate consequences of interactions among organisms has made chemical ecology one of the fastest-growing areas in all of biology. A search for 'chemical ecology' or 'chemical communication' in the Web of Knowledge shows an exponential growth in the number of studies published over the

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past two decades. Chemical ecology has been the focus of several reviews (e.g. Isaacs 1998, Mitchell-Olds *et al.* 1998, Pichersky, Noel & Dudareva 2006), editorials, commentaries and special issues in generalist journals such as *PNAS* (see vol. 92(1), 1995; 105(12), 2008), *Nature* (see the feature 'Insight: Chemical Sensing', vol. 444(7117), 2006) and *Science* (Eisner & Berenbaum 2002), and several outstanding books (e.g. Albone 1984; Müller-Schwarze 2006; Zhang & Hong 2010).

The continual improvement of highly sophisticated and sensitive technologies now allows the separation of very small quantities of complex mixtures into their individual compounds, and the quantification and chemical characterization of each. Along with these technological innovations, chemical ecologists now face a wide range of unexplored molecules, some potentially involved in the mediation of chemical communication and/or chemical defence. In our opinion, these rapid analytical advances have often outpaced the chemical expertise of biologists interested in chemical ecology, resulting in errors ranging from mistaken or incomplete identifications to interpretations that do not withstand critical attention. This is particularly true for studies of mammalian chemical communication, for several reasons. First, the currently available analytical tools have been designed mainly for plants and insects, the most frequent subjects of studies in chemical ecology. These tools may also serve to characterize mammalian chemical mediators and are, therefore, probably not well suited for mammalian chemical molecules. Secondly, interpretation of results depends on the chemical stability of molecules under the experimental conditions of their capture and analysis, and this is often a trickier process in studies of mammals than in investigations of plants or insects. Procedures that are currently frequently used underestimate the experimental rigour required; this is especially true for field studies. Thirdly, behavioural tests aimed at validating signalling compounds of interest, routinely used for insects, are often hard to design in studies of mammals. Consequently, studies in mammalian chemical ecology often are limited to analyses of the overall odour bouquet produced by the organism (or the particular tissue or organ) being studied. This global approach has likely engendered errors and imprecisions, both in chemical characterization of molecules and in the interpretation of their role as chemical mediators of communication. Finally, many environmental factors, such as diet, the action of microorganisms, or pollution, may all affect the identities of molecules produced, further complicating interpretation of the roles of molecules in conveying information. This last difficulty is reinforced in mammalian chemical ecology, owing to the lack of a comprehensive knowledge of molecules of interest.

Aim of the article and selected studies

The aim of this article is to bring to the attention of chemical ecologists on some methodological and analytical pitfalls that reflect misunderstanding of chemistry and of

mammalian biochemistry and to provide a basis for better understanding of chemical mediation of communication in mammals. To do so, we compiled the recent literature treating molecules found in mammalian secretions and having putative roles in communication using Web of Knowledge (© 2011 Thomson Reuters) with different sets of keywords (e.g. mammal and chemical communication or chemical analyses or GC-MS analyses). We restricted our survey to the years 2000–2010 (March), a period marked by a net rise in the number of published articles on chemical ecology and to the primary literature (book chapters and review articles were excluded). We further selected only the articles that clearly reported molecules of interest in separate tables. Following these different steps, we retrieved 41 studies, published in seven different journals, on 33 mammal species (Table S1, Supporting Information). Most of these studies concerned novel reports of compounds in a given mammalian species. However, for a subset of them, the ones that analysed odours from the same species, several molecules were found identical. From these 41 articles, we therefore compiled a list of 857 different molecules (appearing a total of 1370 times in the 41 selected studies), excluding those that were explicitly not fully characterized.¹ These studies followed different approaches to the analysis of odours. Most of them studied the relationships between the full odour bouquets of the studied animals and individual or population parameters (24 studies; Table S1, Supporting Information), while others only aimed at chemically characterizing secreted molecules without proposing any functional relationship between the molecules and individual or population parameters (17 studies, Table S1, Supporting Information). It is important to note that our aim is not to assess the efficiency of each of these studies individually in answering questions about mammalian communication, but rather to identify general problems. We therefore chose not to cite specifically the studies involved except in Table S1, Supporting Information.

Defining five biosynthetic pathways and nine corresponding chemical rules

First, we classified the 857 different molecules according to their biosynthetic pathways following two outstanding reference books (Mann, 1994; Mann *et al.* 1994) and a review (Rodríguez-Concepción, 2006). These pathways are subsumed under what has been termed 'secondary metabolism'. The use of secondary metabolites as chemical mediators of intra- and interspecific interactions is at the root of chemical communication. Secondary metabolites are produced by a relatively small number of essential intermediates deriving from five main biosynthetic

¹Examples of not fully named molecules include 'Unidentified, branched fatty acid', 'a trimethylpyrazine', '3(?)-methyl dodecanoic acid', 'heptadecenoic acid (branched)', '2-nonen-4-one (homologs)', '3- or 4-propyl-1,2-dithiacyclopentane' and 'pentanol+toluol'.

pathways (Mann, 1994; Mann *et al.*, 1994; Rodríguez-Concepción, 2006):

1. the shikimate pathway allows the biosynthesis of some aromatic hydrocarbons.

2. the acetate (or acetyl coenzyme A) pathway is at the source of the production of polyketides, which are the precursors of phenolic derivatives (a benzene core functionalized with an alcoholic function) and fatty acids.

3. the amino acid pathway is the precursor of most nitrogenous heterocyclic compounds.

4. the mevalonate pathway derives from the acetate pathway and leads mainly to the production of isoprenoids (terpenoids, steroids and carotenoids).

5. the methylerythritol 4-phosphate pathway allows plants and bacteria to produce the same precursors as the mevalonate pathway, via an independent pathway localized in different cell compartments.

Secondly, this classification allowed us to define nine chemical rules designed to help clarify the origins of the molecules reported and aid in evaluating their putative roles as chemical mediators of communication in mammals. In brief, the first four rules cover aromatic compounds. The following four rules concern mono- or polyunsaturated and saturated fatty acids. Finally, the last rule we proposed concerns terpenoids and steroids (Table 1, and see Data S1, Supporting Information for more details). Some of these rules were straightforward to establish and did not cast any doubt on the interpretation of the role of some molecules involved in mammalian communication. For example, rule 1 states that the shikimate pathway is a prerogative of micro-organisms, fungi, algae and plants. Consequently, when such molecules deriving from the shikimate pathway were found in the 41 studies selected (e.g. 3-phenylpropanoic acid, found in four different studies), we questioned both their origin and, more importantly, their biological relevance as mammalian chemical mediators. By contrast, other rules were more ambiguous to define and bear exceptions. For example, in rule 5, we propose that whether mammals can produce heterocyclic aromatic molecules is not yet clearly established. Indeed, we know that mammal organisms are able to metabolize nitrogenous heterocyclic aromatic molecules acquired from exogenous sources, such as food. However, we still have no clear examples of mammal species directly producing such nitrogenous molecules. The fact that in some, albeit rare, cases these molecules are also known to act as mammalian chemical mediators obscures the interpretation (for more details, see Data S1, Supporting Information and see below).

Classification of molecules

These nine chemical rules allowed classifying the 857 molecules identified in the 41 studies we reviewed into five categories, as follows. First, we retrieved molecules the names of which have no chemical meaning either because they are incorrectly named or because their name is not precise enough to unambiguously attribute a chemical structure to

the molecule (Table S2, Supporting Information). We found a total of 77 different misnamed molecules in the 41 selected studies (9%).

Secondly, we retrieved molecules that are not produced by any living organism: they are not derived from one of the five biosynthetic pathways presented above, but are rather synthetic chemicals industrially produced by humans (Table S3, Supporting Information). A total of 38 different non-natural molecules have been reported in the 41 studies as possible mammalian molecules (4.4%). Analysis of each study separately, however, shows that the proportion of non-natural molecules described ranges from 0 (23 studies) to values ranging from 15% to 42% (four studies) of the total number of molecules described. We found that several of these non-natural molecules are also toxic or irritating for any living organism. For example, 2-methyl-2-propenal is a non-natural molecule found in cigarettes and used in the past as a chemical weapon (Rose & Cohnsen, 2011); o-toluidine is carcinogenic to humans at 2 p.p.m. (for references, see Table S3, Supporting Information). Some of these non-natural molecules are known to bioaccumulate in living organisms exposed to industrial pollution. For example, the Norwegian killer whale (*Orcinus orca*) is considered to be the most toxic mammal in the Arctic ('dethroning' the polar bear; Bernhoft, Wiig & Skaare 1997) because it accumulates various industrially produced pesticides and PCB (polychlorinated biphenyl), a persistent organic pollutant (e.g. Ross *et al.* 2000; and see for review Muir *et al.* 1999). Their presence in high concentrations raises the real possibility that some of these toxic molecules could interfere not only with physiological functions but also with chemical communication. To sum up, these molecules likely originate from exogenous contaminations and their presence in mammalian secretions is more likely to reflect processes of excretion for detoxification than a role as chemical mediators involved in communication. Consequently, the interpretations for their occurrence put forward in the surveys studied must be re-examined.

Thirdly, we found molecules known in nature but that are certainly not directly produced by any mammal organism, owing to the absence in this taxon of the biosynthetic pathway involved in their production (Table S4, Supporting Information). While these molecules do not necessarily represent identification errors, their presence, if verified, certainly originate from exogenous sources such as food or activities of microflora. We classified 109 different molecules into this category (12.7%). For example, we found reports of molecules that originate from plants but that are known to be toxic for mammals. Nicotine is a nitrogenous heterocyclic compound produced by some solanaceous plants. At large doses, nicotine is highly toxic to humans. The presence of nicotine in human body secretions may possibly result from cigarette contamination. While mammals are unlikely to directly produce these molecules, we do not exclude that some of them may have been further modified by mammalian metabolism. Clear examples include indole and skatole, both of which are

Table 1. Synthetic description of the nine chemical rules

	Rules	Relevance in mammals	Examples of molecules
Aromatic compounds	1. The shikimate pathway is a prerogative of micro-organisms, fungi, algae and plants	No mammalian origin	4-Coumaric acid Caffeic acid Sinapyl alcohol
	2. Aromatic hydrocarbon compounds derived from the acetate pathway are a prerogative of micro-organisms, fungi, algae and plants	No mammalian origin	Methyl salicylate Phloroglucinol
	3. Non-oxidized aromatic derivatives are not of natural origin*	Not natural	Mesitylene Naphthalene
	4. Whether mammals can produce heterocyclic aromatic molecules is not established	Certainly no mammalian origin	Castoramine Muscopyridine
Mono- and polyunsaturated fatty acids	5. Trans monounsaturated fatty acids are extremely rare in the living world	Natural origin is exceptional	Methyl-(E)-9-octadecenoate
	6. The position of the oxidative desaturation in monounsaturated fatty acids indicates their biological origin	Possible mammalian origin	Oleic acid Palmitoleic acid Angelic acid
	7. The position of a double bond in polyunsaturated fatty acids indicates their plant or animal origin	Possible mammalian origin	Arachidonic acid Linoleic acid
	8. Saturated fatty acids are ubiquitous in all living organisms	Possible mammalian origin	Decanoic acid Eicosanoic acid Heptadecanoic acid
Steroids and terpenoids	9. The case of steroids and terpenoids	Possible mammalian origin	Cholesterol Squalene Lanosterol

*Except for phenylpropanoids (see for more details Data S1, Supporting Information)

derived from an amino acid not produced by mammalian metabolism (tryptophan), but several mammalian species are also known to secrete them. Moreover, even molecules of exogenous origin may convey information about the animal. There are in fact numerous examples of exogenous molecules that have become signalling compounds over evolutionary time or that are used by others ('the receivers') for their own benefit. One of the best known examples comes from visual communication: in birds, exogenous carotenoids acquired from food are moved unchanged to the tegument and their effects on the animal's appearance may be used by potential mates to evaluate its foraging ability (e.g. Endler, 1980). Similar phenomena are thus not unexpected in chemical communication. For example, the African crested rat (*Lophiomys imhausi*) has evolved a suite of adaptive traits aimed at advertising deterrent toxins to predators. These toxic compounds are plant secondary metabolites found in roots and bark of a particular tree species. The rats slaver the toxins onto their fur after masticating the roots and bark (Kingdon *et al.* 2011). This mammal has possibly evolved digestive-tract specializations for detoxification and/or specialized salivary secretions to process the toxin (see discussion in Kingdon *et al.*, 2011). This example highlights the possibility that exogenous molecules have acquired adaptive roles in chemical defence and communication in a mammal species. In another type of example, microflora, including infectious

bacteria, may produce specific compounds that convey information about the bearer's health status (Dawkins, 1982; Penn & Potts, 1998; Kavaliers, Choleris & Pfaff, 2005). In murine scent marks, the microbial community is determined by the host's genetic characteristics (Lanyon *et al.* 2007). Consequently, chemicals produced by the microbial community alone may convey information about the animal's traits. Such compounds are part of the extended phenotype *sensu* Dawkins (Dawkins, 1982) of the mammal and may be produced by pathogenic, mutualistic or commensal microflora. It may sometimes be difficult to determine whether adaptive benefits are conferred on both bearers and receivers, in which case the compounds can be regarded as signals and the bearers as 'senders', or whether only receivers gain benefits by their ability to perceive these cues. We do not question that some molecules of natural but non-mammalian origin could act as cues used by mammals to locate and identify conspecific individuals, or assess their quality as potential mates. For example, indole has been repeatedly connected to chemical communication in numerous mammal species (indole has been reported in 12 of the studies analysed; Table S4, Supporting Information). We propose that the putative roles in mammalian communication that have been ascribed to such molecules of exogenous origin should, however, be carefully examined, as both food and microflora may be of only transient occurrence in the animal. The exogenous origin of some

molecules seems often to have escaped the attention of those who have reported their occurrence, and we propose that proper interpretation requires distinguishing such cases from those in which interactions are mediated by molecules produced directly by mammals.

Fourthly, based on our literature selection, we compiled 198 different molecules (23.1%) possibly directly produced by a mammal because they result from biosynthetic pathways known to be present in such organisms (Table S5, Supporting Information). However, it is important to bear in mind that not all of these mammalian molecules are directly involved in communication. Some proteins may function to bind chemical mediators, without bearing any communicatory meaning in themselves. The lipocalin family includes such odour-binding proteins. These molecules, however, may also convey information about the bearer (Flower, 1996), complicating interpretation of their role in chemical communication. Mammalian odours also include excretory products of catabolism such as urea. Their ubiquity makes it difficult to understand how they could play roles in communication.

Finally, we did not attempt to classify 435 different molecules (50.8%; Table S6, Supporting Information) because most of these are structurally so simple that they could have originated from multiple pathways in many organisms. Molecules for which we were unable to ascribe to one of the five biosynthetic pathways were also grouped within this category.

To summarize, 38 of the 41 analysed studies included between 1.2% and 100% of molecules that either were not correctly named, are not natural products of living organisms, or are likely not to have been directly produced by mammals but by other organisms. On average, 29.8% of the reported molecules in all 41 studies belonged to these three questionable categories. Yet, several of the 41 analysed studies adopt conservative approaches that we think are appropriate. First, some authors clearly flag some of the reported molecules as exogenous (acquired from food or resulting from bacterial fermentation). Others 'tentatively' identify the molecules of interest they discuss: some only provide the class of the molecule found, such as 'fatty acid' or 'ester', or even list some molecules as 'unknown'. Such conservative practices will limit the replication of inaccuracies. Cases are not unknown in which chemical ecologists have validated their own findings by referring to other studies that mistakenly implicate molecules well recognized to be contaminants. Furthermore, some authors also indicate the mass spectrum profiles of the compounds they report by providing the mass of characteristic fragments (with or without their relative abundance) for the unknown compounds. Some authors also restrict their analyses to an odour bouquet composed of the major well-characterized molecules, especially those shared by numerous studied individuals. Thereby, they avoid including rare compounds with a greater probability of resulting from exogenous contaminations. In our opinion, such conservative approaches are the best way to avoid errors of identification and/or interpretation.

Recommendations

To complete this opinion paper, we propose recommendations to ecologists who aim to use analytical chemistry in mammalian chemical communication, a very young field of investigation compared with chemical communication between insects, between plants, and between these plants and animal partners.

1. Chemical ecologists need the technical assistance of competent chemists to insure the appropriate use of new instruments and analytical methods, which are being constantly improved and updated. While this seems an obvious recommendation, it is crucial that chemically minded ecologists establish cooperative partnerships with analytical chemists interested in the study of chemical communication. We further recommend that chemical ecologists make controls at each step of their work: when collecting odorant from their model, when extracting these odorants and when running them on the appropriate apparatus. While this recommendation also seems an obvious one, we noticed that of the 41 studies, only 15 of them mentioned the use of controls (and only a subset of six studies clearly specified that controls were collected when odorants were collected).

2. Concerning the analysis and interpretation of chemical data, we first recommend that ecologists attempt to classify their candidate molecules and use the nine rules we have proposed as a first step in evaluating the relevance of these molecules as chemical mediators. We further stress that chemical ecologists should adopt a critical attitude when using the available computerized libraries and not use them as the only tool to identify molecules. These libraries are highly generalist, designed to be suitable for all kinds of chemical analyses from industrial polymers to pharmaceutical products, and therefore include many compounds of non-natural origin as well as natural products of organisms. While their generalist nature may also help in identifying pollutants, the available libraries should be regarded as a secondary helping tool. We suggest use of the data sheets provided by specialized organizations, such as INRS (National Institute for Research and Security, France; <http://www.inrs.fr/accueil.html>), or HSE (Health and Safety Executive, UK; <http://www.hse.gov.uk/hid/haztox.htm>), and freely available online, which can help determine the toxicity, and consequently the possible non-natural origin, of some molecules. Moreover, specialized data bases such as the one proposed by Adams (Adams, 2007), especially designed for essential oils, or those featured in the pherobase (<http://www.pherobase.com/>) and flavornet websites (<http://www.flavornet.org/>), designed for volatile compounds found in insects and plants, respectively, allow rapid identification of numerous, albeit not all, molecules. Such specialized data bases could prove useful in helping identify some molecules of mammalian origin. Currently, the only alternative is manual interpretation of the chromatograms and the creation of specific data bases for each study organism. This approach is,

however, fastidious and potentially complicated. Alternatively, the use of authentic standards is a very powerful identification method. Moreover, for each compound, we recommend that investigators report the number of individual animals studied. This will help in evaluating the relevance of the molecule for mammalian communication. We further recommend that the methods used to characterize each compound should be clearly indicated to permit evaluation of the accuracy of identifications. Among the 41 studies, seven did not refer to any method of identification, nine used only a library or matched the mass spectra and retention times with those found in the literature, eight used only reference compounds. Only 17 used both information from the literature or a library coupled with reference compounds. Finally, behavioural bioassays constitute the necessary step to definitively validate a molecule as a chemical mediator of communication. Such tests may be a real challenge to set-up, especially for the study of large mammalian species. Whenever possible, efforts should be made, however, to design such bioassays.

3. Finally, concerning statistical analysis, we recommend that chemical ecologists compare the effect of including, or not, in their analyses molecules that are of natural but non-mammalian origin. Such comparisons would give invaluable information about the functional and evolutionary relevance of molecules not directly produced by the studied organism.

To date, chemical ecologists have successfully clarified the chemical composition of numerous natural chemical mediators and the behavioural responses associated with these stimuli in numerous taxa of plants, insects and micro-organisms. The recent exponential increase in the number of studies on mammalian chemical communication will allow us in the near future to obtain better knowledge about molecules of interest. Strengthening the communication networks among chemical ecologists, for example, through specialized conferences or by the creation of a website specially focused on the study of mammalian chemical mediators (such a website is not yet available) would also greatly improve the quality of future studies.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Species studied in the 41 selected original papers.

Data S1. Nine chemical rules and representative figures.

Table S2. Names of molecules reported that are incorrect or insufficiently precise to identify the molecule.

Table S3. Molecules of non-natural origin.

Table S4. Molecules of natural, but non-mammalian, origin.

Table S5. Molecules of mammalian origin.

Table S6. Non-classified molecules.

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