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Chemical Signals in Terrestrial Vertebrates: Search for Design Features

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1

Chemical Signals in Terrestrial Vertebrates: Search for Design Features

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1 Introduction
1.1 Olfaction and odor
1.2 Status as signals and validity of identifications
2 Amniote signal chemistry
3 Discussion
3.1 Are there patterns, and at what scale?
3.1.1 Exploratory statistical analysis
3.1.1.1 Variables, classes, and categories
3.1.1.2 Chi square (χ^2) analysis
3.1.1.3 Analysis of variance (ANOVA) for influences on molecular weight
3.1.1.4 Discriminant analysis
3.1.1.5 Stepwise discriminant function analysis
3.1.1.6 Comparison with whole odors
3.2 Signal detectability
3.2.1 Scope and sensitivity of amniote olfactory detection
3.2.2 Access to sensory epithelia
3.2.3 Selectivity of olfaction
3.2.3.1 Species specificity
3.3 Transmission
3.3.1 Active space and detection at a distance
3.3.2 Persistence
3.4 Costs
3.5 Information content
3.5.1 Chemical diversity and signal diversity
3.5.2 Index signals - potential templates for signal patterns
3.5.2.1 Metabolic and physiological indicators
3.5.2.2 Dual-trait pheromones

1 Introduction

Chemical signalling is the oldest and the most widespread mode of communication between organism,[1] and has evolved over more than 3.5 billion years. As a result, the chemicals used to communicate would be expected to exhibit patterned relationships with the messages they transmit, the environmental conditions under which they operate, and the biology of signal emitters and receivers, because signals must be detectable, discriminable, transmissible, informative, and cost effective. In addition, mechanistic links between signals and the metabolism and physiology of the signaller may be reflected as patterns in signal chemistry. Overall, similar messages sent under similar circumstances would be expected to involve chemicals with similar properties.

The relationship of signal function to the physico-chemical properties of pheromones was first examined by Bossert and Wilson,[2] who focused on the active space, speed of dispersion, and persistence of airborne insect pheromones. Alberts[3] examined chemical signalling by terrestrial vertebrates and found that the chemical composition of signalling odors was related to the type of message and environmental conditions. Mean molecular weight (MW) of odor components differed with the type of message transmitted, and increased in the order of sex attraction, recognition, alarm/threat, and range marking. Range marks contained more compounds with aromatic rings; range marks from hotter and moister habitats had higher mean MWs; and recognition odors and range marks had more components than did sex and alarm/threat odors, possibly

3

because they code individual identity. Alberts[3] concluded that the compositions of mammalian chemical signals are adapted to their function and to the ecology of the communicating organisms.

Here, we review current information on intraspecific chemical signals and search for patterns in signal chemistry among modern terrestrial vertebrates (Amniota), including tortoises, squamate reptiles (amphisbaenians, lizards, and snakes), birds, and mammals. The closest living relatives of birds are crocodilians, and birds are now classified as reptiles. Limited data constrained Alberts[3] to assign signalling functions to whole odors and secretions, rather than to specific compounds. More compounds with demonstrated signalling functions subsequently have been characterized, and our search for patterns focuses on these characterized signal compounds.

Our treatment of reptiles complements recent reviews of pheromones[4] and natural products from the integument of nonavian reptiles,[5] and of olfactory signalling[6] and potential semiochemicals in birds.[7] Our treatment of mammals complements reviews emphasizing receiver responses at the neuronal and physiological levels,[8] the role of chemical signals in reproduction,[9] ecological constraints on olfaction,[10] primer pheromones in domestic ungulates,[11] vertebrate pheromones in general,[12] and olfaction across the animal kingdom.[1] Wyatt[13,14] discusses chemical signal design in general terms, and surveys signal design among invertebrates and vertebrates. A special issue of *Hormones and Behavior* on "Chemosignals and Reproduction"[15] contains several up-to-date and detailed reviews.

1.1 Olfaction and odor

We use the term "olfaction" in a broad sense to refer to the sensory detection of chemicals that originate outside the detector's body, including dissolved, surface-bound, and airborne compounds, but we exclude gustation. "Odor" refers to substances that are detected by olfaction. Odors are emitted from an animal's body, including its breath, glandular and skin odors, and from scent marks deposited into the environment. Scent marks include the trails deposited by snakes and other squamates, as well as the discrete urine, fecal, and glandular deposits left by mammals and some lizards.

Terrestrial vertebrates employ three main sensory systems to detect chemicals: the main olfactory system (MOS), the vomeronasal system (VNS), and the gustatory system. The VNS is absent or non-functional in crocodylians, birds, and some mammals. The VNS detects both volatile and non-volatile compounds. No known amniote pheromones are detected by gustation.

1.2 Status as signals and validity of identifications

We restrict the term "chemical signal" to compounds or mixtures whose structures have been elucidated, and that elicit responses at the whole animal level that are similar to those elicited by the natural signal when presented at concentrations similar to those found naturally.[16,17]

Our requirement that a chemical signal has been both bioassayed and characterized excludes hundreds of candidate mammalian signalling compounds for which confirmed structures and bioassays are still required.[18] For example, olfactory communication

between mother and young is universal among mammals,[19] but the only characterized

semiochemicals that mediate mother-offspring interactions are 2-methylbut-2-enal (**1**) in

the milk of European rabbits (*Oryctolagus cuniculus*), which stimulates nipple search;[20]

dodecyl propionate (**2**) in the preputial secretion of rat pups (*Rattus norvegicus*), which

regulates maternal licking;[21] and corticosterone (**3**) in the milk of rats and mice (*Mus*

musculus), which primes the hypothalamic-pituitary-adrenocortical axis of

sucklings.[22,23,24] Among primates, despite detailed work on chemical fingerprints,[25,26] the

COMPOUNDS **1, 2,** AND **3** GO HERE

only signals to have been chemically characterized and bioassayed are three active

compounds in thick-tailed bushbabies (*Galago crassicaudatus*), benzyl cyanide, 2-(4-

hydroxyphenyl)ethanol, and *p*-hydroxybenzyl cyanide,[27] and the free acid "copulins" of

rhesus monkeys (*Macaca mulatta*),[28] whose biological role has been seriously

questioned.[29] Males of the >3 500 snake species likely use odor to find females, but the

only mate-attracting pheromone characterized from snakes are long-chain ($C_{29} - C_{37}$)

saturated (**4-11**) and *Z*-monounsaturated methyl ketones (**12-17**) from female red-sided

garter snakes (*Thamnophis sirtalis parietalis*).[30-32]

COMPOUNDS **4-17** GO HERE

With the exception of the major urinary protein (MUP) mixtures from male mice,

which are unique to individuals and thus constitute signature mixtures according to

6

Wyatt's[33] definition, the signals that we discuss are pheromones according to the
original definition by Karlson and Lüscher [34] (p. 55): **"…** substances which are secreted
to the outside by an individual and received by a second individual of the same species,
in which they release a specific reaction, for example, a definite behaviour or a
developmental process," and to Wyatt's[14] (p. 9) recent operational definition: "fully
identified molecule(s), the same across a species, ... which when synthesized elicit the
same characteristic response in the conspecific receiver as the natural stimulus**.** The
signals discussed here have been chemically characterized by methods that meet
criteria for rigorous semiochemical identification.[17,35]

2. Amniote signal chemistry

If the MUPs, whose signalling roles depend on their being a mixture, are counted as
one compound, there are 63 characterized compounds with known signalling roles in
mammals (Table 1), and 39 in reptiles (Table 2). Some of compounds are parts of
multicomponent signals; some have more than one role.

Table 1 HERE

Table 2 HERE

3. Discussion

3.1 Are there patterns, and at what scale?

Considering how few amniote signal compounds have been characterized, they are
remarkably diverse. Their MWs range from 59.1 Da (trimethylamine, **18**) to 18 893 Da
(darcin), and their structures incorporate 15 functional groups and carbon skeletons with
straight and branched chains; various sites of unsaturation and asymmetry; and

aliphatic, aromatic, and heterocyclic rings. Different compounds are used to send similar signals, and different signals are sent by similar compounds.

COMPOUND **18** GOES HERE

3.1.1 Exploratory statistical analysis

Statistical analysis can sometimes reveal otherwise obscured patterns. Our questions were 1) are certain chemical categories associated with certain kinds of signals ?, and 2) what influences the MWs of pheromones? We confined our statistical analysis to mammals because too few signals are known from reptiles. Even for mammals, limitations of the data set obliged us to take an exploratory approach. The tests are correlated, with the same data used for different analyses, but because we aim to discover new patterns, we did not lower p values to control for experiment-wise error. As "positive controls" we included tests of associations that are known to be present *a priori*; for instance, that between signal context and signal type, and between MW and compound class*.

3.1.1.1 Variables, classes, and categories

This meta-dataset, like most, suffers from biases inherent when research results are used for analyses not anticipated by the original investigators.   Rodents are greatly over-represented, the contexts in which chemical signals are employed are unevenly covered, and the classes of compounds are biased by analytical technique. Our inference space is thus limited, and results are suggestive rather than definitive.

The small numbers of characterized signal compounds, signals, and taxa force us to collapse natural categories into just a few classes for each variable, in order to have sufficient members in each class. We constructed seven classes: chemical class, taxon, signal context, signal type, signal source, carrier, and mode of transmission. Categories of chemical class are "proteins" (including peptides), "hydrocarbons" (including terpenoids), "steroids", "heteroatomic compounds" (nitrogen and sulfur compounds), and "other" (including carboxylic acids, aldehydes, ketones, esters, phenols, and ketals). In addition to being categorized on the basis of overall structure, signal compounds are categorized according to functional group and core structures: steroid nucleus, alcohol, amine, nitrogen, sulfur, nitrogen-heterocycle, sulfur-heterocycle, cyclic, aromatic, lactone, ketone, aldehyde, unsaturated, ester, straight chain, oxygen-heterocycle, hydroxyl, and terpenoid.

Categories of taxa are "rodents," "ungulates," and "other" (lagomorphs, carnivores, and primates). Species also are categorized by diet as "herbivores" or "carnivores and insectivores." Categories of signal context are "dominance/territoriality," "sex," and "other" (which includes mother-offspring interactions). Categories of signal type are "primer", "releaser", and "food mnemonic". Categories of signal effect are "behavioral" and "physiological." Signals in the "behavioral" category include both the classical releaser pheromones and signals with mnemonic effects. The "physiological" category corresponds to the classical "primer" pheromones with developmental and hormonal impacts. Categories of signal source were "body" and "mark." The transmission category entails signal compounds that are airborne and those that are detected by direct contact. The carrier category is divided into "liquid" (urine, tears, and milk) and

"solid" (glandular secretions and feces). The signals carried by breath were too few for inclusion.

3.1.1.2 Chi square (X^2) analysis

A χ^2 analysis detected significant interactions between biological categories that were expected *a priori* (Table 3). The data for testing interactions of chemical and biological classes are given (Table 4). We found four significant interactions involving signal chemistry; different signal compounds are found in different taxa, in herbivores *versus* carnivores and insectivores, in airborne *versus* contact signals, and in different carriers.

Table 3 HERE

Table 4 HERE

The standardized residuals of each cell in the two-way table for the compound class and taxon interaction show that the three categories of taxa use the compound classes with different frequencies. Although this effect and the association of taxon with signal source likely are artifacts of unequal research effort across signal classes in different taxa, it also may reflect a strong association of diet with taxon, and the possible effects of diet on substrates for signal biosynthesis (see section **3.5.2.1**). The association of chemical class with mode of transmission is due to steroids and proteins being detected during contact with the signal source (see section **3.3.1**). The marginally significant association of chemical class with carrier probably is an artifact arising from lumping a heterogeneous group of compounds into the "other" category.

3.1.1.3 Analysis of variance (ANOVA) for influences on molecular weight

We examined by ANOVA the relationship between the chemical and biological categorical variables and signal compound MW, log-transformed to stabilize the variance. We started with a model with all main effects and two-way interactions, and used a stepwise procedure to reduce the model using the step function in R (R Core Team 2014).[36] This yielded a model with four main effects and one interaction: compound class ($p < 0.001$), taxon ($p = 0.035$), signal context ($p = 0.008$), signal type ($p < 0.001$), and the compound class by signal context interaction ($p < 0.001$). To understand the relative contribution of each of the independent variables, we did a variance decomposition using the lmer function of the R lme4 package (Bates et al. 2014),[37] estimating each component as a random effect. By far the largest contributor to predicting log (MW) is compound class (83.8% of the total variance), because proteins and steroids have high MWs by definition.   Since the dominance signal context includes territoriality, and territories often are demarcated by scent marks whose persistence requires signal compounds with low vapor pressures, the significant signal context effect (1.1 % of the total variance) was expected (see further discussion in section **3.3.2**). The compound class by signal interaction was driven entirely by one observation, where the prediction of protein pheromone with the lowest log (MW) was adjusted downward (15.0% of the total variance).

3.1.1.4 Discriminant analysis

A standard linear discriminant analysis was conducted to see if the biological variables would separate any of the pheromone classes. This differs from the χ^2 analysis because it shows both which biological variables are the most useful for

discriminating among the pheromone classes and which of the classes separate out.

We were able to use a slightly less condensed classification of pheromones (although

results were almost the same with the coarser classification defined above). The

following pheromone classes were used: acid, carbonyl, heteroatom, hydrocarbon,

other, protein, and steroid.

The proteins separate from most other compounds on the first discriminant axis (Fig.

1), which is loaded most heavily by transmission (airborne *versus* contact) and carrier.

Grouped with the proteins are the two farnesene mixtures that induce estrus and

accelerate puberty in female mice, and (*Z*)-7-dodecen-1-yl acetate (**19**), which signals

estrus in female Asian elephants (*Elephas maximus*) and which is bound to a carrier

protein (see section **3.3.2**). This grouping corresponds with signalling biology and signal

chemistry; the grouped compounds have low vapor pressures, are detected during

contact with scent marks, and are associated with reproduction. Steroids clump at the

lower right, separating from the remainder on the second discriminant axis; two of them

signal sex (signal) and two are carried in milk (carrier), both with negative coefficients.

COMPOUND **19** GOES HERE

3.1.1.5 Stepwise discriminant function analysis

 The previous discriminant analysis asked whether the pheromone classes could be

discriminated. Here we ask if the levels of each variable representing a biological class

(e.g. carrier, taxon, etc.) could be discriminated based on the molecular composition of

the pheromones. We used the R klaR package[38] and the lda function of the R MASS
package.[39]

A few of the molecular features were useful for discriminating among the biological
classes (Table 5). The scarcity of relationships suggests that many of these molecular
features have little impact on pheromone use, at least for the categories of biological
classes we investigated.

Table 5 HERE

Only a few of the associations between signal chemistry and signal biology have
plausible explanations in terms of signal function: amines, proteins, farnesenes, and **19**
with detection by direct contact; aromatic rings with dominance signal class; and
aldehydes with body sources. For the remainder of the associations, the most
parsimonious explanation is that they are artifacts of the unequal coverage of taxa and
signals, and the bias inherent to analytical techniques. The influence of the small,
biased sample is illustrated by the associations between steroid pheromones and signal
carrier. The associations are negative with urine because, although steroids are
excreted in large quantities in urine, the role of urinary steroids as pheromones has
been neglected (see section **3.5.2.1**), and positive with milk and breath because the
only known steroid pheromones are carried by breath or milk; this in turn produces a
positive association between steroids and signals emitted from the body. Also, primer
pheromones have been characterized only from rodents and ungulates, despite

13

evidence that they are widespread among mammals.[22,26,40,41] Such problems are common where results are extracted from the literature.

Interactions among some biological categories were expected *a priori*, and their significance in the analyses establishes that where relationships exist they were detected. This, in turn, suggests that if there are any other interactions between signal class and chemical class they would have been detected, deficiencies in the data notwithstanding.

3.1.1.6 Comparison with whole odors

The only statistical analysis similar to ours is by Alberts,[3] who used whole odors rather than signal compounds. As a comparison with her results, we recognized the same chemical classes that she did: carbonyl (aldehyde, ketone, and ester), carboxyl (acid), and hydroxyl (alcohol and phenol). However, due to small sample size in our data set, we included only two of her signal contexts, "sex attractant" (intersexual signals) and "alarm-threat" signals (signals observed in aggressive or fear-inducing social interactions). The data set for this analysis contained 20 carbonyl, 8 carboxyl, and 11 hydroxyl compounds, in 15 alarm/threat and 22 sex (both releaser and primer) signals.

Alberts[3] found that mammalian odor composition was related to signal context, but our analysis of signal compounds failed to indicate a significant interaction between her compound classes and signal contexts (X^2 = 0.1612, df = 2, p = 0.9225). Nevertheless, in our stepwise discriminant analysis (see above), aromatic signal compounds most likely were found in the dominance signal class, which includes territorial signals that

would have been classified as range marks by Alberts. As Alberts noted, this may be related to the stability of signal compounds (see section **3.3.2**).

In addition to the patterns that were testable, there are other trends and hints of patterns for which sample sizes are too small. The only feature that generalizes across all characterized amniote chemical signals is that they are coded by a small number of compounds embedded in complex mixtures with diverse chemical characteristics.[18]

Seven peptide or protein signals have been documented for mammals, and none for reptiles. In mammals, five volatile N-containing compounds occur in five signals, and seven volatile S-containing compounds occur in nine signals. Both nitrogen and sulphur occur in seven peptides or proteins that transmit nine signals. Neither N- nor S-containing signal compounds have been found in reptiles.

The non-peptide signals used by mammals have lower MWs than those used by nonavian reptiles, and there are proportionately more multicomponent signals in reptiles than in mammals. Each multicomponent signal in a reptile is a mixture of a single chemical class; either ketones, alcohols or acids, while some multicomponent signals in mammals contain a diversity of compound types.

Ketones are the single most common class of signal compounds in mammals, with 13 compounds identified in five signals, followed by sulphur compounds with seven compounds in nine signals. These numbers of compounds are not significantly different from the occurrence of these compound classes among the components of mammalian odors in general.[42] Strikingly, the rare bicyclic ketals 7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]oct-3-ene (dehydro-*exo*-brevicomin, **20**) and 1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane (frontalin, **21**) signal male breeding status in mice and Asian

elephants, respectively (Table 1). Until more species have been examined there is no way to judge whether this is a coincidence.

COMPOUNDS **20** AND **21** GO HERE

Overall, there are few links between signal chemistry and biology, and no extensive patterns. Patterns may be obscured by or be artifacts of small sample size and/or uneven coverage of species and types of signals. Only a small fraction of the compounds that are involved in amniote signalling have been characterized, and they are drawn from only 20 (0.09%) of a total of about 23 000 species. The taxonomic coverage is biased by far more work having been done on mammals than on other vertebrates; of the 20 species with characterized signal compounds, 15 are mammals, three are birds, and four are nonavian reptiles.

Within the mammals there is a bias towards laboratory rodents; of the 62 identified mammal pheromone components, 13 are from rats and 22 from mice. Consequently, a large fraction of what we think we know about mammals in general is based on a few inbred strains of two species of small rodents. There is too little detailed work on chemical signalling in other species to assess to what extent laboratory rodents are representative of mammals in general, but there are some indications that they may not be typical. Laboratory rodents have olfactory receptor genomes that differ from those of other mammals.[43,44] Rats and mice have dozens of intact VNO V2R genes, while dogs (*Canis familiaris*), cows (*Bos taurus*), and primates probably have none. Mice and rats have 187 and 106 functional VNO V1R genes, respectively, while dogs have eight or nine, humans have two (but do not have a VNO), chimpanzees (*Pan troglodytes*) have

none, cows have 32, and opossums (*Monodelphis domestica*) have 49.[44-46] Bats, the

second most speciose and abundant mammalian order after rodents, do not have

VNOs. Between mice and rats, 85 – 90% of V1R genes are functionally species-

specific, but cow V1R genes are 59% and 69% orthologous with sheep (*Ovis aries*) and

goat (*Capra hircus*), respectively, and sheep and goat have 97% sequence identity.[47]

Mice have 15 trace amine associated receptor (TAAR) genes, dogs have two,

opossums have 21, and platypusses (*Ornithorhynchus anatinus)* have four. With three

TAARs, chickens (*Gallus gallus domesticus)* are more similar to dogs than dogs are to

mice or opossums.[44,46] Mice may be outliers in terms of signal composition; MUP type

proteins are widespread in rodents, but most species do not show the diversity that

makes them into signature mixtures in *M. musculus domesticus*.[48] We have a very

detailed map of one small corner of amniote semiochemistry, and a few landmarks are

visible in the distance, but the landscape in between is *terra incognita*, and this very

uneven coverage is expected to make patterns difficult to discern.

Patterns in signal chemistry may be obscured by limitations in chemical analyses. In

terrestrial vertebrate semiochemistry, the most commonly applied analytical technique is

gas chromatography – mass spectrometry (GC-MS), usually with methods that exclude

compounds with MWs above about 400 Da or that need derivatization. Analyses of

peptide and protein semiochemicals have been applied to far fewer species than GC-

MS, and have characterized chemical signals only in mice and golden hamsters

(*Mesocricetus auratus*).

It is tempting to dismiss the scarcity of discernible patterns among amniote chemical

signals as an artifact of small, biased samples and analytical technique. Alternatively,

17

the scarcity of patterns may accurately represent the chemical diversity of amniote

chemical signals because, as we argue below, the properties of terrestrial vertebrate

olfactory systems and the chemistry of their odors frees signalling compounds from

many of the constraints that might otherwise generate patterns in signal chemistry.

3.2 Signal detectability

3.2.1. Scope and sensitivity of amniote olfactory detection

The amniote olfactory system has a remarkably wide scope. The MWs of molecules

that amniotes are known to detect range from 17 Da for ammonia to 7.4 KDa for

crotatoxins, which are detected by rattlesnakes (*Crotalus* spp.).[49] The smallest signal

molecule is **18** (59.1 Da)[50] and the largest is darcin (1.8893 KDa).[51] The huge diversity

of molecules detectable by mammals includes every known naturally occurring organic

functional group.

Contrary to presumptions that only the VNO detects pheromones, and that only

pheromones are detected by the VN0,[52] signal compounds are not constrained to be

detectable by the VNO. In both reptiles and mammals the VNO detects molecules with

no known signalling function,[53,54] and in mammals the MOS detects pheromones.[55] The

few pheromones that have been identified from squamates appear to be detected by

the VNO, but , long, up-wind excursions by male rattlesnakes to females indicate their

use of airborne pheromones possibly detected by the MOS.[56] The copulatory fluids of

male red-sided garter snakes emit airborne pheromones that terminate courtship in

prospective rivals.[57]

Chemical signals might be expected to be compounds whose receptors exhibit particularly low limits of detection (LODs). This expectation is not borne out; receptors with unusually low LODs (Table 6) are scattered throughout olfactory space and are not confined to detectors of chemical signals. For example, the lowest measured olfactory LOD in domestic dogs is 5×10^{-14} mol/mol for α-ionone,[58] and in mice it is 10^{-16} mol/mol for bourgeonal (3-(4-tert-butylphenyl)propanal),[59] but neither compound is a chemical signal. Male golden hamsters respond to 500 fg of dimethyl disulfide,[60] which is a general attractant but not a hamster pheromone.[61] Compared to the LODs for general odors the olfactory LODs for some known pheromones are not particularly low: sows (*Sus scrofa*) detect the pheromone 5α-androst-16-en-3-one (**22**) at 3×10^{-4} M and geraniol (a terpene alcohol with a floral odor) at 3.5×10^{-7} M.[62] Female mice respond to the male pheromone (methylthio)methanethiol (**23**) spiked into castrate urine at 0.2 μM [63] and to aliphatic acids, which are not pheromones, at 1 nmol/mol to 3 pmol/mol in the gas phase.[64]

COMPOUND **23** GOES HERE [THAT'S RIGHT. **23** HERE]

Table 6 HERE

Comparisons between whole animal and neurophysiological LODs are complicated by the fact that animals have been tested with airborne odors whose concentrations are expressed as mole fractions, or as mass fractions of the solution with which they are equilibrated, while physiological preparations have been tested by directly applying solutions, with concentrations usually expressed in molarity. With that limitation, LODs

are similar for general odors and signal compounds (Tables 6, 7), and low LODs do not necessarily point to a signalling role. For example, the TAAR5 receptor in rats has a LOD of 150 nM for **18**, which is not a rat pheromone.[50] Sulphur compounds with no known semiochemical activity are detected by the mouse olfactory receptor MOR244-3 at 10 nM, which is similar to its LOD for the female-attracting pheromone **23**.[65,66]

Table 7 HERE

Compared to the natural concentrations of signal compounds, mammalian olfaction has sensitivity to spare. The mouse VNO responds to urine diluted by a factor of 100 000, and its most sensitive neurons respond to dilutions of 100 000 000.[67] The LOD of TAAR5 for **18** is 100 000 times lower than the concentration of **18** in male mouse urine,[50] and the LOD for ESP22 is 20 000 times lower than its concentration in the tears of juvenile mice.[68] The concentration of **19** in the urine of female Asian elephants reaches 0.146 mM just before ovulation,[69] steroid pheromones in boar (*Sus scrofa*) saliva occur at low µg/ml,[70] and active constituents in the urine of male red foxes (*Vulpes vulpes*) occur at µg/ml.[71] All these concentrations are at least three orders of magnitude above the LODs for mammalian olfaction. In sharp contrast, the quantity of pheromone on one female garter snake is only just above the LOD of male garter snakes.[31] Clearly, signal compounds are not constrained to have chemical structures that allow them to be detected by sensory neurons with unusually low LODs.

3.2.2 Access to sensory epithelia

An obvious constraint on detectability is imposed by the need for signal compounds to reach chemosensory epithelia. The sensory epithelium of the MOS, the main olfactory epithelium (MOE), is exposed to inhaled and exhaled air, and is most accessible to airborne substances. The VNO sensory epithelium is on the roof of the mouth in most reptiles, and in a liquid-filled diverticulum of the nasal or oral cavities in mammals. Molecules are transferred to it by tongue flicking in squamate reptiles, by flehmen in ungulates and carnivores, and by a vascular pump in rodents.[72-74] Patterns in signal chemistry might be generated by constraints on what types of compounds can reach the sensory epithelia, for instance, if only hydrophilic molecules can reach the VNO, and only volatiles can reach the MOE. Adaptations to circumvent these chemical limitations occur in snakes and mammals. In red-sided garter snakes, the lumen of the VNO is filled with liquid that solubilizes the hydrophobic long-chain methyl ketones of the male attracting pheromone.[75] In Asian elephant bulls, the trunk mucus contains an 18.5 KDa odorant-binding protein that binds the hydrophobic estrus pheromone **19** from female urine. The trunk transfers **19** in the free form to the MOE and as the protein-**19** complex to the VNO. In the VNO ducts, **19** transfers from the odorant-binding protein to a 60 KDa albumin that shuttles it to the sensory neurons.[76] Like **19**, the rat pup preputial pheromone **2** is very hydrophobic and is detected by the VNO,[77] but whether a carrier protein is involved has not been established. Vigorous sniffing and licking of female urine by male mice transfers non-volatile major histocompatability complex peptides to their MOEs.[78] Thus, although there is a trend for non-volatile signals to be detected by the VNO, and more volatile ones by the MOE, signal chemistry is not narrowly constrained by access to the sensory epithelia.

21

3.2.3 Selectivity of olfaction

The olfactory system's responses to chemical stimuli, including chemical signals, are "selective" and "specific" in the same sense that these terms are used in analytical chemistry.[79] A specific response occurs only to one particular substance, while a selective response occurs to two or more substances, which usually have some property in common. Specificity is the highest level of selectivity.

Chemical communication requires chemical signals to stand out from the "chemical cacophony"[13] of general chemical background, the "olfactory cocktail party"[80] of odors from other animals, and the other constituents of the odor in which it is embedded. Chemical differences between signal compounds and potential interferences must be large enough for the olfactory system to respond selectively to the signal compounds. The more selective the olfactory system is, the smaller the structural differences between compounds that can be discriminated, and the larger the number of compounds that can be used as signals. If olfaction is not selective, each compound in the chemical background is surrounded in chemical space by a large number of structurally similar compounds that cannot be used as signals, and signals will be absent from parts of chemical space that are occupied by background compounds. Such gaps in signal space could give rise to recognizable patterns in signal chemistry.

Amniote olfaction is exceptionally selective, as well as having a very wide scope. Even humans, with no functional VNO and only 350 intact olfactory receptor (OR) genes, are estimated to discriminate the odors of over one trillion chemical mixtures.[81] Mammals routinely make fine olfactory discriminations between complex mixtures that

have no connection with signalling,[80,82] showing that the mammalian olfactory system has more selectivity and scope than are required for signal discrimination. Consequently, signal chemistry is not significantly constrained by discriminability, and the selectivity of olfactory detection, combined with its wide scope, is too high to have generated recognizable patterns in signal chemistry.

Sex-specific responses to female sex pheromones in red-sided garter snakes are generated at the receptor level; only males have VNO receptors that respond to the relevant methyl ketones.[75] Specificity and selectivity in the mammalian VNO also reside mainly at the receptor level, mediated by a mixture of specific and broadly selective detectors.[83,84] In the mouse VNO, there are specific detectors for the MUP3 pheromone,[84] and sensory neuron V2Rp5 is specific for the peptide pheromone ESP1.[85] Selectivity in the MOS is generated mainly by combinatorial coding of signals from diverse broadly-tuned receptors;[86] each olfactory sensory neuron in the MOE expresses one olfactory receptor protein that is broadly selective for molecules within a particular range of structures. The MOE also has receptors that are specific for amines,[50] and the breath-borne pheromone carbon disulfide (**24**) has a dedicated olfactory subsystem in mice.[87]

COMPOUND **24** GOES HERE

It is important to note that broadly-tuned receptors can be specific for single components of socially relevant odor mixtures. For instance, the mouse olfactory receptor MOR244-3 is broadly selective for a suite of sulphur compounds,[65,66] but only

23

one of these, the male pheromone **23**, [63] occurs in mouse urine and, under natural conditions the broad selectivity generates a pheromone-specific response. Although the main olfactory receptors (MORs) of mice are broadly selective when exposed to general odors, 80% of them are specific to single compounds among the subset of volatiles that are emitted by mouse urine.[63] MOR Olfr288 responds to several odorants, but the only one of them that occurs in mouse urine is (*Z*)-5-tetradecen-1-ol, which attracts females.[88] This suggests that the structural differences between signal compounds and the other components of the odors in which they are embedded are a potential source of patterns in signal chemistry that is worth investigating.

3.2.3.1 Species specificity

In the wild, in contrast to the single-species systems of laboratory and domestic animals where semiochemical research is focused, most vertebrates share their habitats with thousands of individuals from dozens of other vertebrate species. All of these individuals emit odors and leave scent marks, but only those from an individual's own species are socially or reproductively relevant. Specific mate-recognition signals must self-evidently be species-specific,[89] and so also must most social signals. Odors from other populations are a major source of potential interference with chemical signals, and on this basis, chemical signals would be expected to be unique to a species or a population. This is especially true for scent marks, which must operate in the absence of the animals that deposit them.[90]

All amniote chemical signals are embedded in complex mixtures of other odors. No two species have been found to emit the same mixture of compounds, and there are

numerous examples of interspecific differences that have not yet been shown to be signals of species identity (reviewed in Apps[18]). Other compounds have established roles as species-specific signals. **18** is a species-specific attractant for mice, and male urine from *Mus musculus musculus* (strain C57BL/6) contains about 1 000 times as much **18** as does the urine of male rats, and approximately 300 times as much as urine from male *M. spretus*, *M. m. domesticus* and *M. spicilegus*.[50] *Mus musculus domesticus*, *M. m. musculus*, and *M. m. castaneus* have three different alleles (*Abpa^a* in *domesticus*, *Abpa^b* in *musculus*, and *Abpa^c* in *castaneus*) of the gene that codes for androgen-binding protein in saliva.[91] Males mark their territories with the protein and its influence on female mate choice may maintain reproductive isolation among the three subspecies (Laukaitis et al 1997).[92] The long-chain ketone pheromone profiles of garter snakes (*Thamnophis* spp.) differ more between sympatric than allopatric species (Uhrig et al. 2014),[93] presumably a reflection of reproductive isolation maintained among overlapping congeneric populations.

Despite the expectation of species-specific signal chemistry, several signal components are not species-specific (Tables 1 and 2). 2-Heptanone is part of a multicomponent signal in female mouse urine that delays puberty in immature mice,[94] and part of a multicomponent signal in male rat urine that attracts female rats.[95,96] Another component of the male rat urine signal, 4-ethylphenol, also occurs in beaver (*Castor canadensis*) castoreum as part of a multicomponent signal of range occupation.[97] Both castoreum and fox urine contain acetophenone as a component of multicomponent signals that stimulates overmarking.[71,97] 2,5-Dimethylpyrazine is part of the multicomponent puberty-delaying signal in the urine of female mice[94] and is one of

three volatiles in the urine of male tree shrews (*Tupaia belangeri)* that stimulate overmarking.[98,99] Squalene and cholesterol are widespread in vertebrate secretions. Squalene is part of a female-attracting mixture in the urine of male rats[95,96] and part of a male signal in the red-sided garter snake;[29] both compounds elicit aggression in squamates.[100]

24 facilitates social transmission of food preferences in both rats and mice.[87,101] **3** primes developmental trajectories in mice, rats, and European starlings (*Sturnus vulgaris*).[22-24] 4-Methylphenol is an almost ubiquitous component of mammalian urines, and acts as a signal of estrus in mares (*Equus caballus*)[102] and a female attractant in male rat urine.[96] A mixture of five short-chain aliphatic acids whose concentration in feces declines when female rats, horses (*Equus ferus caballus*), or red foxes are in estrus, shows an inverted-U-shaped dose-response curve in eliciting erections in male rats when spiked into female rat feces.[103] The male effect in sheep (*Ovis aries aries*) and goats (*Capra aegagrus hircus*), in which estrus in ewes or nannies is stimulated by luteinizing hormone (LH) pulses that are triggered by a male's body odor, is not species specific; billy goat odor triggers LH pulses in ewes[104] and ram odor triggers gonadotropin releasing hormone/LH release in nanny goats.[105]

In some of these instances, species specificity would be redunant. Two of the signal compounds that are not species-specific -- **3** in milk and **24** in breath -- are transferred directly from one individual to another, mares urinate in response to investigation by stallions, and the ancestors of sheep and goats would not have associated closely enough for interspecific cross-talk to have occurred. It is when signal compounds are desposited as components of scent marks, and emitted in the absence of the marker,

that additional features of each species' signals are needed to inform conspecifics that

the signal is relevant. Correspondingly, there is a pattern in signal design; all signals

that are emitted from scent marks and that contain a compound emitted by another

species are multicomponent signals, and with the exception of the acid mixture that

signals estrus, at least one of the components in each of them is species-specific.

Nevertheless, because a difference in only a single compound is sufficient to chemically

differentiate two species,[106] species specificity imposes no practical constraint on signal

chemisty.

Even when odors are not species-specific, species-specific responses can be

generated at the level of the olfactory receptors or by processing at higher levels in the

central nervous system. Grus and Zhang[46] found that VNO receptor genes differed

between mouse, rat, dog, opossum, and platypus. There are also large differences in

main olfactory receptor (MOR) genes between species. The number of known receptor

proteins varies from 296 in orangutans (*Pongo pygmaeus abelii*) to 1 948 in African

elephants (*Loxodonta africana*).[107] Among mice, dogs, and humans, out of 412 MOR

gene subfamilies, 34 subfamilies occur only in dogs, 60 only in humans, and 69 only in

mice,[108] but mice have only four intact MOR genes that are not found in rats, and rats

have only one intact MOR gene that is not found in mice.[109] V2R VNO receptors in *Mus

musculus* are specific for the odors of species and subspecies of *Mus*.[110]

Species specificity can reside at levels above the receptors. **18** attracts female mice

and repels rats and many other species.[49] Since all of the species can smell it, the

aversive and attractive effects of the same chemical in different species must be due to

27

different processing within the central nervous system. In mice and rats, species-specific processing of sensory input from the VNO occurs in the amygdala.[111]

Species-specific responses provide a straightforward mechanism for species-specific signalling when different species have most of their odor components in common. In principle, it would be possible for different species to read species-specific messages from different components of the same mixture of compounds. If species specificity is unnecessary, or is not conferred by chemical composition, then a further constraint on the chemical composition of signals is removed, and there are less likely to be patterns in signal chemistry.

3.3 Transmission

3.3.1 Active space and detection at a distance

Most scent marks are visually inconspicuous, and if they are detected from a distance it must be through airborne odors. Hard data on the distances over which scent marks can be detected, or how tetrapods find scent marks, are scarce. Mice can detect fresh urine from at least 15 cm away,[112] but indirect evidence from the detection of target odors by search dogs does not support robust scent-mark detection distances of much more than *ca*. 10 m in large mammals.[113] Odor transport at the spatial scale relevant to terrestrial vertebrates depends on advection, not diffusion; signals are blown downwind and dispersed by turbulence.[14] For volatiles, mass transport by advection is at least three orders of magnitude faster than by diffusion[2] and is independent of MW, which raises the upper limit of MWs that can generate realistic active signal volumes. The limit would be raised still further if molecules that are too heavy to be volatile are

adsorbed onto airborne particles and aerosols, which move with air currents but do not diffuse.[114]As a dog sniffs at a scent source, the air flow around its nostrils disturbs particles that are inhaled,[115] mouse MUPs can be airborne,[116] and synthetic boar pheromones to detect estrus in sows are commercially available in aerosol cans, but there have been no systematic studies on whether airborne signal chemicals are transported on particles. There is nothing to suggest that signal compounds in scent marks have special properties that allow long-range transmission.

Because material can be transferred in bulk to both the VNO and the MOE, there are no obvious constraints on MW or functionality for compounds that are detected during physical contact with either the signalling individual or a scent mark. As expected, proteins, farnesene, and **19** which have low vapor pressures are detected during contact (Fig. 1, Table 5), but so is the compound with the second lowest MW and the highest vapor pressure among mammalian pheromones: 76.1 Da **24** in mouse and rat breath.[87,101] Direct contact detection is widespread; squamates sample surfaces by tongue flicking. In ungulates, males and some females nuzzle and lick a female's genitals, sample the urine that she produces in response, and pump it to the VNO using the flehmen grimace.[73] Cats (Felidae), especially males, use flehmen to sample fresh urine.[72] Nocturnal prosimians sniff, lick, flehmen, bite, and even swallow urine scent marks.[117] Corticosteroids in milk are ingested directly by sucklings.[22,23,40]

3.3.2 Persistence

Because body odors can be produced and emitted continuously, any compound that survives even briefly in light and air could serve as a signal. In contrast, scent marks need to be persistent. Aldehydes, which are susceptible to oxidation, are less common

in scent marks and more common in body odors (Table 1). The stability of aromatic compounds may account for their being more common in range marks[3] and in dominance signals, which include territorial scent marks (Table 1). Heavier molecules with low vapor pressures will be emitted more slowly, and for a given quantity of compound in a scent mark, their emissions will persist for longer than those of smaller molecules. Nevertheless, their slow emission produces low gas phase concentrations, and if marks are detected *via* airborne components, this will render them less detectable. These competing requirements for persistence and detectability could produce a pattern in signal chemistry by limiting signal compounds to a narrow range of MWs and functional groups.

Many squamates continually sample substrate-borne chemicals by tongue flicking, and so their signal detection does not depend on compounds in the gas phase. The chemical signals in squamate scent marks can be remarkably persistent; prairie rattlesnakes (*Crotalus viridis*) locate overwintering dens by following trails left by conspecifics months beforehand.[118] The chemical signals in squamate scent marks have higher MWs and lower vapor pressures than do the non-peptide signal compounds in mammalian scent marks (Tables 1 and 2).

Not all scent marks emit signals over very long periods. Interest by male hamsters in the flank and vaginal marks of females declines sharply after 30 min.[119] Female mouse urine elicits unconditioned ultrasonic squeaks from males for 15 - 18 h.[120] The repellent effect of dominant male mouse urine on other males persists for between 1 h and 24 h,[121] but mice respond to the MUPs in the urine for at least seven days and MUPs can be detected analytically weeks after being deposited.[51,112] Klipspringer *(Oreotragus*

oreotragus) marks last for seven days,[122] and follicular phase urine voided by Asian elephant cows attracts maximal interest from bulls for 1 – 2 days.[76]

Although the loss of signal compounds from scent marks is often ascribed to evaporation, it may not be the only mechanism of signal fade-out. Semiochemically active **20** is converted to *exo*-brevicomin as mouse urine dries and ages,[123] and the disappearance of an ephemeral signal chemical from female mouse urine does not occur in the presence of anti-oxidants.[124] These various mechanisms of signal fade-out are an additional source of the chemical diversity that is likely to disrupt patterns in signal chemistry.

Some scent marks emit odors (but not necessarily signals) for much longer than can be accounted for by simple evaporation. Dwarf mongooses (*Helogale parvula*) can detect 20 - 25-day-old anal gland secretion.[125] Male guinea pigs (*Cavia porcellus*) discriminate the sex of the donors of dry urine films that are 40 days old.[126] Interest by male hamsters persists for 40 days for male flank marks and 100 days for vaginal marks.[119] Male Coquerel's mouse-lemurs (*Microcebus coquereli)* discriminate urine films dried on glass after six months, and rehydrated dried films after 20 months.[117] Unless the long-lived emissions are of compounds with very high MWs, these lifetimes are almost certainly due to fixatives that retard the emission of low MW compounds.[127]

A role as fixatives probably accounts for the presence in Asian elephant temporal gland secretion of high MW proteins, lipids, and steroids with no apparent signal activity,[128] and the presence in saddle-back tamarin (*Saguinus fuscicollis*) scent marks of high MW esters and squalene that make up 96% of the mark but which tamarins do not discriminate from odorless controls.[129] Aphrodisin in the urine of female golden

hamsters binds the attractant dimethyl disulfide.[130] Seventeen mouse urine volatiles are bound to MUPs, and individually specific odor signatures are generated by interactions between volatiles and different MUP mixtures.[131] As a result of the volatiles-MUP interactions, one set of compounds (the volatiles) that dissipates too quickly to produce persistent marks with stable signatures combines with another set of compounds (the MUPs) that have no active space to produce scent marks that are attractive, stable, persistent, and specific to their depositor. The boar pheromones, **22** and 5α-androst-16-en-3-ol (**25**), are concentrated into saliva by binding to pheromaxein, a 15 KDa secretoglobin protein.[132] The two pheromones are released from the protein by its microbial degradation.[133]

COMPOUNDS **22** AND **25** GO HERE [ATTACHMENT IS NAMED "androsteroids compounds 22 and 25"]

There are two clear examples of a scent mark matrix being manipulated to change a chemical signal. In European rabbits, dominant males increase the lifetimes of their chin gland secretions by secreting more of the fixative 2-phenoxyethanol, which rabbits cannot smell.[134] Male ring-tailed lemurs (*Lemur catta*) mix volatile brachial gland secretion with heavier antebrachial gland secretion, which acts as a fixative, before depositing the mixture as scent marks.[26]

Although its possible role as a fixative has not been recognized, urea, which occurs in all mammalian urine, can form inclusion complexes with unbranched carbon chains bearing a variety of terminal functional groups. Complexation is remarkably selective

and is reversible at temperatures around 25° C.[135] Urea complexes form only in the solid phase, and this may explain why liquid female guinea pig urine loses its attractiveness to males after 48 h,[136] but as a dry film it is attractive and discriminable from male urine after 40 days.[126] In addition to urea forming inclusion complexes with volatiles, odor-binding proteins might be denatured by increasing urea concentrations as urine dries, giving rise to serial emission of the volatile ligands as their protein carriers are sequentially denatured in drying urine films. The equilibrium concentrations of volatiles above mouse urine change with time due to drying and slow release from MUPs,[123,137] in this context it is interesting that darcin is stable to 7.5 M urea, while MUP 11 is denatured at 6 M.[138]

If the signal compound emissions of a scent mark remain latent until it is encountered and manipulated by a receiver, its lifetime may become largely independent of environmental conditions, and be better measured by how often it can be "read" than by time.[3] The release of volatiles from scent marks in response to sniffing and licking is most likely due simply to the addition of moisture,[127] but there are also specific displacers in saliva, nasal mucus or breath that liberate signals from their carriers. The saliva of Mongolian gerbils (*Meriones unguiculatus)* specifically liberates components from their Harderian gland secretions.[139] The Asian elephant estrus pheromone **19**, which is hydrophobic, is selectively bound by a 66 KDa albumin in urine. The binding depends on pH; each albumin molecule binds four molecules of **19** at pH 8.4, and none at pH 5. When a bull samples a female's urine by placing his trunk tip on it, the acidic trunk mucus displaces **19** from the albumin.[76,140]

Slow release does not necessarily entail lower initial emission rates that reduce detectability. For example, fresh mouse urine contains signal volatiles that are not bound to MUPs and which generate the same gas phase concentrations as if MUPs were not present,[123] and which repel male mice.[121] The presence of fixatives can decouple scent-mark lifetimes from the MWs of their active constituents, allowing marks with very volatile active components to have long lifetimes and disrupting another possible source of patterns in signal chemistry.

Persistent emissions of low MW compounds can be generated by their *in-situ* production. The liberation of odorous, volatile sulphur compounds from odorless, non-volatile precursors is widespread.[141] Felinine in the urine of domestic cats (*Felis catus*) is a precursor of 3-mercapto-3-methylbutanol ("tomcat thiol").[142] In the urine of male maned wolves (*Chrysocyon brachyurus*), the concentrations of 2-methylbut-3-en-2-ol, 3-methyl-1-methylthiobut-2-ene, and six odorous pyrazines increase with time for at least three days.[143] In the urine of both Asian and African elephant bulls, the concentrations of volatile ketones, alcohols, and alkyl phenols increase with standing at room temperature,[144] and in the urine of female African elephants, α-farnesene peaks after three days at room temperature, and *β*-farnesene and *exo*- and *endo*-brevicomin increase for at least five days.[145]

Rather than being a property specifically of scent mark components, signalling persistence may be conferred by detector sensitivity because low LODs prolong the period over which the declining emissions from a scent mark are detectable. Sniffer dogs can detect cadaver odor on carpet 65 days after it was deposited,[146] a period that is similar to the longest lifetimes reported for mammalian scent marks.

Because animals can remember odors and the signals that they carry, the effects of the signal from a scent mark can persist for longer than the lifetime of the scent mark itself. Mammals remember odors for periods that are well into the upper range of the known lifetimes of scent marks. Asian elephants remember odors for 16 weeks,[147] mice for 32 days,[148] and female genets (*Genetta genetta*) for nine weeks.[149] A special role for memory is supported by the presence in mammalian signals of two compounds that act as mnemonic triggers; the rabbit mammary pheromone **1** is a mnemonic trigger for the mother's odor,[150] and darcin in male mouse urine triggers single-trial learning of the site of the urine and its odor.[151] Remembrance of darcin lasts for at least 14 days, which is much longer than the volatile emissions from the scent mark.[151]

In summary, the range of potential signalling compounds that can operate under any given set of conditions is extended by *in situ* production, adsorption and absorption by fixatives, the formation of protected complexes, high detector sensitivity, and persistent effects on receivers, all of which free signal compounds from the need to be robustly stable to environmental conditions.

3.4 Costs

The costs of chemical signalling arise from fuelling the biosynthesis of signal compounds and their fixatives, and their release into the environment. Even the production of odors by commensal microbes requires hosts to provide substrates. If there are significant differences in the costs of biosynthesizing different compounds, selective pressures to generate signals from compounds that are metabolically inexpensive, would generate patterns in signal chemistry.

Using metabolic by-products as signals imposes limited or no production costs. Male mice use both by-products and "purpose-built" signals to attract females; **18** is a microbially transformed by-product[50] and MUPs are synthesized *de novo*.[152] Male mice that scent mark more grow more slowly,[153] and hungry mice produce less MUP,[154] but the metabolic costs of MUPs and **18** have not been measured. We know of no reports on the differential costs of production of different chemical signals.

The MUPs and aphrodisin have a narrow range of MWs close to the lower end of the MW range for lipocalins, which as a class are small proteins.[155] The odor-binding albumin in the trunk mucus of Asian elephant bulls has a similar MW. All of these small proteins bind a single molecule of ligand, while the 66 KDa albumin in the urine of female Asian elephants, binds four molecules of the oestrus pheromone **19**, at a ratio of 16.6 KDa of protein per molecule of ligand. This 16 – 19 KDa range might be the smallest protein motif that can form a lipophillic calyx that is both stable to environmental perturbations and responsive to functional manipulations, such as the change in pH when an Asian elephant bull's trunk mucus mixes with a cow's urine (see section **3.2.2**). The upper limit of the MW range might be the result of selection for low production cost; the smaller the protein, the lower its metabolic cost per unit of bound ligand.

3.5 Information content

3.5.1 Chemical diversity and signal diversity

Complex sociality based on individual identities requires a wide variety of social signals, and some authors have argued that coding a sufficient number of signals is

possible only with multiple components and/or components of high MW.[3,14,99,156] These arguments seriously underestimate chemical diversity. Even the most conservative of *a priori* calculations of the number of possible natural organic compounds yield numbers in the tens of millions, and the universe of small molecules (<500 Da) alone contains 10^{60} possible structures.[157] There is, in principle, a unique chemical compound for every biological message, hence signals are not constrained by chemistry to have more than one component, or components of a particular type.

The need for there to be a biosynthetic pathway leading to a signal or its precursor confines signal composition to a subset of the chemically possible structures, but substantial chemical diversity is still generated by the multiple pathways in amniote metabolism. For example, human breath[158] and urine[159] contain at least 2 000 volatiles and more than 3 100 small molecules, respectively, and the urine of inbred mice contains at least 3 000 volatiles.[160] The urinary peptidome is similarly rich.[161,162]

Contra Charpentier *et al.*,[163] signal components do not have to be animal metabolites. Commensal microorganisms in scent glands and sacs, the surface of the skin, the gut, and the urinogenital tract generate volatiles that could serve as chemical signals (reviewed in Ezenwa and Williams 2014[164]). **18**, a male mouse pheromone, is produced by microbial transformation of choline in the gut.[50] The microbes need not be animal commensals; **18**, ammonia, and species-typical sulfur volatiles are released from cat urine by soil microbes.[165]

This rich biochemical repertoire and an olfactory system with a broad scope and very narrow selectivity allow amniotes to generate robust redundancy in chemical signals by incorporating multiple compounds. In mice, five out of six of the male urine components

that accelerate puberty in females are active alone.[166] The combination of **20** with 2-*sec*-butyl dihydrothiazole, and MUP3 both induce aggression by territorial male mice against castrates.[167] Male mouse urine contains three volatiles that attract females when spiked separately into castrate urine; **18**, **23**, and *Z*-5-tetradecen-1-ol.[50,66,88] Any single MUP of the several that are absent from a male mouse's MUP signature is sufficient to stimulate him to countermark spiked urine.[84] Either **22** or **25** from boars induce lordosis in sows when presented alone.[168] The three signal compounds in the urine of male tree shrews each elicit over-marking when presented singly.[97,98]

Redundancy in signal chemistry, and signals having one or a few components whose ratios are not critical to message integrity[18] are in sharp contrast to the situation in insects, where multicomponent ratio signals are common.[14] Mammals have *ca.* ten times as many different olfactory receptors as insects,[14] and it is possible that the broader olfactory scope and narrow selectivity of mammals allow them to use a wide diversity of compounds as single-component or redundant signals, while insects with a narrower olfactory scope have to generate specificity by combining chemically similar compounds in specific ratios.

3.5.2 Index signals – potential templates for signal patterns

Index signals are mechanistically linked to metabolic processes or other features of the signaller,[169] and these links could generate patterns in signal chemistry . Their components may be either the obligate by-products of metabolism, such as excreted steroid conjugates or the products of catabolism, or chemicals that have other roles besides signalling.[14,170]

3.5.2.1 Metabolic and physiological indicators

There are differences between taxa in signal chemistry; volatile signalling compounds that contain nitrogen or sulphur have been found only in mammals, where they are more common among signals from carnivores and insectivores than in signals from herbivores. This may be linked to their different diets and digestive physiologies providing different substrates for signal biosynthesis.

Excreted steroids and steroid metabolites are mechanistically linked to physiology, especially to stress and reproductive status, and their concentrations in urine and faeces are routinely measured to monitor reproductive condition and stress.[171] However, they have attracted remarkably little attention from semiochemists. Apart from the androstene pheromones in the saliva of pigs (Table 1), and very recent work on sulfated estrogen,[172] there have been so few demonstrations of a semiochemical function for steroids that Baum and Bakker[173] (p. 281) remark, "The possible role of sex steroids as pheromonal signalling molecules remains a matter of speculation, based on a minimal amount of hard data."

Corticosteroids in milk have primer effects on the development of sucklings,[23,24,40] but their status as pheromones has not been recognised. DeCatanzaro[174] presents evidence that 17β-estradiol is a multifunctional primer pheromone in female mice that acts directly on target tissues after being absorbed into the bloodstream through the oral or nasal mucosa. Nevertheless, it is not clear how the females absorb the minimum effective dose of 140 ng/day when the highest concentration in male mouse urine is 20 ng/ml.[175] Two steroids, 5α-androst-2-en-17-one and -17ß-ol, are present (probably as

sulfates) in the urine of female Asian elephants, and their concentrations change with ovarian activity and the reproductive cycle,[176] but whether they act as cues or signals has not been tested. Testosterone and dihydrotestosterone concentrations in the temporal gland secretion of Asian and African elephant bulls fluctuate in parallel with serum concentrations, and are elevated during musth, although in Asian bulls they are not signals.[128] Small peptides are similarly likely to be index signals, and have been shown to signal sexual immaturity in female mice, and sexual maturity in male mice.[68,177] Some MUPs may be index signals in male mice; their production depends on nutritional status, and some of them appear to be involved in metabolic regulation.[154]

Neurophysiological results support a signalling role for excreted steroids; 71 % of female mouse VNO sensory neurons respond selectively to sulphated steroids,[110,178] compared to 0.3 – 0.7 % of VNO sensory neurons that respond to each of six known pheromones.[53] Nodari *et al*.[179] found that a large majority of mouse VNO sensory neurons respond to sulphated steroids, but not to native steroids, and that at physiological concentrations individual sensors are specific or selective. The mouse V1rj2 and V1rj3 VNO sensory neurons respond selectively to 1,3,5(10)-estratrien-3,17β-diol disulfate and 1,3,5(10)-estratrien-3,17β-diol 17-sulfate, which are components of an incompletely characterized mixture that stimulates males to mount receptive females.[177]

3.5.2.2 Dual-trait pheromones

Some compounds that act as pheromones have other concurrent functions. In the crested auklet (*Aethia cristatella*), a colonial seabird, both sexes possess special wick feathers that emit a mixture of even-numbered C_6-C_{12} aldehydes, which repel

mosquitoes and impair lice and ticks (reviewed in Weldon and Carroll 2007[180]) and also attract conspecifics.[181,182] The crested auklet's odor is hypothesized to function as a signal of mate quality related to ectoparasite repellence.[183]

Antimicrobial activity is widespread among vertebrate secretions. Waxes from birds' uropygial glands, for example, inhibit feather-degrading bacteria.[184] During the nesting season, these glands in the green woodhoepoe (*Phoeniculus purpureus*) and European hoepoe (*Upupa epops*) secrete odorous antimicrobial volatiles that also repel predators.[185] (*E*)-3-Tridecen-2-one, a major volatile component of the interdigital gland secretion of black tailed deer (*Odocoileus hemionus columbianus)* inhibits the growth of bacteria and fungi.[186] This compound and other *c*omponents of mammalian odors that exhibit antimicrobial properties[187] are potentially available as honest cues or signals to prospective mates and other conspecifics.

The pheromones of squamates are hypothesized to have evolved from integumentary lipids that contribute to the transepidermal water barrier.[31,118] If these pheromones currently contribute to impeding dessication, they may provide cues denoting this homeostatic imperative to prospective mates.

Index signal compounds that are mechanistically linked to physiology and metabolism are likely sources of patterns in signal chemistry, but their role has been neglected. Monitoring hormones and other compounds in feces and urine, and as metabolic and disease indicators in urine, reveals how they vary with metabolic state, stress, and other factors.[188] Many of the relevant compounds are commercially available, hence their bioassay for signal function would be straightforward.

3.5.3 Chemical mimicry

Some within-species signals are deceptive. The most striking examples of such deception entail sexual mimicry, where males mimic females, and *vice versa*.[189] Sexual mimicry does not itself prescribe broad patterns of signal design. However, mimetic signals are constrained to match the prevailing honest signal patterns at the species or population level.

Sexual mimicry occurs in red-sided garter snakes. After emerging from hibernation, most males pass through a phase in which they are attractive to other males.[190] These so called "she-males" are believed to achieve a mating advantage by distracting other males in the mating balls of courting males that accumulate around females. In addition, the mating balls that form around she-males also transfer heat to them and reduce their exposure to predators.[191] GC-MS analyses of skin surface extracts of snakes during the breeding season reveal that females contain predominantly unsaturated methyl ketones, males contain predominantly saturated methyl ketones, and she-males are intermediate, containing both saturated and unsaturated methyl ketones.[192]

4 Summary and conclusions

The signalling compounds of amniotes are components of complex mixtures with diverse molecular weights and functionalities. The properties of these mixtures and specific components within them free the signalling compounds from some of the constraints imposed by requirements for signals to be stable, species-specific, persistent etc. In particular, the stability and emission of pheromones carried by scent marks are modulated by interactions with the matrix and by *in situ* production.

There are a few-small scale patterns among amniote chemical signals. Signal compounds with high MWs and low vapor pressures, or that are bound to carrier proteins, are detected during direct contact with the source of the signal. Stable compounds containing aromatic rings are more common in signals of social dominance, including territoriality. Aldehyde signal compounds are emitted from the sender's body rather than from scent marks, perhaps because their susceptibility to degradation renders them too short-lived to persist in scent marks. Lipocalin pheromones have a limited range of MWs, possibly to reduce the metabolic costs of their biosynthesis. Species specificity is conferred by multicomponent signals. If this scarcity of patterns in current data is due to small sample size, biased data that are not comparable, and uneven taxonomic representation, then a large body of comparable, taxonomically diverse, unbiased data will reveal patterns that are currently obscured. Alternatively, a larger and better set of data might confirm that patterns really are sparse and small scale because the design constraints that might have channelled signal chemistry into patterns have been relaxed by amniote behavior and biochemistry. Signal detection imposes no practical constraints on the structures of signalling molecules because amniote olfaction has such a high sensitivity, wide range and narrow resolution, and carrier molecules and special behaviors transfer odors from the environment to the olfactory epithelia. The diversity of metabolic pathways in amniotes and their microbial commensals produces a wide variety of signal compounds. Semiochemicals do not have to be the products of amniote metabolism, and there is sufficient chemical and metabolic diversity for signals to be coded by both single compounds and mixtures. Metabolic diversity enables the production of complex mixtures of matrix compounds

that free signal components from the constraints that would otherwise impose patterns on signal chemistry.

If there are any hidden patterns in amniote signal chemistry, they will be revealed by analyzing odors and the signal compounds they contain using methods whose results are comparable. The range of chemistries represented by the known signals confirms the need for correspondingly diverse analytical methods. Finding patterns in signal chemistry will also require investigation of a far wider and more representative range of species, and a far more rigorous approach to the designation of compounds as signals than prevails at present.

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TABLE AND FIGURE HEADINGS

Table 1. Characterized signalling compounds from mammals

Table 2. Characterized signalling compounds from reptiles, including birds

Table 3. Significant p values from chi-squared analysis of relationships between categories

Table 4. Counts from cross-classifying chemical with biological classes

Table 5. Molecular groups whose presence/absence was found useful in a stepwise discriminant analysis to separate levels of one or more biological classes

Table 6. Selected detection thresholds for whole animal olfaction

Table 7. Detection thresholds of vomeronasal organ (VNO) and main olfactory epithelial (MOE) sensory neurons for semiochemicals

Figure 1. Compounds (divided into seven classes) as represented in the first and second linear discriminant space. A small amount of noise was added to both axes to better separate the points