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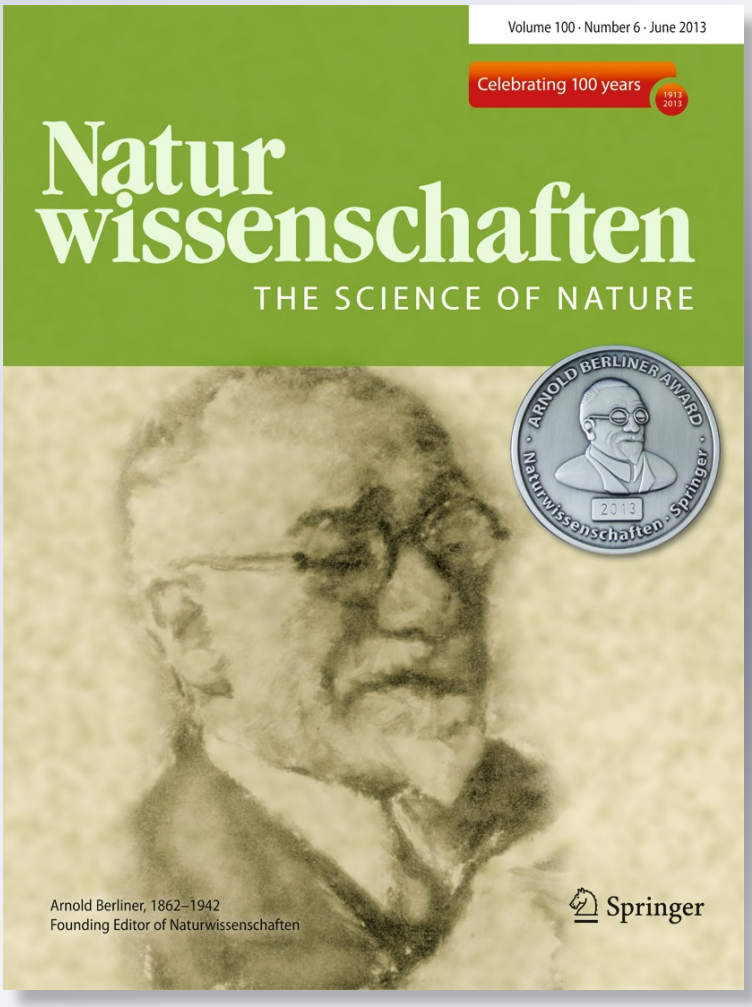

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REVIEW

Are mammal olfactory signals hiding right under our noses?

Peter James Apps

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Abstract Chemical communication via olfactory semiochemicals plays a central role in the social behaviour and reproduction of mammals, but even after four decades of research, only a few mammal semiochemicals have been chemically characterized. Expectations that mammal chemical signals are coded by quantitative relationships among multiple components have persisted since the earliest studies of mammal semiochemistry, and continue to direct research strategies. Nonetheless, the chemistry of mammal excretions and secretions and the characteristics of those semiochemicals that have been identified show that mammal semiochemicals are as likely to be single compounds as to be mixtures, and are as likely to be coded by the presence and absence of chemical compounds as by their quantities. There is very scant support for the view that mammal semiochemicals code signals as specific ratios between components, and no evidence that they depend on a Gestalt or a chemical image. Of 31 semiochemicals whose chemical composition is known, 15 have a single component and 16 are coded by presence/absence, one may depend on a ratio between two compounds and none of them are chemical images. The expectation that mammal chemical signals have multiple components underpins the use of multivariate statistical analyses of chromatographic data, but the ways in which multivariate statistics are commonly used to search for active mixtures leads to single messenger compounds and signals that are sent by the presence and absence of compounds being overlooked. Research on mammal semiochemicals needs to accommodate the possibility that simple qualitative differences are no less likely than complex quantitative differences to encode chemical signals.

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Introduction

Animals in general, and mammals in particular, use chemicals to communicate (Albone 1984; Wyatt 2003). Mammals transmit chemical signals via their breath, urine, faeces, saliva, glandular secretions and body surfaces, and detect them by the main olfactory and vomeronasal systems. These chemical signals, known as semiochemicals, influence behaviour, development, reproduction and social interactions (Doty 1986; Brennan and Zufall 2006; Smadja and Butlin 2009). Their role is especially significant among the small, nocturnal animals that comprise a large majority of mammal species and the vast majority of individual mammals.

Given the importance of chemical signalling and olfaction in the lives of mammals, it is perplexing that of the approximately 3,500 animal semiochemicals that have been identified (El-Sayed 2012), only a few are from mammals (Tirindelli et al. 2009; Fig. 1), and that the rate at which mammal semiochemicals are being identified has not increased despite dramatic improvements in analytical instrumentation (Meinwald 2003), revolutionary new findings in the genetics, biochemistry and neurology of olfaction (Buck and Axel 1991; Zarzo 2007; Zufall and Leinders-Zufall 2007; Keller and Vosshall 2008; Tirindelli et al. 2009; Kaupp 2010; Nara et al. 2011), and a lengthening list of mammals whose scent marking and olfactory behaviour and chemistry have been investigated. The discrepancy between invertebrate and mammalian semiochemistry in the number of identified messenger compounds is striking, and prompts the question, Why are mammal semiochemicals so elusive?

Plausible explanations for the slow rate at which mammal semiochemicals have been identified have already been discussed in the literature. Mammal secretions and the signalling compounds that they (presumably) contain are

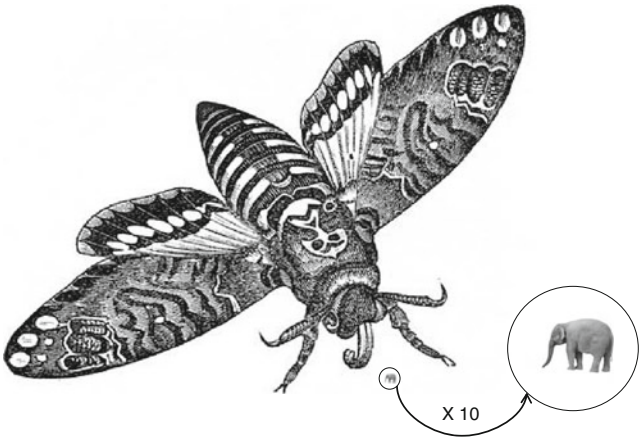

Fig. 1 The moth is 100 times as big (in linear dimension) as the smaller figure of the elephant, approximately the same ratio as the number of semiochemicals that have been characterized from invertebrates and from mammals. The figure was inspired by Fig. 1.1 in Wyatt (2003)

exceptionally analytically challenging (Burger 2005), mammal behaviour is far more complex and variable than behaviour in other taxa (Mackintosh 1985; Johnston 2003), and the closest mammalian equivalent of the insect antennogram; micro-neurophysiology (Lin et al. 2005; Ferrero et al. 2011; Luo et al. 2012) shows little immediate prospect of being applicable beyond laboratory rodents. In addition, insect semiochemistry has been better resourced than its equivalent in mammals (Zimmer and Zimmer 2008).

The analytical challenges of detecting and identifying nanomolar components of complex mixtures, where active compounds range from volatiles with molecular weights of 76 Da (Galef et al. 1988) to 18–22 kDa proteins (Roberts et al. 2010, 2012), and the biological problem of designing and implementing realistic bioassays (Mackintosh 1985; Thom and Hurst 2004) undoubtedly go a long way towards explaining why mammal semiochemicals have been so hard to find. Nonetheless, I will argue that another reason for mammal semiochemicals having been so elusive is that researchers have been looking for the wrong things, and that erroneous expectations about the nature of mammalian chemical signals have led to searches for something that is not there using research strategies that overlook or actively exclude potential semiochemical candidates.

The assumption that mammal semiochemicals are complex mixtures, and speculation that signals are coded by ratios in the abundances of multiple components, developed from some of the pioneering work on mammal chemical communication, which showed that mammal secretions and excretions contain dozens of components (Eisenberg and Kleiman 1972; Goodrich and Mykytowycz 1972). Evans et al. (1978) were the first to use the term "Gestalt" (perceptual wholes that cannot be reduced to or reconstructed from their component parts) in mammalian semiochemistry, and Albone's (1984, 1990) "chemical image" concept, which proposed that every component in a secretion contributes to a chemical signal and that signalling activity depends on both the chemical and biological contexts, was linked to Gestalt psychology by Novotny et al. (1990b). The view that mammal chemical signals in general, and signals of individual identity in particular, are coded by mixtures of compounds in specific ratios has persisted (Singer 1991; Johnston 1998, 2003; Schaefer et al. 2001, 2002; Zhang et al. 2003; Beauchamp and Yamazaki 2005; Müller-Schwarze 2006; Brennan and Kendrick 2006; Hurst 2009; Setchell et al. 2010; Wyatt 2010). Wyatt's (2010) closely argued distinction between classical pheromones and signature *mixtures* (my emphasis) implicitly excludes the possibility that "signatures" may be single-compound "monograms". The search for active mixtures has become the norm, and it is pursued through the use of multivariate statistical analyses (usually of peak areas from gas chromatography) to search among the components of mammal secretions for patterns of abundance that might reveal which components are semiochemicals.

To test the validity of the current narrow focus on multicomponent signals, I have surveyed the literature for evidence on the complexity of mammalian chemical signals, whether they are coded as ratios and whether they depend on a Gestalt or a chemical image. The reported compositions of mammalian secretions in the broad sense and the nature of the mammal semiochemicals that have been chemically identified provide remarkably little empirical evidence that mammal chemical signals are complex mixtures, almost no evidence that they are coded by specific ratios and no evidence at all that they depend on Gestalts or chemical images. This suggests that there are flaws in current approaches to mammalian semiochemistry and in how multivariate analyses of chromatographic data are commonly used to search for semiochemicals, and raises the possibility that unrecognised mammal semiochemicals are hiding in plain sight.

I will sidestep the decades-old dispute about the validity of "pheromone" as a label for chemical signals between mammals (Michael et al. 1976a, b; Smith 1976; Doty 2010; Brennan 2011) and more specifically avoid its being linked to the chemical complexity of a signal (Johnston 2003; Wyatt 2009, 2010) by using the inclusive term "semiochemical" (Regnier 1971), with the understanding that a "chemical" may contain more than one compound. I use the terms "quantitative" and "qualitative" with their usual meanings in analytical chemistry: qualitative analyses determine the presence or absence of a particular substance; quantitative analyses determine how much of it is present. Thus; a qualitative signal is encoded by the presence or absence of one or more chemicals, and a quantitative signal depends on how much of them is present. Both qualitative

and quantitative semiochemicals can be either pheromones or signature mixtures, depending on the response of the receiver. In this sense, qualitative corresponds to "digital" and quantitative corresponds to "analog" as used by Sun and Müller-Schwarze (1998b), Yuan et al. (2004) and Rosell et al. (2010) but avoids the association that these terms have with signalling, which cannot be inferred from chemical composition alone. Qualitative as used here has no connection with "odour quality" as used by Johnston (1998) or "qualitative" as used by Pohorecky et al. (2008) for quantitative features of chemical mixtures. A bioassay tests a chemical's signalling activity by testing how an animal responds to it, whether the response is behavioural, hormonal, developmental or neurophysiological.

Methods

I compiled from the literature the identities of those compounds that have been shown to be mammal chemical signals because these provide the most direct evidence for the chemical nature of mammal semiochemicals in general, and for evidence on whether the activity of multi-component signals depends on the ratios between their components, or on a Gestalt or a chemical image. In addition, I followed the approach pioneered by Brownlee et al. (1969) and Novotny et al. (1986, 1990b) and compiled examples of differences in composition between secretions (in the broadest sense, i.e. including urine, faeces, breath etc.) from different categories of mammal, (for example, males vs. females, dominants vs. subordinates, species etc.), since these differences provide strong evidence about which components of the mixtures are candidates for bioassays as semiochemicals.

Papers on mammal semiochemistry are very widely scattered, and are to be found in journals covering subjects as widely disparate as speleology and industrial engineering (Bullard 1982; Nielsen et al. 2006). Much highly relevant material is in books (e.g. Wyatt 2003; Müller-Schwarze 2006) and symposium series (e.g. the *Chemical Signals in Vertebrates* series) that do not always appear in literature searches and have limited availability on-line. Searches were run mainly on Scopus, Ebsco and journal publisher's web sites. Keywords included pheromone, semiochemical, infochemical, chemosignal, chemical signal, scent mark, olfactory and olfaction, with mammal or the names of families, genera or species. Contents alerts from the *Journal of Chemical Ecology*, *Chemical Senses*, *Ethology*, *Animal Behaviour*, *Behavioral Ecology* and *Sociobiology* and other relevant journals have been monitored for the past 4 years. Books and conference series were browsed "by hand". Chains of references were followed in both directions using citation records, and authors' names were re-entered to track their other contributions. The resulting body of literature can make no claims to be a sample in the statistical sense, and it falls somewhat short of being comprehensive, but there is no reason to suppose that it is not representative or that significant works have been omitted.

I excluded results which could not be replicated and where there are plausible alternative explanations for the reported effects (Michael et al. 1977; O'Connell et al. 1978; Goodwin et al. 1979; Goldfoot 1981; McKenna-Kruse and Howard 1983; Petrulis and Johnston 1995). The established mouse puberty accelerating pheromones have been included, although the work of Flanagan et al. (2011) suggests that the effects of some of them may depend on unidentified experimental details. I also excluded results with insufficient evidence or support [e.g. those reviewed by Frank et al. (2010)], work with an overtly commercial motivation or with clear analytical shortcomings and cases where no systematic difference in chemical composition was detected. I have not included any work on humans, since the role of chemical signalling is so poorly understood (Hays 2003; Wysocki and Preti 2004). Examples are arranged according to whether the signals or chemical differences are qualitative or quantitative, and whether they are single compounds or mixtures.

Results

Signals

Qualitative signals

Single compound qualitative signals Proof positive that not all mammal semiochemicals are mixtures is provided by identifications of single active compounds.

Darcin, one of the Major Urinary Proteins (MUPs) in male house mouse (*Mus musculus*) urine, holds the attention of mice that were attracted to the urine by its odour, and enhances their memory for the odour of the urine (Roberts et al. 2010) and for its spatial location (Roberts et al. 2012). A 7-kDa peptide, exocrine gland-secreting peptide 1 (ESP1), in male tears stimulates vomeronasal sensory neurons and sexual behaviour in female mice (Haga et al. 2010). Female mice are attracted by castrate mouse urine spiked with (methylthio)methanethiol (Lin et al. 2005). Puberty delay in young mice is induced by the adult urine constituent 2,5-dimethyl pyrazine (Jemiolo and Novotny 1994). Carbon disulphide in brown rat (*Rattus norvegicus*) and mouse breath mediates socially transmitted food preferences (Galef et al. 1988; Munger et al. 2010), and dodecyl propionate from rat pups' preputial gland secretion stimulates anogenital licking by females (Brouette-Lahlou et al. 1991). A single 17-kDa protein, aphrodisin, stimulates copulation in male hamsters (Singer et al. 1986; Briand et al.

2004). The rabbit (*Oryctolagus cuniculus*) mammary pheromone is a single compound, 2-methylbut-2-enal (Schaal et al. 2003).

The responses of black-tailed deer (*Odocoileus hemionus columbianus*) to tarsal gland scent are mimicked closely by their responses to the single component *cis*-4-hydroxydodec-6-enoic acid lactone (Brownlee et al. 1969), and specifically to the naturally occurring Z-isomer (Müller-Schwarze et al. 1976), to the racemate and to the naturally predominant (−) enantiomer (Müller-Schwarze et al. 1978).

As female Asian elephants (*Elephas maximus*) pass from anoestrus to pro-oestrus, the concentration of (*Z*)-7-dodecen-1-yl acetate in their urine increases from undetectable to 0.146 mM just before ovulation. This single compound robustly elicits Flehmen responses from male elephants (Rasmussen et al. 1997).

Multi-compound qualitative signals Adult male mice have dehydro-*exo*-brevicomin and 2-(*sec*-butyl)-4,5-dihydrothiazole in their urine; both disappear after castration (Schwende et al. 1986). When spiked together into castrate urine, they elicit aggression from dominant males (Novotny et al. 1985, but see also Chamero et al. 2007) and accelerate puberty in crowded females (Novotny et al. 1999b), and when mixed with water and aged preputial gland secretion, they attract females (Ninomiya and Kimura 1990). There are qualitative differences between male and female mice in which MUPs occur in their urine (Armstrong et al. 2005), and other qualitative differences among males are the basis for recognition of which individual deposited a scent mark (Hurst et al. 2001; Hurst 2009). A mixture of four MUPs or one other unidentified component separately induce aggression in the castrate mouse bioassay (Chamero et al. 2007).

Boar (*Sus scrofa*) saliva contains 3α-androstenol and 5α-androstenone, either of which increases the probability of a sow in oestrus assuming the mating stance (Melrose et al. 1971).

Quantitative signals

Single compound quantitative signals 6-Hydroxy-6-methyl-3-heptanone in male mouse urine accelerates puberty in female mice and declines three- to six-fold in concentration when male mice are castrated (Novotny et al. 1999a). Trimethylamine is a species-specific attractant for mice, and male mouse urine contains about 1,000 times as much of it as the urine of other rodents, and 10–30 times as much as female mouse urine (Li et al. 2013). 2-Heptanone in the odour of rat urine increases in concentration by a factor of approximately four when rats are stressed and increases immobility times in a forced swimming test in rats that inhale it (Gutiérrez-García et al. 2007).

In male Asian elephants, the presence in temporal gland secretion of racemic frontalin above about 14 ng/ml or of either of the isomers above about 7 ng/ml signals musth, but see further discussion below (Greenwood et al. 2005). A twelve-fold increase in 4-methylphenol concentration in mares' urine occurs on the day before oestrus, and spiking dioestrous urine with 4-methylphenol increases its attractiveness to stallions and the extent or penile erections when they smell it (Būda et al. 2012).

Multi-compound quantitative signals When adult female mice are adrenalectomized, the concentrations of six compounds in their urine decline. These six compounds delay puberty in immature females (Novotny et al. 1986). Two farnesene isomers whose concentrations are elevated in the urine of dominant mice are aversive to subordinate males (Novotny et al. 1990a), and accelerate puberty and induce oestrus in crowded females (Ma et al. 1999a; Novotny et al. 1999b). House mouse MUPs are three to four times as abundant in the urine of males as in females (Beynon and Hurst 2003).

Female rats sniff more at male urine than at castrate urine, and Zhang et al. (2008b) found that squalene, 2-heptanone and 4-ethyl phenol spiked together into castrate urine at natural levels increased its attractiveness to females. Zhang and Zhang (2011) partially changed female rats' preferences for urine from different strains of males by manipulating the concentrations of four compounds (note that these authors use "ratio" when referring to concentration). Intriguingly, one of the compounds that they identified as signalling relatedness, 2-heptanone, is also increased in concentration in the urine of stressed rats, and increases immobility times in a forced swimming test (Gutiérrez-García et al. 2007). A mixture of five short-chain aliphatic acids whose concentration in faeces declines when female rats, horses (*Equus caballus*) or red foxes (*Vulpes vulpes*) are in oestrus shows a U-shaped dose–response curve in eliciting erections in male rats (Nielsen et al. 2011).

Mixtures containing six or 24 components of beaver (*Castor canadensis*) castoreum in their natural concentrations elicit reactions that are not significantly weaker than responses to whole castoreum. Interestingly, the response to the 24-component mix was weaker than to the six-component mix (Müller-Schwarze and Houlihan 1991).

Countermarking in red foxes (*V. vulpes*) is elicited by a mixture of eight male urine components at concentrations similar to those in urine (Whitten et al. 1980). The urine of male tree shrews (*Tupaia belangeri*) contains 2,5-dimethylpyrazine at concentrations at least 100 times higher than in the urine of castrates and females, and 3-methylthiopropanoic acid and 3-methyl-2-oxovaleric acid at higher concentrations than in female urine. When any of the three is added to castrate urine, or presented as the pure

substance, it stimulates overmarking by "chinning", which is the normal response to adult male urine. The effect of 3-methyl-2-oxovaleric acid has a quantitative threshold. No pure component stimulates as much overmarking as urine itself (Von Stralendorff 1982, 1987).

Crewe et al. (1979) identified three major components: benzyl cyanide, 2-(hydroxyphenyl)ethanol and *p*-hydroxybenzyl cyanide, in thick-tailed bushbaby (*Galago crassicaudatus*) sternal gland secretion, and found quantitative differences between males and females. In field bioassays with free-ranging bushbabies, benzyl cyanide and 2-(hydroxyphenyl)ethanol alone each elicited no more sniffing than a clean control, but when mixed, they elicited as much sniffing as natural marks. Mixtures of all three compounds, in two different ratios that matched ratios found in male and female secretions, elicited high rates of sniffing (Katsir and Crewe 1980). Artificial marks contained approximately ten times as much of each compound as a natural mark (Crewe et al. 1979).

Other multicomponent signals In mice, up to about 50 peptide ligands of MHC proteins contribute to individual recognition and to mate selection, but whether the signals are qualitative or quantitative has not been established (Leinders-Zufall et al. 2004; but see also Hurst et al. 2004).

Ratio signals I found no study in which ratios per se had been deliberately experimentally manipulated to test whether they encode signals, and so signals that may be coded by ratios are listed under "Ratio candidates" subsection (below).

Candidates

Compounds that differ between individuals or categories, but where no bioassays have been carried out, provide untested candidates for the role of semiochemicals. Certainly, not all of these candidates will pass the bioassay test; Von Stralendorff (1987) showed clear inactivity for compounds whose differences in abundance in the urine of male and female tree shrews suggested that they might be semiochemicals, and although methyl thiolbutyrate increases 1,000-fold in hamster (*Mesocricetus auratus*) vaginal fluid on the day of oestrus, it does not induce sexual behaviour by males (Singer et al. 1983). The three compounds in tamarin scent marks that Smith et al. (1985) found were sufficient to discriminate between males and females were in a fraction of the total volatiles that the tamarins themselves were unable to tell apart.

Qualitative candidates

Single compound qualitative candidates A single compound, 2′-aminoacetophenone, is specific to Mexican free-tailed bats (*Tadarida brasiliensis mexicana*) and absent from other bat species (Nielsen et al. 2006), and accounts for the distinctive (to the human nose) odour of the bats and their roosts. *N*-pentyl acetate appears in the urine of female mice only when they are in oestrus (Schwende et al. 1986). Zhang et al. (2005) found 2-methylquinoline in ferret (*Mustela furo*) urine to be specific to males, and 3-ethyl-1,2-dimethyl-1,2-dithiolane in anal gland secretion to be specific to females. One component of anal gland secretion is present in Siberian weasels (*Mustela sibirica*) and not in steppe polecats (*Mustela eversmanni*) (Zhang et al. 2003). During the breeding season, male red fox (*V. vulpes*) urine contains 2-methylquinoline which is not found in female urine (Jorgenson et al. 1978). 5-Methylhydantoin in giant panda (*Ailuropoda melanoleuca*) anogenital gland secretion is specific to females (Zhang et al. 2008a).

Multi-compound qualitative candidates There are multiple qualitative differences between male and female antechinus (*Antechinus stuartii*) urines (Toftegaards et al. 1999). Zabaras et al. (2005) found qualitative inter-individual differences for all 32 compounds in the sternal gland secretion of tammar wallabies (*Macropus eugenii*), and 18 of the compounds occurred in fewer than five of 25 subjects. In chromatograms from flank gland secretions of male *Crocidura russula*, only a minority of peaks are common to all males (Cantoni et al. 1996). In sac winged bats (*Saccopteryx*), Caspers et al. (2008) found nine male specific compounds (the scent sacs in females are rudimentary), Caspers et al. (2009) found three species-specific compounds in *Saccopteryx bilineata* and two in *Saccopteryx leptura*, and Caspers et al. (2011) found three compounds specific to adult male *S. bilineata*. Numerous components of fishing bat (*Noctilio leporinus*) axial gland secretion differ qualitatively with sex, age and status (Brooke and Decker 1996).

Male and female beavers have different compounds in their anal gland secretions (Sun 1996 cited in Sun and Müller-Schwarze 1998b). Burger et al. (2001) identified 48 compounds and detected several more from ventral gland secretion of male dwarf hamsters (*Phodopus sungorus*) but could not collect enough secretion for analysis from the far less developed glands of females. In *Phodopus* urine, males have seven compounds absent from females in *Phodopus campbelli* and six in *P. sungorus*, and *Phodopus roborowsky* shows multiple qualitative differences from the other two species (Soini et al. 2005). Wellington et al. (1979) found inter-specific qualitative differences in all except three compounds from perineal glands of wild and domestic guinea pigs (*Cavia porcellus* and *Cavia aperea*). In mouse urine, there are two peaks with a qualitative association with H-2 genotype, and eight peaks with qualitative associations with genetic background (Eggert et al. 1996). In deermice (*Peromyscus californicus*), seven urine compounds in males and four in females are present in adults and not juveniles

(Ma et al. 1999b). Two pyrazines disappear from the urine of female pine voles (*Microtus pinetorum*) when they are exposed to the bedding of males (Boyer et al. 1989).

Rabbit chin gland secretion shows numerous qualitative inter-individual differences, and two compounds that are both site-specific and present in the majority of animals at a site (Hayes et al. 2002). There are qualitative inter-individual differences in secretions from five species of deer (Lawson et al. 2000), and of 55 urine volatiles from male white-tailed deer (*Odocoileus virginianus*), 28 showed qualitative differences, nine were present only in dominants during the breeding season and 19 only in subordinates during the breeding season (Miller et al. 1998). In male moose (*Alces alces*) urine, seven compounds are found only during the rut, and two only outside the rut (Whittle et al. 2000). The faeces of feral horses show qualitative changes with sex, season and breeding condition in the presence of free acids, alcohols, phenols and skatole (Kimura 2001). Two compounds occur in the pre-orbital gland secretions of grey duiker (*Sylvicapra grimmia*) and red duiker (*Cephalophus natalensis*) but are absent from blue duiker (*Cephalophus monticola*) (Burger et al. 1988).

Of 22 compounds identified in the anal sac secretions of one male and one female yellow mongoose (*Cynictis penicillata*), only four were present in both animals (Apps et al. 1989). In samples of anal gland secretion from banded mongooses (*Mungos mungo*), no compound occurred in every sample, and the most widespread compound occurred in only 63 of 103 samples (Jordan et al. 2010). In Egyptian mongoose (*Herpestes ichneumon*) anal gland secretion, 2-methyl substituted acids occur only in males (Hefetz et al. 1984). Fossas (*Cryptoprocta ferox*) have three male- and two female-specific compounds in the volatiles from the hair on their tails (Vogler et al. 2008).

Mustelid anal sac secretions show both quantitative and qualitative differences between species (Brinck et al. 1983). In European badgers (*Meles meles*), of 300 compounds tentatively identified in urine, only 33 compounds occurred in at least 25 out of 84 animals (Service et al. 2001), and in subcaudal gland secretion, only 21 of 110 compounds occurred in all of 66 animals (Buesching et al. 2002). In their anal gland secretions, steppe polecats have four compounds not present in Siberian weasels, and 2-ethylthietane is specific to female weasels (Zhang et al. 2002, 2003). 2-Ethylthietane and 3-ethyl-1,2-dithiolane in anal gland secretion are specific to female stoats (Crump 1980).

Of 38 compounds in giant panda anogenital gland secretion, three occur only in females, and two of these differ qualitatively between individuals (Zhang et al. 2008a). Hagey and MacDonald (2003) found 111 out of 951 compounds to occur in at least 10 % of the samples from ten pandas, or in other words that there were 840 compounds that were absent from at least one sample. On average, 5 % of the compounds from females and 13 % of the compounds from males are specific to individual animals. Yuan et al. (2004) tentatively identified 95 compounds from 24 pandas; each had a subset of 33–53 of these, and 39 compounds occurred in three or fewer animals. Burgener et al. (2009) found up to four (but usually zero) individually unique peaks from spotted hyaena (*Crocuta crocuta*) pastings.

Three compounds, 3-methyltetrahydrothiophene, dodecanal and 3-methylbutyl sulphide, in female coyote (*Canis latrans*) urine appear and disappear quasi-periodically, with a peak in abundance at the peak of oestrus (Schultz et al. 1988). Wolf (*Canis lupus*) anal gland secretion shows multiple qualitative and quantitative changes with sex and reproductive condition (Raymer et al. 1985). Out of 77 identified compounds in wolf faeces, Martín et al. (2010) found 55 compounds in faeces from adults that were not present pup faeces, and four compounds in pup faeces were not found in adults.

Numerous compounds in the anogenital gland secretions of ring-tailed lemurs (*Lemur catta*) and Coquerel's sifaka (*Propithecus verreauxi coquereli*) are qualitatively different between the species, several are qualitatively different between the sexes in the sifaka and a large number are qualitatively different between individuals in both sexes of both species (Hayes et al. 2004). While male and female ring-tailed lemurs share about 170 of the compounds in their genital gland secretions, females have an average of 83 more compounds than males (Boulet et al. 2009), and each sex has compounds not found in the other (Scordato et al. 2007). Owl monkey (*Aotus nancymaae*) subcaudal gland secretion differs qualitatively between sexes and age groups (Macdonald et al. 2007). Belcher et al. (1988) found that of 144 compounds in scent marks and suprapubic gland secretion from three female cotton top tamarins (*Sanguinus o. oedipus*), only three occurred in all of the subjects; the other compounds gave each individual a unique qualitative profile.

Quantitative candidates

Quantitative differences are universal at all levels of comparison from physiology within an individual, through inter-individual to inter-specific. They are also generated from identical samples by analytical variability. Given the intrinsic variability of biological materials and the complexity of mammal secretions, it would be surprising to find materials that were closely similar in composition. Only those cases where quantitative differences were linked to a biological difference (e.g. sex or social status) are listed here. Only under exceptional circumstances (all components having the same proportional change in concentration) will a quantitative change in one component not be accompanied by a change in its ratio to other components. Therefore, all instances of quantitative differences are also examples of differences in ratios, but in addition, there are two special

candidate ratios that are listed separately because they have been explicitly identified as potential ratio signals.

Single-compound quantitative candidates The concentration 3-nonene-2-one in whole body odours discriminates between the cryptic rodent species *Mastomys coucha* and *Mastomys natalensis*, despite large differences in concentration between individuals within each species (Apps et al. 1990). 16-Methyl oxacyclohexadecan-2-one is significantly more abundant in male crested porcupines' (*Hystrix cristata*) perianal gland secretion than in females' (Massolo et al. 2009). If male pine voles are castrated, the only detectable change in their urine volatiles is a decrease in the concentration of phenylacetone (Boyer et al. 1989).

Multi-compound quantitative candidates Numerous components of fishing bat (*N. leporinus*) axial gland secretion differ quantitatively with sex, age and status (Brooke and Decker 1996).

Pohorecky et al. (2008) report quantitative differences in rat preputial gland volatiles due to dominance and housing conditions (they use "qualitative" in the sense of different relative amounts). Jemiolo et al. (1987) found only quantitative differences in the urine volatiles of pregnant and lactating mice compared to non-reproductive controls, which were statistically significant for 14 out of 26 compounds. In deermouse urine, there are only quantitative differences between males and females and between virgin and pregnant females, and both quantitative and qualitative differences with age (Jemiolo et al. 1994; Ma et al. 1999b). *P. campbelli* and *P. sungorus* have quantitative differences between males and females for 10 and 11 compounds, respectively (Soini et al. 2005). In CH3 congenic mice, differences in t-complex genes are associated with quantitative differences in seven urine compounds in males and three in females (Jemiolo et al. 1991b). Five peaks from mouse urine have quantitative associations with H-2 genotype, and four have quantitative associations with genetic background (Eggert et al. 1996). Several studies reviewed by Kwak et al. (2010) have found multi-compound associations with major histocompatibility (MHC) genes in mice but in no case have the chemical differences been shown by bioassay to constitute a signal. For example, MHC genotype has a quantitative effect on urine volatiles in inbred mice (Novotny et al. 2007), and Singer et al. (1997) found seven volatile acids in mouse urine whose concentrations differed significantly between MHC types, and demonstrated that trained mice could discriminate the MHC type of urine donor mice when presented with fractions that contained these acids. The dominance status of male mice affects both the overall quantity of volatiles, and the sizes and relative sizes of individual peaks in their whole body odour (Apps et al. 1988), bladder urine and preputial gland secretions (Novotny et al. 1990a, b). Exposure of female pine voles to male bedding increases the concentration of two compounds and decreases the concentration of four compounds in their urine (Boyer et al. 1989).

Volatiles from beaver anal gland secretions can be classified according to family relationships using three chromatographic peak areas from males, or two from females. Classifications based on the three male peaks were 66 % accurate, and including 22 peaks increased the accuracy to 96.5 %, but including more than three but fewer than 13 peaks actually decreased accuracy. Similarly, for females, two peaks gave 89.7 % accuracy, three peaks gave 69.2 % accuracy and 31 peaks gave 100 % accuracy. These classifications were run on peaks that occurred in at least 50 % of samples, after exclusion of up to three peaks from male profiles, and four to nine peaks from females (Sun and Müller-Schwarze 1998b).

In male white-tailed deer, 11 compounds in inter-digital gland secretion are more abundant in dominants (Gassett et al. 1996), and nine compounds that occur in both dominants' and subordinates' urine during the breeding season show significant quantitative differences; three are higher in dominants and six higher in subordinates (Miller et al. 1998). Two compounds in the preorbital secretions of grey duikers (*Sylvicapra grimmia*) occur at higher concentrations in males than in females (Burger et al. 1988).

Anal gland secretion fatty acid profiles are individually different in Indian mongooses (*Herpestes auropunctatus*) (Gorman 1976), red foxes (Albone and Perry 1976) and brown hyaenas (*Hyaena brunnea*) (Mills et al. 1980). The quantities of two compounds in female coyote urine fluctuate quasi-periodically, with a clear peak in abundance at the peak of oestrus (Schultz et al. 1988). Giant panda anogenital gland secretion from males has five short-chain fatty acids at four times the concentration found in females (Hagey and MacDonald 2003), and Yuan et al. (2004) classified giant panda anogenital gland secretions by age and sex from differences in normalised peak areas among the subset of compounds that occurred in more than three animals. Steppe polecats and Siberian weasels have multiple quantitative differences between species in the sulphur compounds of their anogenital gland secretions (Zhang et al. 2002, 2003), and in ferrets, five major anal gland secretion components differ in concentration between individuals (Clapperton et al. 1988).

Smith et al. (2001) demonstrated that the chemical profiles of (pooled) scent marks from five female marmosets (*Callithrix jacchus*) were qualitatively similar; 36 of 42 pooled samples contained the same 162 chemicals, and the other eight samples contained a subset of those. Linear discriminant analysis of peak areas correctly assigned at least 80 % of pooled samples to their donors.

Ratio candidates By selective weighting of ln-transformed peak areas, Willse et al. (2006) found 43 pairs of compounds in which the ratio differed between MHC genotypes in mice, but their signalling role has not yet been verified. The isomeric ratio of frontalin in the temporal gland secretion of male Asian elephants in musth is closer to the 1:1 racemic mixture in mature animals (Greenwood et al. 2005).

Gestalts or chemical images?

If a single compound or simple mixture is active in the absence of the other components of the mixture in which it naturally occurs, the signal cannot depend on a Gestalt or a chemical image.

Active out of context

Six compounds that delay puberty in crowded female mice are active when presented together at their natural concentrations in water or glycol as well as in urine (Novotny et al. 1986, 1990a). Five out of six of the male urine components that accelerate puberty in female mice are active alone in water or castrate urine (Novotny et al. 1999b). The male mouse preputial gland compounds E,E,-α-farnesene and E-β-farnesene, which discourage subordinates from investigating dominant male urine and from scent marking, are active when spiked together into bladder urine, female urine or water (Novotny et al. 1990a; Jemiolo et al. 1992). 2,5-Dimethylpyrazine delays sexual maturation in mice when presented in polyethylene glycol (Jemiolo and Novotny 1994), and 6-hydroxy-6-methyl-3-heptanone accelerates puberty in female mice when presented in water (Novotny et al. 1999a). The male mouse semiochemicals dehydro-*exo*-brevicomin and 2-*sec*-butyl-4,5-dihydrothiazole will induce the Whitten effect in crowded females when presented together in castrate urine or in water (Jemiolo et al. 1985, 1986). The darcin-containing fraction from male mouse urine is as attractive as whole urine (Roberts et al. 2010), and recombinant darcin alone enhances memory (Roberts et al. 2012). Trimethylamine attracts mice when dispensed as a pure vapour (Li et al. 2013), and ESP1 stimulates female mouse sexual activity when presented in buffer on cotton wool (Haga et al. 2010). Unlike dehydro-*exo*-brevicomin and 2-*sec*-butyl dihydrothiazole (see below), recombinant MUPs do not have to be in urine to induce aggression by territorial male mice against castrates (Chamero et al. 2007).

Carbon disulphide, which mediates socially derived food preferences in rats (Galef et al. 1988), improves the acceptance of baits by black rats (*Rattus rattus*) when applied directly to food, instead of being in its natural context of rat breath (Parshad 2002). Although six compounds from castoreum are needed to elicit a full overmarking response from beavers, four of them, 4-ethylphenol, 1,2-dihydroxybenzene, acetophenone and 3-hydroxyacetophenone, each elicit immediate overmarking when presented alone (Müller-Schwarze and Houlihan 1991). The rabbit mammary semiochemical is active in aqueous solution on the end of a glass rod, and in gas chromatography-sniffing tests (Schaal et al. 2003). The deer lactone from black-tailed deer tarsal tufts attracts sniffing and licking when presented on Teflon rods (Müller-Schwarze et al. 1976, 1978).

Even when a boar is absent, the odour of either 3α-androstenol or 5α-androstenone increases the probability of an oestrous sow standing in lordosis, and activity is not enhanced by mixing both compounds (Melrose et al. 1971). Frontalin from Asian elephant bulls in musth is active at 100 μM in pH 8 phosphate buffer (Rasmussen and Greenwood 2003). (*Z*)-7-Dodecen-1-yl acetate, a signal of approaching oestrous in Asian elephants, is approximately twice as active in urine as in water, but this effect is primarily, or only, due to pH; its activity in buffer is the same as in urine (Rasmussen et al. 1997).

The three signal compounds identified by Von Stralendorff (1982, 1987) in male tree shrew urine each elicit overmarking when applied as an ether solution to wire mesh. The major components of thick-tailed bushbaby chest gland secretion elicit as much sniffing as natural marks when they are applied to polyethylene pipes as solutions in dichloromethane (Katsir and Crewe 1980).

Inactive out of context

Three mammal semiochemicals have been explicitly shown to be inactive when presented out of context. To elicit aggression from dominant male mice, dehydro-*exo*-brevicomin and 2-*sec*-butyl dihydrothiazole have to be painted together in castrate urine onto a subordinate male (Novotny et al. 1985); if they are dissolved in water, they are inactive. Castrate mouse urine spiked with (methylthio)methanethiol (MTMT) at the concentration at which it occurs in normal male urine was rendered twice as interesting to female mice, and about two thirds as interesting as intact male urine, while MTMT in water is only slightly more interesting than water alone (Lin et al. 2005). Tamarins are able to discriminate the sex and subspecies of a scent mark, but do not discriminate when presented with either the light or the heavy fraction of pooled scent mark volatiles (Belcher et al. 1986). Whether any of these three cases demonstrate a Gestalt or a chemical image signal is discussed below.

Discussion

Table 1 summarizes the results of the literature search on the nature of mammal chemical signals. There are four identifiable sources of bias in these published findings. The first is towards single-compound signals because it is technically easier to

Table 1 A summary of the results of the literature search on the nature of chemically characterized mammalian chemical signals, showing the numbers of identified mammal semiochemicals and candidates for bioassay that contain one or more than one component, and that are coded by qualitative or quantitative differences

		Qualitative	Quantitative	Total
Signals	Single-component	10	5	15
	Multi-component	6	10	16
	Total signals	15	16	31
Candidates	Single-component	7	3	10
	Multi-component	58	25	83
	Total candidates	65	28	93

Individual studies are compiled and referenced in the "Results" section

identify one chemical than several. Nonetheless, this technical challenge should not be exaggerated; multiple active compounds and candidates have been found in some materials. The second bias, which to some extent counteracts the first, is towards multi-compound signals; when bioassays have been applied to mixtures, definitive tests of whether the same result could be achieved with a single compound have rarely been conducted. For example, Whitten et al. (1980) have not reported on the activity of subsets of the eight components of their fox urine mixture, Eggert et al. (1996) did not test any subsets of the 19 mouse urine compounds associated with mouse genotype, and Singer et al. (1997) concluded that "differences in H-2 are signalled by different relative amounts of the components of a mixture of acids", but did not test any of the seven acids singly. An exception is the finding by Novotny et al. that five of the six puberty accelerating compounds in male mouse urine are active individually (Novotny et al. 1999b). Third, apparent qualitative differences can be generated when a compound is always present, but in some samples is at a concentration below the analytical detection limit (e.g. Wellington et al. 1979). Fourth, the large numbers of minor components that some studies report to be present in certain individuals or classes and not in others (e.g. Service et al. 2001; Buesching et al. 2002; Hayes et al. 2002; Hagey and MacDonald 2003; Yuan et al. 2004; Jordan et al. 2010) are prime candidates as qualitative semiochemicals or labels of identity (although this conclusion is seldom drawn), but may well be analytical artefacts; identification by mass spectrum library search can generate a spurious diversity in chemical identities due to variable overlap between incompletely resolved peaks that vary in size—as a neighbouring peak contributes more or less to the mass spectrum at a peak apex, the closest library fit changes.

Origin and persistence of the view that mammal semiochemicals are complex and quantitative

The assumption that mammal semiochemicals are complex mixtures, and speculation that signals are coded by the ratios in abundance of multiple components, date from the early development of mammalian semiochemistry (Eisenberg and Kleiman 1972; Goodrich and Mykytowycz 1972). Evans et al. (1978), working on aggression reducing signals in female mouse urine, speculated that "the reaction may depend on the Gestalt of the whole urine", and the concept of irreducible quantitative complexity in mammal chemical signals reached an apogee with Albone's "chemical image", which proposed that every component in a secretion contributed to the chemical signal, and that signalling activity depended on both the chemical and biological contexts (Albone 1984, 1990). Novotny linked Albone's chemical image to Gestalt psychology, and cautioned that the chemical image strategy's assumption of "infinite chemical complexity" would "discourage enquiries into mechanisms" (Novotny et al. 1990b) but, nonetheless, the assumption of complexity underlay criticisms of the use of bioassays on progressively finer fractions of mammal secretions—the "response-guided strategy" (Albone 1984; Doty 1986; Johnston 2003), and Johnston (2003) explicitly ascribed the slow pace of identification of mammalian semiochemicals to the search for single active compounds.

Actual evidence that a mammal chemical signal (as opposed to a mammal secretion) had multiple components was first provided by Beruter et al. in 1973, who drew a contrast with insect sex pheromones which at time were known mostly as single compounds or simple blends of closely related compounds (Regnier and Law 1968; Beruter et al. 1973). This ground-breaking work showed not only that a mammal chemical signal had multiple active components but also (and much more importantly) that the various components required correspondingly diverse chemical analyses. Nonetheless, for reasons that are obscure, it did not contribute to the development of mammalian semiochemistry; it remained uncited for three decades and prior to its citing here has been cited only once (Scopus). In contrast, the use of a single analytical technique, gas chromatography, by Michael et al. (1971) to show that rhesus monkey "copulins" consisted of mixed short-chain fatty acids (but see also Goldfoot 1981) and by Gorman to demonstrate that individual Indian mongooses

(*H. auropunctatus*) have quantitatively different mixtures of short-chain fatty acids in their anal sacs (Gorman 1976), were widely cited and have undoubtedly been seminal influences since a majority of analytical semiochemistry uses gas chromatography exclusively to search for active mixtures of volatiles.

The early speculations on the complexity of mammal signals had some empirical support. Even with the relatively low resolution separations that were then available, it was apparent that the secretions and excretions of mammals were much more complex than the sex pheromones of insects (Regnier and Law 1968; Goodrich and Mykytowycz 1972; Beruter et al. 1973; Smith 1976; Müller-Schwarze 2006). In hamsters, both a single compound candidate semiochemical and a mixture of 13 compounds were inactive in a realistic bioassay (Macrides et al. 1977; O'Connell et al. 1978), and the reactions of black tailed deer to fractionated tarsal gland secretion grew stronger as more fractions were recombined (Müller-Schwarze 1969). What is puzzling is that the emphasis on quantitative complexity and ratio coding has persisted, and more recently grown, despite all the mammal semiochemicals that have been chemically characterized being either chemically simple or qualitative, or both (Table 1), and all of them being a tiny minority among the hundreds of other compounds in the materials in which the semiochemicals are deposited. Of 31 mammal semiochemicals that have been chemically characterized, only one possibly depends for activity on the ratio between compounds, 28 identified semiochemicals retain activity out of context, while one single compound and one mixture of two compounds have been shown to be inactive out of context, and a sex and subspecies discrimination no longer occurs if a scent mark is separated into two fractions. Nonetheless, as discussed below, none of these chemical signals have been shown to depend on a Gestalt or a chemical image. Between them, the untested candidates contain several hundred compounds, and clearly, bioassays are lagging behind chemistry.

The persistence of the view that mammal chemical signals are complex and quantitative may have been a response to the slow rate of discovery of new mammal semiochemicals; the difficulty of finding simple, qualitative signals by gas chromatography was ascribed to the signals being complex and quantitative (Setchell et al. 2010); Smith et al. (1985) state explicitly that their testing of a hypothesis of complex quantitative signals was a response to the absence of detectable qualitative differences. The alternative explanations for simple signals being cryptic, that they are obscured by the chemical noise of more abundant components or are analytically intractable or incompatible with gas chromatography, have rarely been recognised (for an exception, see Boyer et al. 1989) except as a rationale for using bioassays rather than chemical analysis (Célérier et al. 2010) and as evidence that signals are carried by volatiles (Kwak et al. 2009). The demonstration by Beruter et al. (1973) that signal components may occur among the non-volatile fraction of mammal secretions is now the first of several; aphrodisin proteins in hamsters (Singer et al. 1986; Briand et al. 2004), MUPs in mice (Hurst et al. 2001; Beynon and Hurst 2003; Armstrong et al. 2005), other rodents (Beynon and Hurst 2008) and predators (Papes et al. 2010), and the oestrus inducing semiochemical in male opossums (*Monodelphis domestica*) sternal gland secretion (Harder et al. 2008) are all incompatible with gas chromatography.

Are signals coded as ratios?

Explicit tests for the semiochemical significance of ratios are conspicuously lacking. Clearly, if test compounds are presented at their natural concentrations, they will also be at their natural ratios, but the converse is not true; the natural ratio can be generated by unnatural concentrations (Katsir and Crewe 1980). I could find no explicit test of whether a ratio per se is a signal. Nielsen et al. (2011) tested only natural ratios of free acids from female rat faeces on the frequency of erections in male rats. To simulate the "scent of age", Osada et al. (2008) spiked three compounds into male mouse urine at their natural concentrations and therefore natural ratios. Dehydro-*exo*-brevicomin and 2-*sec*-butyl-4,5-dihydrothiazole from male mouse urine induce the Whitten effect (synchronization of oestrous cycles) when presented together (Jemiolo et al. 1985) but whether they have to be present in a specific ratio has apparently not been established. In the mixture of the seven acids that Singer et al. (1997) found to differ between mouse MHC types, there were two pairs of peaks whose area ratios were sufficient to discriminate between MHC types, and the normalized area of one of these four peaks differed significantly between the MHC types, and so could alone have been the basis of the discrimination by the trained mice. The possibilities that MHC type was signalled by the ratios within the pairs, or the concentration of a single compound was not tested.

The only convincing case of a ratio signal is the changes in the isomeric ratio of frontalin in the temporal gland secretion of male Asian elephants in musth (Greenwood et al. 2005). Nonetheless, the concentration of frontalin as well as its isomeric ratio fluctuate widely in adolescent bulls, and the possibilities that the signal of mature musth is simply the presence of racemic frontalin at stable concentrations above about 14 ng/ml or the presence of either of its isomers above about 7 ng/ml are yet to be tested.

There are four mammal chemical signals that are definitely not coded by ratios. The signal of male dominance in mouse urine cannot depend on the ratio between farnesene isomers because all reports of the activity of synthetic farnesenes (Novotny et al. 1990a; Jemiolo et al. 1992; Ma

et al. 1999a) have used a 1:1 ratio, which does not occur naturally (Harvey et al. 1989). Adult female mice showed no preference for male urine over a mixture of farnesenes, 2-*sec*-butyl-4,5-dihydrothiazole and dehydro-*exo*-brevicomin at unnatural ratios (Jemiolo et al. 1991a), or for male urine over castrate urine spiked with double the natural concentration of dihydrothiazole and dehydrobrevicomin (Ninomiya and Kimura 1990). That saddleback tamarins can detect the presence of male scent marks mixed with various ratios of female scent marks (Epple et al. 1980) and domestic dogs (*Canis familiaris*) can detect female urine diluted with male urine (Dunbar and Buehler 1980) proves that the sex-specific characteristics are not encoded by different ratios of compounds that are common to urines from both sexes, since such ratios must change as the mixing ratio changes.

Are mammal chemical signals Gestalts or chemical images?

The two mouse semiochemicals that are inactive unless mixed with urine (Novotny et al. 1985; Lin et al. 2005) could depend on a chemical image, but their needing to be in urine is much more likely to be due to adsorption of the semiochemicals to urinary lipocalins (Marchlewska-Koj et al. 2000; Beynon and Hurst 2003; Hurst and Beynon 2004) which may be semiochemicals in their own right (Chamero et al. 2007), or to the presence of semiochemicals from preputial gland secretion in castrate urine (Ninomiya and Kimura 1990). Tamarins' inability to discriminate the sex and subspecies of scent donors in either the light or the heavy fraction of pooled scent marks (Belcher et al. 1986) could be due simply to rapid evaporation of a semiochemically active lighter fraction in the absence of fixatives in the heavy fraction. Both fractions contributing components to the signal does not imply that all the components of both fractions are necessary to the signal, which is required for the signal to be a chemical image. Unless the previously identified semiochemicals from mouse urine that they bioassayed really are inactive, the failure by Flanagan et al. (2011) to replicate their puberty accelerating effects implies that details of context may be important, as required by the concept of a chemical image. Nonetheless, since the activity was present in a narrowly specified fraction of the whole urine, the signal does not meet the definition of a chemical image or a Gestalt.

Complexity

The secretions and excretions in which mammals embed their chemical signals are unquestionably extremely complex (Fig. 2), but this does not necessarily mean that the chemical signals themselves share that complexity, and without exception, the mammal semiochemicals that have been identified constitute a tiny numerical minority among the hundreds of other compounds present; the only chemically characterized signals with more than ten components are the mouse urine MUPS.

Complexity, individuality and signature mixtures

The earliest work on the chemistry of mammalian secretions showed that secretions were sufficiently complex and quantitatively variable to code individual identity by quantitative differences in the composition of mixtures (Berüter et al. 1974; Gorman 1976). Subsequently, complexity being sufficient to code individual identity has metamorphosed into assertions that complexity is necessary to code identity. Arguments that generating large numbers of different signals requires chemical blends rather than single compounds (Von Stralendorff 1987; Johnston 2005) seriously underestimate chemical diversity; among potentially airborne organics up to about the size of squalene ($C_{30}H_{50}$), there are many orders of magnitude more chemicals are available than individuals in any mammal species, and the number of possible biological macromolecules is pseudo-infinite. That every individual of a species has to have a unique odour (Brahmachary 1986) is a misconception; it is necessary only that an individual can recognise the members of its social group as individuals and distinguish them from all the rest (Alberts 1992), or in an extreme case, that a mother recognise her own offspring (Beecher 1989).

Clearly, metabolic pathways constrain what chemicals organisms can produce to a subset of those that are chemically possible. The full potential of mammalian metabolic pathways to generate diverse chemical compounds has not been investigated, but if insect physiology is used as a surrogate, there are tens of thousands of possible isomers within the strictly constrained functional groups and restricted range of chain lengths of real insect pheromones (Byers 2005). This is more than the number of individuals that any one mammal will interact with in a lifetime (Thom and Hurst 2004); even bat mothers finding their pups in roosting crèches only have up to 4,000 pups to choose from (Gustin and McCracken 1987; McCracken 1993). In any case, it is probably not mammalian metabolism that constrains the range of compounds that might be in a mammal's odour; scent glands and sacs, the surface of the skin, the gut and the urinogenital tract are populated by commensal microorganisms which are either suspected (Ware and Gosden 1980; Albone 1984; Nordstrom et al. 1989; Alexy et al. 2003) or proven (Bullard 1982; Voigt et al. 2005; Archie and Theis 2011; Goodwin et al. 2012; Li et al. 2013) to generate odorous volatiles that could act as chemical messengers.

Even if a very wide diversity of compounds can be produced, the utility of single compounds as signals could be constrained by limits to their perception and discrimination. Figures for the total number of odours that can be

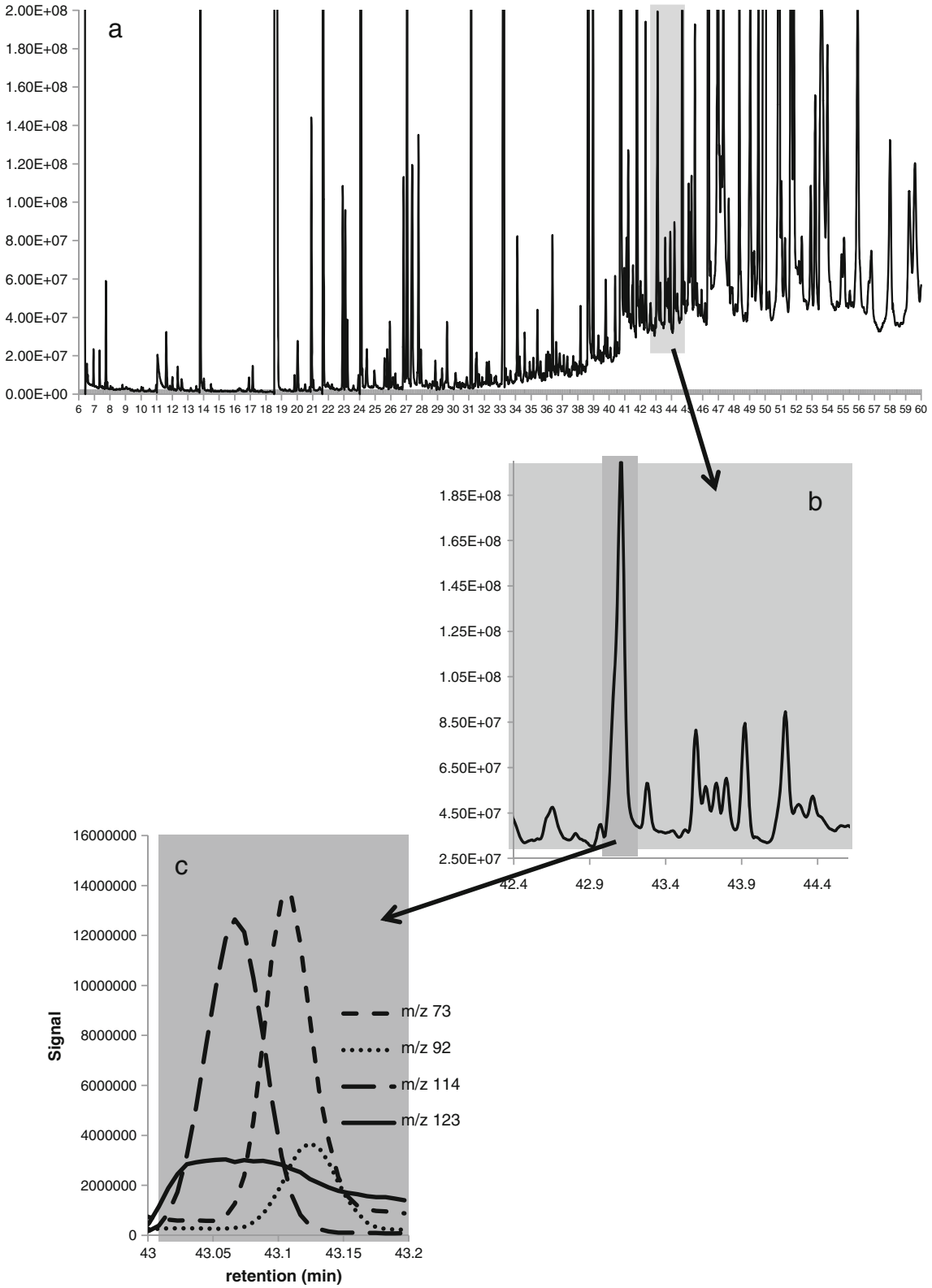

Fig. 2 The complexity of mammal secretions (in the broad sense) is illustrated by a capillary gas chromatogram (**a**) of a methanol extract from the preputial tuft of a dominant male African wild dog (*Lycaon pictus*). The components of the extract are separated as they pass along the capillary column, and as a compound elutes from the column, it generates a peak on the chromatogram. The size of the peak depends on the quantity of the compound. This separation ran for 60 min; expanding a 2-min slice of the chromatogram (*light grey rectangle*) shows that it contains about a dozen visible peaks (**b**). Extracting the traces for single ion fragments detected by a mass spectrometer at the end of the capillary column reveals that one of those dozen peaks (*darker rectangle*) contains at least four different compounds (**c**). Further details of the chemistry can be found in Apps et al. (2012, 2013)

discriminated are largely speculative (Mori and Yoshihara 1995) and are based for obvious reasons on humans. Even if no two chemicals have exactly the same odour (Turin and Yoshii 2003), how easily different compounds can be distinguished from one another depends on the structural differences between them (Laska and Freyer 1997; Laska et al. 1999; Laska 2005). Olfactory discriminating power is related to the number of functional olfactory receptor genes (Laska and Shepherd 2007); non-human mammals have up to three times as many functional olfactory genes as humans (Young and Trask 2002), and can smell compounds that are odourless to humans; for example, mice can smell odourless mineral oil (Gamble and Smith 2009), and rabbits can smell their chin gland secretions which to humans have no odour (Goodrich and Mykytowycz 1972). In addition, most non-human mammals have an extra olfactory sensor, the vomeronasal organ, which extends their sensory range even further. Thus, there is no empirical evidence that limited odour discrimination would prevent the use of single compound identity labels in mammals.

The effects of various manipulations of mouse amniotic fluid on newborn mouse pups' orientation to their mother's nipples and their first sucking have been interpreted as evidence that the fluid contains a signature mixture (Logan et al. 2012), but although pups learn diet-induced differences in the composition of amniotic fluid, the composition of the amniotic fluid is the same in females of different strains, and pups do not differentiate between lactating females. A signature mixture would differ between females, and pups would differentiate. Complexity per se is not necessary to the signal; the response to amniotic fluid can be replicated by pairing the odour of single compounds that do not naturally occur in amniotic fluid with gentle brushing that simulates the mother's licking. Intriguingly, two out of three molecular weight fractions of amniotic fluid have to be combined to produce the signal, so there must be more than one active component, but one of the fractions has components with molecular weights of 3–10 kDa whose vapour pressures must be below the detection limit of mammalian olfaction.

Complexity and data analysis

There appears to be a particularly close (but implicit) link between the concept of a holistic chemical image and the use of principal components analysis (see below) to reduce the dimensionality of multiple chromatographic peak areas (Lawson et al. 2000; Service et al. 2001; Bloss et al. 2002; Safi and Kerth 2003; Burgener et al. 2009; Setchell et al. 2010, 2011; Jordan et al. 2010). The chemical image concept also apparently underpins the use of the number or diversity of detectable peaks on a gas chromatogram as a signal parameter (Caspers et al. 2009; Boulet et al. 2009, 2010; Burgener et al. 2009; Rosell et al. 2010; Setchell et al. 2011; DelBarco-Trillo et al. 2012), the use of multidimensional distances between chromatograms (Service et al. 2001; Safi and Kerth 2003; Caspers et al. 2009; Burgener et al. 2009) and the use of the term semiochemical for every peak on a chromatogram (Boulet et al. 2009).

There can be no objection to a search for active mixtures as long as it does not exclude the possibility of discovering single active compounds, or to a search for quantitative signals that does not exclude signals encoded by presence and absence. However, as argued below, in the current focussed search for multi-component quantitative semiochemicals, signalling mixtures are being sought by means that actively exclude the possibility of finding qualitative differences and simple signals. It has been common practise for peaks that are absent from some chromatograms to have their areas in chromatograms where they do appear excluded from the statistical analysis (Sun and Müller-Schwarze 1998a, b; Service et al. 2001; Yuan et al. 2004; Macdonald et al. 2007; Arnold 2009; Boulet et al. 2009; Jordan et al. 2010; Martín et al. 2010), thus excluding the possibility of finding signals that are coded qualitatively, since qualitative signalling necessarily requires that signalling components be absent from some samples and present in others. If this approach had been followed with mouse MUPS, which are a paradigm case of signature mixtures, their role in individual recognition could not have been detected because some MUPs are not expressed at all by some individual mice (Hurst et al. 2001).

Inevitably, data processing that requires components to be present in all samples will not find chemical signals that are encoded by compounds' presence and absence. If only those components that are common to both of two classes of scent are included in data processing (Osada et al. 2008; Boulet et al. 2009), it is inevitable that any differences between the classes that are detected will be quantitative and not qualitative. If labels of individual identity or correlates of genotype are the target, then even ostensibly conservative exclusions which require only that peaks occur in 50 % (Sun and Müller-Schwarze 1998b), or 10 % of samples (Hagey and MacDonald 2003; Massolo et al. 2009) still exclude the possibility of finding qualitative individual identity labels, whether single- or multi-compound, because each label will occur in only one animal. Paradoxically, excluding components deletes parts of the holistic chemical image, which depends on every component having a signalling role. Figure 3 shows a simplified schematic of the characterization of semiochemicals via chemical analysis and multivariate statistics or other data processing methods.

One reason that mammal semiochemicals have been hard to find may be that they have been hiding in plain sight among the chromatographic peaks that were excluded from multivariate statistical analysis because they did not occur in

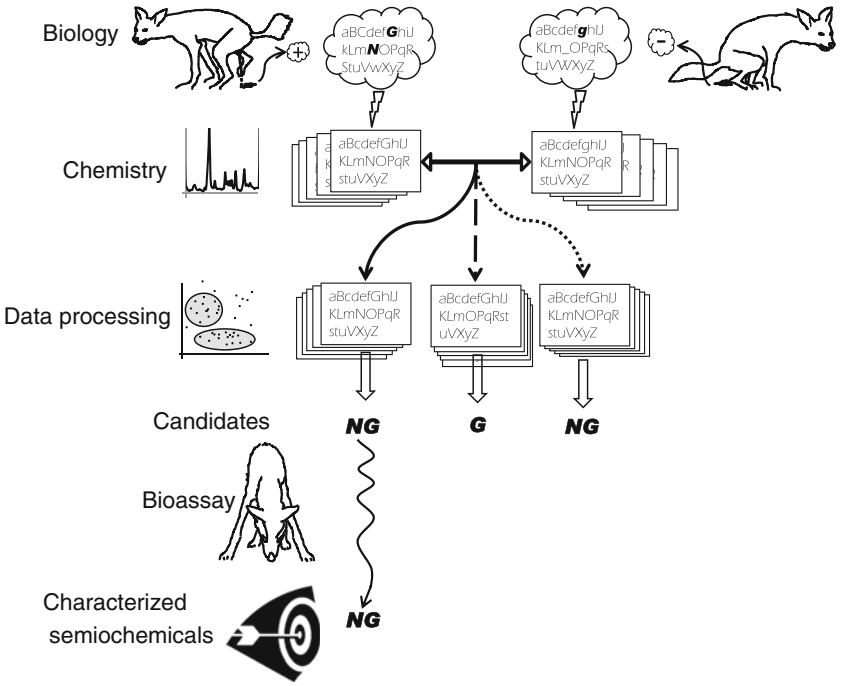

Fig. 3 Simplified schematic of the process from the biology of scent marking to the chemical characterization of a semiochemical. Dominant African wild dogs (*Lycaon pictus*) produce semiochemically active scent marks by urinating with a cocked leg (*top row left*), faeces deposited in a squat by subordinates are semiochemically inactive (*top row right*; Jordan et al. 2013). *Each letter* signifies one, a few or several chemical compounds; and the active compounds are in *bold face*. *Upper and lower case* signify a difference in quantity, the *letter being replaced by an underscore* signifies presence and absence, respectively. Differences in chemical composition between active urine and inactive faeces give clues as to which compounds from the urine are carrying signals; chemical analysis (*second row*) of multiple samples reveals the composition of the active and inactive mixtures but with losses of some compounds and some changes in quantity. In the analytical results, there is nothing about the chemistry of the active components that distinguishes them from the chemical background; to identify and characterize the actives, the analytical results from multiple samples of different types have to be compared (*double-ended arrow*). Multivariate statistics as commonly applied (*dashed arrow*) prunes the analytical data and excludes qualitative differences (*third row*), which removes qualitative signals from the data while retaining only quantitative signals. Other data processing methods of various kinds (*solid arrow*) and more recent applications of multivariate statistics that are robust to the properties of semiochemical data (e.g. Caspers et al. 2011; *dotted arrow*) have retained the active components in the data and have generated both qualitative and quantitative candidates for bioassay (*fourth row*), as listed in the "Candidates" section of the "Results". So far, only the candidates identified by the non-multivariate methods have generated positive bioassays (*fifth row*, *wavy arrow*) and been confirmed as semiochemicals (*bottom row*); these are listed in the "Signals" section of the "Results"

some samples. Rather than excluding chromatographic peaks that do not occur in a subset of samples, an alternative first step, which is at least as logically parsimonious, would be to exclude every peak that occurs in more than one animal when searching for labels of individual identity, and to exclude all peaks that occur in both sexes when searching for signals of sex. If multivariate statistics are employed, the tests must be robust to zero values and to the number of variables being larger than the number of samples, so that no peaks have to be excluded from statistical analysis.

Multivariate analysis can detect differences that have no signalling role; for example, principal components analysis of gas chromatography peak areas can classify the subspecies and sex of tamarins (*Saguinus fuscicollis*) from the butyrate ester and squalene composition of their scent marks, but the tamarins themselves do not discriminate the ester and squalene fraction of scent marks from different subspecies and sexes, or from clean surfaces or control odours (Smith et al. 1985; Belcher et al. 1986). Even if animals can discriminate between chemically classified scents, it does not mean that a real signal is the basis for the discrimination; mammals can be trained to discriminate between arbitrary odours that have no functional significance (Beauchamp et al. 1985; Thom and Hurst 2004; Laska et al. 2008). The two sets of compounds in male mouse urine that Osada et al. identified as indicators of donor age on the basis of trained discriminations (Osada et al. 2003) and innate preferences (Osada et al. 2008) were completely different. References to volatile "fingerprints" in this context are particularly (though probably unintentionally) apposite, since as Sun and Müller-Schwarze (1998b) point out, real fingerprints are individually unique but are not used for individual recognition. Although multivariate analyses very often demonstrate that chromatographic profiles can be grouped according to features of the animals that produced

the samples, so that the corresponding differences in chemical composition could serve as signals, multivariate analysis of chromatographic data has not yet led to the chemical characterization of a mammal semiochemical.

Conclusion

The quantitative complexity of mammal chemical signals has been frequently affirmed, but not confirmed. Expectations that mammal chemical signals are encoded by complex quantitative mixtures have led to single compounds and qualitative differences being overlooked as candidate semiochemicals, and the rate at which new mammal chemical signals are characterized is likely to increase if research strategies and methods accommodate the possibility that signals are coded by qualitative differences or by single compounds and simple mixtures. Eliminating chromatographic peaks because they do not occur in some samples is equivalent to eliminating eye and hair colour as cues in human face recognition because not everybody is a blue-eyed blonde. Perhaps we have not seen the trees because we have been looking for forests.

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