When is a butterfly like an elephant?

David R Kelly

The behaviours of organisms as diverse as elephants and butterflies are affected by pheromones with identical or similar structures. Recent developments in the molecular biology of pheromone detection suggest why.

Address: Department of Chemistry, University of Wales, College of Cardiff, P.O. Box 912, Cardiff CF1 3TB, Wales, UK.

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The recent report that female Asian elephants release (*Z*)-7-dodecenyl acetate in their urine, to signal that they are ready to mate, is by any measure an extraordinary discovery [1]. Who would have predicted that the largest living land animal would use the same pheromone as the turnip looper and the cabbage looper and at least 100 other species of butterflies and moths (Lepidoptera) [2]? (*Z*)-7-dodecenyl acetate is the fifth most common attractant for Lepidoptera and has structural features that are typical of the majority of Lepidopteran pheromones ([3], Table 1).

This is not the first 'coincidence' of this kind. There are several cases scattered throughout the literature in which organisms from diverse parts of the natural world use similar or identical compounds for communication. Are they truly coincidences, or are there deeper reasons for the similarities across species? Here I discuss several particularly striking examples of such similarities, and the clues that they may offer us about pheromone signalling pathways.

Beetles, trees, moths and mice

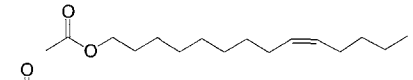
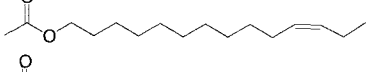
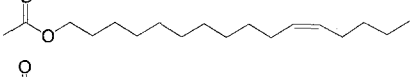
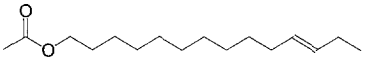
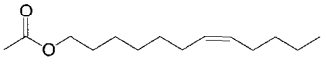
Bark beetles have sophisticated chemical signalling systems which control tree colonization within [4] and between [5] species. *exo*-Brevicomin (compound **1**, Fig. 1) is a male attractant produced by female western pine beetles, *Dendroctonus brevicomis*. It was the first of a series of bark beetle pheromones [6] with the 6,8-dioxa-bicyclo[3.2.1]octane motif [7], which includes *endo*-brevicomin (compound **2**) [8], frontalin (compound **3**) [9] and multistriatin (compound **4**) [10]. A similar spiroacetal (compound **5**) is produced by Norway spruce in response to attack by the ambrosia beetle, *Typodendron lineatum* [11] and the unsaturated analogues (compounds **6**, **7**) are male-released attractants of the swift moth, *Hepialus hecta* [12,13].

Structures of this kind are also found in mouse pheromones. The odour of mouse urine is extremely important in regulating the reproductive status of mice. For example, groups of female mice isolated from males lose their estrus cycle (this is called the Lee–Boot effect), but this can be restored by dosing their bedding with male urine [14]. When young female mice are housed together away from adults, puberty is delayed. Again, normal development can be restored by the application of adult urine to their bedding. Mice live in highly structured social groups, in which hierarchical position is determined by aggressive displays between males. During these displays they discharge unusually pungent urine [15]. Levels of dehydro-*exo*-brevicomin (compound **8**), *p*-toluidine, *sec*-butyl (compound **9**) and *iso*-butylthiazolines (compound **10**) in adult male mouse urine are reduced in castrates, but only dehydro-*exo*-brevicomin and *p*-toluidine levels are restored by treatment with testosterone [16]. A mixture of dehydro-*exo*-brevicomin and the *sec*-butylthiazoline **9** spiked into the urine of castrated mice elicits intermale aggression [17], female attraction, estrus acceleration and synchronization [18]. Either compound alone in castrate urine or the mixture in water has no effect, indicating that other compounds in the urine are essential for full activity. This bioassay work was performed with racemic dehydro-*exo*-brevicomin [19], but recently it has been shown that the biological activity in fact resides in the enantiomer shown as compound **8** [20].

The chirality of the *sec*-butylthiazoline **9** does not affect biological activity, probably because it racemizes quickly in solution. This compound has not yet been found to be important elsewhere in nature, but the *iso*-butylthiazoline **10** and the corresponding thiazole are components of the

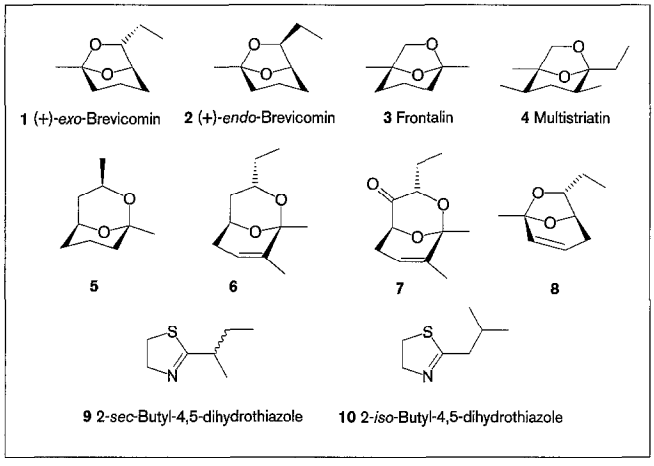
Table 1

Lepidopteran and elephant pheromones.

Structure	Name occurences, % occurrence
	*Z*-9-Tetradecenyl acetate 168, 7.2%
	*Z*-11-Tetradecenyl acetate 157, 6.8%
	*Z*-11-Hexadecenyl acetate 133, 5.7%
	*E*-11-Tetradecenyl acetate 124. 5.3%
	*Z*-7-Dodecenyl acetate 111, 4.8%

Data are taken from an analysis of 2 292 attractants from 1068 species using the computer program PherFind (D.R.K., unpublished). The structures are aligned to show the most common position for alkene bonds, which is at Ω-5 and Ω-3.

Figure 1



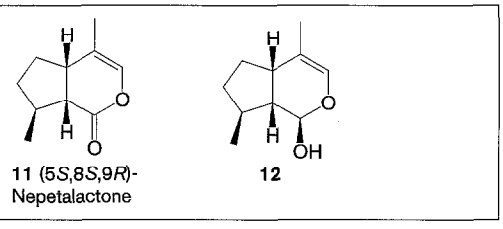
Beetle (**1–4**), spruce (**5**), moth (**6,7**) and mouse (**8–10**) signalling chemicals.

preorbital gland secretion of two African antelopes, the grey duiker *Sylvicapra grimmia* and the red duiker *Cephalophus natalensis*. The secretion is used for marking territory and more of the thiazoline **10** is produced by males, which have more closely defined territories than females [21]. So in this case three mammals and an insect have overlapping complements of two families of closely related compounds that are used for similar purposes.

Lions and tigers and ... aphids ...

Catnip is an extract of the catmint plant *Nepeta cataria* which elicits a dramatic response in domestic cats [22,23], lions [24] and tigers. Initial attraction is followed by behaviour very similar to the cat mating ritual. This involves chin and cheek marking, followed by rolling on to the back and body rubbing [25]. Indeed, it is very difficult to grow catmint in a suburban garden because cats constantly roll on it and destroy it. The active constituent of catnip is (4a*S*,7*S*,7a*R*)-nepetalactone (compound **11**, Fig. 2), which elicits the catnip response at concentrations as low as parts per billion to parts per trillion [25]. Nepetalactone and other stereoisomers [26] are found in many *Nepeta* species and are formed biosynthetically [27] from nerol (or a closely related monoterpene) by terminal hydroxylation, oxidation and cyclization [28].

Figure 2



The active constituent of catnip extract (**11**), which is also an aphid pheromone, as is **12**.

Cats respond equally well to both enantiomers of nepetalactone, although there are reports that racemic nepetalactone is weakly active or inactive to cats ([29], and references therein). Frustratingly, the group which tested enantiomerically pure material did not test the racemate. If all of the published data are correct, this would be the first case where both enantiomers have activity and the racemate does not [30]. However, the response to catnip is rather variable and highest in cats of reproductive age, so a mundane explanation is more likely.

Aphids feed on the sap of plants through fine tubes. They reproduce asexually during the summer, but sexually during the winter [31]. The females release a male-attractant pheromone from their hind legs. In the vetch aphid, *Megoura viciae*, and the wheat aphid, *Schizaphis gramium*, this is a mixture of nepetalactone **11** and the nepetalactol **12** [32,33] and these compounds also act as an attractant for the black bean aphid, *Aphis fabae* [34]. Females of several *Cryptomyzus* species [35] release both the lactol **11** and the lactone **12** (in the ratio 30:1), although males are only weakly attracted to the pure compounds, indicating that further components are required for full activity. The lactol **12** alone is the sex pheromone of the damson-hop aphid, *Phorodon humuli* [36]. It is very common for species from closely related phyla to use mixtures with the same components, but with different ratios or extra components which give species specificity and maintain reproductive isolation. Aphid parasitoids also respond to these compounds, but are more strongly attracted to the lactone **11** [37].

Catmint is a perennial with soft grey or green leaves that are ideal for aphid attack. It is possible that the catmint releases nepetalactone **11** to attract aphid parasitoids and reduce the level of aphid infestation. But the effect on cats cannot be explained so easily. The lack of enantioselectivity in the cat response suggests that nepetalactone is mimicking some other effector, but if so the fact that cats are very sensitive to nepetalactone is surprising. Cats have an array of marking glands which have been barely investigated. In particular, glands on the front and side of the head are used in 'affectionate' behaviour in which cats rub the cheek, shoulder or sometimes the whole body against each other, inanimate objects or human legs. Perhaps compounds that are secreted by these glands are similar to nepetalactone. It is also conceivable that catmint seeds are dispersed from the cat's coat, and that the advantage of this mechanism of dispersal is sufficient to compensate for the damage done to the plant by a rolling cat.

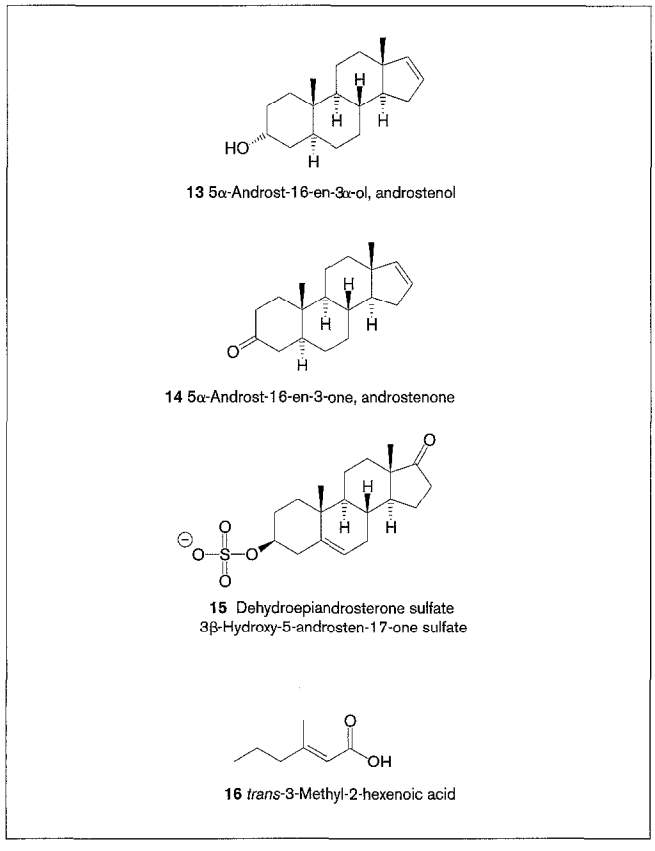
Pigs and people

When a boar is sexually aroused or displaying aggression, characteristic chomping jaw movements cause the secretion

of a viscous, frothy saliva (from the submaxillary glands [38]), with a repugnant odour. The boar snorts and blows the saliva close to the head of the sow. If the sow is receptive (because she has ovulated recently), she will adopt an immobile mating stance, known as 'standing', and mating follows. In traditional farming practice, a 'teaser' boar is used to detect 'standing', in preparation for the expensive services of a stud boar. Saliva production is an important component of the mating ritual, although it is not essential as some sows will 'stand' in response to the sound of the boar's snort alone [39]. Surgical removal of the submaxillary glands of the boar causes loss of libido and passive behaviour.

The semiochemically active components of the saliva are androstenol (compound **13**, Fig. 3), which has a musky odour, and androstenone **14**, which has a urinous odour and is present in smaller amounts [40,41]. The proportions are age-dependent. The compounds are produced in the testes [42], and either one can elicit the 'standing' response. A mixture of androstenol **13** and androstenone **14** in an aerosol can (Boarmate) can be used instead of 'teaser' boars to detect ovulation. Androstenone **14** [43], skatole and to a lesser extent indole [44,45] are responsible for the unpleasant smell of cooked boar meat [46].

Figure 3



Pheromones of pigs (**13**, **14**) and odorant molecules produced by humans (**15**, **16**).

The remarkable aphrodisiac effect of these steroids on pigs led to a search for them in the vertical pig — man. The most likely site for pheromone production in man is the axillae, or arm pits. Axillae have a particularly high concentration of apocrine glands, which only become active with the approach of puberty. Androstenol **13** [47], and androstenone **14** [48,49] were detected in human axillae sweat, saliva, urine, semen and the blood stream [50], but only in trace amounts. The predominant steroid in axillae sweat is the dehydroepiandrosterone sulfate **15** [51]. Many other steroids are secreted onto the skin as sulfates and glucuronides which are cleaved by bacteria to give 'free' steroids [52].

There have been many attempts to show that pig pheromones affect human behaviour, but it is difficult to obtain convincing data and to interpret it. In one experiment, toilet cubicles were sprayed with androstenol **13**. Men avoided the treated cubicles, but women were unaffected [53]. In another experiment, female students exposed overnight to androstenol participated in more social exchanges with men the next morning, whereas men treated similarly were unaffected [54]. Although these results are interesting, they do not make a compelling case for a pheromone-like response to these steroids in man.

It is, however, notoriously difficult to show that humans respond to pheromones at all. The much-quoted McClintock study apparently showed that women living together in dormitories synchronized the onset of their menstrual cycles, and that women who had more contact with men had shorter cycles [55]. In the context of the behaviour of other primates, this is wholly reasonable, but although two studies confirmed these findings [56,57] two others did not [58,59].

These steroids are also found in plants, but their roles are unclear. Androstenol **13** is found in the ultimate delicacy, truffles, and this is why pigs are able to locate truffles even when they are growing up to half a metre below ground [60]. Caviar, celery and young parsnips also contain androstenone **14** [61] as do many of the exotic foods prized by epicureans [62]. Even though excesses of these steroids smell unpleasant, it appears that humans respond favourably to them in small quantities. These steroids are not, incidentally, responsible for the unpleasant odour of unwashed people. In fact the major malodorous component of armpit odour [63] is *trans*-3-methyl-2-hexenoic acid **16** [64], which is found in the axillae of both males and females [65] and was at one time thought to be specific to the characteristic smell of schizophrenics [66].

The detection of steroids which have pheromonal activity in pigs and are found in trace amounts in humans and vegetables might not seem much of a coincidence, but similar steroids are also major pheromones of fish [67]. These are

frequently released as glucuronides or sulfates in the urine and as free steroids from the gills [68].

The existence of chemical signals between fish was demonstrated as long ago as 1932, but it is only in the last 10 years that the compounds responsible have been identified. The systems employed are best exemplified by the goldfish, which has been studied in the most detail. Goldfish do not have defined territories, are promiscuous and have no parental behaviour. The females spawn amongst vegetation followed by a small shoal of suitors who fight to get close enough to fertilize the eggs. About 10 hours before spawning, female goldfish start to produce large quantities of a constellation of steroids **17abc**, **18abc**, **19ac** and **20**. These promote oocyte maturation and, when released into the water, increase gonadotropin levels in males. This in turn promotes the formation of milt (seminal fluid and sperm) in males and increased intermale aggression during spawning [69]. Oocyte final maturation is induced by the dihydroxypregnene **17a**, whereas the most important pheromonal components are the free and sulphated di- and trihydroxypregnenes **17ab**, **18ab** and the androstendione **20** [70], which are detected by the medial portion of the olfactory bulb [71]. Androstendione **20** blocks the milt and gonadotropin promoting effects of the dihydroxypregnene **17a**, but the biological relevance of this is unknown at present.

Another intriguing fish pheromone is the prostaglandin $F_{2\alpha}$, (PG-$F_{2\alpha}$, compound **21a**, Fig. 4), which circulates in the blood stream of female goldfish and is associated with follicular rupture and ovulation. When it is released into the water together with a metabolite, 15-keto-PG-F2a **21b**, it induces increased (but short-lived) aggression amongst males and courtship behaviour [72]. As prostaglandins are normally short-range and short-lived effectors, it is astonishing to find PG-$F_{2\alpha}$ being used in this way.

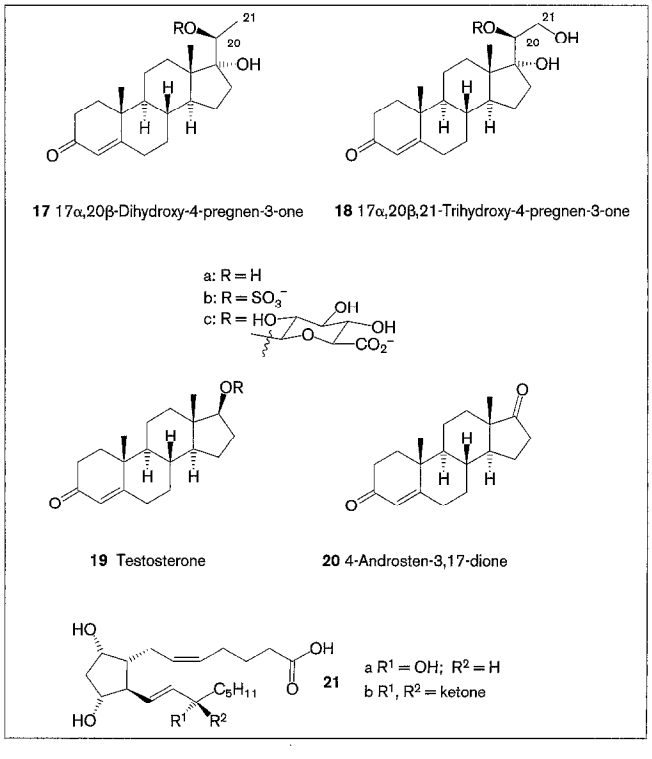
Pheromone transport and release

Organisms have developed a range of strategies for the release of pheromones. The highly volatile pheromones used by Lepidoptera are usually dispersed by wing fanning. Some species of insects have specialized glands (such as hair pencils) with a large surface area, which can be inverted to present the inner secretory surface to the outside world. In fish, free steroids are released from the gills, which have a large surface area washed by a continuous flow of water. Mammals release steroids in comparatively small volumes of urine or through the skin, however, requiring that the released compounds be much more soluble; they are therefore conjugated as sulfates or glucuronides.

In mammals, however, evidence is emerging that specialized proteins are used to transport odorous compounds. The first indications came from work on the Syrian golden hamster, which is unusually dependent on olfaction for normal sexual behaviour [73]. The night before the onset of oestrus, the female hamster lays down a trail to her underground burrow using a watery vaginal secretion which attracts the male. This vaginal deposit contains >200 compounds. The major pheromonal activity was initially suggested to be due to dimethyl disulfide [74,75], but these results were called into question by later studies which showed that although males investigate vaginal odours more frequently than females, there is no difference between the sexes in the investigation of dimethyl disulfide [76]. Dimethyl disulfide is extremely common in nature; it deters feeding on brassicas by sheep [77], it acts as a nipple attachment pheromone in pup rats [78] and is the principal malodorant in human tooth disease.

Whatever signal initially attracts the male golden hamster to the female's burrow, it is a protein (aphrodisin, [79]) that induces the later stages of courtship and copulation. Aphrodisin is found on the genital region of the female, which is licked by the male and detected by the vomeronasal organ [80]. The behaviour it induces is sufficiently stereotypical that an extract painted onto the hind quarters of an anaesthetised male will induce mounting attempts by another male [81]. Aphrodisin [82] is a member of the lipocalin (lipocalcyin) family of 20-kDa soluble extracellular proteins [83]. This is a diverse family, including odorant-binding proteins (OBPs) in the

Figure 4



Fish pheromones.

nasal mucus [84], major urinary protein (MUP), retinol and retinoic acid binding proteins, β-lactoglobulin, α1-microglobulin and quiescence-specific protein [85].

Another indication that lipocalins are involved in the function of pheromone-like compounds came with the finding that the human armpit odour compound *trans*-3-methyl-2-hexenoic acid **16** binds to two proteins (of molecular weights 25 and 46 kDa) which appear to be involved in transportation from the site of biosynthesis [86]. The smaller of these has the amino acid sequence of apolipoprotein D, but has a different glycosylation pattern from plasma apolipoprotein D [87]; this protein is also a member of the lipocalin family. There is no known role for plasma apolipoprotein D, although it binds progesterone and pregnelone *in vivo* and there are indications that it might also bind bilirubin and cholesteryl esters. The boar pheromones androstenol **13** and androstenone **14** are released from a 20-kDa carrier protein (pheromaxein), which is produced in the submaxillary glands [88]. Very little is known about its structure, but its molecular weight is consistent with it being a lipocalin [89].

The lipocalins appear to be generally used as transport proteins for small hydrophobic molecules, although in many cases the ligand for a particular lipocalin has not been identified. No enzymatic activity has been demonstrated for any lipocalin, except for the facile isomerization of PGH2 by PGD synthase to PGD2, which acts as a sleep promoter [90]. The lipocalins have low sequence homology, but essentially identical three-dimensional structures, in which an α-helix and two orthogonal β-sheets form a β-barrel with a hydrophobic interior, which is the putative binding pocket [91]. A three-residue invariant sequence is found at one end of the β-barrel at the start of the A β-strand, and another is found at the end of the F β-strand. These may be part of a binding site for common cell surface receptors; it is plausible that the other, more variable, end of the β-barrel binds the ligand [92]. A lipocalin with 31% homology to apolipoprotein D is expressed by *Escherichia coli* in the stationary growth phase [93]. It is tempting to suggest that its role is to bind ligands which signal that growth and division should resume.

Dehydro-*exo*-brevicomin **8** and *sec*-butylthiazoline **9** are both selectively bound by mouse major urinary protein (MUP), which is expressed in the liver [94]. The X-ray crystal structure has been determined and shows a typical lipocalin structure [95]. MUPs differ between different strains of mice, but the variations are generally conservative (e.g. Lys to Gln or Glu) [96]. The amino-terminal sequences of mouse OBPs are very similar to those of the MUPs [97], which is significant because the levels of sequence homology among the lipocalins are generally low. The idea that similar if not identical proteins are involved in pheromone release and detection is appealing on the grounds of molecular economy [98]. However, recent results indicate that the MUPs may be directly involved in pheromone signalling. The puberty retardation seen in young female mice when they are housed together cannot be overcome by dehydro-*exo*-brevicomin **8** or *sec*-butylthiazoline **9**, yet MUP alone or a truncated MUP hexapeptide (NH_2-Glu-Glu-Ala-Arg-Ser-Met), delivered intranasally, accelerated puberty as assessed by uterus weight [99]. This is a startling result wholly at odds with the notion that MUP is merely a transport protein. Moreover it should be noted that despite over 10 years of work no ligand for aphrodisin has been identified. This raises the question of what role, if any, dehydro-*exo*-brevicomin **8** or *sec*-butylthiazoline **9** play in this system; is the volatile ligand an attractant for the involatile lipocalin, in other words a pheromone's pheromone?

The use of common compounds for signalling between organisms raises important questions about how selectivity is achieved. In some cases there is none. Cross-species mating attempts have been observed between *Arctiidae* (Tiger and Footman moths) and between other moths [100] — but presumably such attempts are not made between moths and elephants. In other species slight shifts in the composition of a blend of pheromones result in large differences in attraction [101]. All that can be said with certainty is that this will be a fertile area for further investigation.

Pheromone signalling

A picture is beginning to emerge of the molecular mechanisms by which odorants are detected by mammals and pheromones by insects. In Lepidoptera, olfaction and pheromone detection are performed by different sensilla (hair-like projections) on the antenna; mammals detect odorants in the olfactory tract and the vomeronasal organ. The exact role of the latter is still a cause of conjecture, but many believe that it is the vomeronasal organ that is responsible for the detection of pheromones (see below).

There are at least two distinct odorant detection pathways in the olfactory tract of mammals (see [102] for review). In the first, guanine nucleotide binding protein (G protein) coupled receptors modulate adenylate cyclase to increase cAMP levels; this opens cyclic nucleotide gated ion channels which produce the action potential in the neuron. In the second, the sequence of events is similar except that inositol 1,4,5-triphosphate (IP_3) is used as the 'second messenger'. The two pathways use different receptors and ion channels. In both cases sensitivity after stimulus is reduced by phosphorylation of the G proteins, by either protein kinase A for the cAMP system or protein kinase C for the IP_3 system. In addition, increased Ca^{2+} levels elicited by odorants reduce sensitivity by inhibiting adenylate cyclase and strong odorants activate NO synthase and cGMP production, which also reduces sensitivity. Any given odorant only activates one of these two

systems, but there is no relationship between the class of odorant and the system activated [102,103].

Very little is known about insect olfaction, but more is known about how insect pheromones are detected. They are initially bound and transported by specific pheromone-binding proteins (SPBP) [104,105]. The binding of the pheromone or the SPBP–pheromone complex [106] to a G protein coupled odorant receptor, activates a specific phospholipase C; inositol 1,4,5-triphosphate is released and this gates the opening of an ion channel, which produces the action potential [107,108]. As with mammalian olfaction, the signal is 'turned off' by phosphorylation of the receptor [109] and high cGMP levels.

Role of the vomeronasal organ

The evidence that the vomeronasal organ (also known as Jacobson's organ) is responsible for pheromone detection is at present confusing. The organ is sexually dimorphic and found in most animals, but is apparently absent in birds, crocodiles, cetaceans, bats and some primates [110]. Teleosts (bony fish) have no anatomically distinct vomeronasal organ; instead, the medial portion of the olfactory tract has pheromone receptors. In animals that do have a vomeronasal organ, its surgical removal reduces investigatory and sexual behaviour, but this can be restored by luteinising hormone releasing hormone [111] and (in rodents) has less profound effects on animals that have prior sexual experience. Nevertheless, the balance of evidence appears to favour the notion that the vomeronasal organ is involved in pheromone detection.

There is growing evidence that the signal transduction pathways in the vomeronasal organ are different from those in the olfactory epithelium. The vomeronasal organ has distinct OBPs [112], G protein coupled receptors [113] and adenylate cyclase, although both tissues express the oCNC2 ion channel [114,115]. In the turtle, the vomeronasal gland has both cAMP and IP_3 gated ion channels, but the cAMP pathway is not involved in transduction of common odorants [116]. It is an appealing idea that the vomeronasal organ may exclusively or primarily use the IP_3 pathway to transduce odorant detection [117], and that the receptors used may be homologous to those used by insects to detect pheromones. Homology between these receptors and signalling pathways might go some way towards explaining the extraordinary coincidences in pheromone structures used by different organisms discussed above.

Ways forward

To determine whether the insect and mammalian pheromone response pathways are indeed similar, we need a better understanding of the components of both pathways, particularly the mammalian one. At present, progress is blocked by the lack of a high affinity ligand for a mammalian vomeronasal receptor. A 70-kDa odorant-binding glycoprotein (vomeromodulin), which is only expressed by the vomeronasal gland, has been suggested to be a pheromone transporter, but again no ligand or receptor has been identified [118]. Two recent developments offer hope for future progress: the cloning of aphrodisin [82], which will allow the expression of large quantities of this protein, and the observation that MUP, a protein that is already available in quantity, accelerates puberty [99]. If receptors for these proteins can be found in the vomeronasal organ, the elucidation of the molecular mechanisms responsible for the fascinating behavioural effects of pheromones in mammals will be a large step closer.

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