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Proteins and peptides as pheromone signals and chemical signatures

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Proteins and peptides are used as pheromones by many invertebrates and vertebrates, on land as well as underwater. These molecules are used as pheromones by aquatic animals such as squid, sea hares, and newts. Males in some terrestrial salamander species use high molecular weight glycopeptide pheromones transferred directly to the nostrils of the female. In *Drosophila* fruit flies, sex peptides in the male's seminal fluid change the female's behaviour so that after mating she rejects other males and starts to lay eggs. The rapid evolution of *Drosophila* sex peptides and salamander peptide pheromones suggests that sexual conflict may be involved. In mice, exocrine gland-secreting peptide 1 (ESP1) secreted from the male's tear glands is transferred to the female's nose through physical contact during investigation of the facial areas during courtship. ESP1 activates a narrowly specific vomeronasal receptor, leading to receptive behaviour in the female. The urine marks of male territorial house mice contain high concentrations of highly variable major urinary proteins (MUPs). The MUPs bind small molecule pheromones and other odorants, slowly releasing them and thus greatly prolonging the attractive volatile lifetime of the signal. One of the MUPs, darcin, is the same in all male house mice: it is a pheromone in itself. When the female comes into contact with darcin in a scent mark it prompts a learned attraction to both the male's individual chemical signature mixture and the location of the scent mark. Proteins and peptides may also contribute to the highly variable chemical profiles that differ between individual mammals, for example, and that are used as cues to allow individuals to be distinguished.

Pheromones are molecules that have evolved into chemical signals between organisms of the same species, in which they cause a specific reaction, such as a stereotyped behaviour or a developmental process, for example (Wyatt, 2010, after Karlson & Lüscher, 1959). Although the first pheromones to be identified were small volatile molecules such as bombykol, the female sex pheromone of the silk moth *Bombyx mori*, a wide variety of molecules have now been found to be used as pheromones (Wyatt, 2009, 2014). These include high molecular weight proteins and peptides, the subject of this short review.

Proteins and peptides are not volatile, but despite their relatively large size they can be highly soluble. Soluble proteins and peptide molecules are used as pheromones by aquatic organisms such as the mollusc *Aplysia* and aquatic amphibians. Terrestrial animals may also use these molecules as pheromones, but in some cases the molecules are transferred directly from the signaller to the receiver, for example by the male red-legged salamander, *Plethodon shermani*, to the nostrils of the female. These and other examples are discussed in more detail below.

Proteins and peptides may also contribute to the highly variable chemical profiles that differ between individual mammals, for example, and that are used as cues to allow individuals to be distinguished. These nonpheromone cues will be discussed in the concluding sections of this review.

PROTEINS AND PEPTIDES AS PHEROMONES

Peptides are linear molecules made of a series of amino acid subunits covalently bonded to each other (Berg, Tymoczko, & Stryer, 2002). The smallest peptides are dipeptides, consisting of two amino acids. The distinction between polypeptides and proteins on the basis of size is ambiguous and somewhat arbitrary: polypeptides are chains of up to about 50–70 amino acids; proteins are peptides with a larger number of amino acids. More important is that proteins have a stable three-dimensional (3D) structure (conformation). For the 3D structures of some typical proteins used as pheromones see, for example, Figure 7 in Yoshinaga et al. (2013).

Developments in proteomics, the characterization and identification of all the proteins in a biological sample (Wilkins et al., 1996), are likely to lead to the discovery of many more protein and peptide pheromones across a wide range of taxa. Beynon et al. (2013) and Drea et al. (2013) discuss techniques for proteomic analysis of

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mammalian scent marks and secretions; their observations may be relevant to studies of other taxa.

How Pheromones Evolve

Pheromone signals can evolve from any reliable chemical cue(s) to the sender's condition if this gives both the receiver and the sender a selective advantage (Bradbury & Vehrencamp, 2011, p. 377 ff; Wyatt, 2014). Like other pheromones, peptide and protein pheromones are usually co-opted from molecules with other functions. For example, male and female *Nereis succinea*, a marine polychaete worm, use a chemical duet to synchronize the release of their eggs and sperm into the surrounding sea water (Breithaupt & Hardege, 2012; Hardege, Bartels-Hardege, Müller, & Beckmann, 2004). The pheromones appear to have evolved from molecules that would have leaked from sexually mature adults or would have been released along with eggs or sperm stored in the body cavity. Attracted by low concentrations (10^{-10} M) of the female tetrapeptide pheromone (cysteinyl-glutathione), the male releases small quantities of sperm and his pheromone (inosine and glutamic acid), which prompts the female to release her eggs together with large quantities of cysteinyl-glutathione, which in turn prompts the male to release his sperm.

The contact pheromone protein on *Loligo pealeii* squid egg masses, which provokes intense male–male competition for females, is added by exocrine glands in the female's reproductive system (Cummins et al., 2011). The selective advantage for males may be that competitive fighting is initiated where females laying eggs are present, and for females the benefit may be access to the fittest males. Cummins et al. (2011) note that the protein is a distant member of the chordate β-microseminoprotein family found in mammalian reproductive secretions.

Another route to the evolution of pheromone signals is by sender exploitation of molecules to which the receiver is already sensitive (Bradbury & Vehrencamp, 2011, p. 377 ff; Wyatt, 2014). This may underlie the evolution of male sex peptides in the ejaculate of male *Drosophila* (Avila, Sirot, Laflamme, Rubinstein, & Wolfner, 2011). These sex peptides appear to target receptors in the female's uterus and oviduct that are involved in controlling reproduction (Häsemeyer, Yapici, Heberlein, & Dickson, 2009; Rezával et al., 2012; Yang et al., 2009). Among the effects of the sex peptides are that the female rejects other males and starts laying eggs instead. The arms race between male and female for control of her reproduction is discussed below.

Peptide Pheromones and Speciation

When populations of a species diverge into separate species, their pheromones usually change as part of reproductive isolation (Smadja & Butlin, 2009; Wyatt, 2014). Typically, animals that use a multicomponent pheromone with a combination of small molecules, such as moths, diverge by adding or losing molecules from the blend. Animals using peptide or protein pheromones can diverge by changing the sequence of amino acids in the pheromone. With peptide or protein pheromones, organisms can have unique sequences of amino acids as their species-specific pheromone. For example, the related species of Japanese newt *Cynops pyrrhogaster* and *C. ensicauda* have male decapeptide pheromones that differ by just two amino acids (Toyoda et al., 2004).

As populations undergo incipient speciation it is sometimes possible to see the divergence in pheromone signal. In one part of Japan, males of the local population of *C. pyrrhogaster* produce the decapeptide sex pheromone with one amino acid changed from the sequence used by the main population. Females of the main population do not respond to the new decapeptide variant (Nakada et al., 2007).

In another example of incipient speciation, two subspecies of the European house mouse, *Mus musculus*, *M. m. domesticus* and *M. m. musculus*, meet in a hybrid zone across central Europe (Karn & Laukaitis, 2014; Laukaitis & Karn, 2012). Female mice show a preference for partners of the same subspecies, and part of this choice is based on androgen-binding proteins (ABPs) secreted in the males' saliva. Strong positive mate choice has driven selection in the hybrid zone for a rapid accumulation of amino acid site differences in the salivary ABPs of the two subspecies. Increased production of these proteins has also been selected for.

Androgen-binding proteins are heterodimers (composed of a combination of two different polypeptides). Many peptide pheromones act as single molecules (Table 1). However, some peptide pheromones are effectively multicomponent, along the lines of moth multicomponent pheromones, consisting of a number of molecules working together to give the signal. For example, a combination of protein pheromones released by egg laying attracts the sea slug *Aplysia* to mating and egg-laying aggregations (Table 1; Cummins et al., 2007). *Aplysia* is a simultaneous hermaphrodite, which needs another individual to mate with. The four pheromones attractin, enticin, seducin and temptin are produced by the albumin gland, which packages the eggs. The proteins form multimeric combinations that are synergistic (attractin plus any of the other three molecules are more attractive than attractin alone). There are differences between the peptide pheromones used by different species of *Aplysia*, but there is some cross attraction between species' long-distance pheromones. As long as mating mistakes are not made at the time of copulation (which may rely on different cues from long-distance attraction) species specificity in the long-distance pheromone may not be selected for. The advantages of aggregation include reducing individual risk of predation (Wyatt, 2014).

MODES OF DELIVERY OF PHEROMONES

Proteins and peptides can be delivered in a variety of ways. These include contact pheromones exposed on the surface of the ‘signaller’, which act via contact with the chemosensory cells of the receiver. Alternatively, proteins and peptides may be secreted, then dissolved in an aquatic medium and carried in currents to the receiver. In a terrestrial environment, proteins and peptides may be deposited in scent marks, for example in urine or vaginal secretions, or, in some species, delivered directly to the receiver's sense organs. Finally, proteins and peptides may be allohormones, delivered directly to the receiver but bypassing the conventional chemosensory systems of the receiver.

Contact Pheromones

Peptides and proteins bound to the body surfaces can also be important in many types of animal as contact pheromones, detected only on contact or at very short range. The contact pheromone on squid egg masses, mentioned above, is one example (Cummins et al., 2011).

In the termite *Reticulitermes speratus*, workers recognize eggs by shape and size and by a surface contact ‘termite egg recognition pheromone’ (TERP) consisting of an antibacterial protein called lysozyme (Matsuura, Tamura, Kobayashi, Yashiro, & Tatsumi, 2007) and a digestive ß-glucosidase enzyme (Matsuura, Yashiro, Shimizu, Tatsumi, & Tamura, 2009). Although volatile molecules attract workers towards the eggs, it is egg morphology and nonvolatile egg recognition proteins that prompt the workers to pick up and groom eggs (Matsuura, 2012).