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Pheromone isoform composition differentially affects female behaviour in the red-legged salamander, *Plethodon shermani*

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Pheromones are a diverse class of biological molecules that play critical roles in mediating social and sexual behaviours. In many systems, pheromones exist in complex mixtures, with the precise composition and ratios of the different components essential for bioactivity. The interactive effects of complex pheromone mixtures, however, have been minimally studied in vertebrates. In the red-legged salamander, male salamanders use nonvolatile proteinaceous pheromones to modify female courtship behaviour and mating receptivity. One component of this pheromone mixture is a hypervariable 7 kDa protein, plethodontid modulating factor (PMF). Within a single population, individual male salamanders express more than 30 variants (isoforms) of PMF. While the complete pheromone secretion increases female mating receptivity, a subset of PMF isoforms was demonstrated to reduce receptivity. In the current study, we demonstrated that a single PMF isoform had no effect on female mating behaviour, while a more complete mixture of PMF variants recapitulated the effect of the whole pheromone mixture and increased female receptivity. From these data, we hypothesize (1) that female preference and sexual selection have promoted the rapid gene duplication of PMF over ~20 MY, resulting in the complex mixture we observe today and (2) that PMF isoforms act synergistically through complex neurophysiological pathways to modulate female courtship behaviour. These studies help define a framework for further investigations of the complex interactions and molecular mechanisms by which protein pheromones modulate female mating behaviour.

Communication in animals is, by and large, driven by multimodal signals that provide tremendous breadth and context towards individual decision making and evocation of specific behaviours (Hebets & Rundus, 2011). With auditory and visual cues, the timing, order and composition all influence the transmission of information and can alter a receiver's behaviour (Taylor & Ryan, 2013). Pheromones are an important class of conspecific signal that can regulate behaviour directly through well-defined neural circuits or indirectly through hormonal regulation (Dulac & Torello, 2003). For receivers, pheromones can provide information about the sender such as species, sex, reproductive status and/or disease state (Albone, 1984; Johnston, 2000; Melrose, Reed, & Patterson, 1971). In line with the original definition of 'pheromones' coined by Karlson and Lüscher (1959), a major emphasis has been placed on identifying specific chemicals (i.e. single-component signals) that elicit well-defined responses. However, as with other types of signals, most if not all pheromone molecules are delivered as complex mixtures, and their bioactivity has often been tied to both the presence and relative proportions of different components (Legrand, Botton, Coracini, Witzgall, & Unelius, 2004; LeMaster & Mason, 2002). Synergy between components of pheromone mixtures and their effects on behaviour have been well characterized in many invertebrate systems. In social insects, complex mixtures of 'primer pheromones' are delivered to maintain social harmony through regulation of physiology and, indirectly, behaviour. Different species of ants and wasps vary the branching patterns in different cutaneous hydrocarbons to identify task assignment (Conte & Hefetz, 2008). Nemotode ascarosides are produced through species-specific biosynthetic pathways that yield unique mixtures that aid in mate finding, aggregation and developmental

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diapause (Choe et al., 2012). Emerging evidence in some vertebrate pheromones suggests a similar dependence on synergy. In common carp, elevated prostaglandin $F_{2\alpha}$ in plasma stimulates the release of itself in addition to other currently unknown metabolites that together attract males more effectively than exogenous prostaglandin $F_{2\alpha}$ alone (Lim & Sorensen, 2012). Multicomponent pheromones may be common because of the modular nature of glomeruli in the brain, where all of the appropriate neurons are required to fire simultaneously in order to activate a particular circuit and elicit a specific behaviour or endocrine response (Wyatt, 2014).

Pheromone molecules are not restricted to purely volatile compounds, and many vertebrates rely on water-soluble proteins to provide information and elicit specific behavioural responses (Wyatt, 2010). For example, male firebelly newts secrete a peptide pheromone (termed sodefrin) that can attract gravid females (Kikuyama et al., 1995); in male mice, proteins are released as part of tear composition that increase female receptivity (Haga et al., 2010); and also in mice, the many isoforms of the major urinary protein (MUP) family perform several social functions, including promotion of male aggression towards other males, regulation of female receptivity and learning of individual scent profiles (Chamero et al., 2007; Mudge et al., 2008; Roberts, Davidson, McLean, Beynon, & Hurst, 2012). Compared to their volatile counterparts, mixtures of protein pheromones seem particularly well suited for the study of interactive effects for three reasons. First, protein pheromone genes often comprise multigene families that are products of exacerbated gene duplication and positive selection, allowing examination of their evolutionary histories by molecular phylogenetics (Mudge et al., 2008; Watts et al., 2004). Second, as direct gene products, their synthesis and expression levels are generally regulated through well-characterized molecular processes (transcription and translation), permitting correlation between changes in pheromone levels and composition by gene expression analyses. Third, recombinant pheromones can be prepared using heterologous expression systems to control the exact composition of experimental mixtures (Houck et al., 2008; Roberts et al., 2010). Even with the many advantages of protein pheromones, relatively few studies have examined protein interactions in complex mixtures. This dearth of studies is surprising because the prevailing opinion is that interactions are a central property of many pheromone communication systems (de Bruyne & Baker, 2008; Cardé & Haynes, 2004; Lassance & Lofstedt, 2009; Novotny, Harvey, Jemiolo, & Alberts, 1985; Sorensen & Stacey, 1999).

For more than 100 million years, mixtures of non-volatile proteinaceous courtship pheromones have regulated female behaviour and mating receptivity in plethodontid salamanders (Houck, Bell, Reagan-Wallin, & Feldhoff, 1998). Because amphibians are basal tetrapods, salamanders provide an excellent model system for studying the evolution of pheromone signalling. Courtship pheromones, unlike many chemoattractants, are a special type of sex pheromone that are privately delivered during courtship and influence associated behaviours (Houck & Arnold, 2003). The annual mating season for many plethodontid salamanders occurs during a few months in late summer or early autumn. Before this mating season, plasma androgen levels are elevated in adult male salamanders. The increased androgen induces the development of a specialized chin gland (termed a 'mental' gland) (Sever, 1976; Woodley, 1994). Based on molecular, proteomic, and behavioural studies, the only currently defined function of the mental gland is the production of courtship pheromones (Feldhoff, Rollmann, & Houck, 1999; Rollmann, Houck, & Feldhoff, 1999; Wilburn et al., 2012). In a successful courtship between a male and female, the female typically straddles the male's undulating tail and the pair walks forward in unison. This behaviour was described as a tail-straddling walk (TSW) by Arnold (1976). At the end of this walk, the male deposits a spermatophore, the female walks forward until her cloaca (between her hindlegs) is positioned above it, and then she presses down to collect the apical sperm mass on the spermatophore (Arnold, 1976). In our principal model, the red-legged salamander, *Plethodon shermani*, the male periodically turns and delivers courtship pheromones to the female by 'slapping' his mental gland against the female's nares. These pheromones then travel along the female's nasolabial grooves, which deliver the aqueous pheromone to neural receptors in the vomeronasal organ (VNO). Ultimately, pheromone stimulation results in activation of specific brain regions that modify female courtship behaviour (Laberge, Feldhoff, Feldhoff, & Houck, 2008; Rollmann et al., 1999; Wirsig-Wiechmann, Houck, Feldhoff, & Feldhoff, 2002). In laboratory trials, the pheromone extract reduces the time females spend in TSW. We interpreted the decrease in time in tail-straddling walk as an increase in female mating receptivity (Houck et al., 1998).

Chemical analysis of the *P. shermani* pheromone extract revealed that more than 85% of the mixture was composed of two major proteins (Feldhoff et al., 1999). The first identified component was a 22-kDa protein termed plethodontid receptivity factor (PRF), which is related to IL-6 cytokines. Similar to the complete pheromone extract, purified PRF also increased female receptivity (Rollmann et al., 1999). Through continued biochemical analysis, three sequence variants (isoforms) of PRF were identified. These variants were termed B, C1 and C2. This nomenclature was based on the relative charge and elution conditions when the pheromones were analysed by high-performance liquid chromatography (HPLC) (see Fig. 1, Table 1). All three PRF isoforms shared a sequence identity greater than 95%. Individual analysis of pheromone extracts from more than 100 male *P. shermani* revealed significant variability in the relative ratios of these isoforms, with ~20% of animals expressing only two of the three isoforms. Notably, all current evidence suggested that these isoforms were the products of gene duplication, and not allelic variation or alternative splicing (Chouinard, Wilburn, Houck, & Feldhoff, 2013). Courtship trials testing the efficacy of a recombinant PRF-C2 elicited the same response as the complete mixture (increased female receptivity), suggesting some redundancy in function between the isoforms (Houck et al., 2008).

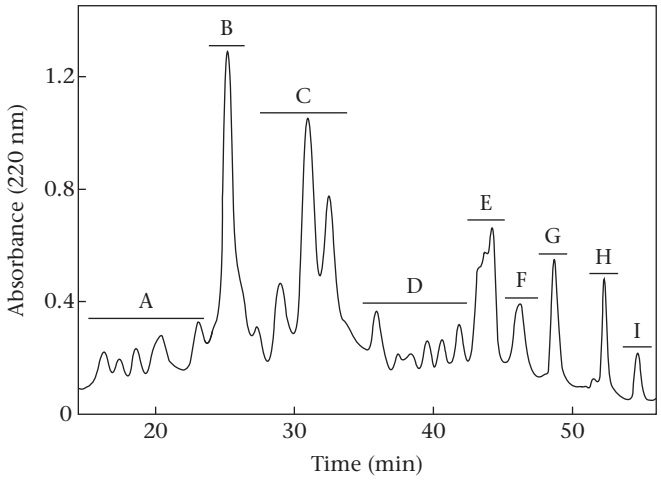

Figure 1. Separation of *P. shermani* pheromones by anion exchange high-performance liquid chromatography (HPLC). Negatively charged pheromones are separated by an increasing salt gradient, and fractions are labelled based on relative negative charge. Plethodontid receptivity factor (PRF) primarily comprises the B and C fractions, while plethodontid modulating factor (PMF) is primarily found in E, F, G, H and I. Adapted from Wilburn et al. (2012).