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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).

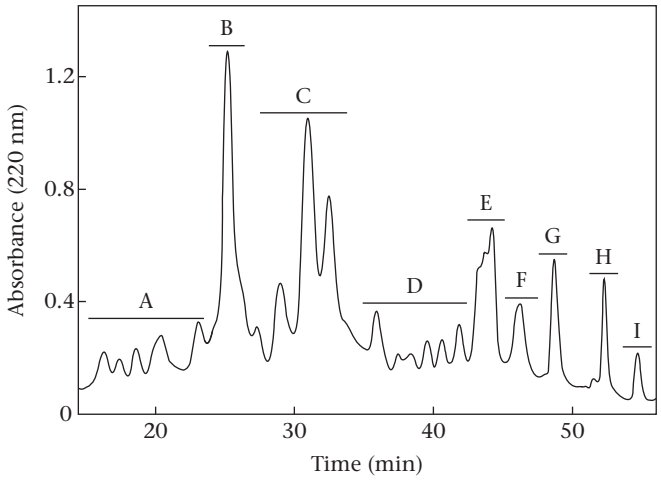

Figure 1. Separation of *P. shermani* pheromones by anion exchange high-performance liquid chromatography (HPLC). Negatively charged pheromones are separated by an increasing salt gradient, and fractions are labelled based on relative negative charge. Plethodontid receptivity factor (PRF) primarily comprises 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).

Table 1
Summary of pheromone isoforms by high-performance liquid chromatography (HPLC) fraction

HPLC fraction	Pheromone	No. of isoforms	Effect on female receptivity
A	*	*	*
B	PRF	1	+18% (Rollmann et al., 1999); +27% (Houck et al., 2008)
C	PRF	2	+18% (Rollmann et al., 1999); +27% (Houck et al., 2008)
D	*	*	*
E	PMF	~20–25	-23% (Houck et al., 2007)
F	PMF	~10–15	-23% (Houck et al., 2007)
G[1]	PMF	1–3	*
H[1]	PMF	1–3	*
I[1]	PMF	1–3	*

PRF: plethodontid receptivity factor; PMF: plethodontid modulating factor.
*Indicates uncharacterized composition and/or no behavioural tests prior to this study.
[1] Fractions are >90% for a single isoform, with one or two minor isoforms.

The second pheromone component identified from male mental glands was the 7-kDa protein, plethodontid modulating factor (PMF). PMF is related to the three-finger protein (TFP) superfamily that includes many snake venom neuro- and cytotoxins (Palmer et al., 2007). Proteomic and molecular analyses for *P. shermani* revealed that, when compared to the three highly conserved PRF isoforms, individual males expressed more than 30 unique PMF isoforms that only shared ~30% average amino acid identity. PMF was observed in five major HPLC fractions (termed E, F, G, H and I; Fig. 1); further analysis revealed that nearly all of the PMF isoforms were found in the E and F fractions (≥30 isoforms total; Table 1), while the G, H and I fractions were highly enriched for individual isoforms (each >90% purity). Consequently, these three isoforms (G, H and I) were the most abundant PMFs and comprised ~25% of the total PMF mixture (Wilburn et al., 2012). In an earlier study (Houck et al., 2007), a mixture of PMF-EF was tested in staged courtship trials. In contrast to both whole pheromone and PRF, PMF-EF actually decreased female receptivity (based on an increase in TSW time). It was hypothesized that, because of its homology with snake venom neurotoxins, PMF may be acting to relax the female, thereby facilitating completion of the courtship.

However, as already noted for many insect systems, both the presence and ratios of particular pheromone components are often critical for proper biological activity. As an incomplete mixture of PMFs was used in the previous assay, further study on the impact of these highly abundant isoforms was warranted. Using a yeast expression system, a recombinant PMF-G (rPMF-G) was prepared that was biochemically identical to the natural pheromone (Wilburn et al., 2014). Importantly, PMF-G was the most abundant PMF isoform in nearly all male *P. shermani* (Chouinard et al., 2013; Wilburn et al., 2012). While a single PRF isoform was sufficient to stimulate female salamanders (Houck et al., 2008), it was unknown whether the same phenomenon would occur with PMF, which has more than10× the isoform diversity. To assess the potential interactive and synergistic effects that may underlie the PMF complex, we determined the effects of rPMF-G and PMF-EFGHI on female mating receptivity in *P. shermani*.

METHODS

Plethodon shermani *Courtship Behaviour*

The courtship ritual of *P. shermani* involves many characterized behaviours and occurs in five well-stereotyped stages (Arnold, 1976). (1) Orientation: the male faces towards the female and follows her. (2) Persuasion: the male presents visual (and likely chemical) cues to attempt to persuade the female to court. The performance of one persuasion behaviour (foot dancing) is positively correlated with advancement to the next phase (Eddy, Kiemnec-Tyburczy, Uyeda, & Houck, 2012). (3) Tail-straddling walk: the male and female walk in unison while the female straddles the male's tail, maintaining her snout near the base of his tail. During this lengthy phase, the male periodically turns and 'slaps' the female's snout with his mental gland to apply courtship pheromones. Stimulation from these pheromones decreases the length of TSW (Houck et al., 1998). (4) Deposition: the male periodically pauses during TSW and, if the female does not try to advance further (possibly implying ample female receptivity), he presses his body to the ground and deposits a spermatophore while continuing to undulate his tail. The deposition process consistently requires about 7 min. The spermatophore is composed of a small sperm cap that sits atop a gelatinous base. (5) Insemination: the male lifts his vent off of the deposited spermatophore and walks forward. The female typically follows until her vent is positioned above the spermatophore. She then lowers her vent, removes the sperm cap (leaving the gelatinous base behind) and departs from the male, thus completing courtship.

Animal Collection, Maintenance and Pre-screening

Plethodon shermani salamanders were collected during their August breeding season from a single site in Macon Co., North Carolina, U.S.A. (35°10′48″N, 83°33′38″W), and sexed based on the presence of a mental gland in males or large ova visible through the ventral body wall in females. Initially, salamanders were maintained at Highlands Biological Station. All animals were individually housed in clean plastic boxes (17 × 9 × 13 cm) each lined with a damp paper towel and supplied with a second damp crumpled paper towel for a refuge. Once per week, animals were transferred to clean boxes with fresh substrate and fed two waxworms (*Galleria mellonella*). The temperature and humidity were maintained at 15–18 °C and ~70%, respectively. Some *P. shermani* do not mate under laboratory conditions. For larger observational studies, animals must be pre-screened to determine mating propensity (Houck et al., 2007). Briefly, male and female salamanders were randomly paired in clean plastic boxes with a damp paper towel substrate (no refuge) and left together overnight. The following morning, we determined whether the pair mated by (1) examining the box for the presence of the spermatophore gelatinous base and (2) checking the female's cloaca for a visible sperm cap. Following successful insemination, females were removed from pre-screening for 1 week to allow the sperm cap to dissolve before being presented with another mating opportunity. From the pre-screening data, 160 males and 160 females were selected for observed courtship trials and shipped to Oregon State University. Animals were then housed at the same temperature and humidity, and maintained on a North Carolina photoperiod.

Gland Removal and Pheromone Preparation

To prevent interference from endogenous male pheromones, all male salamanders had their mental glands surgically removed, based on the methods of Rollmann et al. (1999). Briefly, males were anaesthetized in a mixture of 7% ether in water and the exterior pad-like mental gland was removed from the dermis. Following surgery, each male was placed in a clean box and the chin rested on a small piece of gauze containing an antibiotic ointment. Males were allowed 2 weeks to heal before being used in any behavioural experiments. The mental gland is found immediately under the two to three cell layers of the epidermis and its removal is minimally

invasive. While infections from gland removal are generally rare (>99% survival rate over ~20 years; L. D. Houck & R. C. Feldhoff, personal observations), a few animals did present with an infection such that they were immediately removed from the experiment and treated as recommended by an Oregon State University veterinarian. These animals were not used in any additional courtship observations. Gland removal surgery had no effect on male courting behaviour, including slapping during TSW (Eddy et al., 2012; Houck et al., 1998, 2007, 2008; Kiemnec-Tyburczy, Woodley, Feldhoff, Feldhoff, & Houck, 2011; Rollmann et al., 1999; Rollmann, Houck, & Feldhoff, 2003). Pheromones were extracted from the excised mental glands following the methods of Rollmann et al. (1999). Briefly, mental glands were induced to secrete pheromones by incubation with 0.8 mM acetylcholine chloride in Amphibian Ringer's Solution for ~60 min. Whole pheromone extract was centrifuged at 10 000 × *g* for 10 min, the supernatant was collected and the centrifugation was repeated before storage of supernatant at −80 °C. Methods and animal care were approved by Oregon State University's Institutional Animal Care and Use Committee (ACUP 3007 and 4053 to L. D. Houck). Based on the methods of Wilburn et al. (2012), a natural mixture of PMFs containing fractions E through I (PMF-EFGHI) was purified from whole pheromone extract using strong anion exchange HPLC. As described by Wilburn et al. (2014), rPMF-G was expressed using the methylotrophic yeast *Pichia pastoris*. As a eukaryote, *P. pastoris* can perform many post-translational modifications, including production of disulphide bonds, which do not naturally occur in *Escherichia coli* expression systems. All biochemical assays (reverse phase HPLC, tandem mass spectrometry, disulphide bond analysis, far-UV circular dichroism, multidimensional NMR) demonstrated that rPMF-G has a three-dimensional structure identical to that of natural PMF-G. Both PMF-EFGHI and rPMF-G were prepared at 0.5 μg/μl in 0.5× phosphate buffered saline (PBS), the same concentration used for the PMF-EF behavioural trials in Houck et al. (2007).

Staged Courtship Trials

Effects of courtship pheromones on female receptivity were assayed by recording the courtship behaviour of females treated with exogenous application of one of the two pheromone solutions (PMF-EFGHI, rPMF-G), or a vehicle control (0.5× PBS). Methods were adapted from those used by Houck et al. (2007). Because of the limited number of successful courtship encounters in the laboratory, previous experiments could reliably test only two treatments. To maximize statistical power and test three different solutions, we used a mixed effects design. On each observation night, male and female salamanders were paired as described during pre-screening trials. Salamanders were equally divided into four groups of 40 pairs, with each group observed one night per week for 5 weeks. During the first week of observations, within each group, male and female salamanders were randomly paired, and allowed 3 h to initiate TSW. Once a pair entered TSW, if the male attempted to apply pheromone by slapping the female with his chin, we applied 5 μl of a random treatment to the female's nares by micropipette. Subsequently, the female would receive additional 5 μl aliquots of the same treatment after 5 min and 10 min following the initial slap (15 μl total). To partially control for the physical stimulus of slapping, we only considered the data point valid if the male slapped the female at least three times (corresponding to the three pheromone applications). Time was recorded from the initiation of TSW to spermatophore deposition. Occasionally, application of the pheromone would 'startle' the female salamander, causing her to disengage momentarily from TSW, but she subsequently resumed courtship with little-to-no persuasion from the male. Courtship time is defined as time in TSW (excluding interruptions), and total time is defined as time in TSW plus any TSW interruption times. For a given courtship encounter to be included in the analysis, (1) the pair had to have entered TSW and proceeded to spermatophore deposition, (2) the male had to have slapped the female's snout at least three times, (3) the female had to have received all three 5 μl aliquots of treatment and (4) the total TSW interruption time had to be less than 40 min. If a pair successfully completed a courtship encounter meeting these criteria, that male and female remained paired for all subsequent weekly observations. If that pair entered courtship a second time, the female would randomly receive one of the two remaining treatment groups, providing a partial repeated measures design. No pairs engaged in courtship three times or received all three treatments. For salamanders that had yet to mate successfully, the pairs were randomized each week until (1) they successfully mated and were paired for the remainder of the experiment or (2) the experiment was terminated. Pheromone treatments were coded prior to the start of the experiment such that all observers were blind to the treatment during observations. The purpose of these criteria was to maintain consistency with previous studies, to best control for properly observing effects of pheromone treatment (i.e. ensuring females received sufficient dosage, physical stimulus of slapping corresponded to minimum number of pheromone applications) and to maximize statistical power. While no pair received all three treatments, 10 out of 32 pairs received two separate treatments.

Data were analysed using linear mixed-effects models with the R package 'nlme' with parameter estimation by maximum likelihood. Both courtship time and total time were analysed with treatment (0.5× PBS, rPMF-G, PMF-EFGHI) as a fixed effect and salamander pair as a random effect. The significance of treatment was tested by likelihood ratio test against an intercept-only null model, with individual effects of the three solutions examined post hoc by *t* test with corrected standard errors.

RESULTS

A total of 32 salamander pairs engaged in courtship successfully and met the aforementioned criteria. Ten of these pairs received two of the three possible treatments (42 total courtship encounters). The distribution of the data for the three treatments was approximately equal ($N = 15$ for 0.5× PBS, $N = 15$ for rPMF-G, $N = 12$ for PMF-EFGHI). The data for both courtship time (TSW without interruption time) and total time (TSW with interruption time) were fitted by maximum likelihood to mixed effect models with and without treatment as a fixed effect. The effect of PMF-EFGHI over 0.5× PBS was not significant at $P < 0.05$ ($P = 0.1027$). However, courtship behaviour in *P. shermani* is highly variable, and examination of the data by box plot revealed three outliers outside the mean ± 1.5 × interquartile range (Fig. 2a). Outliers were removed in subsequent analyses. In support of this decision, we note that, minus the outliers, all courtship times for pairs treated with PMF-EFGHI were less than the median time with 0.5× PBS. With the outliers removed, treatment was significant in both cases (likelihood ratio test: courtship time: $P = 0.0394$; total time: $P = 0.0469$). Because courtship time is the measure used in previous behavioural studies (Houck et al., 1998, 2007, 2008; Rollmann et al., 1999, 2003), this is the more relevant measure of comparison and the primary focus of our analysis.

For the 10 pairs that received two treatments, there was a significant effect from the inclusion of the random effect of pair ($P = 0.026$). The mean courtship time for the negative control of 0.5× PBS was 55.9 min (range 28–87 min). Post hoc *t* tests between comparing rPMF-G, with mean 56.0 min (range 23–107 min), to 0.5× PBS revealed no significant difference ($P = 0.9930$). In contrast, PMF-EFGHI significantly decreased courtship time to a

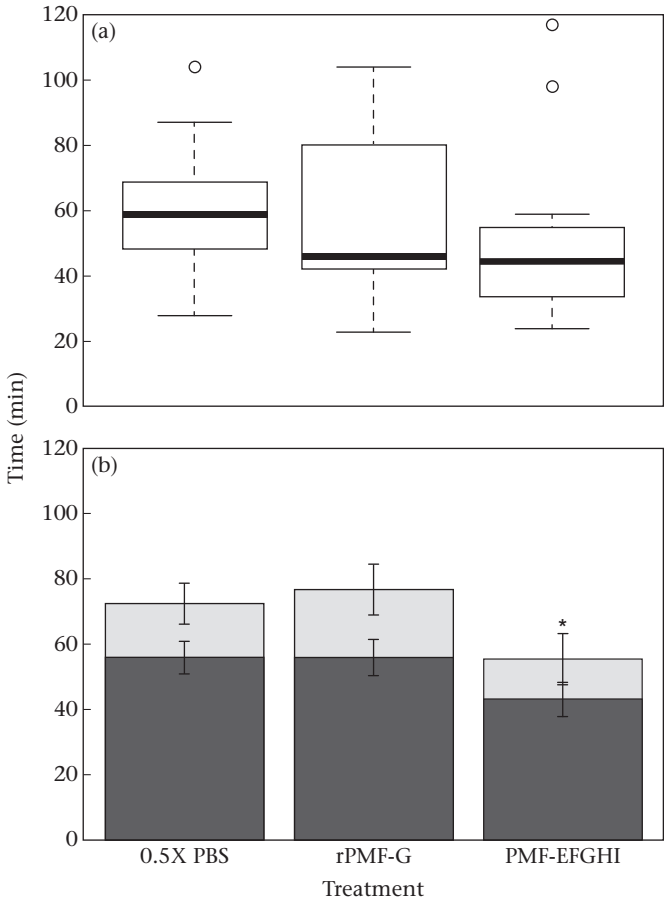

Figure 2. Effect of plethodontid modulating factor (PMF) components on female receptivity. (a) Box-and-whisker plot of courtship time for each treatment group (total $N = 42$), with whiskers defining the range of the data, and the box denoting the middle 50% (25th percentile, Q_1, to the 75th percentile, Q_3). Outliers are defined as values outside $Q_1 - 1.5 \times (Q_3 - Q_1)$ or $Q_3 + 1.5 \times (Q_3 - Q_1)$. (b) Maximum likelihood means of courtship time (dark grey) and total time (light grey) ± SE for each treatment group excluding outliers (total $N = 39$). $^*P < 0.05$.

mean of 43.1 min (range 24–59 min), implying an increase in female receptivity by ~23% ($P = 0.0425$, Fig. 2b). As with previous studies, there was no significant difference in initiation frequency, number of interruptions, interruption time or insemination success between the three treatment groups (data not shown).

DISCUSSION

The aim of this study was to compare the effects of different PMF isoform mixtures on female behaviour in *P. shermani*. We now report that the most abundant PMF isoform (PMF-G) had no effect on female mating behaviour, but a more complete mixture of PMF isoforms (PMF-EFGHI) increased female receptivity, with an effect similar to that of whole pheromone extract and PRF. Both of these effects are in contrast to the previous study (Houck et al., 2007), which demonstrated that PMF-EF decreased female receptivity. It should be reiterated that while PMF-E and PMF-F together represent a highly complex mixture with more than 30 isoforms, the G, H and I peaks are highly enriched for the three most abundant PMF isoforms. Therefore, the only difference between PMF-EF and PMF-EFGHI are these three major PMFs, with PMF-G in isolation having no measurable effect.

We propose two competing hypotheses to explain how PMF-EFGHI increased female receptivity: (1) additive effects from PMF-HI and (2) synergistic effects in the PMF isoform complex. For the first time, the effects of isoforms PMF-G, PMF-H and PMF-I on female mating receptivity were evaluated, using the two mixtures PMF-EFGHI and rPMF-G. With regard to hypothesis 1, since the mean courtship time for rPMF-G and 0.5× PBS were nearly identical (~0.1 min difference), it is possible that isoforms H and I (either independently or in combination) caused a large increase in female receptivity, overcoming the negative effects of PMF-EF. According to hypothesis 2, the PMF isoforms functionally complement one another such that PMF-EF interacts with isoforms G, H and I in a manner that increases female receptivity. The first hypothesis seems unlikely: in an additive effect model, with PMF-EF producing −23%, PMF-EFGHI producing +23% and PMF-G having no effect, PMF-HI would generate +46%, nearly twice what PRF or whole pheromone elicited. Also, in this model, it seems surprising that PMF-G, as the most abundant PMF isoform (Wilburn et al., 2012), would be functionally inert with regard to female receptivity. The other hypothesis is that PMF isoforms act synergistically to enhance female receptivity. In this scenario, multiple isoforms of PMF are required to functionally complement one another, explaining why rPMF-G had no effect on female receptivity. At the same time, a mixture excluding the three most abundant isoforms may have a negative effect on female receptivity (i.e. females may perceive this as an abnormal/atypical signal and be 'dissuaded' from courting with that particular male). Other pheromone systems, as well as the study of communication at large, provide evidence for this second hypothesis. In ermine moths, females synthesize hydrocarbon chains modified with acetate or alcohol groups that attract potential mates; strong synergistic effects were observed when the alcohol and acetate versions were tested in combination (Löfstedt, Herrebout, & Menken, 1991). Similar effects were observed in leafrollers (*Argyrotaenia sphaleropa*) where alcohol- and acetate-diene pheromones have synergistic interactions, which were further enhanced by specific ratios between the two versions (Legrand et al., 2004). In túngara frogs, the whine-chuck system of auditory communication has been a well-documented example of multimodal components functioning better in combination than individually (Ryan & Rand, 2003). Recently, this signal was demonstrated to be further modulated by both the presence and sequential order of a visual cue, vocal sac inflation (Taylor & Ryan, 2013). Consequently, we hypothesize that PMF isoforms function synergistically to enhance female receptivity.

While synergism may be a required component of the PMF complex and may partially explain the many PMF gene duplications, the same is not clear for the other *P. shermani* pheromone, PRF. In a previous study (Houck et al., 2008), a single PRF isoform was sufficient to increase female receptivity similar to the complete mixture of all three PRF isoforms (Rollmann et al., 1999). While both PRF and PMF have experienced rapid evolution across the plethodontid phylogeny (Palmer, Picard, Watts, Houck, & Arnold, 2010; Watts et al., 2004), the three PRF isoforms in *P. shermani* are much more highly conserved (>95% amino acid identity) compared to the more than 30 PMF isoforms (~30% average amino acid identity). In addition, the single PRF isoform used in the Houck et al. (2008) study is the most abundant isoform and expressed by nearly every male in the population (Chouinard et al., 2013). While there is variation in the ratios of the three PRF isoforms between individual males, their sequence similarity may permit functional complementarity such that, at sufficient protein concentrations, any one of the PRF isoforms may be able to sufficiently act in place of the others. This is likely less plausible for PMF, given its tremendous diversity, such that the two pheromones have evolved under different selection constraints. Consequently, the isoform composition and ratios for PRF and PMF may independently be allowing

females to infer and assess different qualities about the males, and would be consistent with the redundant signals hypothesis (Zuk, Ligon, & Thornhill, 1992).

Whether these effects are additive or synergistic, the mechanism by which the interactive effects may be mediated remains unknown. All data to date suggest that PMF regulates female behaviour by binding to vomeronasal type-2 receptors (V2Rs) in the female VNO: (1) PMF-EF activated neurons in the female VNO (Wirsig-Wiechmann, Houck, Wood, Feldhoff, & Feldhoff, 2006); (2) in rodents, V2Rs are primarily responsible for binding peptide or protein pheromones (Herrada & Dulac, 1997; Isogai et al., 2011; Yoshinaga et al., 2013); and (3) V2Rs are highly expressed in the *P. shermani* VNO (Kiemnec-Tyburczy, Woodley, Watts, Arnold, & Houck, 2012). It has been hypothesized that VNO receptors (like olfactory receptors) display monoallelic expression such that each neuron in the VNO expresses only a single receptor, limiting it to recognizing only a small number of potential pheromone ligands (Dulac & Axel, 1995; Dulac & Torello, 2003; Herrada & Dulac, 1997). Vomeronasal receptors are generally highly sensitive, often requiring sub-picomolar concentrations. However, in mice, there is evidence that individual VNO neurons express multiple V2R receptors, and even neurons with the same receptors respond to different pheromone molecules (Leinders-Zufall, Ishii, Mombaerts, Zufall, & Boehm, 2009). In *P. shermani*, PMF-responsive VNO neurons may be expressing different receptors that each bind different PMF isoforms, and stimulation of multiple receptors by these different isoforms is necessary for proper signalling. Alternatively, individual VNO neurons may be independently activated by different PMF isoforms and the proposed interactive effects are mediated by downstream processing in the central nervous system. Recently, we determined the three-dimensional structure of PMF-G by multidimensional NMR, and homology modelling suggested that most of the PMF sequence hypervariability was manifested in one loop of the protein structure. Consequently, we hypothesized that this loop is critical for receptor interactions (Wilburn et al., 2014). While PMF is monomeric in solution, it is also plausible that activation of female receptors may require forming a complex with multiple PMF isoforms. None of these hypotheses are mutually exclusive, but all align with the general model that PMF probably influences reproductive success by binding to vomeronasal receptors (Wirsig-Wiechmann et al., 2006), activating regions of the brain involved in reproductive behaviour (Laberge et al., 2008) and altering the timing of courtship (Houck et al., 2007). Over the last 100 million years, the PMF complex has undergone extensive gene duplication and pervasive positive selection (Palmer et al., 2010). With likely synergism between the many isoforms being required to increase receptivity, we propose sexual selection from female receptors as the cause of this rapid evolution. Future next-generation sequencing studies identifying potential pheromone receptors in *P. shermani* should enhance our understanding of the mechanism(s) by which PMF activates VNO neurons and modulates female behaviour.

In conclusion, we have demonstrated that the hypervariable vertebrate pheromone plethodontid modulating factor differentially regulated female mating behaviour based on isoform composition. In contrast to PRF (Houck et al., 2008), a single isoform of PMF was not sufficient to affect female receptivity, while different combinations of more complex mixtures may have opposing effects based on the presence or absence of the three most abundant isoforms. We hypothesize that synergistic effects have promoted the large PMF isoform diversity present in *P. shermani* and that female salamanders may evaluate isoform complexity through V2R receptors in their VNO. The requirement of a complete PMF mixture to increase female receptivity provides a functional basis for the perpetuation of extensive PMF gene duplication in *Plethodon* salamanders for the past ~100 million years (Palmer et al., 2010). Future studies will investigate the mechanistic basis by which female *P. shermani* react to differences in PMF isoform composition.

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