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Co-option and evolution of non-olfactory proteinaceous pheromones in a terrestrial lungless salamander

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ABSTRACT

Gene co-option is a major force in the evolution of novel biological functions. In plethodontid salamanders, males deliver proteinaceous courtship pheromones to the female olfactory system or transdermally to the bloodstream. Molecular studies identified three families of highly duplicated, rapidly evolving pheromones (PRF, PMF, and SPF). Analyses for *Plethodon* salamanders revealed pheromone mixtures of primarily PRF and PMF. The current study demonstrates that in *Desmognathus ocoee* – a plesiomorphic species with transdermal delivery – SPF is the major pheromone component representing >30% of total protein. Chromatographic profiles of *D. ocoee* pheromones were consistent from May through October. LC/MS-MS analysis suggested uniform SPF isoform expression between individual male *D. ocoee*. A gene ancestry for SPF with the Three-Finger Protein superfamily was supported by intron-exon boundaries, but not by the disulfide bonding pattern. Further analysis of the pheromone mixture revealed paralogs to peptide hormones that contained mutations in receptor binding regions, such that these novel molecules may alter female physiology by acting as hormone agonists/antagonists. Cumulatively, gene co-option, duplication, and neofunctionalization have permitted recruitment of additional gene families for pheromone activity. Such independent co-option events may be playing a key role in salamander speciation by altering male traits that influence reproductive success.

1. Introduction

Novel phenotypes are rarely thought to evolve through *de novo* synthesis, but more commonly through the co-option of extant structures that can be repurposed for new functions [1,2]. At the genomic level, gene co-option can occur through multiple mechanisms, with the most common route being gene duplication followed by mutation accumulation that can drive neofunctionalization [3,4]. Genes that are co-opted for new functions typically possess characteristics that already make them well-suited for their new roles: for example, α-crystallins in the lens of the eye form transparent arrays that refract light and focus it on the retina. These liquid crystal arrays require that the proteins remain stable at very high concentrations, a quality of the heat shock protein family from which α-crystallins were co-opted [5,6]. While innovation and co-option through natural selection have been widely studied in many systems [7–10], it has been minimally examined in the context of sexual selection.

For more than 100 million years, plethodontid salamanders have utilized a system of non-volatile proteinaceous courtship pheromones that regulate female mating receptivity and behavior [11,12]. Part of the courtship ritual includes a highly specialized behavior (tail straddling walk, TSW) that coordinates and facilitates sperm transfer from males to females. Preceding the annual courtship season, plasma androgens rise in male salamanders and induce development of a submandibular mental gland dedicated to the synthesis of non-volatile proteinaceous courtship pheromones that reduce the length of TSW [12]. In the red-legged salamander (*Plethodon shermani*) and other closely related species, a male salamander delivers pheromones by "slapping" the mental gland to the female's snout, where pheromones diffuse into the female olfactory chamber, bind to receptors on vomeronasal neurons, and activate regions of the brain involved in mating behavior [13,14]. This olfactory form of pheromone perception closely resembles that of most other animals [15]; however, only 27 out of ~355 plethodontid species utilize this form of delivery. The majority of plethodontid species (~300 spp.) apply pheromones transdermally by "scratching" a female's dorsum using enlarged premaxillary teeth [16]. Subsequent rubbing of the mental gland over the abraded site presumably allows pheromones to diffuse into the bloodstream [17]. These pheromones do not activate neurons in the vomeronasal organ [18], and must influence female physiology and behavior through alternative, but currently unknown, pathways.

Three major plethodontid pheromones have been biochemically purified and assayed for behavioral effects: Plethodontid Receptivity

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Factor (PRF) and Plethodontid Modulating Factor (PMF) in *P. shermani*, and Sodefrin Precursor-like Protein (SPF) in *Desmognathes ocoee*. All three proteins persist within the mental gland as multi-isoform blends, and when delivered as highly complex mixtures, decrease the duration of TSW. As male salamanders can mate several times, reduced TSW time may increase the number of courtship encounters which result in successful sperm transfer. Behavioral studies indicated that the application of fewer isoforms of each pheromone can have different effects on female behavior [11,19–21]. All three pheromone families have unique evolutionary origins and have been co-opted from different gene families: PRF is a 22 kDa protein related to IL-6 cytokines, PMF is a 7 kDa protein related to the highly diverse three-finger protein (TFP) superfamily, and SPF is a 20 kDa protein related to γ-type phospholipase A2 inhibitors [11,22,23]. Although the three are considered discrete gene families, it has been hypothesized that the γ-type phospholipase A2 inhibitors may have arisen from the TFP superfamily [24]. Phylogenetic studies using RT-PCR in 28 plethodontid species revealed that PMF and SPF are relatively ancient pheromones (>100 million years old), while PRF is a more recent adaptation found only in the genus *Plethodon* (where some species employ olfactory delivery). Using these cDNA sequences, analysis of the d_N/d_S ratios by PAML revealed all three families have undergone rapid evolution, likely due to sexual selection [23,25,26]. It is noteworthy that in newts (family Salamandridae), a homolog of SPF is cleaved to liberate a decapeptide (sodefrin) that is released by male aquatic newts to attract females [27], suggesting that the SPF family may have been co-opted multiple times within amphibians for pheromone activity.

Comparison of pheromone composition across different species and modes of delivery suggest that numerous pheromone gene co-option events have occurred across the plethodontid phylogeny. Recent proteomic analyses in the redback salamander (*Plethodon cinereus*) revealed that, despite using the transdermal mode of delivery, its pheromone composition was principally composed of PRF and PMF, similar to that of *P. shermani* (which uses slapping delivery) [28]. In a cDNA library screen comparing *P. shermani* with two distantly related scratching species (*D. ocoee*, and *Eurycea guttolineata*), SPF was suggested to be the major pheromone component. Additionally, three putative pheromones were identified with sequence similarity to peptide hormones (glucagon, natriuretic peptide, and relaxin). There were fewer PMF isoforms in these species and no expression of PRF. Therefore, although *P. cinereus*, *D. ocoee*, and *E. guttolineata* all use transdermal delivery, their pheromones may target unique receptors and produce genus-specific physiological effects [29]. To better understand changes in pheromone composition across plethodontid salamanders utilizing transdermal delivery, and gain insight into the forces that contribute to gene co-option, more comprehensive proteomic studies were required. Towards these goals, we characterized the composition of the *D. ocoee* mental gland extract using contemporary biochemical and mass spectral methods. Deeper cDNA sequencing and targeted PCR analyses permitted identification of previously uncharacterized putative pheromone genes and greater SPF isoform variation. Finally, pheromone composition was assessed between male salamanders and across different times throughout the courtship season to assess individual and temporal variation.

2. Materials and methods

2.1. Animal collection, tissue removal, and pheromone extraction

Male *D. ocoee* salamanders were collected from Deep Gap in Clay County, North Carolina (35°02′20″N 083°33′08″W) during the August breeding season. Animals were anesthetized in a mixture of 7% ether in water and mental glands surgically removed. Ten glands were immediately incubated in RNAlater (Ambion, Austin, TX) at 4 °C overnight before long-term storage at −20 °C. The remaining glands were incubated in 0.8 mM acetylcholine chloride (in amphibian Ringer's solution; 20 glands per 1 mL) for 1 h to induce secretion of protein pheromones. The solution was centrifuged at 10,000 *x g* for 10 min, the supernatant collected, and re-centrifuged prior to storage at −80 °C. In order to examine pheromone expression across the complete courtship season, male *D. ocoee* were also collected from the same location at approximately 3 week intervals from late May until early October, and pheromone extracted from ~20 glands at each time point. Methods and animal care were approved by the University of Louisville's Institutional Animal Care and Use Committee (IACUC #12,041 to P.W. Feldhoff).

2.2. Preparation of a mental gland cDNA library

RNA was extracted from two male mental glands using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. Double-stranded cDNA was synthesized using the Creator SMART cDNA Library Construction Kit (Clontech, Palo Alto, CA), cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and transformed into One Shot chemically competent *E. coli* cells (Invitrogen). Cells were plated on LB/agar plates with 50 μg/mL kanamycin to select for resistant clones. A total of 864 clones were isolated, inoculated into 150 μL Circlegrow broth (MP Biomedical, Irvine, CA) and cultured overnight at 37 °C. From each culture, glycerol freezer stocks were prepared and 1 μL aliquots used for colony PCR with M13 primers. PCR products were screened by gel electrophoresis, with those greater than 500 base pairs purified using the QIAquick PCR Purification kit (Qiagen), and supplied to the University of Louisville DNA Core Facility for automated Sanger-based DNA sequencing. To more thoroughly analyze SPF and additional pheromone diversity, gene specific primers were designed, targets amplified by PCR from five individual *D. ocoee* mental glands, and cloned and sequenced using the methods described above.

2.3. DNA sequencing, processing, and analysis

Nearly full-length cDNA sequences were processed using the DNASTAR package (Lasergene Version 7.1: DNASTAR, Madison, WI). Low quality sequences were filtered and contigs assembled using Seqman Pro. The open reading frame of each sequence was identified, translated using EditSeq, and full-length translations aligned with MegAlign using the ClustalW algorithm. All sequences were deposited into NCBI GenBank (Accession # KP410906 - KP410997). Unique sequences were compiled into a database for peptide mass fingerprinting analyses. The mean nucleotide and amino acid distances were calculated in MEGA 5 using a JTT matrix-based model, and analyses conducted using a maximum composite likelihood model with 500 bootstrap replicates.

2.4. Purification and mass spectral analysis of major pheromone components

Pheromone components were purified through a combination of strong anion-exchange-HPLC (Mono Q; Pharmacia, Piscataway, NJ), reverse phase-HPLC (RP-HPLC) (C-18; Grace Davison Discovery Sciences, Deerfield, IL), and size exclusion chromatography (G-75 Superfine; Pharmacia, Piscataway, NJ). All chromatographic separations were accomplished on a 2695 Alliance HPLC System equipped with a 2487 dual wavelength absorbance detector and Empower software (Waters Division, Milford, MA). The Mono Q column (0.5 × 5.5 cm) was eluted at 1 ml/min with a NaCl gradient in 50 mM Tris/HCl buffer, pH 8.0 (mixed gradient: 5 mM NaCl/min for 30 min, 10 mM NaCl/min for 15 min, 20 mM NaCl/min for 10 min). The C-18 column (0.5 × 15 cm) was eluted with a linear acetonitrile (ACN) gradient in 0.1% trifluoracetic acid. The G-75 column (1.6 × 15.5 cm) was eluted at ~10 mL/h with 0.5 X phosphate-buffered saline, pH 7.4 (PBS). *D. ocoee* pheromone extract was initially separated by size exclusion chromatography, pooled into three fractions which were then subjected to anion-exchange HPLC, with pooled anion-exchange fractions in turn analyzed