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Evolutionary shifts in courtship pheromone composition revealed by EST analysis of plethodontid salamander mental glands

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ABSTRACT

Courtship behavior in salamanders of the family Plethodontidae can last more than an hour. During courtship, males use stereotyped behaviors to repeatedly deliver a variety of proteinaceous pheromones to the female. These pheromones are produced and released from a specialized gland on the male's chin (the mental gland). Several pheromone components are well characterized and represented by high frequency transcripts in cDNA pools derived from plethodontid mental glands. However, evolutionary trends in the overall composition of the pheromonal signal are poorly understood. To address this issue, we used random sequencing to survey the pheromone composition of the mental gland in a representative species from each of three distantly related plethodontid genera. We analyzed 856 high-quality expressed sequence tags (ESTs) derived from unamplified primary cDNA libraries constructed from mental glands of *Desmognathus ocoee*, *Eurycea guttolineata*, and *Plethodon shermani*. We found marked differences among these species in the transcript frequency for three previously identified, functional pheromone components: Plethodontid Receptivity Factor (PRF), Sodefrin Precursor-Like Factor (SPF), and Plethodontid Modulating Factor (PMF). In *P. shermani* mental glands, transcripts predominately encoded PMF (45% of all ESTs) and PRF (15%), with less than 0.5% SPF. In contrast, in *D. ocoee* and *E. guttolineata* the proportions were ~20% SPF, 5% PMF, and PRF was absent. For both *D. ocoee* and *E. guttolineata*, peptide hormone-like transcripts occur at high frequency and may encode peptides that change the physiological state of the female, influencing the female's likelihood to complete courtship. These and previous results indicate that the evolution of courtship pheromones in the Plethodontidae is dynamic, contrasting with the predominant mode of evolutionary stasis for courtship behavior and morphology.

1. Introduction

The best understood pheromones of amphibians are proteinaceous compounds that mediate sexual communication in salamanders (Kikuyama et al., 1995; Rollmann et al., 1999) and mate attraction in anurans (Wabnitz et al., 1999). Several proteinaceous pheromones of amphibians have been characterized and range in size from 10 to over 200 aa (reviewed in Kikuyama et al., 2002). Some of these pheromones are prone to rapid diversification via amino acid substitutions, which may promote speciation (Palmer et al., 2005, 2007a, 2007b; Watts et al., 2004). In contrast, pheromones used by salamanders in other sexual and nonsexual contexts (e.g., species and sex identification) have been detected by behavioral assays but have not been characterized (Dantzer and Jaeger, 2007; Dawley, 1986).

Plethodontid salamanders use protein pheromones during an intricate courtship that accomplishes indirect sperm transfer via a spermatophore that is attached to the substrate (Houck and Arnold, 2003). In most of the 300+ species of plethodontid salamanders, the male's chin (mental) gland seasonally hypertrophies and produces multiple proteins during a lengthy courtship season (Houck and Sever, 1994; Lanza, 1959; Sever, 1975). During courtship, males deliver these mental gland proteins to the female using one of two, stereotyped behaviors (Arnold, 1977). In 'scratching delivery', the male swabs the female's dorsum with his mental gland and then abrades the swabbed site with specialized premaxillary teeth, apparently introducing pheromones into the superficial vessels of her circulatory system. In 'olfactory delivery', the male repeatedly touches the secretory surface

Abbreviations: aa, amino acid(s); ANP, atrial natriuretic peptide; BLAST, basic local alignment and search tool; BNP, natriuretic peptide precursor type B; bp, base pair; cDNA, DNA complementary to RNA; DNA, deoxyribonucleic acid; DNP, *Dendroaspis* natriuretic peptide; EST, Expressed Sequence Tag; GLP, glucagon-like peptide(s); GLP-1, glucagon-like peptide 1; GLP-2, glucagon-like peptide 2; GO, gene ontology; HPLC, high performance liquid chromatography; kDa, kilodaltons; mRNA, messenger ribonucleic acid; Myr, million years; NLP, natriuretic-like peptide(s); ORF, open reading frame; PCR, polymerase chain reaction; PMF, Plethodontid Modulating Factor; PRF, Plethodontid Receptivity Factor; RNA, ribonucleic acid; RT-PCR, reverse-transcription polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPF, Sodefrin Precursor-Like Factor; TNP, taipan natriuretic peptide; UV, ultraviolet; VNO, vomeronasal organ.

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of his mental gland to the female's nares, with the consequence that pheromones are introduced into the female's nasal cavity and reach her vomeronasal organ (VNO). Scratching delivery is the ancestral delivery mode, found in all major clades and a majority of species, while olfactory delivery is a derived mode, restricted to a clade of about 30 species in the genus *Plethodon* (Fig. 1) (Houck and Arnold, 2003).

Partial biochemical characterization of the major gland proteins has been accomplished for two plethodontid species: *Plethodon shermani* (olfactory delivery) and *Desmognathus ocoee* (scratching delivery). Analysis using NH_2-terminal protein sequencing, SDS-PAGE and HPLC revealed three structurally unrelated proteins that constitute the major components of the male courtship pheromones in these two species (Feldhoff et al., 1999; Houck et al., 2008b; Rollmann et al., 1999). The first pheromone that was isolated, Plethodontid Receptivity Factor (PRF), is related to the interleukin-6 family of cytokines and reduces the duration of courtship in *P. shermani* (Rollmann et al., 1999). PRF expression appears to be limited to a single genus, *Plethodon* (Palmer et al., 2005).

The second pheromone, Plethodontid Modulating Factor (PMF), is expressed in *Aneides*, *Desmognathus*, and *Plethodon*, based on RT-PCR isolation from mental glands (Palmer, 2004). Plethodontid Modulating Factor (a 7 kDa protein) has an effect on female courtship behavior opposite that of PRF. When delivered alone to *P. shermani* females, PMF increases total courtship duration. Since these components are delivered together during courtship, as part of the total pheromone mixture, this apparently enigmatic effect may be an experimental artifact. In the natural mixture, PMF may have synergistic interactions with other pheromone components. For example, PMF may calm the female and enhance the effects of PRF on the female's courtship behavior (see Houck et al., 2007 for additional discussion).

The third protein pheromone identified for plethodontid salamanders, Sodefrin Precursor-Like Factor (SPF), is similar to the uncleaved precursor protein of the salamandrid (newt) sex attractant, a decapeptide termed sodefrin (Palmer et al., 2007b). Like PRF, SPF decreases total courtship time when it alone is delivered to the female (Houck et al., 2008a). Sequences of SPF have been amplified from the cDNA of mental glands of four genera of plethodontid salamanders: *Aneides*, *Desmognathus*, *Eurycea* and *Plethodon* (Palmer et al., 2007). To date, SPF has been validated behaviorally only in *D. ocoee* (Houck et al., 2008a). Studies using codon-substitution models to estimate the nonsynonomous/synonomous substitution rates of PMF, PRF and SPF show that all three have experienced positive selection across lineages at a subset of amino acid sites (Palmer et al., 2007a). These sites may be co-evolving with binding sites on receptors in females (Watts et al., 2004).

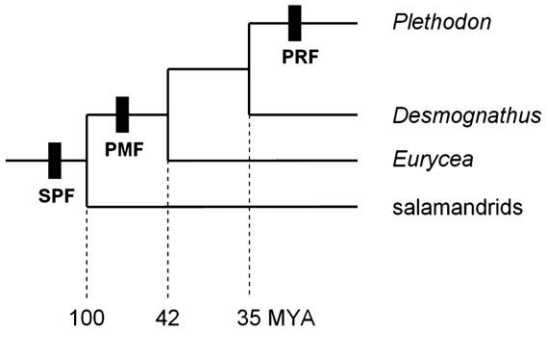

Fig. 1. Cladogram showing the relationships of three genera of plethodontid salamanders (*Eurycea*, *Desmognathus*, and *Plethodon*) and origin of three courtship pheromones. The outgroup (salamandrids) represent a second family of salamanders (the Salamandridae) that includes *Cynops* and *Salamandra*. Relationships shown here are concordant across studies using both morphological and molecular characters (Chippindale et al., 2004; Macey, 2005; Min et al., 2005, Vieites et al., 2007). Approximate divergence times (shown at bottom) are based on albumin immunology (Larson et al., 2003). Small rectangular boxes show the point of origin of three pheromones: Sodefrin-like Precursor Factor (SPF), Plethodontid Modulating Factor (PMF), and Plethodon Receptivity Factor (PRF). Basal clades within the genus *Plethodon* (not shown) do not possess PRF (see Palmer et al., 2007b for details).

In sum, previous work demonstrated that proteins secreted by plethodontid mental glands influence female behavior and have experienced rapid evolution. However, HPLC and SDS-PAGE analyses have shown that many proteins expressed in male mental glands remain uncharacterized in *P. shermani* (Feldhoff et al., 1999) and in *D. ocoee* (Houck et al., 2008b). Thus, the complex protein profiles revealed by initial analyses may include other molecules that persuade courting females to mate with particular males. Unfortunately, evolutionary trends in the overall composition of the mental gland secretions are poorly understood. It is unknown whether overall pheromone composition is evolutionarily conserved or prone to rapid diversification that parallels documented diversification of particular pheromone components (PMF, PRF, and SPF). PCR isolation has revealed a shift from SPF to the dominant use of PRF within the genus *Plethodon* (Palmer et al., 2007b), but no other shifts have been documented. Evolutionary inferences are also limited because protein screening is partial and limited to only two plethodontid species. To address these broader evolutionary issues, we used an EST approach to compare the mental gland expression profiles of three distantly-related plethodontid genera. We predicted that mental gland proteins corresponding to high frequency transcripts are likely to have functional effects during courtship because specialized glands are known to contain large amounts of pheromone RNA in both vertebrates (newts; Iwata et al., 2000) and invertebrates (sea hares; Fan et al., 1997).

We compared the ESTs from mental glands of three diverse members of the family Plethodontidae: *D. ocoee*, *P. shermani*, and *Eurycea guttolineata*. These three taxa represent three of four major plethodontid lineages (Chippindale et al., 2004; Macey, 2005; Mueller et al., 2004). The genera *Desmognathus* and *Plethodon* represent two sister clades, while *Eurycea* is more distantly related (Fig. 1). Our primary goals were to (1) determine the overall composition of the mental gland secretion for each species, (2) examine the complexity of expression in the male mental glands (including investigating novel high-frequency transcripts that might encode functional proteins) and (3) compare the composition of the mental gland proteins between the three species. We hope that these comparisons will begin to elucidate the evolutionary history of pheromone composition in this family. In addition, because previous RT-PCR was unable to distinguish low and high frequency transcripts, our final aim was to establish whether differences in sequences previously obtained by RT-PCR reflect actual sequence variation in mental gland transcripts.

2. Materials and methods

2.1. Tissue collection and RNA isolation

Adult males in breeding condition (indicated by enlarged premaxillary teeth and/or a visible mental gland) were collected for each of the three study species. Because previous studies of courtship pheromones have been focused on *D. ocoee* and *P. shermani* (e.g., Houck et al., 2008b; Rollmann et al., 1999), one of our goals was to compare the EST pheromone sequences to those obtained by PCR from these focal species. Male *D. ocoee*, *E. guttolineata*, and *P. shermani* were collected from Macon County, North Carolina (35°02′20″N 083°33′08″W, 35°02′40″N 083°19′17″W, and 35°19′48″N 083°33′38″W, respectively).

To collect gland tissue, all animals were anesthetized in 4% ethyl ether in water. The mental gland of each male was then surgically ablated with iridectomy scissors (Houck et al., 1998, 2008a). Males fully recover from this procedure in approximately one week (Rollmann et al., 1999). For each species, glands from multiple

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males were stored as pooled samples in RNA*later* (Ambion, Austin TX). RNA from the *D. ocoee* glands ($n=20$) and *E. guttolineata* glands ($n=7$) was extracted using Trizol reagent (Invitrogen, Carlsbad CA) according to the manufacturer's protocol. RNA from *P. shermani* ($n=10$ glands) was extracted from the pooled glands using an mRNA isolation kit (Stratagene, La Jolla CA).

2.2. cDNA library construction and plasmid sequencing

The *P. shermani* library was constructed using the ZAP Express XR Library Construction Kit (Stratagene, La Jolla CA) that utilized the Lambda-ZAP Vector. The library was generated using standard protocols supplied by the manufacturer. The *D. ocoee* and *E. guttolineata* gland cDNA libraries were synthesized using the Creator Smart cDNA Library Construction Kit (BD Biosciences Clontech, Palo Alto CA) according to the manufacturer's instructions. Briefly, long-distance PCR was used to generate cDNA inserts that were run through column fractionation to exclude small products (≤500 bp). The size exclusion biased the libraries toward larger sequences, deemed appropriate because these smaller sequences were assumed to be mostly 3′ and 5′ untranslated regions. Although the use of differing methodologies to construct libraries may have biased the representation of some ESTs in the libraries, general information about the percentage of transcripts is nevertheless informative for our purposes.

In order to obtain a representative sample of ESTs from each library, primary clones were chosen randomly from each of the three libraries. Plasmid DNA was isolated from each clone using the Qiaquick miniprep kit (Qiagen, Valencia CA) and the DNA was then sent away for sequencing. Clones from the *P. shermani* library were sequenced using the T3 primer at the DNA core facilities at the University of Louisville. Plasmid DNA from the *D. ocoee* and *E. guttolineata* libraries were sequenced with the M13 Forward primer at the Nevada Genomics Center. An additional screening step was used on the primary clones from *E. guttolineata* library because unlike the other two libraries, this one had a smaller proportion of clones containing inserts. Primary clones were screened by isolating plasmid DNA from each clone using the Qiaquick miniprep kit (Qiagen, Valencia CA) and digesting with *Sfi*I. Agarose (1%) gel electrophoresis followed by ethidium bromide staining was used to visualize the digested DNA under UV light. Clones containing inserts over 500 bp were then sent for single-read sequencing.

2.3. EST assembly and identification

SeqManII (DNAstar Version 5.0) was used to identify and exclude low quality sequences, cluster sequences, and assemble contigs. High-quality sequences were sorted into contigs of 80% identity. The consensus sequence from each contig was then compared to known sequences in GenBank using translated BLAST searches (ww.ncbi.nlm.nih.gov/BLAST) against the translated sequence database. GO (Gene Ontology)-slim functions were recorded as a way to classify the salamander ESTs into putative functional categories based on their similarity to previously identified genes (e.g., (Wagstaff and Harrison, 2006). The ESTs were sorted into GO-slim categories if they had a BLAST *e* value $<e^{-10}$ to a known gene product. ESTs from all three species were deposited in the NCBI EST database (http://www.ncbi.nlm.nih.gov/dbEST). The *D. ocoee* ESTs were archived under the accession nos. FK700083–FK700535, the *E. guttolineata* ESTs under FG985087–FG985271, and the *P. shermani* ESTs under FK253129–FK253344, FK703776 and FK703777.

Our primary interest in constructing these libraries was to identify cDNAs (generated from RNA) that likely transcribed proteins secreted into the lumen of the male mental gland that might act as functional pheromones. We had two criteria for identifying a contig as a potential pheromone component. First, it had to contain more than three ESTs (and was therefore relatively highly transcribed). Second, it had some region of homology to gene products that were physiologically active (but the BLAST score was not necessarily below the *e*-value of e^{-10}). Therefore, highly transcribed contigs not identified in the initial BLAST search (described in previous paragraph) were subjected to an additional screening process that looked more closely at the BLAST matches to determine if there were any regions of homology to physiologically active gene products. Once potential candidates were identified, NCBI Unigene gene expression profiles were examined to determine whether the gene was normally expressed in the blood, skin, or connective tissues of other vertebrates. Those predicted ORFs that were determined to be pheromone candidates were aligned with those of other vertebrates using BioEdit version 7.0.9 (Hall, 1999). Additionally, each ORF was checked for the presence of a signal peptide using SignalP 3.0 (Bendtsen et al., 2004) and biochemically relevant information about cleavage sites was used to predict whether the salamander ORF might transcribe a functional peptide.

2.4. Calculation of pheromone sequence dissimilarity

To compare the DNA sequence diversity of PMF, PRF, and SPF between our ESTs and the previously published sequences, we compiled and aligned our ESTs and those sequences in GenBank separately. We used ClustalW implemented in MegAlign (DNAstar Version 5.0) to align the pheromones from each species. To maximize the number of ESTs included in the analysis, we used the first 114 bp of each pheromone ORF to estimate nucleotide dissimilarity. The same 114 bp fragment was used from published pheromone sequences in order to directly compare sequence diversity. Average nucleotide sequence dissimilarity was measured using the Tamura–Nei method (calculated in MEGA, Version 4.0; Tamura et al., 2007) as the number of unique substitutions per nucleotide site for a pair of sequences with a correction for multiple hits.

2.5. Comparison of unidentified ESTs in all libraries

We also checked for similarity of the unidentified sequences between the three libraries. Because it is known that *P. shermani* and *D. ocoee* both share the pheromone component PMF, the three species may have other proteins in common. To investigate this possibility, we compiled all ESTs from all three species that did not have an identified function and used SeqManII to cluster these unknown ESTs into contigs of 75% identity. Each of these contigs was checked manually to determine whether ESTs from multiple species were present in the same contig.

3. Results

3.1. Overview of mental gland libraries

The average EST length for all male mental gland library sequences was approximately 500 bp. The total number of high-quality ESTs obtained was 856 (453 from *D. ocoee*, 185 from *E. guttolineata*, and 218 from *P. shermani*). As expected, transcripts encoding previously identified pheromones were the most prevalent transcripts in all of the libraries (Table 1). In fact, all the libraries were roughly equivalent in general composition. Aspects similar in every library included the percentage of ESTs assigned to multi-sequence or single-sequence contigs, number of sequences grouped into all GO-slim categories (except unclassified), and average length of ESTs (Fig. 2). However, there were also some striking differences among the three libraries. When the ESTs were classified into GO-slim functions, the number of ESTs grouped into the general pheromone and unclassified categories differed among the salamander taxa. The percentages of ESTs identified as pheromone transcripts in the *P. shermani* library was about double of that found in the *D. ocoee* and *E. guttolineata* libraries

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Table 1
Summary statistics of mental gland EST libraries from three salamander species

	D. ocoee	*E. guttolineata*	*P. shermani*
Total no. of sequences in multi (2+) sequence contigs	283	112	181
Known pheromone	127	52	135
Function classified	21	11	9
Function unclassified	135	49	37
Total no. of single sequence contigs	170	73	37
Known pheromone	0	0	1
Function classified	19	33	8
Function unclassified	151	40	28
Totals	453	185	218

(Fig. 2). In comparison, the *D. ocoee* and *E. guttolineata* libraries had a much higher percentage of unclassified ESTs.

3.2. Comparison of pheromone ESTs across the three plethodontid genera

The three species showed differences in the number of known pheromone ESTs (PMF, PRF, or SPF) found in each of the male gland libraries (Fig. 3). In *P. shermani*, the pheromone ESTs were predominately identified as PMF and to a lesser extent, PRF. The *D. ocoee* and *E. guttolineata* mental gland EST compositions were similar, each having approximately equal proportions of SPF and PMF ESTs. The DNA sequence variation present in the ESTs was generally higher in the libraries than that obtained by RT-PCR. In *P. shermani*, the average nucleotide dissimilarity was the same (0.01%) for the PRFs obtained by both methods, but the ESTs showed a higher dissimilarity (0.25%) for PMFs than did the sequences obtained by RT-PCR (0.01). In *D. ocoee* the dissimilarity was higher in the ESTs in both PMF (0.08% vs. 0.01%) and SPF (0.23% vs. 0.02%). Because so few sequences were obtained by RT-PCR for *E. guttolineata*, we were unable to do any dissimilarity comparisons with that species.

3.3. Identification of novel highly-expressed transcripts

ESTs that were identified at low levels (less than three ESTs) and that were similar to genes expressed in blood, skin, or connective tissue were considered to be part of the normal housekeeping repertoire of gland cells and associated tissues. An EST from *E. guttolineata* had short regions of identity to relaxin, a hormone with

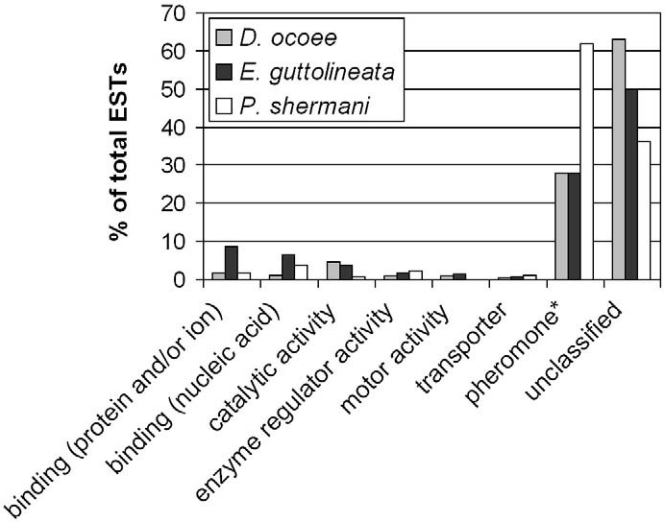

Fig. 2. Summary of the GO-slim molecular functions of ESTs from *D. ocoee*, *E. guttolineata*, and *P. shermani*. *Pheromone is not a GO-slim function, but this term is used for convenience.

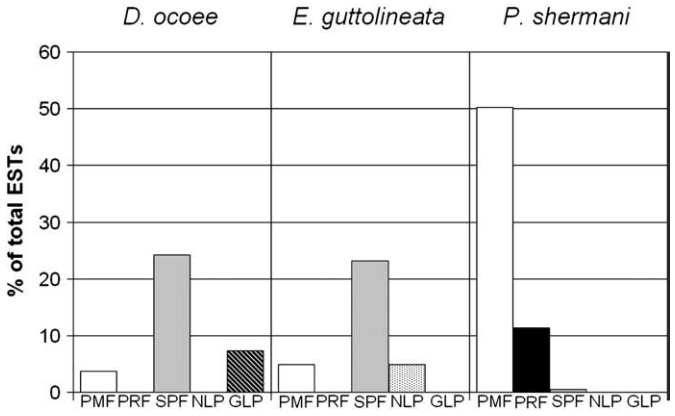

Fig. 3. The percentage of total ESTs that were identified as the three known pheromones (PMF, PRF, or SPF), natriuretic-like peptides (NLP), or glucagon-like peptides (GLP).

pleiotropic effects produced primarily by reproductive organs in male and female animals (Bani, 1997). This hormone may be secreted and delivered to the female during courtship, but because there was only a single EST, it is unlikely that it is highly transcribed by the mental gland. Other highly expressed transcripts were mostly attributable to general cellular maintenance and did not appear to function as pheromones. A few of the highly expressed transcripts may instead encode enzymes used to process excreted pheromone components. For example, in both *D. ocoee* and *E. guttolineata*, several of the ESTs encoded a transcript that showed identity to the M3 Thimet peptidase family, a family that cleaves circulating peptides (Lew, 2004). The *P. shermani* and *E. guttolineata* libraries both contained ESTs that were identified as cystatins. Some members of the cystatin family have cysteine protease inhibiting functions. PMF and SPF have multiple conserved cysteines. Thus, these cystatins may function to protect these pheromones from being degraded in the lumen of the mental gland.

One of the two candidate pheromone transcripts represented ~7% of all ESTs from the *E. guttolineata* library and encoded a predicted protein that showed similarity to the natriuretic peptide family. This family of small hormones (~29 aa) that mainly stimulate sodium excretion and vasorelaxation (Bovy, 1990; Matsuo, 2001). The predicted ORF from *E. guttolineata* appears to be about 50 aa shorter than natriuretic peptide precursors produced in the mammalian heart, but contains multiple sites known to be necessary for functionality (Fig. 4). These sites include two cysteine residues that disulfide bond to form a cysteine ring (salamander residues 7 and 23), and a phenylalanine at residue 8 (Bovy, 1990). The ORF also contained a predicted signal peptide of 22 aa and four basic amino acids that may be used as a mono- or dibasic cleavage site to generate the mature peptide from the precursor. Since the length of the peptide is unknown, we cannot be certain of the cleavage site. We defined the first arginine as a likely cleavage site and used this assumption to generate the predicted bioactive peptide in Fig. 4. The predicted ORF from the consensus sequence has been deposited in GenBank (accession no. EU797453).

The second predicted ORF was encoded by 5% of all ESTs from the *D. ocoee* library (Fig. 3). The consensus sequence was similar to that of the glucagon superfamily, which are 28–38 aa hormones primarily expressed in the pancreas and intestine. The glucagon-like peptide from the salamander (Fig. 4) contained the conserved residues known to be necessary for the glucagon-like peptide 1 to fully function: 1 (Histidine), 4 (Glycine), 6 (Phenylalanine), 7 (Threonine), 9 (Asparagine) 22 (Phenylalanine), and 23 (Isoleucine) (reviewed in Kieffer and Habener, 1999). As in mammalian glucagon, the predicted glucagon-like ORF from *D. ocoee* contained a single arginine that is likely the N-terminal cleavage site (Irwin, 2001) and a predicted

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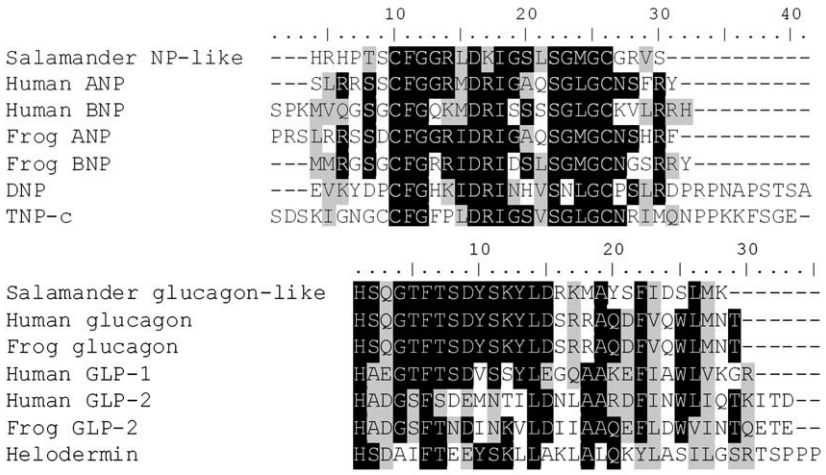

Fig. 4. Comparison of the mature hormone-like peptides aligned using ClustalW implemented in MegAlign. Upper panel contains the alignment of salamander (*E. guttolineata*) natriuretic peptide, human atrial natriuretic peptide (ANP) (GenBank accession no. NM_006172), human peptide precursor type B (BNP) (NM_002521), African clawed frog ANP (AF287050), anuran BNP (AY660659), *Dendroaspis* natriuretic peptide (DNP) (Schweitz et al., 1992), and taipan natriuretic peptide (TNP)-c (P83230). The lower panel contains the salamander (*D. ocoee*) glucagon-like peptide, human glucagon, glucagon-like peptide 1 (GLP-1), and glucagon-like peptide 2 (GLP-2) (NP_002045), African clawed frog glucagon and GLP-2 (NP_001079142), and helodermin (P04204). Black shading indicates identical aa and gray shading indicates similar aa that are conserved in three or more peptides (shading executed in BioEdit; Hall, 1999).

signal peptide (22 aa). We hypothesized that the lysine at position 28 was the last aa on the c-terminal and functions as the cleavage site since glucagon-like peptides in *Xenopus* have lysine as their final aa (Irwin et al., 1997). The *D. ocoee* glucagon-like ORF has been deposited in GenBank (accession no. EU797454).

3.4. Similarity of unidentified ESTs in all libraries

When we compiled all ESTs (from all three species) that showed no significant similarity to known genes in the initial BLAST search, we found that only a single contig contained ESTs from two species. Some of these unknowns were present at relatively high levels (up to 5% of total ESTs in *D. ocoee* and 7% in *E. guttolineata*) and later identified as potential pheromone components. Thus, each species apparently had a large proportion of unique transcribed sequences, even though they all transcribed the known pheromone components PMF and SPF.

4. Discussion

Our EST analysis revealed extensive differences in mental gland pheromone composition between genera, based on the assumption that message frequency can be used as a proxy for protein frequency in the pheromone blend itself. The changes in pheromone composition inferred from the EST data contrast with the evolutionary conservatism of the behavioral and morphological components of the courtship pheromone delivery system. Plethodontid salamanders are an ancient but morphologically and behaviorally conservative group of salamanders that contain many cryptic species described recently with genetic techniques (Highton and Peabody, 2000; Houck and Arnold, 2003). Against this generally static background, an important transition in behavior – from scratching delivery to olfactory delivery – occurred about 19 Myr ago within the genus *Plethodon* (Houck and Arnold, 2003; Palmer et al., 2005; Picard 2005). Studies of the process of molecular evolution of the three pheromone components (PRF, PMF, SPF) revealed that although there is stasis in morphology and delivery behavior, the pheromone components have evolved rapidly (Palmer et al., 2007a, 2007b; Watts et al., 2004). Our EST data confirm that the variation seen in PCR amplification of the previously identified pheromone components is present in transcripts in the male mental gland. Our results also demonstrate considerable differences in mental gland ESTs underlying the apparent conservatism in behavior in the two genera with scratching delivery, *Desmognathus* and *Eurycea*.

Both SPF and PMF are expressed by members of all three divergent clades of plethodontid salamanders, but SPF appears to be the primary component in *D. ocoee* and *E. guttolineata*. The PMF and SPF components apparently originated early in the plethodontid lineage (Palmer et al., 2007b). Palmer et al. (2007b) proposed that sodefrin evolved as a pheromone before the split of salamandrids and plethodontids, but a shift occurred within the plethodontids to a SPF/PMF blend. It appears that at least three distinct shifts in pheromone composition have occurred after the evolution of the SPF/PMF blend, during the differentiation of *P. shermani*, *D. ocoee*, and *E. guttolineata*. Two shifts have occurred during the differentiation of *D. ocoee* and *E. guttolineata*. Each appears to have independently recruited a hormone-like component as part of their pheromone secretion. It appears that in the 42 Myr since these two genera diverged (Fig. 1), each has evolved different pheromone components while the morphology of the animals has remained relatively static. Both of these lineages may have recruited hormone-like compounds because the males' scratching delivery facilitates rapid entry of gland secretions into the female circulatory system. Finally, our data suggest there was a shift from the SPF-dominated pheromone to a PMF/PRF-dominated pheromone in the eastern *Plethodon* lineage, as originally postulated by Palmer et al. (2007b) by showing that *P. shermani* male mental glands express predominately PMF and PRF.

The unanswered question about mental gland expression of natriuretic- and glucagon-like peptides is whether these peptides affect the sexual behavior of the plethodontid female. Such effects are plausible given the diversity of physiological effects that these peptides are known to possess. In particular, such physiological effects may mediate a change in female behavior during courtship. For example, natriuretic peptides are known to regulate pathways that reduce stress in rats (Franci et al., 1992) and so might make a female less prone to startle and exit courtship. There is precedent for expression of this peptide family in exocrine glands. Natriuretic-like peptides isolated from snake venoms are functionally and structurally similar to peptides expressed in the heart of mammals (e.g., Fry et al., 2005; Schweitz et al., 1992).

Members of the glucagon superfamily, helodermin and helospectins, are secreted in the venom of a lizard and reduce the blood pressure of rats (Grundemar and Högestätt, 1990). Novel glucagon-like peptides identified in anuran amphibians are related to the well-

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known proglucagon-derived hormone found in mammals, glucagon-like peptide 1(GLP-1) (Irwin et al., 1997). These anuran peptides act as potent agonists to the human glucagon receptor, even though there are nine aa differences between the anuran glucagon-like peptides and human glucagon. The efficacy of the frog glucagon-like peptides suggests that even though the salamander glucagon-like peptide has numerous aa substitutions, it may still be physiologically active. Because glucagon mediates satiation in other vertebrates (Chelikani et al., 2005), it is possible that glucagon-like peptides in plethodontid pheromones decrease a female's perceived hunger or otherwise affect her sexual behavior.

To understand how different pheromone components evolve, one can use a simple resource allocation model to describe different selective pressures that may affect a female's likelihood to mate. The probability of a male inseminating a female will not only be a function of her reproductive status and how attractive she finds the male, but will also be affected by her need to forage and her need to engage in predation avoidance (such as moving to a less exposed environment). A first order model of the probability of insemination (P_i) given encounter between sexual partners can be written as

$$P_i = \delta(M)(1-P_d)$$

where P_d is the probability of the female departing prior to insemination and $\delta(M)$ describes the relative ability of the male to persuade the female to mate. Thus, P_d is the sum of the probabilities of the female departing to forage or avoid predators, and can be thought of as the balance of her time allocation trade-off. Male persuasiveness (M) ranges from 0 to some maximum, M_{max}, and so $\delta(M)=M/M_{max}$. The equation describes one quadrat of a hyperbolic paraboloid, a surface which contains no local minima or maxima so two pheromone functions could evolve independently. This model suggests that males have two independent and nonexclusive ways to increase insemination success. First, males can increase their persuasiveness by manipulating females such that $\delta(M)$ increases. This aspect of persuasiveness was investigated in behavioral assays that manipulated pheromone composition (Houck et al., 2007, 2008b; Rollmann et al., 1999). A second strategy is to manipulate females such that P_d decreases. A male might use courtship pheromones (in particular the natriuretic- and glucagon-like peptides) to alter the balance of the trade-off the female makes towards courtship by reducing female vigilance or increasing her perceived satiation. In other words, the female might be more likely to invest time in courtship and insemination because she is less likely to depart from a sexual encounter.

The resource allocation model and the presence of the natriuretic- and glucagon-like transcripts in the male gland raise the possibility of a previously unrecognized pheromone action — that a courting male can modify female likelihood to successfully complete courtship by influencing the factors that determine whether a female engages and remains in courtship. However, although it is plausible that the natriuretic- and glucagon-like peptides we identified affect female behavior, either directly or in combination with pheromones, such effects remain to be verified. To date, the only direct tests of the effects of male pheromones on female plethodontids during courtship have used courtship duration as an indicator of female receptivity. Tests for behavioral and physiological effects of these peptides should be a focus of future efforts conducted with plethodontids.

In conclusion, our study has (1) provided a framework for conceptualizing how males use chemical communication to influence the sexual response of potential mates and (2) expanded our understanding of how the composition of mental gland secretions evolves. At the same time, our EST analysis highlights both the need to survey mental gland composition in a broader array of plethodontid taxa and to assay the behavioral effects of additional pheromone components.

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