Journal of Molecular Evolution
https://doi.org/10.1007/s00239-021-10026-0

ORIGINAL ARTICLE

Pheromone Gene Diversification and the Evolution of Courtship Glands in Plethodontid Salamanders

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Received: 27 November 2020 / Accepted: 28 July 2021

Abstract

Proteinaceous pheromones that diversify through gene duplication can result in shifts in courtship cocktails that may serve as a mechanism for reproductive isolation. The molecular evolution of pheromones has been extensively studied in salamanders, but how these genes and associated novel courtship glands have codiversified has not been evaluated. In this study we used transcriptional analyses to examine the relationship between pheromone diversification and gland type in three divergent lineages of plethodontid salamanders. Our results revealed that plethodontid salamanders express up to eight divergent Sodefrin Precursor-like Factor genes (*spf*, representing both alpha and beta subfamilies) along with Plethodontid Modulating Factor (*pmf*) and Plethodontid Receptivity Factor (*prf*). Expression of pheromone genes is tissue specific with *pmf*, *prf*, and some *spf* genes restricted to the mental gland. In contrast, the caudal gland shows strong expression of the other *spf* genes. We found evidence for punctuated changes in pheromone cocktail composition related to the loss of metamorphosis, and subsequent extreme reduction of the mental gland, in a paedomorphic lineage. Our study provides insight into how pheromone diversification can be partitioned into unique glands, which may lead to cocktail specificity in behavioral modules during courtship.

Keywords Caudal gland · Life cycle · Mental gland · Metamorphosis · Paedomorphosis · Subfunctionalization

Introduction

Gene duplication can be the source for diversification of molecular signaling pathways and the foundations of functional innovation (Thornton 2001; Zhang 2003; Huminieck and Wolfe 2004; Li et al. 2005; Bridgham et al. 2006; Eick and Thornton 2011). For example, the proliferation of genes that encode for reproductive pheromones can result in saltatory shifts in mate recognition cocktails (Symonds and Elgar 2008; Doty et al. 2016). This has been studied extensively in insects, but much less focus has been given to other taxa that utilize reproductive pheromones (Symonds and Elgar 2008). It remains unclear how gene duplications that give rise to gland-specific expression influence the evolution and specialization of pheromone delivery systems.

Pheromone signaling facilitates salamander courtship (Houck 2009; Woodley 2010; Woodley and Staub 2021). Even though male pheromone excreting glands vary widely across lineages (Arnold 1977; Houck and Arnold 2003; Sever 2003) the prevailing hypothesis is that their products are generally used to entice females to accept a sperm packet (Arnold 1977; Houck and Arnold 2003). Courtship behavior and gland morphology have been well characterized across families (Houck and Arnold 2003; Sever 2003; Sever et al. 2016; Arnold et al. 2017), but analyses of salamander courtship pheromones have been primarily restricted to a few glands and lineages. These include the mental gland (below the chin) of a few genera of lungless salamanders (Plethodontidae; Rollmann et al. 1999; Palmer et al. 2007a, b, 2010; Kiemnec-Tyburczy et al. 2009), and the cloacal glands of a few genera of aquatic breeding newts (Salamandridae; Kikuyama et al. 1995; Janssenswillen et al. 2015a; Van Bocxlaer et al. 2015), and axolotls (Ambystomatidae; Maex et al. 2016). Males of all three of these families are known to express *Sodefrin Precursor-like Factor* (*spf*), and SPF proteins have been shown to accelerate courtship in representative species (Kikuyama et al. 1995; Houck et al. 2008; Janssenswillen et al. 2015a). It is now appreciated

Handling editor: **Willie Swanson**.

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Published online: 14 August 2021

that *spf* is in fact a multigene family with at least two major groups (*spfα* or *spfβ*) that diverged in the Paleozoic (Van Bocxlaer et al. 2015). Subsequent duplications have led to further diversification of the *spfα* and *spfβ* subfamilies within urodele clades (Bossuyt et al. 2019). Thus far *spfβ* has only been identified in salamandrids and ambystomatids (Bossuyt et al. 2019). In addition to *spf*, plethodontid salamanders also express two other pheromones, *Plethodontid Modulating Factor* (*pmf*; Houck et al. 2007; Palmer et al. 2007a, b, 2010) and *Plethodontid Receptivity Factor* (*prf*; Rollmann et al. 1999; Watts et al. 2004). Multiple copies of each of these genes are known to be expressed in the mental glands of plethodontids (Palmer et al. 2005; Kiemnec-Tyburczy et al. 2009; Wilburn et al. 2012, 2017). As of yet, we lack a comprehensive understanding of pheromone diversity, particularly within different courtship glands, and how they have evolved in tandem.

Most plethodontid salamanders utilize a stereotypical breeding behavior known as the tail straddle walk (Arnold 1977; Arnold et al. 2017), which involves the mental gland (Sever et al. 2016) and the caudal gland (located at the base of the tail; Rupp and Sever 2017). To date, pheromone research on plethodontids has been restricted to mental glands of two genera (*Plethodon* and *Desmognathus*) (Doty et al. 2016; Palmer et al. 2005, 2007a, 2007b, 2010; Wilburn et al. 2012, 2014; Wilburn and Feldhoff 2019).

In this study we use comparative transcriptome analyses to evaluate pheromone diversity in mental and caudal glands of three divergent plethodontids: *Desmognathus brimleyorum, Plethodon albagula*, and *Eurycea tynerensis*. All three of these taxa metamorphose into a more terrestrial adult salamander and exhibit terrestrial courtship. However, *Eurycea tynerensis* exhibits an alternative life history mode (Emel and Bonett 2011), paedomorphosis, where reproductive adults retain aquatic larval characteristics. The significance of including paedomorphs is because they appear to have prominent caudal glands, but drastically reduced mental glands (this study). Variation in this system provides an opportunity to analyze how pheromones have subfunctionalized in parallel with courtship glands. Analyzing pheromone diversity within specialized glands contributes to the understanding of signal evolution, which is thought to drive diversification of the most species rich family of salamanders, the Plethodontidae.

Methods

Animals

Our analyses focus on three plethodontid salamanders, which span the breadth of divergence in the family. The Western Slimy Salamander (*Plethodon albagula*) is a completely terrestrial plethodontid. The Ouachita Dusky Salamander (*Desmognathus brimleyorum*) and some populations of the Oklahoma Salamander (*Eurycea tynerensis*) are biphasic with terrestrial adults. There are also fully aquatic (paedomorphic) populations of *Eurycea tynerensis.* Images and glands were taken from adult males near the midpoint of the reproductive season, when mental and caudal glands are most prominent. All analyzed males had well-developed testicular lobes indicating full maturity. Tissues from mature paedomorphic and metamorphic females, and larvae of *E. tynerensis* were also used to test whether pheromone genes are male and gland specific. All specimens were handled in accordance with IACUC protocols at the University of Tulsa (TU-0028 and TU-0029).

Gland Morphology

Specimens were anesthetized by submersion in a 0.1% solution of tricaine methanesulfonate (MS-222). Dorsal, ventral, and lateral digital images were taken of each reproductive male with a Pentax K-7 (14.6 megapixel) camera or a Dino-Lite Edge digital microscope. This included close-up images of the mental gland (ventral portion of the head) and caudal gland (lateral view of the tail).

ImageJ (Abramoff et al. 2004) was used to measure the mental gland (ventral view) and the caudal glands (lateral view) from close-up digital images. Gland area was normalized by body size (snout vent length). Normalized gland areas were then log transformed for One-way Analyses of Variances (ANOVA) using the package *stats* in *R* and Fisher's Least Square Difference with Bonferroni correction using the package *agricolae* in *R* to determine if gland size varied significantly among the four groups (*D. brimleyorum*, *P. albagula* and metamorphic and paedomorphic *E. tynerensis)*.

We used histology to assess the cellular-level presence/absence of gland types. Mental glands and caudal glands were dissected from representative *D. brimleyorum*, *P. albagula* and metamorphic and paedomorphic *E. tynerensis*. Glands were fixed in 5% paraformaldehyde, rinsed with phosphate buffered saline, and placed in a 30% sucrose solution. A Microm HM550 Cryostat Microtome (Thermo Fisher) was used to section the glands at 10 microns and the sections were then stained using hematoxylin and eosin (adapted from Fischer et al. 2008). Sections were imaged with an Olympus DP72 camera mount on an Olympus BX53 compound microscope and then processed using the software CellSens.

Transcriptome Sequencing

Mental glands and caudal glands of adult males were dissected from five paedomorphic, five biphasic (completely

metamorphosed) *E. tynerensis,* three *D. brimleyorum,* and three *P. albagula* for transcriptomic analyses. RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA), generally following the manufacturer's protocol, except that the isopropanol precipitation step was performed at − 20 °C. RNA libraries were prepared using Illumina TruSeq RNA prep kit and sequenced for 300 cycles on a MiSeq at the University of Tulsa (Clay et al. 2019 for additional details). The runs on average yielded 1,917,617 reads per sample. Reads with a quality score less than 30 were discarded. Paired end reads were imported directly into CLC Genomics Workbench (version 12.0). De novo assemblies were made for each species and gland. Consensus sequences were extracted from the de novo assemblies and then BLASTx searched against a local database of known SPF, PMF and PRF amino acid sequences. Sequences with strong identity (based on low e-value) were also searched on NCBI GenBank using BLASTx to confirm our identification. We also isolated seven housekeeping genes (*eef1a1*, *hsp90ab1*, *rplp0*, *rpl7*, *rpl27a*, *rpl8*, and *rps8*) from the transcriptomes to normalize our expression values (Table S1). Once the pool of candidate pheromone and housekeeping genes was identified, ExPASy translate tool (Artimo et al. 2012) was used to identify open reading frames (ORFs). Our transcriptomic mining identified complete open reading frames of several *spf* genes in each species, two *pmf* genes and *prf*, which was only identified in *P. albagula*. All genes identified in this study were based on thousands of reads and had more than a 100-x coverage. This supports the accuracy of the assemblies. Even though these sequences may not be an exact representation of the expressed pheromone proteins in the cocktail, for the purpose of this study we refer to our identified sequences as genes because our phylogenetic evidence suggests that they reflect ancient duplications (see below). These genes are highly variable at the nucleotide level even within populations, likely reflecting allelic variation, but this does not confound the deep divergence we identified between genes.

Phylogenetic Analysis of SPF

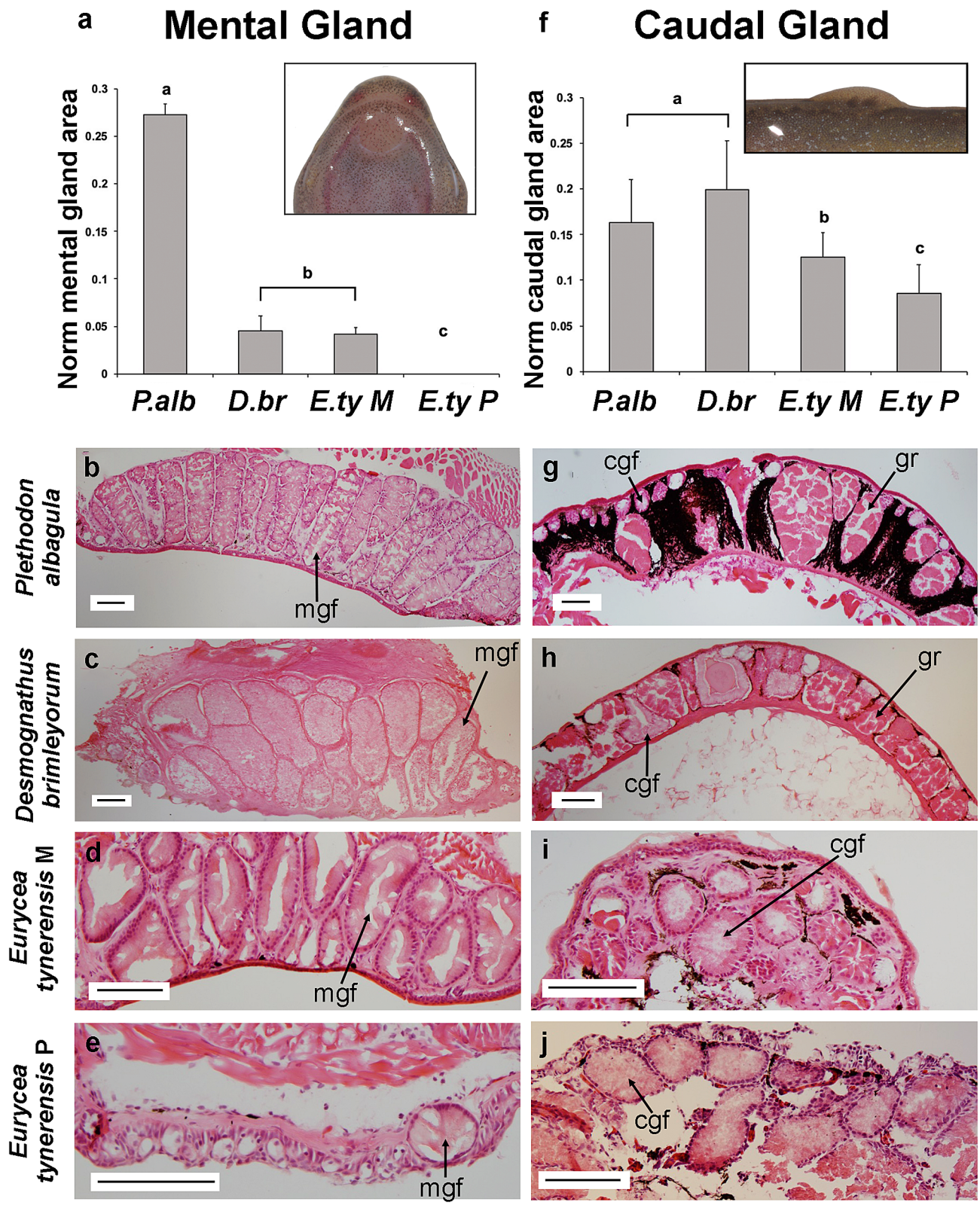
The amino acid sequences were aligned using the BLOSSUM protein weight matrix in Clustal Omega (Sievers et al. 2011). We included representatives of all other major salamander *spf* sequences that have been identified from plethodontids (other *Desmognathus*, *Eurycea,* and *Plethodon* plus *Aneides;* Rollmann et al. 1999; Palmer et al. 2007a, b, 2010; Kiemnec-Tyburczy et al. 2009; Doty et al. 2016), salamandrids (*Ichthyosaur*, *Lissotriton*, *Notophthalmus*, and *Pleurodeles;* Kikuyama et al. 1995; Janssenswillen et al. 2015a; Janssenswillen et al. 2015b; Van Bocxlaer et al. 2015), and ambystomatids (*Ambystoma;* Maex et al. 2016). The final alignment included 52 sequences and was trimmed to 236 amino acid positions (Fig. S1). We used SignalP to predict the signal peptide and cleavage site in the SPF amino acid sequences (Petersen et al. 2011).

Bayesian analyses implemented in BEAST version 2.4 (Bouckaert et al. 2014) were used to reconstruct the phylogeny of amphibian *spf*, under the Blosum62 amino acid substitution model (Henikoff and Henikoff 1992). An uncorrelated lognormal molecular clock and Yule speciation process were used as priors. The analysis was run for 25 million generations, sampling every 1000 generations. Stationarity was assessed by viewing likelihood values across generations in Tracer version 1.5 (Rambaut and Drummond 2007). The first 20% of generations (5000 trees) were discarded as burn-in and median branch lengths were calculated from the 20,001 post-burn-in trees. Node support was evaluated Bayesian posterior probabilities ≥ 0.95. The eight divergent *spf* genes are referred to as: *spfα1*, *spfα2*, *spfα3*, *spfβ1*, *spfβ2*, *spfβ3*, *spfβ4*, *spfβ5* (see Phylogenetic Results). We identified *pmf* in all three species including two divergent genes in *D. brimleyorum,* which we tentatively refer to as *pmf1* and *pmf2*. One of the genes (*pmf1*) also occurs in *P. albagula* and is similar to those previously identified in Palmer et al. (2010). *pmf2,* found in *D. brimleyorum* and *E. tynerensis,* is more divergent. Uncorrected pairwise differences were calculated for the eight SPF amino acid sequences using PAUP v4.0a (Swofford 2003).

Transcriptomic Analyses

We compared relative expression levels in the tissues and individuals for every pheromone gene (*prf*, *pmf1*, *pmf2*, *spfα1*, *spfα2*, *spfα3*, *spfβ1*, *spfβ2*, *spfβ3, spfβ4*, *spfβ5*) that each species had based on total read counts that were calculated using RNA-seq analysis in CLC Workbench. For the RNA-seq analysis the open reading frames were used as a reference to map raw paired end reads. Total read counts of the pheromone genes and the seven housekeeping genes (Table S1) were normalized using *DESeq2* (Love et al. 2014). Normalized read counts were log transformed before performing One-way Analyses of Variances (ANOVA) and Fisher's Least Square Difference with Bonferroni correction to determine if the pheromone genes were differentially expressed among groups (*P. albagula, D. brimleyorum,* metamorphic *Eurycea tynerensis,* and paedomorphic *Eurycea tynerensis*). A Multivariate Analysis of Variances (MANOVA) using *stats* in *R* was also performed to evaluate the expression difference between glands.

We reconstructed ancestral pheromone expression using maximum likelihood with the Asymmetrical 2-parameter Markov k-state model in Mesquite v3.61 (Maddison and Maddison 2018). The phylogeny was pruned from Bonett and Blair 2017 to include *D. brimleyorum, P. albagula* and metamorphic and paedomorphic *E. tynerensis*. Pheromone expression data for each gene and gland were treated as



◂**Fig. 1** Average gland area normalized by snout vent length (SVL) for *Plethodon albagula* (P. alb), *Desmognathus brimleyorum* (D. br), metamorphic *Eurycea tynerensis* (E. ty M), and paedomorphic *Eurycea tynerensis* (E. ty P) (**a**, **f**). **a** Normalized mental gland area. **f** Normalized caudal gland area. Insets are of *Eurycea tynerensis* glands. The letters on the graphs represent significantly different groups as determined by a Fisher's least square difference test. Transverse sections of tissue taken from ventral to the lower jaw (**b**–**e**) and the dorsal base of the tail (**g**–**j**) of reproductive males. Scale bars on lower left corners of images are 200 microns. Sections were cut at ten microns and stained with hematoxylin and eosin. *cgf* Caudal gland follicle, *gr* Granular glands, *mgf* Mental gland follicle

categorical with high expression considered "present" and low/no expression considered "absent".

Evaluation of Pheromone Gene Expression

We used qPCR on *E. tynerensis* to evaluate whether *spf* genes were restricted to expression in male reproductive glands, which were compared to additional tissue types (ventral skin, liver, and head glands), females, and larvae. Head glands in the cheek area were included because of their similar morphology to other courtship glands (Siegel et al. 2020). For gene expression analyses, cDNA was synthesized with SuperScript II (Invitrogen) using random hexamer. Taqman (BHQ1a-6FAM) gene expression assays were developed for *pmf and* seven paralogous *spf* genes (see phylogenetic analyses): *spfα1*, *spfα2*, *spfα3*, *spfβ1*, *spfβ2*, *spfβ3*, *spfβ4* (Table S2). To do this, first we identified the exons within each pheromone gene using genomic sequence data derived from anchored enrichment (Phillips et al. 2017). The OligoAnalyzer PrimerQuest tool was used to identify optimal primer/probe combinations. We chose assays that spanned exon boundaries to avoid genomic contamination. For each gene, primer and probe binding sites were conserved across both paedomorphic and metamorphic *E. tynerensis* (no SNPs), but were highly divergent among genes (many SNPs among pheromone genes). In other words, binding affinity should have been the same between paedomorphs and metamorphs, but assays were likely gene specific. Quantitative PCR reactions were run using ABI TaqMan Gene Expression Master Mix on an ABI StepOne Plus qPCR machine at the University of Tulsa. All compared samples for a given gene were run simultaneously with a six-point standard curve, negative RT reactions, and negative controls. Expression quantity (Qty) values were interpolated from CT values (number of cycles) based on the standard curves for each gene. Qty values for pheromone genes were normalized with the gene coding for ribosomal protein L8 (*rpL8*; Aran et al. 2014), which is commonly used for normalization in amphibian gene expression studies. Normalized Qty values (from qPCR) for the pheromone genes were highly correlated with normalized read counts across samples and tissues (Table S3). This demonstrated the effectiveness of both the pheromone gene assays and the *rpL8* assay as a normalizing quantity for qPCR. For each gene, One-way Analyses of Variances (ANOVA) were performed on the log transformed Qty values, with Fisher's Least Square Difference with Bonferroni correction to determine significant differences among groups (males, females, larvae, life histories, and tissue types).

Results

Gland Morphology

The areas of mental glands ($F_{3,14} = 3833$; $P < 0.001$) and caudal glands ($F_{3,14} = 7.437$; $P < 0.01$) differed significantly among groups. Histologically, both mental and caudal glands are composed of individual gland follicles that often cluster together. *Plethodon albagula* had the largest relative mental gland given body size followed by *Desmognathus brimleyorum* and metamorphic *Eurycea tynerensis* with similarly sized mental glands (Fig. 1a, b). Macroscopically, paedomorphic *Eurycea tynerensis* have no obvious mental gland. However, after histological analyses of the ventral chin area, small individual mental gland follicles were found in a few specimens (Fig. 1). The individual follicles are around fifty times smaller than the average mental gland aggregation in metamorphic *Eurycea tynerensis. P. albagula* and *D. brimleyorum* had the largest caudal glands given body size followed by metamorphic *E. tynerensis* and then paedomorphic *E. tynerensis* with the smallest. Our histological examination of the caudal glands allowed us to determine their follicular composition. *Plethodon albagula* and *D. brimleyorum* caudal glands are composed mainly of granular glands with caudal gland follicles that are smaller and more widely dispersed (Fig. 1). In contrast, *E. tynerensis* caudal glands are comprised mainly of caudal gland follicle aggregations (Fig. 1).

Phylogenetic Analysis of SPF

Across plethodontids we identified at least eight divergent *spf* genes (Fig. 2). Two highly divergent versions of *spf* have been previously identified in newts (referred to as *spfα* and *spfβ*; Janssenswillen et al. 2015a; Van Bocxlaer et al. 2015). We also found strong support for two major clades of SPF (Fig. 2). At least three of the genes are in the *spfα* clade and five are in the *spfβ* clade. We refer to these as *spfα1*, *spfα2*, *spfα3*, and *spfβ1*, *spfβ2*, *spfβ3*, *spfβ4* and *spfβ5*, respectively. The majority of research on *spf* in plethodontids has been with *spfα1* but more recently *spfα2* was identified in *Desmognathus ocoee* mental glands (Doty et al. 2016; Fig. 2) In addition we identified *spfα3,* which is related

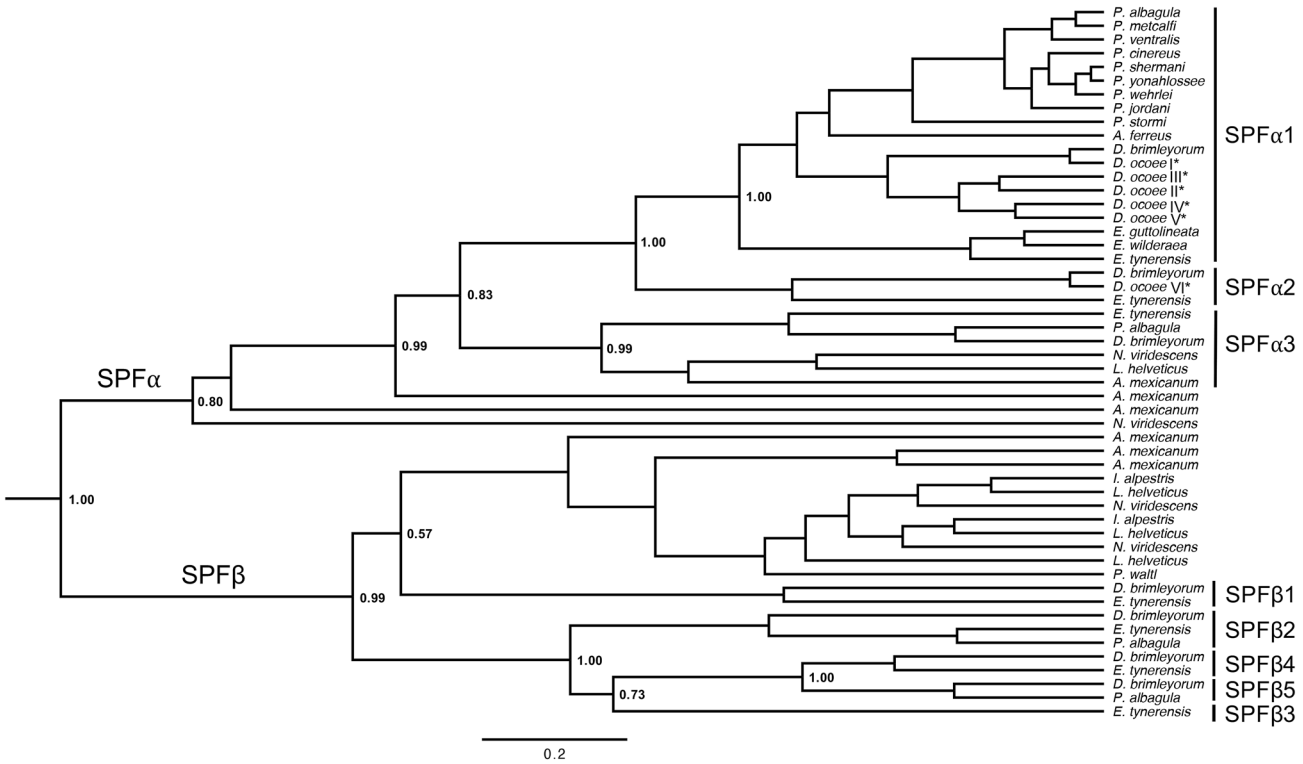


Fig. 2 Bayesian phylogenetic analysis of SPF proteins indicating the eight different genes identified in plethodontids (three *α* and five *β*). Asterisks indicate previously identified *spfα1* and *spfα2* from *Desmognathus* mental glands (Doty et al. 2016). Bayesian posterior probabilities indicate support for major nodes. Scale bar represents number of substitutions per site

to *spfα* previously known from newts and ambystomatids (Janssenswillen et al. 2015a; Van Bocxlaer et al. 2015; Maex et al. 2016). This indicates at least two duplications of *spfα,* one prior to the divergence of salamandroids (150 MYA; Anderson 2012; Gao and Shubin 2012) and the other prior to the deepest divergence within plethodontids giving rise to *spfα1* and *spfα2* (66 MYA; Shen et al. 2016). We identified several plethodontid specific duplications of *spfβ* that are sister to the *spfβ* previously identified in salamandrids and ambystomatids (Fig. 2). The uncorrected pairwise differences between SPF amino acid sequences ranged from ~ 50 to 80% (Table S4), which matches phylogenetic divergence (Fig. 2). All identified SPFs are predicted to have a signal peptide except for *spfβ3*, which has thus far only been identified in *E. tynerensis* (Table S5). The signal peptide length ranged from 17 to 39 amino acids (mode = 19). There were a few ambiguous amino acids (< 1%; Xs in sequences) within our ORFs due to intraspecific variation at the nucleotide level but this is minimal compared to the amino acid divergence among genes (Table S4).

Differential Expression of Pheromone Genes

Overall expression of pheromone genes differed between mental and caudal glands (MANOVA, $F_{1,30} = 6.9239$; $P < 0.001$). The pheromone genes mainly expressed in the mental gland were *prf*, *pmfs*, *spfα1*, and *spfα2* (Fig. 3). All of these genes, as well as *spfα3*, and *spfβ2* had significantly different expression levels among species and life cycle modes (Table S6). *P. albagula* was the only species to express *prf* and it is the most highly expressed pheromone transcript in their profile. *D. brimleyorum* expressed both *pmf* genes identified, while the other species only expressed one. The pheromone transcript most highly expressed in *D. brimleyorum* mental glands was *spfα1,* while *spfα2* had the highest expression in *E. tynerensis* mental glands (Fig. 3). Paedomorphic *E. tynerensis* had significantly lower levels of expression in their mental gland area as compared to metamorphic *E. tynerensis, D. brimleyorum,* and *P. albagula* (ANOVA, $F_{1,14} = 10.337$; $P < 0.01$) (Fig. 3).

The five *spfβ* genes were mainly expressed in the caudal glands and at significantly different levels among species and life cycle modes (Table S6). The caudal glands of paedomorphic and metamorphic *E. tynerensis* had very similar expression profiles to each other as compared to *P. albagula* and *D. brimleyorum* (ANOVA, $F_{1,14} = 453.19$; $P < 0.01$) (Fig. 3). Unlike the other two species, *D. brimleyorum* only expressed *spfβ2* in the mental gland (Fig. 3). *D. brimleyorum* and *P. albagula* caudal glands expressed *spfβ5*, while *spfβ3* was

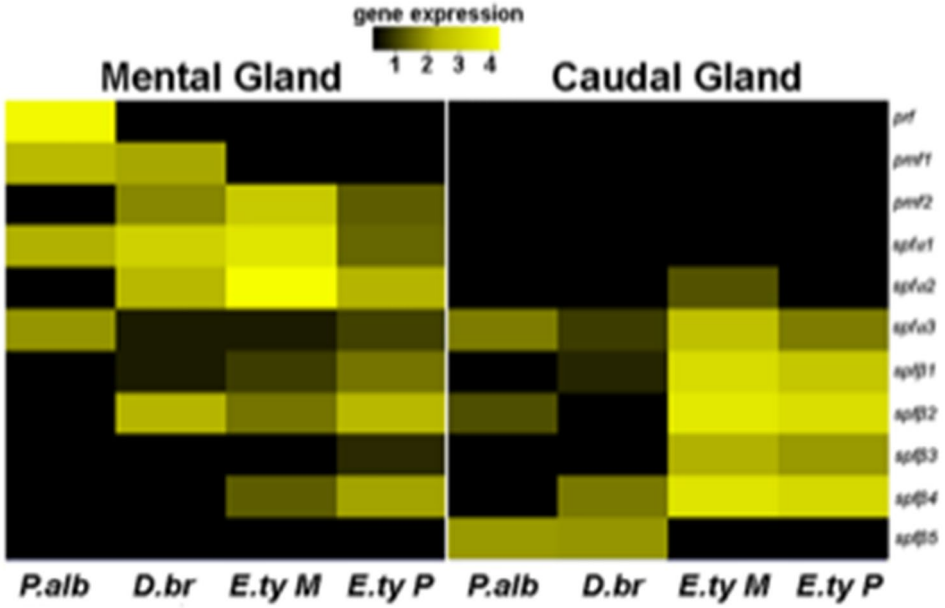


Fig. 3 Heatmap depicting log10 gene expression levels of eleven different pheromone genes in the mental and caudal glands of adult male *Plethodon albagula* (P. alb), *Desmognathus brimleyorum* (D. br), metamorphic *Eurycea tynerensis* (E. ty M), and paedomorphic *Eurycea tynerensis* (E. ty P)

only in *E. tynerensis* (Fig. 3). *P. albagula* lacked expression of both *spfβ1* and *spfβ4* (Fig. 3).

Further evaluation of putative pheromone genes in *Eurycea tynerensis* using quantitative PCR analyses show expression values highly consistent with normalized results from RNA-seq. There were significant differences in variation among groups for all eight pheromone genes: *pmf* ($F = 4.423$); *spfα1* ($F = 4.427$); *spfα2* ($F = 16.791$); *spfα3* ($F = 3.842$); *spfβ1* ($F = 8.613$); *spfβ2* ($F = 6.311$); *spfβ3* ($F = 9.247$); *spfβ4* ($F = 6.724$); $P < 0.001$ for all genes. Post hoc comparisons with Bonferroni correction showed that *pmf*, *spfα1* and *spfα2* had significantly higher expression in mental glands of metamorphs compared to all other groups (paedomorphs, females, larvae, and other tissues) (Fig. S3). Metamorphic males expressed *spfα3* at significantly higher levels in their caudal glands compared to all other groups (Fig. S3).

As for the *spfβ*s, *spfβ1* and *spfβ3* were more highly expressed in the caudal glands of both paedomorphic and metamorphic males compared to all other groups. However, the expression levels of both these genes in the mental glands and caudal glands of paedomorphs and metamorphs were statistically the same. Paedomorphic males had the highest expression of *spfβ2* in their caudal glands compared to all other groups (Fig. S3). Overall, *spfβ4* is expressed at much lower levels but was highest in the caudal glands of metamorphic and paedomorphic males. Despite its close topographical location to the mental gland, quantitative PCR analyses demonstrated that pheromone gene expression in the lateral head glands is more similar to the caudal gland. Furthermore, pheromone genes were not (or only negligibly) expressed in control male tissues (ventral skin and liver), adult paedomorphic females, adult metamorphic females, or larvae in regions equivalent to where these genes are expressed in males (below the chin and above the base of the tail or tailfin). In summary, it is most likely that ancestral plethodontids expressed *pmf, spfα1,* and *spfα2* in the mental gland and *spfβ1, spfβ2,* and *spfβ3* in the caudal gland (Fig S2). We didn't find evidence of *prf* outside of *Plethodon* mental glands (Fig. S2).

Discussion

The duplication of molecular signaling pathway components can result in the divergence of expression levels and spatial distributions (Hughes 1994; Prince and Pickett 2002; Makova and Li 2003; Huminiecki and Wolfe 2004; Li et al. 2005; Proulx 2012). In salamanders, extensive pheromone diversification has led to variation in cocktail combinations, which has been considered an impetus for lineage proliferation (Palmer et al, 2005, 2007b 2010; Wilburn and Swanson 2016). Our phylogenetic analyses of *spf* genes sampled from divergent plethodontids revealed multiple duplications within both the alpha and beta subfamilies. We found gland specific expression of

three pheromone gene families (*prf*, *pmf*, and *spf*; Fig. 4). Loss of the expression of these genes coincides with the reduction of a mental gland in the absence of metamorphosis in paedomorphic *Eurycea tynerensis* (Fig. 4). The proliferation of pheromone coding genes in plethodontids generates diverse pheromone profiles, and we found that their subfunctionalization (described below) among glands provides opportunity for modular segregation of signaling during courtship.

Pheromone Evolution and Gland Diversification

Following gene duplication, the divergence of expression from an ancestral gene to the daughter genes (subfunctionalization) provides opportunity for tissue specificity and diversification of expression profiles (Hughes 1994; Makova and Li 2003; Gu et al. 2004; Huminiecki and Wolfe 2004; Li et al. 2005; Freilich et al. 2006). This is particularly evident in large gene families (Huminiecki and Wolfe 2004). In salamanders, *Sodefrin Precursor-like Factor* and *Plethodontid Modulating Factor* are large gene families with numerous duplicates (Palmer et al. 2010; Wilburn et al. 2012, 2017; Van Bocxlaer et al. 2015; Wilburn and Swanson 2016). Some copies of *spf* have broad expression patterns across unique male reproductive glands within and between species (Janssenswillen et al. 2015b). For example, we found that *spfα3* along with the *spfβ* genes are expressed across multiple tissue types. Our phylogenetic analyses revealed that *spfα3* emerged before the divergence of salamandroids and is therefore more widely expressed across lineages. In

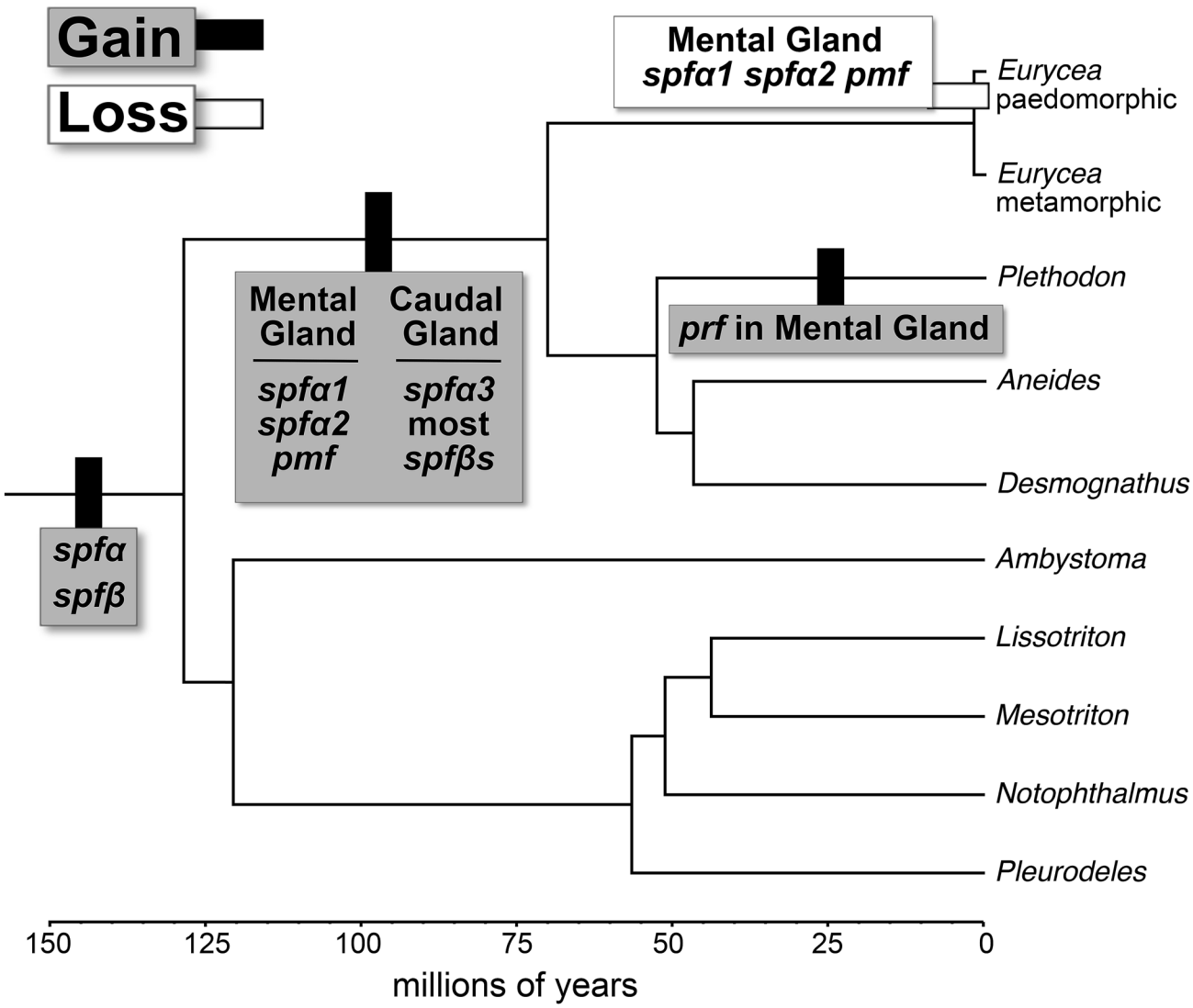


Fig. 4 Phylogeny of representative salamander genera depicting the gains (black hashes) and loss (white hash) of recognized pheromone genes and associated male reproductive glands in plethodontids

at least one widespread species of newt, *Notophthalmus viridescens*, many *spf* copies are expressed in both cheek and cloacal glands, demonstrating broad tissue expression (Janssenswillen et al. 2015b).

In contrast, we found evidence of gland specificity within each of the pheromone gene families. Expression of *pmf, prf, spfα1* and *spfα2* is specific to the mental gland and is even absent from adjacent lateral head glands (Fig. S3). Mental glands are an ancestral character (Sever et al. 2016) of plethodontids and this novel gland type may have presented an opportunity for tissue specificity of the duplicated pheromone genes. Caudal glands also seem to be unique to plethodontids, but their appearance is much more subtle in many species (Rupp and Sever 2017). The *spfβ* genes are largely restricted to the caudal gland as well as lateral head glands (with the exception of *spfβ2* in *D. brimleyorum*). These two novel glands provided the framework for the co-option of gene duplicates into different gland types.

Subfunctionalization of duplicate genes can not only lead to tissue specificity but also specialization of function within the tissue (Huminiecki and Wolfe 2004; Li et al. 2005; Freilich et al. 2006). Salamander courtship is comprised of a series of behavioral modules and each gland is associated with a different piece of the repertoire (Arnold et al. 2017). The evolution of gland-specific pheromone cocktails may allow for specialization of signals within the courtship ritual. During stereotypical salamander courtship of plethodontids and rhyacotritonids (a related family), males utilize a tail straddle walk (Arnold et al. 2017) where females could come into contact with SPFβs exuding from the caudal gland. The mental gland evolved before the diversification of plethodontids and is thought to be associated with terrestrial courtship (Sever et al. 2016). The ancestral delivery mechanism involves males scraping the female with enlarged premaxillary teeth and delivering the mental gland pheromones transdermally through abrasions (Arnold et al. 2017), which is coincident with the expression specificity of *prf, pmf,* and *spfα1* and *spfα2*. In our study we observe an intriguing loss of the mental gland and associated pheromones in paedomorphic *Eurycea tynerensis* (Fig. 4). The presence of this gland varies among plethodontids and could have consequences for pheromone diversification, reproductive isolation, and speciation.

Consequences for Speciation

Differences in mate recognition signals can instigate and maintain reproductive isolation, which is an important avenue for speciation (Nei et al. 1983; Symonds and Elgar 2008; Wicker-Thomas 2011; Allison and Carde 2016; Treer et al. 2018). It has been hypothesized that the diversification and rapid evolution of the salamander pheromone families is due to a "molecular tango", meaning, coevolution between male pheromones and female receptors (Palmer et al. 2005, 2007a, b). The two major avenues that still need to be addressed regarding this hypothesis are: (1) identifying candidate female receptors for SPF, PMF, and PRF (Palmer et al. 2005); (2) testing for a relationship between sexual selection, pheromone evolution, and species diversity (Woodley et al. 2010; Woodley and Staub 2021). For instance, recent examination of two closely related but reproductively isolated newts showed distinct male pheromone cocktails (Treer et al. 2018). However, it is unclear whether or not these species-specific pheromone blends contributed to reproductive isolation (Treer et al. 2018).

In our study we observed saltatory shifts in pheromone gene blends between paedomorphic and metamorphic *E. tynerensis.* The reduction of the mental gland in paedomorphic *E. tynerensis* has led to a loss of pheromones (*pmf*, *spfα1* and *spfα2*) in the chin. *Eurycea tynerensis* utilize the ancestral tail straddle walk, but only metamorphs utilize mental gland transdermal pheromone delivery (personal observation). In newts and ambystomatids there is little evidence for reproductive isolation between paedomorphs and metamorphs (Denoël et al. 2001; Krenz and Verrell 2002; Whiteman et al. 2006; Oromi et al. 2016). We have population genetic evidence of isolation between paedomorphic and metamorphic *E. tynerensis* in sympatry (Bonett unpublished), but it still needs to be determined whether this is driven by a saltatory loss of pheromones and a mental gland delivery mechanism. Exploration of pheromone diversity across glands and life cycle modes in plethodontids is needed to understand how development influences pheromone expression and speciation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00239-021-10026-0.

Acknowledgements We thank N. Ledbetter and B. Molone for assisting with field work and salamander collection. We would also like to thank A. Trujano, who helped with RNA extractions and cDNA synthesis and C. Burleson who annotated gene transcripts. Finally, we thank M. Howery of the Oklahoma Department of Wildlife Conservation and K. Irwin of the Arkansas Game and Fish Commission for permitting logistics.

Author Contributions MAH, MAS, and RMB designed the study and performed analyses. All authors participated in collecting data and preparing the manuscript.

Funding This study was in part funded by the National Science Foundation (DEB1050322 and DEB1840987 to RMB; DEB1210859 to RMB and MAS) as well as by the University of Tulsa through the Student Research Grant program.

Data Availability Gene transcripts are available on GenBank. Histological slides, specimens, and tissue samples are available from RMB.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Consent for Publication All authors have approved of the manuscript prior to submission.

Ethical Approval Specimens were handled in accordance with Institutional Animal Care and Use Committee (IACUC) protocols at the University of Tulsa (TU-0028 and TU-0029).

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