CHEMICAL COMMUNICATION IN A DECLINING NORTH AMERICAN ANURAN AMPHIBIAN, THE NORTHERN LEOPARD FROG (*LITHOBATES PIPIENS*)

BY

BRITTNEY GRAHAM

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Natural Resources and Environmental Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2017

Urbana, Illinois

Master's Committee:

Adjunct Professor Jinelle H. Sperry, Advisor
Associate Professor Michael P. Ward
Assistant Professor Alison M. Bell
Dr. Irene E. MacAllister

Abstract:

Chemical signaling is known to be an important communication mechanism for amphibians. However, very few studies have directly investigated chemical signaling in anuran groups (frogs and toads), particularly adult anurans. Previous work has focused primarily on salamander and larval anuran behavioral responses. Additionally, chemoreceptor (CR) genes have only been identified for *Xenopus* species with no previous identifications for any other adult anuran species. Here, I examine the evolutionary and functional implications of northern leopard frog (*Lithobates pipiens*) CRs, identified through collaboration, by conducting a phylogenetic analysis for each CR type identified using a Maximum Likelihood approach (Chapter 1). I incorporated amino acid CR sequences spanning aquatic, semiaquatic, and terrestrial vertebrate species, and the resulting trees indicate split divergence patterns across CR types. More specifically, olfactory receptors (ORs) and trace amine associated receptors (TAARs) appear to be most closely related to transitional vertebrate and terrestrial species' orthologs, and vomeronasal receptors (VRs Types I and II) appear to be most closely related to transitional vertebrate and aquatic vertebrate orthologs, hinting at both volatile and aquatic (water soluble and nonvolatile) detection of chemicals.

In this study, I also investigate terrestrial, sex-biased anuran behavioral responses to conspecific chemical cues in adult *L. pipiens*, comparing time spent on cue and non-cue sides of an experimental terrarium (Chapter 2) to expand on current knowledge of chemical detection in a declining, North American species. Results show nuanced response profiles for males and females, based on familiarity (odors of individuals housed together and those that were housed in separate enclosures) and same-sex vs. opposite-sex cues.

Females were more responsive overall (P <0.005), investigating unfamiliar, opposite sex and familiar, same-sex odors. Though not statistically significant (P=0.40), the data (see Figure 7.) suggest that male investigation of cue and non-cue sides differed slightly for familiar, same-sex conspecifics. Neither sex appeared to respond to unfamiliar, same-sex odors. Intuitively, ecological benefits associated with familiar odor detection and mate localization are likely responsible for these trends.

Collectively, my results expose CR divergence patterns across aquatic, semiaquatic, and terrestrial species and help elucidate conspecific ligand functional roles that influence *L. pipiens* behavior.

Dedicated to Diane Nicolette Graham

Acknowledgments:

To begin with, I would like to sincerely thank my project advisors, Drs. Jinelle Sperry and Irene MacAllister, for granting me this tremendous educational opportunity. I have learned a great deal about the investigative process and problem-solving in research while employed at the U.S. Army Engineer Research and Development Center (ERDC) Construction Engineering Research Laboratory (CERL) and throughout my University of Illinois' Graduate College attendance. I have also gained critical proposal writing and scientific mentoring experience, crucial guidance on experimental design and statistical methodology in ecological research, and, lastly, invaluable molecular and chemical characterization laboratory experience. Furthermore, Jinelle and Irene have both gone above and beyond the call-of-duty for any typical academic advisor. They supported and encouraged me through a tremendously difficult transition in my personal life this past year after I lost my mother, and their patience, understanding, and flexibility ultimately facilitated my completion of this work. For that, I am inexpressibly grateful. Their compassion and dedication to their students is inspiring, and I hope to emulate these qualities myself as I progress as a scientist.

I would also like to thank my collaborators on the project, Dr. Ping Gong and Jessica Kirkpatrick for their expertise in bioinformatics, as well as my dedicated team of laboratory technicians: Clayton Dilks, Dylan O'Hearn, Abigail Brake, Zach Williams, Brennan Wright, and Kapil Thatcher for contributing their time and intellectual resources to the project. Clayton Dilks and Dylan O'Hearn in particular dedicated a great deal of time and effort assisting with a number of significant equipment-related hurdles along the way, and I could not have completed all necessary project tasks without their help. Additionally, I would like

to thank Dr. Hugh Robertson, for his expertise and guidance on phylogenetic analysis, and all of the Sperry lab members for supporting me and offering helpful advice, both scholastically and otherwise throughout my stay at the University of Illinois. Valerie Buxton deserves a special thank you, for going out of her way to provide comfort and relatable counsel after my mother passed.

Of course, committee members, DAR animal care staff, Anita Purves Nature Center staff, the Carl R. Woese Institute for Genomic Biology at UIUC staff, and the U.S. Army Corps of Engineers Basic Research program facilitators all deserve thanks for their cooperation, involvement, and support of this research as well. The U.S. Army Corps of Engineers Basic Research Program, specifically, funded this research and made the entire experience possible.

Furthermore, I would like to express my sincerest gratitude towards my friends and family for their encouragement, patience and essentially for putting up with my long nights, busy weekends, and a slew of equipment-related frustrations for the past two years. I was certainly not the easiest person to be around at times, and all of them stuck by me regardless. To my husband who pulled countless all-nighters along with me—going on supply runs for food and caffeine and keeping me motivated to push through the exhaustion, I could not be more grateful for your presence in my life. And I could not have gotten through this past year without you.

Finally, I would like to dedicate this Masters Thesis to my mother, Diane Nicolette Graham, who passed away very suddenly in March of 2017. She was my biggest cheerleader, and she inspired me every single day to work towards making this world a

better place. She fiercely believed in me, and I would not be where I am today without her.

I love you, mom, and I will carry your love and memory with me always.

TABLE OF CONTENTS

Chapter 1: Identification and Phylogenetic Analysis of Putative Chemosensory Receptors in Adult Northern Leopard Frog (*Lithobates pipiens*) Olfactory Epithelium

Abstract:

Animals rely on their sense of smell to detect chemical messages in their environment. This chemical language informs individuals on the presence of food/prey, potential mates, suitable habitats, and predators. G-protein coupled receptors, which bind chemical cues and transmit the message to olfactory neurons, are the basis of olfaction, and few studies have identified full-length chemoreceptor proteins in adult anuran olfactory epithelium. Here we performed an RNAseq (i.e., sequencing of transcriptome-wide transcripts) of Northern Leopard Frog olfactory epithelium to identify four types of candidate chemosensory receptors: olfactory receptors (ORs), vomeronasal type 1 and type 2 receptors (V1Rs and V2Rs) and trace amine associated receptors (TAARs). By comparing the identified receptors with other vertebrate orthologs via phylogenetic analysis, we elucidated the evolutionary trajectory of vertebrate chemoreception across terrestrial and aquatic groups. Finally, we identified the transcripts putatively coding for chemosensory receptor accessory proteins Gαolf and transient receptor potential channel 2 (trpc2).

Key words: amphibian, olfactory epithelium, G-protein, vomeronasal organ, olfactory receptor, pheromone receptor

Introduction:

Chemical signaling is a well-studied aspect of animal communication and has been shown to impact numerous facets of reproduction and survival (Wyatt 2003; 2010). Within this context, amphibians present a unique and interesting case of chemical signaling due to their biphase life history where they likely retain the ability to sense waterborne, volatile, and nonvolatile odorants (Belanger and Corkum 2009; Woodley 2014; Woodley 2015). For instance, a variety of salamanders and frogs have been observed producing water-soluble and nonvolatile pheromones (Belanger and Corkum 2009; Houck 2009; Wyatt 2003; Woodley 2010; Cummins and Bowie 2012), and other amphibians are known to emit volatile constituents; which, intuitively, may act as a form of chemical signaling (Poth et al. 2012; Poth et al. 2013; Starnberger et al. 2013). Behavioral assays have also demonstrated amphibian use of olfaction to sense both volatile and waterborne cues from various sources (Mason et al. 1998; Belanger and Corkum 2009). Although the vast majority of amphibian chemical sensing research has thus far targeted salamander clades; there is growing recognition of the importance of chemoreception in other amphibian groups, even for some anuran amphibians previously presumed to rely exclusively on auditory signaling (Kiemnec 2009; Belanger and Corkum 2009; Poth et al. 2012; Woodley 2015).

Although these studies have helped elucidate the chemical cues used by amphibians and their associated behavioral responses, very few studies have attempted to examine the molecular underpinnings of olfaction, especially for anuran groups (Mezler et al. 2001). A molecular-level understanding of how amphibian chemoreception functions, across clades, could drastically improve our knowledge of the evolution and functional importance of amphibian chemoreception. Thus, here we identify candidate chemoreceptor (CR) genes within the olfactory epithelium of the northern leopard frog (*Lithobates pipiens*) and

conduct phylogenetic analyses to explore the evolutionary trajectories of these proteins across vertebrate groups and generate predictions regarding possible ligand groups. We aim to provide a platform for future research attempts to deorphan anuran CRs. The northern leopard frog, *L. pipiens,* was selected as our focal species because it inhabits both terrestrial and aquatic environments (MacAllister et al. 1999; Kendell 2002), which makes the odorant space it can potentially sense quite large. Also, previous studies indicate a variety of olfactory responses by both southern and northern leopard frogs (*L. pipiens* and *Lithobates sphenocephalus*) during various stages of development (Shinn and Dole 1978; Glennemeier and Denver 2002; Johnson et al. 2003).

Broadly, the molecular basis of olfaction is the interaction between a chemoreceptor and its cognate ligand or set of ligands (Buck and Axel 1991;1992). The binding event of a compound to the CR initiates a series of Guanine nucleotide binding protein (G-protein)-mediated signal transduction cascades, leading to a cation influx via cyclic nucleotide-gated ion channels and Ca^{2+} activated Cl^- channels (Touhara et al. 2006). The end result is a depolarization of the neuron and transmission of information to the brain.

In vertebrates, there are three primary types of receptors that detect odorants (Touhara and Vosshall 2009). These are olfactory receptors (ORs), and vomeronasal receptors, type 1 and type 2 (V1Rs and V2R respectively), and each belong to a G protein-coupled receptor (GPCR) super family of integral membrane proteins (Touhara and Vosshall 2009). There are also a few trace amine associated receptors (TAARs) thought to be specific for amine chemical cues (Borowsky et al. 2001). Despite extraordinary sequence diversity, all are presumed to have a similar tertiary structure (Freitag et al. 1998; Niimura 2013). The binding specificities of CRs can vary considerably. For example, an individual CR

can bind multiple different odorants (i.e. exhibit broadly tuned binding) and an individual odorant can be bound by multiple *different* receptors (Malnic et al. 1999; Grus and Zhang 2008; Hashiguchi et al. 2008; Spehr and Munger 2009). In contrast, pheromone receptors tend to be narrowly tuned to an individual pheromone (Grus and Zhang 2008; Touhara and Vosshall 2009).

As GPCRs, ORs are coexpressed with specific G-proteins that serve to mediate the odorant signaling cascade. Current evidence suggests that ORs are typically coexpressed with Gαs/Gαolf proteins; V1Rs with Gαi2 proteins, and V2Rs with Gαo proteins (Dulac 2000; Kajiya et al. 2001; Kiemnec-Tyburczy et al. 2011). V1R and V2R expressing sensory neurons are also known to depend on the phospholipase C- and the diacylglycerol-mediated transduction pathway that ultimately leads to the activation of the canonical Transient receptor potential cation channel, subfamily C, member 2 (trpc2) protein (Inamura et al. 1997; Lucas et al. 2003; Liberles 2014). The trpc2 is a cation channel critical for signal transduction in the VNO of rodents, and trpc2 specifically is almost ubiquitous across vertebrate species (Liman 1999; Leypold et al. 2002; Stowers et al. 2002; Kiemnec-Tyburczy et al. 2011). This suggests a highly conserved functional role in odorant detection.

For terrestrial vertebrates, evidence suggests that both ORs and TAARs are broadly tuned receptors, while V1Rs and V2Rs are more narrowly tuned binders, as they appear to be more divergent (Grus and Zhang 2008; Hashiguchi et al. 2008; Spehr and Munger 2009). Additionally, a variety of small volatile odorants have been shown to bind V1Rs in mammals, while V2Rs have been hypothesized to bind larger water-soluble molecules; specifically peptides and proteins (e.g. ESP1 in mice; Boschat et al. 2002; Leinders-Zufall et

al. 2004; Spehr and Munger 2009; Haga et al. 2010; Wyatt 2010; Kiemnec-Tyburczy et al. 2011). However, this trend does not appear to hold for squamate reptiles (Brykczynska et al. 2013), since they are known to detect volatile odorants through the vomeronasal system (VNS) but their V2R repertoire is much more expansive than their V1R repertoire. Whether or not this hypothesis is supported across amphibian clades remains unknown, as even primarily terrestrial amphibian species respond to waterborne chemical cues through the VNS (Kiemnec-Tyburczy et al. 2011).

Additionally, at least for one salamander species, the red-legged salamander (*Plethodon Shermani*), it appears that the majority of known V2R receptor proteins more closely resemble African Clawed Frog (*Xenopus laevis)* rather than zebrafish (*Danio rerio*) receptors, suggesting that the divergence of CR proteins dates back to a common tetrapod ancestor that may have begun to adopt a partially terrestrial lifestyle (Kiemnec-Tyburczy et al. 2011). Again, it is presently unknown if this conservation pattern holds for other amphibian species. If validated, as Kiemnec-Tyburczy et al. suggested, these V2R trends may accompany adaptations related to a transitional lifestyle for amphibians (2011).

Importantly, the OR, V1R, V2R, and TAAR chemoreceptor-coding scheme allows a limited number of receptors to cover a vast odorant landscape, which makes inferring the ligand specificities of each receptor difficult across animal groups. Furthermore, despite great strides made in the identification of olfactory receptor genes through the advent of genome sequencing (Niimura and Nei 2006; Shi and Zhang 2007; Nei et al. 2008), few CR-ligand pairs have been uncovered, and, to our knowledge, none have been identified for amphibians (Woodley 2010). Still, increasing the number of identified chemoreceptor sequences for different amphibian species, especially anuran species--considering the

current gap in the literature, can help generate evolutionary inferences based on known orthologs, hinting at trace chemoreceptor divergence and possible function. A comparison can allow for indirect ligand-class inferences, which in turn may facilitate more efficient screening of suspected ligand compounds with behavioral or physiological relevance. Identifying full-length chemoreceptor proteins and their linked G-proteins (e.g. coexpressed Gα and trpc2 genes) can also help clarify if the functions of these receptor proteins are likely conserved in particular amphibian groups evolutionarily and hint at its overall importance to individual survival.

In this study, our objective was to identify putative OR, V1R, V2R, and TAAR sequences in *L. pipiens* olfactory epithelium through RNA sequencing, along with putative G-proteins and trpc2 like proteins, and to phylogenetically screen the receptor sequences against known vertebrate sequences in terrestrial, semiaquatic, and aquatic species. If the receptor proteins discovered in our study are shown to be functional, a comparison to other vertebrate orthologs could narrow down which class of compounds likely bind identified *L. pipiens* receptors, along with their environmental origins (i.e. aquatic vs terrestrially derived cues); which ultimately helps elucidate the evolutionary relationships of these receptors.

Methods:

Tissue Collection and RNA Extraction Protocol:

Adult *L. pipiens* individuals were obtained from Kons Scientific Co. (Germantown, WI). The specimens were anesthetized using Pharmaceutical grade tricaine

methanesulfonate (MS-222) and sacrificed in accordance with approved protocols: IACUC number 15013 and USAMRMC proposal number ERDC-FY-16-002.

Olfactory epithelium from each individual was excised and submerged in RNALater solution (Ambion). Samples were immediately placed on ice and then transferred to cold storage within 4 hours of collection. The olfactory epithelium from nine *L. pipiens* frogs (N=9) was pooled and total RNA was isolated using the RNeasy Plus Universal Mini Kit (Qiagen) as per the manufacturer's instructions. Total RNA was quantified via the Quant-iT Broad Range assay kit (ThermoFisher) and the integrity was assessed via denaturing gel electrophoresis.

Transcriptomic Sequencing and Data Analysis:

Sequencing: The pooled total RNA was analyzed at the University of Illinois at Urbana-Champaign's (UIUC's) Roy J. Carver Biotechnology Center (RJCBC). An RNAseq library was prepared using a TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA) and quantitated by qPCR. The library was sequenced in a 161-cycle, paired-end run using a HiSeq SBS Kit V4 on a HiSeq 2500 platform. Sequencing reads were demultiplexed, adaptor-trimmed and converted into FASTQ format using Illumina's bcl2fastq Conversion software (version 2.17.1.14). Subsequent data processing and bioinformatics analysis were performed on Biocluster (24 Intel Xeon 2.7 GHz CPU cores and 384 GB of RAM per node), a high performance computational cluster available at the Carl R. Woese Institute for Genomic Biology in UIUC's RJCBC.

Pre-processing: FASTQC (version 0.11.2) was used to assess the quality of the sequencing data before and after further cleaning. Trimmomatic (version 0.33) was used to

trim and filter any residual adaptor content and low quality bases with the following settings: ILLUMINACLIP:TruSeq3-PE-2.fa:2:15:10 LEADING:28 TRAILING:28 SLIDINGWINDOW:3:15 MINLEN:30.

De novo transcriptome assembly: The cleaned reads were normalized and assembled into a transcriptome using Trinity (version 2.0.6) on 24 CPU cores with 300GB of RAM. Quality assessment and filtering of the assembly was performed using Transrate (version 1.0.1) by mapping normalized reads back to the assembly.

Preliminary CR identification: We curated functionally characterized or computationally inferred putative OR, VR and TAAR gene sequences in up to 26 fish, amphibian, and reptile species deposited in NCBI's GenBank (see Table 1.2 below). BLAST+(version 2.2.31) was utilized to construct a subject nucleotide database of the filtered assembly by running the makeblastdb script. Next we queried the known CRs against the subject database using tblastx under the following settings: low-complexity filtering turned off, *E*-value threshold = 1e-5, and output in BLAST archive format. The blast_formatter script was run to convert the output format to tabular. Only the top hit for each subject sequence was retained. These BLAST hits were further filtered by setting a threshold of alignment length >= 250 amino acids.

Transmembrane domain identification: The remaining contigs were run through OrfPredictor (Min et al. 2005) to identify their protein-coding region (i.e., Open Reading Frame or ORFs). The ORFs were further analyzed using TMHMM (Krogh et al. 2001) to predict transmembrane domains (TMDs). Contigs containing both complete ORFs and 7 TMDs (Shi and Zhang 2009) were considered putative OR, V1R, V2R or TAAR genes in the adult *L. pipiens* olfactory epithelium.

In parallel, we also used SOAPdenovo-Trans v.1.03 (Xie et al. 2014) to assemble another transcriptome from the same RNA sequencing dataset (Gong et al. Manuscript in preparation) and followed the same procedure as described above to identify and annotate putative CRs. This was necessary because significant discrepancies often exist between transcriptomes assembled using different assemblers, leading to significant differences in both the number and the sequences of putative CRs inferred.

Transient receptor potential channel trpc2 and coupled G-protein transcripts were also identified from both Trinity- and SOAPdenovo assemblies using the same pipeline, specifying *E*-value thresholds of 1e-20 and 1e-10, respectively.

Phylogenetic Analysis of Putative Chemosensory Receptor Genes:

Putative CR contigs shared between the Trinity- and the SOAPdenovo-assembled transcriptomes (full-length identity >= 95%) were selected to create a shortlist of sequences for each type of CR identified (i.e., 93 ORs with detailed 3' and 5' ends, 2 V1Rs, 4 V2Rs, and 1 TAAR). Geneious version 10.1.3 (http://www.geneious.com, Drummond 2012; Kearse et al. 2012) was then used to create separate CR BlastP databases for ORs, VRs, and TAARs incorporating known CR gene ORF sequences across 26 species (for a total of 4605 OR ORFs, 541 VR ORFs, and 571 TAAR ORFs; see Table 1.2 below) for direct screening against the shortlist of *L. pipiens* CR sequences.

A BlastP search was conducted for each *L. pipiens* CR group using these custom OR, TAAR, and VR databases to identify the single best translated ORF sequence match per each *L. pipiens* OR and the top 10 best matches for each *L. pipiens* V1R, V2R, and TAAR for identification of the optimal ortholog candidates across all 26 species. Geneious "grade"

percentages (combining E-value, Percent-Identity, and Query-Cover; Drummond 2012; Kearse et al., 2012) were utilized to make these selections. Subsequently, a separate BlastP search was conducted per *L. pipiens* CR group (incorporating the shortlist 93 ORs, 2 V1Rs, 4 V2Rs, and 1 TAAR) to identify the cumulative 10 best mouse (MUS taxid) translated ORF matches per CR type for inclusion in the alignment and the phylogenetic analysis as the mammal outgroup (*see supplementary file for all sequences included*). Thus, a total of 177 ORs (based on shared matches), 32 V1Rs, 52 V2Rs, and 21 TAARs, were chosen for subsequent alignment and analysis.

The shortlisted translated putative *L. pipiens* CR ORFs were aligned to their top orthologs using CLUSTALX (Version 2.1), and *TrimAl_1.4* was employed to eliminate alignment segments where positions with greater than or equal to 80% gaps were present across all sequences. After trimming, a maximum likelihood approach for phylogenetic analysis with 1000 bootstraps specified was performed using *PhyML* 3.0 (Felsenstein 1981; 1985; Stéphane et al. 2005; Guindon et al. 2010). *FigTree* (Version 1.4.2) and *Adobe illustrator CC 2017* were then utilized for tree construction and display. All trees were midpoint rooted for display purposes.

Results:

Northern Leopard Frog Putative Chemosensory Transcripts:

Total RNA isolated from pooled olfactory epithelium tissue resulted in 632 ng and had a high purity as reflected by its A_{280}/A_{260} and A_{260}/A_{230} ratios. Both the quality and quantity of this RNA sample met the requirements for Illumina sequencing. We obtained 273,573,153 raw reads of 160 bp (see Table 1.1 for statistics on sequencing data,

processing, and *de novo* assembly). After pre-processing (trimming and filtering of adaptors and low-quality bases), we obtained 252,411,921 clean reads, which were *de novo* assembled into a transcriptome of 1,475,831 contigs using Trinity. After removing contigs shorter than 200 bp, Transrate determined 377,187 of the remaining contigs as trustworthy contigs with sufficient sequencing coverage depth. These good-quality contigs were compiled to serve as the subject database for BLAST search of potential CR transcripts.

The curated query database consisted of 5717 known putative CRs in amphibian, fish and reptile species (see Table 1.2 for statistics on species and group breakdown of CRs). Using BLAST+'s TBLASTX program (with a similarity threshold of $E<10^{-5}$ and a subject sequence length threshold of 750 nt), we identified 189 Trinity-assembled contigs as putative OR transcripts, 15 contigs as putative VR transcripts, and 1 as a putative TAAR transcript. Among the OR and VR transcripts, multiple isoforms per gene were identified and the best functional receptor candidates were selected based on comparison with an alternative transcriptome assembly (Gong et al. Manuscript in preparation). These included the following full-length putative transcripts: 103 ORs, 1 TAAR, 2 V1Rs, and 4 V2Rs. Transcripts which did not have clearly identifiable start and stop codons for the OR group were not included in the phylogenetic analysis.

Northern Leopard Frog Putative Chemosensory Accessory Transcripts:

A similarity search revealed a transcript for the transient receptor potential channel trpc2, an indicator of vomeronasal neurons (Liberles 2014) which has been shown to be expressed in both the main olfactory epithelium (MOE) and the vomeronasal organ (VNO)

in *X. lavevis* (Sansone et al. 2014). Despite the anatomical segregation of VNO and MOE in the mostly aquatic *Xenopus*, expression of VRs are not limited to the VNO. The predicted amino acid sequence of the *L. pipiens* trpc2 was similar to frog (*S. tropicali* and *Nanorana parkeri* 94% and 99% amino acid identity respectively), zebrafish (*D. rerio*; 74%) identity and mouse (*Mus musculus*; 78% identity).

In addition, we identified homologs of olfactory specific G-proteins (G_{olf} and G_s) required for the signal transduction of most ORs (Berghard and Buck, 1996).

Phylogenetic analysis:

Total numbers of putative *L. pipiens* CRs included in phylogenetic analyses are as follows: 93 ORs, 2 V1Rs, 4 V2Rs, and 1 TAAR, and their orthologs span 11 different species (177 ORs, 32 V1Rs, 52 V2Rs, and 21 TAARs overall). The species identities included for all phylogenetic analyses (# of species represented by each tree: 5 OR, 5 V1R, 5 V2R, and 7 TAAR) and the number of ortholog-representatives included, per species, are provided in Table 1.3.

Amino acid substitution models selected for each tree were as follows: OR=*JTT+G+I+F*, V1R=*JTT+G+F*, V2R=*JTT+G+I+F*, and TAAR=*JTT+G+F* (Vincent et al. 2017; *see supplementary files for further details*). Bootstrap branch support values out of 1000 were calculated (shown in Figures 1.1, 1.2, 1.3, and 1.4 in Appendix A Chapter 1 Figures Supplementary File; support values of 700/1000 and higher alone are shown for the OR tree in Figure 1.1 in Appendix A Chapter 1 Figures Supplementary File), where the majority of *L. pipiens* CRs cluster with *S. tropicalis* and other transitional vertebrate orthologs, specifically reptile orthologs.

Discussion:

To our knowledge, this is the first study that has reported full-length CRs in a non-*Xenopus* anuran amphibian. We also identified up to 36 Golf candidates and a trpc2 candidate (Kajiya et al. 2001; Stowers et al. 2002; Kiemnec-Tyburczy et al. 2011. These full-length CRs and associated linked-G-proteins (*see supplemental materials*) indicate that the first stages of the vertebrate CR transduction pathway are likely conserved in *L. pipiens* genome, hinting at a degree of chemosensory functional relevance for the northern leopard frog and/or its recent common ancestors. The phylogenetic analyses we conducted with these putative CRs have generated a number of hypotheses regarding the evolutionary pressures that shaped them, in addition to predictions surrounding receptor function.

First, a large proportion (64/93) of the *L. pipiens* ORs included in this analysis appear to be most closely related to orthologs of the primarily aquatic Western Clawed frog (*Xenopus Silurana Tropicalis;* Hellsten et al. 2010). However, our comprehensive list of *L. pipiens* top matching ORs includes both semiaquatic and terrestrial species, suggesting that waterborne and volatile chemical cues could be relevant for adult *L. pipiens* natural history. In fact, of all 26 species queried for this analysis, the top orthologs selected (see Table 1.3) belong to *S. tropicalis*, the American alligator (*Alligator mississippiensis*), the Chinese alligator (*Alligator sinensis*), and the arboreal lizard/Carolina anole (*Anolis carolinensis*). All but one of these species, *A. carolinensis*, are semi-aquatic vertebrates (*S. tropicalis, A. mississippiensis*, *and A. sinensis)* which spend variable durations in water and on land (Weldon and Ferguson 1993; Thorbjarnarson et al. 2002; Herrel et al. 2012). Therefore, it is more than reasonable to assume that these OR divergence patterns (see Figure 1.1 in

Appendix A Chapter 1 Figures Supplementary File) accompany adaptations associated with a transitional lifestyle, possibly resulting in the detection of a variety of cue classes (water soluble, volatile, and nonvolatile odorants). Meanwhile, *L. pipiens* ORs that most closely resemble the terrestrial, *A. carolinensis* ORs (Lovern et al. 2004) are more narrowly predicted to detect terrestrially derived, volatile cues (e.g. OR7030549NLF and gi|637331092|ref|XM_003224539.2|_1847_Ac; See Figure 1.1 in Appendix A Chapter 1 Figures Supplementary File).

With regard to the best *L. pipiens* V1R matches, the organisms for which top orthologs were discovered include a variety of fish species (see Table 1.3), *S. tropicalis*, *A. mississippiensis,* and *A. sinensis*. Mammalian (MUS taxid) V1R divergence appears to predate the divergence of these transitional vertebrate and fish orthologs (See Figure 1.2 in Appendix A Chapter 1 Figures Supplementary File), which suggests that aquatic environmental pressures likely contributed to the recent evolution of *L. pipiens* V1Rs*.* Thus, the 2 *L. pipiens* V1Rs included in the analysis likely detect water-soluble pheromones or other nonvolatile environmental cues associated with primarily aquatic habitats. This prediction supports the emerging theory that anuran V1Rs could be an exception to the 'rule' of terrestrial vertebrate V1R volatile odorant detection (Woodley 2010). However, it is also possible that these V1R divergence patterns indicate a separate evolutionary emergence of volatile-detecting V1Rs in transitional vertebrate clades, in response to an increasingly terrestrial lifestyle (see *Xenopus*, *Alligator*, and *L. pipiens* representatives in Figure 1.2 in Appendix A Chapter 1 Figures Supplementary File).

Concerning the 4 putative *L. pipiens* V2Rs incorporated in our phylogenetic analysis, a variety of fish species' orthologs (see Table 1.3), *S. tropicalis* orthologs, and a singular *A.*

carolinensis ortholog were identified as the best matches. The divergence pattern observed across all species (see Figure 1.3 in Appendix A Chapter 1 Figures Supplementary File) indicates that *L. pipiens* V2Rs are most closely related to *S. tropicalis* orthologs for all but one receptor, which resembles the divergence pattern observed by *P. Shermani* in relation to *Xenopus laevis* and other vertebrate receptors (*P. Shermani;* Kiemnec-Tyburczy et al. 2011). The remaining *L. pipiens* V2R included in this analysis appears to be most closely related to a variety of fish orthologs. Collectively, this divergence pattern and the ecologies of *Xenopus* and fish, respectively, suggest that all the *L. pipiens* V2Rs included in this analysis likely detect aquatically derived pheromones (water-soluble or nonvolatile); as was posited by Kiemnec-Turburczy et al. (2011) regarding *P. shermani* V2R function. Alternatively, these *L. pipiens* V2Rs might well detect more general (aquatic) environmental odorants (Woodley 2010). Regardless, since the V2Rs expressed in *L. pipiens* vomeronasal epithelium were not targeted in this study, transcriptome sequencing of vomeronasal tissue is required before any encompassing hypotheses can be generated surrounding V2R function in *L. pipiens*.

Finally, the TAAR divergence patterns observed across top matching species (see Figure 1.4 in Appendix A Chapter 1 Figures Supplementary File) indicate that the *L. pipiens* TAAR included in our analysis is most closely related to a recent common ancestor of known mouse and reptile TAAR orthologs. Fish orthologs appear to have diverged prior to this split, and the majority of the closest *L. pipiens*' orthologs belong to MUS, an entirely terrestrial group. Of the closely related reptile orthologs, *A. mississippiensis* and *A. sinensis* are non-exclusively terrestrial species. Still, these divergence patterns indicate that a terrestrial lifestyle shaped the evolution of this *L. pipiens* TAAR, suggesting that it likely

detects volatile amine chemical cues. This is further supported by *A. mississippiensis'* capacity for volatile odorant detection (Weldon et al. 1990; Hansen 2007; Mason and Parker 2010.

Collectively, our results suggest that anuran amphibians (e.g. *L. pipiens*) retain the capacity to detect a variety of terrestrial and aquatically derived chemical cues, consistent with previous organismal studies demonstrating behavioral responses in *L. pipiens* and the closely related *L. sphenocephalus* (Shinn and Dole 1978; Glennemeier and Denver 2002; Johnson et al. 2003). Futher research is needed to validate these speculations and elucidate the significance of this chemical sensing capacity. The discovery of putative CR genes and phylogenetic analysis conducted in this study facilitate future research aimed at deorphaning *L. pipiens* CRs and offer predictions to help streamline ligand-screening.

Tables:

Table 1.1: Statistics for RNAseq data, pre-processing and de novo assembly (Trinity) to establish reliability of data and CR identification.

Contig metrics:		
	n seqs	1475831
	smallest	224
	largest	29971
	n bases	783617848
	mean len	530.97
	n under 200	0
	n over 1k	136058
	n over 10k	324
	n with orf	97780
	mean orf percent	49.02
	n90	262
	n70	364
	n50	587
	n30	1255
	n10	3265
	gc	0.43
	gc skew	-0.04
	at skew	0.01
	cpg ratio	1.41
Read mapping metrics:		
	fragments	31105623
	fragments mapped	23243455
	p fragments mapped	0.75
	good mappings	15781036
	p good mapping	0.51
	bad mappings	7462419
	potential bridges	462241
	bases uncovered	119108626
	p bases uncovered	0.15
	contigs uncovbase	955034
	p contigs uncovbase	0.65
	contigs uncovered	318958
	p contigs uncovered	0.22
	contigs lowcovered	1297058
	p contigs lowcovered	0.88
	contigs segmented	119635

Table 1.1 (cont.)

	p contigs segmented	0.08
Summary Statistics		
	TRANSRATE ASSEMBLY SCORE	0.049
	TRANSRATE OPTIMAL SCORE	0.2703
	TRANSRATE OPTIMAL CUTOFF	0.4983
	good contigs	377187
	p good contigs	0.26

Table 1.2: Statistics for species and group breakdowns for collated known chemosensory receptors in fish, reptile, and amphibian species available for comparison.

NCBI taxonomy ID	Scientific name	Common name	OR	VR	TAAR
8496	Alligator mississippiensis	American alligator	489	1	9
38654	Alligator sinensis	Chinese alligator	580	2	7
28377	Anolis carolinensis	arboreal lizard/Carolina anole	212	65	6
7994	Astyanax mexicanus	Mexican tetra or blind cave fish	109	5	41
244447	Cynoglossus semilaevis	tongue sole (bony fishes)	92	3	22
7955	Danio rerio	Zebrafish	478	50	98
8010	Esox Lucius	northern pike (bony fishes)	133	4	23
8153	Haplochromis burtoni	Burton's mouthbrooder (bony fishes)	98	12	20
7998	Ictalurus punctatus	channel catfish (bony fishes)	16	0	0
215358	Larimichthys crocea	large yellow croaker (bony fishes)	207	4	25
7897	Latimeria chalumnae	coelacanth (fish)	163	30	26
7918	Lepisosteus oculatus	spotted gar (bony fishes)	168	7	44
106582	Maylandia zebra	zebra mbuna (bony fishes)	100	5	25
32507	Neolamprologus brichardi	Fairy cichlid (bony fishes)	75	4	14
8208	Notothenia coriiceps	black rockcod (bony fishes)	38	6	5
8022	Oncorhynchus mykiss	rainbow trout	8	1	0
8128	Oreochromis niloticus	Nile tilapia (bony fishes)	182	12	43
8090	Oryzias latipes	Japanese medaka (bony fishes)	87	6	30
48698	Poecilia Formosa	Amazon molly (bony fishes)	109	9	35
8081	Poecilia reticulate	guppy (bony fishes)	99	13	30
303518	Pundamilia nyererei	Victorian cichlid (bony fishes)	95	8	18
8030	Salmo salar	Atlantic salmon (bony fishes)	83	13	0

Table 1.2 (cont.)

144197	Stegastes partitus	bicolor damselfish (bony fishes)	68	3	25
31033	Takifugu rubripes	torafugu (bony fishes)	112	5	18
8364	Xenopus (Silurana) tropicalis	Western clawed frog	765	260	7
8355	Xenopus laevis	African clawed frog	39	13	0
		total no. of genes	4605	541	571
		total no. of species	26	25	22

Table 1.3: Number of Northern leopard frog CRs and their top orthologs used for phylogenetic analysis.

Species	OR	V1R	V2R	TAAR
Northern leopard frog (*Lithobates pipiens*)	93	2	4	1
Mouse (*MUS* taxid)	10	10	10	10
Western Clawed frog (*Xenopus Silurana tropicalis*)	45	3	28	0
Chinese alligator (*Alligator sinensis*)	11	1	0	1
American alligator (*Alligator mississippiensis*)	13	1	0	1
Arboreal lizard/Carolina anole (*Anolis carolinensis*)	5	0	1	1
Spotted gar (*Lepisosteus oculatus*)	0	0	1	1
Mexican tetra or blind cave fish (*Astyanax mexicanus*)	0	0	0	3
Coelacanth fish (*Latimeria chalumnae*)	0	13	1	2

Table 1.4: Anticipated CR numbers based on those found in other frog species (Shi and Zhang 2009). Additional Note: we likely missed many true CRs because we only considered those appearing in both SOAP and Trinity assemblies to be reliable candidates, including 103 ORs, 2 V1Rs, 6 V2Rs, and 1 TAAR. In addition, our pooled tissue samples were dissected from adult individuals. It is likely that we failed to capture a number of L. pipiens CRs expressed in other life stages (Shi and Zhang 2009; Zhou et al. 2009; Woodley 2010; Kiemnec-Tyburczy et al.). Finally, the fact that we only analyzed samples from the main olfactory epithelium could explain why we identified so few V1Rs and V2Rs because they may be expressed primarily in the vomeronasal organ epithelium (Woodley 2010).

Anticipated CR numbers prior to analysis	OR	V1R	V2R	TAAR
	410	21	249	2

Chapter 2: Behavioral responses by northern leopard frog adults to conspecific chemical cues

Abstract:

Chemical signaling is an important facet of vertebrate communication, and amphibians are not the exception. However, very little is known about adult anuran (frog and toad) chemical communication and the few studies that have investigated chemical signaling in this group have discovered species- and sex-biased behavioral responses by adult anurans to chemical signals. Varied life histories and reproductive pressures likely dictate these responses, but to better understand the prevalence and purpose of adult anuran olfaction across species, information on chemical signaling across a large number of species is needed. We investigated sex-biased adult northern leopard frog behavioral responses to familiar and unfamiliar conspecific chemical cues to determine the importance of olfaction in conspecific recognition. Our results indicate that adult females respond favorably to conspecific cues with females spending significantly more time investigating cue vs. control sides of the testing-arena. Overall, results for males were equivocal with males responding much more variably. Analyses comparing familiar versus unfamiliar indicate that both sexes show a slight preference for familiar cues. Females respond to unfamiliar, opposite-sex conspecific cues, indicating chemical cues are likely involved in mate identification for females. Unfamiliar, same-sex conspecific odors did not significantly influence behavior for either sex. Our results indicate that adult anurans use chemical signaling for identification of conspecifics. Future research should investigate aquatic vs. terrestrial and learned vs. innate odors of significance to northern leopard frogs.

Introduction:

Chemical signaling is a dynamic and versatile communication mechanism, spanning the entire gamut of living organisms in both terrestrial and aquatic habitats (Gleeson, 1978; Wyatt, 2003). This well-studied and fundamental aspect of vertebrate communication is important for a wide range of taxa, providing critical information regarding the presence of predators and prey, locations of mates and offspring, and allowing for territory and breeding site recognition (Wyatt, 2003, 2005, 2010, 2014; Brennan and Zufall, 2006; Muller-Schwarze, 2006). For amphibians the majority of work has focused on salamander groups and the tadpole/juvenile stage of anurans (Belanger and Corkum, 2009; Woodley, 2014, 2015). Relatively little is known about adult anuran chemical communication, especially terrestrial communication (Woodley, 2014, 2015), with auditory and visual signaling taking precedence in anuran research (Belanger and Corkum, 2009; Kiemnec, 2009; Poth et al., 2012; Woodley, 2015). A great deal of information is still needed regarding the types of chemical cues that induce behavioral responses in adult anurans, and information on olfaction in tadpoles is not likely to be sufficient for understanding the breadth and purpose of adult olfaction.

One research area that has received extensive interest is individual response to conspecific cues. Conspecific cues can be used to find mates and suitable habitat as well as to assess habitat quality (Elliott et al., 1993; Aragón et al., 2000, 2001;Muller-Schwarze, 2012). Numerous studies have demonstrated adult anuran responses to conspecific auditory cues (Loftus-Hills and Johnstone,1970; Ryan, 1988; Wilczynski and Endepols, 2007; Belanger and Corkum, 2009; Bee et al. 2013) but relatively little work has focused on

conspecific chemical cues. Recent efforts to fill this knowledge gap have documented varied responses with some studies showing conspecific attraction and others demonstrating avoidance (Belanger and Corkum, 2009; Schulte and Rössler, 2013) or no response (Gonzalo et al., 2006). Not surprisingly, responses often depended on whether the cue was from individuals of same or opposite sex (Hamer et al., 2011, Asay et al., 2005). Strength of response often differed by sex with some studies showing stronger responses by males (Hamer et al., 2011; Asay et al., 2005) and others showing stronger responses by females (Pearl et al., 2000; Wabnitz et al., 1999, 2000). In the Australian terrestrial toadlet (*Pseudophryne bibronii)*, females and males both responded to opposite-sex conspecific volatile cues, but only females responded to both opposite- and same-sex conspecific cues (Byrne and Keogh, 2007).

Species- and sex-biased behavioral responses to chemical signals are likely a function of the varied life histories and reproductive pressures on anurans (Arak, 1983; Werner, 1986; Duellman, 1986, 1989; Starnberger et al. 2014). Males that need to compete for mates, for example, may benefit more than females from utilizing conspecific chemical information, allowing them to better assess physical risks before engaging in competition with other males (e.g. Hamer et al., 2011). Conversely, responses may be similar between males and females if conspecific cues are used primarily to identify breeding habitat (Schulte et al., 2011). However, conspecific cues may also be avoided for species where density dependent larval survival is present (Spieler and Linsenmair, 1997) and field trials examining conspecific attraction or repulsion to non-chemical cues have been equivocal, with some studies showing avoidance and some attraction (Buxton and Sperry, 2016).

Finally, strength of response may also vary with familiarity of the conspecific, as

anurans have been shown to be attracted both to familiar conspecifics and familiar environmental cues (i.e. pond water; Aragón et al., 2003; Belanger and Corkum, 2009). A learning component to conspecific recognition or identification, associated with positive or negative conditioning (Grubb 1976; Schoenbaum et al. 1999), might thus be responsible for some of the variable sex-biased and species-biased responses observed by anurans to conspecific chemical signals. Species are known to differ in their behavioral plasticity (West-Eberhard 1989; Laurila et al. 2002; Teolitsky et al. 2005), and learned odor recognition for plastic species might be adaptive under conditions where environmental pressures fluctuate over generations. For example, some frogs are known to utilize olfactory learning and social learning for predator avoidance (Ferrari and Chivers 2008; Ferrari et al. 2009). Alternatively, innate olfactory responses can signify a critical role in olfactory recognition regardless of environmental fluctuations (Spehr et al. 2006). The Dusky Gopher frog (*Lithobates sevosus*) for example displays innate recognition of two turtle species' chemical cues, as it relies on these odors to locate suitable burrows (Thurgate and Pechmann 2006). Regardless, understanding the importance of innate vs learned chemical signaling to adult anurans and how responses to conspecific cues vary across species and sexes requires research across a wide range of species, which is currently lacking (Belanger and Corkum, 2009; Woodley, 2014, 2015). Here we examine sex-biased responses to conspecific volatile cues in adult Northern Leopard frogs (*Lithobates pipiens*) and determine whether those responses differ based on familiarity and/or opposite-sex vs same-sex conspecific cues.

Methods:

Test Subject Housing Conditions:

A total of 43 northern leopard frog adults, 22 male and 21 female, were collected from semi-natural environments (maintained outdoor breeding ponds) at Kons Scientific Co. (Germantown, Wisconsin USA). The frogs were separated by sex upon arrival in the lab and maintained in water held at a constant temperature throughout all trials in an effort to retain courtship and breeding receptivity (Kendell, 2002). The frogs were housed communally, with either 4 or 5 individuals per tank (Dimensions: 13.5" x 19.75" x 15"; 4 female tanks with 4 individuals each, 1 female tank with 5 individuals, 3 male tanks with 4 individuals each, and 2 male tanks with 5 individuals each). All of the frogs were maintained in reverse osmosis (RO) water with added salt, held within a range of 16.67 to 18.33 degrees Celsius, and each tank was positioned with a slight tilt, establishing a partially aquatic, partially terrestrial environment. The elevated half of each of the tanks incorporated stacked, cut, black rubber floor mats for separation from the water. All tanks were kept in the same room, where the lights were set on a timer for 12 light and 12 dark hours (dark hours: 7 PM to 7 AM), and the tanks were washed once a month and syphoned one hour after every feeding. Tanks were also automatically flushed for 3 minutes, four times each day. The frogs were checked daily and fed live crickets 3-6 times per week.

Behavioral Trial Procedures:

Behavioral trials were designed to evaluate response to the following cues: 1) familiar same-sex conspecific odors (conspecifics housed together), 2) unfamiliar same-sex conspecific odors (conspecifics housed in different enclosures), and 3) unfamiliar opposite-sex conspecific odors (conspecifics housed in different enclosures). The order of

experiments was randomized for all individuals, and each individual was only tested once for any given cue-type. All subjects were distinguished from one another via unique dorsal spot patterns for testing purposes, tested alone, and the minimum duration between experiments for any individual was 5 days; in an effort to avoid procedural habituation. Because the frogs needed to be separated by sex to preserve breeding receptivity for opposite-sex conspecific response trials, familiar opposite-sex trials were not conducted.

The testing area was located in a room separate from the housing tanks to reduce disturbances to non-test animals. Room temperature was held constant at 20-21.1 degrees Celsius for all trials, and lighting was eliminated during habituation and trial periods to prevent visual stimuli from biasing the results and to mimic nocturnal activity patterns observed in this species (Kendell, 2002). Behavioral trials were performed using a rectangular, glass arena (Dimensions: 30'' x 11.5'' x 11.5'') with point source cues positioned at either end of a 3-compartment grid (Right and Left sides= 8.5'', Middle= 13''); with moist, unscented, lotion-free paper towel substrate--one experimental and one control. Researchers were not present in the room during the trial and trials were recorded using an infrared video recorder. All human intervention between habituation and trial periods was done with a single red-light flashlight to reduce disturbance to the animal (Bouchard et al., 2009). All containers and experimental surfaces were thoroughly cleaned with *VIRKON Aquatic Solution* before, after, and between every behavioral trial to prevent the introduction of any contamination-source odors.

Prior to each trial period, the individuals chosen at random for testing on that day were isolated and placed in plastic, cylindrical containers [diameter: 4.75'', height: 8''; with a large 4'' x 4'' Mirasorb BAND-AID gauze pad lining the bottom, 100 ml of standing, clean

RO--with salt added--water, and a 1'' diameter ventilation hole in the lid]--for a minimum of 3 hours in the testing room before trials commenced to acclimate the individuals to the testing room. The containers were obscured from the experimental arena via an opaque curtain, all human personnel left the room, and the lights were maintained with a timer for normal light and dark hours (dark hours: 7 PM to 7 AM) until the isolation period was completed.

At the start of the trail, test subjects were placed into the testing arena (Right and Left sides= 8.5'', Middle= 13'') containing moist substrate paper-towels and allowed a 30-45 minute acclimation period under dark conditions. The side of the arena, which was to receive the cue, and the source tanks for the cue was randomized for every trial. The cues themselves were added to the cue holding container by pouring a scoop (roughly 1 cup/236.588 ml) of tank water with associated skin, fecal, and urine cues into a plastic container (dimensions: 9" x 3" x 1.75") with 90 3/16th" holes drilled in the top for aeration. The cue container was placed on one side of the arena with another, identical container, containing only clean RO (with salt added) water, placed on the far side of the arena as a control. Video recordings of the trials were later scored by a blind observer with the amount of time in seconds a given individual spent within the cue and control sides of the testing arena (within that 20-minute/1200 second period) recorded for all trials, where expected time for each side was 340 seconds and expected time spent in the center was 520 seconds (based on size).

Statistical Procedure:

We first tested for overall response to the cues for each sex using linear mixed

models fit with the *nlme package* in *RStudio version 1.0.143* (Pinheiro et al., 2017; RStudio, 2016). The difference in time each individual spent on the cue vs control side of the testing arena was treated as the response variable, where expected time spent on both sides under the null hypothesis is equal and the difference zero (e.g. 340 seconds-340 seconds = 0). SubjectID was incorporated as a random effect to account for pseudoreplication, as some individuals were tested with more than one of the three cue-type combinations (1) familiar, volatile, same-sex conspecific odors, 2) unfamiliar, volatile, same-sex conspecific odors, and 3) unfamiliar, volatile, opposite-sex conspecific odors. A significant intercept (i.e. where the intercept is estimated to be significantly different than zero) in this case signifies a significant difference in time spent on the cue and control sides across all males and females, respectively. No fixed variables were added to these intercept-only models.

After separately evaluating female and male northern leopard frog responses to introduced cues across all groups, we conducted an unbalanced repeated measures ANOVA with both male and female trials combined, to evaluate differences based on sex, familiarity and opposite- vs same-sex cues. The *anova.lme* function in the *nlme R package* was employed to evaluate whether differences in time spent on cue vs control sides of the testing-arena existed for each cue-type. Trials where individuals did not move from the middle section of the testing arena (2 of 55 female and 2 of 53 male) were interpreted as a lack of response to the experiment and removed from analyses.

Results:

Frogs spent more time on the cue side of the tank in 62% of trials (67/108), compared to 34% of trials where frogs spent more time on the non-cue side and 4%

(4/108) where no choice was detected (frog did not move from middle of tank). However, this preference varied by gender with female leopard frogs spending more time on the cue side for 69% of trials (38/55; average differential time = 246.60 seconds, SE= 65.65, *P* <0.005; Table 2.1, Figure 2.1) for an average of roughly 21% greater time spent investigating cue vs control side of the testing arena, and male preference for the cue side was only demonstrated in 55% of trials (29/53) with similar time spent on the cue and non-cue sides (average differential time = 83.81 seconds, SE= 98.29, *P*=0.40) for an average of roughly 7% greater time spent investigating the cue side. Preference did not consistently vary by date of the trial (P = 0.86), by frog body size (P = 0.48), or mass (P = 0.15), and preference did not depend on the order of experimental treatments either (P= 0.1231).

For females, preference for cue side was exhibited for familiar (71% of trials), unfamiliar (68% of trials), opposite sex (79% of trials) and same sex cues (64% of trials), although none of these factors were significant in the repeated measures ANOVA (Table 2.2). See Figure 2.2 for summary figures. Conversely, males showed much more variation in responses with preferences less consistent and pronounced (Figure 2.2, Table 2.2). For males, preference for the cue side was exhibited in 71% of familiar cue trials, 47% of unfamiliar cue trials, 56% of opposite and 54% of same sex trials (Figure 2.2), where no significance was detected.

Discussion:

Our results demonstrate that adult northern leopard frogs exhibit behavioral attraction to conspecific chemical cues, although the strength of response varied by sex.

Females were much more consistent in their behavior whereas male response was highly variable. Females exhibited preferences, albeit in some cases slight, to cues from familiar and unfamiliar conspecifics as well as to cues from both males and females. Male response was equivocal across all trials, although they also demonstrated slight preference for conspecific, particularly familiar, cues.

Previous work has demonstrated positive phonotaxis response by adult female leopard frogs to male conspecific calls (Pace 1974; Larson 2004) but this is the first to demonstrate response of this species to conspecific chemical signaling. Much of what is known about conspecific attraction in amphibians is based on phonotaxis trials. A very large body of work, focused on female response, has shown that females exhibit phonotaxis with strength of response (i.e. mate choice) affected by various acoustic parameters of the male call (Pröhl, 2003; Leary, 2009; Bee et al. 2013). However, phonotaxis trials designed to assess male responses have been rare, in large part because most female anurans do not advertise vocally. Very little is known about conspecific attraction via chemical signaling in anurans but the few studies that have undertaken this question have found varied responses (Asay et al., 2005; King et al., 2005; Gonzalo et al., 2006; Byrne and Keogh, 2007; Belanger and Corkum, 2009; Hamer et al., 2011). Our work demonstrates that northern leopard frogs likely use chemical communication to detect conspecifics and that the attraction exhibited by females to conspecific chemical cues is not purely a function of mate detection, as females appeared to respond to cues from both conspecific males and females.

Broadly, recognition and attraction to conspecific cues likely serves a variety of roles including aiding in habitat selection, predator avoidance, mediation of intraspecific competition, and improved reproductive success via mate localization (Belanger and

Corkum, 2009; Woodley, 2014). Evidence for each of these has been found across amphibians groups, and since northern leopard frogs are known to congregate for breeding (McAllister et al., 1999; Kendell, 2002) and cluster while in captivity (personal observation), detection of conspecific odors may contribute to individual fitness for a number of reasons. Additionally, as is true for other species across animal groups, isolation can leave individuals vulnerable to predation (Jennions and Backwell, 1992) and conspecific odors can be used to locate foraging and breeding sites, increasing individual search efficiency (Secondi et al., 2005; Grueter and Leadbeater, 2014). The attraction to conspecific chemical cues we have demonstrated in female leopard frogs likely serves mating and other social or navigation functions. The strongest preference (quantified as time spent on cue side of testing arena vs non cue side) was documented for females presented with unfamiliar male cues (where these females did not receive prolonged exposure to these males), which is likely a function of mate recognition. This attraction could be innate, but since all the frogs were shipped together (overnight) it is impossible to determine whether or not this is the case. Still, whether learned or innate, females demonstrated a preference for male odors over a control with very little olfactory conditioning involved, suggesting a clear role in mate localization. We also saw a positive response to familiar female chemical cues, indicating that other aspects of conspecific attraction likely play a role.

Additionally, both females and males responded more positively to familiar versus unfamiliar same sex cues. This could be due to a preference for familiar individuals or a preference for familiar environmental odors (learned odors associated with conspecifics that they were housed with). Regardless, learned familiar cues may be a proxy for home

range and/or breeding site recognition, with numerous studies showing that anurans orientate towards home pond water and other associated environmental cues (Grubb 1975; Belanger and Corkum, 2009). Conspecific cues may also elicit an aggregation response by both females and males for the purpose of breeding (Secondi et al., 2005), possibly aiding in localization of breeding sites. Although we cannot differentiate responses to self versus responses to other familiar individuals, previous work with *Leiopelma hamiltoni* demonstrated attraction to both self and non-self familiar cues (Waldman and Bishop 2004), suggesting that familiarity plays a role in conspecific attraction. It should be noted that the cues used in these experiments could have contained minor prey (i.e. cricket) particulates. However, the sex-biased differences in behavioral response across cue-types indicate that prey particulates are not likely to be the cause of response.

Overall, the necessity for additional studies dedicated to evaluating the role and importance of chemical signaling in amphibians is growing, as a large proportion of amphibian populations have experienced massive declines over the past several decades (Collins and Storfer, 2003; Polo-Cavia et al., 2016). Research on chemical communication in amphibians, especially within more natural settings, could help conservationists determine the influence of chemical detection and signaling on overall amphibian survival and inspire more effective management techniques.

Tables and Figures:

Table 2.1: Linear Mixed Model results for both females and males; Mean Difference (in seconds) of time spent on cue minus control side of test-arena where expected time spent on both sides is 340 seconds overall; associated standard error, degrees of freedom, sample size per analysis, and t-value test statistics shown. Bold text indicates statistically significant values ($p<0.05$).

	Mean Difference	SE	DF	N	t- value	*p*
Female	246.60	65.65	32	53	3.76	**<0.005**
Male	83.81	98.29	29	51	0.85	0.40

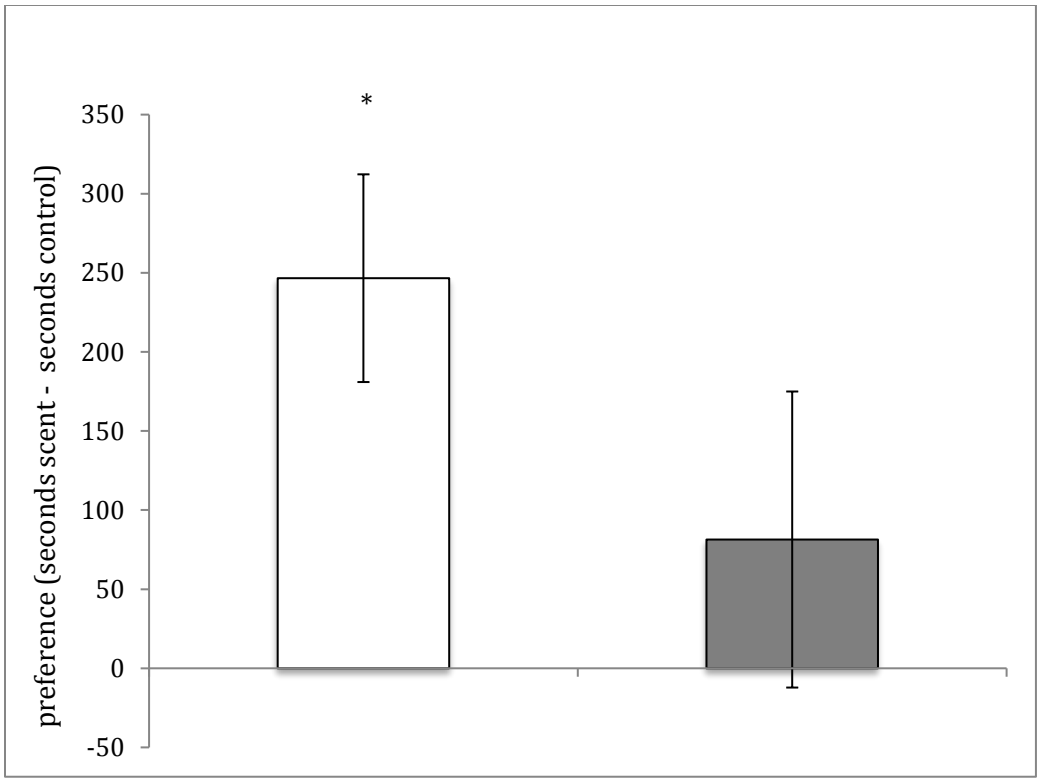

Figure 2.1: Female and male *L. pipiens* responses to conspecific odor cues. Open bar: female. Filled bar: male. Values represent mean preference for scented side (time spent on cue side in seconds minus control side in seconds). Asterisk denotes significant preference based on separate linear mixed model results. N=53 for females, and N=51 for males.

Table 2.2: Repeated Measures ANOVA results; Evaluations of significance at each level of familiarity, same vs opposite-sex cue, and sex fixed treatments; along with their associated interaction terms; Numerator and denominator degrees of freedom, F ratios, and p values displayed. Statistically significant values considered ($p<0.05$). Number of groups: 43 and total observations: 104.

Source	num d.f.	den d.f.	*F ratio*	*P*
Sex	1	41	0.14	0.71
Familiarity	1	57	0.35	0.56
same vs opposite-sex cues	1	57	1.09	0.30
sex*familiarity	1	57	0.27	0.60
sex*same vs opposite-sex cues	1	57	0.12	0.74

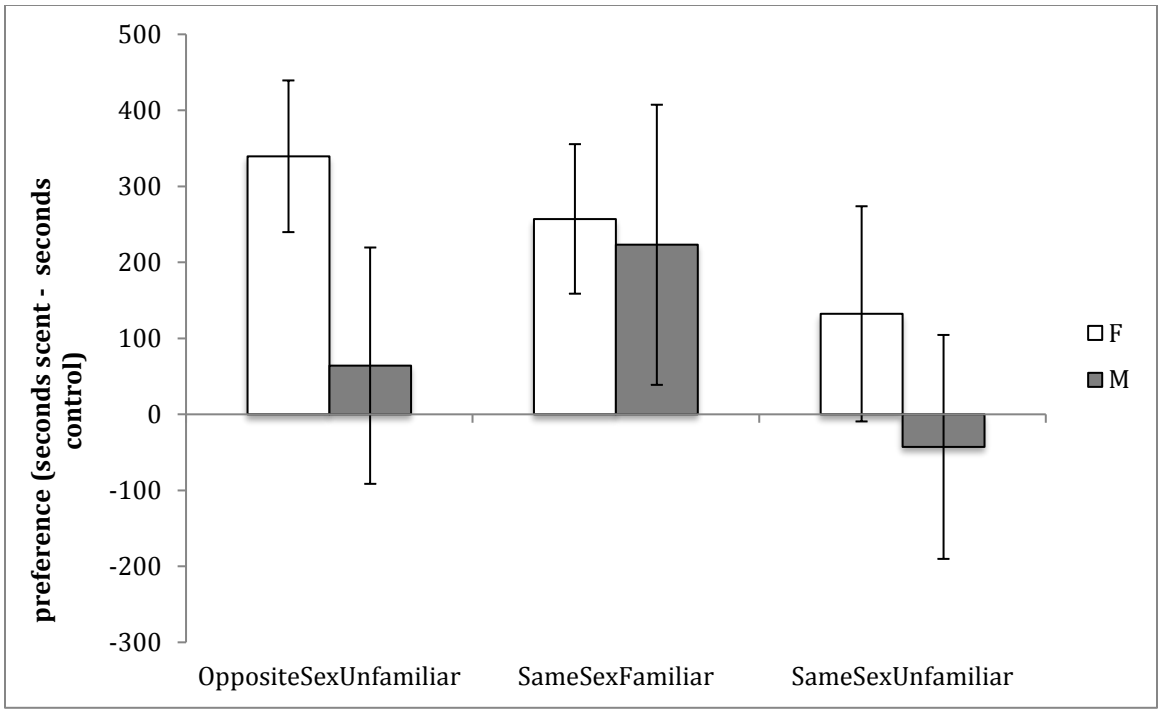

Figure 2.2: Female and male *L. pipiens* responses to conspecific odor cues (expected time on each side is 340 seconds total). Open bars: female. Filled bars: male. Values represent mean preference for scented side (time spent on cue side in seconds minus control side in seconds) per each cue-type combination (1.Unfamiliar, Opposite-sex 2. Familiar, same-sex, and 3. Unfamiliar, same-sex). N=19 for females exposed to unfamiliar, opposite-sex cues. N=17 for males exposed to unfamiliar, opposite-sex cues. N=17 for females exposed to familiar, same-sex cues. N=17 for males exposed to familiar, same-sex cues. N=17 for females exposed to unfamiliar, same-sex cues. Finally, N=17 for males exposed to unfamiliar, same-sex cues.

Chapter 3: Summary

Though chemical communication is known to provide critical information to amphibian species, adult anuran chemical communication remains largely a mystery, especially for declining species. Insights into its molecular components, its influence on adult behavior, and its overall significance to anuran survival is thus sorely lacking, and our research represents a necessary first step towards bridging this critical gap in current knowledge. The full-length CR proteins and coupled G-proteins, we identified provide a platform for future studies to directly examine receptor function across receptor classes in adult *L. pipiens* olfactory epithelium (i.e. ORs, TAARs, V1Rs, and V2Rs). The presence of full-length CR genes and their associated G-proteins suggests that the first step(s) of the CR signaling transduction pathway is likely intact (i.e. these CR genes are probably not dysfunctional, evolutionary relics). Furthermore, the distribution of these receptors among other known aquatic, semiaquatic, and terrestrial vertebrate receptors indicates divergence of function for the detection of aquatic and terrestrially derived chemical cues across CR types, sometime within the 'recent' evolutionary past. Whether or not these CRs retain similar functions to their orthologs included in this analysis is presently unknown, but the genetic information collected suggests a degree of conserved receptor function in adults *L. pipiens*.

Intuitively, considering the semiaquatic nature of *L. pipiens* and other anurans, detection of a variety of cues would be adaptively advantageous for seasonal transitions towards more or less aquatic lifestyles. However, future studies targeting vomeronasal organ tissue and those directly aimed at testing receptor function are needed to further

elucidate the binding specificities of different receptor classes for adult anuran chemoreception.

Regarding the influence of chemical detection on adult *L. pipiens* behavior, our research shows that detection of familiar (or learned), same-sex conspecific cues in both male and female adult northern leopard frogs could be beneficial, possibly acting as proxy home-range or familiar environmental cues associated with quality habitat. These familiar odors would help them return to known and/or quality habitat after foraging or migration attempts. Our findings also suggest that chemical detection of unfamiliar (likely innate), opposite-sex conspecific cues is of significance to adult female northern leopard frogs, presumably for localization of mates during courtship. No apparent detection of unfamiliar, same-sex conspecific cues by either sex was found. Further research is needed to accurately identify the significance of these nuanced behavioral responses by northern leopard frog adults but, collectively, our results indicate that chemical signaling could play a significant role in mediating adult *L. pipiens* behavior, whether or learned or innate.

Chapter 4: Literature Cited

Aragón P, López P, Martín J, 2000. Conspecific chemical cues influence pond selection by male newts Triturus boscai. *Copeia* 2000:(3):874-878.

Aragón P, López P, Martín J, 2001. Effects of conspecific chemical cues on settlement and retreat-site selection of male lizards Lacerta monticola. *Journal of Herpetology* 35:(4):681-684.

Aragón P, López P, Martín J, 2003. Differential avoidance responses to chemical cues from familiar and unfamiliar conspecifics by male Iberian rock lizards (Lacerta monticola). *Journal of Herpetology* 37:(3):583-585.

Arak A, 1983. Male-male competition and mate choice in anuran amphibians. *Mate choice*:181-210.

Asay MJ, Harowicz PG, Su L, 2005. Chemically mediated mate recognition in the tailed frog (Ascaphus truei). *Chemical Signals in Vertebrates* 10:24-31.

Bee MA, Schwartz JJ, Summers K, 2013. All's well that begins Wells: celebrating 60 years of Animal Behaviour and 36 years of research on anuran social behavior. *Animal Behaviour* 85:5-18.

Belanger RM, Corkum LD, 2009. Review of Aquatic Sex Pheromones and Chemical Communication in Anurans. *Journal of Herpetology* 43:184-191.

Berghard A, Buck LB, 1996. Sensory transduction in vomeronasal neurons: evidence for G alpha o, G alpha i2, and adenylyl cyclase II as major components of a pheromone signaling cascade. *Journal of Neuroscience 16:*(3):909-918.

Bishop DC, Haas CA, and Mahoney, LO, 2012. Response of Lithobates okaloosae, L. clamitans and L. sphenocephala tadpoles to chemical cues of snake and fish

predators. *Florida Scientist* 75(1).

Borowsky B, Adham N, Jones KA, Raddatz R, Artymyshyn R, Ogozalek KL., Durkin MM, Lakhlani PP, Bonini, JA, Pathirana, S, Boyle, N, 2001. Trace amines: identification of a family of mammalian G protein-coupled receptors. *Proceedings of the National Academy of Sciences 98:*(16):8966-8971.

Boschat C, Pélofi C, Randin, O, Roppolo, D, Lüscher, C, Broillet, MC, Rodriguez I, 2002. Pheromone detection mediated by a V1r vomeronasal receptor. *Nature neuroscience 5*:(12):1261.

Bouchard J, Ford A, Eigenbrod F, Fahrig L, 2009. Behavioral responses of northern leopard frogs (Rana pipiens) to roads and traffic: implications for population persistence. *Ecology and Society* 14:(2).

Brennan PA, Zufall F. 2006. Pheromonal communication in vertebrates. *Nature* 444:(7117):308.

Brykczynska U, Tzika AC, Rodriguez I, Milinkovitch MC, 2013. Contrasted evolution of the vomeronasal receptor repertoires in mammals and squamate reptiles. *Genome biology and evolution* 5:(2):389-401.

Buck L, Axel R, 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell 65:*(1):175-187.

Buck LB, Axel R, 1992. A novel multigene family may encode odorant receptors. *Soc. General Physiol. Series 47*:39-51.

Buxton VL, Sperry JH, 2016. Reproductive Decisions in Anurans: A Review of how predation and competition affects the deposition of eggs and tadpoles. *BioScience 67:*(1):26-38.

Byrne PG, Keogh JS, 2007. Terrestrial toadlets use chemosignals to recognize conspecifics, locate mates and strategically adjust calling behaviour. *Animal Behaviour* 74:(5):1155-1162.

Collins JP, Storfer A, 2003. Global amphibian declines: sorting the hypotheses. *Diversity and distributions* 9:(2):89-98.

Cummins SF, Bowie JH, 2012. Pheromones, attractants and other chemical cues of aquatic organisms and amphibians. *Natural product reports* 29:642-658.

Drummond A, 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics 28:*(12):1647-1649.

Duellman WE, Trueb L, 1986. Biology of amphibians. JHU press.

Duellman WE, 1989. Alternative life-history styles in anuran amphibians: evolutionary and ecological implications. *Alternative life-history styles of animals* 101-126.

Dulac C, 2000. Sensory coding of pheromone signals in mammals. *Current opinion in Neurobiology 10:*(4):511-518.

Elliott SA, Kats LB, Breeding JA, 1993. The use of conspecific chemical cues for cannibal avoidance in California newts (Taricha torosa). *Ethology* 95:(3):186-192.

Felsenstein J, 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach, *Journal of molecular evolution* 17:368-376.

Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:(4):783-791.

Ferrari MC, Brown GE, Messier F, Chivers DP, 2009. Threat-sensitive generalization of predator recognition by larval amphibians. *Behavioral Ecology and Sociobiology*

63:(9):1369-75.

Ferrari MC, Chivers DP, 2008. Cultural learning of predator recognition in mixed-species assemblages of frogs: the effect of tutor-to-observer ratio. *Animal Behaviour* 75:(6):1921-5.

Freitag J, Ludwig G, Andreini I, Rössler P, Breer H, 1998. Olfactory receptors in aquatic and terrestrial vertebrates. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 183*:(5):635-650.

Gleeson RA, 1978. Functional adaptations in chemosensory systems. *In Sensory Ecology*: Springer US; 291-317.

Glennemeier KA, Denver RJ, 2002. Role for corticoids in mediating the response of Rana pipiens tadpoles to intraspecific competition. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 292:*(1):32-40.

Gong P, Kirkpatrick JR, Maxwell AD, Graham BM, Fields CJ, Zhang C, Sperry JJH, MacAllister IE. 2017. Bioinformatic identification of putative olfactory receptors in six anuran species from RNA-Seq data-assembled transcriptomes (In preparation).

Gonzalo A, Cabido C, Galan P, Lopez P, Martín J, 2006. Predator, but not conspecific, chemical cues influence pond selection by recently metamorphosed Iberian green frogs, Rana perezi. *Canadian journal of zoology* 84:(9):1295-1299.

Grubb JC, 1975. Olfactory orientation in southern leopard frogs, rana utricularia. *Herpetologica* 31:(2):219-221.

Grubb JC, 1976. Maze orientation by Mexican toads, Bufo valliceps (Amphibia, Anura, Bufonidae), using olfactory and configurational cues. Journal of Herpetology 26:97-104.

Grueter C, Leadbeater E, 2014. Insights from insects about adaptive social information use. *Trends in ecology & evolution* 29:(3):177-184.

Grus WE, Zhang J, 2008. Distinct evolutionary patterns between chemoreceptors of 2 vertebrate olfactory systems and the differential tuning hypothesis. *Molecular biology and evolution* 25:(8):1593-1601.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O, 2010. New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Systematic Biology* 59:(3):307-21.

Haga S, Hattori T, Sato T, Sato K, Matsuda S, Kobayakawa R, Sakano H, Yoshihara Y, Kikusui T, Touhara K, 2010. The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. *Nature* 466:(7302):118-122.

Hamer R, Lemckert FL, Banks PB, 2011. Adult frogs are sensitive to the predation risks of olfactory communication. *Biology letters* 7:(3):361-363.

Hansen A, 2007. Olfactory and solitary chemosensory cells: two different chemosensory systems in the nasal cavity of the American alligator, Alligator mississippiensis. *BMC neuroscience* 8:(1):64.

Hashiguchi Y, Furuta Y, Nishida M, 2008. Evolutionary patterns and selective pressures of odorant/pheromone receptor gene families in teleost fishes. *PLoS One* 3:(12):e4083.

Hellsten U, Harland RM, Gilchrist MJ, Hendrix D, Jurka J, Kapitonov V, Ovcharenko I, Putnam NH, Shu S, Taher L, Blitz IL, 2010. The genome of the Western clawed frog Xenopus tropicalis. *Science 328*:(5978):633-636.

Herrel A, Gonwouo LN, Fokam EB, Ngundu WI, Bonneaud C, 2012. Intersexual differences

in body shape and locomotor performance in the aquatic frog, Xenopus tropicalis. *Journal of Zoology* 287:(4):311-316.

Houck L, D. 2009. Pheromone Communication in Amphibians and Reptiles. *Annual Review of Physiology* 71:161-176.

Inamura K, Kashiwayanagi M, Kurihara K, 1997. Blockage of urinary responses by inhibitors for IP3-mediated pathway in rat vomeronasal sensory neurons. *Neuroscience Letters* 233:129-132.

Jennions MD, Backwell PRY, 1992. Chorus size influences on the anti- predator response of a Neotropical frog. *Animal Behaviour* 44:(5):990-992.

Johnson JB, Saenz D, Adams CK, Conner RN, 2003. The influence of predator threat on the timing of a life-history switch point: predator-induced hatching in the southern leopard frog (Rana sphenocephala). *Canadian Journal of Zoology* 81:(9):1608-1613.

Kajiya K, Inaki K, Tanaka M, Haga T, Kataoka H, Touhara K, 2001. Molecular bases of odor discrimination: reconstitution of olfactory receptors that recognize overlapping sets of odorants. *Journal of Neuroscience* 21:(16):6018-6025.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P, Drummond A, 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:(12):1647-1649.

Kendell K, 2002. Survey protocol for the northern leopard frog. Fish & Wildlife Division, Resource Status and Assessment Branch, Alberta Sustainable Resource Development.

Kiemnec KM, 2009. *Chemical cues and the molecular basis of olfactory chemoreception in*

caudate amphibians (Doctoral dissertation).

Kiemnec-Tyburczy KM, Woodley SK, Watts RA, Arnold SJ, Houck LD, 2011. Expression of vomeronasal receptors and related signaling molecules in the nasal cavity of a caudate amphibian (Plethodon shermani). *Chemical senses* 37:(4):335-346.

Krogh A, Larsson B, Von Heijne G, Sonnhammer EL, 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of molecular biology* 305:(3):567-580.

King JD, Rollins-Smith LA, Nielsen PF, John A, Conlon JM, 2005. Characterization of a peptide from skin secretions of male specimens of the frog, Leptodactylus fallax that stimulates aggression in male frogs. *Peptides* 26:(4):597-601.

Larson KA, 2004. Advertisement call complexity in northern leopard frogs, Rana pipiens. *Copeia* 2004:(3):676-682.

Leary CJ, 2009. Hormones and acoustic communication in anuran amphibians. *Integrative and comparative biology* 49:(4):452-470.

Laurila A, Karttunen S, Merilä J, 2002. Adaptive phenotypic plasticity and genetics of larval life histories in two Rana temporaria populations. *Evolution* 56:(3):617-27.

Leinders-Zufall T, Brennan P, Widmayer P, Maul-Pavicic A, Jäger M, Li XH, Breer H, Zufall F, Boehm T, 2004. MHC class I peptides as chemosensory signals in the vomeronasal organ. *Science 306*:(5698):1033-1037.

Leypold BG, Yu CR, Leinders-Zufall T, Kim MM, Zufall F, Axel R, 2002. Altered sexual and social behaviors in trp2 mutant mice. *Proceedings of the National Academy of Sciences* 99:6376–6381.

Liberles SD, 2014. Mammalian pheromones. *Annual review of physiology* 76:151-175.

Liman ER, Corey DP, Dulac C, 1999. TRP2: a candidate transduction channel for mammalian pheromone sensory signaling. *Proceedings of the National Academy of Sciences* 96:5791–5796.

Loftus‐Hills JJ, Johnstone BM, 1970. Auditory Function, Communication, and the Brain‐Evoked Response in Anuran Amphibians. *The Journal of the Acoustical Society of America* 47:(4B):1131-1138.

Lovern MB, Holmes MM, Wade J, 2004. The green anole (Anolis carolinensis): a reptilian model for laboratory studies of reproductive morphology and behavior. Ilar Journal 45:(1):54-64.

Lucas P, Ukhanov K, Leinders-Zufall T, Zufall F, 2003. A diacylglycerol-gated cation channel in vomeronasal neuron dendrites is impaired in TRPC2 mutant mice: mechanism of pheromone transduction. *Neuron* 40:551–61.

Malnic B, Hirono J, Sato T, Buck L, B. 1999. Combinatorial receptor codes for odors. *Cell 96*:(5):713-723.

Mason RT, Chivers DP, Mathis A, Blaustein AR, 1998. Bioassay methods for amphibians and reptiles. In *Methods in Chemical Ecology Volume* 2:271-325. Springer US.

Mason RT, Parker MR, 2010. Social behavior and pheromonal communication in reptiles. *Journal of Comparative Physiology A*, 196:(10):729-749.

McAllister KR, Leonard WP, Hays DW, Friesz RC, 1999. Washington State status report for the northern leopard frog. *Washington Department of Fish and Wildlife, Wildlife Management Program, Olympia, WA*.

Mezler M, Fleischer J, Breer H, 2001. Characteristic features and ligand specificity of the two olfactory receptor classes from Xenopus laevis. *Journal of Experimental Biology*

204:(17):2987-2997.

Muller-Schwarze D, 2006. *Chemical ecology of vertebrates*. Cambridge University Press.

Muller-Schwarze D, (Ed.), 2012. *Chemical signals in vertebrates*. Springer Science & Business Media.

Nei M, Niimura Y, Nozawa M, 2008. The evolution of animal chemosensory receptor gene repertoires: Roles of chance and necessity. *Nature Reviews Genetics* 9:951-963.

Niimura Y, Nei M, 2006. Evolutionary dynamics of olfactory and other chemosensory receptor genes in vertebrates. *Journal of human genetics* 51:505–517.

Niimura Y, 2013. Identification of chemosensory receptor genes from vertebrate genomes. *Pheromone Signaling: Methods and Protocols* 95-105.

Pace AE, 1974. Systematic and biological studies of the leopard frogs (*Rana pipiens* complex) of the United States.

Pearl CA, Cervantes M, Chan M, Ho U, Shoji R, Thomas, EO, 2000. Evidence for a mate -attracting chemosignal in the dwarf African clawed frog Hymenochirus. *Hormones and Behavior* 38:(1):67-74.

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team, 2017. nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-131, https://CRAN.R project.org/package=nlme.

Polo-Cavia N, Burraco P, Gomez-Mestre I, 2016. Low levels of chemical anthropogenic pollution may threaten amphibians by impairing predator recognition. *Aquatic Toxicology* 172:30-35.

Poth D, Peram PS, Vences M, Schulz S, 2013. Macrolides and alcohols as scent gland constituents of the Madagascan frog Mantidactylus femoralis and their Intraspecific

diversity. *Journal of natural products* 76:1548-1558.

Poth D, Wollenberg KC, Vences M, Schulz S, 2012. Volatile Amphibian Pheromones: Macrolides from Mantellid Frogs from Madagascar. *Angewandte Chemie International Edition* 51:2187-2190.

Pröhl H, 2003. Variation in male calling behaviour and relation to male mating success in the strawberry poison frog (Dendrobates pumilio). *Ethology 109:*(4):273-290.

RStudio Team, 2016. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.

Ryan MJ, 1988. Constraints and patterns in the evolution of anuran acoustic communication. *The evolution of the amphibian auditory system* 637-677.

Sansone A, Syed AS, Tantalaki E, Korsching SI, Manzini I, 2014. Trpc2 is expressed in two olfactory subsystems, the main and the vomeronasal system of larval Xenopus laevis. *Journal of Experimental Biology* 217:(13):2235-2238.

Saenz D, Johnson JB, Adams CK, Dayton GH, 2003. Accelerated hatching of southern leopard frog (Rana sphenocephala) eggs in response to the presence of a crayfish (Procambarus nigrocinctus) predator. *Copeia* 2003:(3):646-649.

Schoenbaum G, Chiba AA, Gallagher M, 1999. Neural encoding in orbitofrontal cortex and basolateral amygdala during olfactory discrimination learning. *Journal of Neuroscience* 19:(5):1876-84.

Schulte LM, Rössler DC, 2013. Do poison frogs recognize chemical cues of the other sex or do they react to cues of stressed conspecifics? *Behavioural processes* 100:32-35.

Schulte LM, Yeager J, Schulte R, Veith M, Werner P, Beck LA, Lötters S, 2011. The smell of

success: choice of larval rearing sites by means of chemical cues in a Peruvian poison frog. *Animal Behaviour* 81:(6):1147-1154.

Secondi J, Haerty W, Lode T, 2005. Female attraction to conspecific chemical cues in the palmate newt Triturus helveticus. *Ethology* 111:(8):726-735.

Shi P, Zhang JZ, 2007. Comparative genomic analysis identifies an evolutionary shift of vomeronasal receptor gene repertoires in the vertebrate transition from water to land. *Genome Research* 17:166-174.

Shi P, Zhang J, 2009. Extraordinary diversity of chemosensory receptor gene repertoires among vertebrates. In *Chemosensory systems in mammals, fishes, and insects* 57-75. Springer Berlin Heidelberg.

Shinn EA, Dole JW, 1978. Evidence for a role for olfactory cues in the feeding response of leopard frogs, Rana pipiens. *Herpetologica* 167-172.

Spehr M, Kelliher KR, Li XH, Boehm T, Leinders-Zufall T, Zufall F, 2006. Essential role of the main olfactory system in social recognition of major histocompatibility complex peptide ligands. *Journal of Neuroscience* 26:(7):1961-70.

Spehr M, Munger SD, 2009. Olfactory receptors: G protein-coupled receptors and beyond. *Journal of neurochemistry* 109:(6):1570-1583.

Spieler M, Linsenmair KE, 1997. Choice of optimal oviposition sites by Hoplobatrachus occipitalis (Anura:Ranidae) in an unpredictable and patchy environment. *Oecologia* 109:(2):184–199.

Starnberger I, Preininger D, Hödl W, 2014. From uni- to multimodality: towards an integrative view on anuran communication, *Journal of Comparative Physiology A* 200:777–787

Starnberger I, Poth D, Peram PS, Schulz S, Vences M, Knudsen J, Barej MF, Rödel MO, Walzl M, Hödl W, 2013. Take time to smell the frogs: vocal sac glands of reed frogs (Anura: Hyperoliidae) contain species-specific chemical cocktails. *Biological Journal of the Linnean Society* 110:(4):828-838.

Stéphane G, Franck L, Patrice D, Olivier G, 2005. PHYML Online—a web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Res*, 33 (suppl_2): W557-W559.

Stowers L, Holy TE, Meister M, Dulac C, Koentges G, 2002. Loss of sex discrimination and male-male aggression in mice deficient for TRP2. *Science*, 295:1493–500.

Teplitsky C, Plénet S, Léna JP, Mermet N, Malet E, Joly P, 2005. Escape behaviour and ultimate causes of specific induced defences in an anuran tadpole. *Journal of Evolutionary Biolo*gy 18:(1):180-90.

Thorbjarnarson J, Wang X, Ming S, He L, Ding Y, Wu Y, McMurry ST, 2002. Wild populations of the Chinese alligator approach extinction. *Biological Conservation* 103:(1):93-102.

Thurgate NY, Pechmann JH. Chemical cues and burrow choice in newly metamorphosed Dusky Gopher frogs (Rana sevosa). The Ecology of the Endangered Dusky Gopher Frog (Rana Sevosa) and a Common Congener, the Southern Leopard Frog (Rana Sphenocephala). 2006 May;5:14.

Touhara K, Katada S, Nakagawa T, Oka Y, 2006. Ligand screening of olfactory receptors. *G Protein-Coupled Receptors: Structure, Function, and Ligand Screening*, 85-109.

Touhara K, Vosshall LB, 2009. Sensing odorants and pheromones with chemosensory receptors. *Annual review of physiology 71*:307-332.

Lefort V, Longueville JE, Gascuel O. "SMS: Smart Model Selection in PhyML." *Molecular*

Biology and Evolution, msx149, 2017.

Wabnitz PA, Bowie JH, Tyler MJ, Wallace JC, Smith BP, 1999. Animal behaviour: Aquatic sex pheromone from a male tree frog. *Nature* 401:444-445.

Wabnitz PA, Bowie JH, Tyler MJ, Wallace JC, Smith BP, 2000. Differences in the skin peptides of the male and female Australian tree frog Litoria splendida. *The FEBS Journal 267:*(1):269-275.

Weldon PJ, Ferguson MW, 1993. Chemoreception in crocodilians: anatomy, natural history, and empirical results. *Brain, behavior and evolution* 41:(3-5):239-245.

Weldon PJ, Swenson DJ, Olson JK, Brinkmeier WG, 1990. The American alligator detects food chemicals in aquatic and terrestrial environments. *Ethology* 85:(3):191-198.

Werner EE, 1986. Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. *The American Naturalist* 128:(3):319-341.

West-Eberhard MJ, 1989. Phenotypic plasticity and the origins of diversity. *Annual review of Ecology and Systematics* 20:(1):249-78.

Wilczynski W, Endepols H, 2007. Central auditory pathways in anuran amphibians: the anatomical basis of hearing and sound communication. In *Hearing and sound communication in amphibians:* Springer New York; 221-249.

Woodley SK, 2010. Pheromonal communication in amphibians. *Journal of Comparative Physiology A* 196:(10):713-727.

Woodley S, 2015. Chemosignals, hormones, and amphibian reproduction. *Hormones and behavior 68*:3-13.

Woodley SK, 2014. Chemical signaling in amphibians. *Neurobiology of chemical communication* 255-285.

Wyatt TD, 2003. *Pheromones and animal behavior: communication by taste and smell*. (Cambridge, United Kingdom): Cambridge University Press.

Wyatt TD, 2005. Pheromones: convergence and contrasts in insects and vertebrates. In *Chemical Signals in Vertebrates* 10: Springer, Boston, MA; 7-19.

Wyatt TD, 2010. Pheromones and behavior. In *Chemical communication in crustaceans:* Springer New York; 23-38.

Wyatt TD, 2014. *Pheromones and animal behavior: chemical signals and signatures*. Cambridge University Press.

Xie Y, Wu G, Tang J, Luo R, Patterson J, Liu S, Huang W, He G, Gu S, Li S, Zhou X, 2014. SOAPdenovo- Trans: de novo transcriptome assembly with short RNA-Seq reads. *Bioinformatics* 30:(12): 1660-1666.

Zhou S, Stone EA, Mackay TF, Anholt RR, 2009. Plasticity of the chemoreceptor repertoire in Drosophila melanogaster. *PLoS genetics* 5:(10): e1000681.

Zucchi R, Chiellini G, Scanlan TS, Grandy DK, 2006. Trace amine-associated receptors and their ligands. *British journal of pharmacology* 149:(8):967-978.

Appendix A: Supplementary Files

1. *Chapter 1 Figures Supplementary File* (phylogenetic tree figures associated with Chapter 1)
2. *Known OR-TAAR-VR Supplementary File* (details about the 26 amphibian, reptile, and fish source species used to identify best orthologs)
3. *OR 4605 ORF-TMH-Prediction Supplementary File* (complete list of the olfactory receptor open reading frames known for 26 amphibian, reptile, and fish source species)
4. *VR 541 ORF-TMH-Prediction Supplementary File* (complete list of the vomeronasal receptor open reading frames known for 26 amphibian, reptile, and fish source species)
5. *TAAR 571 ORF-TMH-Prediction Supplementary File* (complete list of the trace amine-associated receptor open reading frames known for the 26 amphibian, reptile, and fish source species)
6. *110 Common Northern Leopard Frog CRS_annotation-ORF-TMH –SOAP-Trinity Supplementary File* (identified putative northern leopard frog chemoreceptor sequences and details)
7. *Northern Leopard Frog Common TRPC-nt-aa-SOAP-Trinity Supplementary File* (identified putative northern leopard frog trpc gene sequences and details)
8. *Northern Leopard Frog Common Golf-nt-aa-SOAP-Trinity Supplementary File* (identified putative northern leopard frog coupled g-protein gene sequences and details)
9. *Sum of Best ORF Matches Supplementary File* (summary statistics for top selected open reading frame ortholog matches)