CHARACTERIZATION OF TWO LARGE GENE FAMILIES IN THE SEA LAMPREY

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Fisheries and Wildlife – Doctor of Philosophy

2013

ABSTRACT

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This dissertation employed molecular biology and bioinformatics to examine two large gene families in the sea lamprey, *Petromyzon marinus*. An integrative approach was used to define these gene families in order to ensure the validity of the size and members of each gene family. There are two chapters: Chapter 1 examines chemosensory gene expression in a specialized part of the olfactory system and Chapter 2 studies the expression of detoxification genes in the liver and gills in response to the lampricide, 3-trifluoromethyl-4-nitrophenol (TFM).

CHEMORECEPTOR GENES

For this dissertation, I will restrict chemoreception to the detection of chemical signals in the nose (note: chemoreception includes taste), and is accomplished by detection of odorants in the environment by specialized sensory cells in the main olfactory epithelium (MOE). In certain tetrapods, a second sensory epithelium is also found in the nose, called the vomeronasal organ (VNO). Canonically, each epithelium represents the start of different olfactory pathways, which govern different behavioral responses. Each epithelium expresses different classes of chemoreceptor (CR) genes; the MOE expresses odorant receptors (ORs) and trace amine-associated receptors (TAARs), while the VNO expresses ORs, vomeronasal type-1 and type-2 receptors (V1Rs and V2Rs). The sea lamprey olfactory organ has one nostril and so has one nasal capsule, which is divided into two spatially distinct regions: the main olfactory epithelium

(MOE) and the accessory olfactory organ (AOO). The MOE has been well studied in lampreys but the function of the AOO has eluded description for over 100 years. Based on other research and due to its proximity to the MOE, we hypothesized that the AOO represents an ancestral VNO. If this AOO is indeed an ancestral VNO, we expect a different connectivity to the central nervous system than from the MOE, and would expect expression of pheromone receptors (V1Rs and V2Rs). CR expression in the MOE and AOO of sea lamprey were examined. The differential expression of CR genes between the two epithelia was determined and the connectivity of the main and accessory epithelia was determined using neural tract tracing. Quantitative PCR confirmed and quantified the differential expression of specific genes in the main and accessory olfactory epithelia.

CYTOCHROME P450 GENES

The second gene family to be explored is the cytochrome P450 family. P450 genes encode for steroidogenic or detoxification enzymes that are inducible by a substrate. As part of the strategy for controlling sea lamprey populations TFM is applied to streams. Very little is known at the molecular level of how TFM works to kill sea lamprey larvae, but based on responses by other organisms to xenobiotic substances, our hypothesis is that P450 genes are induced by exposure to TFM. P450 genes were predicted from the sea lamprey genome and larvae were exposed to TFM and gill and liver tissues were harvested over an 8-hour time course. Expression was confirmed using high-throughput sequencing and quantitative PCR. The immediate goal was to determine which P450 genes are induced by exposure to TFM. Alternatively, we generated a list of predicted Phase II detoxification enzymes in the event that P450 genes showed no difference in expression. The long-term goal is to use that knowledge to design more efficient and specific lampricides.

This dissertation is dedicated to my parents, Suk-Jin and Young-Ja Chang, who instilled in me a desire and passion for education. I also dedicate this to my partner, Bob Coffey, who has supported me throughout this process.

ACKNOWLEDGEMENTS

I would like to thank my graduate committee including Dr. Weiming Li, Dr. Titus Brown, Dr. Colleen Hegg and Dr. Laura Smale for their help and guidance. Additional thanks go to Kaben G. Nanlohy, Dr. Juan Pedro Steibel and Pablo Reeb for help with bioinformatics and statistical analyses.

Funding was provided by US NIGMS grant 5R24GM83982 (to WL), NSF grant IOB0517491 (to WL), Great Lakes Fishery Commission Grants (to WL), and USDA NIFA AFRI Competitive Grant no. 2010-65205-20361 (to CTB).

Logistical support was provided by the Hammond Bay Biological Station (part of the U.S. Geological Survey – Biological Resources Division) in Millersburg, MI. Special thanks to Karen Slaght for helping set up the TFM experiments.

Dr. Yu-Wen Chung-Davidson also contributed to this research and was a source of tremendous support.

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KEY TO ABBREVIATIONS

VNO – vomeronasal organ

AOE - accessory olfactory epithelium

MOE – main olfactory epithelium

DTN – dorsomedial telencephalic neuropil

OR – odorant receptor

V1R – vomeronasal receptor, type 1

V2R – vomeronasal receptor, type 2

TAAR – trace amine-associated receptor

GnRH – gonadotropin-releasing hormone

LCM – laser capture microdissection

GO – gene ontology

PCR – polymerase chain reaction

CR – chemoreceptor

INTRODUCTION

The sea lamprey is a jawless vertebrate of the superclass Agnatha, currently represented by lampreys and hagfish, that diverged from jawed vertebrates (Gnathostomes) approximately 560 million years ago [1]. Two important ways that lampreys differ from other vertebrates are they are jawless and their skeleton is cartilaginous. Divergence of Agnathans from other vertebrates has been inferred by anatomy [2] and by examination of certain genes, such as ribosomal RNA [3, 4]. Currently, there are 38 recognized species of lamprey worldwide: 34 in the northern hemisphere and 4 in the southern hemisphere [1]. Differentiation of lamprey species is presently determined by a variety of factors, including dentition, anatomy and life history. This dissertation will focus on the North American species, *Petromyzon marinus*. As a model organism, the sea lamprey has been used to study topics as diverse as olfaction [5–7] and locomotion [8], however, the lack of a completed genome has meant that past studies were limited to a few genes at a time.

The genome of the sea lamprey has recently been sequenced with 70% coverage and a depth of 9x, comprising about 26,000 genes [9]. A whole genome "shotgun" approach was used to sequence the DNA from liver cells of an adult sea lamprey. While the examination of the sea lamprey genome is in its nascent stages, some interesting features have been identified, including 2 rounds of whole genome duplication (2R), a rich G-C content (~46%) and high content of repetitive elements [9]. Having a completed genome of this early vertebrate fills a gap in the body of literature concerning evolution of whole genomes as well as specific gene families. Before high-throughput sequencing, a handful of studies used commercially available microarrays to discover the gene profiles of specific organs after exposure to a micro-organism [10–13]. With the power of next-generation sequencing technology, it is now possible to know

all genes expressed in a particular organ or in a particular state or treatment. Annotation of the genome, coupled with high-throughput sequencing technologies such as Illumina/Solexa have permitted a richer description of the content of the sea lamprey genome and ultimately permit whole genome or transcriptome analyses.

This dissertation is composed of two manuscripts describing two large gene families in the sea lamprey genome: chemoreceptor (CR) and cytochrome P450 genes. Here, I define chemoreceptor genes as chemosensory genes that are expressed in the olfactory or vomeronasal epithelia of vertebrates, and include odorant receptors (ORs), trace amine-associated receptors (TAARs), and vomeronasal type-1 and type-2 receptors (V1Rs and V2Rs). The CR and P450 gene families were chosen because of their different histories in vertebrate evolution. Olfactory receptor genes have undergone significant expansions during vertebrate evolution, now comprising the largest gene family in the genome [14]. The P450 gene family is also large and diverse, but when compared to chemoreceptors, this family comprises a smaller portion of the genome. While P450 gene complements may be similar between organisms with respect to gene families represented or even in total genes, important differences between organisms arise in the responsiveness of certain P450 genes to xenobiotic compounds and contribute to the differential survival of organisms in the same environment. A better understanding of these large gene families will give clues as to their role in the evolution of sea lamprey and of vertebrates in general. Recent work has confirmed that the sea lamprey genome has undergone a 2R duplication [9]; therefore, I speculated that the chemoreceptor and P450 gene families have undergone a similar expansion. As well, a deeper understanding of the makeup of these gene families would have significant implications for management of this invasive species. I hypothesized that sea lamprey chemoreceptor and P450 gene families would share some

characteristics with homologous gene families in other vertebrates, but also would exhibit some species- or lineage-specific characteristics to distinguish them from other vertebrates, which would reflect the sea lamprey's basal phylogeny within vertebrates and the sea lamprey's life history and environment. My goal was that through examination of these gene families in sea lamprey, insight into the evolution of these gene families in the vertebrate lineage could be obtained.

OVERVIEW OF OLFACTION

Organisms gain information about their environment via their senses, including olfaction. Chemical information is detected in the air or water by inspiration of signals into the nasal cavity. In some tetrapods, there are two sensory epithelia in the nasal cavity with distinct sensory cells. In the main olfactory epithelium are specialized ciliated cells called olfactory sensory neurons (OSNs), which are responsible for detection of regular odors. In the vomeronasal organ (VNO) are specialized microvillar cells called vomeronasal sensory neurons (VSNs), which are responsible for detection of pheromones. On the cilia and microvilli are receptors, which are part of the 7-transmembrane family of G-protein coupled receptors, and the transmembrane part of the receptor is where the variability in receptors exists to match the various odorants in the external environment. Chemoreceptor (CR) genes encode for a large group of G-protein coupled receptors that are expressed in olfactory or vomeronasal sensory neurons [14, 15]. Within CR genes are 4 main subtypes that are grouped together by function: olfactory receptors (OR), trace amine-associated receptors (TAAR) and vomeronasal type 1 and type 2 receptors (V1R and V2R). The OR family was characterized by Buck and Axel [14] and has since been shown to be largely expanded in vertebrates [16]. The V1R family is expanded in tetrapods and the V2R family is expanded in teleost fish [17]. G-proteins can bind guanine,

which gives them their name. G-proteins are linked to the ORs, and are comprised of 3 subunits; alpha, beta and gamma [18]. Different G-proteins are distinguished by their alpha subunit and olfactory related G-proteins include G_o, G_{olf}, $G_{\alpha i2}$ and $G_{\alpha o}$. Binding of an odorant to a G-protein coupled OR induces an exchange of receptor-bound guanosine diphosphate (GDP) for guanosine triphosphate (GTP), which then activates the alpha subunit to dissociate and bind to a second messenger; either adenylyl cyclase, which converts ATP to cyclic AMP (cAMP) or phospholipase C, which then cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diaglycerol [18, 19]. In the cAMP pathway, a rise in the intracellular concentration of cAMP then triggers the opening of cyclic nucleotide gated calcium channels, which allows an influx of Ca^{++} into the OSN, which causes depolarization; in the IP_3 pathway, the rise in intracellular concentration of IP_3 triggers the release of Ca^{++} from the endoplasmic reticulum, which depolarizes the vomeronasal sensory neuron [18, 19].

Signals from the MOE and VNO follow segregated pathways to the brain. In the MOE, the chemical information (now converted to an electrical signal) is transmitted through the axons of OSNs, which terminate in the first part of the brain, called the olfactory bulb (OB) [19]. Specifically, the axons of OSNs terminate in discrete, densely packed areas of neuropil called glomeruli. In glomeruli, incoming olfactory information is received, integrated and relayed via mitral cells to higher order integrative centers in the brain such as the habenula, hypothalamus, thalamus and olfactory cortex [19, 20]. In the vomeronasal system, axons of the vomeronasal sensory neurons terminate in glomeruli in the accessory olfactory bulb and are relayed via mitral cells to areas such as the amygdala and hypothalamus [21, 22].

SEA LAMPREY – MAIN OLFACTORY PATHWAY VS. PUTATIVE VOMERONASAL PATHWAY

The sea lamprey has a single nostril, which contains a single, recognized olfactory epithelium, the MOE. The MOE is lined with a pseudo-stratified ciliated columnar epithelium, which projects to the main olfactory bulb [5, 20, 23]. At the base of the MOE are small vesicles that are lined with a cuboidal, ciliated epithelium, termed the accessory olfactory organ (AOO) or accessory olfactory epithelium (AOE). The function of this structure is unknown, but recent research (Ren et al. 2009) has suggested that the sea lamprey AOE is connected to the medial portion of the olfactory bulb [6]. Recent work has discovered 59 chemoreceptor genes, including ORs, TAARs and V1Rs in the genome of the sea lamprey [24].

Olfaction in certain tetrapods is governed by two parallel pathways that have overlapping functions: the main olfactory pathway detects regular, non-volatile odorants and the vomeronasal pathway detects pheromones [21]. The vomeronasal pathway runs in parallel to the main olfactory pathway, with a separate sensory epithelium, separate projections to the forebrain and a separate olfactory integration center [19, 21].

In Chapter 1, expression profiling was paired with neural tract-tracing and molecular biology to discover a partial division of the primary olfactory pathway in the sea lamprey which may be related to the tetrapod vomeronasal pathway. This work was published in 2013 (Chang et al. *BMC Evolutionary Biology* 2013, **13**:172 doi:10.1186/1471-2148-13-172).

Additionally, it is known that sea lamprey mating is mediated by pheromones [25, 26] and is currently exploited as part of the integrated pest management strategy used by the U.S. Geological Survey to control sea lamprey populations in the Great Lakes. A better

understanding of the expression of chemoreceptors in sea lamprey is the first step to identifying which are responsible for mediating pheromone detection and so can be targeted for future works to block detection, which would reduce mating.

OVERVIEW OF DETOXIFICATION

Organisms must possess a mechanism for elimination of chemical compounds that are detrimental to their ongoing survival. Detoxification of either endogenous or exogenous toxic substances is accomplished by a suite of two large groups of enzymes that are grouped according to substrate and mode of action. These two enzyme groups are termed Phase I and Phase II enzymes and work together to clear toxic substances from an organism [27–30].

Detoxification can be divided into two phases or steps: modification and conjugation. Modification is accomplished by the Phase I enzymes which include the cytochrome P450 enzymes. The cytochrome P450 superfamily is a large group of genes that are responsible for synthesis of endogenous steroids and metabolism of xenobiotic compounds [31–33] and is primarily expressed in the liver, but expression in extra-hepatic tissues in other vertebrates is known. For example, pregnane X receptor, a detoxification related gene, is expressed in liver, eye, brain, intestine, heart and kidney of zebrafish [34]. As well, the olfactory epithelium in various organisms from insects to mammals is known to express cytochrome P450 detoxification genes in response to a toxic compound [35–37]. In humans, there are 18 families of P450 genes, grouped into numbered families based on sequence similarity and functional similarity [32, 38–41]. With the advent of next-generation high-throughput sequencing technologies such as Solexa/Illumina, entire genomes from multiple organisms (pufferfish [42]; mouse [43]; sea urchin [44]; rat [45]; and zebrafish http://www.sanger.ac.uk/Projects/D_rerio/wgs.shtml) have

been sequenced, allowing for evolutionary and phylogenetic analyses on a whole genome scale. Sea lamprey have a reduced repertoire of P450 genes compared to other vertebrates such as zebrafish and humans [39, 46], but still maintain representatives of all four detoxifying P450 families in the genome. This large family of genes is highly conserved and is found in diverse organisms such as plants, insects, fish and humans. Their mode of action is primarily to make a substrate more polar in order to facilitate excretion of the compound [47]. One example is oxidation of a toxic compound, R, in the equation $RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$. The toxic substance is metabolized into a form that is more readily excreted by the organism, but if this metabolite cannot be excreted, Phase II enzymes then act on this metabolite to ultimately clear this substance from the organism [30, 48]. Phase II enzymes are grouped as transferases and include methyltransferases, sulfotransferases and glutathione-S-transferases. These enzymes work by adding a side-group to a metabolite to increase its solubility and facilitate excretion [48].

SEA LAMPREY AND DETOXIFICATION OF 3-TRIFLUOROMETHYL-4-NITROPHENOL (TFM)

The control of sea lamprey populations is accomplished through a multidisciplinary strategy that includes treatment of infested streams with the selective lampricide 3-trifluoromethyl-4-nitrophenol (TFM) to kill sea lamprey larvae. Little is known about how TFM is processed by larvae, but it is known that TFM is glucuronated and that sea lamprey larvae are unable to quickly rid themselves of TFM-glucuronide [49–51]. Formation of TFM-glucuronide would imply a Phase II UDP-glucoronosyltransferase in the pathway of TFM detoxification. There is some research that suggests TFM interferes with oxidative phosphorylation, meaning

that the supply of ATP does not match the demand, essentially starving the larvae to death [52, 53]. What is not known is what role P450 enzymes have in TFM detoxification.

The exact mechanism of TFM toxicity is unknown, but given that it is a xenobiotic compound, we hypothesized that expression of select P450 genes is induced by TFM exposure. Obtaining a complete survey of the P450 complement in sea lamprey as well as identification of candidate genes that respond to TFM exposure will help to improve the current practice of lampricide treatment as well as help design new and more effective lampricides.

In Chapter 2, I have determined the complete predicted P450 complement of sea lamprey, including representatives of the four known detoxification families. Further analysis has identified several candidate P450 genes in liver and gill that respond to TFM exposure.

This dissertation began as our efforts to sequence the sea lamprey genome began. The integrated pest management strategy employed by the United States Geological Survey to control sea lamprey populations has two main arms: treatment of infested streams with the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) to kill larval sea lamprey and baiting of traps with the pheromone 3-keto petromyzonol sulfate (3-kPZS) to trap migrating adults as they travel to spawning grounds. Genes related to detoxification and olfaction are implicated in the effectiveness of these two strategies and the P450 and chemoreceptor gene families were chosen for study. This dissertation furthers our understanding of the evolution of these gene families in the vertebrate lineage and helps to identify gene targets for future control strategies of sea lamprey populations.

CHAPTER 1

A Primordial Vomeronasal System in a Jawless Vertebrate

Abstract

Background: A dual olfactory system, represented by two anatomically distinct but spatially proximate chemosensory epithelia that project to separate areas of the forebrain, is known in several classes of tetrapods. Lungfish are the earliest evolving vertebrates known to have this dual system, comprising a main olfactory and a vomeronasal system. Lampreys, a group of jawless vertebrates, have a single nasal capsule containing two anatomically distinct epithelia, the main (MOE) and the accessory olfactory epithelia (AOE). Given that the sea lamprey AOE function has remained elusive, we hypothesized that the AOE may represent part of a primordial vomeronasal system in sea lamprey and we examined the AOE projections to the forebrain and compared the projection pattern to that of the tetrapod vomeronasal epithelia.

Results: To test this hypothesis, we characterized the neural circuits and molecular profiles of the accessory olfactory epithelium in the sea lamprey (*Petromyzon marinus*). Neural tract-tracing revealed direct connections from the AOE to the dorsomedial telencephalic neuropil (DTN) which in turn projects directly to the dorsal pallium and the rostral hypothalamus. High-throughput sequencing demonstrated that the main and the accessory olfactory epithelia have virtually identical profiles of expressed genes. Real time quantitative PCR confirmed expression of representatives of all 3 chemoreceptor gene families identified in the sea lamprey genome.

Conclusion: Anatomical and molecular evidence shows that the sea lamprey AOE may serve a chemosensory function.

Background

A dual olfactory system is thought to be unique to tetrapods. The two distinct sensory epithelia of this system, the main olfactory epithelium (MOE) and the vomeronasal organ (VNO), heterogeneously express families of chemoreceptor genes, with some overlap [21]. These epithelia have anatomically distinct projections to different parts of the forebrain. These dichotomous molecular and anatomical profiles led to the hypothesis that the VNO is specialized to detect pheromones [14, 15, 54–56] whereas other research has suggested overlapping functions for the MOE and VNO [57–61]. Amphibians were thought to be the earliest evolving animals with a complete vomeronasal system [62, 63], however recent work has shown that lungfish possess cellular (microvillous cells) and molecular (vomeronasal receptors, VNRs) components of a typical vomeronasal system [64]. It should be noted that although they do not possess a canonically recognized VNO, molecular components of a vomeronasal system exist in elephant shark [65] and teleost fish [16, 17]. Therefore, the vomeronasal system is presumed to have evolved after the main olfactory system in the vertebrate lineage [66].

Although a distinct vomeronasal system had not been identified in teleost fish [67, 68], a recent study has found a vomeronasal system in a sister group to tetrapods, the lungfish [64, 69]. Moreover, vomeronasal-type receptors have been identified in a basal vertebrate, the sea lamprey (*Petromyzon marinus*) [24, 65]. Although goldfish do not have a VNO, Dulka [67] suggested that different subdivisions of the olfactory tract respond to odorants of different functions. Interestingly, the sea lamprey, like some tetrapods, has two separate and distinct olfactory epithelia. The AOE was discovered by in 1887 by Scott [70], but its function had eluded description. In 2009, Ren et al. showed that lamprey AOE is lined with a simple cuboidal ciliated epithelium and projects to the medial olfactory bulb [6]. In addition, another structure with

elusive function in the sea lamprey brain, the dorsomedial telencephalic neuropil (DTN) [71], is located in a similar position to the mammalian AOB [72]. The sea lamprey DTN is dorsomedially situated, immediately caudal to the olfactory bulb, receives input from the olfactory bulb and projects to the hypothalamus [73, 74].

We reasoned that if the AOE was chemosensory, it should express at least some of the chemoreceptor (CR) genes encoded in the lamprey genome [24]. We further reasoned that the AOE projects to a separate telencephalic region, possibly the DTN, in addition to the known projections to the medial olfactory bulb [6]. Here we present evidence that AOE expresses all known families of lamprey CR genes and projects to the DTN. We conclude that the AOE-DTN-hypothalamic pathway in lamprey is a partial segregation of the olfactory pathway from the known MOE and AOE projections to the OB.

Results

AOE projects to the DTN and other telencephalic areas

Figure 1 is an atlas to provide reference for the tract-tracing images. Relevant structures to this study as well as reference landmarks are provided. Injections of biocytin to the AOE vesicles revealed labeling in the olfactory system and the telencephalon. Neurons with wide, thick cell bodies with a dendritic knob and cilia extending into the lumen of the accessory olfactory organ were evident (Fig. 2A). Labeled cells in the MOE showed a retrograde connection from the AOE, however this could be due to leakage of dye from the AOE to en passant olfactory nerve fibers rather than a neural connection between the AOE and MOE. Tall, thin neurons were labeled in the basal lamellae of the MOE (Fig. 2B). Labeled cells lining the MOE were pseudo-stratified ciliated columnar cells and those lining the AOE were ciliated, round cells. The dorsal half of the olfactory nerve was more strongly labeled than the ventral part

(Fig. 2C). Labeled fibers and cells were observed in the medial olfactory bulb, at the ventral aspect of the DTN as well as the preoptic area and striatum (Fig. 2 D, E, F). Coarse fibers were visible in the DTN and cell bodies were seen at the ventral DTN (Fig. 2G). A bundle of thick fibers was seen between the medial pallium and the DTN, as well as cell bodies in the dorsal pallium and ventral DTN (Fig. 2H). The dorsal pallium (Fig. 2 I) showed a grouping of coarse fibers and some cell bodies. In summary, the AOE has connections to the medial olfactory bulb, the DTN and pallia, and indirectly to the rostral hypothalamus.

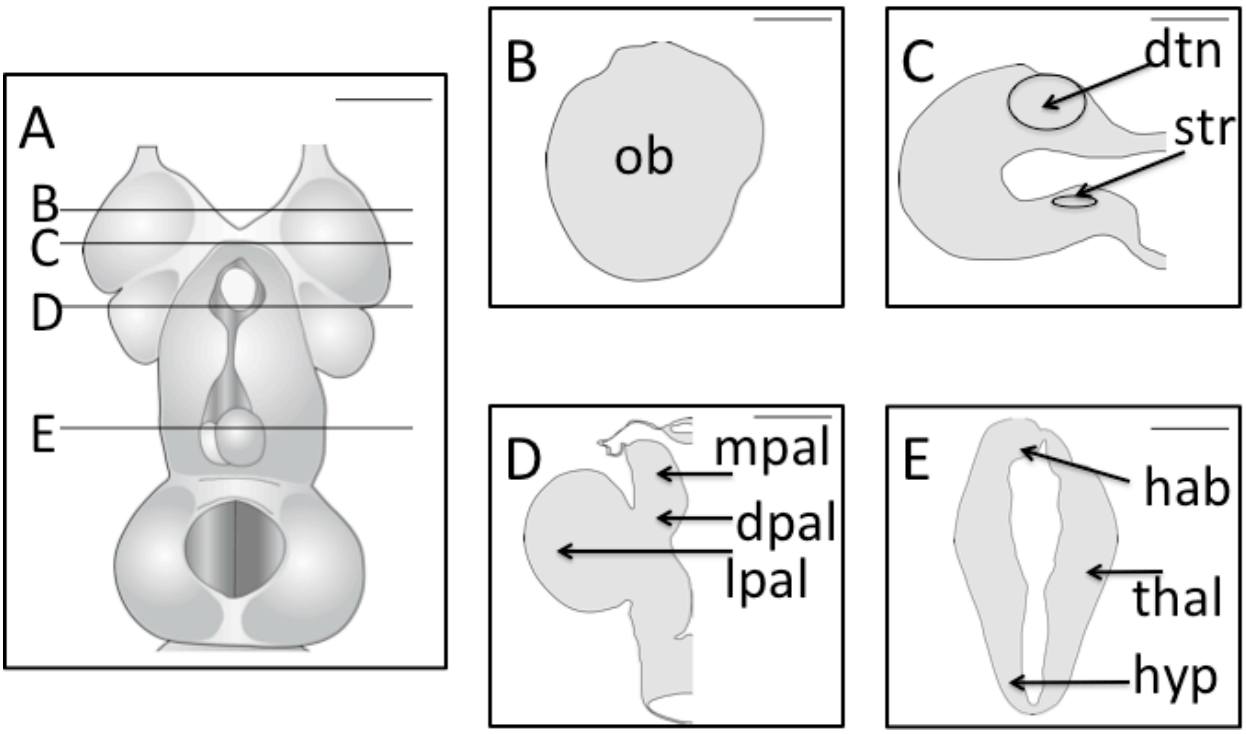


Figure 1: Diagram of dorsal view of adult lamprey brain and coronal plane slices. **A,** The adult lamprey brain is shown in dorsal view with lines representative of coronal sections indicated by lines and letters. **B,** Olfactory bulb. **C,** Rostral telencephalon with the dorsomedial telencephalic neuropil and striatum. **D,** Telencephalon with lateral, medial and dorsal pallia. **E,** Caudal telencephalon with habenula, thalamus, hypothalamus. Scale bar in all pictures is 100 µm. dpal: dorsal pallium; dtn: dorsomedial telencephalic neuropil; hab: habenula; hyp: hypothalamus; lpal.: lateral pallium; mpal.: medial pallium; ob: olfactory bulb; str: striatum; thal: thalamus.

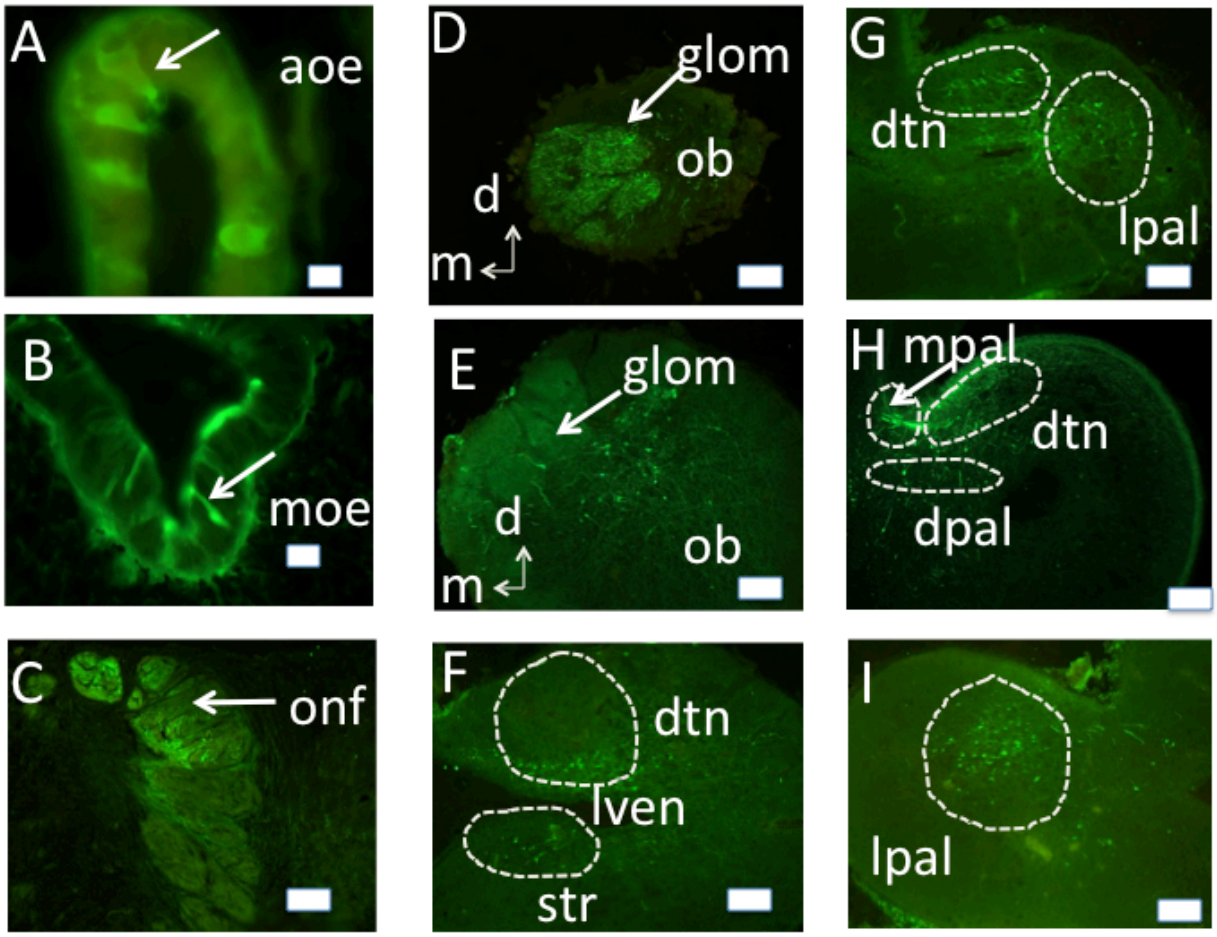


Figure 2: Anterograde and retrograde connections of the lamprey accessory olfactory organ. Biocytin injections were made to AOE vesicles and representative pictures are shown here. **A**, following dye loading, short stout cells with cilia extending into the lumen of the AOE are visible (red arrows). Scale bar: 10 μm. **B**, olfactory sensory neurons with long thin axons are retrogradely labeled in the valleys of main olfactory lamellae (red arrows). Scale bar: 20 μm. **C**, the dorsal half of the nerve is preferentially labeled, reflecting the dorsal pathway of axons from the AOE to the telencephalon. Cell bodies and fibers are seen in the medial (**D**) and central (**E**) part of the olfactory bulb. The ventral portion of the DTN and the striatum (**F**) have short thin fibers and cell bodies. The DTN has coarse, thick fibers and the lateral pallium has short, coarse fibers and cell bodies (**G**). A bundle of thick fibers is visible from the medial pallium to the DTN (**H**) and cell bodies are visible in the dorsal pallium and the ventral border of the DTN. The lateral pallium has a grouping of cell bodies and a mixed population of thin and thick fibers (**I**). aoe: accessory olfactory epithelium; dpal: dorsal pallium; dtn: dorsomedial telencephalic neuropil; glom: olfactory glomerulus; lpal: lateral pallium; lven: lateral ventricle; ob: olfactory bulb; onf : olfactory nerve fascicle; str: striatum. Scale bars for **C**-**I**: 100 μm.

For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

DTN connects to the AOE and the hypothalamus

Injections of biocytin to the DTN revealed labeling of cells in the AOE as well as direct projections to various regions of the telencephalon. Labeling revealed round cells in the AOE (Fig. 3A**)** and tall elongate cells in the MOE (Fig. 3B**)**. Fibers and cell bodies were labeled in the medial olfactory bulb (Fig. 3C), similar to the results shown in Derjean et al., 2010 [7]. The rostral DTN was densely labeled with fibers (Fig. 3D, E). Within the DTN were some cell bodies oriented dorsoventrally with at least 1 process extending dorsally toward the DTN (Fig 3E). At the caudal end of the DTN, the fiber population was smaller than at the rostral end. As well, the fibers were coarse and grouped at the dorsal part of the DTN (Fig 3F). The thalamus had coarse fibers bilaterally located (Fig. 3G). The rostral hypothalamus had coarse fibers and some cell bodies bilaterally at the level of the mammillary recess (Fig. 3 H). In the habenula, a mixed population of thin and thick fibers was seen (Fig 3 I). In summary, sea lamprey DTN has connections to the AOE as well as multiple integrative centers (dorsal pallium, lateral pallium, thalamus and hypothalamus).

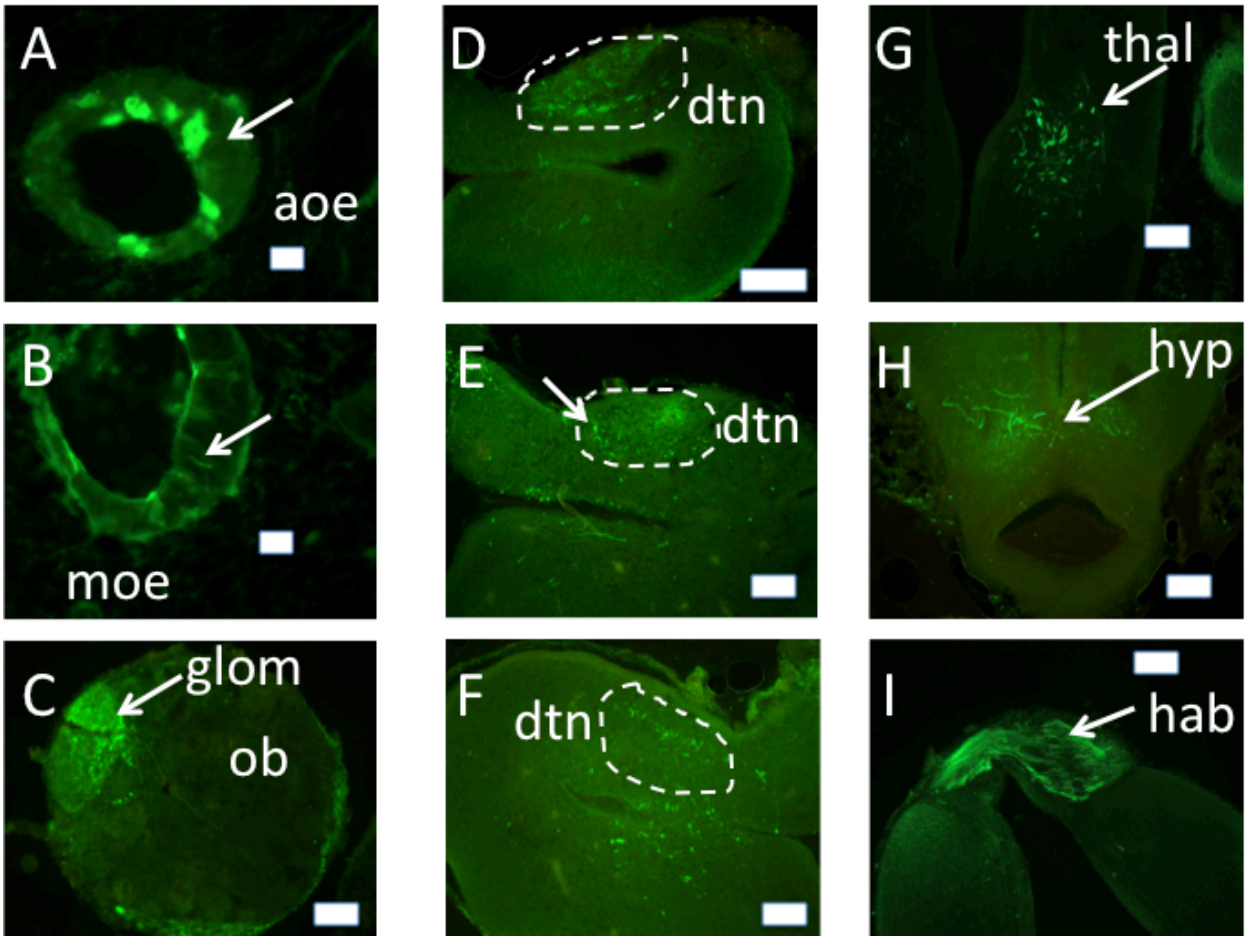


Figure 3: Anterograde and retrograde connections of the lamprey dorsomedial telencephalic neuropil (DTN). Biocytin injections were made to the DTN and representative pictures are shown here. **A**, following dye loading, round or ovoid shaped cells are labeled in AOE vesicles (arrow). Scale bar: 20 μ **B**, olfactory sensory neurons with long thin cell bodies are seen in the main olfactory epithelium (red arrow). Scale bar: 20 μm. **C**, medial glomerular territories are labeled. Scale bar: 100 μm. **D**, the DTN has a dense population of fibers and a smaller population of coarse thick fibers at the ventral portion, as well as some thick short fibers in the striatum. Scale bar: 200 μm. **E**, the caudal aspect of the DTN has a sparse population of short fibers, both coarse and thin. Labeled cells are dorsoventrally oriented, located proximate to the DTN (arrow) (**F**) and visible throughout the entire rostrocaudal extent of the DTN. **G**, coarse, thick fibers are labeled in the thalamus. Scale bar = 100 um. **H**, coarse, thick fibers are seen bilaterally in the hypothalamus. Scale bar = 100 um. **I**, the habenula is densely labeled with thin and thick fibers.

aoe: accessory olfactory epithelium; dtn: dorsomedial telencephalic neuropil; glom: glomerular territory; hab: habenula; hyp: hypothalamus; moe: main olfactory epithelium; ob: olfactory bulb; thal: thalamus;

MOE and AOE have virtually identical gene expression profiles

The first attempt to discover gene categories that differed between the MOE and AOE by 2 fold ($\log_2 2 = 1.0$) failed to show any differences in gene expression between the two epithelia. Therefore, the threshold for differential gene expression was lowered ($\log_2 1.414 = 0.5$) which corresponds to a 1.414 fold change in expression. 31 of 11,225 gene ontology (GO) categories were shown to be differentially expressed between the two epithelia, which represent less than 0.3% of the Gene Ontology (GO) categories compared. A heat map of the 31 GO categories changed is shown in Figure 4. The majority of GO category differences are due to cell maintenance or receptor trafficking (e.g. GO0010970: microtubule based transport or GO0048193: Golgi vesicle transport).

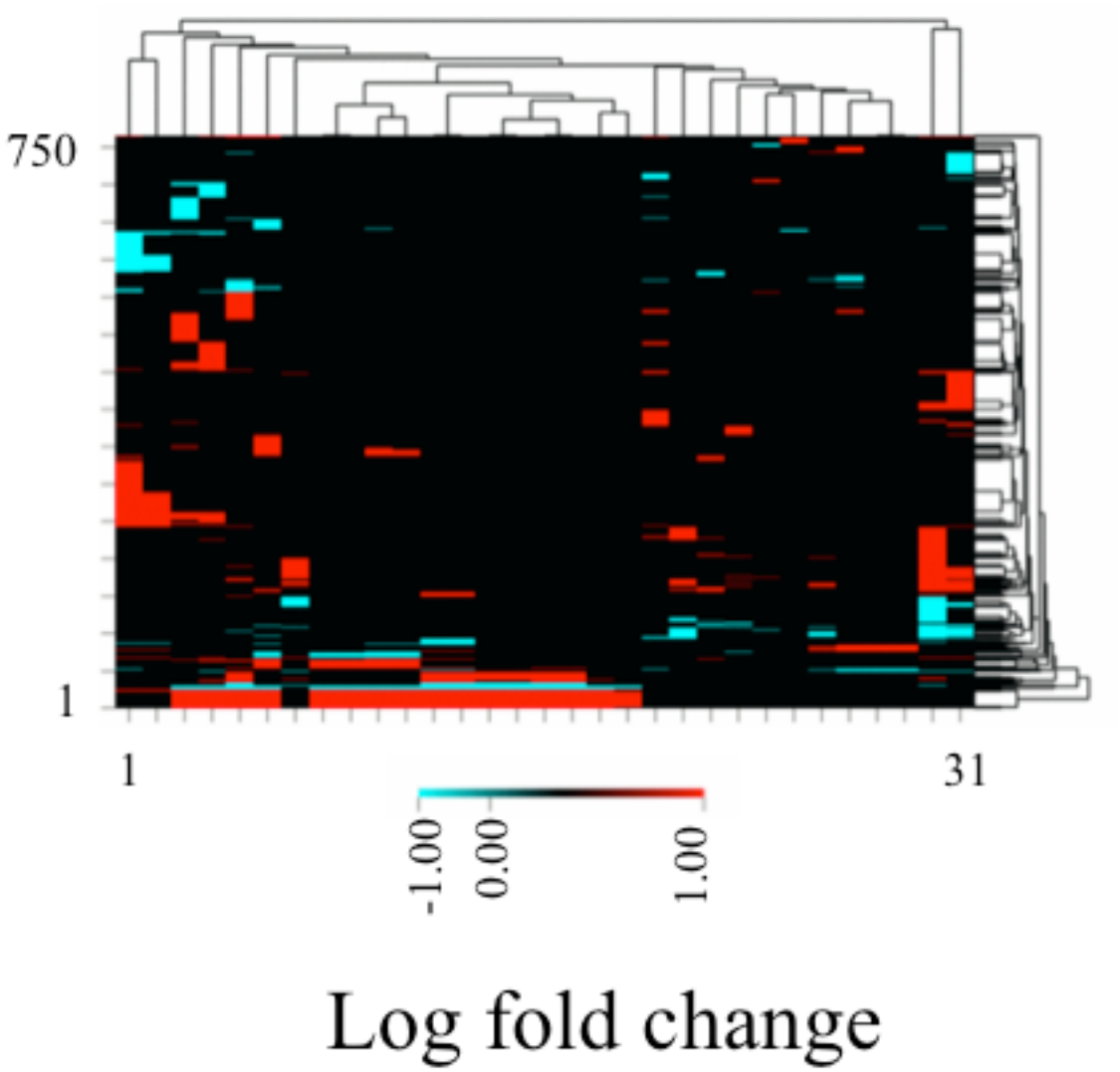


Figure 4: Comparisons of the transcriptomes (accessory vs. main olfactory epithelium) using gene ontology (GO) analyses. Transcriptomes were obtained using Illumina DGE sequencing technology. X-axis represents the GO categories and Y-axis represents gene clusters. Color scale represents the Log_2 (transcript number in accessory/transcript number in main olfactory epithelium). X-axis: 1.GO0044429 mitochondrial part, 2.GO0005759 mitochondrial matrix, 3.GO0030529 ribonucleoprotein complex, 4.GO0006412 translation, 5.GO0034621 cellular macromolecular complex subunit organization, 6.GO0016032 viral reproduction, 7.GO0006887

Figure 4 (cont'd)

exocytosis, 8.GO0019080 viral genome expression, 9.GO0019083 viral transcription, 10.GO0022415 viral reproductive process, 11.GO0019058 viral infectious cycle, 12.GO0022411 cellular component disassembly, 13.GO0071845 cellular component disassembly at cellular level, 14.GO0043624 cellular protein complex disassembly, 15.GO0043241 protein complex disassembly, 16.GO0032984 macromolecular complex disassembly, 17.GO0034623 cellular macromolecular complex disassembly, 18.GO0006415 translational termination, 19.GO0022626 cytosolic ribosome, 20.GO0003924 GTPase activity, 21.GO0048193 Golgi vesicle transport, 22.GO0008565 protein transporter activity, 23.GO0031902 late endosome membrane, 24.GO0033044 regulation of chromosome organization, 25.GO0080008 CUL4 RING ubiquitin ligase complex, 26.GO0051648 vesicle localization, 27.GO0007018 microtubule-based movement, 28.GO0010970 microtubule-based transport, 29.GO0030705 cytoskeleton-dependent intracellular transport, 30.GO0016192 vesicle-mediated transport, and 31. GO0031988 membrane-bounded vesicle.

Expression of chemoreceptor genes is sexually dimorphic

Sequences generated from high-throughput sequencing were aligned to the mouse RefSeq mRNA database. Using these sequences in combination with those identified by Libants et al. [20], representative chemoreceptor and chemoreceptor-related genes were selected to confirm the Solexa sequencing results and to further examine the chemoreceptor gene expression of the MOE and AOE. Real time quantitative PCR confirmed expression of six chemoreceptor and chemoreceptor-related genes (OR 3267, OR 6425, TAAR 3721, V1R 18775, CASR and adenylate cyclase) in both the MOE and AOE. Collectively, MOE and AOE displayed a sexually dimorphic pattern in expression of CR and CR-related genes. OR 3267 ($p < 0.0001$), TAAR 3721 ($p < 0.0001$) and adenylate cyclase ($p = 0.0319$) were expressed higher in adult females than in males (Fig. 5) while OR 6425 ($p < 0.0001$), V1R 18775 ($p = 0.0029$) and CASR ($p < 0.0001$) were expressed higher in adult males than in females (Fig. 6). The expression levels of these genes did not differ between MOE and AOE.

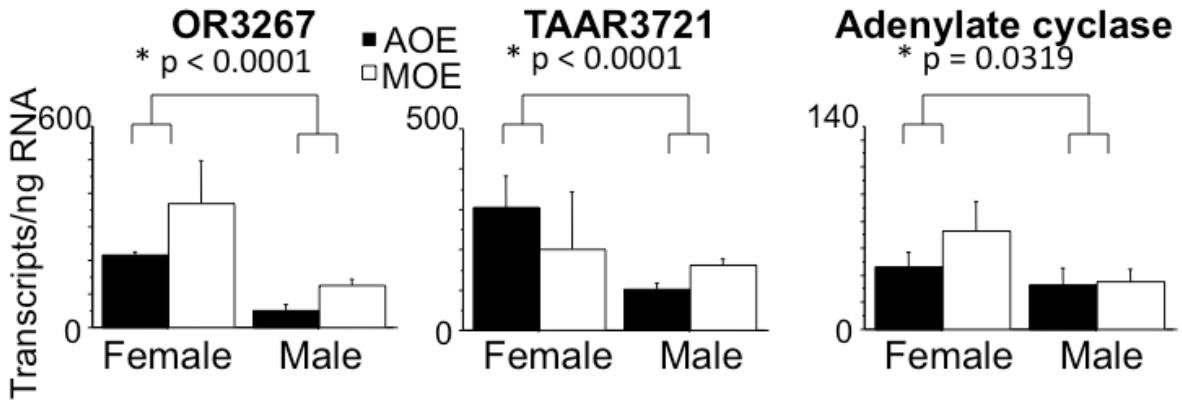


Figure 5: OR 3267, TAAR 3721 and adenylate cyclase are expressed significantly higher in adult female than in adult male sea lampreys. SYBR green real time quantitative PCR reveals olfactory receptor 3267 ($p < 0.0001$), trace amine-associated receptor 3721 ($p < 0.0001$) and adenylate cyclase ($p = 0.0319$) are expressed significantly higher in adult female lampreys than in adult males.

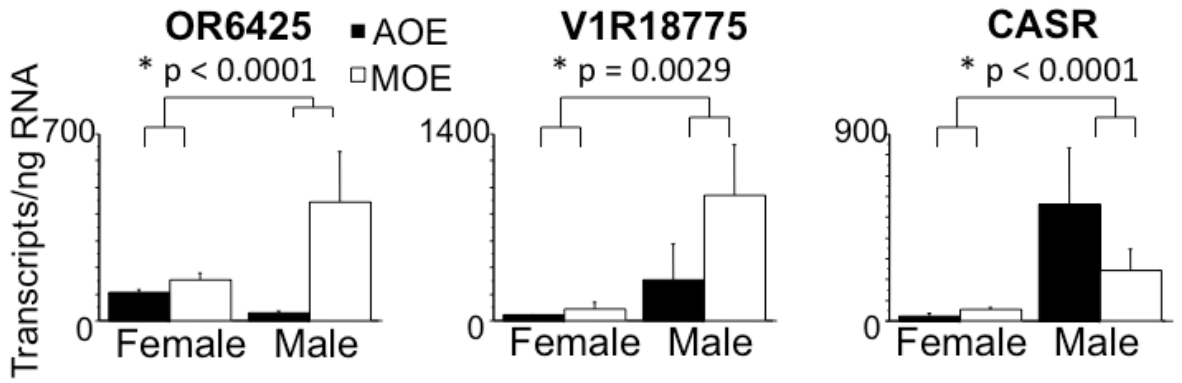
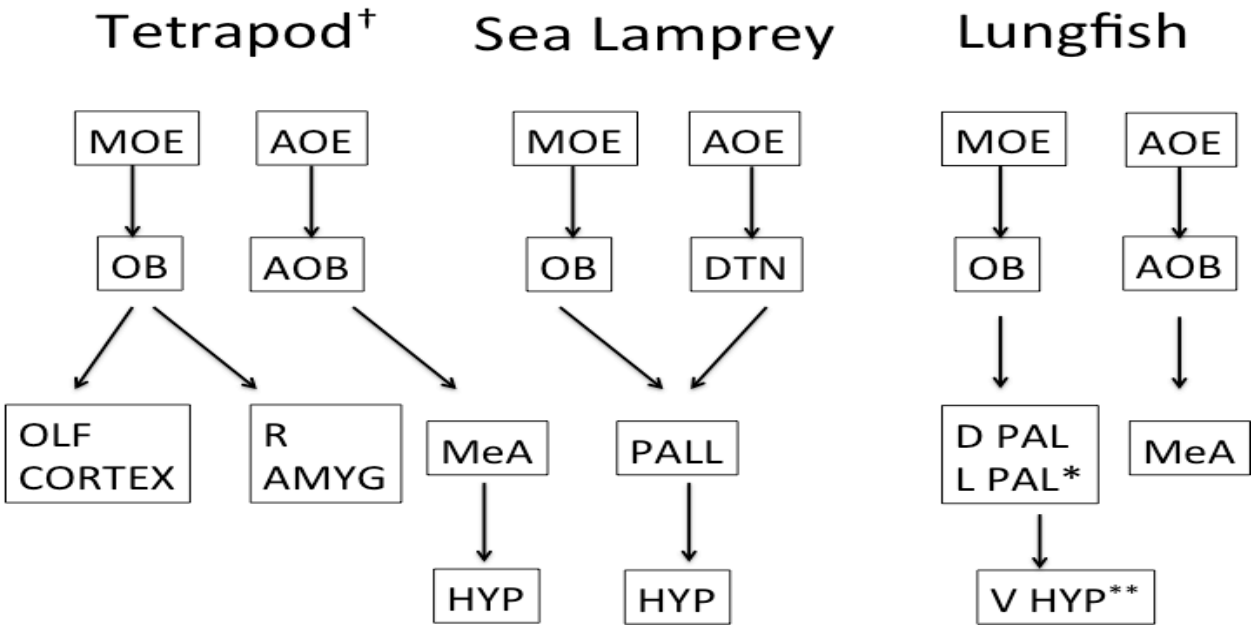


Figure 6: OR 6425, V1R 18775 and CASR are expressed significantly higher in adult male lampreys than in adult female lampreys. SYBR green real time quantitative PCR reveals olfactory receptor 6425 ($p < 0.0001$), vomeronasal type-1 receptor 18775 ($p = 0.0029$) and a calcium sensing receptor ($p < 0.0001$) are expressed significantly higher in adult male than in adult female sea lampreys.

Discussion

This study discovered similarities between the tetrapod vomeronasal pathway and a lamprey accessory olfactory pathway containing the AOE and DTN, as shown in Table 1 and Figure 7. GO analysis coupled with real-time quantitative PCR demonstrated that lamprey MOE and AOE gene expression profiles are similar. Lamprey AOE expresses all known families of lamprey chemoreceptor genes. Taken together, results suggest that the sea lamprey possesses a chemosensory accessory olfactory system.



*[75]

**[76]

†[77]

Figure 7: Connectivity of tetrapod and lamprey olfactory systems. Output from the main (MOE) and accessory olfactory epithelium (AOE) of lamprey is not segregated as seen in tetrapods. Secondary and tertiary outputs from lamprey more closely resemble secondary and tertiary outputs from vomeronasal organ (VNO) in tetrapods. AMYG: amygdale; AOB: accessory olfactory bulb; DTN: dorsomedial telencephalic neuropil; HYP: hypothalamus; OB: olfactory bulb; OLF CORTEX: olfactory cortex; PALL: pallial areas.

Table 1: Comparison of main and accessory olfactory system components in rodent, frog, zebrafish and lamprey *note – teleost fish do not have a recognized vomeronasal organ nor an accessory olfactory bulb; author contributions to this table are italicized.

		MOE			MOE/AOE	AOE		
		Rodent	Frog	Lamprey	Zebrafish	Rodent	Frog	Lamprey
Peripheral	Cell Type	Pseudostratified Ciliated Columnar [78]	Ciliated [79, 80]	Pseudostratified Ciliated Columnar [5, 81]	Pseudostratified Ciliated Columnar and Microvillous [82]	Microvillous [83]	Microvillous [80]	Simple Ciliated Cuboidal [6, 70, 84]
Peripheral	Genes	OR, TAAR [60]	OR [85], V1R [59]	OR, TAAR, V1R [24]	OR [86], TAAR [87], V1R, V2R [88]	OR, V1R, V2R [60]	V2R [89]	OR, TAAR, V1R [24, 65]
Peripheral	Targets	To MOB [90]	To MOB[80], AOB[91] and below MOB [92]	To MOB and DTN. (in larvae, to hypothalamus[93])	To MOB and ventral telencephalon [82, 94]	To AOB [22]	To AOB [80, 95]	*To MOB and DTN*
Central	Cell Type	Mitral cells [96]	Mitral cells [91, 97]	Mitral cells [6, 81]	Mitral cells [98, 99]	Mitral cells [100]	Mitral cells [77, 95]	Mitral cells
Central	Glomeruli	Yes [19]	Yes, [77, 91]	Yes, loosely defined [20]	Yes [82, 98, 101, 102]	Yes [22]	Yes [95]	None
Central	Targets	To olfactory cortex [16]	To olfactory amygdala [103]	To pallial areas, hypothalamus, medial habenula [73, 74, 104]	To habenula/limbic system output pathway [102]	To amygdala [105]	To piriform cortex [106]	*To pallial areas and hypothalamus*

Our neural tract-tracing results show direct connections between the AOE and the DTN. Injections of biocytin to the AOE revealed connections to the medial olfactory bulb similar to pallial areas of the telencephalon and the DTN, which was similar to the results of Derjean et al., [7] in the same species of lamprey. Labeling of cells in the MOE after injection to the AOE was unexpected as the MOE and AOE are anatomically separate, however, this may be due to piercing of olfactory nerve fascicles during injection, which are in close proximity to the AOE vesicles [6, 70]. Alternatively, some AOE vesicles have been observed to be connected to the MOE by ducts at the ventrolateral aspect of the nasal capsule, though this assertion could be an artifact of the plane of sectioning [107]. Moreover, dye could have been transported anterogradely to the MOE from the AOE via the olfactory nerve axons that are in close proximity to the AOE. Injections of biocytin to the DTN revealed connections with the AOE and the MOE. While the connections we observed between the AOE and DTN in lamprey are very similar to the primary projections of the VNO to the AOB in tetrapods, a difference is that in lamprey, the AOE has direct projections to the MOB [7]. We were not able to discern a laminar organization to the DTN similar to that seen in the AOB of tetrapods, though it should be noted that in sea lamprey OB, glomeruli are not as discretely identified, compared to other vertebrates [20]. In tetrapods, the MOE and VNO have segregated outputs to the MOB and AOB, respectively [105]. Therefore, the lamprey pathway is less segregated than those in adult tetrapods.

Interestingly, the lamprey system shares similarities with the system in developing tetrapods. Previous studies in sea lamprey have already demonstrated anatomical evidence that MOE and AOE both project to the medial olfactory bulb and functional evidence that the medial olfactory bulb activates locomotor brain regions [7]. In sea lamprey larvae, there is evidence that

some projections from the MOE bypass the OB and directly contact the hypothalamus [93]. Our work builds on these findings via anterograde and retrograde tracings from the AOE and the DTN of lamprey to show partial segregation at the peripheral level. The vomeronasal system recently discovered in lungfish is also a less segregated system [64], as molecular markers for a VNO are expressed in the MOE. Taken together, we believe our evidence shows the sea lamprey possesses anatomical and chemical precursors to an accessory olfactory system, however, we cannot discount the possibility that the pathway we have discovered is a functional link to the medial olfactory bulb and as such, is a part of the classically recognized olfactory pathway in sea lamprey. The question of the ancestral vertebrate condition with respect to olfactory projections (mixed or segregated outputs) requires further investigations.

Another similarity seen between the lamprey and tetrapod pathways is in their projections to higher centers. A recent study found that in sea lamprey, the medial OB projects to the medial habenula, an integrative center [104]. The lamprey DTN has direct connections to a putative amygdala homolog as well as the hypothalamus and thalamus. Dye injections to the DTN revealed labeling in the dorsal pallium, the hypothalamus and the thalamus. This confirms previous discoveries by Northcutt and Puzdrowski [73] who demonstrated DTN connectivity to the hypothalamus. Polenova and Vesselkin [74] also demonstrated connectivity of the DTN to the pallial areas of the telencephalon. Our work provides further information on the telencephalic pathways with respect to the main and accessory olfactory epithelia. The bi-directional connectivity between the medial pallium and striatum has been demonstrated in silver lamprey by Northcutt and Wicht [108]. Furthermore, the pallial areas are likely homologs of the tetrapod amygdala because of GABA-ergic projections from the medial pallium to the striatum [8].

Taken together, the target areas of the sea lamprey AOE are similar to those of the VNO in tetrapods, however, the pathways and neural networks are less complex.

The pathway seen in our study flows from the AOE to the DTN to the pallial areas and the hypothalamus. In tetrapods, the MOE and VNO have anatomically distinct primary projections. The MOE projects primarily to the main olfactory bulb and the VNO projects to the accessory olfactory bulb. In mice, there is a further segregation of output from the VNO. Specifically, sensory neurons in the anterior and posterior VNO express V1R and V2R receptors, respectively, and project to the anterior and posterior AOB, repeating the anatomical division seen at the periphery [21, 22]. Output neurons from the AOB in turn project to limbic areas of the brain including the amygdala, which in turn has projections to the hypothalamus [21]. From the AOB, there are two distinct populations of output neurons that project to the rostral and caudal regions of the amygdala, which in turn project to rostral and caudal regions of the hypothalamus which mirrors the segregated inputs from the vomeronasal organ [72, 105]. In sea lamprey, there is a convergence of output from the MOE and the AOE. Both the MOE and AOE have connections to the OB and the DTN, and so there is not a clear division of output from the MOE and AOE to their respective olfactory integration centers.

The sea lamprey AOE has cellular and molecular characteristics of an olfactory sensory epithelium. Since its discovery in *Petromyzon* by Scott in 1887 [70], AOE has been suggested to function as Jacobsen's organ [70], nasal sac rudiments [109], part of the pituitary [110] and Bowman's glands [111]. Recently, Ren et al. [6] demonstrated retrograde connectivity from the medial olfactory bulb to the AOE and concluded that the AOE and its projections are a distinct division within the olfactory pathway. Our data complements this conclusion by demonstrating

anterograde connectivity from the AOE to the medial OB. In addition, we have shown connectivity between the AOE and the DTN. Morphologically, the retrogradely labeled sensory neurons from both MOE and AOE in lamprey are ciliated. Molecular level analysis revealed further evidence that the lamprey AOE is a sensory epithelium. As expected, the overall gene categories expressed in MOE and AOE are virtually identical, furthering the case of the AOE as a chemosensory structure. Expression of chemoreceptor genes from all three of the families of chemoreceptor genes (ORs, TAARs and V1Rs) identified in the lamprey genome was confirmed [24]. In tetrapods, the VNO expresses V1Rs, V2Rs and ORs [55, 58, 60, 112, 113] while the MOE expresses ORs, TAARs and V1Rs [59]. While the MOE and VNO are anatomically separate in tetrapods, there is overlap with respect to chemoreceptor gene expression, secondary projection pathways and neural connectivity [58, 61, 89, 114]. The similarities in chemoreceptor gene families expressed in lamprey MOE and AOE may be explained by the status of the lamprey as a basal vertebrate [1, 115]. Moreover, during embryological development, the MOE and AOE of vertebrates both arise from the olfactory placode [116, 117]. At the neural circuit level, as well as the molecular level, it appears that the lamprey dual system is not as segregated as the tetrapod dual olfactory system.

Chemoreceptor genes were found to have a sexually dimorphic pattern of expression in lamprey MOE and AOE. In vertebrates, sexually dimorphic gene expression is usually linked to sex determination. For example, in rainbow trout, sox9a1 is expressed in male gonads and cyp19a1 is expressed in female gonads [118]. In the sea lamprey, the gene expression pattern observed in this study may be related to its sexually dimorphic behavior. While both males and females can detect the pheromone 3-keto petromyzonol sulfate (3kPZS), only females show a strong locomotor response [25]. However, this speculation requires further examinations.

Conclusion

Anatomical and molecular evidence shows that the sea lamprey has physical and chemical components that may represent a primordial accessory olfactory system.

Methods

Experimental Animals: Migrating adults (n = 93) were obtained from the St. Mary's River in Sault Ste. Marie, Michigan from the Hammond Bay Biological Station with mean length ± s.d. (48.3 cm ± 0.4 cm) and mean weight ± s.d. (237.4 g ± 5.0 g). Animals were handled according to guidelines provided by the Institutional Animal Care and Use Committee at Michigan State University.

Neural Tract Tracing: Animals were euthanized in tricaine methanesulfonate (MS-222, 100 mg/L, Sigma). The olfactory epithelium and brain were rapidly exposed by dorsal dissection, removing any surrounding muscle or cartilage. The tissue was rinsed in aerated cold Ringer's solution (pH 7.4) with the following composition: 130 mM NaCl, 2.1 mM KCl, 2.6 mM $CaCl_2$, 1.8 mM $MgCl_2$, 4 mM HEPES, 4 mM dextrose and 1 mM $NaHCO_3$. Glass capillaries with a diameter of 50 μm were filled with 2 μl of 2% biocytin [in 0.1M phosphate buffer saline (PBS), pH7.2] and inserted into either multiple accessory olfactory vesicles or the DTN (see Figure 8), and the tracer was applied to the lesion. Tissue was rinsed and incubated in lamprey Ringer's for 10 minutes before being placed in a flow-through chamber held at 7^{o}C. The tissue was continuously perfused with cold aerated Ringer's solution during the entire incubation period. After 4 hours, the tissue was fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Tissue was then immersed in Sakura Tissue-Tek O.C.T. compound (VWR) and frozen with a combination of liquid nitrogen and dry ice. Thin sections (20 μm) were collected on Superfrost Plus slides

(VWR) and stored at -20°C. Slides were washed in 0.1 M PBS (pH 7.4) and biocytin signal was visualized by addition of Alexa 488 Streptavidin (1:100, Invitrogen). Slides were examined on an upright Zeiss Axioskop 2, equipped with fluorescence and a CCD camera. Images were captured using Axiovision software (Zeiss). Samples with clear leakage from the intended injection site were rejected.

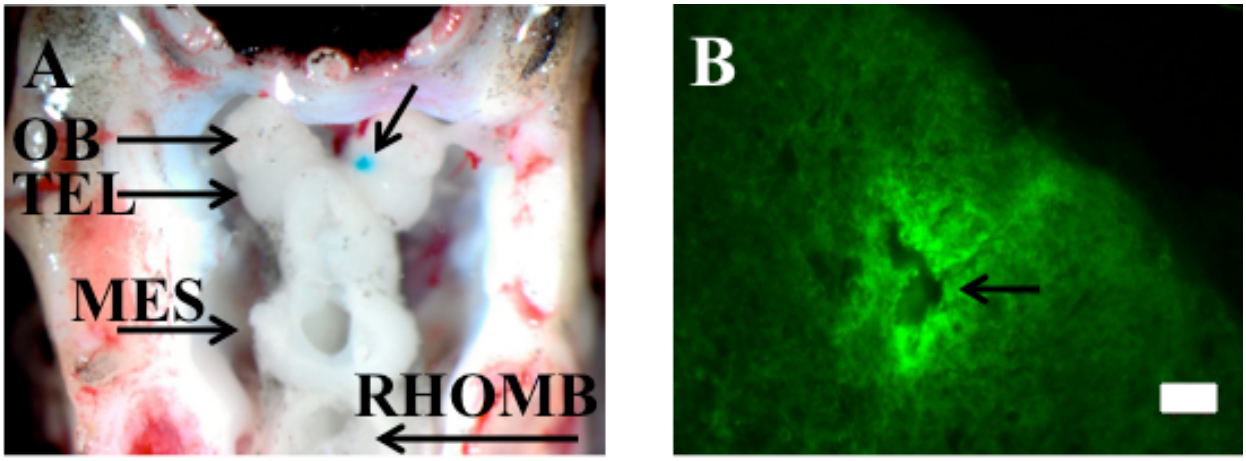


Figure 8: Biocytin injections to dorsomedial telencephalic neuropil and accessory olfactory epithelium. **A**, Sea lamprey brain exposed in cranium. Injection site at dorsal and medial at the margin of the olfactory bulb and telencephalon (blue dot/arrow). **B**, Lesion in lateral DTN shown. Fluorescence is shown around the lesion, indicating it as the site of injection of biocytin.

Laser Capture Microdissection (LCM) and mRNA-Seq Preparation: Olfactory organs from mature males and females were dissected out, embedded in O.C.T. compound and frozen with a combination of dry ice and liquid nitrogen. Seven-µm frontal sections were collected on non-charged glass slides (VWR) and stored at -80°C. Slides were then passed through an ascending alcohol series and rinsed with xylene to dehydrate the tissue and remove the alcohol. Slides were then viewed under an inverted Nikon Eclipse microscope outfitted with the Arcturus Pixcell II/e Laser Capture Microdissection System and Arcview software (Arcturus). The MOE and the AOE are not distinguishable with the naked eye, but are easily distinguished when

viewed under a microscope (data not shown). Cells from the MOE and AOE were lifted under the following conditions (duration: 20.0 ms, repeat: 0.4 s, spot size: 7.5 μm, power: 100 mW). Because of the anatomical separation of the MOE and AOE, we were absolutely sure that we were lifting cells from the appropriate epithelium. RNA was extracted using TRIZOL reagent (Invitrogen) and stored at -80^{O}C. Quality of samples was verified using an Agilent 2100 Bioanalyzer before submission for high-throughput sequencing.

GO Analyses: MOE and AOE RNA samples were sequenced at the Michigan State University Research Technology Support Facility, using the Illumina DGE kit according to manufacturer's instructions. 64,141,260 reads were obtained and 58% (37,785,187) passed a quality filter. The filtered reads were aligned, using Bowtie software [119], to our assembly of the sea lamprey transcriptome to obtain transcript expression count information for each lane, which were then quantile-normalized. The transcriptome assembly was, in turn, aligned to mouse RefSeq protein sequences, providing a putative orthology with which mouse protein annotations were assigned to corresponding lamprey transcripts, and these annotations were combined with transcript expression counts to infer expression information for putative lamprey-mouse orthologs. This information was used to infer putative ortholog differential expression between MOE and AOE. Using inferred expression ratios, significantly enriched or depleted gene ontology categories were identified, with the help of GoMiner software [120].

SYBR Green Real-Time Quantitative PCR: Cells from MOE and AOE of six individuals (four male, two female) were collected using LCM. RNA from these cells were extracted and used for real-time quantitative PCR (methods followed Chung-Davidson et al. [121]). Solexa DGE reads were aligned to the mouse refseq mRNA database [13] and chemosensory and

chemosensory-related genes were selected from the putative mouse orthologs. Only full-length, intact sequences were used for primer design using Primer Express software (Applied Biosystems) (Table 2). The sea lamprey genome does not possess vomeronasal type-2 receptors (V2R), but does contain calcium-sensing receptors (CASRs), which are V2R-like (Libants et al., 2009). The genes monitored were: OR 3267, OR 6425, TAAR 3721, V1R 18775, CASR and adenylate cyclase.

Table 2: Primers used for SYBR green qPCR

GENE	FWD (SENSE)	REV (SENSE)	REV (5'-3')
OR3267	aaccgggctgagcaagaac	cgagggagcgagaaacttca	tgaagtttctcgctccctcg
OR6425	gaagaacatctgtgccatgca	gcagaacgtcgcgtcctt	aaggacgcgacgttctgc
TAAR3721	tctgcagctgcctgaagtagag	ccatcgcgggcaaca	tgttgcccgcgatgg
V1R18775	attggcacgtgtcacatgaga	gagagaacgcgaggcttatcag	ctgataagcctcgcgttctctc
CASR	ttttgaccaagatgcaagacaag	cccgccagccctttt	aaaaagggctggcggg
AC9	cgccataggtatccacatcttca	tggcccaccttgaggaaag	ctttcctcaaggtgggcca
GP	ccaggccagggaaatgc	tgagctgaggcaagaagtaatcag	ctgattacttcttgcctcagctca

CHAPTER 2

The Origin of the P450 Superfamily in a Jawless Vertebrate

Abstract

Background: The sea lamprey (*Petromyzon marinus*) is native to the coastal region of Eastern North America but is invasive in the Great Lakes. Efforts to control sea lamprey populations include treatment of infested streams with the selective lampricide, 3-trifluoromethyl-4-nitrophenol (TFM). Treatment is almost 100% effective; however, there are non-target species effects. As well, TFM is expensive and requires application over several years and subsequent monitoring. To date, the exact mechanism of how TFM kills sea lamprey larvae is unknown.

Organisms respond to chemical insults mainly in two ways: induction of either cytochrome P450 enzymes (AKA phase I enzymes) or of phase II detoxification enzymes. We hypothesized that larval sea lamprey undergo detoxification of xenobiotic compounds using cytochrome P450 enzymes, as other organisms, including fish, metabolize xenobiotics via P450 enzymes. A survey was undertaken of the genome of sea lamprey to determine the complement of P450 and phase II genes. Sequencing of liver and gill RNA following exposure to a chemical toxicant was undertaken to obtain the gene profiles of these tissues, especially with respect to detoxifying genes. Our goal was to determine which phase I or phase II genes, if any, are induced by exposure to TFM. This study is the first attempt to characterize the detoxifying enzymes (phase I or phase II) that are activated in larval lamprey in response to lampricide TFM exposure.

Results: The sea lamprey genome contains 7 of the 18 known vertebrate P450 families, including the detoxifying families 1 through 4. Analysis of transcriptome profiles of gill or liver demonstrated minimal (0.1% - 1.2%) differential expression of genes when different dosages of TFM were compared. A further analysis that contrasted gill and liver at the same dosage demonstrated a more robust differential expression (12% - 14.7%) of several genes. From this list of differentially expressed genes, several P450 and phase II genes were chosen for further analysis. Quantitative PCR experiments confirmed differential expression analysis and identified several candidate detoxification genes (CYP 1a1, CYP 2j6, CYP 3a9, CYP 3a13, CYP 4v2, ALDH 8a1, EPHX2, and NADPH-cytochrome P450 reductase).

Conclusion: The sea lamprey genome contains more cytochrome P450 families compared to sea urchin, but less families than zebrafish or humans. High-throughput sequencing and quantitative PCR has confirmed expression of several cytochrome P450 and phase II genes that are known to detoxify xenobiotics in other organisms.

Background

Sea lamprey (*Petromyzon marinus*) is an invasive species to the Great Lakes ecosystem that has devastated native fish stocks. Part of the integrated pest management program is treatment of infested streams with the selective lampricide 3-trifluoromethyl-4-nitrophenol (TFM) which kills sea lamprey larvae [122–124]. The specific toxicity of TFM on sea lampreys was discovered by Applegate et al. in 1958 [124]. Subsequent studies discovered that TFM is glucuronidated in the liver of rats [125] and rainbow trout [126]. In a comparison of sea lamprey larvae and rainbow trout juveniles, it was discovered that sea lampreys are not able to efficiently conjugate TFM to the glucuronide form and so are not able to excrete the toxicant quickly [127]. More recent research has attempted to elucidate the biochemical and energetic mechanisms by

which TFM exerts its effects on sea lamprey larvae. A pair of more recent studies suggests that TFM interferes with mitochondrial oxidative phosphorylation which results in a depletion of ATP [52, 53]. The purpose of this study was to determine the complement of P450 genes in sea lamprey and to discover whether P450 or phase II genes were induced in sea lamprey larvae by exposure to the lampricide TFM. While highly selective, as TFM use increased in the Great Lakes, there was concern over unintended effects on non-target species [123, 128–130]. One early study investigated the effects of TFM on rainbow trout and found minimal effects on adults [126]. The aquatic midge, *Chironomus tentans*, was found to be able to safely eliminate biotransformed versions of TFM [131]. Plants are similarly able to process and eliminate TFM [129]. Further research on the effects of TFM on non-target fish species showed that while TFM is taken up, it is quickly eliminated within 12 hours of exposure [130, 132–135].

In 1967, Williams proposed that metabolism of foreign compounds (or xenobiotics) is accomplished in two steps, or phases, which correspond to groups of genes [27, 28]. Metabolic genes were grouped according to action on a substrate. Phase I genes (i.e., cytochrome P450 genes) oxidize or reduce a compound by introducing or exposing a functional group. Phase II genes (e.g., epoxide hydrolases or glutathione-S-transferases, etc.) then act on these metabolites, making them more soluble and readily excretable. The cytochrome P450 superfamily is comprised of genes grouped into families and sub-families by sequence identity and shared function. These genes encode for enzymes responsible for detoxification as well as steroid biosynthesis. Of particular interest are four sub-families of P450 termed CYP 1, 2, 3 and 4. These enzymes are known to have detoxifying effects of xenobiotic substances in invertebrates [136–138] and vertebrates [29–32, 39, 40, 46, 137, 139–141]. The sea urchin (*Strongylocentrotus purpuratus*) has 120 CYP genes representing 6 families [136], zebrafish

(*Danio rerio*) has 94 genes representing 18 families [46] and humans (*Homo sapiens*) have 57 genes representing the same 18 families [39].

The sea lamprey P450 complement has not been studied and given the basal position of the sea lamprey in vertebrate phylogeny, knowing which P450 genes are present in the genome would add to the body of knowledge of the evolution of P450 genes in vertebrates. We also felt that using high-throughput sequencing was the most effective way to examine the P450 complement and analyze the mode of action of TFM. Although transcriptome analysis of fish is still in the early phases of research, prior studies have examined the transcriptomes of fish. Early works have monitored a salmonid response (gill or liver tissue) to an environmental toxicant, and these studies used commercially available microarrays in their analysis [10–13]. With the advent of high-throughput sequencing technologies such as Solexa sequencing, large transcriptome libraries could be generated and analyzed very quickly. More recent studies have looked at the transcriptomes of teleost fish to discover genes involved in immune response after infection by a microorganism [142–144]. A recent study used the transcriptomes from various fish species to construct an evolutionary history of basal jawed vertebrates that matched the fossil record [145].

Given the basal phylogenetic position of the sea lamprey and because of the selective toxicity of TFM, we speculated that the sea lamprey has an overall P450 complement similar to other vertebrates and that select P450 genes would play a role in TFM detoxification. Using high-throughput sequencing paired with molecular biology, here, we report that the sea lamprey genome has 11 of the 18 known vertebrate P450 gene families, and includes all four detoxifying P450 families.

Results

A Limited P450 Complement in Sea Lamprey

Inspection of the sea lamprey genome [9] revealed 56 predicted P450 genes and gene fragments representing 11 CYP families (Table 3). Compared to sea urchin, the sea lamprey has less total P450 genes but more families represented in the genome [32]. Comparing sea lamprey to zebrafish [46] and humans [32, 39], the sea lamprey genome has fewer families and fewer P450 genes than zebrafish and humans (Table 3). Examination of the sea lamprey P450 complement did not detect the dramatic expansion of the CYP 2 family that is seen in sea urchin [32], zebrafish [46] or human [32, 39]. All 4 families known to be associated with detoxification in other organisms, however, are represented in the sea lamprey genome (Table 4, Table 5).

Table 3: Summary of gene complements by family in sea urchin, sea lamprey, zebrafish and human. Author contributions are italicized.

	Sea Urchin[136]	***Sea Lamprey***	**Zebrafish**[46]	**Human**[39]
# of families	6	*11*	18	18
# of genes	120	*56*	94	57 (+59 pseudogenes)

Table 4: P450 gene complements for CYP families 1-4 in sea urchin, sea lamprey, zebrafish and human. Author contributions are italicized.

Family	**Sea Urchin**[136]	***Sea Lamprey***	**Zebrafish**[46]	**Human**[39]
1	11	*4*	5	3
2	73	*5*	48	20
3	10	*3*	5	4
4	10	*2*	4	11

Table 5: Comparison by CYP gene family representation in sea urchin, sea lamprey, zebrafish and human. Author contributions are italicized.

	Sea Urchin[136]	***Sea Lamprey***	**Zebrafish**[46]	**Human**[39]
CYP 1	✓	✓	✓	✓
CYP 2	✓	✓	✓	✓
CYP 3	✓	✓	✓	✓
CYP 4	✓	✓	✓	✓
CYP 5			✓	✓
CYP 7		✓	✓	✓
CYP 8		✓	✓	✓
CYP 11			✓	✓
CYP 17	✓	✓	✓	✓
CYP 19			✓	✓
CYP 20			✓	✓
CYP 21		✓	✓	✓
CYP 24			✓	✓
CYP 26		✓	✓	✓
CYP 27		✓	✓	✓
CYP 39			✓	✓
CYP 46			✓	✓
CYP 51	✓	✓	✓	✓

Differential Expression (DE) Analysis Reveals Several Candidates for TFM Detoxification

A summary of differentially expressed tags for each contrast is shown in Table 6. Tags were blasted against the refseq mouse database to obtain ortholog information (Table 7) and several candidate genes for detoxification were identified based on significant P-values and by keyword search.

Table 6: Number of differentially expressed tags (FDR, 0.05) obtained in each contrast. [-1; down-regulated, 0: no change, 1: up-regulated]

Contrast	G1vsG2	G1vsG3	G2vsG3	L1vsL2	L1vsL3	L2vsL3	G1vsL1	G2vsL2	G3vsL3
-1	187	100	55	56	54	15	1107	1894	2411
0	30299	30408	30456	24993	25042	25083	18355	21648	20790
1	177	95	92	85	47	36	2707	2802	3034

Table 7: Total and differentially expressed tags with ortho information in the mouse files (FDR < 0.05, adjusted using the information of matching tags)

Contrast	G1vsG2	G1vsG3	G2vsG3	L1vsL2	L1vsL3	L2vsL3	G1vsL1	G2vsL2	G3vsL3
Total	7196	7196	7196	6135	6135	6135	5813	6674	6469
DE	88	39	45	39	7	9	696	898	954

Nine pair-wise comparisons of the transcriptome libraries were performed, contrasting dosage levels within gill (contrasts 1-3), liver (contrasts 4-6), and between liver and gill at each dose of TFM (contrasts 7-9). Contrasts 1 through 6 revealed minimal differential expression of genes within gill or liver and failed to show differential expression of any phase I or phase II genes (Table 8).

Table 8: Summary of differential expression of genes (Contrasts 1-9)

CONTRAST	Total Genes	# of Differentially Expressed Genes
c1 (Gill 0.6 vs 0 mg/L)	7197	87 (1.21%)
c2 (Gill 1.2 vs 0 mg/L)	7197	38 (0.53%)
c3 (Gill 1.2 vs 0.6 mg/L)	7197	44 (0.61%)
c4 (Liver 0.6 vs 0 mg/L)	6136	38 (0.62%)
c5 (Liver 1.2 vs 0 mg/L)	6136	6 (0.10%)
c6 (Liver 1.2 vs 0.6 mg/L)	6136	8 (0.13%)
c7 (Liver vs Gill @ 0 mg/L)	5814	695 (11.95%)
c8 (Liver vs Gill @ 0.6 mg/L)	6675	897 (13.44%)
c9 (Liver vs Gill @ 1.2 mg/L)	6470	953 (14.73%)

When we compared expression levels between tissues at the same dosages (contrasts 7 through 9) we found that several phase I and phase II genes are differentially expressed.

Liver-Gill Comparison (0 mg/L TFM)

In gill and liver treated with 0 mg/L TFM, we found significant differential expression ($P<0.05$) of ten P450 genes, eight of which are found in the detoxifying families of P450 genes (CYP 2c50, CYP 2d22, CYP 2j9, CYP 2j11, CYP 2t4, CYP 3a13, CYP 4b1, and CYP4f17). Three phase II genes were also differentially expressed (ALDH 8a1, EPHX2, MGST3). All of the genes were expressed higher in liver than in gill except for CYP 2d22, CYP 2t4 and MGST3. Results are summarized in Table 9.

Table 9: Differentially expressed phase I and Phase II genes between liver and gill (0 mg/L dosage)

Gene Name	Up (+) or Down (-) regulated in Liver vs Gill	P-Value
CYP 2c50 isoform 2	+	1.44E-15
CYP 2d22	-	6.32E-21
CYP 2j9	+	5.41E-04
CYP 2j11	+	2.57E-20
CYP 2t4	-	2.01E-03
CYP 3a13	+	4.68E-08
CYP 4b1	+	2.54E-16
CYP 4f17	+	1.37E-12
ALDH 8a1	+	1.04E-07
EPHX2	+	9.10E-05
MGST3	-	5.67E-08

Liver-Gill Comparison (0.6 mg/L TFM)

In gill and liver treated with 0.6 mg/L TFM, we found significant differential expression (p value of less than 0.05) of the same P450 genes that were differentially expressed at 0 mg/L TFM. The same phase II genes were also found to be significantly differentially expressed (p value of less than 0.05). Two new significantly differentially expressed genes appeared at this

dosage level; one P450 gene, CYP 4v3 and one phase II gene, GSTT3. All of the genes were expressed higher in liver than in gill except for CYP 2d22, CYP 2t4 MGST3 and GSTT3. Results are summarized in Table 10.

Table 10: Differentially expressed phase I and Phase II genes between liver and gill (0.6 mg/L dosage)

Gene Name	Up (+) or Down (-) regulated in Liver vs Gill	P-Value
CYP 2c50 isoform 2	+	2.45E-26
CYP 2d22	-	5.04E-24
CYP 2j9	+	4.12E-03
CYP 2j11	+	2.12E-23
CYP 2t4	-	7.81E-04
CYP 3a13	+	1.85E-08
CYP 4b1	+	1.06E-20
CYP 4v3	+	4.61E-03
CYP 4f17	+	2.53E-15
ALDH 8a1	+	9.02E-08
EPHX2	+	5.86E-03
GSTT3	-	6.27E-07
MGST3	-	3.42E-07

Liver-Gill Comparison (1.2 mg/L TFM)

In gill and liver treated with 1.2 mg/L TFM, we found significant differential expression (p value of less than 0.05) of the same P450 genes that were differentially expressed at 0.6 mg/L TFM. The same phase II genes were also found to be significantly differentially expressed (p value of less than 0.05). One new P450-related gene was significantly differentially expressed at this dosage level, NADPH-cytochrome P450 reductase. Results are summarized in Table 11.

Table 11: Differentially expressed phase I and Phase II genes between liver and gill (1.2 mg/L dosage)

Gene Name	Up (+) or Down (-) regulated in Liver vs Gill	P-Value
CYP 2c50 isoform 2	+	6.65E-17
CYP 2d22	-	3.12E-19
CYP 2j9	+	5.96E-07
CYP 2j11	+	1.02E-23
CYP 2t4	-	2.03E-03
CYP 3a13	+	5.38E-08
CYP 4b1	+	5.60E-22
CYP 4v3	+	2.29E-03
CYP 4f17	+	8.99E-12
ALDH 8a1	+	1.41E-12
EPHX2	+	4.23E-04
GSTT3	-	1.18E-06
MGST3	-	3.43E-06
NADPH-cytochrome P450 reductase	+	5.88E-03

Inspection of the differential expression analysis revealed an increasing pattern of expression when the different dosage levels were contrasted. At 0 mg/L dosage of TFM, there were 11 phase I and phase II enzymes differentially expressed which indicated that these genes are constitutively expressed (Table 9). When the dosage was increased to 0.6 mg/L, the same genes were differentially expressed with the addition of one other CYP gene (CYP 4v3) and one phase II enzyme (GSTT3). At 1.2 mg/L dosage, we found the same genes differentially expressed at 0 mg/L and 0.6 mg/L, with the addition of NADPH-cytochrome P450 reductase. Our results suggest that as TFM dosage increases, more detoxification genes are expressed in the liver.

Analysis and Quantification of Select Phase I and Phase II Genes

Seven of the 8 genes monitored showed a differential expression between liver and gill tissue. ALDH8a1, CYP2j6, CYP4v2, EPHX2, AND NADPH-cytochrome P450 reductase were expressed 2-5x higher ($P<0.05$) in liver than in gill. CYP1a1 and CYP3a9 were significantly

(P<0.05) higher in gill than in liver. There was no significant difference of expression of CYP3a13 between gill and liver (Figure 9).

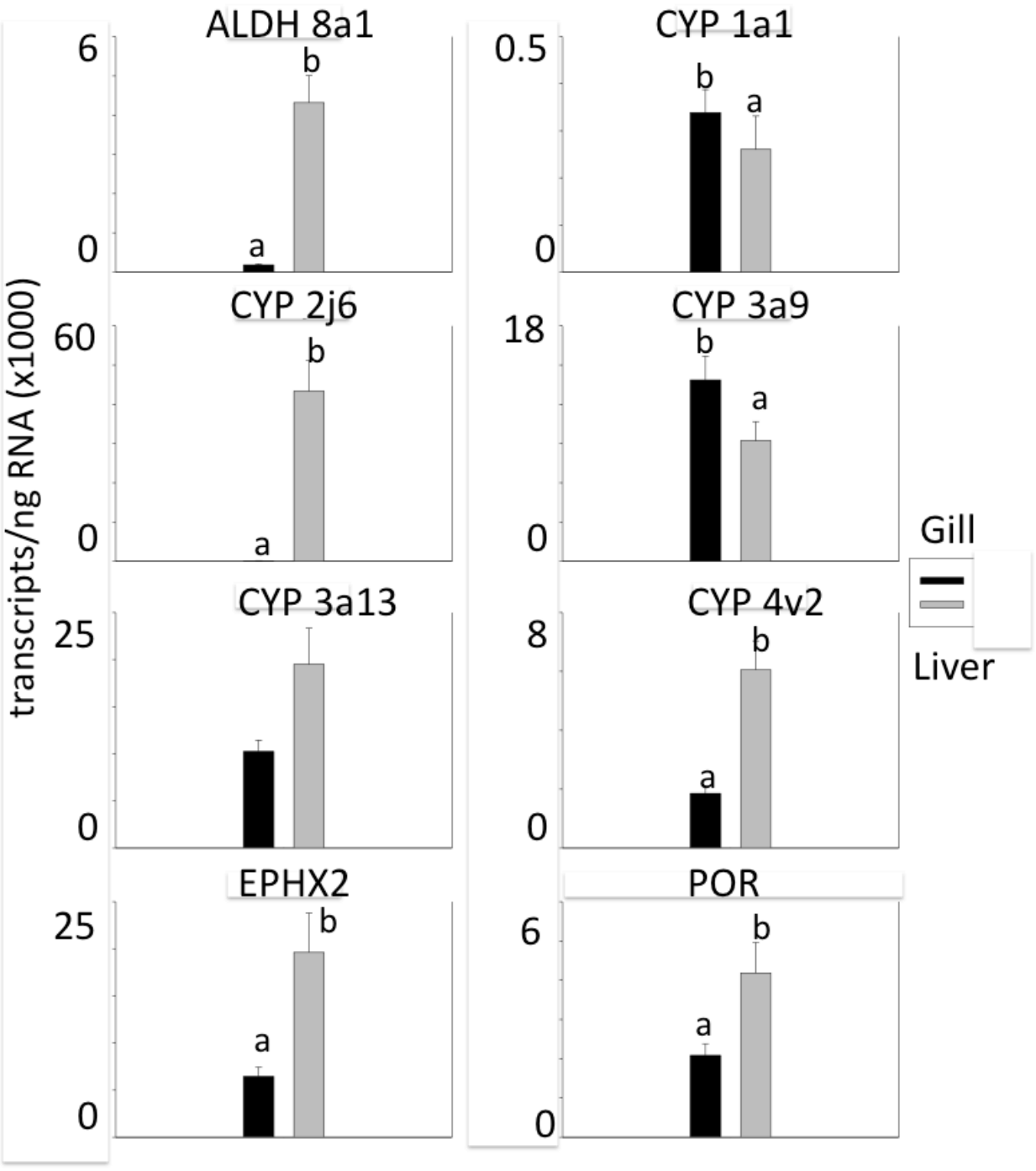


Figure 9: Differential expression of P450 and phase II genes in gill and liver of sea lamprey larvae in response to TFM treatment, after 8 hours. One-way ANOVA was run on logarithmically normalized data. Letters indicate significant differences (P<0.05).

ANOVAs - Gill

Six of the 8 genes monitored showed a significant ($P<0.05$) difference in expression in 1 or more of the 3 possible dose comparisons (0 mg/L vs 0.6 mg/L, 0 mg/L vs 1.2 mg/L, 0.6 mg/L vs 1.2 mg/L).

ALDH8a1, CYP3a13 and NADPH-cytochrome P450 reductase showed a significantly higher expression at 0.6 mg/L that is similar at 1.2 mg/L. CYP1a1 and EPHX2 showed a significantly higher expression at 0.6 mg/L and lower expression at 1.2 mg/L to control values. CYP4v2 showed a significantly higher expression at 0.6 mg/L and significantly lower expression at 1.2 mg/L but does not reach control values. There was no dose effect for CYP2j6 or CYP3a9 in gill. (Figure 10)

Of the 6 genes that showed a dose effect in gill (ALDH8a1, EPHX2, NADPH-cytochrome P450 reductase, CYP 1a1, CYP3a13 and CYP 4v2), all showed an increase in expression at 0.6 mg/L. Two (CYP1a1 and EPHX2) then showed a decreased expression at 1.2 mg/L. Three (ALDH8a1, NADPH-cytochrome P450 reductase and CYP3a13 showed a stable expression when the dose was increased to 1.2 mg/L and one (CYP4v2) showed a significant decrease in expression at 1.2 mg/L.

ANOVAs - Liver

Four of the 8 genes monitored showed a significant ($P<0.05$) difference in expression in 1 or more of the 3 possible dose comparisons (0 mg/L vs 0.6 mg/L, 0 mg/L vs 1.2 mg/L, 0.6 mg/L vs 1.2 mg/L). There was no dose effect for ALDH8a1, CYP3a9, EPHX2 or NADPH-cytochrome P450 reductase. CYP1a1 and CYP3a13 showed a significantly higher expression at 0.6 mg/L and lower expression at 1.2 mg/L to control values. CYP2j6 and CYP4v2 showed a significantly higher expression at 0.6 mg/L that is similar at 1.2 mg/L. (Figure 11)

Of the 4 genes that showed a dose effect in liver (CYP1a1, CYP 2j6, CYP 3a13 and CYP4v2), all of them showed an increase in expression at 0.6 mg/L. Two (CYP1a1 and CYP3a13) then showed decreased expression at 1.2 mg/L and two (CYP2j6 and CYP4v2) showed a stable expression when the dose was increased to 1.2 mg/L.

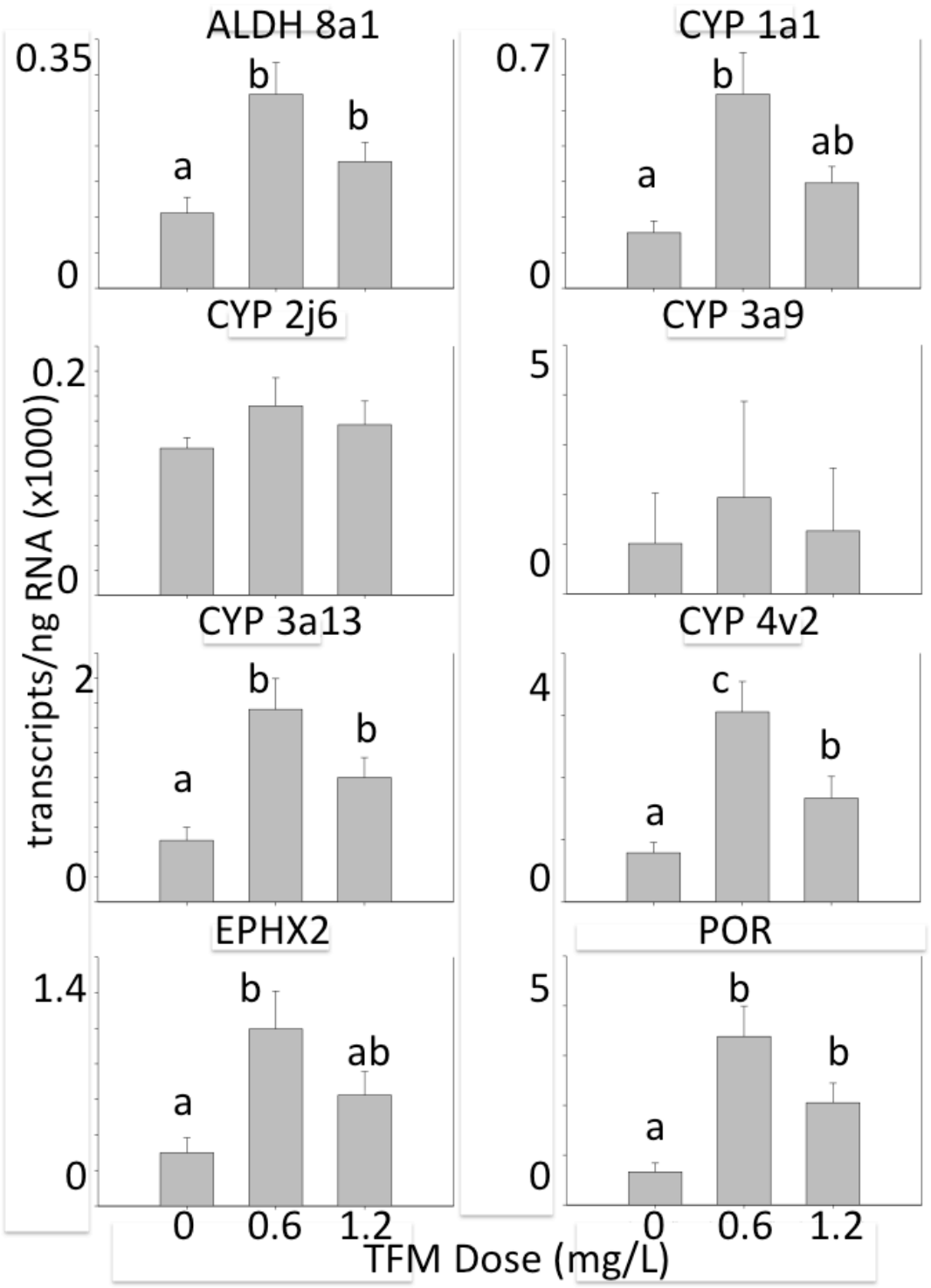


Figure 10: Differential expression of P450 and phase II genes in gill of sea lamprey larvae in response to TFM treatment, after 8 hours. One-way ANOVA was run on logarithmically normalized data. Letters indicate significant differences ($P<0.05$).

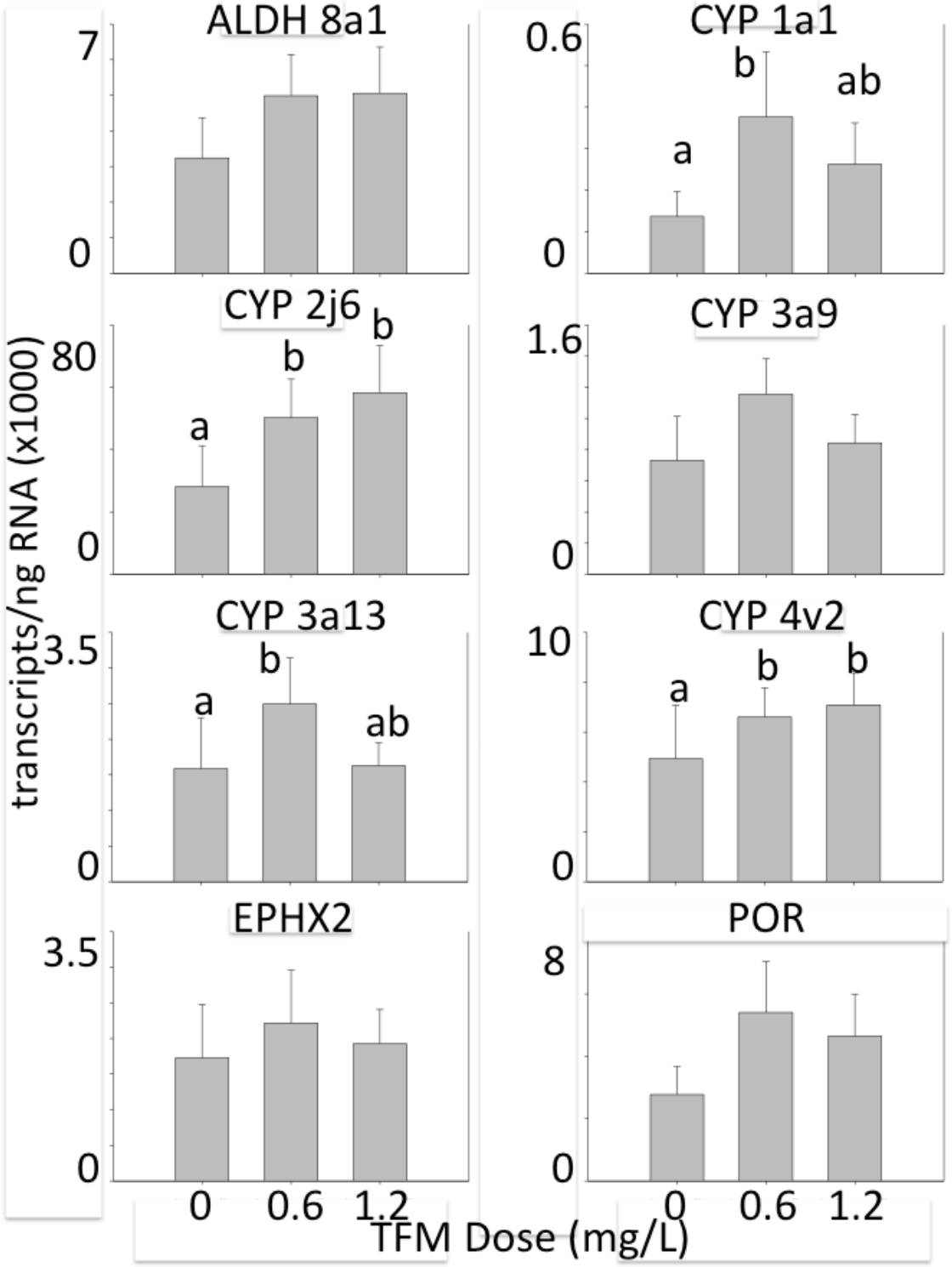


Figure 11: Differential expression of P450 and phase II genes in liver of sea lamprey larvae in response to TFM treatment, after 8 hours. One-way ANOVA was run on logarithmically normalized data. Letters indicate significant differences ($P<0.05$).

Discussion

A thorough search of the sea lamprey genome revealed several characteristics of the P450 complement and included representatives of all known detoxifying families (CYP 1-4). All of the P450 families found in sea urchin [136] are represented in sea lamprey, however the sea lamprey has less total P450 genes. This difference in total number of P450 genes is likely due to the lineage-specific expansion of what are termed chemical defensome genes in the sea urchin [136]. The sea lamprey genome possesses less P450 gene families and less total P450 genes than zebrafish and humans [39, 46]. We failed to find representatives of 7 of the 18 gene families that are common to zebrafish and human genomes. The sea lamprey genome was derived from liver tissue, which is known to express significant amounts of P450 genes, so we believe that the 70% coverage is representative of the genome and of P450 genes in sea lamprey [9].

Our results show that several phase I and phase II genes in gill and liver of larval sea lamprey are transcriptionally activated after 8 hours of treatment with TFM. More of the genes we examined for expression demonstrated a dose effect in gill versus liver, suggesting that the gill may be an important extra-hepatic site for TFM detoxification. The increased differential expression of genes in the gill after exposure to a stressor (including xenobiotic substances) has been seen in other fish [12, 13], suggesting that the gill as an extra-hepatic site for detoxification is not unique to the sea lamprey. None of the phase II enzymes we monitored showed a dose effect in liver. Only P450 genes showed a dose effect in liver.

Our differential expression analysis demonstrated that on a transcriptome level, a small percentage of genes were differentially expressed after exposure to TFM in both liver and gill. This result is consistent with other studies, as not all P450 genes in an organism would be

required to metabolize individual xenobiotics [11, 12, 146–148], and in fact, the small percentage of genes involved indicates specificity in the response of sea lamprey to TFM exposure. In this study, more phase I/cytochrome P450 genes than phase II genes are differentially expressed (Tables 9-11) which suggests the importance of these genes over phase II enzymes in detoxification of TFM

Quantitative PCR confirmed our differential expression results. Five of the 8 representative genes we monitored showed a higher expression level in liver over gill, which follows the larger number of differentially expressed genes seen in liver when compared to gill. Recruitment of P450 and phase II genes increased as TFM dose increased as well. Our results also showed that select P450 genes are expressed at high doses of TFM at 8 hours. The stable expression of CYP 2j6 and CYP 4v2 in liver indicates that the mid-level dose (0.6 mg/L) is sufficient to induce expression of these detoxification-related genes.

Phase I – Cytochrome P450 Genes

Sea lamprey CYP 1a1 was the sole representative of the CYP 1 detoxification family and though it was deemed to be an incomplete gene, it was included in this analysis due to its important role in detoxification in other organisms. In zebrafish exposed to benzo[a]pyrene, CYP 1a enzymatic activity in the liver was increased about 3-fold compared to control samples [30]. In sea bream, CYP 1a mRNA was found in the olfactory bulbs of control fish, but after treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), expression was found throughout the entire brain [139] and in lake trout, CYP 1a expression increased in brain after exposure to β-naphthoflavone (BNF) [149]. While the complement of CYP genes in the genomes of teleost fish differs by species, it is known that the zebrafish genome contains representatives from all

four detoxifying CYP families [46], as does pufferfish [32]. Our analysis showed the CYP 1a1 gene was incomplete in the sea lamprey genome (assembly 7.0) however, we were able to demonstrate an increased expression of this gene in both liver and gill.

As expected, CYP 2j6 showed an increase in expression after exposure to TFM. In all organisms studied, the CYP 2 family is known to detoxify various xenobiotic compounds; moreover, this family is expanded in the genomes of mammals [32, 39, 46, 136, 150]. Expansion in sea urchin appears to be lineage specific [136], and the expansion in mammals appears to be an independent lineage-specific expansion [32, 39, 150]. While we cannot confidently state that the CYP 2 family is as greatly expanded in sea lamprey as it is in other vertebrates we can point to the differential expression analysis, which showed expression of more CYP 2 members at all treatment levels, than any other CYP family (Tables 9-11).

Though CYP 3a9 expression levels did not change in response to TFM treatment, another member of the same sub-family, CYP 3a13, was responsive to TFM treatment. The CYP 3a family is known to metabolize endogenous compounds [38, 151] as well as for xenobiotic compounds [147, 152]. The differential response of these two members of the CYP 3a subfamily to TFM is perhaps a feature related to the vulnerability of the sea lamprey to TFM.

Based upon membership in the CYP 4 family, CYP 4v2 was expected to be responsive to TFM treatment. Both liver and gill showed an increase in expression of CYP 4v2 after TFM exposure. The CYP 4 family is responsible for metabolism of fatty acids, but can also metabolize xenobiotic compounds [41]. Because the CYP4 family has dual metabolic functions, the increased expression of CYP 4v2 cannot be resolved to be due to the effect of TFM alone. A substrate for CYP 4v2 has not been identified and mutations in this gene are implicated in a

condition known as Bietti's crystalline corneoretinal dystrophy which causes abnormal lipid metabolism and is linked to crystal and lipid deposits in the cornea and retina, leading to loss of vision [153]. Increased expression of CYP 4v2 in response to TFM exposure could be attributed to metabolism of lipids as an alternate source of ATP, as ATP has been shown to be depleted in sea lamprey after exposure to TFM [52, 53].

NADPH-cytochrome P450 reductase was responsive to TFM treatment in gill but not in liver. This difference in tissue responsiveness was unexpected as the liver is metabolically active and the primary site for detoxification of xenobiotics. NADPH-cytochrome P450 transfers an electron from NADPH to a cytochrome P450 enzyme and is a crucial first step for P450 function [154]. In the liver, expression was non-zero and constant across TFM treatment levels, however in gill, expression increased with increased TFM dosage, which supports our findings in the differential expression analysis that the gills are a capable of extra-hepatic detoxification.

Phase II Genes

While quantitative PCR did not show a significant dose effect for ALDH 8A1 in liver, there was a increase in expression in gill and so, this gene may still play an important role in detoxification of TFM. In rats, ALDH 8A1 is highly expressed in liver and kidney and is responsible for metabolism of aldehydes to carboxylic compounds [155]. This gene is particularly induced by peroxisome proliferator-activated receptor alpha (ppar-α) ligands [155]. In mice, PPAR-α gene is activated in response to fasting conditions and is responsible for stimulating metabolism of fatty acids in the liver as an alternative energy source in starvation conditions [156]. The recent works by Birceanu et al. demonstrated that TFM interferes with and in fact uncouples mitochondrial oxidative phosphorylation, blocking ATP production, which

results in depletion of energy stores [52, 53]. The increased expression of ALDH 8A1 may be a secondary or phase II response that is induced in sea lamprey to metabolize potentially toxic aldehydes formed when fatty acid stores are metabolized as an alternate energy source.

In liver, EPHX2 did not have an increase in expression, however there was an increased expression in gill, which is the same pattern seen for ALDH 8a1. Epoxide hydrolases have two functions and are termed bi-functional. The N-terminal domain metabolizes lipids [157], while the C-terminal domain metabolizes harmful epoxides [158]. In detoxification, the function of epoxide hydrolases is to add a water molecule to a potentially harmful epoxide, thus rendering it more soluble and readily excreted by an organism [158]. Though we did not see a differential expression in liver, the similar expression pattern of this phase II enzyme again suggests that the gill is an important site for extra-hepatic detoxification.

Given the broad substrate specificity of CYP genes, it is likely that more than 1 CYP or phase II gene is activated in response to TFM exposure in sea lamprey larvae, however, given that TFM is such an effective lampricide, we cannot discount the possibility that lethality is due to the absence of a phase I or phase II gene in the sea lamprey genome that is capable of binding and metabolizing TFM. It is possible that the selective effect of TFM on sea lamprey larvae mortality is due to the absence of an appropriate detoxification system. Other studies that have examined the biochemical consequences of TFM exposure in sea lamprey do suggest that mortality is caused by uncoupling of oxidative phosphorylation [52, 53], which has been shown to be a factor in cell death in rat hepatocytes [159]. We have demonstrated that of the 8 representative genes we monitored, there is a significant effect of dose on expression in the gill of 6, which suggests the gill plays a role in detoxification of TFM. In liver, only P450 genes from our candidate list demonstrated a dose effect on expression. The four P450 genes that show

a response to TFM dosage are candidates for further studies and are likely to be involved in TFM detoxification.

Conclusion

The sea lamprey P450 family is not as expanded, compared to other vertebrates. After exposure to sub-lethal concentration of the lampricide TFM, expression of six candidate detoxification genes (ALDH8a1, EPHX2, NADPH-cytochrome P450 reductase, CYP 1a1, CYP3a13 and CYP 4v2) were confirmed in gill and expression of 4 candidate genes (CYP1a1, CYP 2j6, CYP 3a13 and CYP4v2) were confirmed in liver.

Methods

Experimental Animals: Sea lamprey larvae (n=144) were obtained from the United States Geological Survey Hammond Bay Biological Station (Millersburg, MI) with mean length ± s.e.m (10.58 cm ±0.26 cm) and mean weight ± s.e.m. (1.74 g ± 0.15 g). Animal use was approved by the MSU Institutional Animal Care and Use Committee (AUF #06/09-095-00).

Search for P450 Sequences in Sea Lamprey Genome: Twelve cytochrome P450 sequences from amphioxus, tunicate, zebrafish, pufferfish and sea urchin were used to create position specific scoring matrices (PSSMs). These matrices were then used in a reverse psi blast where assembly 2.0 of the sea lamprey genome was used to query the PSSMs to find homologous sequences in the lamprey genome [160]. Analysis of gene structure was performed on nucleotide sequences using GENSCAN (http://genes.mit.edu/GENSCAN.html), which resulted in 54 matches (29 full length intact sequences and 25 partial sequences). All 54 matches were then aligned to the sea lamprey genome which resulted in 23 full length intact sequences and 31 partial sequences [9]. A keyword search in the genome browser

(http://petromyzon.msu.edu/fgb2/gbrowse/sea_lamprey/) supplemented our search for cytochrome P450 genes and phase II genes. Select search terms were selected from a list of phase II genes in a commercially available mouse cDNA microarray (Mouse Drug Metabolism RT^2 Profiler^{TM} PCR Array, Qiagen). Search terms included CYP, P450, EPHX, MGST, GSTT, ALDH and AHR. The list of genes returned from these keywords was compared to the list generated from comparison of assemblies 2.0 and 7.0.

TFM Treatment: Larvae were acclimated for 24 hours in 9 identical 19 L aerated aquaria (n=16 per tank). Tanks were then treated with 0, 0.6 or 1.2 mg/L of TFM (3 replicate tanks per treatment level). 4 larvae were removed from each tank at each of the four time points (1, 2, 4 and 8 hours) and anaesthetized with tricaine methanesulfonate (MS-222, 100 mg/L, Sigma) and decapitated. The head of 1 larva was fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) and the livers, gills and brains of the other 3 were removed and snap-frozen in liquid nitrogen. RNA was extracted using TRIZOL reagent and stored at at -80^{O}C. Quality of samples was verified using an Agilent 2100 Bioanalyzer before submission for high-throughput sequencing.

High-throughput Sequencing, Assembly and Alignment to Mouse Refseq Database: Liver and gill samples were sequenced at the Michigan State University Research Technology Support Facility, using the Illumina DGEx kit according to manufacturer's instructions. 817 156 282 reads were obtained and 81.72% (667 813 367) passed a quality filter. The filtered reads were aligned, using Bowtie software [119], to our assembly of the sea lamprey transcriptome to obtain transcript expression count information for each lane. The transcriptome assembly was, in turn, aligned to mouse RefSeq protein sequences, providing a putative orthology with which mouse protein annotations were assigned to corresponding lamprey transcripts, and these annotations

were combined with transcript expression counts to infer expression information for putative lamprey-mouse orthologs. This information was used to infer putative ortholog differential expression between gill at all levels of TFM treatment and between liver at all levels of TFM treatment.

Differential Expression (DE) Analysis: Originally our experiment consisted of 21 sequenced libraries from 18 samples. These samples represented liver and gill tissue treated at all three doses of TFM (0, 0.6 and 1.2 mg/L) and at 8 hour time point and were from nine different individuals (three biological replicates for each dose level). Three liver samples were represented by two libraries as technical replicates and were introduced as a between-plate internal standard. We chose to sequence samples from the 8-hour time point because we reasoned that differential expression would be greatest at the last time point of treatment, based on CYP 1a mRNA expression in rainbow trout after exposure to a xenobiotic [149]. Samples 'G81-a8' and 'L83-a6' were set aside from the analysis due to be putative outliers/mislabeled. Additionally, the two technical replicates within each flow cell were added to circumvent the problem of technical replication. Consequently, the dataset analyzed consisted of counts for 136,667 tags and 17 libraries.

We kept for analysis tags with at least 2 counts per million in at least 3 samples [161]. To account for differences in library sizes and composition, samples were normalized using the TMM (trimmed mean of M values) method proposed by Robinson and Oshlack [162] and implemented in edgeR [163].

Statistical Analysis: According to our experimental design an ideal statistical model to analyze the counts would include: dose, tissue, and flow cell as fixed effects, and animal as

heteroskedastic random effect. To the best of our knowledge, none of the available packages for RNA-seq data analysis can incorporate random effects. Consequently, we decided to split the set of samples into smaller partitions that could account for effect model implementing the analysis tools of edgeR v.2.4.6 [163].

Nine contrasts of the transcriptome libraries were defined and are shown in Table 12.

Table 12: Partitions and contrasts of transcriptome libraries for differential expression analysis

Partition	Contrast	Transcriptome Libraries
1	1	G1vsG2
1	2	G1vsG3
1	3	G2vsG3
2	4	L1vsL2
2	5	L1vsL3
2	6	L2vsL3
3	7	G1vsL1
4	8	G2vsL2
5	9	G3vsL3

G:Gill; L:liver; 1-2-3:Dose 1,2 or 3

Partition 1 was used to test contrasts 1-3 and allowed for comparing the effect of TFM dose in gill. Partition 2 allowed testing contrasts 4-6 and allowed for comparing the effect of TFM dose in liver. The statistical model to analyze these partitions is:

$$Y_{ij} = \mu + D_i + B_j + \varepsilon_{ij} \qquad \text{[Model 1]}$$

where: Y_{ij} =normalized counts, D_i = Dose effect, B_j =Block effect (combination of Flow effects)

Partition 3 included contrast 7 and allowed for comparing the expression between liver and gill at TFM dose of 0 mg/L. Partition 4 included contrast 8 and allowed for comparing the expression between liver and gill at TFM dose of 0.6 mg/L. Partition 5 included contrast 9 and allowed for comparing the expression between liver and gill at TFM dose of 1.2 mg/L. The statistical model to analyze these partitions is:

$$Y_{ij} = \mu + T_i + B_j + \varepsilon_{ij} \qquad \text{[Model 2]}$$

where: Y_{ij} =normalized counts, T_i = Tissue effect, B_j =Block effect (combination of Flow cell and animal effects)

We defined these contrasts as they provided the best ways to determine differential expression in a biologically relevant manner. For instance, contrasts 1 through 6 allowed us to determine dose effects within individual tissues and contrasts 7 through 9 allowed us to determine differential expression by direct comparison of tissues at the same TFM dosage level. We speculated that gill and liver would have different metabolic capacities in response to TFM exposure.

We evaluated the tags for each contrast and adjusted the p-values for false discovery rates using the Benjamini and Hochberg method [164] using only tags that that had a blast match to the mouse refseq database.

SYBR Green Real-Time Quantitative PCR: RNA from liver and gill (n = 348) was used for real-time quantitative PCR (methods followed Yeh et al. 2012) [165]. Samples were from 15 TFM dosage levels x time points for each tissue type. Solexa DGE reads were aligned to the mouse refseq mRNA database and P450 and phase II genes were selected from the putative mouse orthologs. Only full-length, intact sequences were used for primer design (except where indicated) using Primer Express software (Applied Biosystems) (Table 13). Select genes were chosen from the list of predicted genes we generated as well as from lists generated by differential expression analysis. The genes monitored were: CYP 1a1, CYP 2j6, CYP 3a9, CYP 3a13, CYP 4v2, NADPH-cytochrome P450 reductase, ALDH8a1, and EPHX2. To determine whether there was a tissue effect on gene expression of the genes we selected, all responses in

gill were grouped together and the same was done for liver samples. A separate one-way ANOVA was run on log normalized data for each gene. To determine if there was a dose effect, gill and liver responses at all three doses for each gene were analyzed by one-way ANOVA.

Table 13: Primers used for SYBR green qPCR

GENE	FWD (SENSE)	REV (SENSE)
CYP 1A1	cttcctcaccgagatgttcc	aggccgaagatctgaacctt
CYP 2J6	ggcgcttcacccttatgatg	cagcgatcttctcctcaatgc
CYP 3A9	atccgaaatgtgctgactcc	ttggggttggtctgagaatc
CYP 3A13	cagagagcagagggaccagt	atttcagccgggatctttgt
CYP 4V2	cgcgcaggaagatgatcac	tccaggaagtccacgaggat
ALDH 8A1	tggactcgtttgaaccatca	tccactgttctcgcatgttc
EPHX2	ataactgggtggacgacagc	gtcgtccagaaacaccacct
NADPH-cytochrome P450 reductase	aagtacgcggtgtttggtct	ccacgtgatgaagtcctcct

SUMMARY

This dissertation has examined two large gene families contained in the genome of an early vertebrate, the sea lamprey. Integration of molecular biology and bioinformatics has permitted a more robust examination of the recently released sea lamprey genome. The chemoreceptor gene family (CR) is not as expanded in sea lamprey, as it is in jawed vertebrates. The CR gene family has expanded in the vertebrate lineage, as organisms transitioned from an aquatic to a terrestrial environment [166, 167]. The CR complement of Actinopterygians (ray-finned fishes) is larger than that of sea lamprey, which could be explained by the 210 million years that intervened between the appearance of lampreys and the appearance of fish in the vertebrate lineage [1–4, 168]. The expanded CR complement in fish could alternately be explained by the additional round of genome duplication (3R) of ray-finned fishes [167, 168]. Coupled with the recent discovery that the sea lamprey genome has undergone two rounds of duplication (2R) [169], the smaller size of the CR gene family in sea lamprey supports the phylogenetic position of lampreys. Additionally, I have shown expression of specific CR genes in an anatomical region of the sea lamprey nasal capsule (the accessory olfactory epithelium, AOE) with no previously known function. The expression of representatives from all three known families of CR genes in the AOE strongly suggests a chemosensory function for the AOE which includes pheromone detection. Through reciprocal neural tract-tracing, I have demonstrated a novel neural pathway from the AOE to a region of the brain that is distinct from the projections of the main olfactory epithelium, that is proposed as a primordial accessory olfactory bulb (AOB). These anatomical and molecular lines of evidence suggest the components for a complete vomeronasal system were present in the sea lamprey, suggesting that a dual olfactory system is the plesiomorphic condition for vertebrates. Given that the sea

lamprey employs pheromones for mating and given that sea lamprey control includes baiting of traps with synthetic pheromone, knowledge of the genes expressed in a putative vomeronasal organ homolog and knowledge of the pathway from this organ is important in understanding how pheromone information is processed in the sea lamprey brain. Moreover, the expressed genes and pathway can then be exploited to block this pathway, either at the ligand-binding level employing antagonists to specific receptors or by ablation of the pathway itself to block processing of pheromone signals.

The P450 gene family in the sea lamprey genome is also not as expanded, compared to other vertebrates. The smaller overall size of the P450 gene family in the sea lamprey genome compared to other vertebrates (about 50% smaller), as well as the smaller number of genes within each family, further supports the evolutionary position of sea lamprey in vertebrate phylogeny [9, 32, 39, 46, 168]. The greater number of P450 genes in zebrafish is likely due to a lineage-specific 3R genome duplication in fish [168]. While humans have about the same number of functional P450 genes as sea lamprey (56 in sea lamprey vs. 57 in humans), we have not verified functionality of each of the 56 full-length predicted genes in sea lamprey nor have we done any analysis to identify of any are pseudogenes. Humans have 59 P450 pseudogenes [39], with a total of 116 P450 sequences, which is closer to the 120 sequences seen in sea urchin [136]. The large number of P450 genes in sea urchin is proposed to be a consequence of the sea urchin embryo's need to detoxify any chemical challenges during early development, including an extended (5 month) free-swimming pelagic stage in a marine environment, suggesting this expansion of P450 genes may be lineage-specific [136, 170].

It should be noted that the precise mechanism of how TFM exerts its lethal effect on sea lamprey larvae has not been elucidated. While the work by Birceanu et al. [52, 53] strongly

suggests that TFM uncouples oxidative phosphorylation, causing death, a link to specific phase I or phase II genes has not been established. The lethality of TFM on larvae may be due to the absence of appropriate phase I or phase II genes in the sea lamprey genome meaning that sea lamprey are not able to process/detoxify this xenobiotic chemical. To determine whether there is a pseudo-selective pressure of TFM on sea lamprey populations, comparisons of genomes, life cycles and behaviors between Atlantic (TFM-naïve) and Great Lakes populations could be accomplished.

I have identified several candidate P450 genes whose expression is induced by exposure to TFM. A thorough search of the sea lamprey genome coupled with comparisons of P450 complements in other organisms, supports the current evolutionary position of sea lamprey in the vertebrate lineage. Knowledge of the P450 genes that are involved in TFM detoxification can be used to design new lampricides that cannot be induced by sea lamprey P450 genes, or to optimize the dose of TFM, reducing cost and non-target species effects.

Both the CR and P450 gene families in the sea lamprey genome are not as expanded when compared to other vertebrates. Though there are likely species-specific differences in expression of select genes within each family, the quantification of the sizes of these two important gene families in a basal vertebrate gives further credence to the evolutionary position of the sea lamprey in the vertebrate lineage and allows resolution at the genome level.

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