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EVOLUTION, CLASSIFICATION, AND BIOCHEMICAL FOUNDATIONS OF TOLL-LIKE RECEPTORS (TLRS) AND THEIR ROLE IN CHYTRIDIOMYCOSIS RESISTANCE

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EVOLUTION, CLASSIFICATION, AND BIOCHEMICAL FOUNDATIONS OF TOLL-LIKE RECEPTORS (TLRS) AND THEIR ROLE IN CHYTRIDIOMYCOSIS RESISTANCE

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ABSTRACT

EVOLUTION, CLASSIFICATION, AND BIOCHEMICAL FOUNDATIONS OF TOLL-LIKE RECEPTORS (TLRS) AND THEIR ROLE IN CHTRIDIOMYCOSIS RESISTANCE

Joseph De Leon

Chytridiomycosis, a disease caused by the fungus Batrachochytrium dendrobatidis (*Bd*), is a major concern for Amphibian populations. Over the past thirty years, global declines related to *Bd* infection have been observed, particularly in neotropical amphibians. Although *Bd* is endemic to many amphibian species, a significant proportion of amphibian populations have shown evidence of resistance to the pathogen. The precise reasoning as to why there is resistance variation across amphibian taxa remains to be elucidated, but many hypotheses have been suggested. In particular, immunogenetic variations in the innate immune system among amphibians are potential indicators to this dilemma. Toll-Like Receptors (TLRs), a family of innate immune receptors that recognize pathogens, are crucial in the first line of defense against foreign invaders and regulate both the innate and adaptive immune response. TLRs have been shown to be upregulated as a consequence of *Bd* infection and have also been confirmed to recognize fungal pathogens. Thus, TLRs are likely to play a key role in the defense against *Bd*. The aims of this study are to sequence the TLR repertoire of amphibians with an emphasis on neotropical taxa, as well as to provide the selection landscapes of all TLRs expressed in the amphibians studied. We preformed RNA extractions of a diverse array of amphibians and also used TLR sequences available from the ncbi. The species

under analysis belonged to Anurans, Caudata, and Gymnophiona. We carried out transcriptome assemblies and annotated genes encoding TLRs, as well as phylogenetic techniques to align and provide selection landscapes of amphibian TLRs. For receptors involved in *Bd* infection, the hypothesis is that positively selected sites (PSSS) in codons associated with non-redundant functions can lead to defective changes that impact the immune response. With our results, we provided evidence of positive selection occurring on TLRs which is indicative of pathogen-mediated evolution, which could have implications in resistance to *Bd*. Our work provides a platform for future research in chytridiomycosis resistance and is also the largest attempted transcriptome analysis of amphibian TLRs to date.

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INTRODUCTION

The innate immune system is an ancient form of host defense against pathogens in multicellular organisms, which functions as the first line of defense against foreign pathogens and invaders. Specific to the innate immune system is the use of a diverse array of pattern recognition receptors (PRRs) that are displayed on the cell surfaces, intracellular membranes, and tissue fluids (Janeway & Medzhitov, 2002). PRRs recognize pathogen-associated molecular patterns (PAMPs), which are small molecular motifs that are associated with a particular microbe. Some of these PAMPs include lipopolysaccharide, peptidoglycan, lipopeptides, flagellin, RNA (single and double stranded), etc. (Varga, Bui-Marinos, & Katzenback, 2019). The binding of PRRs to PAMPs lead to an intracellular signaling cascade that codes for the expression of essential innate immunity genes (Janeway & Medzhitov, 2002; Varga et al., 2019). Toll-Like Receptors (TLRs) are essential PRRs in the immune system, as they recognize a wide variety of pathogens and are expressed in many cells involved in both innate and adaptive immunity, as well as non-immune cells such as epithelium and fibroblasts (Richmond, Savage, Zamudio, & Rosenblum, 2009)

TLRs are type I integral membrane glycoproteins and are part of a larger superfamily of proteins that includes interleukin receptors (Akira & Takeda, 2004). There are many different types of TLRs, ranging from 10 in humans to at least 17 in amphibians. Each TLR is unique with different properties that allow them to recognize distinct ligands. In recent years, TLRs have been a very intense topic of research (Luther & Ebel, 2006). TLRs are expressed in many cell types and tissues, and they recognize ligands of bacteria, fungi, and other microorganisms capable of transmitting disease. In

particular, the focus has been on TLRs and whether they are relevant in disease susceptibility. What is known, however, is that TLRs have a special role in the activation and regulation of the immune response. The main questions hovering around are as follows: 1) Do TLRs, whether it be deficiency in expression or non-synonymous amino acid changes, contribute to susceptibility and resistance to disease and 2) How can scientists manipulate TLR signaling to improve the immune response (El-Zayat, Sibaii, & Mannaa, 2019). In this paper, I will focus on the former of the two questions. I will first provide a review of vertebrate TLRs, which includes the evolutionary origin, diversification, and biochemical foundations of vertebrate TLRs as well as the role of TLRs in fungal infections. I will then discuss the implications of evolutionary forces acting on amphibian TLR genes in the fight against Chytridiomycosis, a fungal disease that is responsible for global decline in amphibian populations. Lastly, I will present the work carried out by our research team, and how this work can provide framework for future research.

CHAPTER 1: EVOLUTION AND CLASSIFICATION OF TOLL-LIKE RECEPTORS

Prototypical TLRs

Toll is a family of protein receptors that function in the development and immune response in animals and plants (Dembic, 2005). Toll was first discovered in *Drosophila melanogaster* as a protein involved in development of the embryonic dorsal-ventral polarity specification (Brennan & Gilmore, 2018). In vertebrates, Toll-like receptors (TLRs) mediate and implement an immune response to counteract foreign invaders.

A prototypical TLR is defined as having three functional domains: an extracellular domain (ECD) containing leucine-rich repeats (LRRs), a transmembrane domain, and an intracellular Toll/IL-1 (TIR) domain (Botos, Segal, & Davies, 2011; Liu, Zhang, Zhao, & Zhang, 2019). TLRs are not the only protein containing LRRs (Leulier *et al*., 2008). Other proteins containing LRR motifs are prevalent among prokaryotes and eukaryotes (Brennan & Gilmore, 2018). LRR-containing proteins are found in NOD receptors, secreted proteins, membrane-spanning proteins, and GPI-anchored proteins (Brennan & Gilmore, 2018). In the human proteome, 375 LRR-containing proteins exist, however many have been uncharacterized (Ng *et al*., 2011). Approximately half of these LRR proteins in humans have no other domain apart from the LRR, whereas the others contain transmembrane regions or signal peptides (Ng *et al*., 2011).

The TIR domain, which is displayed on the cytosolic face of the membrane, is an

evolutionarily conserved region (Botos *et al*., 2011). TIR proteins, like LRR-containing proteins, can exist without the association of other domains. TIR proteins have been identified in plants, metazoans, bacteria, and viruses (Leulier *et al*., 2008). In the phyla Porifera and Cnidaria, the TIR proteins identified do not contain an LRR domain, meaning these proteins most likely function in intracellular signaling transduction without the recognition of PAMPs (Brennan & Gilmore, 2018). In mammals, the TLR adaptor proteins Mal and TRAM are evolutionarily similar to the TIR-only proteins found in basal invertebrates, which suggests that mammalian TLR adaptor proteins may have evolved from these TIR-only proteins (Ve *et al*., 2015; Brennan & Gilmore 2018). In vertebrates, TIR domains are also found in receptors other than TLRs that function in the immune response. The interleukin-1 and interleukin-18 receptors (IL1R and IL18R) contain TIR domains, however their extracellular regions contain immunoglobular-like domains rather than LRR (Leuiler *et al*., 2008). Since these interleukin receptors are only found in deuterostomes, it is likely that the divergence between TLRs and IL1R/IL18R occurred immediately after the emergence of deuterostomes (Leulier *et al*., 2008).

Emergence of TLRs

The existence of both TIR and LRR proteins that exist independently of one another led to the hypothesis that TLRs originated from the association of TIR proteins and LRR proteins through a transmembrane domain (Beutler *et al*., 2004; Brennan and Gilmore, 2018). Since these TLR-related genes have been found in more divergent species of Cnidaria and Porifera, it is suggested that these genes are homologous to all animal phyla and originated roughly 700 mya (Leulier *et al*., 2008).

Extensive genomic data suggests that prototypical TLRs originated in the eumetazoan ancestor more than 581 mya (Liu *et al*., 2019; Leulier *et al*., 2008). This stems from the fact that no prototypical TLRs have been identified in non-animal phyla or in the phylum Porifera, which would lead to a point of TLR emergence right before the separation of bilaterians and cnidarians (Weins *et al*., 2007; Gauthier *et al*., 2010; Hentschel *et al*., 2012; Leulier *et al*., 2008). Interestingly, Cnidarians display structurally different TLRs than other phyla, which suggests that Cnidarian TLRs may have evolved different functional mechanisms of pathogen recognition (Leulier *et al*., 2008).

Further along the evolutionary time scale marks the branching point of deuterostome and protostome divergence. In this branching point, TLR3 emerged in deuterostomes about 570 mya (Leulier & Lemaitre, 2008). Protostome and deuterostome TLRs share the same common ancestor, but phylogenetic analysis reveals that these TLRs evolved independently by gene duplication, which suggests a divergence of protostome and deuterostome TLRs due to functional differences (Roach *et al*., 2005; Zheng *et al*., 2005; Kanzok *et al*., 2004).

Evolution of Vertebrate TLRs

TLRs are classified based on the number of cysteine clusters in their extracellular domains (ECD) (Liu *et al*., 2019). Multiple cysteine cluster TLRs (mccTLRs) contain two cysteine clusters at the carboxy terminus of the LRR (LRR-CT). Single cysteine cluster TLRs, however, contain only one LRR-CT. MccTLRs have been shown to be present in more ancient species, with sccTLRs emerging later in the evolutionary tree (Brennan & Gilmore, 2018). The emergence of sccTLRs is suggested to have occurred in

Mollusks (Brennan & Gilmore, 2018). Evidence for this is supported by the fact that mccTLRs are the predominant TLR type in cnidarians and nematoda, and since mollusks underwent a huge expansion in TLR genes, sccTLRs most likely originated in this phylum (Brennan & Gilmore, 2018).

All vertebrate TLRs are classified as sccTLR, whereas some invertebrates are sccTLRs (most invertebrates are mccTLR) (Liu *et al*., 2019). This suggests that somewhere along the evolutionary history, the emergence of vertebrate TLR were associated with a loss of mccTLRs. Although some invertebrates have sccTLRs, researchers have struggled to establish an orthologous relationship of TLR genes between vertebrates and invertebrates. This suggests that the majority of TLR genes emerged shortly after the emergence of vertebrates, and then rapidly diversified (Liu *et al*., 2019)

Diversification of Vertebrate TLRs

Currently there are at least 29 classified TLRs in vertebrates (Liu *et al*., 2019). However, the TLR repertoire in each class of vertebrates varies numerously. For example, transcriptome analysis has revealed that mammals have at least 13 TLRs (TLR1-13) whereas the TLR repertoire of amphibians showed 16 distinct TLRs *(*Liu *et al*., 2019). Difference in TLR amount between species is suggested to be a product of environmental variation, where species with more complex habitats had to expand their TLR genes to co-exist with diverse pathogens (Liu *et al*., 2019). For the most part, the evolutionary rates of vertebrate TLRs are relatively slow. This suggests that there is strong selection acting on TLRs to maintain their function (Roach *et al*., 2005).

Classification of Vertebrate TLRs

Vertebrate TLRs are essentially classified based on the type of ligand they recognize and their localization in the cell. The two types of vertebrate TLRs are viral and non-viral TLRs. Non-viral TLRs are typically displayed on the plasma membrane and recognize various non-nucleic acid-containing ligands (Liu *et al*., 2019). Viral TLRs are mainly displayed in endosomes and recognize nucleic acids of viruses (Kawai and Akira 2010). TLRs are a family of type-I integral membrane glycoproteins (Leulier & Lemaitre, 2008), but they can be further divided into subfamilies based on their evolutionary similarities and function. Roach *et al* (2005) studied the evolution of vertebrate TLR genes and identified six major TLR subfamilies: TLR1, TLR3, TLR4, TLR5, TLR7, and TLR11. In each subfamily of TLRs, there exists independent TLR genes. For example, the TLR1 subfamily contains the TLR genes (which code for the TLR proteins) TLR1, TLR2, TLR6, TLR10, and TLR14 (Roach *et al*., 2005). In the context of TLRs, it is important to refer to them as either a TLR gene/protein or a TLR subfamily. Liu *et al* (2019) also studied the evolution of vertebrate TLR genes and identified *eight* TLR subfamilies instead of six: TLR1, TLR3, TLR4, TLR5, TLR7, TLR11, TLR13, and TLR15 subfamilies. This study was more inclusive of a larger number of vertebrate species relative to Roach *et al* (2005). Liu *et al* (2019) classified TLR15 as a separate subfamily, whereas Roach *et al* suggested that TLR15 deviated from the TLR1 subfamily. The study done by Liu *et al* (2019) most likely provides a more accurate representation of TLR15 since they sequenced the transcriptome of multiple species of birds and reptiles. Liu *et al* (2019) also identified TLR13 as a separate

subfamily from TLR11 due to the remarkable differences in their ECD structure.

TLR1 Subfamily

The TLR1 subfamily is composed of TLR1, TLR1L, TLR1A, TLR1B, TLR2, TLR2A, TLR2B, TLR6, TLR10, TLR14, TLR18, TLR25, and TLR27 (Liu *et al*., 2019). Together, these groups of TLR genes comprise the largest TLR subfamily. Of the TLR genes in this subfamily, fish contain TLR1/2/18/25/27, amphibians contain TLR1/1L/2/14, reptiles contain TLR1/2/14, birds contain TLR1/1A/1B/2A/2B, and mammals contain TLR1/2/6/10 (Liu *et al*., 2019). The TLR genes in the TLR1 subfamily differ greatly between species. This can be perhaps be attributed to class-specific adaptations and evolutionary differences relative to other subfamilies (Liu *et al*., 2019). TLR14 is only present in amphibians and reptiles, which suggests that TLR14 emerged roughly 365 mya after the divergence of fishes and amphibians (Carrol 2009). TLR1A/B and TLR2A/B in birds formed a clade, and TLR1/6/10 in mammals also clustered together, which indicates a paralogous relationship of these genes within their respective species (Liu *et al*., 2019).

TLR2 is displayed on the plasma membrane and recognizes lipid containing ligands such as lipoproteins, peptidoglycan, lipoteichoic acid, lipoarabinomannan, lipopolysaccharides, and other ligands (Takeda, Kaisho, & Akira, 2003). The main reason why TLR2 recognizes numerous pathogens is because TLR2 confers the ability to associate with TLR1 and TLR6 through heterodimerization. Through this dimerization with different TLRs, TLR2 heterodimers can distinguish between structurally different PAMPs, giving them the capacity to initiate a response to various pathogens.

When TLR2 associates with TLR1, this heterodimer recognizes triacylated lipopeptide PAMPs (Jin *et al*., 2007). However, when TLR2 forms a heterodimer with TLR6, it recognized diacylated lipopeptide PAMPs (Botos *et al*., 2011). This difference in recognition is due to the fact that the TLR2-TLR1 dimer can accommodate an extra peptide tail in the ECD binding pocket because of the difference in structural conformation in the ECD between TLR1 and TLR6. The conformation of TLR6 does not allow for such an accommodation, so this TLR recognizes peptide PAMPs with two tails (Kang *et al*., 2009). TLR2 has also been shown to form homodimers in vitro in response to diprovocim, a synthetic agonist of TLR activation (Su *et al*., 2019).

TLR10 is evolutionarily similar to TLR1, TLR2, and TLR6, however, the specific ligands to which TLR10 binds to are currently unknown (Fore *et al*., 2020). TLR10 is displayed on the plasma membrane and forms TLR10 homodimers, TLR10-TLR2 heterodimers, TLR10-6 heterodimers, and TLR10-1 heterodimers (Fore *et al*., 2020). However, the function of each individual dimer is yet to be determined. As for TLR10 ligands, studies have shown that ligands that interact with TLR2 are likely to be ligands for TLR10 (Fore *et al*., 2020). It has been suggested, through computational modeling, that the TLR10 homodimer recognizes diacylated lipopeptides (Verma *et al*., 2014, Tarlinton *et al*., 2016). Other ligands for TLR10 include the following: TLR2-TLR10 recognizes several PAMPs of TLR2-TLR1 (Verma *et al*., 2014), TLR2-TLR10 potentially recognizes lipopolysaccharide (LPS) ((Verma *et al*., 2014), HIV-gp41 is recognized by TLR10 (Henrick *et al*., 2019).

TLR14 has not been identified in mammals and is one of the unique TLR types that can recognize both viral and non-viral ligands (Hwang *et al*., 2010). TLR14 is most

similar to TLR1/2/6/10, and since TLR14 is only expressed in species lacking TLR6 and TLR10, then it is probable that TLR14 serves as a substitute for those TLRs. The function of TLR14 is currently unknown, however, results from Hwang *et al*., (2010) indicate that TLR14 responds against gram-negative/gram positive bacteria and viruses.

TLR18 is only found in fishes, and these TLRs recognize bacterial pathogens. Shan *et al* (2018) introduced flagellin, LPS, and polyinosinic-polycytidylic acid to TLR18-expressing cells, and TLR18 was upregulated in response to all of these ligands. This strongly suggests that TLR18 functions in the immune response to bacterial ligands.

TLR25 is a TLR unique in fish that localizes to intracellular compartments and recognizes both bacterial and viral components (Lee *et al*., 2020; Li *et al*., 2018). Similar to TLR1, TLR25 lacks an LRR-NT, which perhaps indicates that TLR25 functionally associates with TLR2 to expand the array of potential TLR2 PAMPs (Lee *et al*., 2020). Upregulation of TLR25 occurred upon introduction of LPS, LTA, zymosan, *A. hydrophila,* and *S. agalactiae* (Lee *et al*., 2020). This indicates that these are all potential ligands of TLR25, and since LTA and zymosan are ligands of TLR2 as well, TLR25 and TLR2 may functionally form a heterodimer to recognize these PAMPs (Lee *et al*., 2020). Introduction of Poly (I:C) also led to increased expression of TLR25 (Li *et al*., 2018). This could potentially mean that TLR25 recognizes bacterial as well as viral PAMPs, however, future work needs to be done to further confirm this phenomenon. TLR27 has been identified in fish species (Liu *et al*., 2019). However, the ligands for TLR27 are currently unknown.

TLR3 and TLR4 Subfamilies

The TLR3 Subfamily only contains the TLR3 gene (Liu *et al*., 2019; Roach *et al*., 2005). TLR3 exists as a single gene across all vertebrate species without any losses or polymorphisms (Liu *et al*., 2019). Because of this, TLR3 is considered the most conserved TLR subfamily within vertebrates, which indicates a strong preservation of TLR3 function.

TLR3 is responsible for the recognition of viral double-stranded RNA (dsRNA) (Takeda *et al*., 2003). TLR3 is expressed on the surface of endosomes (Liu *et al*., 2019) and recognizes nucleic acids upon phagocytosis (Bell *et al*., 2005). TLR3 binds to dsRNA oligonucleotides of 40-50 base-pairs in length under acidic conditions, and upon recognition to the ligand, TLR3 undergoes homodimerization (Liu *et al*., 2009; Wang *et al*., 2010). This dimerization brings the TIR domains within proximity to initiate downstream signaling. TLR3, unlike other nucleic acid sensing TLRs, does not show specificity to a particular sequence. This is most likely because TLR3 interacts with the sugar-phosphate backbones of the RNA and not the individual nitrogenous bases (Liu *et al*., 2009).

Similar to the TLR3 subfamily, the TLR4 subfamily is composed of only the TLR4 gene. TLR4 is generally evolutionarily conserved among vertebrate species, however there is some evidence for gene loss in fishes and amphibians (Liu *et al*., 2019). On the other hand, some species contain multiple copies of TLR4, such as four copies in *Astyanax mexicanus* and *Cyprinus carpio* (Liu *et al*., 2019).

The main ligands of TLR4 are Lipopolysaccharides (LPS), which are cytotoxic components located in the outer membrane of Gram-negative bacteria. Additional ligands

include taxol, heat shock proteins, and extracellular matrix components (Takeda *et al*., 2003). TLR4 binds to LPS with the help of Myeloid differentiation factor 2 (MD-2), which is a co-receptor that binds to TLR4 before recognition of the PAMP (Kim *et al*., 2007). Ligand recognition leads to the homodimerization of two TLR4-MD-2 complexes and sequential downstream signaling.

TLR5 Subfamily

The TLR5 subfamily includes TLR5, TLR5S, and TLR5L (Liu *et al*., 2019). TLR5 pseudogenization has occurred independently in birds, reptiles, and amphibians (Bainova *et al*., 2014; Velova *et al*., 2018). TLR5S and TLR5L are short soluble forms of TLR5. TLR5S and TLR5L lack a transmembrane domain and an intracellular domain and are structurally similar to one another (Liu *et al*., 2019). Considering this, the homologous relationship between these genes is unclear. Liu *et al* (2019) proposed that TLR5S and TLR5L arose independently via gene duplication of TLR5-ECD in fish and amphibians. This is because these short soluble forms of TLR5 are not found in birds and mammals, and these genes are highly similar with one another within each species. Of the TLR genes in this subfamily, fishes contain TLR5/5S, amphibians contain TLR5/5L, reptiles contain TLR5/5L, birds contain TLR5, and humans contain TLR5 (Liu *et al*., 2019).

TLR5 is displayed on the plasma membrane of immune cells. TLR5 recognizes flagellin of both gram negative and gram positive bacteria (Takeda *et al*., 2003), which is responsible for cellular locomotion. The structure of TLR5 is similar to TLR3, which is expected due to the close evolutionary relationship of the two proteins. TLR5 interacts

with flagellin via salt bridges and hydrogen bonding (Yoon *et al*., 2012). The activation of TLR5 leads to homodimerization.

TLR7 Subfamily

The TLR7 subfamily is composed of TLR7, TLR8, and TLR9 (Liu *et al*., 2019). These TLRs recognize viral nucleic acids. In the phylogenetic analysis of Liu *et al* (2019) the TLR7 subfamily emerged at the root of the vertebrate tree, which indicates that TLR7 subfamily divergence occurred before the divergence of fishes. Of the TLR genes in this subfamily, fishes, amphibians, reptiles, and mammals contain TLR7/8/9, but birds contain only TLR7 (Liu *et al*., 2019). Even though birds do not contain TLR8 and TLR9, extensive gene duplication of TLR7 in birds have been observed (Velova *et al*., 2018; Grueber *et al*., 2012; Liu *et al*., 2019). The reason TLR7 is under this type of selection is because avian TLR7 has been shown to recognize a highly pathogenic influenza virus (HPAIV) (Chen *et al*., 2013). This pathogen poses a lot of threat to many species of birds, which can potentially explain the need for gene duplication of TLR7.

TLR7 recognizes single-stranded RNA (Zhang *et al*., 2018). It has been shown that guanosine (G) and 2'-deoxyguanosine, and polyuridine (polyU) ssRNA are agonists of TLR7 (Shibata *et* al., 2016; Zhang *et al*., 2017; Zhang *et al*., 2018). TLR7 exists as a monomer in solution, and the dimerization of TLR7 is activated by ligand-binding (Zhang *et al*., 2016). TLR7 binds to its ligands using hydrophobic, hydrophilic, and protein-protein interactions.

TLR8, like TLR7, recognizes ssRNA (Tanji, Ohto, Shibata, Miyake, & Shimizu,

2013). TLR8 selectively binds to uridine-rich ssRNA, whereas TLR7 selectively recognizes guanosine-rich ssRNA. Tanji *et al* (2013) determined the crystal structure of TLR8 bound to its ligand, specifically TLR8 bound to resiquimod (R848), an antiviral agonist that targets TLR8. The unliganded form of TLR8, which is structurally different than most TLRs, exists as a preformed dimer (Tanji *et al*., 2013). TLR7 and TLR8 interact with their ligands in similar ways. A major difference is the type of ligand each one identifies; TLR7 recognizes guanosine-rich ssRNA and GS9620, and TLR8 recognizes uridine-rich ssRNA (Zhang *et al*., 2018). Both receptors, however, can recognize IQDs such as R848 and CL075. Zhang *et al* (2018) suggested that the reason for this specificity is because of differences in volume of ligand-binding pocket and electrostatic potentials.

TLR9 recognizes unmethylated cytosine-phosphate-guanine (CpG) single-stranded DNA (ssDNA) (Ishida, Ohto, Shibata, Miyake, & Shimizu, 2018). Like TLR8, TLR9 also exists as a preformed dimer (Latz *et al*., 2007). TLR9 recognizes the first 3 sequences in the CpG oligonucleotide with great affinity.

TLR11 Subfamily

The TLR11 subfamily comprises TLR11, TLR12, TLR19, and TLR20 (Liu *et al*., 2019). Of the TLR genes in this subfamily, mammals contain TLR11/12, fishes contain TR19/20, amphibians contain TLR12/19, and no TLR11 subfamily genes were observed in birds or reptiles (Liu *et al*., 2019).

TLR11 and TLR12 are found in mammalian species. These proteins are homologs that have been shown to recognize *Salmonella spp.* and *E. coli* flagellin as well as *Toxoplasma gondii* profilin-like protein (Hatai, Lepelley, Zeng, Hayden, & Ghosh, 2016). TLR11 is equipped with unique ability to bind to two distinct PAMPs using different mechanisms and protein domains. TLR11 and TLR12 are also unique in that they are localized to endosomes, which is typically rare among non-viral TLRs (Raetz *et al*., 2013). TLR11 can form both homodimers and heterodimers, but the binding to profilin-like protein requires association with TLR12 in a pH-dependent manner (Raetz *et al*., 2013). The binding of TLR11 to profilin-like protein is optimized at neutral pH's. However, TLR11 binds to flagellin at low pH's (Hatai *et al*., 2016). Because of this pH-dependency, TLR11 most likely preferentially recognizes flagellin in endolysosomal compartments (low pH), and TLR11 binding to profilin-like protein should occur prior to cleavage outside of endolysosomal compartments (Hatai *et al*., 2016).

TLR19 is expressed in fishes and amphibians. TLR19 localizes to endosomes, binds to dsRNA, and utilizes TRIF (adaptor protein) to initiate downstream signaling (Ji *et al*., 2018). TLR19 is functionally similar to TLR3 in that they are both intracellular TLRs recognizing dsRNA. Why amphibians and fishes have two endosomal dsRNA recognizing TLRs is unclear.

Evidence indicates that TLR11 is an ortholog for TLR20. TLR20 localizes to endosomes, and although the exact ligands for TLR20 are unknown, it is suggested to be involved in the carp immune response to protozoan parasites (*Trypanoplasma borreli)* (Pietretti *et al*., 2013).

TLR13 Subfamily

The TLR13 subfamily contains TLR13, TLR21, TLR22, and TLR23 (Liu *et al*., 2019). Fish contain TLR13/21/22/23, amphibians contain TLR13/21/22, reptiles contain TLR13/21/22, birds contain TLR21, and humans contain only TLR13 (Liu *et al*., 2019).

TLR13 is expressed in mammalian species and recognizes ribosomal RNA (rRNA), specifically the 23S ribosomal RNA of both gram-negative and gram-positive bacteria (Li & Chen, 2012). TLR13 is localized to endosomes (Li & Chen, 2012).

There have not yet been any crystal structures reported of TLR13 or the TLR13 ligand-binding complex. However, Li & Chen *et al* (2012) have identified a specific sequence in the rRNA that is involved in activation of TLR13 known as the Immune Stimulatory RNA from 23S rRNA (ISR23). Mutations in this sequence are suggested to be an evasion mechanism by bacteria to avoid detection by TLR13 (Li & Chen, 2012).

TLR21 is found in all vertebrates except mammals and is functionally similar to mammalian TLR9 (Keestra *et al*., 2010). TLR21 is localized in endosomes and recognize CpG DNA and bacterial genomic DNA (Keestra *et al*., 2010). Since birds lack TLR9, TLR21 likely emerged as a separate TLR with similar functions. TLR21 has a much broader ligand-specificity than TLR9, as TLR21 recognizes CpG DNA ODNs as well as bacterial DNA, whereas TLR9 only binds to hexameric CpG-DNA motifs.

TLR22 is found in fishes, amphibians, and reptiles. This receptor is exceptional in that it recognizes viral dsRNA and localizes to the cell membrane, which is uncommon in viral TLRs (Matsuo *et al*., 2008). The use of a receptor to recognize dsRNA at the cell surface could be due to the complex aquatic environments that fishes, amphibians, and

reptiles are faced with. The mechanism of how TLR22 binds to its ligand is currently unknown. For the last member of this subfamily, TLR23, no expression analysis or known ligands have been studied. However, this TLR is only found in fishes (Liu *et al*., 2019).

TLR15 Subfamily

TLR 15 is uniquely found in birds and reptiles, and it recognizes lysates from yeast and RNA viruses (Boyd *et al*., 2012; Chen *et al*., 2013). Boyd *et al* (2012) determined that yeast lysates are ligands of TLR15 by introducing *Saccharomyces cerevisiae* lysates to TLR15 transfected HEK293 cells and observed an increase in TLR15-dependent transcription factors. TLR15 has also been shown to function in the antiviral response. In chickens, IL-1b can be significantly upregulated through a MyD88-dependent TLR15 response (Linger *et al*., 2012). Also, the expression of TLR15 was significantly increased in birds after infection to Marek's disease virus (Jie *et al*., 2013). Although this data cannot give us a definitive answer that TLR15 recognizes viral nucleic acid oligomers, it gives us strong indication that TRL15 is involved in the immune response against viruses.

TLR	Class	Ligand	Vertebrate groups present
TLR1	Non-viral	Triacylated lipopeptide	All vertebrate groups
TLR1L	Non-viral	unknown	Amphibians
TLR2	Non-viral	Lipopeptides, peptidoglycan, lipotechoic acid, lipoarabinomannan, lipoplysaccahride, Porins, Zymosan (fungi)	All vertebrate groups
TLR2L	unknown	unknown	Amphibians
TLR3	Viral	Double-stranded RNA	All vertebrate groups
TLR4	Non-viral	Lipoplysaccharide (LPS) Taxol	All vertebrate groups
		Envelope proteins HSP 60/70 Fibronectin Oligosaccharides hyaluronic acid Polysaccharides Fibrinogen	
TLR5	Non-viral	Bacterial flagellin	All vertebrate groups
TLR5L	unknown	unknown	Amphibians, Reptiles
TLR5S	Non-Viral	Bacterial flagellin	Fishes
TLR6	Non-viral	Diacylated lipopeptides	Mammals
TLR7	Viral	Single-stranded RNA	All vertebrate

			groups
TLR8	Viral	Single-stranded RNA	Fishes, Amphibians, Reptiles, Mammals
TLR9	Viral	Cytosine-phosphate-guanine (CpG) DNA	Fishes, Amphibians, Reptiles, Mammals
TLR10	Non-viral	Similar ligands to TLR1/2/6	Mammals
TLR11	Non-viral	Protozoan profilin like protein, flagellin	Mammals
TLR12	Non-viral	Protozoan profilin like protein, flagellin	Amphibians, Mammals
TLR13	Viral	Bacterial 23S ribosomal RNA	Fishes, Amphibians, Reptiles, Mammals
TLR14	“Hybrid”	Hemorrhagic septicemia virus, *Streptococcus iniae, Edwardsiella tarda*	Amphibians, Reptiles
TLR15	“Hybrid”	RNA viruses and Lysates from yeast	Reptiles, Birds
TLR18	Non-viral	bacteria	Fishes
TLR19	Viral	Double-stranded RNA	Fishes, Amphibians
TLR20	Viral	Protozoan profilin like protein	Fishes
TLR21	“Hybrid”	CpG oligodeoxynucleotides, Bacterial genomic DNA	Fishes, Amphibians, Reptiles, Birds
TLR21L	Unknown	Unknown	Reptiles
TLR22	Viral	Double-stranded RNA	Fishes, Amphibians, Reptiles
TLR23	Unknown	unknown	Fishes

TLR25	“hybrid”	Bacterial LPS and double-stranded RNA	Fishes
TLR27	Unknown	unknown	Fishes

Table 1. Summary of TLRs

Summary of TLRs, their ligands, and respective species (Jin et al., 2007; Botos et al., 2011; Su et al., 2019; Fore et al., 2020; Hwang et al., 2010; Shan et al., 2018; Lee et al., 2020; Wang et al., 2010; Kim et al., 2007; Yoon et al., 2012; L

Evolutionary Rates of TLRs

TLRs are at the front line of innate immune defense, making their function incredibly important for vertebrates. Multiple studies have sequenced the repertoire across classes of vertebrates, and the consensus is that vertebrate TLR genes are largely under purifying selection (Velova 2018, Dannemann 2016, Kloch *et al* 2018, Ferrer-Admetlla 2008, Key 2014, Liu *et al*., 2019). Due to the important role of TLRs, it is not surprising organisms have acquired evolutionary mechanisms to conserve the structural framework of these proteins. Even though purifying selection is the main selection acting on TLRs, there exists instances of positive selection across all vertebrate TLRs (Liu *et al*., 2019, velova 2018, greuber 2012, Areal 2011). Although these positively selected sites are relatively minute, they may be significant in susceptibility to disease.

Selection Differences Between Viral and Non-Viral TLRs

The force of selection differs between viral and non-viral TLRs, with viral TLRs exhibiting more purifying selection relative to non-viral TLRs, and non-viral TLRs evolving faster than viral TLRs (Liu *et al*., 2019). This is indicative of the difference in PAMPs between the two classes of TLRs, since non-viral pathogens display more complex patterns (Barreiro *et al*., 2009). Because of the complexity of non-viral PAMPs, pathogenic pressures act on TLRs to duplicate and expand their repertoire to recognize a diverse array of ligands. Also, different non-viral TLRs can recognize the same pathogen, which makes the function of one or more TLRs redundant. Thus, many non-viral TLRs are tolerant to non-synonymous mutations that lead to positive selection since another TLR can preserve the recognition of the mutual pathogen. Viral TLRs recognize nucleic acids, which have relatively low structural variation compared to non-viral microbes, and viral TLRs tend to exert higher specificity to their ligands. Areal *et al*, however, says there is not much difference in the selection landscape between viral and non-viral TLRs in mammals. He explains this by using the analogy of an "arm's-race" between TLRs and their pathogens. Pathogens evolve in a way to avoid detection of pattern-recognition receptors (PRRs), which includes structural changes in their motifs that bind to these receptors. TLRs therefore co-evolve with these pathogens to keep up with their structural changes by changing their ligand-binding domain (Areal *et al* 2011). The fact that viral nucleic acids undergo mutations faster than bacteria or yeast, then viral TLRs will have to evolve at a fast rate to keep up with these changes. However, the majority of studies have showed that there are in fact differences in selection between viral and non-viral TLRs. The selection landscape between viral and non-viral TLRs is most-likely specific to

vertebrate groups or classes, rather than a dichotomy that exists in all vertebrates.

Evolutionary Rates of Class-Specific TLRs

The orthologous genes shared by all vertebrate members are TLR3, TLR4, TLR5, and TLR7. Out of these TLRs, TLR3 and TLR7 are the most evolutionarily conserved (Liu *et al*., 2019). Genes that are under large positive selection include avian TLR1B, TLR4, and TLR5 as well as fish TLR23 (Liu *et al*., 2019, Velova *et al*., 2018). The results of Liu *et al*. also detected differences in natural selection across vertebrate classes. The proportion of positively selected sites (PSSs) ranges from 2.2%- 25.0% (in amphibians and birds respectively) (Liu *et al*., 2019). These differences in selection indicate class-specific evolutionary patterns such as adaptations to varying environments (Liu *et al*., 2019). It was also found that higher rates of evolution occur in the TLR-ECD compared to the TIR domain (Wlasiuk and Nachman 2010, Liu *et al*., 2019). This illustrates the importance of ligand-recognition in the evolutionary trajectory of TLRs and could be explained by the diversification of pathogens.

In fishes, the TLR genes that have been shown to be under the most evolutionary constraint are TLR18, TLR7, TLR25, and TLR3 (Liu *et al*., 2019). The TLR genes under most positive selection are TLR20 and TLR23. Interestingly, even though TLR7 is one of the more constrained TLRs in fishes, the %PSSs has been shown to be higher than in other TLRs with higher dN/dS values (Liu *et al*., 2019).

In Reptiles, TLR14 and TLR21 are the most conserved. TLR2 is under the most positive selection out of the reptile TLR repertoire, which could indicate the intense

pathogenic pressures that act on TLR2 since it recognizes a diverse array of pathogens.

Bird TLR21 is the most constrained out of the avian TLR repertoire (Liu *et al*., 2019). The results of Velova *et al* (2014found that the %PSSs in TLR21 is very low, which solidifies the purifying selection acting on TLR21. Avian TLRs display the most positive selection out of all classes of vertebrates, with the mean dN/dS values higher than all other vertebrates, and the value of %PSSs ranging from 20.8%-42.9% (excluding TLR21). It is unclear why TLR21 is the only avian TLR under substantial purifying selection, but it could be because TLR21 intracellularly recognizes conserved CpG oligonucleotide sequences.

In Mammals, TLR9 is one of the most constrained, and TLR4/8/12 have some of the highest number of positively selected sites (Areal *et al*., 2011; Liu *et al*., 2019) Mammalian TLRs are generally constrained, with a few TLRs displaying patches of positively selected sites.

Many codons under positive selection are in close proximity to ligand-binding sites (Tschirren *et al*., 2011). This indicates that TLRs co-evolve with structural changes in pathogens, and most of the positive selection is occurring as a result of ligand-mediated changes in TLRs.

Amphibian TLRs

In the literature, there have 16 distinct TLR genes identified in amphibians: TLR1, TLR1L, TLR2, TLR3, TLR4, TLR5, TLR5L, TLR7, TLR8, TLR9, TLR12, TLR13, TLR14, TLR19, TLR21, and TLR22 (Liu *et al* 2019). The first attempt to

sequence the transcriptome of amphibian TLRs came from the work of Ishii *et al*., (2007). They sequenced the transcriptome of *Xenopus laevis* and found 23 TIR-containing proteins. 19 of these proteins were identified as TLRs, and the other 4 were adaptor molecules (Ishii, Kawasaki, Matsumoto, Tochinai, & Seya, 2007). These TLRs included TLR1/2/3/5/6/7/8/9/10/11/12/13/14/15/21/22/23. In a previous study (Roach *et al*., 2005), TLR4 and TLR16 were predicted to exist, but Ishii *et al* did not identify the complete TLR4 contig in the *Xenopus laevis* transcriptome, even though the tlr4 gene was found in the assembly. TLR4 mRNA was also identified in frogs and tadpoles, so Ishii predicted that the TLR4 protein must exist in amphibians. They identified three TLR1/6-like proteins, and hypothesized that these variations may have been increased after diverging from fish.

The next attempt to sequence the TLR repertoire of amphibian species was Babik *et al* (2014), who analyzed the transcriptome of urodele amphibians, specifically *Lissotriton* newts. The TLR repertoire of newts is structurally similar to that of *Xenopus spp.* They found 16 distinct TLR genes in *Lissotriton,* which differs from the results of Ishii *et al* who found 19 TLR genes in *Xenopus.* There were no TLR4 genes identified, however TLR5L was found (Babik *et al*., 2014). The domain structure of newt TLRs is similar to *Xenopus,* but newts contain TLR19, have three transmembrane domains in TLR12, and do not contain a transmembrane domain in TLR22 (Babik *et al*., 2014). These differences in TLR composition are due to lineage-specific gene duplications and losses. All TLR genes were expressed in both the spleen and the liver, however, remarkable differential expression was observed. TLR5, TLR5L, and TLR22 had higher expression in the liver whereas TLR12 and TLR19 had higher expression in the spleen

(Babik *et al*., 2014).

Unlike Ishii *et al*., (2007), Babik *et al*., (2014) analyzed the selection landscaped acting on these TLRs. Overall, they found that TLRs undergo purifying selection with lineage-specific adaptation occurring in some codons. These lineage-specific adaptations in TLRs have been hypothesized to be the backbone of vertebrate TLR dynamics (Babik *et al*., 2014, Tschirren *et al*., 2011, Grueber *et al*., 2014). Differences in selection between viral and non-viral TLRs were not observed in this transcriptome analysis (Babik *et al*., 2014). The strongest mode of positive selection was observed acting on TLR5L, which is consistent with previous findings that TLR5 is usually under positive selection (Wlasiuk *et al*., 2009). This is most likely due to structural changes in bacterial flagellin that help evade an immune response. This study showed that anuran and urodele amphibians have very similar TLR repertoires, however, lineage-specific duplications and adaptation led to changes in the composition between these two species.

The latest and perhaps most accurate amphibian transcriptome analysis of TLRs came from Liu *et al*., which was inclusive of over 90 amphibian species. This analysis also identified 16 distinct TLRs, which includes TLR4. The identification of TLR4 confirms previous predictions that TLR4 does exist in amphibians. According to Liu, amphibian TLR7, TLR8, TLR9, and TLR14 are the most constrained with low percentage of PSSs (Liu *et al*., 2019). Out of all the classes of vertebrates, amphibian TLRs have the lowest mean values of dN/dS, with TLR19 having the highest dN/dS and a relatively low percentage of PSSs. This means that amphibian TLRs are under the most purifying selection out of all vertebrate classes. This is inconsistent with the general consensus that positive selection in TLRs is correlated with complex environments and

pathogens (Roach *et al*., 2005; Liu *et al*., 2019). Since amphibians are both terrestrial and aquatic vertebrates, the ecological environments of these organisms provide complex pathogenic pressures, which should indicate higher site variations and selection in amphibian TLRs. However, Liu *et al* (2019) did not observe high selection acting on amphibian TLRs, and instead, the diversification and expansion of the amphibian TLR repertoire may be a result of complex pathogens rather than site variations. Amphibian TLR2 and TLR1 do have the highest percentage of PSSs, which could perhaps have functional implications in susceptibility to disease (Liu *et al*., 2019).

CHAPTER 2: BIOCHEMICAL FOUNDATIONS OF TLRS

Structure

A prototypical TLR contains three domains: an extracellular domain, a transmembrane domain, and an intracellular domain (Botos *et al*., 2011). The extracellular domain (ECD) of the TLR is involved in ligand recognition. The ECD faces either the extracellular environment or endosomal lumens, depending on if the TLR is displayed on the plasma membrane or endosomes. The intracellular domain of TLRs, also known as the Toll/IL-1R (TIR) domain, is responsible for the intracellular signaling cascade that eventually leads to the expression of innate immunity genes. Lastly, the transmembrane domain is embedded in the membrane and allows for the connectivity of the ECD and TIR domains.

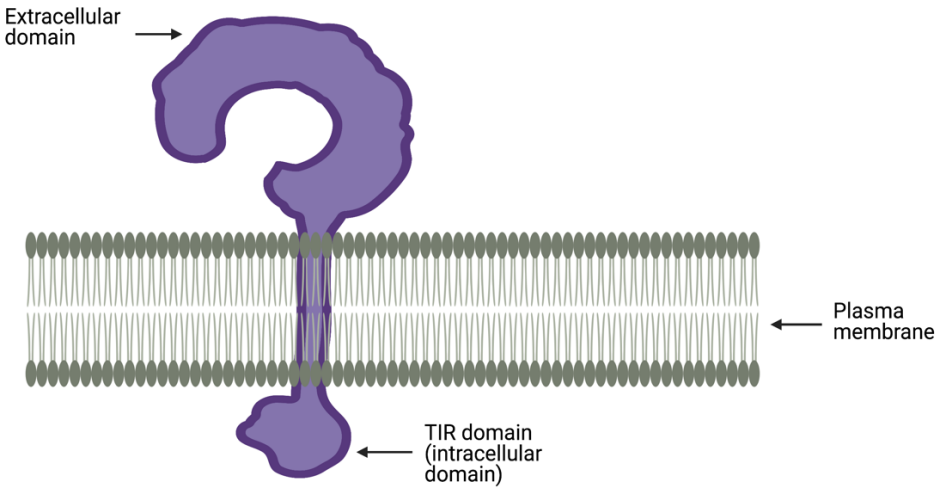

Figure 1. Prototypical TLR Structure

A simplistic view of a prototypical TLR. The extracellular domain recognizes ligands and is displayed on the outer region of the membrane. The TIR domain faces the cytoplasmic side of the cell and initiates downstream signaling. The transmembrane domain associates the two domains.

The Extracellular Domain

The ECD of TLRs is composed of motifs known as Leucine-rich repeats (LRR), which are hydrophobic amino acid sequences of roughly 20-29 residues in length (Botos *et al*., 2011). The ECD is on the N-terminus of the TLR protein and contains about 550-

800 amino acid residues, and the LRRs adopts a loop structure in three-dimensions (Bell *et al*., 2003). When the LRR is assembled into a protein, consecutive LRRs take on a solenoid configuration, where the hydrophobic residues face the interior region to form a stable core and adjacent β-strands align to form a hydrogen-bonded parallel β-sheet (Botos *et al*., 2011). The solenoid structure of the LRR is forced into a curved configuration where the concave surface is formed by the β-sheet (Botos *et al*., 2011). This is because the β-strands are packed much tighter than the non-β portions of the loop. These hydrophobic interactions lead to LRR proteins having a concave surface, convex surface, ascending lateral surface, and a descending lateral surface (on the opposite side of the ascending surface) (Bella *et al*., 2008).

ECD-LRRs of TLRs consist of 19-25 amino acid residues, with the average length being 24 (Botos *et al*., 2011). TLR-ECDs are typically classified as having a "horseshoe" structure, because of the fact that the LRRs do not form a complete circle (Akira & Takeda, 2004; Botos *et al*., 2011). Because there are roughly only 24 residues in the LRR of ECDs, the formation of multi-turn helices on the convex surfaces does not occur. Therefore, the inner-strand distances on the convex sides are relatively short, which give TLR-ECDs a lower curvature and larger exterior diameter than other proteins with LRR motifs (Botos *et al*., 2011). The LRRs of TLRs contain a variety of secondary structures on their convex sides, such as β-strands, 3_{10} helices, and polyproline II helices (Botos *et al*., 2011). TLR-ECD structures are relatively planar compared to other LRR proteins, and this planarity is hypothesized to be important for ligand binding and recognition (Botos *et al*., 2011). The ECD contains structures that cap the N and C-terminal side of this region known as the LRR-NT and LRR-CT. Disulfide bonds link the

LRR-NT amino acids, whereas the LRR-CT are globular and contain two α-helices stabilized by two disulfide bonds (Botos *et al*., 2011). Ligand binding mainly occurs on the ascending lateral surface of the ECD (Jin 2007; Kang 2009; Liu 2008; Park 2009; Botos *et al*., 2011). This surface is free to interact with a ligand because it lacks N-linked glycan.

The Intracellular Domain (TIR) and Transmembrane Domain

The TIR domain faces the cytoplasmic region of the cell when the TLR is expressed on the cell surface. TLRs activate a signaling cascade through their TIR domains. The TIR domain interacts with other TIR-domain-containing adaptor proteins via TIR-TIR interactions, and consequently leads to a cytosolic signaling cascade (Jang & Park, 2014). In all TIR domains, β-strands and α-helices arrange as a central five-stranded parallel β-sheet surrounded by five α-helices (Botos *et al*., 2011). Important to the dimeric surfaces of the TIR domain is the BB-loop that connects strands β-B and α-B, and also contains residues from the DD-loop and the α-C helix (Botos *et al*., 2011). The BB-loop is not only important for TIR dimerization, but it is also available to interact with adaptor molecules during signal transduction, making it important in TLR signaling as well (Botos *et al*., 2011).

The transmembrane domain is a single helix which consists of roughly 20 neutral, hydrophobic residues that run through the membrane (Botos *et al*., 2011). The transmembrane domain helps different TLRs interact with one another through the use of membrane spanning proteins, which direct nucleic acid recognizing TLRs to endocytic

compartments.

There are many different types of TLRs, and each TLR is equipped with different properties that make their crystal structures significantly different. These distinct properties allow some TLRs to carry out specific functions, such as TLR2 being able to dimerize with TLR1 and TLR6, or TLR4 recognizing lipopolysaccharide and fungal ligands. The TIR domain, however, is an evolutionarily conserved region, and in general TLR proteins do not vastly differ in the structure and sequence of their TIR domain.

Ligand-Binding

TLRs recognize ligands with strong specificity due to structural and physiochemical compatibility between the TLR-ECD and PAMP. Each TLR protein recognizes a distinct PAMP due to intrafamily structural and biochemical differences in the ECD. Upon recognition of the ligand, TLRs undergo dimerization with another TLR protein to form homodimers and/or heterodimers (Sun *et al*., 2008). Depending on the TLR type, the product could be a heterodimer with an evolutionarily related TLR, or a homodimer with another protein of the same TLR type. For example, TLR2 forms heterodimers with either TLR1 or TLR6 upon ligand recognition (Jin *et al*., 2007, Kang *et al*., 2009). TLR2 differentially associates with TLR1 or TLR6 to recognize structurally different ligands, which allows for a diverse array of potential pathogens for recognition. Another example is TLR9, which forms homodimers with another TLR9 protein to recognize cytosine-phosphate-guanine (CpG)-DNA (Ishida *et al*., 2018; Wei *et al*., 2009).

Activation of TLRs is dependent on non-covalent, hydrophobic, and electrostatic

interactions between the TLR-ECD and the PAMP of the respective pathogen (Botos *et al*., 2011). TLRs are lined with hydrophobic residues in the LRR that directly bind to recognition sites on PAMPs. Depending on the TLR type, the chemical interaction with the ligand differs. The TLR2-TLR1 heterodimer contains deep hydrophobic pockets that accommodate peptide tails of lipopeptide ligands (Su *et al*., 2019). This allows for compatible non-covalent, protein-protein interactions which further enhance the specificity for that ligand. On the other hand, TLR3 homodimerizes to recognize double-stranded RNA (dsRNA) (Wang *et al*., 2010; Bell *et al*., 2005). The LRRs of TLR3 binds to oligonucleotides of the dsRNA virus by electrostatic interactions between the phosphate backbone and hydrogen-bonding between base pairs (Liu *et al*., 2008). These vast differences in interactions between TLR types and ligands are present in all TLRs, and it is this quality of TLRs that make them essential players in the immune system due to their ability to recognize an incredible array of pathogens.

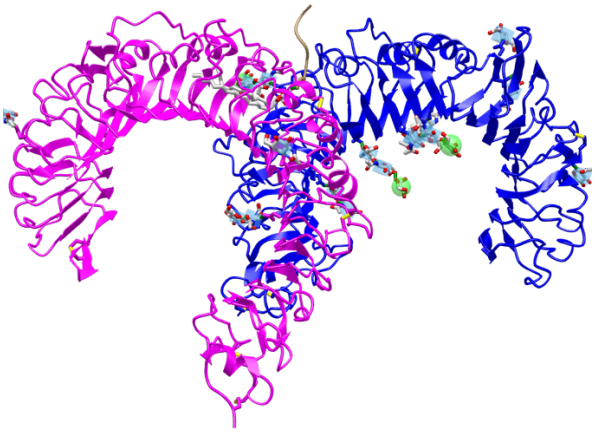

Figure 2. Structure of TLR2-TLR6 Heterodimer

Crystal structure of TLR2-TLR6 heterodimer bound to lipopeptide ligand. Figure taken from ncbi (PDB ID: 3A79, MMDB ID: 78279).

TLR Signaling

Upon TLR activation, the TLR-ECD undergoes conformational changes that causes the TIR domains to be in proximity for initiation of downstream signaling (Akira *et al*., 2004). This downstream signaling involves the recruitment and activation of adaptor proteins, signaling molecules, and molecular complexes in an intracellular signaling cascade. As a result of this cascade, transcription factors NF-kB and IRF-3 translocate to the nucleus for the expression of essential innate immunity genes such as pro-inflammatory cytokines, type I interferons, and co-signaling molecules (Kawai *et al*., 2007). Pro-inflammatory cytokines promote inflammation by activating immune cells to fight against infection as well as produce more cytokines (Charles 2002). Type I interferons are mainly produced as a result of viral infection and are key regulators in

innate and adaptive immunity by activating B and T cells (McNab *et al*., 2015).

Depending on the TLR type, TLRs differentially express immunity genes through the utilization of different signaling pathways. There are two main pathways of TLR signaling: MyD88-dependent and MyD88-independent pathway (Akira *et al*., 2003). The MyD88-dependent pathway recruits Myeloid differentiation primary response (MyD88) adaptor protein to the TIR domain. MyD88-independent pathways do not recruit MyD88 to the TIR domain, but rather the recruitment of other adaptor proteins such as TIRAP, TRAM, and TRIF to the TIR domain. In TLRs studied, the main products in MyD88-dependent pathways are inflammatory cytokines, and in MyD88-independent pathways type I interferons are produced (Akira *et al*., 2003). However, some pathways can produce both products depending on the TLR type. Excess production of inflammatory cytokines causes harmful effect in tissues, so organisms have developed mechanisms to counteract this by negatively regulating TLR signaling. This occurs via inhibitory molecules binding to upstream adaptor proteins (MyD88, TRIF, TRAM, TIRAP) and preventing their association with downstream signaling molecules (Kondo *et al*., 2013).

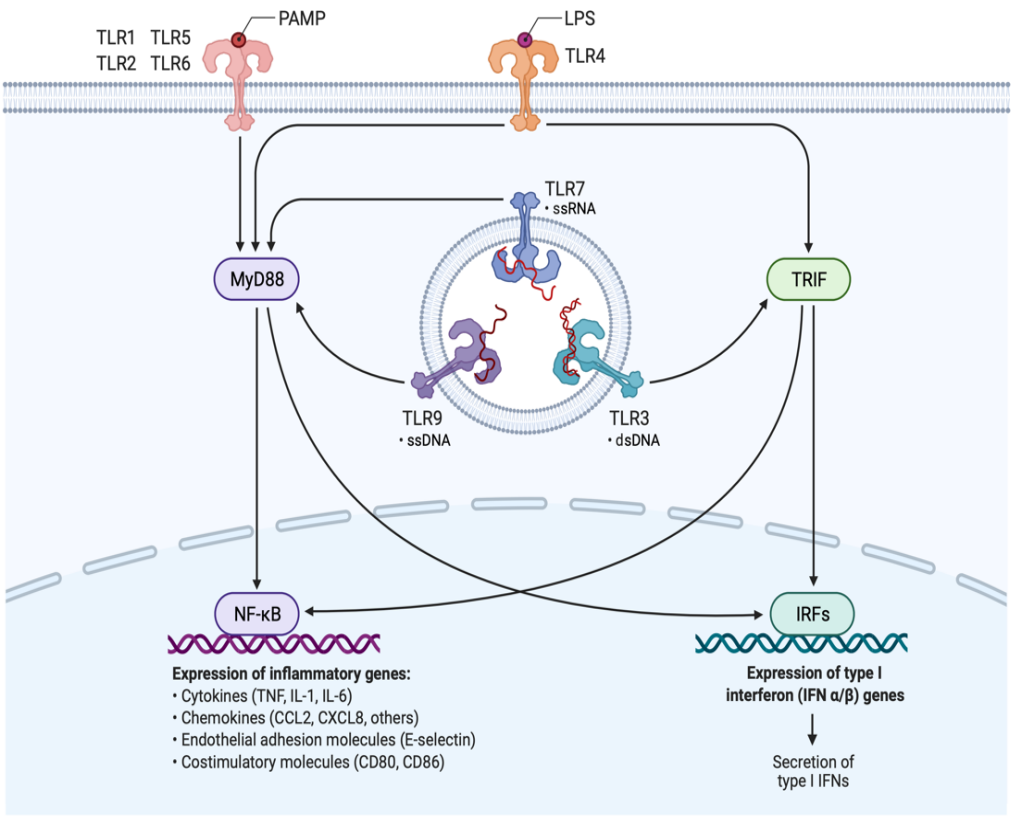

Figure 3. TLR Intracellular Signaling Cascade

This figure illustrates the basic intracellular signaling cascade upon TLR activation. Binding of TLR to PAMPs causes adaptor proteins to signal downstream molecules until the activation of transcription factors NF-kB and IRF3, which express innate immunity genes.

TLR Type	Pathway	Dimerization	Gene product
TLR1	MyD88-dependent	With TLR2	Inflammatory cytokines
TLR2	MyD88-dependent	With TLR1, TLR2, and TLR10, and homodimerization	Inflammatory cytokines
TLR3	MyD88-independent	Homodimerization	Type I interferons
TLR4	MyD88-dependent MyD88-independent	Homodimerization with MD-2	Type I interferons Inflammatory cytokines
TLR5	MyD88-dependent	Homodimerization	Inflammatory cytokines
TLR6	MyD88-dependent	With TLR2	Inflammatory cytokines
TLR7	MyD88-dependent	Homodimerization	Inflammatory cytokines Type I Interferons
TLR8	MyD88-dependent	Homodimerization	Inflammatory cytokines Type I interferons
TLR9	MyD88-dependent	Homodimerization	Inflammatory cytokines Type I interferons

Table 2. TLR Pathways, Dimerization, and Products

Summary of studied TLRs and their known pathways, dimerization, and products (Akira and Takeda, 2004; Jin et al., 2007; Kang et al., 2009; Liu et al., 2008; Park et al., 2009; Yoon et al., 2012)

CHAPTER 3: TLRS IN FUNGAL DISEASE

Function of TLRs Upon Fungal Infection

TLRs are regulators of the immune response by activating the expression of essential innate immunity genes. TLRs also function in mediating the adaptive immune system by activating expression of genes that activate T-cells and TLR co-receptors, which serves as positive feedback for further immune response. There are many differences between TLR recognition of viruses, bacteria, and fungi, with fungi adopting multiple evasion strategies to avoid recognition (Bourgeois & Kuchler, 2012). Many fungi are dimorphic, can undergo morphogenesis, and have cell walls with complex structures that are very resistant to environment stresses including host immune attack (Bourgeois & Kuchler, 2012). To understand the significance of TLRs in the amphibian disease Chytridiomycosis, it is important to understand the function of TLRs in fungal infections. In this section, I provide a review of what is known about TLRs and fungal diseases.

The PRRs that recognize fungal ligands are TLRs, C-type lectin receptor family (Dectin-1/2, SIGNR), CD5/36, and Galectin-3 (Romani 2011). The ligand-binding activation of TLRs to fungal PAMPs are difficult to analyze, as the precise mechanism to how TLRs elicit an immune response to fungi is quite complex. Not only are the cell walls of fungi incredibly diverse, but surface TLRs associate with fungal PAMP-bound co-receptors to initiate downstream signaling. Endosomal TLRs (TLR3/7/9) also recognize intracellular fungal nucleic acids from the engulfment and degradation of fungal pathogens. Upon activation of endosomal TLRs, they activate the expression of

genes that further enhance the binding of surface TLRs to fungal pathogens.

TLRs regulate hematopoietic rates in the body by modulating proliferation and differentiation upon interaction with fungal PAMPs (Boiko and Borghesi *et al*., 2012). It has been shown that cells of the bone marrow lacking TLR4, TLR9, and/or MyD88 display enhanced reconstruction of blood cellular components (Massberg and Von Adrian *et al*., 2009), and that mutations in Drosophilia *Toll* cause deregulation of hematopoiesis (Qui *et al*., 1998). This indicates that TLRs in hematopoietic stem cells (HSCs) participate in maintenance of hematopoiesis and activates hematopoiesis upon microbial infections, which has been shown in *C. albicans* stimulation of TLR2 in mice. This stimulation drove differentiation of HSCs (Basu *et al*., 2000).

TLR2 deficiency has been shown to affect the recruitment of leukocytes (neutrophils, monocytes, dendritic cells) to the primary site of infection, as well as affect the cytotoxicity of neutrophils in *Aspergillus fumigatus* infections (Meier *et al*., 2003; Bellocchio *et al*., 2004). TLR2 deficiency in *Candida albicans* infections has been shown to have no effect on early phagocyte recruitment but greater macrophage recruitment in late-phase of infection (Netea *et al*., 2004), which shows that TLR2 differentially modulates phagocyte recruitment depending on the fungal strain. TLR4 deficiency, however, leads to a dysfunction of neutrophil effectors in both *A. fumigatus* and *C. albicans*, and TLR9 deficiency enhances the function of neutrophils and macrophages (Gasparoto *et al*., 2010; Kasperkovitz *et al*., 2011).

TLRs regulate the adaptive immune response by activating antigen presenting cells (APCs), or by acting as co-receptors for T-cell receptors on T-cells (Jin *et al*., 2012). TLR2 promotes T-reg differentiation in *Paracoccidoides brasiliensis,* and promotes

production of IL-10 in *Candida* infections (Netea *et al*., 2004; Loures *et al*., 2009). TLR6 promotes IL-23 release and a Th17 response (Moreira *et al*., 2011). TLR3-deficient mice have been showed to fail in the activation of CD8+ T-cells following vaccination by *A. fumigatus.*

In epithelial tissue, TLR expression is significantly altered upon fungal infection, which indicates that TLRs are essentially upregulated in fungal infection of mucous membranes. TLR4 has been shown to be essential in fighting fungal invasion in mice (Weindl *et al*., 2007), and TRIF deficient cells are less resistant to *A. fumigatus* (De Luca *et al*., 2010). Overall, TLRs modulate the ability of epithelial cells and immune cells to respond to signals outside of TLR ligands (Bourgeois & Kuchler, 2012).

TLR-Dependent Susceptibility to Fungal Infections

Many studies have identified correlations between TLR/adaptor deficiencies and susceptibility to fungal infections. A study in mice lacking MyD88 were highly susceptible to *C. albicans* (Bellocchio *et al*., 2004). This is due to an impaired fungal clearance as a result of MyD88 deficiency (Bellocchio *et al*., 2004). TRIF deficient and TLR3 deficient mice have been shown to be highly susceptible to pulmonary aspergillosis, but TRIF deficiency has no effect on susceptibility in corneal aspergillosis (Leal *et al*., 2010; De Luca *et al*., 2010; Carvalho *et al*., 2012). These findings indicate that adaptors have distinct functions in TLR signaling.

Mice lacking TLR2 have a lower number of CD4+ and CD25+ cells, which improves fungal clearance in *Candida* infections (Bellocchio *et al*., 2004). The opposite

effect is observed in TLR2-deficient mice upon infection with *P. jirovecii*, which results in an increased fungal burden and higher severity in symptoms (Wang *et al*., 2008). TLR2 deficiency results in an increased susceptibility to intranasal and intraperitoneal cryptococcal infections, but no change was observed in TLR2 deficiency in intratracheal cryptococcus infections (Yauch *et al*., 2004; Nakamura *et al*., 2006). These findings indicate that susceptibility to infection due to TLR2-deficiency depends on the distinct fungal strain as well as the mode of entry.

TLR4 deficiency is similar to TLR2 in that susceptibility depends on the fungal strain and the infection route (Bourgeois & Kuchler, 2012). Lack of TLR4 enhances the response to *Pneumocystis jirovecii* and *Coccidioides posadasii* in mice, but fungal clearance is impaired in *Aspergillus spp.* infections (Ding *et al*., 2005; Awasthi 2010; Leal *et al*., 2010). Interestingly, variability in fungal cell wall glycosylation can affect TLR4 activation. *Candida albicans* mutants lacking O-glycosylation are specifically recognized by TLR4, and consequently elicits an enhanced inflammatory response (Lewis *et al*., 2012).

In TLR3-deficient mice, memory-CD8+ T cells were failed to be activated following *Aspergillus* vaccination (Carvalho *et al*., 2012). This indicates that TLR3 is essential in the activation of the memory cells during *Aspergillus* infection. TLR7 is utilized in *C. albicans* infections as it has been shown that mice lacking TLR7 are more susceptible to systemic infections to this pathogen (Biondo *et al*., 2012).

TLR9 deficiency in mice shows conflicting results. In *Cryptococcus neoformans* infections (both intranasal and intratracheal), TLR9 deficiency results in higher susceptibility (Wang *et al*., 2011; Zhang *et al*., 2010). The opposite effect is observed in

A. fumigatus infections, where TLR9 deficiency results in delayed mortality and improved fungal clearance (Ramaprakash *et al*., 2009; Bellocchio *et al*., 2004). In *C. albicans*, no change in survival and/or fungal clearance is observed in TLR9 deficient mice, however this may be due to concentration dependent TLR9 activation of the fungal load, since lower fungal doses lead to increased susceptibility in *Candida spp*. infections (Miyazato *et al*., 2009; Biondo *et al*., 2012).

Due to the contrasting evidence of the role of TLRs in susceptibility to fungal diseases, the role of TLRs most likely depends on the fungal species, mode of infection, infectious doses, and collaboration between PRRs (Bourgeois & Kuchler, 2012). An explanation for why TLR deficiency leads to either resistance or susceptibility is due to the specific role the TLR plays in the infection model. A certain TLR can impair fungal toxicity, but simultaneously affect host tolerance which would lead to a more severe infection.

TLR Type	Effect of Deficiency	Fungal strain
TLR2	Impaired leukocyte recruitment Greater macrophage recruitment Promotion of T-reg differentiation Promotion of IL-10 induction, lower number of CD4+ and CD25+ cells and impaired fungal clearance Increased susceptibility to intranasal and intraperitoneal infections; no change in intratracheal infections	*A. Fumigatus* *C. albicans* *P. brasiliensis* *Candida spp.* *Cryptococcus spp.*
TLR4	Enhanced immune response Enhanced immune response Impaired fungal clearance	*P. jirovecci* *C. posadasii* *Aspergillus*
TLR3	Failed activation of CD8+ cells	*Aspergillus*

TLR7	Increased susceptibility to systemic infections	*C. albicans*
TLR9	Higher susceptibility in intranasal and intratracheal infections	*C. neoformans*
	Delayed mortality and improved fungal clearance	*A. fumigatus*
	No change	*C. albicans*

Table 3. Function of TLRs in Fungal Infection

Table summarizing effect of host TLR deficiency and fungal infections. These results emphasize the varying functions of TLRs in fungal disease and the dependency on fungal strain and mode of fungal entry. (Bellochio et al., 2004; Wang et al., 2008; Bou

Single-Nucleotide Polymorphisms (SNPs)

In mice, MyD88 deficiency leads to susceptibility to fungal infections, but this is not the case for humans (Bourgeois & Kuchler, 2012). Susceptibility to fungal infections in mice have been studied by the immunosuppression or gene-knockout of TLRs, but research carried out in humans have utilized single nucleotide polymorphisms (SNPs) in TLR genes to identify patterns in susceptibility. TLR1 SNPs have been shown to lead to increased susceptibility to candidemia by affecting the release of inflammatory cytokines (Plantinga *et al*., 2012). The TLR1 Arg80Thr, TRL1 Asn248Ser, and TLR6Ser249Pro SNPs are linked to increased susceptibility to *Aspergillus spp*. (Kesh *et al*., 2005). In TLR3, the +95C/A polymorphism is associated with susceptibility to aspergillosis, and TLR3 L412F SNP leads to impaired TLR3 signaling and increased prevalence of

cutaneous candidiasis (Carvalho *et al*., 2012: Nahum *et al*., 2012). The TLR4 Asp299Gly polymorphism is linked to greater risk of contracting chromic pulmonary aspergillosis (Carvalho *et al*., 2008). Both the TLR4 Asp299Gly and TRL4 Thr399Ile SNPs lead to increased IL-10 release upon *C. albicans* (Van der Graaf *et al*., 2006). On allele C of TLR9 T-1237C SNP, higher susceptibility to allergic bronchopulmonary aspergillosis (ABPA) was identified (Carvalho *et al*., 2008). These findings emphasize the role of SNPs in susceptibility to disease, and selection landscapes across species may serve as good indicators to predict susceptibility and resistance to a fungal disease. Transcriptome and phylogenetic analyses of TLRs across species can help identify SNPs and amino acid substitutions in TLRs, and depending on the results one can predict which group of organisms is considered "at-risk" to a particular pathogen.

Chytridiomycosis

Chytridiomycosis, a fungal disease that affects the skin integrity of amphibians, is linked to global decline in amphibian populations. This disease is caused by the fungal pathogen *Batrachochytrium dendrobatidis (Bd)* (Martel *et al*., 2018). This pathogen is part of the class *Chytridiomycota*, and they infect their host by binding zoospores to the epithelium. This pathogen widely affects populations in central and south America, and is only prevalent in some parts of Australia, Europe, Asia, and Africa (Martel *et al*., 2018). The importance in studying this disease is rooted in conserving biodiversity across amphibian populations. By understanding the mechanisms that are causing the decease of world-wide amphibian populations, scientists can find indicators of susceptible species and can help preserve their populations.

Mechanism and Pathophysiology of *Bd* Infection

The zoospores released by *Bd* are attracted to keratinized epithelium and carbohydrate components of mucus membranes and epidermis (Van Rooji *et al*., 2015). The attached zoospores then develop into cysts which anchor to the skin surface using adhesions proteins, while also utilizing chitin binding molecule (CBM18) to facilitate survival on the host skin (Rosenblum *et al*., 2012; Martel *et al*., 2018). *Bd* further develops endobiotically via germ tubes extending from the cyst. This tube enters the host cytoplasm, which allows *Bd* to introduce genetic material into the host. The distal region of the germ tube produces an intracellular thallus. The thallus then repeats the same mechanisms as above to dig deeper into the epithelium, and the zoospores are free to infect multiple cells (Martel *et al*., 2018).

As a result of *Bd* infection, the epidermal lining is largely disrupted (Martel *et al*., 2018). *Bd* proteases (serine-type proteases and fungalysin metallopeptidases) disrupt host intracellular junctions, which causes damage to skin integrity (Martel *et al*., 2018; Rosenblum *et al*., 2013). This damage affects the osmoregulatory functions of the skin such as electrolyte transport, ion balancing, and hydration. These effects are harmful on other physiological processes such as cardiac electrical conductivity and blood plasma osmolality. *Bd* also suppresses host immune responses (Ellison *et al*., 2014). Physical symptoms of *Bd* infected frogs are hyperkeratosis, disordered epidermal cell layers, erosions, and spongiosis (Martel *et al*., 2018).

Chytridiomycosis Susceptibility

An interesting phenomenon of chytridiomycosis is the variation in susceptibility and resistance across individuals and populations. Some frogs exhibit severe clinical symptoms and high mortality rate, whereas other frogs are essentially unaffected upon infection. The factors affecting this variation can be divided into both extrinsic and intrinsic factors.

Extrinsic host factors are variables such as environmental habitat and behavior (Martel *et al*., 2018). It has been shown that species located in tropical, high-altitude, and wet environments are declining at a faster rate, such as in central and south America (La Marca *et al*., 2005). Endangered species include those of *Atelopus*, Salamanders, and Hylid frogs (La Marca *et al*., 2005; Cheng *et al*., 2011; Fisher *et al*., 2009; Skerratt *et al*., 2016). Resistant frogs include species such as *Xenopus laevis, Lithobates pipiens, Pseudacris regilla,* and *Crinia signifera*. Behavioral factors also increase susceptibility to *Bd* such as contact with contaminated water, thermoregulatory behaviors, and use of retreat sites (Martel *et al*., 2018).

Life stage has been reported as an intrinsic factor affecting susceptibility, with infection increasing with larval development (Smith *et al*., 2007). Other intrinsic factors include nutritional level, stressors, and the immune system (Martel *et al*., 2018). The variation in the immune response may be associated with evolved differences in innate immunity that make certain frogs susceptible to the pathogen.

Role of TLRs in Chytridiomycosis Susceptibility

Evaluating the variation in the efficacy of host immune responses is crucial in understanding how *Bd* differentially affects populations. One potential explanation to the differences in chytridiomycosis susceptibility are differences in host immune responses (Savage and Zumido 2011). However, transcriptomic data on amphibians have been very limited up to date, so the assessment of disease susceptibility regarding transcriptomics in immune systems has not been clearly elucidated. In order to provide an explanation for the variation in susceptibility to chytridiomycosis, there needs to be a clear picture of the evolution of acquired and innate immune responses to *Bd* across species. TLRs are suggested to a play a key role in the defense against *Bd.* Because TLRs recognize a diverse array of PAMPs and link early innate immunity to adaptive immunity, they are a probable regulator in the inflammatory response. Whether or not immunogenetic differences in TLRs between species is correlated to susceptibility to *Bd* is yet to be elucidated.

Ellison *et al* (2014) identified more than 300 differentially expressed genes in both infected and uninfected frogs to *Bd.* Interleukin (IL) genes such as IL-10 and IL-17 had the greatest expression changes between these two groups of frogs (Ellison *et al*. 2014). All IL genes had significantly lower expression in frogs previously exposed to *Bd*, which indicates damage to the immune response after previous infection. This could also indicate that downregulation of the immune response is a by-product of *Bd* reinfection. In addition to IL genes, cytokines (CCL4, CCL19, CXCL10, CXCL14) are highly expressed as a result of infection. Ellison *et al.,* (2014) suggests that skin inflammatory tissue is a major factor in the defense against *Bd,* and modifications to the inflammatory response in

frogs could help scientists understand levels of susceptibility in different species.

In *Atelopus zeteki,* infection results in increased expression of seven TLR genes, the highest being TLR2 and TLR5 (Ellison *et al*., 2014). The increased expression of TLR2 is significant since this receptor has been reported to recognize fungal ligands. TLR2 has also been reported to recognize chitin in association with TLR1(Fuchs *et al*., 2018), which is a significant component of fungal cell walls. Thus, TLR2 and TLR1 may play a role in the initial recognition of *Bd.* The increased expression of TLR5 can be explained because of secondary bacterial infections (Ellison *et al*., 2014). Since TLR5 recognizes bacterial flagellin, it has been suggested that the upregulation of TLR5 is to protect frogs from mortality due to these bacterial infections.

Cause of Variation in Susceptibility to *Bd*

One of the biggest dilemmas in Chytridiomycosis studies is to answer why some frogs are resistant and why some are susceptible. As of now, studies regarding this topic have led to conflicting data, and scientists are merely left with suggestions and hypotheses based off limited experimental data. Rosenblum *et al* analyzed the expression patterns of innate immunity genes of susceptible species upon infection. They concluded that susceptibility to the pathogen is due to a lack of a robust immune response (Rosenblum *et al*., 2009; Rosenblum *et al*., 2012). Experimental data from Ellison *et al* (2014) shows that this is not necessarily true, as *Bd*-infected *Atelopus zeteki* (a highly susceptible species) showed increased expression of all key innate immunity genes. Thus, the explanation to the variation in susceptibility is not simply a weak front-line defense, but quite possibly immuno-specific genetic variations in immune genes across species.

Many of these studies have focused on expression patterns of a single species. Now that it is known that differential expression is not enough to explain susceptibility, other approaches need to be utilized in studying the immune system of amphibians. Species-wide comparative transcriptomics/phylogenetics between resistant and susceptible species can potentially elucidate this variation in susceptibility. Single nucleotide polymorphisms (SNPs) have been linked to susceptibility to a variety of fungal infections in humans. Thus, positive selection occurring at individual codons in amphibians can perhaps lead to susceptibility as well. The opposite effect may be observed as well for genes under purifying selection. Since genes under purifying selection indicate conservation of function, then species containing a higher number of TLR codons under purifying selection may be more resistant.

Lau *et al* (2018) sequenced multiple isolates of TLR2 and TLR4 from resistant Japanese Ranidae frogs. These two TLRs recognize fungal ligands, making them potential players in the *Bd* immune response. They found that these genes are largely under purifying selection, which is indicative of the resistant nature of these frogs. However, they also identified evidence of positive selection occurring at individual codons. These positively selected sites (PSSs) may be indicative of pathogen-mediated adaptive evolution (Lau *et al*., 2018).

It is very likely that by comparing the transcriptome of resistant frogs to the transcriptome of susceptible frogs, there will be differences in the selection landscape between the two groups. Pathogen-mediated selection may be acting on the TLRs of susceptible frogs, resulting in a defect in changes in the TLR sequence that originally elicit an effective response to the pathogen. Even though TLRs are under sequence-wide

purifying selection, these small episodes of positive selection can be located on significant codons involved in TLR ligand-binding, dimerization, or activation, which would result in changes to the amino acid sequence that responds to *Bd*. These changes could affect the inflammatory response to *Bd* and result in severe symptoms and death. The hypothesis is that the TLRs of susceptible species will contain significant codons under positive selection, and resistant species will not have positive selection in these codons and display purifying selection as an indication to conserve the function of recognizing *Bd*.

Up to this point, I have reviewed what is known about vertebrate TLRs. The important aspects of this review are the evolution, classification, and biochemistry of TLRs, as well as the role of TLRs in fungal diseases. Because we are interested in studying TLRs and their significance in resistance to Chytridiomycosis, I dedicated a significant portion of the review to discuss the literature of amphibian TLRs. In this next section, I will outline the process of our conducted study, including the methodology, results, and discussion of our findings. Our aims for this project are to sequence the TLR repertoire of amphibians, as well as provide a selection landscape of all amphibian TLRs. The selection landscape will tell us whether or not there is evidence of sequence-wide positive selection occurring on TLRs across amphibian species, which would confirm the hypothesis that amphibian TLRs are subjected to pathogen-mediated selection. As mentioned previously, this selection could perhaps be indicative of susceptibility to *Bd.* We also aim to provide an evolutionary relationship among amphibian TLRs to depict the trajectory of the TLR family. This is currently the largest attempt to sequence the transcriptome of amphibian TLRs as we were inclusive of over 100 amphibian species.

CHAPTER 4: METHODS, RESULTS, AND DISCUSSION

Methodology

RNA Sequencing, Transcriptome Assembly and Annotation

Samples were obtained by dissection of amphibians into separate organs, as well as from public SRRs in the NCBI-SRA. We did RNA-seq-experiments with 41 species, and data from 35 species were pulled from the NCBI. Specifically, we stored the liver, spleen, gut, skin, eggs and testes of our sample organisms at -20°C. We performed RNA extractions of each tissues using TRIzol protocol, and the RNA samples were sent for Illumina sequencing. We received the complementary-DNA (cDNA) sequences of the RNA transcriptome and the reads were then processed using trimmomatic v0.39. Trimmomatic removes illumina adaptors and low-quality bases from sequencing. Further correction and editing are done by Rcorrector v1.0.4 which corrects the sequencing based on low quality scores. After sequence editing, assemblies are done using RNAspades v3.14.1, transabyss v2.0.1, all at default settings. BUSCO v4.0.1 is used to assess the quality of our assemblies. For the post-assembly process, we used transrate v1.0.3 which creates a consensus transcriptome based off the three assemblies generated. We then use bash script to remove sequences lower than 300 base pairs (bp) from the assembly. This allows us to have two files: one file containing sequences greater than 300 bp (consensus transcriptome) and another file with sequences lower than 300 bp. The assembly with the larger bp are then subjected to cd-hit v4.8.1, which lowers sequence redundancy by removing duplicate reads from the assembly.

For gene annotation, the assemblies are matched against uniprot libraries using ncbi-blast v2.10.0. This takes nucleotide sequences of the assembly and translates it into 6 different reading frames, where it is then compared against protein sequences from the uniprot libraries. Blast n is then used to compare against keg libraries.

Phylogenetic Analysis

The amino acid site numbers discussed in the results section are based on TLRs from the analysis of Liu *et al*., 2020. After annotation of the TLR genes, each gene set was aligned using DECIPHER R-package, and amino acid site number was obtained by referring to the alignment of each gene. We used the phylogeny of all amphibians for the protein evolution analyses, which were derived from the two largest phylogenetic reconstructions of amphibians that provide an associated chronogram and DNA alignments (Pyron 2014; Jetz and Pyron 2018). With these published data, we derived a single chronogram by pruning the tree using "ape" R-package (Paradis, 2012) to contain only Anurans. We then used the resulting chronogram as the backbone for five types of site-based selection analyses in Hyphy v.2.5.0 (Pond *et al*., 2005): FEL, which calculates the rate of synonymous (dS) and non-synonymous (dN) substitutions per site with maximum likelihood (Pond *et al*., 2005); MEME, a mixed-effects maximum likelihood approach to test for episodic selection at individual sites (Murrell *et al*., 2012); FUBAR, a fast, unconstrained Bayesian approximation to infer nonsynonymous and synonymous substitution rates on a per-site basis (Murrell *et al*., 2013); BUSTED, a branch-site unrestricted statistical test for episodic diversification that provides a gene-wide test for

positive selection (Murrell *et al*., 2015) and aBSREL, an adaptive branch-site random effects likelihood approach to test for proportion of sites under positive selection (Smith *et al*; 2015).

Tables were constructed by pooling together the results given from the above programs and matching them with respective TLR subfamilies. Number of codons and structures of the TLRs were predicted using the SMART tool (Liu *et al*., 2019). We were able to predict the number of LRRs as well as the division between extracellular and intracellular domains with this tool.

Results

Our transcriptome analysis revealed 692 unique TLR sequences from 106 species. Of these 692 species, 77 of them were Anurans, 22 were salamanders, and 6 were caecilians. The mean number of TLRs expressed in the skin for Anurans was 11 TLRs (ranged from 6 TLRs to 16 TLRs). For Caudata, the mean number was 8 TLRs (ranged from 6 TLRs to 15 TLRs), and 11 TLRs was the average number for Gymnophiona (ranged from 6 TLRs to 15 TLRs). We found a total of 17 distinct TLR proteins (TLR1/1L/2/2L/3/4/5/5L/7/8/9/12/13/14/19/21/22) across amphibian genera, in which all grouped into 7 subfamilies based on our phylogenetic analyses. These 7 subfamilies are TLR1, TLR3, TLR4, TLR5, TLR7, TLR11, and TLR13. The latest transcriptome analysis of vertebrate TLRs done by Liu *et al*., 2020 found 8 distinct subfamilies with the inclusion of a TLR15 subfamily. However, amphibians have been shown to not express TLR15, which is confirmed based on our results. In the TLR1 subfamily, we identified 5 distinct TLR genes: TLR1, TLR1L, TLR2, TLR2L, and

TLR14. Interestingly, TLR2L has not been previously identified in vertebrates, making it a newly identified TLR gene. Our discovery of this new gene could be due to our inclusion of a greater number and variation of amphibian species. This TLR2L was only identified in Anurans. The TLR3 subfamily only contained the TLR3 gene, and the TLR4 subfamily, similar to TLR3, only contained the TLR4 gene. The TLR5 subfamily contained TLR5 and TLR5L in our analysis. The TLR7 subfamily was comprised of TLR7, TLR8, and TLR9 genes, and the TLR11 subfamily consisted of TLR12 and TLR19. Lastly, the TLR13 subfamily consisted of TLR13, TLR21, and TLR22.

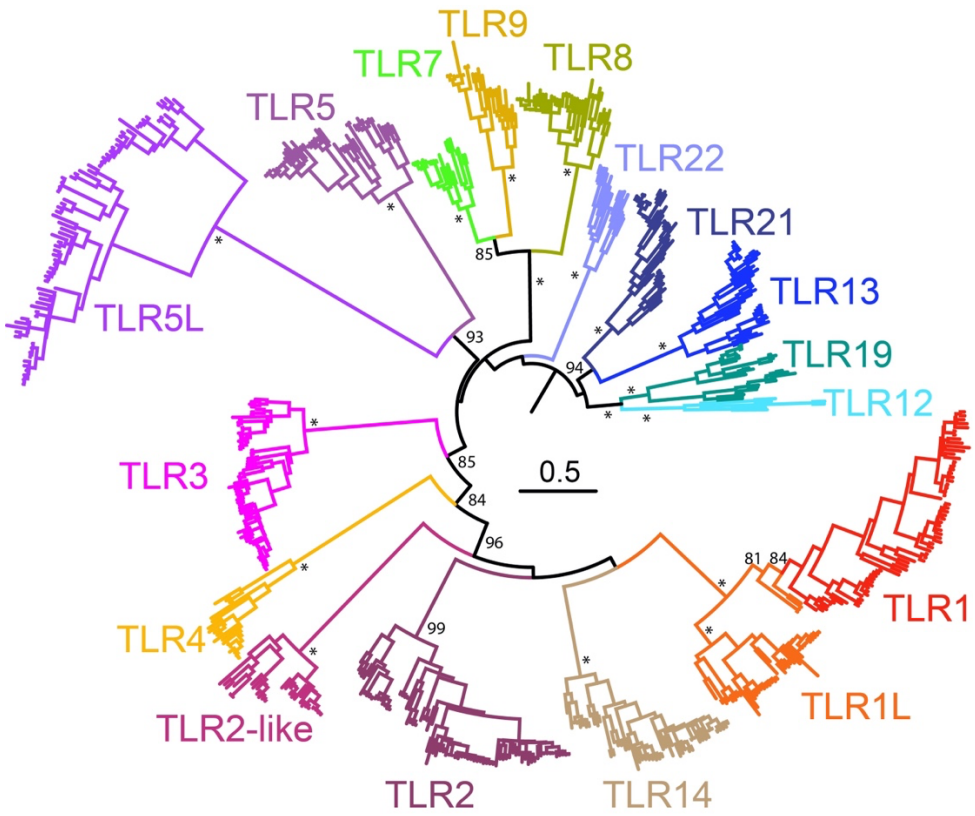

Figure 4. Evolutionary Relationship of TLRs

All TLRs in amphibians and their evolutionary relationship. The TLR2 subfamily in amphibians contains TLR1/TLR1L/TLR2/TLR2LTLR4/TLR14. The TLR3 subfamily only contains TLR3, and the TLR4 subfamily also contains only TLR4. The TLR5 subfamily contains TLR5. And TLR5L. The TLR7 subfamily contains TLR7, TLR8, and TLR9. The TLR11 subfamily consists of TLR12 and TLR19, and the TLR13 subfamily contains TLR13, TLR21, and TLR22

Our selection analysis was done through the computation of 5 programs: aBSREL, BUSTED, FUBAR, MEME, and FEL. aBSREL tells us whether or not positive selection is occurring in TLRs by analyzing the branching between clades. BUSTED tells us if positive selection is occurring as well as the percentage of positively selected sites (%PSSS). FUBAR and MEME finds evidence of positive selection at individual codons and provides the number of sites under positive selection. Lastly, FEL generates number of sites under negative selection. Through the analysis of the data given, we were able to find more positive selection occurring in non-viral TLRs relative to viral TLRs, as well as positive selection occurring in TLRs relevant to *Bd* infection.

aBSREL

In the TLR1 subfamily, aBSREL identified evidence of positive selection occurring in TLR1(4 branches), TLR1L (1 branch), and TLR2 (3 branches). No evidence of positive selection was observed for TLR2L and TLR14. All Anurans had positive selection in TLR1 except *Xenopus laevis/tropicals* and *Bombina variegata.* A couple species of Gymnophiona had positive selection in TLR1 such as *Typhlonectes compressicuada* and *Microaecilia dermatophaga.* In TLR1L, the only species with episodic selection detected was *Xenopus tropicalis.* Many taxa with positive selection in TLR2 were observed by aBSREL. These include *Boana pugnax, Limnodynastes dumerilii, Uperoleia mahinyi, Spea multiplicate, Pelobates cultripes, Xebophrys sangzhiensis, Oreolalax rhodostigmatus,* and *Leptobrachium boringi/leishanese.*

The only species identified by aBSREL with positive selection in TLR3 was *Atelopus ignescens*. In TLR4, aBSREL found that *Dendrobates sirensis/pumilio, Epipedobates*

anthonyi, and *Hyloxalus jacobuspetersi* had episodic selection. TLR5 displayed positive selection in *Scaphiopus couchi, Spea multiplicate, Agalychnis callidryas,* and *Litoria verreauxii.* ABSREL found TLR5L positive selection in node 136.

The TLR7 subfamily displayed the least amount of positive selection according to aBSREL. The only TLR in this subfamily to have episodic positive selection was TLR8. However, only two species had selection in TLR8: *Andrias davidianus,* and *Hynobius retardatus.* ABSREL did not identify any branches under positive selection in either TLR7 or TLR9.

Selection was observed in *Dendrobatidae spp.* and node 31 for TLR19. Interestingly, TLR13 had many taxa with positive selection. Every species had positive selection for TLR13 in our analysis except All species except *Rhinatrema bivittatum, Microacaecilia spp., Geotrypetes seraphini, Andrias davidianus, Hynobius retardatus, Bombina orientalis/variegata, Xenopus tropicalis/laevis, Spea multiplicate, and Leptobrachium leishanense*. TLR21 only had *Andrias davidianus* with positive selection. And lastly, aBSREL did not identify positive selection in any species for TLR22.

BUSTED

BUSTED identified positive selection occurring in TLR1, TLR1L, and TLR2, but no positive selection was observed in TLR2L and TLR14. The highest percentage of positively selected sites (%PSSS) were found in TLR2, with 1.33 %PSSs. TLR1 and TLR1L had 0.98% and 0.2% PSSS respectively. BUSTED found 0.28% PSSs in TLR3, and no selection was identified in TLR4. TLR5, interestingly, had a relatively high

number of PSSs. BUSTED found 7.04 %PSSs in TLR5 with a p-value of 0.0375, and 0.6% PSSs were found in TLR5L.

Although the TLR7 subfamily showed large purifying selection according to aBSREL, BUSTED identified positive selection occurring in all three TLRs. TLR7, TLR8, and TLR9 had 3.31, 3.66, and 3.16% of PSSs, respectively. Similar %PSSs for the TLR7 subfamily indicates the evolutionary similarity of these TLRs. TLR19, part of the TLR11 subfamily, had no indication of positive selection. For the TLR13 subfamily, BUSTED only identified positive selection in TLR22, with 0.95% PSSs. According to BUSTED, the most conserved TLR out of the whole analysis was TLR21, with 0% PSSs.

FUBAR and MEME

In the TLR1 subfamily, FUBAR identified codons under positive selection in all TLR members apart from TLR1. TLR1L, TLR2, TLR2L, and TLR14 had 2, 1, 1, and 2 sites under positive selection, respectively. Interestingly, although FUBAR did not identify selection in TLR1, MEME found 12 individual codons under positive selection in TLR1. For TLR1L/2/2L/14, MEME identified 8, 8, 3, and 7 sites under positive selection, respectively. FUBAR recognized positive selection in 1 codon for TLR3, and MEME identified 12 codons for TLR3. FUBAR did not identify any codons under positive selection in TLR4, and MEME only found 1 site under positive selection in TLR4.

FUBAR identified 3 codons under positive selection in TLR5 and MEME found 8 sites under positive selection. FUBAR did not find any indication of positive selection in

TLR5L, but MEME found 6 sites under positive selection. For the TLR7 subfamily, FUBAR results display the evolutionary conservation of this subfamily. The only TLR member of this subfamily to show evidence of positive selection was TLR8, with 8 sites under positive selection. This is consistent with aBSREL results which found TLR8 to be the only TLR member in this subfamily with positive selection. MEME, however, found PSSs in TLR7, TLR8, and TLR9, with 5, 5, and 2 sites respectively. The only TLR with PSSs observed by FUBAR in the TLR11 and TLR13 subfamilies was TLR21. TLR21 had 2 sites displaying positive section. TLR13, TLR19, and TLR22 had no PSSs according to FUBAR. MEME did not find any PSSs in TLR19, but found 6, 7, and 7 PSSs in TLR13, TLR21, and TLR22 respectively.

FEL

FEL gave us the number of codons under negative selection in both the extracellular and intracellular domains. TLR1 had 190 sites in the extracellular domain (ECD) and 121 sites in the intracellular region under negative selection. The following members of the TLR1 subfamily also contained the following sites under negative selection: TLR1L (154 ECD sites, 93 intracellular sites), TLR2 (232 ECD sites, 119 intracellular sites), TLR2L (97 ECD sites, 9 intracellular sites, and TLR14 (279 ECD sites, 142 intracellular sites). FEL found 265 ECD sites and 95 intracellular sites under negative selection in TLR3, and 42 ECD sites and 41 intracellular sites in TLR4. TLR5 had 150 ECD sites and 109 intracellular sites under negative selection, and TLR5L had 279 ECD sites only since TLR5L does not contain any intracellular domains.

The following members in the TLR7 subfamily contain negatively selected sites:

TLR7 (322 ECD sites, 65 intracellular sites), TLR8 (182 ECD sites, 98 intracellular sites), and TLR9 (266 ECD sites, 73 intracellular sites). In the TLR13 subfamily, these TLRs had negatively selected sites in the following regions: TLR13 (176 ECD sites, 81 intracellular sites), TLR21 (117 ECD sites, 120 intracellular sites), and TLR22 (223 ECD sites, 97 intracellular sites). Keep in mind that although the extracellular domains have generally more negatively selected sites than the intracellular domains, this does not indicate that the ECD is a more conserved region, but rather that the ECD contains more codons relative to the intracellular region of the TLR protein.

	TLR1	TLR1L	TLR2
Total species	67	44	80
Taxonomic Orders	All	All	All
N Codons	778-829	773-827	770-800
aBSREL (branches with + selection)	YES	YES	YES
aBSREL (N branches)	4	1	3
aBSREL (Taxa with evidence)	All anurans except *Xenopus* and *Bombina*; *Typhlonectes compressicuada*, *Microaecilia dermatophaga*	*Xenopus tropicalis*	*Boana pugnax*, node 109, *Limnodynastes dumerilii*, *Uperoleia mahinyi*, node 53, *Spea multiplicate*, *Pelobates cultripes*, *Xebophrys sangzhiensis*, *Oreolalax rhodostigmatus*, *Leptobrachium boringi/leishanese*
BUSTED (alignment-wise with + selection)	YES	YES	YES
BUSTED P-value	P = 0.0001	P = 0.002	P < 0.0001
BUSTED % of sites with dN/dS > 1	0.98	0.2	1.33
FUBAR (N sites with + selection; PP > 0.91	0	2	1
MEME (N sites with + selection; p <0.01	12	8	8
FEL (N sites with w < 1 in extracellular	190	154	232

domain			
FEL (N sites with w < 1 in intracellular domains	121	93	119

Table 4. TLR1 Subfamily Selection Landscape

Summary of selection landscape in TLR1 subfamily members (TLR1, TLR1L, and TLR2). The table above shows results for aBSREL, BUSTED, FUBAR, MEME, and FEL. (dN/dS > 1 = positive selection)

	TLR2L	TLR14
Total species	29	50
Taxonomic orders	Anura	All
N Codons	459-532	784-854
aBSREL (for branches with + selection)	NO	NO
aBSREL (N branches)	None	None
aBSREL (Taxa with evidence)	None	None
BUSTED (alignment-wise with + selection)	YES	YES
BUSTED P-value	P = 0.0001	P = 0.002
BUSTED % of sites with dN/dS > 1	0.98	0.2
FUBAR (N sites with + selection, PP > 0.91)	0	2
MEME (N sites with + selection; p < 0.01	12	8
FEL (N sites with w < 1 in extracellular	190	154

domain)		
FEL (N sites with w < 1 in intracellular domains)	121	93

Table 5. TLR1 Subfamily Selection Landscape Continued

Summary of selection landscape in TLR1 subfamily members (TLR2L and TLR14). The table above shows results for aBSREL, BUSTED, FUBAR, MEME, and FEL. (dN/dS > 1 = positive selection)

	TLR3	TLR4
Total species	66	18
Taxonomic orders	All	Anura
N Codons	896-926	796-872
aBSREL (for branches with + selection)	YES	YES
aBSREL (N branches)	1	1
aBSREL (Taxa with evidence)	*Atelopus ignescens*	*Dendrobates sirensis/pumilio, Epipedobates anthonyi, Hyloxalus jacobuspetersi*
BUSTED (alignment-wise with + selection)	YES	NO
BUSTED P-value	$P < 0.0001$	0.1125
BUSTED % of sites with $dN/dS > 1$	0.28	0.51
FUBAR (N sites with + selection; $PP > 0.91$)	1	0
MEME (N sites with + selection; $p < 0.01$)	12	1
FEL (N sites with $w < 1$ in extracellular domain)	265	42
FEL (N sites with $w < 1$ in intracellular domains)	95	41

Table 6. TLR3 and TLR4 Selection Landscape

Summary of selection landscape in the TLR3 and TLR4 subfamilies. The table above shows results for aBSREL, BUSTED, FUBAR, MEME, and FEL.

	TLR5	TLR5L
Total species	45	74
Taxonomic orders	All	All
N Codons	845-891	624-699
aBSREL (for branches with + selection)	YES	YES
aBSREL (N branches)	2	2
aBSREL (Taxa with evidence)	*Scaphiopus couchi, Sea multiplicate, Agalychnis calidryas, Litoria verreauxii*	Node 136
BUSTED (alignment-wise with + selection)	YES	YES
BUSTED P-value	0.0375	$P < 0.0001$
BUSTED % of sites with $dN/dS > 1$	7.04	0.6
FUBAR (N sites with + selection; $PP > 0.91$)	3	0
MEME (N sites with + selection; $p < 0.01$)	8	6
FEL (N sites with $w < 1$ in extracellular domain)	150	279
FEL (N sites with $w < 1$ in intracellular domains)	109	-

Table 7. TLR5 Subfamily Selection Landscape

Summary of selection landscape in the TLR5 subfamily. The table above shows results for aBSREL, BUSTED, FUBAR, MEME, and FEL.

	TLR7	TLR8	TLR9
Total species	22	35	22
Taxonomic orders	All	All	All
N Codons	1,025-1,109	1,026-1,094	1,010-1,045
aBSREL (for branches with + selection)	NO	YES	NO
aBSREL (N branches)	None	1	0
aBSREL (Taxa with evidence)	None	*Andrias davidianu*, *Hynobius retardatus*	None
BUSTED (alignment-wise with + selection)	YES	YES	YES
BUSTED P-value	P = 0.0071	P = 0.0158	P = 0.0438
BUSTED % of sites with dN/dS > 1	3.31	3.66	3.16
FUBAR (N sites with + selection; PP > 0.91)	0	8	0
MEME (N sites with + selection; p < 0.01)	5	5	2
FEL (N sites with w < 1 in extracellular domain)	322	182	266
FEL (N sites with w < 1 in intracellular domains)	65	98	73

Table 8. TLR7 Subfamily Selection Landscape

Summary of selection landscape in the TLR7 subfamily. The table above shows results for aBSREL, BUSTED, FUBAR, MEME, and FEL.

	TLR12	TLR19
Total species	Only found in 12 taxa	23
Taxonomic orders	-	All
N Codons	894-954	937-959
aBSREL (for branches with + selection)	-	YES
aBSREL (N branches)	-	1
aBSREL (Taxa with evidence)	-	Node 31, *Dendrobatidae spp.*
BUSTED (alignment-wise with + selection)	-	NO
BUSTED P-value	-	P = 0.4813
BUSTED % of sites with dN/dS > 1	-	14.08
FUBAR (N sites with + selection; PP > 0.91)	-	0
MEME (N sites with + selection; p < 0.01)	-	0
FEL (N sites with w < 1 in extracellular domain)	-	10
FEL (N sites with w < 1 in intracellular domains)	-	62

Table 9. TLR11 Subfamily Selection Landscape

Summary of selection landscape in the TLR11 subfamily. Most analyses failed for TLR12. The table above shows results for aBSREL, BUSTED, FUBAR, MEME, and FEL.

	TLR13	TLR21	TLR22
Total species	43	46	20
Taxonomic orders	All	All	All
N Codons	901-968	890-969	928
aBSREL (for branches with + selection)	YES	YES	NO
aBSREL (N branches)	3	1	None
aBSREL (Taxa with evidence)	All species except *Rhinatrema bivittatum, Microacaecilia spp., Geotrypetes seraphini, Andrias davidianus, Hynobius retardatus, Bombina orientalis/variegata, Xenopus tropicalis/laevis, Spea multiplicata,* and Leptobrachium leishanense	*Andrias davidianus*	None
BUSTED (alignment-wise with + selection)	NO	NO	YES
BUSTED P-value	P = 0.1590	P > 0.5000	P = 0.0157
BUSTED % of sites with dN/dS > 1	14.97	0	0.95
FUBAR (N sites with + selection; PP > 0.91)	0	2	0
MEME (N sites with + selection; p < 0.01)	6	7	7
FEL (N sites with w < 1 in extracellular domain)	176	117	223
FEL (N sites with w < 1 in intracellular domains)	81	120	97

Table 10. TLR13 Subfamily Selection Landscape

Summary of selection landscape in the TLR13 subfamily. The table above shows results for aBSREL, BUSTED, FUBAR, MEME, and FEL.

Discussion

One of our biggest findings from this project was the identification of TLR2-like (TLR2L), a gene found only in amphibian species. Our transcriptome analysis was similar to Liu *et al* (2019) transcriptome analysis of the amphibian TLR repertoire, in which Liu *et al* (2019) identified 16 distinct TLR genes that grouped into 7 subfamilies. Our inclusion of a higher number and greater diversity of amphibian species could account for this discovery. TLR2L also differed drastically in the number of codons relative to TLR2, with TLR2L having ~500 codons and TLR2 having ~800 codons. The SMART tool was not able to identify a TIR domain, but the extracellular and transmembrane domains were present. Since the TIR domain was not present in TLR2L, it is likely that this receptor cannot initiate downstream signaling and perhaps serves as a co-receptor for other TLRs since it contains regions for ligand-binding and membrane-spanning. We do not know the exact reason and mechanism to the emergence of TLR2L, but given that gene duplication is common in vertebrate TLRs, TLR2L likely emerged via the same manner. TLR1L has also been identified in a previous transcriptome analysis (Liu *et al*., 2019) and only belongs to amphibians. It is interesting to note that both TLR2L and TLR1L are only present in amphibians, which could indicate heavy pathogenic pressures acting on the TLR2 and TLR1 heterodimer. This is because pathogen-mediated evolution of PRRs can cause respective amino acid sequences to change, leading to redundancy of the original sequence and newly emerged proteins. It is possible that such pressures are acting on amphibian TLR1 and TLR2 which have led to these duplications.

As expected, we also found that most positively selected sites are located in the

extracellular domain. This is consistent with the literature which suggests that evolutionary rates of TLRs are dictated by their ligand-recognition regions. Positive selection in the ECD can occur in the following ways. Firstly, the TLR-ECD changes to be able recognize a diverse array of ligands. This is illustrated by the vast differences in LRR modules, 3D conformation, and non-covalent interactions of the ECD among TLR proteins. Because of these changes, TLR repertoire expansion occurs in vertebrates to allow recognition for a variety of pathogens. The other way the ECD can evolve is through evasion mechanisms and mutations of pathogens. These pathogens undergo changes in their recognition sites in order to avoid recognition of PRRs. As a result, TLRs undergo similar changes in their amino acid composition in order to counteract these evasion mechanisms, leading to this "co-evolutionary" arms-race between pathogens and TLRs. The TIR and transmembrane domains, however, have no necessity to undergo non-synonymous mutations and consequential amino acid changes, since their function in downstream signaling is important for eliciting a proper immune response regardless of pathogen evolution. This is illustrated by the lack of positively selected sites that we found in these intracellular domains.

We also found that the selection landscape differed between viral and non-viral TLRs. The viral TLRs in this study were TLR3/7/8/9/19/22 and the non-viral TLRs were TLR1/1L/2/2L/4/5/13. This is consistent with previous selection analyses of vertebrate TLRs, however, there remains to be some controversy of whether or not a discrepancy exists between these two groups of TLRs. The general consensus of most studies is that non-viral TLRs are under greater positive selection due to the pressure of diverse ligand recognition. Because of this many non-viral TLRs bind to similar ligands, which means

that the accumulation of non-synonymous mutations in one of these TLRs are relatively tolerated as another TLR can bind to that same ligand. This leads to a more redundant TLR-ligand specificity compared to viral TLRs, as viral ligands are more often than not recognized by one specific TLR. Another reason why viral TLRs are more conserved than non-viral TLRs is because viruses display less complex PAMPs, which leads to conservation of the ECD to recognize the viral sequence. However, Areal *et al*., 2011 analyzed the selection landscape in mammalian TLRs and found similar positive selection occurring in both viral and non-viral TLRs. The authors explained this by suggesting that mutation rates of DNA and RNA are faster than those of bacteria and yeast. Because of this, viral TLRs need to undergo changes in their ECD to keep up with viral mutations that change their recognition sequence. The dichotomy of whether or not a selection landscape exists between these two groups in all vertebrates is unclear. Our results suggests that amphibian non-viral TLRs are under more positive selection than their viral TLR counterparts, and this phenomenon may pertain to vertebrate classes rather than vertebrates as a whole.

Interestingly, both TLR14 and TLR21 had very similar selection analyses. BUSTED identified 0% PSSs, and FUBAR and MEME identified 2 sites and 7 sites under positive selection, respectively, in both of these TLRs. These TLR members are not part of the same subfamily, but they are the only "hybrid" TLRs present in amphibians. The fact that they recognize both viral and bacterial components could be indicative of conservation of function, which is illustrated by their similar selection analyses. It is also interesting to note that amphibians contain two out of the three "hybrid" TLRs that exist in vertebrates. The fact that Amphibians contain TLRs that simultaneously recognize

viral and bacterial components illustrates the complex pathogenic environments of Amphibians, and also correlates environmental pressures with TLR expansion.

Based on our results and of Liu *et al* (2019), both TLR1L and TLR2L have only been identified in amphibians. This could indicate significance in amphibian TLR1 and TLR2. The TLR-like proteins (TLR1L, TLR2L, TLR5L, TLR21L) emerged as a result of gene duplication, and the fact that TLR1L and TLR2L are only present in amphibians could suggest paralogous specialization of these proteins in amphibian species. The function of TLR1L and TLR2L has not been clearly studied but being that the TLR1-TLR2 heterodimer recognizes chitin, a polysaccharide component in the cell walls of *Bd*, then TLR1L and TLR2L could either have some role in *Bd* defense or be a product of duplication as a result of pathogenic pressure of amphibian TLR2 and TLR1. The specific pathogens that are creating these pressures is not known, but considering that *Bd* is a potential ligand to TLR1 and TLR2, *Bd* could be causing changes in these TLRs of susceptible species that result in higher ω values and a less effective immune response. Further studies would need to be carried out to confirm whether the amphibian TLR1 and TLR2 heterodimer recognizes *Bd*.

We found that TLR5, another potential TLR in the *Bd* immune response, showed high instances of positive selection. Notably, TLR5L exists in amphibians, which also indicates the selective pressures acting on TLR5. Since TLR5L is also present in reptiles, and TLR5S is present in birds, TLR5L did not arise as a result of speciation in amphibians but rather as a result of gene duplication in TLR5 somewhere along the evolutionary tree between fishes, amphibians, and reptiles (Liu *et al*., 2019). TLR5 is predicted to be involved in the delayed mortality in Chytridiomycosis (Ellison *et al*.,

2014). This is because TLR5 is upregulated as a result of *Bd* infection, and since it recognizes bacterial flagellin, TLR5 likely defends against secondary bacterial infections in *Bd* infected frogs. The presence of TLR5L in amphibians could perhaps be significant to *Bd* defense, but the conclusion cannot be made until further studies elaborate on the function of both amphibian TLR5 and TLR5L.

Given the evidence of previous studies showing upregulation of TLRs in response to *Bd* infection, and the role of TLRs in fungal infection and clearance, it is highly likely that TLRs, specifically TLR1/2/5, are involved in Chytridiomycosis defense. The deeper question is whether episodic positive selection in the TLR-ECD (in TLRs involved in *Bd* defense) is responsible for the dichotomy between *Bd*-resistant and *Bd*-susceptible amphibian species. Amino acid changes, particularly in codons within close proximity to binding regions and/or dimerization interfaces, that are associated with positive selection can lead to a defect in the ability of TLRs to bind to *Bd* or form dimers. This has been shown in human research, where single-nucleotide polymorphisms (SNPs) in TLRs lead to susceptibility to a variety of fungal infections. SNPs change the original TLR sequence that recognizes the pathogen, and this results in failure of TLR activation and a consequential weak immune response.

The problem with amphibians is that no crystal structure of amphibian TLRs or TLRs bound to *Bd* exist. So even though we provided a selection landscape and individual codons under positive selection, we do not know the implications of a certain codon under positive selection because we do not know the exact ligand-binding regions in the amphibian ECD, which would not allow us to correlate the significance of amino acid substitutions at a particular site and defective activation of the TLR. We also do not

have enough data regarding amphibian species and susceptibility/resistance to chytridiomycosis, which hinders our ability to associate patterns of amino acid substitutions with susceptible species. What we were able to show, however, was that amphibian TLRs, particularly those that are potential players in *Bd* defense, do show instances of positive selection in the extracellular domain. This leaves the hypothesis open for future researchers that changes in the ligand-binding regions of TLRs in susceptible species lead to either a failed recognition of *Bd* or reduced recognition of secondary bacterial pathogens, which causes higher frequency of death in amphibian populations compared to resistant populations. Of course, in order to answer these questions more problems need to be elucidated such as crystal structures of amphibian TLRs, differential expression analyses during infection, and more data regarding resistant and susceptible species to Chytridiomycosis.

Conclusion

In this study, we provided a review of what is known about vertebrate TLRs, sequenced the transcriptome repertoire of amphibian TLRs and classified them according to their evolutionary similarities. We discovered a new TLR protein (TLR2-like), which totaled to 17 distinct TLRs which grouped together in 7 TLR subfamilies. We provided the selection landscapes of each amphibian TLR, which revealed higher positive selection occurring in the extracellular domain relative to the intracellular domain, and also found higher positive selection occurring in non-viral TLRs than viral TLRs. So far, this has been the largest attempt to sequence the transcriptome repertoire of amphibian TLRs, as

well as the largest selection analysis of amphibian TLRs.

Perhaps most importantly, we confirmed positive selection across sites in amphibian TLRs, particularly those likely involved in chytridiomycosis defense. Confirming this leaves open the possibility that polymorphisms in TLRs are causing species of amphibians to be susceptible. Taking this into consideration, our work provides framework for future research regarding amphibian TLRs and chytridiomycosis resistance.

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