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Sequencing and functional characterization of a Latrodectus geometricus defensin, Lg-defensin

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By

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Sequencing and functional characterization of a *Latrodectus geometricus* defensin, Lg-defensin

By

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Thesis Abstract

Defensins are small, cysteine-rich, immune related proteins described to be the most widespread family of invertebrate antimicrobial peptides. Presently, there is limited information regarding spider defensins. In this study, a new defensin peptide was described in *Latrodectus geometricus*, Lg-defensin. The full open reading frame was resolved using RACE and *de novo* assembly, followed by a bioinformatical analysis and phylogenetic alignment to determine conserved sequence patterns and structural similarities to other defensins. Next, the functionality of Lg-defensin was determined using *in vitro* RNA interference to assess what impact silencing had on the spider's ability to control a gram-positive and gram-negative bacterial infection, whose level was determined via qPCR analysis. Results indicated Lg-defensin to be a highly conserved defensin with similar structure and sequence patterns to other related taxa. Additionally, Lg-defensin was found to have higher antibacterial activity towards the gram-positive bacteria in contrast to its effect on gram-negative bacteria. This is the first report regarding the completed characterization of a defensin peptide in *L. geometricus* and provides a basis for future research regarding Lg-defensin and its clinically relevant capabilities.

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LITERATURE REVIEW

Introduction

While there are many ways in which organisms fight off invading microbes, there is one that has sparked great interest for the scientific community over the past decade: the synthesis of antimicrobial peptides. One of the main motives for researching these immune-related proteins is the increasing concern in protecting the human population from antibiotic-resistant bacteria. Due to the overuse and misuse of antibiotics, resistance from both gram-positive and gram-negative bacteria has led to a drastic reduction in the antibacterial action achieved by antibiotics (Sierra et al., 2017). In fact, the Center for Disease Control and Prevention (CDC) reported that ~2.8 million people become infected, and more than 35,000 people die, from antibiotic-resistant bacteria in the United States each year, and it is predicted the numbers will continue to rise (CDC, 2019). Therefore, the discovery of new antimicrobial agents, less likely to promote more microbial resistance, are necessary to prevent future catastrophes.

Current research shows that naturally synthesized antimicrobial peptides can be effective alternatives to antibiotics (Lei et al., 2019; Dijksteel et al., 2021; Moretta et al., 2021). Antimicrobial peptides (AMPs) are small proteins that play an important role in the innate immune response for vast number of organisms (Huan et al., 2020). These AMPs can be characterized in several ways including the organism

synthesizing them, their secondary structure, their mode of action, how often they are synthesized, and the location where they are synthesized within the organism (Erdem Büyükkiraz and Kesmen, 2022). Generally, the main functional role of AMPs is to disrupt and permeabilize foreign microbial membranes (Moretta et al., 2021). This interference is made possible due to the AMPs amphipathic structure; meaning they have cationic and hydrophobic faces, permitting the AMP to interact with the negatively charged bacterial cell membranes allowing for complete permeabilization into the microbe (Bahar and Ren, 2013). As compared to traditional antibiotics, this unique ability makes it difficult for bacteria to build resistance towards AMPs (Boparai and Sharma, 2020).

Different ways in which AMPs target the bacterial cell membrane have been described through four known models: the "aggregate" model, the "toroidal" pore model, the "barrel-stave" model, and the "carpet" model (Huan et al., 2020; Erdem Büyükkiraz and Kesmen, 2022). The "aggregate" model is the AMPs ability to insert itself within the membrane through reorientation and form a sphere-like structure to disrupt the membrane's integrity. The "toroidal" pore model describes how an AMP penetrates the membrane by interacting with the lipid bilayer through its hydrophobic regions, perpendicularly, while the hydrophilic regions face the pores. The "barrel-stave" model is the AMPs ability to form staves parallel to the membrane followed by the creation of barrels that are inserted within the membrane perpendicularly. Lastly, the "carpet" model is the peptide's ability to coat the membrane in a specific area and penetrate the bilayer,

creating a pore and causing permeation (Huan et al., 2020; Erdem Büyükkiraz and Kesmen, 2022). However, not all AMPs are strictly antibacterial. In fact, many of these peptides have also been found to possess antifungal, antiviral, antiparasitic, and even antitumoral properties (Bahar and Ren, 2013; Moretta et al., 2021). Moreover, some AMPs have also been found to do more than just disrupt microbial membranes including inhibiting macromolecular synthesis, translocating intracellular peptides, and even inhibiting DNA/RNA/protein synthesis depending on the AMP and the organism (Huan et al., 2020; Erdem Büyükkiraz and Kesmen, 2022).

With over 5,000 naturally occurring AMPs described, these specialized proteins have endured throughout the evolution of life, with a vast number having been characterized in invertebrates (DRAMP.org, 2019; Lei et al. 2019; Manniello et al., 2021). Although, out of the many invertebrates noted in databases such as the Data Repository of Antimicrobial Peptides (DRAMP.org, 2019) discoveries from order Araneae seems few and far between. While this is not ideal, the AMPs that have been described in spiders have promising future clinical applications, demonstrating that its highly plausible for other species of Araneae to have the potential in harboring other AMPs with therapeutic utilization (Santos et al., 2010; Ayroza et al., 2012; Rossi et al., 2012; Tan et al., 2013; Troeira Henriques et al., 2017; Reis et al., 2018; Abdel-Salam et al., 2019; Wadhwani et al., 2021). In addition, new AMP discovery in different species could also greatly contribute to broadening researchers' understanding of the spider immune system.

Study Species: Latrodectus geometricus

One such group in the order Araneae, already known for harboring many toxins and molecules with therapeutic potential, is the genus *Latrodectus*. This genus resides in the family Theridiidae, which makes up the comb-footed or cob weaver spiders (Hannum and Miller, 2010). Currently, there are 32 known species of *Latrodectus* around the world (World Spider Catalog, 2021), with five located within the United States: *Latrodectus bishopi* or the red widow spider, *Latrodectus hesperus* or the western black widow spider, *Latrodectus variolus* or the northern black widow spider, *Latrodectus mactans* or the southern black widow spider, and *Latrodectus geometricus* or the brown widow spider (Hannum and Miller, 2010). Of all these species, *L. geometricus* is one of the most established widows in the United States (Vincent et al., 2008). Due to their prevalent abundance in the East Texas area, medical significance, and potential for harboring AMPs with the therapeutic application, *L. geometricus* is an ideal candidate for new AMP discovery.

Population Distribution of L. geometricus

L. geometricus was first described in Colombia by C.L. Koch in 1841, which had lead researchers to believe that the species originated within South America; however, it is also suggested to have originated in Africa due to the wide distribution and closest sister species having been found there (Garb et al., 2004). Throughout the centuries it has also been found in other areas of the world including India, Japan, Australia, Turkey,

Indonesia, Israel, and Papua New Guinea (Simo et al., 2013). In the United States, *L. geometricus* was first described in Florida in 1936 in the peninsular portion of the state (Pearson, 1936). However, within a decade of the 21st century, *L. geometricus* has since expanded from Florida all the way across the southern border to southern parts of California, and it is possible the species has continued to spread North (Schraft et al., 2021). In contrast to black widows, *L. geometricus* is known for commonly residing in more urbanized areas such as in the corners of buildings and homes and is hypothesized to be an invasive species in the United States (Vincent et al., 2008; Hannum and Miller, 2010; Vetter et al., 2012). While its expansion is prodigious, the most fascinating trait of this species, and more specifically this genus, is the venom that they possess.

Medical Significance of L. geometricus

Commonly, when the general population thinks of widow spiders, the first thing to come to mind is their venomous nature, which can cause symptoms frequently referred to as latrodectism (CDC.org, 2018; Khamtorn et al., 2020). While this venom can be toxic, it is also a source of many biologically active compounds capable of having great therapeutic potential (Reyes-Lugo et al., 2009). For example, α-latrotoxin (α-LTX), the most well studied latrotoxin and the main cause of latrodectism, was successfully given as a therapeutic agent in treating botulinum poisoning, which ultimately can cause neuromuscular paralysis if left untreated (Yan and Wang, 2015). In addition, since α-LTX is specific in targeting vertebrate neurotransmitters, it has been found to be a useful

tool in studying the molecular mechanisms involved in regulating neurotransmitter release (Ushkaryov et al., 2004). In addition, insect-specific latrotoxins have also shown promising applications for being potent, highly specific insecticides to many different pests (Rivera de Torre et al., 2019; Khamtorn et al., 2020). Aside from the well described toxins, while it is known that spiders do have AMPs, one of the least studied components of *Latrodectus* venom are the possible circulating AMPs within it (Kuhn-Nentwig & Nentwig, 2013).

Khamtorn et al. (2020) sought to describe the numerous bioactive components that make up the *L. geometricus* venom. While there were a wide range of proteins and molecules, a discovery that stood out was the observation of AMP activity. In fact, the venom exhibited activity against gram-positive *Bacillus subtilis* and not the gram-negative *Pseudomonas aeruginosa,* but no isolated component behind the activity was resolved (Khamtorn et al., 2020). Other studies from other species also share similar findings outside of the venom, such as high AMP activity observed in the eggs of *L. tredecimguttatus* from an isolated toxic proteinaceous component named Latroeggtoxin-IV (Lei et al., 2015). Although, no studies have successfully isolated and characterized an AMP within the *Latrodectus* genus; either in the venom or circulating throughout the body. Nevertheless, while these AMPs may not have been discovered yet, the possibility for widow spiders, such as *L. geometricus,* to possess an AMP with great therapeutic potential is high. However, before discussing these possible AMPs in further detail, it is

important to first understand where AMPs come from and how they work in the spider's immune system.

Biology of the Spider Immune System

The biology of a spider's immune system starts in their open circulatory system, which is made up of the hemolymph vascular system in combination with an intricate system of sinuses and lacunae where hemolymph can be distributed directly into the tissues rather than directed through vessels (Wirkner and Huckstorf et al., 2013). Hemolymph can best be equated to blood in vertebrates in that it is the central hub for the transportation of important nutrients, oxygen, and hormones to maintain proper homeostasis within the spider (Kuhn-Nentwig & Nentwig, 2013; Bednaski et al., 2015). A specific component of hemolymph are the hemocytes, or specialized cells capable of carrying out immune responses and/or maintaining proper homeostasis within the spider (Kuhn-Nentwig & Nentwig, 2013). Presently, there have been six different types of hemocytes identified in spiders: prohemocytes, granulocytes, plasmatocytes, cyanocytes, leberidocytes, and adipohemocytes (Sherman et al., 1973; Fukuzawa et al., 2008; Kuhn-Nentwig & Nentwig, 2013; Soares et al., 2013; Kuhn-Nentwig et al., 2014; Bednaski et al., 2015; Soares et al., 2015). However, the spider's immune system is aided by only two kinds of hemocytes, plasmatocytes and granulocytes, which orchestrate and aid in both cellular immune responses and humoral immune responses (Kuhn-Nentwig & Nentwig, 2013; Fukuzawa et al., 2008; Kuhn-Nentwig et al., 2014).

The cellular immune response is described as specific actions carried out by immune-related hemocytes that can prevent further invasion and infection from pathogens (Rowley & Powell, 2007). These specific actions performed by hemocytes include phagocytosis, nodulation, and encapsulation (Kuhn-Nentwig & Nentwig, 2013). Phagocytosis is the action of engulfing large particles such as an invading microbe by forming a phagosome, or an intracellular vesicle made from the plasma membrane of the hemocyte, to ingest and destroy the microbe (Abbas et al., 2019). Nodulation is the formation of melanized or non-melanized nodules in response to recognizing invading pathogens such as fungi or bacteria (Satyavathi et al., 2014). Encapsulation is the process of hemocytes binding to a large foreign target, such as a protozoan, and forming a multilayer capsule around the invader ultimately leading to melaninization and destruction of the target (Satyavathi et al., 2014). While these actions are directly performed by the hemocytes, there are also other indirect activities initially carried out by hemocytes, collectively known as the humoral response.

The humoral response includes mechanisms such as melaninization, the clotting cascade, and the production of AMPs (Kuhn-Nentwig & Nentwig, 2013). Melaninization is the process of destroying pathogens through the production of a polymer of dihydroxyindole carboxylic acid, or melanin. The melanin is deposited directly onto the foreign microbe, or onto its capsule, and engulfs the microbe by hardening into a gel leading to the termination of the microbe within minutes (Kuhn-Nentwig & Nentwig, 2013). The clotting cascade has not been described in detail within spiders but rather in a

distant relative, *Tachypleus tridentatus* (the horseshoe crab), that could model at least a somewhat partial picture for what is happening in the spider. Essentially, this series of events is triggered by identifying the invading microbe from compounds expressed on the surface such as lipopolysaccharides (LPS), a major component for the outer membrane of gram-negative bacterium (Kuhn-Nentwig & Nentwig, 2013). The LPS is then attached to a molecular component called factor C, which triggers serine protease zygomen to autocatalytically activate factor C´, which itself can activate factor B to B´ that can initiate a proclotting enzyme necessary to synthesize coagulogen into noncovalent coagulin homopolymers needed for clotting to occur (Osaki & Kawabata, 2004). So far, only transcripts for factor C precursors, coagulation factor B precursors, and proclotting enzyme precursor-like components have been identified in the tarantula *Acanthoscurria gomesiana* (Lorenzini et al. 2006).

Lastly, AMPs are the immune-related proteins synthesized mostly by granules from granulocytes in spiders (Kuhn-Nentwig & Nentwig, 2013). Due to their open circulatory system, these AMPs can be found throughout the body or in specific tissues depending on the species. These AMPs play an important part in protecting the spider against future and present infections. Presently, in spiders, there are three traditional classes of AMPs distinguished by their secondary structure and amino acid makeup: I) linear amphipathic, α-helical AMPs, II) linear peptides enriched with particular amino acid residues, and III) cyclic or open-ended cyclic AMPs. Furthermore, these traditional classes can also be found in other distant relatives, such as in the subphylum Hexapoda,

highlighting evolutionary conservation across taxa. Although, it is important to note that there are still many AMPs that do not fall into any of these categories.

Traditional AMP Classes

I. Linear Amphipathic, α -helical

The class of linear amphipathic, α-helical AMPs (LAαAs) are a common class of AMPs typically less than 40 amino acid residues and have a relatively broad activity spectrum (Bulet et al., 2004). In invertebrates, they tend to be produced by hemocytes, epithelial tissue, fat bodies (equivalent to the liver in vertebrates), and more exclusively in the venom glands of many species (Bulet et al., 2004; Corzo et al., 2002). Prominent examples of invertebrate LAαAs include melittin of *Apis mellifera* (honeybee), ponericins of *Pachycondylas goeldii* (predatory ants), and cecropins of the orders Diptera, Lepidoptera, and Coleoptrea (review by Memariani et al., 2019; Orivel et al., 2001; Brady et al., 2019). The main function most LAαAs possess pertains to the disruption of cell membranes; however, the mode of action they take can vary (Giangaspero et al., 2001; Corzo et al., 2002).

LAαAs described within spiders have been found almost exclusively within venom glands. Currently, the LAαAs and isoforms characterized within spiders are cupiennin 1a – 1d of *Cupinnieus salei* (tiger wandering spider), latarcins of *Lachesana tarabaevi* (ant spider), lycotoxins I and II of *Lycosa carolinensis* (wolf spider), lycocitins and lycosin-I of *Lycosa singorienis*, LyeTx I of *Lycosa erythrognatha* (wolf spider), cyto-insectotoxin

1a of *Lachesana tarabaevi*, rondonin of *Acanthoscurria rondoniae* (tarantula), OtTx1a of *Oxyopes takobius*, and oxyopinins 1, 2a-2d, and 4a of *Oxyopes kitabensis* (wolf spider) (Kuhn-Nentwig et al., 2002; Kozlov et al., 2006; Yan & Adams, 1998; Corzo et al., 2002; Budnik et al., 2004; Santos et al., 2010; Dubovskii et al., 2011; Polina et al., 2012; Riciluca et al., 2012; Tan et al., 2013; Vassilevski et al., 2013). Shared characteristics between these peptides include their mature peptide size ranging from 20 to 48 amino acid residues, apart from rondonin which only contains 10 amino acids (aa) corresponding to a C-terminal fragment of the "d" subunit of hemocyanin (Riciluca et al., 2012). Additionally, there is usually an absence of cysteine residues, apart from oxyopinin 4a which contains a single disulfide bridge (Dubovskii et al., 2011), and they typically have a cationic, α-helical structure with a C-terminal amide, similar to those found in other invertebrate species (Kuhn-Nentwig et al., 2002; Kozlov et al., 2006; Yan & Adams, 1998; Corzo et al., 2002; review by Memariani et al., 2019; Brady et al., 2019). Cupiennin 1a and latarcin 2a have also been characterized to have a non-specific hinge located between the α-helical structure described to aid in the peptide's mode of action (Pukala et al., 2007; Kozlov et al., 2006).

Through further structural investigation, researchers were also able to hypothesize the specific types of modes of action for cupiennin 1a, lycotoxins, and oxyopinin 1 as being pore-forming, like the previously described toroidal pore model (Pukula et al., 2007; Yan & Adams, 1998; Belokoneva et al., 2004). Additionally, most laractins were described to possess carpet-like mechanisms, unique to this class of AMPs, in infiltrating invading

microbes (Kozlov et al., 2006). The antimicrobial activity of most spider LAαAs inhibits the action of gram-positive bacteria, gram-negative bacteria as well as possessing hemolytic activity involving the disruption of red blood cells. However, aside from antibacterial activity, cupiennin I, oxyopinins, and cyto-insectoxin 1a were also found to have insecticidal activity (Kuhn-Nentwig et al, 2002; Corzo et al., 2002; Polina et al., 2012), while latarcins, lycotoxins I and II, LyeTx I, and lycocitin were shown to have antifungal activity (Kozlov et al., 2006; Yan & Adams, 1998; Santos et al., 2010; Budnik et al., 2004). The exception of these AMPs is rondonin, which was specifically fungicidal towards different species of fungi (Riciluca et al., 2012).

II. Linear Peptides Enriched with Particular Amino Acid Residues

Linear peptides enriched with particular amino acid residues (LPEPAAR) are those AMPs defined by their high proportions of a specific amino acid such as proline, arginine, tryptophan, and glycine (Brady et al., 2019). This class of AMPs also contains no cysteine residues, and do not have any common secondary structures present within the mature peptides like the other classes of AMPs (Balandin and Ovchinnikova, 2016). Most notably, proline and glycine are the highest reoccurring amino acid in this class of AMPs (Balandin and Ovchinnikova, 2016). Common Hexapoda representatives include metchnikowin of *Drosophila melanogaster* (fruit fly), apidaecins of *Apis mellifera* (honeybee), and attacins of *Hyalophora cecropia* (cecropia moth) (Hultmark et al., 1983; Casteels et al., 1989; Levashina et al., 1995). Metchnikowin and apidaecins are rich in

prolines while attacins are rich in glycine. Each AMP was also identified to be active against bacteria as well as fungus and were typically only expressed during infection (Hultmark et al., 1983; Casteels et al., 1989; Levashina et al., 1995).

LPEPAARs have also been described in spiders. Examples include ctenidins of *Cupiennius salei* (wandering spider) and acanthoscurrin I and II of *Acanthoscurria gomesiana* (Brazilian tarantula) (Baumann et al., 2010b; Lorenzini et al., 2003a; Kuhn-Nentwig & Nentwig, 2013). There are 3 isoforms of ctenidins, all of which have a mature peptide of about 109 to 120 amino acid residues long with a high glycine content of >70% (Baumann et al., 2010b). Similarly, both acanthoscurrin I and II are about 130 to 132 amino acid residues long with a high glycine content of 72–73% (Lorenzini et al., 2003a). Interestingly, the only difference between the mature peptide regions of acanthoscurrin I and II is the absence of two glycines in isoform two (Lorenzini et al., 2003a). Ctenidins are bacteriostatic towards gram-negative bacteria and somewhat towards gram-positive bacteria (Baumann et al., 2010b) while acanthoscurrin is active against gram-negative bacteria and yeast (Lorenzini et al., 2003a). Both these AMPs were also found to be constitutively expressed in the hemocytes of the respective species it was isolated from (Fukuzawa et al., 2008; Lorenzini et al., 2003a; Baumann et al., 2010b). The most significant difference between these two AMPs was ctenidins contained a central sequence, which interrupted the glycine repeats in the sequence, VIDGKDDVGL, however, the purpose of this sequence is still unknown (Baumann et al., 2010b).

III. Cyclic or Open Ended Cyclic AMPs

Cyclic, or open-ended cyclic, peptides are unique from all other types of AMPs for their possession of cysteine residues, which become oxidized to form internal disulfide bridges (Bulet et al., 2004; Balandin and Ovchinnikova, 2016). The term cyclic refers to the peptides' structural makeup in that they take on a cyclic ring structure due to the cysteine residues forming disulfide bonds (Joo et al., 2012). The number of cysteine residues determines the number of disulfide bridges the AMP will have, which further divides cyclic peptides into several subclasses. Cyclic AMPs with one disulfide bridge are the least common subclass in invertebrates, containing only two cysteine residues. A prominent example is thanatin of *Podisus maculiventris* (spined soldier bug) having only 21 amino acid residues, forming a two-stranded β-sheet stabilized by the single disulfide bridge (Fehlbaum et al., 1996). However, no representatives from this subclass have been found in Araneae.

Another subclass of cyclic AMPs contain peptides with two disulfide bridges with adopted 2-β-sheet, antiparallel folds connected by either a variable or β turn. Common examples include androctonin of *Androctonus australis* (fattail scorpion), tachyplesin I and II of *Tachypleus tridentatus* (Japanese horseshoe crab), and polyphemusins I and II of *Limulus polyhemus* (American horseshoe crab) (Ehret-Sabatier et al., 1996; Nakamura et al., 1988, Miyata et al., 1989). The common pathogens most all cyclic peptides with four cysteine residues attack include gram-positive bacteria, gram-negative bacteria, and

fungi (Ehret-Sabatier et al., 1996; Nakamura et al., 1988, Miyata et al., 1989). Tachyplesin and polyphemusins were found to be synthesized in the hemocytes (Nakamura et al., 1988, Miyata et al., 1989), while androctonin was found in the hemolymph of breeding adult scorpions (Ehret-Sabatier et al., 1996).

Currently, the most recognized cyclic peptide of this subclass within spiders is a small, open-ended cyclic peptide characterized within the uninfected mygalomorph spider *A. gomesiana* called gomesin (Silva et al., 2000). The mature cationic peptide has a total of 18 aa, including four cysteine residues that form two internal disulfide bonds, as well as a glycine residue involved in the C-terminal amidation of arginine, necessary for its antimicrobial activity (Fázio et al., 2006). The expression profile of gomesin is like that of other open-ended cyclic peptides in that it was primarily observed within the hemocytes of naïve spiders with absent to weak expression everywhere else (Bulet et al., 2004; Lorenzini et al., 2003b). Along with many other cyclic peptides, gomesin has broad-spectrum activity. Targeted pathogens include both gram-positive and gram-negative bacteria, filamentous fungi and yeast, and even the protozoan *Leishmania amazonesis*, responsible for chronic cutaneous leishmaniasis (Silva et al., 2000). Apart from this group of cyclic peptides, the last subclass, and currently the most widespread AMP in the animal kingdom, are collectively known as defensins (Balandin and Ovchinnikova, 2016).

Defensins: The AMP of Interest

While there are many kinds of defensins, each group of defensins can be distinguished by the specific patterning of cysteine residues, their secondary structure, and most importantly the type of organism producing them (Bulet et al, 2004; Balandin and Ovchinnikova, 2016). Compared to the small role defensins have in the innate immune response of larger animals like mammals, invertebrates rely a great deal on defensins and are considered an essential, evolutionary conserved element in responding to invading pathogens (Contreras et al., 2020). Invertebrate defensins are normally 18 to 45 amino acid residues long containing six to eight cysteine residues, making three to four disulfide bridges, and exhibit a unique cysteine-stabilized α-helix/ β-sheet motif (CS-αβ) (de la Vega & Possani, 2005; Wu et al., 2018). In addition, they are typically most effective against gram-positive bacteria as compared to gram-negative bacteria, with exceptions being particularly effective against fungi (Bulet et al., 2004; Wu et al., 2018). However, a contrasting feature of invertebrate defensins across taxa is the variance amongst their mature peptide sequences. Thus, terrestrial invertebrate defensins can be further divided into two groups. The first group can be found in insect orders such as Diptera, Hymenoptera, Coleoptera, Hemiptera, and Lepidoptera; while the second group is found in the orders Odonata and Arachnida (de la Vega & Possani, 2005; Balandin and Ovchinnikova, 2016).

Examples of the first subfamily of defensins include, but are not limited to, defensin in *Anopheles gambiae* (the African malaria mosquito), *Calomera littoralis* (the tiger beetle), and *Bombus pascuorum* (the European bumblebee) (Rees et al., 1997; Vizioli et al., 2001; Rodríguez-García et al., 2016). Commonalties of these defensins tend to lie within their structural makeup, all of which contain the typical cysteine stabilized αβ (CS-αβ) motif where their N- or C-terminal contain an α-helical region while their disulfide bonds create three β-sheets, making the CS-αβ motif. Of the three examples, *A. gambiae* and *C. littoralis* are the typical size for a mature defensin peptide, consisting of around 30 to 46 residues, including the six cysteine residues (Vizioli et al., 2001; Rodríguez-García et al., 2016). However, exclusive to the *Bombus* genus, the defensin is much larger, consisting of 51 residues due to a conserved 12-residue extension found on the C-terminal end of the AMP (Rees et al., 1997). All insect defensins have strong antibacterial activity, particularly towards gram-positive bacteria, with sparse antifungal activity (Rees et al., 1997; Vizioli et al., 2001; Rodríguez-García et al., 2016). Expression patterns of these defensins are also similar showing low constitutive expression before infection, and a sharp rise in expression after infection occurs (Vizioli et al., 2001; Rodríguez-García et al., 2016; Rees et al., 1997).

In addition to the predominately described antibacterial invertebrate defensins, there are also several outliers such as drosomycin, a defensin characterized in *D. melanogaster* that exclusively possesses antifungal activity (Zhang and Zhu, 2009). This defensin contains a mature peptide of 44 amino acid residues, eight being cysteine residues,

forming four disulfide bonds along with the typical conserved CS-αβ motif most all defensins have (Zhang and Zhu, 2009). While the typical invertebrate cysteine pattern is Cys1-Cys4, Cys2-Cys5, and Cys3-Cys6, the bridge Cys1-Cys8 is exclusive to drosomycin, making its cysteine parring Cys1-Cys8, Cys2-Cys5, Cys3-Cys6, and Cys4-Cys7 (Bulet et al., 2004). In addition to drosomycin, there have only been three other exclusive antifungal defensins characterized in invertebrates: heliomicin of *Heliothis virescens* (tobacco budworm), termicin of *Pseudacanthothermes spiniger* (fungus-growing termite), and gallerimycin of *Galleria mellonella* (greater wax moth larvae) (Bulet et al., 2004). The reason why these peptides only target fungi is still under investigation.

Apart from the first subfamily of invertebrate defensins, the other ancestral subfamily of defensins presents a more conserved sequence homology between species. For Odonata, limited information is known. Only one paper mentions a 38-residue defensin isolated in the hemolymph of *Aeschna cyanea*, with high activity towards gram-positive bacteria (Bulet et al., 1992). However, literature regarding other species of dragonflies and their defensins remains unpublished. Although, in this defensin subfamily, much more information is known about ticks. In fact, defensins and their many isoforms have been described in both hard ticks: *Haemaphysalis longicornis* (Lu et al., 2010; Sun et al., 2017), *Dermacentor variabilis* (Johns et al., 2001; Ceraul et al., 2003), *Dermacentor silvarum* (Wang et al., 2015; Li et al., 2021), *Ixodes scapularis* (Wang and Zhu, 2011), *Ixodes ricinus* (Chrudimksa et al., 2010), *Ixodes persulcatus*

(Saito et al., 2009), *Ixodes holocyclus* (Cabezas-Cruz et al., 2019), *Amblyomma hebraeum* (Lai et al., 2004), *Amblyomma americanum* (Todd et al., 2007) and soft ticks: genus *Ornithodoros* (Nakajima et al., 2002; Chrudimská et al., 2010; Li et al., 2021). All tick defensins are highly similar in structure with a typical CS-αβ motif, and have a length of around 71 aa, with six being cysteine residues, in the mature peptide region (Todd et al., 2007; Chrudimská et al., 2010; Cabezas-Cruz et al., 2019; Li et al., 2021).

Similar to insects, tick defensins also exhibit a prepropeptide region within their open reading frame, unique from other arachnids, which is viewed as a conserved evolutionary protein element of tick defensins (Froy & Gurevitz, 2003). Location of expression for tick defensins can vary between species including the salivary glands, midgut, fat bodies, or hemocytes (Todd et al., 2007; Li et al., 2021; Johns et al., 2001; Nakajima et al., 2002). While it is true these defensins are constitutively expressed, many species show increased expression patterns during feeding, a vulnerable state for ticks (Nakajima et al., 2002; Chrudimksa et al., 2011; Saito et al., 2009; Sun et al., 2017). As for the antimicrobial activity, most tick defensins are active towards gram-positive bacteria; however, some, including their many isoforms, are also effective in targeting gram-negative bacteria and fungi (Lu et al., 2010; Wang et al., 2015; Cabezas-Cruz et al., 2019).

While there is plethora of information regarding defensins in ticks, scorpion defensins also have many interesting features including having a unique evolutionary

relationship with classical neurotoxins found in scorpion venom (Meng et al., 2020). Scorpion defensins such as AbDef-1 in *Androctonus bicolor* and BmKDfsin3 of *Mesobuthus martensii* both contain a mature peptide around 38 aa, six of which are cysteine residues (Zhang et al., 2015; Cheng et al., 2020). While these defensins have the typical CS-αβ motif, this conserved structure can also be found in potassium channel-blocking scorpion toxins (Zhao et al., 2019). In fact, it has been hypothesized that scorpion defensins are evolutionary precursors to scorpion neurotoxins due to their high sequence similarities, including the six cysteine residues and their placement along the mature peptide region (Meng et al., 2020). In addition, just as other AMPs, these defensins have a high affinity in attacking bacteria as well as other pathogens like BmKDfsin3 inhibiting the Hepatitis C virus (Zhang et al., 2015; Cheng et al., 2020). However, while research regarding tick and scorpion defensins seems to be on the rise, research on spider defensins remains limited.

Publications mentioning the identification of defensins in spiders varies between only briefly mentioning their presence within spiders to comprehensive analyses of the peptide. Examples include several papers citing the presence of defensin peptides during transcriptome analysis in the venom glands of several species of spiders, but further investigation into the defensins internal role was not performed (Kuhn-Nentwig et al., 2019; Paiva et al., 2019; Khamtorn et al., 2020). Another example addresses the immune potential from embryos of *Parasteatoda tepidariorum* and *Pardosa sp.* and finds defensins to be a main source of defense but does not discuss the individual peptides in

detail (Czerwonka et al., 2021). More comprehensive studies include the discovery of five putative defensin sequences in five different species of spider: *Cupiennius salei, Meta menardi, Teganaria atrica*, *Phoneutria reidyi,* and *Polybetes pythagoricus* (Baumann et al., 2010a). All the spider defensins showed highly similar characteristics to each other including size, secondary structure, sequence identity, and activity. Furthermore, Baumann et al. (2010a) compared each defensin and found an 89–97% identity between defensins in these species as well as a 70% identity to scorpion and tick defensins. While this is consistent with the theory of types of invertebrate defensins, another defensin in *C. salei*, named defensin-2, was isolated in the venom glands, but only showed a 54% identity to the defensin found by Baumann et al. (2010a) (Kuhn-Nentwig et al., 2019).

In addition, Zhao et al. (2011) isolated and characterized a defensin-like peptide in the venom glands of *Ornithoctonus hainana* named Oh-defensin. While the spider defensins discovered by Baumann et al. (2010a) and Kuhn-Nentwig et al. (2019) all had a mature peptide of around 37 aa residues, Oh-defensin contained 52 aa. However, all defensins contained the typical six cysteine residues and the typical CS-αβ motif (Zhao et al., 2011). In regards to comparing bioactivity, defensins of Baumann et al. (2010a) were not interpreted while Oh-defensin was found to not only be active against gram-positive bacteria, but also against gram-negative bacteria and *Candida albicans* (Zhao et al., 2011). Just as in other AMPs, spider defensins have the potential for therapeutic utilization. Further investigation in other species of spider could lead to new alternatives

in treating and targeting bacterial or fungal infections as well as contributing to a greater understanding of the evolution of invertebrate defensins.

Objectives

Objective 1: Resolving the mRNA transcript for defensin in *Latrodectus geometricus*

While there are several species of spiders with described defensins, none of the species in the genus *Latrodectus* have had a defensin described or characterized, according to the literature. Contributing to the small pool of identified spider defensins will not only broaden the understanding of the spider's immune system but also contribute to understanding the evolutionary relationship between invertebrate defensins. In this objective, the transcript and full open reading frame was resolved for a defensin in *L. geometricus*, Lg-defensin, using rapid amplification of cDNA ends and *de novo* assembly, followed by a bioinformatical analysis and phylogenetic analysis using the mature peptide region.

Objective 2: Assessment of bacterial infection control after RNAi-silencing of Lg-defensin

While putative annotations may be provided by bioinformatic analysis, empirical validation of gene function required additional assays. Therefore, to provide functional evidence of Lg-defensin as an antimicrobial peptide, a target region of the transcript was used to prepare, *in vitro*, dsRNA that was injected into widows followed by a bacterial

infection from either gram-positive or gram-negative bacteria to assess the impact of silencing. To functionally analyze silencing results, qPCR was performed using bacterial–specific primers to analyze differences in the presence of bacterial DNA between Lg-defensin silenced/ infected and unsilenced/ infected spiders.

CHAPTER 1: RESOLVING THE mRNA TRANSCRIPT FOR A DEFENSIN IN *LATRODECTUS GEOMETRICUS*

ABSTRACT

Defensins are a widespread antimicrobial peptide found throughout the animal kingdom. Unpublished data in our lab shows that a defensin homolog in *Latrodectus geometricus* is expressed before and during bacterial infection (unpublished; Lg-defensin). Using our current, limited Lg-defensin sequence data, the remaining transcript regions were resolved using rapid amplification of cDNA ends (RACE) followed by *de novo* assembly through Transcriptome Shotgun Assembly. The deduced amino acid sequence contains a signal peptide of 23 aa residues followed by a mature peptide region of 37 aa residues, six of which were cysteines. The mature peptide region showed high identity to other related organisms such as ticks, scorpions, and other spiders, ranging from 41%–70%. This is the first identified defensin reported in the genus *Latrodectus* adding to the small group of defensins identified in spiders.

INTRODUCTION

As modern technology continues to bring forth new innovations and medicines to cure life-threatening diseases, the immune system remains to be one of the most intricate and intriguing inventions from nature in fighting against pathogens. The immune system of each eukaryotic organism is made up by the innate immune response while some, like

humans, have an adaptive immune response capable of memory and target specificity (Abbas et al., 2019). However, most organisms, like invertebrates, have only been found to have a non-specific targeting, innate form of immune response (Loker et al., 2004; Rowley & Powell, 2007). In narrower terms, the invertebrate innate immune response can be broken down into two different response mechanisms, cellular immune response and humoral immune response. The cellular immune response is defined as specific actions performed by immune effector hemocytes (Loker et al., 2004). These hemocytes are specialized cells found within the hemolymph of arthropods with specific duties of either maintaining the organism's homeostasis or carrying out immune responses, like phagocytosis, nodulation, and encapsulation, to prevent the spread of infection (Lemaitre & Hoffmann, 2007; Satyavathi et al., 2014). In contrast, the humoral immune response includes other mechanisms, also carried out by immune effector hemocytes, such as melaninization, the clotting cascade, and the synthesis of antimicrobial peptides (Osaki and Kawabata, 2004; Loker et al., 2004; Kuhn-Nentwig & Nentwig, 2013).

Antimicrobial peptides (AMPs) are cationic, amphipathic proteins typically 12 to 100 amino acids long (Jenssen et al., 2006; Bahar and Ren, 2013). The main function of AMPs is to disrupt and permeabilize foreign microbial membranes made possible by their amphipathic structure (Bahar and Ren, 2013). Furthermore, AMPs can be broken up into groups based on their secondary structure and deduced amino acid sequence (Jenssen et al., 2006; Bahar and Ren, 2013; Erdem Büyükkiraz and Kesmen, 2022). These groups consist of linear, amphipathic, α-helical AMPs (LAαAs), linear peptides enriched with

particular-amino acid residues (LPEPAARs), and cyclic or open-ended cyclic AMPs. However, there are still many AMPs that do not fall into any of these traditional categories.

Cyclic or open-ended cyclic AMPs are the largest group, which can be divided into subgroups depending on the number of cysteine residues (Bulet et al., 2004; Balandin and Ovchinnikova, 2016). These cysteine residues become oxidized to form internal disulfide bridges, contributing to the peptide's overall mode of action (Isogai et al., 2011). While there are many kinds of cyclic AMPs, one of the most common are defensins (Bulet et al., 2004; Balandin and Ovchinnikova, 2016; Contreras et al., 2020). These cysteine rich AMPs normally have a mature peptide around 18 to 45 amino acids long, with six to eight of them being cysteine residues, making three to four disulfide bridges (de la Vega & Possani, 2005; Wu et al., 2018). While there are many kinds of defensins throughout the animal kingdom, invertebrate defensins are unique from other defensins as they exhibit a special cysteine-stabilized α-helix/ β-sheet motif (CS-αβ) and typically share the same bioactivity (Ganz and Leher, 1995; Froy & Gurevitz, 2003; Bulet et al., 2004; Wu et al., 2018).

In fact, most invertebrate defensins are highly effective against gram-positive bacteria with some exceptions being particularly effective against fungi and gram-negative bacteria (Ganz and Leher, 1995; Froy & Gurevitz, 2003; Bulet et al., 2004; Zhang et al., 2009; Wu et al., 2018). Although, there is variability regarding mature

peptide sequences. Thus, invertebrate defensins can be broken into two groups based on conserved sequence similarity (de la Vega & Possani, 2005; Balandin and Ovchinnikova, 2016). The first group consists only of orders found in class Insecta in orders Diptera, Hymenoptera, Coleoptera, Hemiptera, and Lepidoptera; while the second group is found in orders Odonata, Arachnids, and even distant, unrelated relatives such as mollusks (de la Vega & Possani, 2005; Balandin and Ovchinnikova, 2016). However, many invertebrate defensins remain outliers to these groups simply due to their unique peptide structure.

Many invertebrates have had their defensins isolated and characterized, including organisms like scorpions, ticks, and insects. However, some groups remain with little investigation such as spiders. The first published paper specifically discussing and exploring spider defensins was in 2010, discovering five putative defensins in five different species of spider (Baumann et al., 2010a). The authors found an 89–97% similarity between the defensins in these species as well as a 70% identity to defensins in other arachnids like scorpions and ticks (Baumann et al., 2010a). Each of the described defensins has a mature peptide of 37 aa, six of which were cysteine residues, with the typical CS-αβ motif. However, the bioactivity of these peptides remains unknown (Baumann et al., 2010a). Additionally, only one of these defensins, defensin-1 of *Cupiennius salei*, was used for visualizing expression patterns. Results showed expression in the hemocytes, ovaries, suboesophageal nerve mass, and muscle while

having weak expression in the heart, silk glands, and venom glands (Baumann et al., 2010a).

In a separate study, *C. salei* was also discovered to have a second defensin, defensin-2, exclusively expressed in the venom glands, and has a 54% identity to defensin-1 found by Baumann et al. (2010a), including similar placement of cysteine residues (Kuhn-Nentwig et al., 2019). The activity of defensin-2 is also unknown. Similarly, another study found an exclusively spider defensin-like AMP called Oh-defensin of *Ornithoctonus hainana.* This defensin also has exclusive expression in the venom glands (Zhao et al. 2011). Unlike other invertebrate defensins, Oh-defensin had a mature peptide of 52 aa, six of which were cysteine residues, and was described to be active not only against gram-positive and gram-negative bacteria, but also against *Candida albicans* (Zhao et al. 2011).

While both Baumann et al. (2010a) and Zhao et al. (2011) described defensins within spiders, these defensins appeared to have different properties indicating the possibility that spiders express multiple kinds of defensin peptides. However, little has been done to describe defensins in other species of spiders. Further investigation into spider defensins would not only broaden the understanding of the immune response in spiders, but also provide a supplementary foundation for evolutionary studies regarding the most widely distributed AMP in invertebrates. Previous preliminary experiments in our lab indicated the presence of a defensin-like peptide in *Latrodectus geometricus.* In

this study, the full open reading frame for the defensin gene, Lg-defensin, was resolved in *L. geometricus* using rapid amplification of cDNA ends (RACE) and *de novo* assembly followed by a bioinformatical analysis. Furthermore, a phylogenetic sequence alignment was made, using the mature putative peptide sequences of other invertebrate defensins.

MATERIALS AND METHODS

Spider Collection and Maintenance

Brown widow spiders (*L. geometricus*) were found locally in Nacogdoches County of East Texas and kept in optimal conditions, as described in Baumann et al. (2010a). Habitat conditions included providing each mature spider with a 1oz container with a lid, with the addition of leaves and sticks to provide stability for web making and places to hide. Habitats were kept at room temperature and adult spiders were fed twice a month, using forceps, by placing *Tenebrio molitor* (mealworms) within their webbing. Females were used for research purposes due to their longer life span and larger size than males (Schraft et al., 2021).

Bacterial Culturing

To prepare bacteria for the infection of the spiders, *E. coli* (DH5α cells; New England Biolabs, Ipswich, MA, USA) was inoculated in 5 mL of Luria-Broth (LB) medium. The culture was then placed in the incubator at 37°C on the shaker overnight. The following day, 1 mL of the cultured bacteria was aliquoted into a 1.5 mL microcentrifuge tube and pelleted by centrifuging at 13,000xg for 5 min at room temperature and the supernatant was removed. Cells were then washed with 1 mL of deionized (DI) water and centrifuged at 13,00xg for 5 min to pellet. This process was

repeated twice before quantification. The amount of visible sample was diluted to OD_{600} = ~0.2, using the Beckman Coulter: DU 800 spectrophotometer (Beckman Coulter, Brea, CA, USA). The concentration was previously validated in the lab to result in infection without causing mortality in widow spiders. This bacterial sample was used to infect female brown widow spiders through injection.

Infection of the Brown Widow

To prepare the spiders for injections, the widows were pre-chilled in a container of ice for ~3 min to allow for immobilization, a methodology previously confirmed in our lab to immobilize the spider. Within the container of ice, the ventral side of the cephalothorax was facing anterior, and injections occurred in the membranous area between the second and third coxa using a 10 µl Hamilton syringe with a 33-gauge, 30° beveled needle (Hamilton, Reno, NV, USA). Using the previously prepared *E. coli,* spiders were injected with 0.5 µl of bacteria. Based on previous, unpublished data in our lab, expression of the Lg-defensin was found before and during infection. However, expression of Lg-defensin was highest 24 h post-infection. Therefore, the spider was incubated at room temperature for a 24 h period before total RNA extraction.

RNA Extraction

Following the 24 h period, spiders were cut at the pedicel to separate the cephalothorax from the abdomen, and the abdomen was laterally cut down the middle.

Each half of the spider abdomen was placed in a 1.5 microcentrifuge tube with 1 mL of TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The tissue was homogenized using RNase-decontaminated scissors as well as an RNase-decontaminated glass rod to pulverize the sample. The sample was then placed at -80°C for ~ 1 h. After, RNA was isolated using the TRIzol RNA extraction protocol provided by the manufacturer and resuspended in 20 µl of diethyl pyrocarbonate (DEPC) water. Extracted RNA was then quantified using the NanoVue Plus spectrophotometer (GE Healthcare, Chicago, IL, USA) and stored at -80°C to ensure future viability.

Rapid Amplification of cDNA Ends (RACE) and Sanger Sequencing of the 3'- end

RACE is a method to resolve full transcript sequences from partial mRNA template by adding known sequences to the 5' or 3' end of the mRNA that can subsequently serve as priming sites. Using this method, the 3'- end of the transcript was resolved for Lg-defensin. The protocol was followed according to the manufacture (Takara Bio USA Inc., Mountain View, CA, USA). An aliquot equating to 1 µg of purified RNA was used to prepare RACE-ready first-strand cDNA (Takara Bio USA Inc., Mountain View, CA, USA). For the following steps regarding RACE, the 3'-RACE-PCR gene-specific primer (GSP) was as stated: 5'-GATCATCCATAGCATTTGCAAGTTC-3'. The 3'-RACE-PCR was then prepared according to Table 1, and thermal cycler settings were provided by the manufacturer according to program 2 for the gene-specific primer (GSP) with annealing temperatures between 60–70°C.

Table 1

The total reaction used to make a 3'-RACE-PCR.

Components	3'-RACE Sample
3'-RACE-Ready cDNA	2.5 µl
10X UPM	5 µl
3' GSP (10 µm)	1 µl
Deionized Water	-
Master Mix	41.5 µl
Total:	**50 µl**

Once the PCR had completed 35 cycles, the 3'-RACE-PCR products were resolved on a 2% agarose gel at 100V for 30 min. Bands observed were excised for gel extraction using the QIAquick Gel Extraction Kit, following the protocol provided by the manufacturer (Qiagen, Hidden, Germany, EU). Final products were then centrifuged from the spin columns into 1.5 mL microcentrifuge tubes using 20 µl of nuclease-free water. The purified PCR product was then quantified on the NanoVue Plus spectrophotometer to determine the product's concentration before being sent off for Sanger sequencing using Eurofins Genomics DNA Tube Sequencing services (https://www.eurofins.com/). Obtained sequences were compared with sequences deposited in GenBank using BLAST N to confirm that they belonged to the 3' end of the Lg-defensin transcript.

De-novo Assembly of the 5' end

The 5'- end of the Lg-defensin transcript was obtained through *de novo* assembly using the Transcriptome Shotgun Assembly (TSA) tool in BLAST N. TSA is a database that contains pre-assembled RNA transcripts by computational analysis using Next Generation Sequencing (NGS). Most of these proteins remain unidentified and uncharacterized in the database, as they come from sequence read archives (SRA) containing raw sequencing data allowing researchers to compare their physical data to TSA data. The partial Lg-defensin nucleotide sequence was used in BLAST N using the TSA tool and was found to have several hits related to *L. geometricus* RNA sequences in the TSA database, with the target setting adjusted for *Latrodectus* (taxid: 6923).

The most similar retrieved sequences were TSA: *Latrodectus geometricus* Lg3tf038393g4u transcribed RNA sequence (Accession: GBJM01068764.1) with a percent identity of 92.18% and TSA: *Latrodectus geometricus* Lg3tf038393g5u transcribed RNA sequence (Accession: GBJM01068763.1) with a percent identity of 97.53%. Sequences were then analyzed and compared to the original, partial Lg-defensin transcript. Through careful comparison using the partial ORF, it was found that GBJM01068764.1 had an almost identical 3'- ORF region as to Lg-defensin as shown in Figure 1, therefore the two sequences were combined and the 5'- end of full transcript for Lg-defensin was obtained.

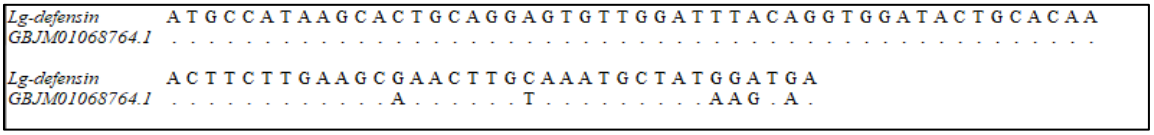


Figure 1: The 3'-ORF region of Lg-defensin in comparison to the almost identical GBJM01068764.1 region. Dots represent conserved residues in relation to the Lg-defensin sequence.

Bioinformatic analysis

Once the 3' and 5' end of Lg-defensin were determined, comparisons to other sequences were performed using BLAST N and BLAST X (https://blast.ncbi.nlm.nih.gov) to identify any other similar transcripts that have been identified within *Latrodectus* (taxid:6932), Araneae (taxid:6893) Acari (taxid: 6933), Scorpiones (taxid: 6855), and Insecta (taxid: 50557). In addition, the TSA tool in BLAST N was used to further assess other possible *Latrodectus* sequences uncharacterized in the database. Information collected from BLAST included percent identity or percentage of bases identical to Lg-defensin, query coverage or percentage of the Lg-defensin length that aligns to the GeneBank hit, accession length or length of the original hits provided by GeneBank, and the accession number or the identifying label for the hit. Conserved domains were determined using a conserved domain database search (.ncbi.nlm.nih.gov/Structure/cdd/). The ORF was found using ORF finder (ncbi.nlm.nih.gov/orffinder/) to determine where the 5'- and 3'-untranslated regions were in the Lg-defensin transcript. Once the ORF was defined, the sequence was then inputted into ExPASy translate (https://web.expasy.org/translate/) to deduce the putative amino

acid sequence of Lg-defensin, and the presence of a putative signal peptide was determined using SignalP 5.0 (cbs.dtu.dk/services/SignalP/). Once the signal peptide was found, the putative mature peptide region was established. Mature peptide tertiary structure was predicted using the default parameters of SWISS-model (https://swissmodel.expasy.org/).

Further analysis of the ORF and the mature peptide region included a phylogenetic alignment of Lg-defensin to other invertebrate defensins using BioEdit v 7.0.5.3. This analysis software was used to identify distinct features found within invertebrate defensins such as the number of cysteine residues, arrangement of cysteine residues, if there was an equal number of residues upstream and downstream from the disulfide bridge and determining other conserved residues using the ClustalW Multiple Alignment tool in BioEdit. The ORF of Lg-defensin was aligned to other ORFs found from the *Latrodectus* TSA data and the BLAST X results using specific parameters to Araneae. Other invertebrate sequences found using BLAST X were inputted in BioEdit using only the putative mature peptide region. This was to observe any residue conservation and variability between the Lg-defensin mature peptide region and other invertebrate defensin mature peptide region, as this is the most conserved region of the defensin.

RESULTS

Lg-defensin sequence characterization

The starting portion of Lg-defensin was obtained through previous experiments performed in our lab (unpublished). Using 3'-RACE PCR, the remaining C-terminal sequence of Lg-defensin was obtained, which included a polyadenylation signal as well as a poly-A-tail. The N-terminal sequence of Lg-defensin was obtained through *de novo* read walking using BLAST as well as the TSA Sequence (Accession: GBJM01068764.1). Combining this data, the full-length transcript yielded 530 nucleotides with a 5'-UTR of 83 bp, an ORF of 186 bp and a 3'-UTR of 161 bp followed by a poly-A-tail, indicated by the polyadenylation as seen in Figure 3. A conserved domain search confirmed a Defensin_2 superfamily domain in the mature peptide region between amino acids 25 to 60. The deduced amino acid sequence of the ORF, found using Expasy translate, yielded 61 aa with a signal peptide comprising amino acids 1–24, obtained using SignalP 5.0 software. The signal peptide contains 24 aa, encoded by 72 nucleotides, followed by a mature peptide of 37 aa, encoded by 111 nucleotides, and ending with a stop codon as shown in Figure 3. No propeptide region was found within the ORF of Lg-defensin. In addition, the mature peptide was found to contain six conserved cysteine residues, all of which were in the mature peptide region. In addition,

SWISS-model predicted the mature peptide of Lg-defensin to have a CS-αβ motif structure, with two anti-parallel beta-strands and one alpha-helix as shown in Figure 2.

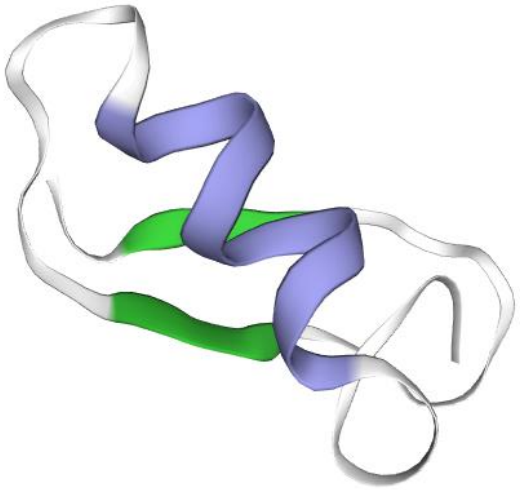


Figure 2: SWISS-model prediction of the Lg-defensin peptide. Lg-defensin was predicted to have a cysteine-stabilized α-helix/ β-sheet motif (CS-αβ) typical of most invertebrate defensins. Highlighted in green are the two anti-parallel beta-strands and highlighted in purple is the alpha-helix.

Figure 3: Nucleotide sequence of Lg-defensin and deduced amino acid sequence. The putative mature peptide is highlighted in blue (nucleotide sequence) and underlined in black (amino acid sequence). The signal peptide is underlined using the dashed line (amino acid sequence). The polyadenylation signal is highlighted in orange, following a poly-A tail.

Phylogenetic Analysis

Lg-defensin showed high homology to other *Latrodectus* nucleotide sequences obtained using TSA in BLAST N as depicted in Figure 4. Of the two species found, *L. hesperus* (Accession: GFDB01026266.1) had a 92.78% identity and 96% query coverage while *L. tredecimguttatus* (Accession: GANL01004664.1) had a 93.89% identity and 96% query coverage to the ORF region of Lg-defensin. Further investigation indicated these uncharacterized sequences both have a Defensin_2 superfamily conserved domain. Furthermore, both sequences had similar conservation in 12 nucleotide positions that were different from Lg-defensin's ORF, as seen in Figure 4. Nucleotide sequences were then placed in Expasy Translate to determine their amino acid sequences. Both hypothetical proteins were 61 aa long, just like Lg-defensin. Sequence comparisons showed that they both shared two of the same residues, different from Lg-defensin, in positions 55 and 61 in the mature peptide region as shown in Figure 5. Furthermore, *L. hesperus* also had a differing residue in the signal peptide region of Lg-defensin in position 12. Cysteine residues were parallel to Lg-defensin with each hypothetical protein containing six cysteines. Both sequences were also found to have a signal peptide 24 aa long followed by a mature peptide of 37 aa, just for Lg-defensin.

A BLAST X search, restricted to organism Araneae (taxid: 6893), retrieved four defensins and one antiviral peptide from four different families in relation to Lg-defensin. The percent identity between all specimens was between 60.66%–70.49%. After aligning

the sequences using BioEdit, the most similar sequence was the defensin of *Stegodyphus mimosarum* while the least similar sequence was the antiviral peptide An1a *Alopecosa nagpag.* Variability was found in the signal peptide region of the AMP, with only three conserved residues between all species, while *Parasteatoda tepidariorum*, a species in the same family as *L. geometricus*, had the most similar signal peptide region to Lg-defensin as shown in Figure 6. Within the signal peptide regions, an indel event was also found in the *S. mimosarum* sequence between positions three and four. The mature peptide regions showed six cysteine residues all of which had a consistent placement to Lg-defensin. Moreover, it was revealed that an additional 19 residues were conserved to the mature peptide sequence of Lg-defensin, as seen in Figure 6.

To find additional, comparable defensin sequences, amongst different invertebrates, three other taxa groups were searched using BLAST X: Acari, Scorpiones, and Insecta. After determining the mature peptide regions for each resulting hit, sequences were inputted into BioEdit for a phylogenetic alignment using the ClustalW Multiple Alignment tool to visualize the evolutionary conservation of the mature peptide region between taxa as shown in Figure 6. The overall observation showed that the position of the cysteine residues remained consistent amongst all representing taxa as well as a glycine, Gly28 to Lg-defensin, aside from the partial sequence of *Rhipicephalus appendiculatus* defensin (Accession: AAX33350.1). Moreover, an additional three residues were shared between the ancestral group of defensins in positions 5, 14, and 23.

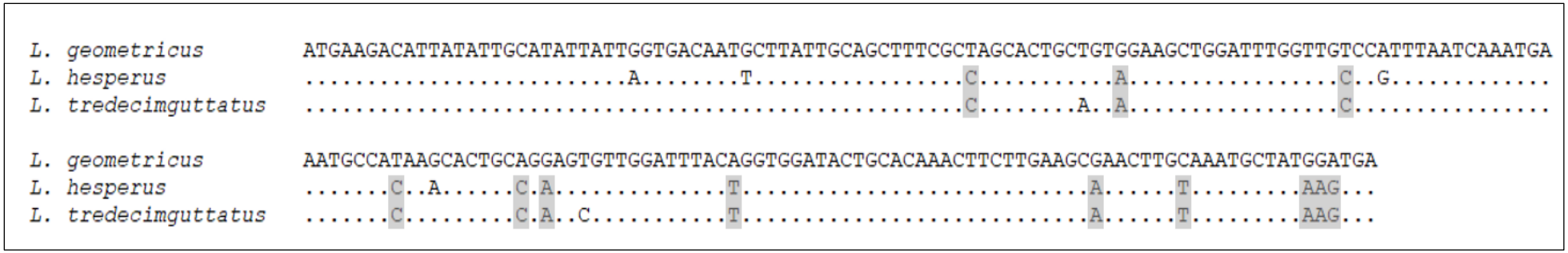


Figure 4. Results from TSA of *Latrodectus* taxid:6923 using the ORF region of Lg-defensin. Nucleotide sequences were aligned using BioEdit to compare ORF regions to the ORF region of Lg-defensin (top line). Dotted lines below *L. geometricus* represent conserved residues as it relates to the Lg-defensin sequence. Conserved nucleotides between found sequences are highlighted in grey as they differ from *L. geometricus*.

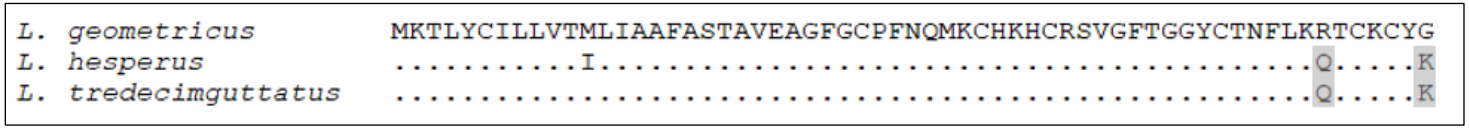


Figure 5. Deduced amino acid sequences using results from TSA of *Latrodectus* taxid:6923. Amino acid sequences were aligned using BioEdit to compare ORF regions to the ORF region of Lg-defensin (top line). Dotted lines below *L. geometricus* represent conserved residues as it relates to the Lg-defensin sequence. Conserved amino acids between found sequences are highlighted in grey as they differ from *L. geometricus.*

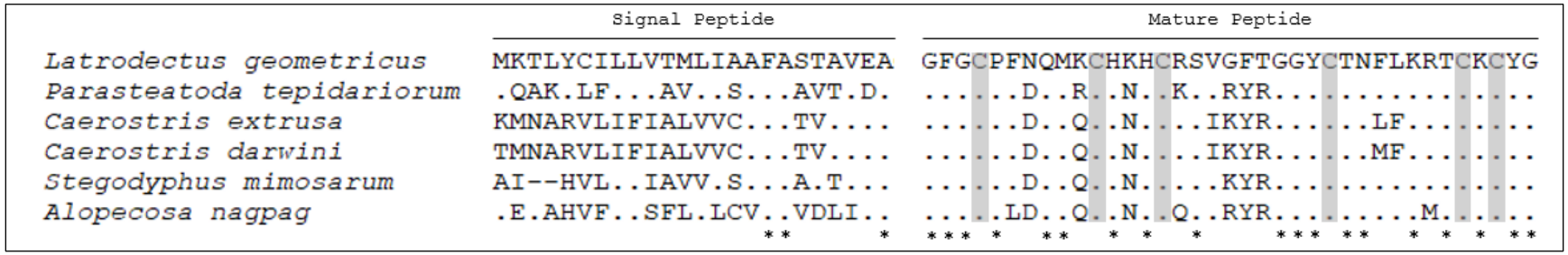
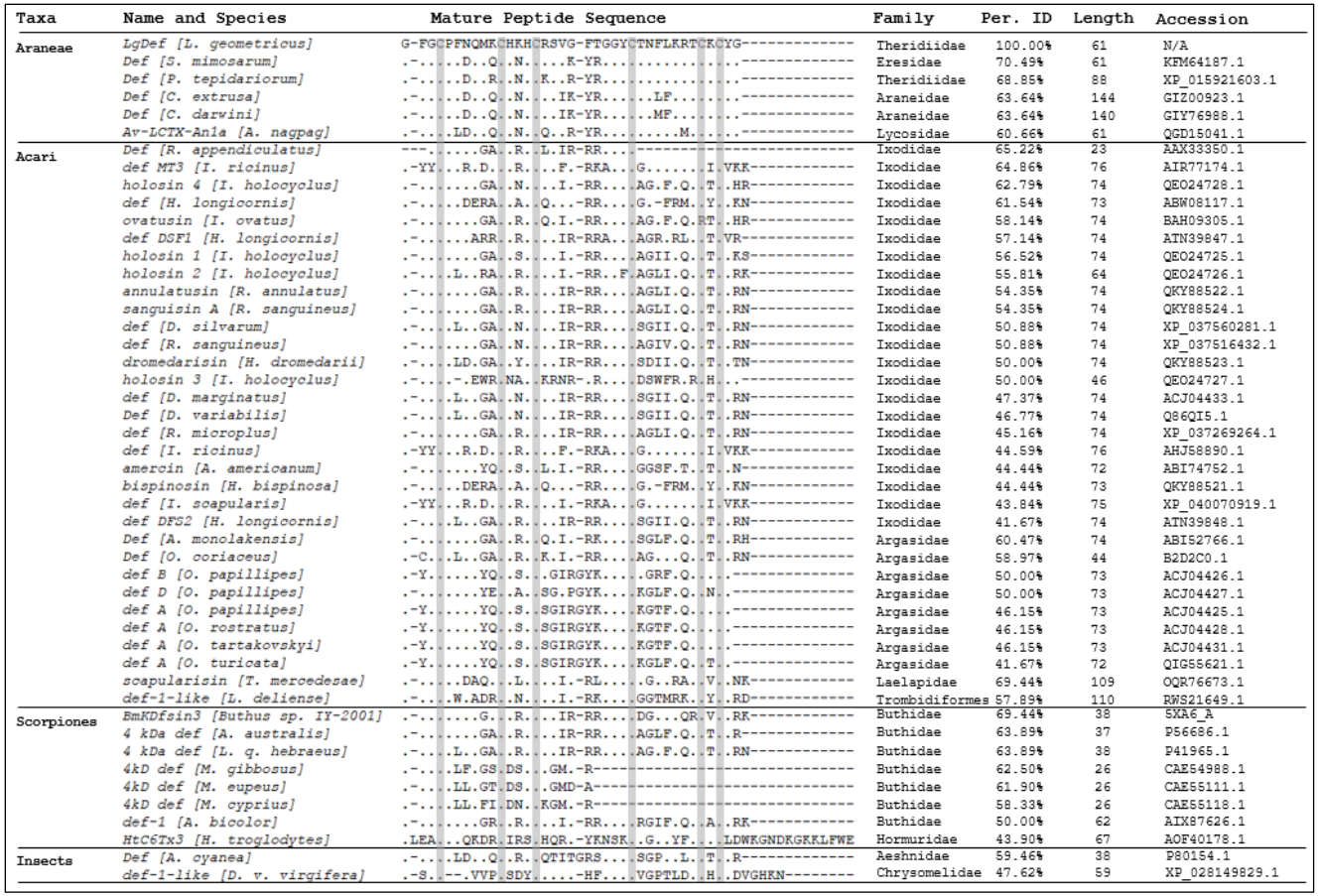


Figure 6. Amino acid sequences of Araneae defensins from BLAST X results as compared to Lg-defensin of *L. geometricus.* Amino acid sequences were aligned using BioEdit to compare Araneae ORF regions to the ORF region of Lg-defensin (top line). Dotted lines below *L. geometricus* represent conserved residues as it relates to the Lg-defensin sequence. Conserved cysteine residues are highlighted in gray. Conserved amino acids in all sequences are denoted with an asterisk. Only a signal peptide and mature peptide region were observed for all sequences.

Of all organisms, the most hits were found in Acari, all of which were related to defensins. Not all data was used for this alignment, but the percentage identity in this comparison ranged from 41.67%–69.44% and the query coverage ranged from 37%–96% with four different represented families, two of which were for sequences from mites as seen in Figure 7. Alignments were made using the putative mature peptide region, and it was found that most shared similarities were found between sequences from the same genus as observed in Figure 7. All mature peptides were parallel in length to Lg-defensin, with a full peptide length average of 72–74 aa. However, an indel event was noted in position 31, indicated by a glycine, in all *Ornithidorus* spp. except for a defensin of *O. coriaceus,* making all *Ornithidorus spp.* mature peptide 38 aa rather than 37 aa.

Scorpiones were found to have the highest percent identity to Lg-defensin of the three other searched taxa groups, all of which were defensins, aside from one toxin of *H. troglodytes*, ranging from 43.90%–69.44% and query coverage ranging from 33%–96%, with two represented families. Like Acari, shared residues were mostly found between same genus such as *Mesobuthus* spp. in positions 6, 12, 13, 17, and 18. The most similar sequence was BmKDfsin3 of a *Buthus spp.* while the least similar sequence was the toxin. Lastly, insects were found to be the least abundant with only two representatives, *A. cyanea,* a dragonfly in the family Aeshnidae and *Diabrotica virgifera virgifera,* a Coleopteran in the family Chrysomelidae. Percent identity for *A. cyanea* was 59.46% with a query coverage of 58% while *D. v. virgifera* was 47.62% with a query coverage of 67%, both of which were annotated as defensins. An indel event was also found for *D. v.*

virgifera at positions 5 and 6 as well as having an additional five residues, totaling 40 aa for its mature peptide region.



Taxa	Name and Species	Mature Peptide Sequence	Family	Per. ID	Length	Accession
Araneae	*LgDef [L. geometricus]*	G-FGCPFNQMKCHKHCRSVG-FTGGYCTNFLKRTCKCYG----------	Theridiidae	100.00%	61	N/A
	Def [S. mimosarum]	.-.....D..Q..N....K-YR.....------------	Eresidae	70.49%	61	KFM64187.1
	Def [P. tepidariorum]	.-.....D..R..N..K..R-YR.....------------	Theridiidae	68.85%	88	XP_015921603.1
	Def [C. extrusa]	.-.....D..Q..N....IK-YR.....LF.....------------	Araneidae	63.64%	144	GIZ00923.1
	Def [C. darwini]	.-.....D..Q..N....IK-YR.....MF.....------------	Araneidae	63.64%	140	GIY76988.1
	Av-LCTX-An1a [A. nagpag]	.-....LD..Q..N..Q..R-YR.....M.....------------	Lycosidae	60.66%	61	QGD15041.1
Acari	*Def [R. appendiculatus]*	---.....GA..R..L.IR-RR...----------	Ixodidae	65.22%	23	AAX33350.1
	def MT3 [I. ricinus]	.-YY..R.D..R....F.-RKA..G.....I.VKK----------	Ixodidae	64.86%	76	AIR77174.1
	holosin 4 [I. holocyclus]	.-.....GA..N....I.-RR....AG.F.Q..T..HR----------	Ixodidae	62.79%	74	QEO24728.1
	def [H. longicornis]	.-.....DERA..A..Q...-RR....G.-FRM..Y..KN----------	Ixodidae	61.54%	73	ABW08117.1
	ovatusin [I. ovatus]	.-.....GA..R..Q.I.-RR....AG.F.Q.RT..HR----------	Ixodidae	58.14%	74	BAH09305.1
	def DSF1 [H. longicornis]	.-.....ARR..R....IR-RRA..AGR.RL..T..VR----------	Ixodidae	57.14%	74	AIN39847.1
	holosin 1 [I. holocyclus]	.-.....GA..S....I.-RR....AGII.Q..T..KS----------	Ixodidae	56.52%	74	QEO24725.1
	holosin 2 [I. holocyclus]	.-....L..RA..R....I.-RR..FAGLI.Q..T..RK----------	Ixodidae	55.81%	64	QEO24726.1
	annulatusin [R. annulatus]	.-.....GA..R....IR-RR....AGLI.Q..T..RN----------	Ixodidae	54.35%	74	QKY88522.1
	sanguisin A [R. sanguineus]	.-.....GA..R....IR-RR....AGLI.Q..T..RN----------	Ixodidae	54.35%	74	QKY88524.1
	def [D. silvarum]	.-....L..GA..N....IR-RR....SGII.Q..T..RN----------	Ixodidae	50.88%	74	XP_037560281.1
	def [R. sanguineus]	.-.....GA..N....IR-RR....AGIV.Q..T..RN----------	Ixodidae	50.88%	74	XP_037516432.1
	dromedarisin [H. dromedarii]	.-....LD.GA..Y....IR-RR....SDII.Q..T..TN----------	Ixodidae	50.00%	74	QKY88523.1
	holosin 3 [I. holocyclus]	.-....-.EWR..NA..KRNR-.R....DSWFR.R.H...----------	Ixodidae	50.00%	46	QEO24727.1
	def [D. marginatus]	.-....L..GA..N....IR-RR....SGII.Q..T..RN----------	Ixodidae	47.37%	74	ACJ04433.1
	Def [D. variabilis]	.-....L..GA..N....IR-RR....SGII.Q..T..RN----------	Ixodidae	46.77%	74	Q86QI5.1
	def [R. microplus]	.-.....GA..R....IR-RR....AGLI.Q..T..RN----------	Ixodidae	45.16%	74	XP_037269264.1
	def [I. ricinus]	.-YY..R.D..R....F.-RKA..G.....I.VKK----------	Ixodidae	44.59%	76	AHJ58890.1
	amercin [A. americanum]	.-.....YQ..S..L.I.-RR....GGSF.T..T..N----------	Ixodidae	44.44%	72	ABI74752.1
	bispinosin [H. bispinosa]	.-.....DERA..A..Q...-RR....G.-FRM..Y..KN----------	Ixodidae	44.44%	73	QKY88521.1
	def [I. scapularis]	.-YY..R.D..R....I.-RKA..G.....I.VKK----------	Ixodidae	43.84%	75	XP_040070919.1
	def DFS2 [H. longicornis]	.-....L..GA..R....IR-RR....SGII.Q..T..RN----------	Ixodidae	41.67%	74	AIN39848.1
	Def [A. monolakensis]	.-.....GA..R..Q.I.-RK....SGLF.Q..T..RH----------	Argasidae	60.47%	74	ABI52766.1
	Def [O. coriaceus]	.-C...L..GA..R..K.I.-RR....AG...Q..T..RN----------	Argasidae	58.97%	44	B2D2C0.1
	def B [O. papillipes]	.-Y.....YQ..S..GIRGYK....GRF.Q.....----------	Argasidae	50.00%	73	ACJ04426.1
	def D [O. papillipes]	.-.....YE..A..SG.PGYK....KGLF.Q..N..----------	Argasidae	50.00%	73	ACJ04427.1
	def A [O. papillipes]	.-Y.....YQ..S..SGIRGYK....KGTF.Q.....----------	Argasidae	46.15%	73	ACJ04425.1
	def A [O. rostratus]	.-Y.....YQ..S..SGIRGYK....KGTF.Q.....----------	Argasidae	46.15%	73	ACJ04428.1
	def A [O. tartakovskyi]	.-Y.....YQ..S..SGIRGYK....KGTF.Q.....----------	Argasidae	46.15%	73	ACJ04431.1
	def A [O. turicata]	.-Y.....YQ..S..SGIRGYK....KGLF.Q..T..----------	Argasidae	41.67%	72	QIG55621.1
	scapularisin [T. mercedesae]	.-.....DAQ...L....I.-RL.....G..RA..V..NK----------	Laelapidae	69.44%	109	OQR76673.1
	def-1-like [L. deliense]	.-....W.ADR..N....I.-RK....GGTMRK..Y..RD----------	Trombidiformes	57.89%	110	RWS21649.1
Scorpiones	*BmKDfsin3 [Buthus sp. IY-2001]*	.-.....G..R....IR-RR....DG...QR.V..RK----------	Buthidae	69.44%	38	5XA6_A
	4 kDa def [A. australis]	.-.....GA..R....IR-RR....AGLF.Q..T..R----------	Buthidae	63.89%	37	P56686.1
	4 kDa def [L. q. hebraeus]	.-....L..GA..R....IR-RR....AG.F.Q..T..RN----------	Buthidae	63.89%	38	P41965.1
	4kD def [M. gibbosus]	.-....LF.GS..DS...GM.-R----------	Buthidae	62.50%	26	CAE54988.1
	4kD def [M. eupeus]	.-....LL.GT..DS...GMD-A----------	Buthidae	61.90%	26	CAE55111.1
	4kD def [M. cyprius]	.-....LL.FI..DN..KGM.-R----------	Buthidae	58.33%	26	CAE55118.1
	def-1 [A. bicolor]	.-.....GR..R....I.-RR....RGIF.Q..A..RK----------	Buthidae	50.00%	62	AIX87626.1
	HtC6Tx3 [H. troglodytes]	.LEA...QKDR.IRS.HQR.-YKNSK..G..YF....LDWKGNDKGKKLFWE	Hormuridae	43.90%	67	AOF40178.1
Insects	*Def [A. cyanea]*	.-....LD..Q..R..QTITGRS....SGP..L..T..R----------	Aeshnidae	59.46%	38	P80154.1
	def-1-like [D. v. virgifera]	.-S..--.VVP.SDY.....-HF....VGPTLD..H..DVGHKN--------	Chrysomelidae	47.62%	59	XP_028149829.1

Figure 7. Phylogenetic alignment using invertebrate defensin mature peptide regions using BioEdit. BLAST X results are grouped by their representative taxa group as shown in the first column. Name of species and BLAST X result are listed in column 2. The mature peptide sequences are listed in column 3. The conserved cysteine residues are highlighted in light grey. Family of species (column 4), percent identity (column 5), original accession length (column 6), and the searchable accession ID (column 7) are listed besides each representative.

DISCUSSION

In this study, a defensin peptide was successfully identified in the brown widow spider, *Latrodectus geometricus,* titled Lg-defensin. This is the first report of a defensin peptide to be molecularly characterized and described in *L. geometricus*, contributing to the small pool of defensin and defensin-like proteins discovered in spiders (Baumann et al., 2010a; Zhao et al. 2011; Kuhn-Nentwig et al., 2019; Czerwonka et al., 2021). As mentioned previously, defensins are a type of cyclic antimicrobial peptide, rich with disulfide bonds widely distributed amongst the animal kingdom and defined as a crucial part of the host's innate immune response (Bulet et al., 2004; Contreras et al., 2020). Invertebrate defensins have been described in two classes, distinguished by their conserved mature peptide sequences. The first class includes defensins of neopteran insects while the second class, sometimes referred to as the 'ancestral' group, includes a more diverse group of invertebrates including orthopterans, arachnids, and even mollusks (Froy & Gurevitz, 2003; de la Vega & Possani, 2005; Balandin and Ovchinnikova, 2016). Lg-defensin would be considered a defensin of the ancestral class as it had matches in the NCBI database to almost exclusively representatives of this group of defensins, apart from a few exceptions.

However, in general, other features that suggested Lg-defensin to be a defensin of *L. geometricus* included its conserved domain, indicating Lg-defensin to be in the

Defensin_2 superfamily, as well as containing six cysteine residues in its mature peptide region, indicating three disulfide bonds. This number of cysteines is specific to defensins rather than the other cyclic peptides, as it contributes to the unique folding and stability of the protein due to the formation of disulfide bonds (Sevier and Kaiser, 2002). Moreover, the bonding pattern of these cysteine residues exhibited C1-C4, C2-C5, C3-C6; a pattern also found in other arthropod defensins (Bulet et al., 2004; Baumann et al., 2010a; Rodríguez-García et al., 2016; Contreras et al., 2020; Cheng et al., 2020). Using the SWISS-model tool, it was also predicted that Lg-defensin has the typical CS-αβ motif, also found in other invertebrate defensins, with one α-helix and two antiparallel β-sheets (Froy & Gurevitz, 2003; Bulet et al., 2004; Contreras et al., 2020). The α-helix and β-sheet regions in the defensin are considered active sites and are vital in contributing to the antimicrobial activity of all invertebrate defensins (Tsuji et al., 2007). Thus, it is indicative to suspect that Lg-defensin most likely possesses some form of antimicrobial activity within *L. geometricus.*

While these features are essential to defensins, one unique trait of Lg-defensin was its genetic structure. Lg-defensin yielded a genetic structure like those of scorpion defensins and spider defensins (de la Vega & Possani, 2005; Baumann et al., 2010a; Cheng et al., 2020). This finding is not surprising considering that scorpions present a closer homology to spiders as compared to other arachnids (Jeyaprakash & Hoy, 2009). Additionally, no propeptide region was found in Lg-defensin, opposite of tick defensins but parallel to scorpion defensins. While exon shuffling was first identified as the reason

for this absence of a propeptide, it is now hypothesized that this diversification is most likely due to multiple, independent corresponding insertions, deletions, or sliding events happening between introns after exon shuffling has occurred (Froy & Gurevitz, 2003; de la Vega & Possani, 2005). It is also confirmed that with the absence of a propeptide, spiders might have also had similar posttranslational processing from a defensin precursor like that in scorpion defensins (Zhang et al., 2015). Further research should be done to determine the absence or presence of introns in the Lg-defensin DNA sequence to better understand its genetic structure in relation to other invertebrate defensins. However, combining the evidence of Lg-defensins genetic structure and characteristics indicates that this gene is most likely an active, cyclic, antimicrobial peptide that assists in the immune response in *L. geometricus.*

While there is more literature describing scorpion and tick defensins, other species of Araneae, found using BLAST X, showed similar gene structure to the Lg-defensin sequence. The majority of these defensins found remain uncharacterized in terms of functionality, with one being hypothesized as having antibacterial properties during embryo development in *P. tepidariorum* (Czerwonka et al, 2021), a species found in the same family as *L. geometricus*. This evidence suggests that it is possible that Lg-defensin may have antibacterial properties as well considering the overall consensus is that defensins possess antibacterial capabilities. However, the most interesting match was Antiviral-LCTX-An1a (An1a), an antiviral toxin identified in the venom of *Alopecosa nagpag* (Ji et al., 2019). While this peptide had the lowest identity to Lg-defensin it

shares high homology to the other defensin sequences. Unlike the antibacterial properties portrayed by most defensins, An1a was found to have protease inhibiting properties targeting viruses such as the anti-dengue serotype-2 virus and zika virus (Ji et al., 2019). As the function of Lg-defensin is still undefined, it is possible that Lg-defensin may have more than just antibacterial properties like that of An1a.

In addition to the findings in BLAST X, sequences published by Baumann et al. (2010a) were also aligned with Lg-defensin to determine conservation of other spider defensins. Although these alignments were not documented, sequence identity was much higher on the peptide level, than spider defensins found in BLAST X, ranging from 69%–77%, apart from the partial defensin sequence of *M. menardi* being 79%. Although, all sequences showed higher homology to Lg-defensin in the mature peptide region rather than in the signal peptide region, just as those found in BLAST X. Parallel to Lg-defensin's genetic structure, full putative defensins from Baumann et al. (2010a) were 61 aa long with a putative signal peptide 24 aa long and a putative mature peptide 37 aa long, apart from *T. arica* only having a putative signal peptide of 23 aa.

Apart from the genetic structure, while Lg-defensin was found to be expressed both before and during infection, information regarding the specific tissues Lg-defensin was being expressed in could not be concluded. Although, Baumann et al. (2010a) was one of the few papers to describe expression patterns for a defensin in a spider, defensin-1 in *C. salei.* Their findings showed constitutive expression in numerous tissue types such

as the ovaries, subesophageal nerve mass, hepatopancreas, muscle tissue, and hemocytes with little to no expression in other observed tissue types like the venom glands, silk glands, and heart tube (Baumann et al., 2010a). In contrast to this observation, as indicated by Khamtorn et al. (2020), while defensin-1 portrays many genetic similarities to Lg-defensin, defensin-like peptides do exist in the venom glands of *L. geometricus.* This finding is similar to the expression of Av1a, defensin-2 of *C. salei*, which was found specifically in the venom glands but showed less homology to other arachnid defensins than to its counterpart defensin-1, or even the previously mentioned Oh-defensin of *O. hainana* also having exclusive expression in the venom glands and was unable to be found in the BLAST search (Ji et al., 2019; Kuhn-Nentwig et al., 2019; Zhao et al. 2011). However, it is more likely this means that *L. geometricus* harbors multiple kinds of defensins. This could mean there are defensins expressed throughout the body of *L. geometricus*, like *C. salei*, to protect against general bacterial infections; in addition to defensins exclusively being expressed in the venom glands to aid in the protection against infection while consuming prey. Future studies should focus on indicating which tissues Lg-defensin is being synthesized in to conclude this hypothesis.

Additionally, while Lg-defensin was the only described *Latrodectus* defensin for this research, TSA returns also showed other highly conserved, uncharacterized defensin sequences found within *L. hesperus* and *L. tredecimguttatus*. In addition, a conserved domain search indicated both sequences were found to be a part of the Defensin_2 superfamily, the same as Lg-defensin. Interestingly however, *L. hesperus* and *L.*

tredecimguttatus were more similar to each other on both a peptide and nucleotide level than to Lg-defensin. The reason for this is most likely due to their phylogenesis, with *L. hesperus* and *L. tredecimguttatus* being more closely related to each other than to *L. geometricus* (Garb et al., 2003). Further investigation of *Latrodectus* defensins could not only provide more data on the diversity of invertebrate defensins but offer a greater understanding of immune-related proteins involved in *Latrodectus* species and their importance during a microbial invasion.

In the phylogenetic alignment, all invertebrate defensins displayed similar residue placements for all six cysteine residues as well as glycine, observed at the beginning of the mature peptide sequence. This glycine has been hypothesized to contribute to the flexibility between the α-helix and β-sheets in invertebrate defensins (Cornet et al., 1995). Moreover, all representatives shown are considered a part of the ancestral class of defensins. The exception came from the family Chrysomelidae *D. v. virgifera,* having only 48% identity to Lg-defensin on the peptide level while also having a significantly longer mature peptide region. It is also interesting to observe the specific amino-acid homology found amongst different families, and even more specifically genera like *Ixodes, Ornithodorus, Rhipicephalus, Caerostris,* and *Mesobuthus*. While these defensins are classified under the ancestral group due to sequence homology, this phylogenetic alignment also shows a vast sequence diversification between these organisms, most likely driven by selective pressures including environment, diet, genetics, and reproduction (Tassanakaion et al., 2015).

Aside from this diversity, the greatest similarities to Lg-defensin, aside from the spiders, were found in scorpions and members of Acari, both of which are arachnids. Scorpions accounted for the highest similarity ranging from 44%–69% identity on the peptide level and a query coverage of 33%–96%, with the lowest identity coming from a venom peptide, HtC6Tx3 of *H. troglodytes.* Interestingly, while this toxin has a mature peptide of 47 aa it shares many features with arthropod defensins and defensin-like peptides, like those found in Lg-defensin, and most likely possesses pharmacological properties as indicated by Zhong et al. (2017). This is not surprising since scorpion defensins have been thought to be the evolutionary precursors to neurotoxins in scorpions (Meng et al., 2020). In contrast, the highest identity to Lg-defensin was an antiviral peptide called BmKDfsin4, derived from the venom glands of *M. martensii,* identified as having similar characteristics to scorpion toxins such as having the ability to block potassium channels, an activity commonly described in invertebrate defensins (Zhao et al., 2019; Cheng et al., 2020). While Lg-defensin was only described bioinformatically, this high identity to AMPs with the ability to block potassium channels provides evidence that Lg-defensin is worth functionally describing to determine what therapeutic capabilities it possesses. However, while defensins are commonly described as antibacterial, BmKDfsin4 was found to have antiviral properties toward hepatitis C virus capable of inhibiting its replication abilities (Cheng et al., 2020). This is another annotated hit with antiviral capabilities further demonstrating that Lg-defensin may possibly possess antiviral capabilities.

Sequences from Acari were more variable than scorpion sequences, between 33%–69% identity on the peptide level and a query coverage of 37%–96% with the highest similarity coming from a mite, *Tropilaelaps mercedesae*. In addition, it was noted that their average peptide length was around 72 to 74 aa, due to their additional propeptide region (Zhang et al., 2015; Chrudimská et al., 2010). Moreover, while representatives for tick defensins were found in both Ixodidae and Argasidae, when families were compared to Lg-defensin, neither showed a more significant identity to Lg-defensin over the other. This finding is not surprising since the diversity between tick species and their defensins is high (Cabezas-Cruz et al., 2016). Tissue expression and function also seemed to vary between species, with most having the typical antibacterial properties, mostly targeted at gram-positive bacteria, like that of most invertebrate defensins and expression being found in most tissue types (Todd et al., 2007; Wang & Zhu, 2011; Cabezas-Cruz et al., 2016; Sun et al., 2017; Li et al., 2021). This is further evidence that Lg-defensin most likely also possesses antibacterial properties, with high specificity to gram-positive bacteria. Furthermore, with annotated tick defensins, like DSF1 and DSF2 of *Haemaphysalis longicornis*, found to target MRSA, this could also mean Lg-defensin could have great therapeutic potential in targeting antibiotic-resistant bacteria (Sun et al., 2017).

The similarities in the mature peptide region to a more distant relative, *Aeshna cyanea* was lower, but still significant at 59% at the peptide level and a query coverage of 58%. This defensin was isolated in the hemolymph and was strongly active toward gram-

positive bacteria (Bulet et al., 1992). As previously mentioned, it is more than likely that Lg-defensin also possesses antibacterial properties towards gram-positive bacteria since many of these annotated defensins share this same bioactivity. In addition, while the search was limited to terrestrial invertebrates for the phylogenetic alignment, different mollusk defensins were also found to have similar mature peptide sequences to Lg-defensin. Percent identity ranged from 46%–61% and all proteins contained the same cysteine bonding patterns as those presented in the alignment. This further confirms the idea that Lg-defensin is an ancestral, invertebrate defensin (Froy & Gurevitz, 2003).

Evidence from this research indicates Lg-defensin to be the first defensin isolated in *L. geometricus.* Not only does this study contribute to the small pool of described Araneae defensins, but also contributes to the further understanding of the evolutionary relationship between the highly diversified innate immune system within invertebrates. Evidence from this study also indicated Lg-defensin to possess antibacterial, specifically targeting gram-positive bacteria, and possibly antiviral properties as suggested by the annotated results found using BLAST X. Additionally, this defensin may also have a unique approach in targeting invading microbes, such as potassium-channel blocking capabilities, considering the many toxins it shared close resemblance to. Research to identify the functional properties of Lg-defensin is underway. Specifically, the defensins significance during bacterial immune response as it compares between gram-positive bacteria and gram-negative bacteria.

CHAPTER 2: ASSESSMENT OF BACTERIAL INFECTION CONTROL AFTER RNAI-SILENCING OF LG-DEFENSIN IN *L. GEOMETRICUS*

ABSTRACT

In the previous study, a defensin peptide from *Latrodectus geometricus*, Lg-defensin, was identified. While this peptide presented a highly conserved open reading frame related to other defensins, from related taxa, the functionality of the peptide requires empirical exploration. Although, it was hypothesized, by the result of chapter, to have antibacterial activity as indicated by the literature of other related defensins. Thus, in this study, to determine the function of Lg-defensin, gene expression was silenced using *in vivo* RNA interference (RNAi) technology and assessed the impact of silencing on the spider's ability to control a bacterial infection via qPCR quantification of bacteria post-infection. To determine if Lg-defensin had specific antibacterial activity, both a gram-positive and a gram-negative species were tested. Results from this study indicated that Lg-defensin does have antibacterial activity, with higher specificity in targeting gram-positive bacteria. This research provides a basis for future exploration into the broad immune potential of Lg-defensin in infection defense.

INTRODUCTION

With increasing concerns regarding antibiotic-resistant bacteria, the search for alternative antimicrobial agents is of high priority. Current research has shown that

naturally synthesized antimicrobial peptides can be an effective alternative to antibiotics (Lei et al., 2019; Dijksteel et al., 2021; Moretta et al., 2021). Antimicrobial peptides (AMPs) are small proteins synthesized to play an important role in the innate immune response for a vast number of organisms (Huan et al., 2020). Distinguishing traits of these immune-related proteins include their secondary structure, mode of action, time of production, and location of production (Erdem Büyükkiraz and Kesmen, 2022). While a large number of AMPs have been characterized and isolated from various invertebrate phyla including Hexapoda, Crustacea, and Echinoderms, more specific classes remain limited, such as Araneae (Coates et al., 2022).

While the understanding of how a spider responds to infection is inadequate compared to other invertebrates, spiders have been found to release a variety of AMPs in response to pathogen invasion with 42 AMPs already described (Kuhn-Nentwig and Nentwig, 2013; Czerwonka et al., 2021). The most commonly described AMP in spiders are linear amphipathic, alpha-helical peptides with examples including oxyopinins and OtTx1a of *Oxyeopes kitabensis,* cyto-insectotoxin 1a, LyTx-1a, and latarcins of *Lachesana tarbaevi,* LyeTxI-b and LyeTx I of *Lycosa erythrognatha,* lycosin I-II and lycocitins of *Lycosa singorensis,* and rodonin of *Acanthoscurria rondoniae* to name a few (Corzo et al., 2002; Budnik et al., 2004; Kozlov et al., 2006; Santos et al., 2010; Polina et al., 2012; Riciluca et al., 2012; Vassilevski et al., 2013; Kusmenkov et al., 2013; Tan et al., 2013; Wang et al., 2016; Reis et al., 2018). Other prominent examples include cyclic peptide gomesin of *Acanthoscurria gomesiana* and linear peptides enriched with

particular amino acid residues such as acanthoscurrin I and II of *A. gomesiana* and ctenidins of *Cupiennius salei* (Silva et al., 2000; Lorenzini et al., 2003a; Baumann et al., 2010b). Of these discoveries, several have also been described as having clinical applications such as LyeTx I and LyeTxI-b being described as ideal candidates for alternatives to antibiotics, juruin having antifungal action to clinically resistant fungi strains such as, or gomesin possessing anticancer properties towards melanoma and leukemia cell lines (Santos, et al., 2010; Ayroza et al., 2012; Troeira et al., 2017; Reis et al., 2018). While this evidence demonstrates that AMP discovery in other species of spiders is promising, many spider AMPs remain uninvestigated.

The focus of the study was on a well-characterized group of cyclic peptides collectively known as defensins. These cyclic peptides represent a highly conserved AMP in invertebrates and are considered as a vital element to their innate immune response (Bulet et al., 2004; Contreras et al., 2020). Invertebrate defensins are normally 18 to 45 amino acid residues long, containing six to eight cysteine residues, with three to four disulfide bridges and exhibit a unique cysteine-stabilized α-helix/ β-sheet motif (CS-αβ) (de la Vega & Possani, 2005; Wu et al., 2018). While many AMPs have a wide array of microbial targets, invertebrate defensins are typically most effective against gram-positive bacteria as compared to gram-negative bacteria, with exceptions being particularly effective against fungi (Bulet et al., 2004; Wu et al., 2018). Notable representatives of arachnid defensins, or more broadly the ancestral class of invertebrate defensins, include ticks such as *Dermacentor silvarum* or *Haemaphysalis longicornis* and

even scorpions like *Androctonus australis*, all of which share similar functional and structural qualities (de la Vega et al., 2005; Ehret-Sabatier et al., 1996; Wang et al. 2015; Sun et al., 2017). However, the discovery of spider defensins remains insufficient compared to its close relatives.

Much of the literature regarding defensins in spiders focuses solely on the structure and sequence patterns rather than the functionality of the peptide. Recent examples have briefly described defensin peptides observed during transcriptome analysis of the venom glands in several spiders, but further investigation into these defensins internal role has not been done (Kuhn-Nentwig et al., 2019; Paiva et al., 2019; Khamtorn et al., 2020). Czerwonka et al. (2021) addresses the immune potential from embryos of *Parasteatoda tepidariorum* and *Pardosa sp* and finds defensins to be a main source of defense but fails to address the protein in further detail. However, more comprehensive studies regarding spider defensins include Baumann et al. (2010a), who specifically analyzed defensin peptides in five species of spiders: *Cupiennius salei*, *Phoneutria reidyi*, *Polybetes pythagoricus*, *Tegenaria atrica*, and *Meta menardi*. Not only did they describe the peptide sequences of all five species and their relation between each other and other invertebrate species, but they also investigated the location of defensin expression in *C. salei* (Baumann et al., 2010a).

Zhao et al., (2011) specifically defined the activity and the structure of a defensin-like peptide from the venom glands of *Ornithoctonus hainana* they called Oh-defensin.

Antimicrobial assays using the purified protein found strong antibacterial activity towards *Staphylococcus aureus*, *Escherichia coli*, *Bacillus dysenteriae,* and *Candida albicans* with a MIC of 1.25 µg/ml (Zhao et al., 2011). However, the structure of this defensin more closely resembled those of insects rather than more closely related invertebrates like ticks and scorpions. This finding supports the need and opportunity to identify more spider defensins. Not only is discovering new defensins in spiders important for possible alternatives in medicine, but also to fully understand their diversity and evolutionary relationship amongst other invertebrate defensin peptides and their purpose in the spider's immune system.

While most AMPs have been functionally characterized through purifying the peptide and performing various antimicrobial assays, this research intends to take an alternative approach. In chapter 1, the sequence of a defensin peptide in *Latrodectus geometricus,* Lg-defensin, was identified and bioinformatically characterized. Similarities to other arachnid defensin peptides were high, supporting the hypothesis that Lg-defensin is an antimicrobial peptide that functions in the immune response of *L. geometricus* against bacterial infection (unpublished). To test this hypothesis, the Lg-defensin gene was silenced using RNAi and the impact of silencing had on the spider's ability to control a bacterial infection was assessed by quantifying the presence of bacterial DNA via qPCR analysis. In addition, many invertebrate defensins have high antibacterial activity towards numerous strains of bacteria, therefore the effects of silencing were tested on both a gram-positive and a gram-negative bacterial species.

MATERIALS AND METHODS

Spider Maintenance

Brown widow spiders were maintained as previously described in chapter 1.

Preparation of Lg-defensin dsRNA

The RNAi pathway or post-transcriptional gene silencing is a conserved biological response induced by the presence of dsRNA for a specific gene, such as Lg-defensin. If activated, a specialized RNA III endonuclease, Dicer, will cut this dsRNA into small interfering RNAs (siRNAs). These siRNAs will then form an RNA-induced silencing complex (RISC) that will attach to the mRNA sense strand leading to a translational halt of Lg-defensin. Preparation of Lg-defensin dsRNA was performed using the *in vitro* MEGAscript RNAi kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The target region for silencing was ~356 bp. This region was amplified using Lg-defensin specific primers made with a flanking T7 promoter sequence at the 5' ends of both the sense and antisense primers as shown in Table 1. To generate ~1 µg of template DNA of the selected region, two 50 µl PCRs were made using 2X GoTaq® Green Master Mix (Promega, Madison, WI, USA) and uninfected, female *L. geometricus* cDNA. After PCR assembly, the following two-step PCR protocol was performed in the T100 Thermal

Cycler system (Bio-Rad, Hercules, CA, USA): 1 cycle of 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 55.4°C for 30 sec, 72°C for 1 min, and a final extension step of 72°C for 5 min.

The PCR products were then resolved on a 1.5% agarose gel at 115V for 20 min and the bands were extracted from the gel using the GeneJET Gel Extraction Kit (Thermo Fisher Scientifiic, Waltham, MA, USA). The purified product was then used in the MEGAscript RNAi Kit procedure, which was followed according to the provided protocol. The additional annealing step was skipped since hybridization most likely happened during the incubation period in the transcription reaction since the transcript size was ≤ 800 nt and was made using a single template with opposing T7 promoters. The final dsRNA product was eluted by two separate centrifugations using 100 µl of pre-heated elution solution at 95°C. The purified dsRNA was quantified using the NanoVue Plus spectrophotometer (GE Healthcare, Chicago, IL, USA) and stored in -20°C for later use.

Table 1

RNAi primers for amplification of 356 bp long sequence of Lg-defensin that is used as a template for dsRNA synthesis.

Name	Target	Sequence
RNAi_T7F	*Lg-defensin*	5'-TAATACGACTCACTATAGGGAGAGAGATACTCATAGCCTGAATCTTG-3'
RNAi_T7R	*Lg-defensin*	5'-TAATACGACTCACTATAGGGAGAGACAGTTCATTATAATTTTCC-3'

dsRNA injections and confirmation of Lg-defensin silencing

To determine the time required to achieve complete silencing of the Lg-defensin transcript within female *L. geometricus*, several time points post-dsRNA injection were assessed. Each time point group consisted of four spiders with time points being 48 h, 72 h, 5 days, 7 days, 14 days, and 21 days post-injection of dsRNA. Spiders were injected as previously described in chapter 1. Approximately 10 µg of dsRNA was injected per spider (~2.5 µl of volume), an amount that was used to silence highly conserved transcription factors in *P. tepidariorum* (Schacht et al., 2019).

After the allotted time for each group had passed, spiders were terminated for RNA extraction by cutting each spider in half and homogenizing each half in 1 mL of TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA was then isolated according to the TRIzol Reagent User Guide and eluted using 20 µl of DEPC treated water. Following RNA isolation, RNA was quantified on the NanoVue plus spectrophotometer and used for cDNA synthesis using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). For each cDNA protocol, ~ 1 µg of RNA was used. To validate the silencing of the Lg-defensin transcript by PCR, primers were designed in regions that flanked the dsRNA target region to avoid amplifying the injected dsRNA. Amplification was performed by using 2xGoTaqGreen Master Mix and designed primers shown in Table 2. After PCR assembly, the following two-step PCR protocol was performed: 1 cycle of 95°C for 3 min, followed by 35 cycles of 95°C for 30

sec, 47.1°C for 30 sec, 72°C for 1 min, and a final extension step of 72°C for 5 min. Additionally, to test cDNA synthesis, a portion of the housekeeping gene cytochrome oxidase I (COI) in *L. geometricus* was amplified using primers presented in Table 2 (Torres et al., 2022). The following two-step PCR protocol was performed in the T100 Thermal Cycler system (Bio-Rad, Hercules, CA, USA): 1 cycle of 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec, 50.4°C for 30 sec, 72°C for 1 min, and a final extension step of 72°C for 5 min. PCR products were then resolved on a 1.5% agarose gel at 115V for 20 min using 5 µl per sample. Silencing of Lg-defensin was determined based on the absence or faint presence of bands, at the expected size, resolved on the agarose gel as compared to the COI control PCR.

Table 2

Validating silencing primers by standard PCR methods.

Name	Target	Sequence	Original Reference
Val_F	*Lg-defensin*	5'-TATCCTTTGGCTGATACGC-3'	This study
Val_R	*Lg-defensin*	5'-GCTTTTGCATGAAATTTGTTTCTC-3'	This study
LgeoCoF	*COI*	5'-ACTGCCATAAGAGTTCTTATTCGAATTG-3'	Torres et al., 2022
LgeoCoR	*COI*	5'-AAGTAAAACAGCTGTAATTAACACTGATCAA-3'	Torres et al., 2022

dsRNA injections and infection of L. geometricus

Once the appropriate time of silencing was confirmed, an assessment of immune response following bacterial infection was performed in an RNAi experiment. For this,

four groups of 20 adults, female *L. geometricus* were included: the first two groups served as the control groups, without the silencing of Lg-defensin and the second two groups served as the silenced Lg-defensin groups where one group was subsequently infected with gram-positive bacteria and the other group was subsequently infected with gram-negative bacteria. Before infections, spiders of the silenced/ infected group were injected with 10 µg of dsRNA (~2.5 µl) while the non-silenced/ infected group was injected with the same volume using an elution solution to account for the variable of puncture trauma. Groups were then left to incubate at room temperature until the appropriate time for silencing had passed, approximately 21 days. Next, the four groups were injected with either *Staphylococcus aureus* (gram-positive) or *E. coli* (gram-negative). Each bacterial species was prepared in an overnight culture of LB broth at 37°C, with shaking. The following day, cultures were removed and prepped as previously described in chapter 1 with an OD_{600} = ~0.2. Injections for the bacteria were performed using the same methodology as mentioned in chapter 1. After injections, spiders were then left for 24 h at room temperature before placing them individually in 1.5 mL tubes and storing them at -80°C for DNA extraction.

DNA Extractions

DNA extractions were performed using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany, EU) with a modified version of the "Purification of total DNA from ticks using the DNeasy® Blood & Tissue Kit for detection of Borrelia DNA"

protocol to account for the size of the spiders as well to ensure the specified bacterial DNA could be detected during qPCR. Spiders were first individually thawed for 10 min on ice followed by a series of washes using 1 mL of 70% ethanol and thrice with 1 mL of nuclease-free water, changing the 1.5 mL tube between each wash. After the series of washes, spiders were dried with sterile filter paper, and each spider was placed in a fresh 1.5 mL tube.

For spiders injected with *E. coli*, homogenization was performed using 0.9 mL of ATL buffer and sterilized scissors, followed by adding 100 µl of proteinase K, and incubating the mixture for 3 h at 56°C while being vortexed intermittently every 10 to 15 min. After the incubation, 200 µl of the sample was transferred to a fresh 1.5 mL tube for the continuation of the DNA extraction. In contrast, homogenization for spiders injected with *S. aureus* was performed using 0.9 mL of enzymatic lysis buffer and sterilized scissors followed by incubation for 1 h at 37°C. Enzymatic lysis buffer consisted of 20 µM of Tris-HCl (pH 8.0), 2 µM of EDTA-Na (pH 8.0), 1.2% of Triton X-100, and 20 µg/ mL of Lysozyme. The lysate was then transferred to a larger, 5 mL tube, and 125 µl of proteinase K was added to the sample. The sample was then incubated at 56°C for 3 h while being vortexed intermittently every 10 to 15 min. After the 3-hour incubation for both groups, 200 µl of the sample was transferred to a fresh 1.5 mL for continuation of the DNA extraction. The remaining protocol remained unmodified for both groups,

followed by a final resuspension using 200 µl of AE buffer and storing the extracted DNA at -20°C.

qPCR analysis of bacterial DNA presence

Using extracted DNA from infected spiders, the presence of bacterial DNA was assessed in relation to silenced versus unsilenced Lg-defensin using qPCR. Primers were designed to target highly conserved housekeeping genes specific to the bacteria represented as shown in Table 3. Experiments were performed using ig® SYBR Green qPCR 2x Master Mix (Intact Genomics, St. Louis, MO, USA) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The qPCR reactions consisted of 1 µl of DNA, 10.0 µl of SYBR Green, 0.5 µl of each primer (10 µM), and 8 µl of nuclease-free water. For representatives of the *E. coli* group, the qPCR program consisted of 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 20 sec and 60°C for 60 sec. For representatives of the *S. aureus* group, the qPCR program consisted of 1 cycle at 95°C for 8 min, followed by 40 cycles at 95°C for 15 sec and 59°C for 35 sec. Reactions were performed in triplicate and all quantification cycles (Cq) were represented as averages.

Cq values are determined by the detection of fluorescent signals, which are tagged to the amplified template by SYBR Green. The Cq value is defined as the number of cycles it takes for the reactions fluorescence levels to be higher than the background level of the machine. This value is inversely related to the amount of transcript present within

the sample, thus the higher the Cq value the lower the amount of transcript is available in the reaction and vice versa. Therefore, to determine the difference in relative bacterial load between silenced/ infected individuals and non-silenced/ infected individuals as it related to the type of bacteria groups were infected with, Cq values were averaged and statistically compared using a pooled t-test in JMP®, Pro 15.2.0 (SAS Institute Inc., Cary, NC, USA).

Table 3

Bacterial Primers for qPCR.

Bacteria	Name	Target	Sequence	Original Reference
S. aureus	Staph16S-1	*16S rRNA*	5'-CGGTCCAGACTCCTACGGGAGGCAGCA-3'	Eleaume and Jabbouri, 2004
S. aureus	Staph16S-2	*16S rRNA*	5'-GCGTGGACTACCAGGGTATCTAATCC-3'	Eleaume and Jabbouri, 2004
E. coli	ybbW401F	*ybbW*	5' -TGATTGGCAAAATCTGGCCG-3'	Walker et al., 2017
E. coli	ybbW611R	*ybbW*	5'-GAAATCGCCCAAATCGCCAT-3'	Walker et al., 2017

RESULTS

Validating silencing using dsRNA

To validate silencing, 24 spiders were separated into groups of four to represent various time points including 48 h, 72 h, 5 days, 7 days, 14 days, and 21 days after dsRNA injections. Each representative was injected with dsRNA and left to incubate for the allotted amount of time. For each group, one subject died from either possible injection trauma or a weakened immune system and two died from the 21-day group. Resolved PCR reaction results showed that the effects of silencing were not visualized until 14 days post injections, as seen in Figure 1, where bands began to become slightly fainter than previous time points. Although, complete suppression of Lg-defensin was not achieved even after 21 days. Despite this observation, it was still possible to conclude that the translation of mRNA was significantly decreased after 21 days.

As seen in Figure 1, 21 days post-injection showed only faint expression at the expected size using the validation primers. Furthermore, the quality of cDNA for the 21-day group was validated using the COI primers showing that the cDNA was sufficiently synthesized. Reasons for incomplete silencing could be due to the constitutive expression patterns of humoral immune effector proteins in arachnids, such as Lg-defensin in *L. geometricus* (Bechsgaard et al., 2016). Additionally, due to a limited supply of adult,

female *L. geometricus*, experimenting with additional methodologies to improve silencing could not be performed.

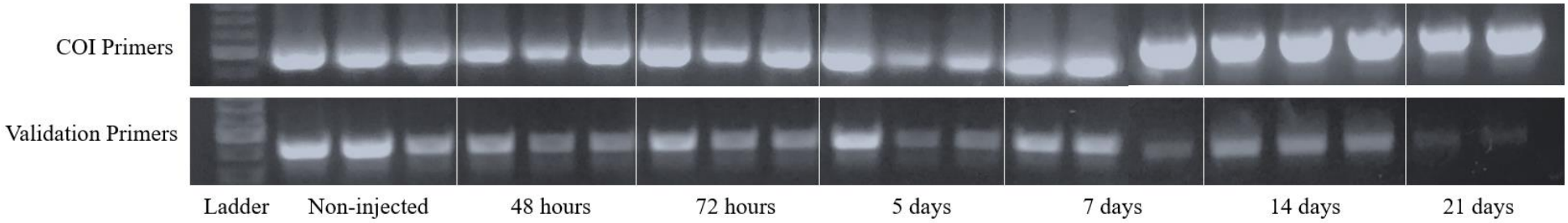


Figure 1. Validating *in vivo* silencing of Lg-defensin over a 21 day period through PCR and gel electrophoresis. Samples are divided by the specific time point they represented. Each pair of bands on both the top and bottom line correspond with the same cDNA sample to test both the quality of the cDNA and if silencing is occurring. The top line represents PCRs using the COI primer set to test the proficiency of the cDNA. The bottom line represents PCRs using the validating primer set to determine if Lg-defensin was silenced.

Functionally characterized Lg-defensin using RNAi and qPCR

To observe if Lg-defensin had antimicrobial activity, the presence of bacterial DNA during infection of Lg-defensin silenced spiders was quantified using qPCR. Average Cq values were used as an indicator of bacterial DNA amount and values were compared between non-silenced/infected individuals and silenced/ infected individuals. High Cq values indicated less bacterial DNA present while low Cq values indicated more bacterial DNA present. Results were then plotted on a box plot using JMP shown in figure 2. For the first round of injections, 40 individuals were subjected to dsRNA injections and 40 individuals were subjected to an elution solution following a 21-day incubation. Survival from both groups averaged ~61%, with 24 surviving after dsRNA injections and 25 surviving after elution solution injections. Groups were then divided into two, with one representing *S. aureus* infections and the other representing *E. coli* infections.

For *S. aureus* infections, 11 of 13 spiders survived in the silenced/ infected group (85% survival rate) and 13 of 14 survived in the non-silenced/ infected group (93% survival rate) after 24 h. Due to limited reagents, 9 individuals were chosen at random from each group for DNA extraction and qPCR. The mean Cq value for silenced/ infected individuals was 15.45 with a standard deviation of 0.70. In contrast, the mean Cq value for non-silenced/ infected individuals was 17.25 with a standard deviation of 0.79. After the pooled t-test in JMP, it was determined that there was a statistically significant

difference between average Cq values of silenced/ infected versus non-silenced/ infected individuals after an *S. aureus* infection ($p<0.0001$) with a 95% confidence interval. This finding indicated that there was a higher presence of bacterial DNA in the silenced/ infected group as compared to the non-silenced/ infected group.

For the *E. coli* infections, 11 of 11 spiders survived in the silenced/ infected group (100% survival rate) and 9 of 11 spiders survived in the non-silenced/ infected group (82% survival rate) after 24 h. As in the *S. aureus* groups, only 9 individuals were chosen at random from the silenced/ infected group for DNA extraction and qPCR. The mean Cq value for silenced/ infected individuals was 19.85 with a standard deviation of 0.30. In contrast, the mean Cq value for non-silenced/ infected individuals was 19.47 with a standard deviation of 0.26. After the pooled t-test in JMP, it was determined that there was a statistically significant difference between average Cq values of silenced/ infected versus non-silenced/ infected individuals after an *E. coli* infection ($p<0.01$) with a 95% confidence interval. Interestingly, this finding was different from *S. aureus* infections with a higher presence of bacterial DNA in the non-silenced/ infected group versus the silenced/ infected group. Although, note the mean Cq values are also much larger than the gram-positive group as well indicating a lower presence of bacteria after 24 h.

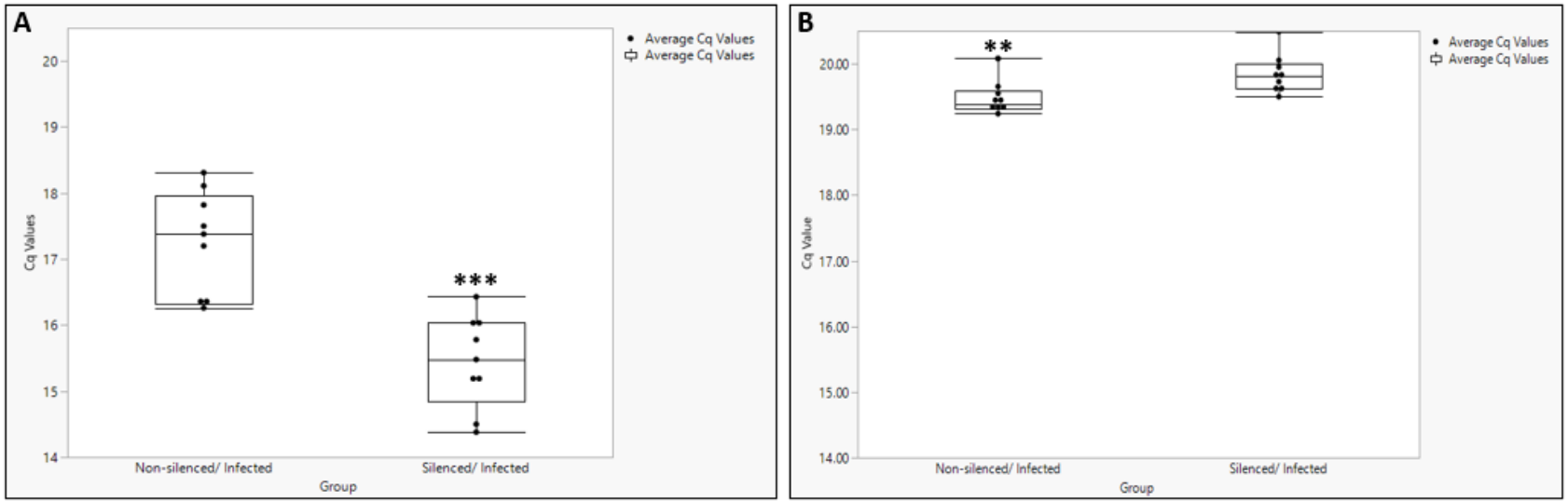


Figure 2. Comparisons of presences of bacterial DNA 24 h post infection in silenced/ infected and unsilenced/ infected spiders via qPCR. Box plots represent each group with individual plot points representing the average Cq value for each spider. ***Panel A*** shows non-silenced/ infected spiders on the left and silenced/ infected spiders on the right after a 24-hour *S. aureus* infection. Differences in average Cq values were determined using a pooled t-test and found presences of bacterial DNA to be significantly higher in the silenced/ infected group versus the non-silenced/ infected group (*** $p<0.0001$). ***Panel B*** shows non-silenced/ infected spiders on the left and silenced/ infected spiders on the right after a 24-hour *E. coli* infection. Differences in average Cq values were determined using a pooled t-test and found the presences of bacterial DNA to be significantly higher in the non-silenced infected individuals than in silenced/ infected individuals (** $p<0.01$).

DISCUSSION

In many invertebrates, defensins have been described to contribute significantly to the immune response against invading pathogens (Contreras et al., 2020). Being a short, cysteine rich peptide, defensins are also one of the best described AMPs within the animal kingdom (Bulet et al.,2004; Contreras et al. 2020). However, information regarding defensins in spiders is scarce. In this study, the Lg-defensin peptide of *L. geometricus* was functionally characterized using RNAi followed by qPCR analysis to test the impact of silencing on bacterial infection. Predictions based on closely related defensins indicated that Lg-defensin played a significant role in bacterial immune response, and if reduced or not present during infection, would cause a significant difference in the presence of bacterial DNA versus if it was left unaffected. Findings from this study suggest that Lg-defensin does play a role in responding to bacteria like other, closely related taxa defensins such as those in ticks, scorpions, and dragonflies, all of which contain representatives of the ancestral class of invertebrate defensins (de la Vega et al., 2005).

Silencing Lg-defensin significantly affected the presence of *S. aureus* DNA versus individuals in the non-silenced/infected group. This finding is not surprising considering Lg-defensin also resembled a similar protein sequence and structure to the other, previously described antibacterial defensins with high affinity to gram-positive

bacteria (unpublished). Notable defensin-like peptides of spiders however, like Oh-defensin, do not match these functional observations indicating a possible divergence between types of defensins that can be found in spiders. This variance could be due to the differences in the location of expression, protein sequence, or the functional purpose within the species. In addition, it was determined by Khamtorn et al., (2020) that *L. geometricus* harbors defensins in the venom glands, like the location of expression for Oh-defensin, but the characterization of these defensins was not presented. Future studies should aim to describe the location from which Lg-defensin is being expressed within *L. geometricus* to further determine functional relevance and establish if *L. geometricus* harbors multiple kinds of defensin peptides possibly in the venom glands and hemocytes.

However, aside from published spider defensins, results from this study complement the findings of previous invertebrate defensin studies. While there are exceptions, many defensins relative to Lg-defensin had a high affinity to targeting bacteria, specifically gram-positive bacteria (Bulet et al., 1992; Ehret-Sabatier et al., 1996; Nakajima et al., 2002; Bulet et al, 2004; Saito et al., 2009; Wang et al., 2015; Sun et al., 2017; Contreras et al. 2020; Li et al., 2021). For example, defensins of *Dermacentor silvarum,* Ds-defensins, showed significant antimicrobial activity against various gram-positive bacteria, but was less effective in inhibiting gram-negative bacteria (Wang et al., 2015; Li et al., 2021). Similar results were also found in other hard tick defensins such as *Ixodes persulcatus* and *Haemaphysalis longicornis* and soft tick defensins such as *Ornithodoros robata*, with more antibacterial activity targeted at gram-

positive bacteria (Nakajima et al., 2002; Saito et al., 2009; Sun et al., 2017). Other representatives include a scorpion defensin of *Androctonus australis* belonging to the family of anti-gram positive invertebrate defensins and a dragonfly defensin of *Aeschna cyaneaa* which was strongly active against gram-positive bacteria (Bulet et al., 1992; Ehret-Sabatier et al.; 1996).

In contrast, the presence of *E. coli* DNA seemed to have an inverse correlation between groups, where more *E. coli* DNA was found in those of the non-silenced/infected group than of the silenced/infected group with a $p<0.01$. Reasons for this could include that Lg-defensin may not even have a significant role in targeting gram-negative bacteria, considering how large the Cq values were as compared to the *S. aureus* groups. One study that successfully silenced varisin, a major tick defensin in *Dermacentor variabilis,* found that while the inhibitory activity of varisin was reduced, silenced ticks were still able to inhibit the growth of the bacteria tested (Hynes et al., 2008). Thus, they assumed that the antimicrobial activity was mediated by circulating lysozymes, a large family of hydrolytic enzymes that target the peptidoglycans of bacterial cell walls (Hynes et al., 2008; Bechsgaard et al., 2015; Coates et al., 2022). Several genes encoding for lysozymes have been found in various spider genomes, including a close relative *P. tepidariorum,* and unlike most invertebrate defensins, lysozymes not only target gram-positive bacteria but also gram-negative bacteria (Bechsgaard et al., 2015; Coates et al., 2022). Therefore, one hypothesis could be that

because there was a decreased presence of circulating defensins it increased the need for other circulating effector molecules like lysozymes.

To add to this hypothesis, it would also be interesting to observe the different interactions that take place within various signaling pathways after silencing Lg-defensin. While it is true that AMPs are released after recognition of intruding pathogens, the expression and release of AMPs in invertebrates are also due to the activation of signaling pathways such as the Toll pathway or the immune deficiency (IMD) pathway, both of which are involved in responding to gram-negative bacteria (Bechsgaard et al., 2015; Coates et al., 2022). Therefore, this upregulation of lysozymes or other unknown AMPs could be due to these pathways detecting an inhibition to express adequate amounts of Lg-defensin. However, not much is known about these pathways in spiders, and many of the essential genes described for these signaling pathways in insects are missing in arachnids, indicating an evolutionary divergence in response to pathogen invasion (Bechsgaard et al., 2015; Viljakainen, 2015). While this problem is out of the scope of this research, it will be interesting to see the development of information regarding the internal molecular immune responses of spiders in the future.

Evidence from this study suggests that Lg-defensin does have antibacterial activity. This is the first functionally characterized defensin in *L. geometricus.* Future projects should aim at purifying the protein and performing antimicrobial assays including antibacterial assays testing more strains of gram-positive and gram-negative

bacteria, such as antibiotic-resistant strains, and antifungal assays to test for a broader antimicrobial spectrum. Results from these studies could predict if Lg-defensin could be a plausible candidate as an alternative to antibiotics.

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