COMPARATIVE IMMUNOLOGY FROM LYMPHOID TISSUES IN CARTILAGINOUS FISH TO BOVINE ULTRALONG IMMUNOGLOBULIN PHYLOGENY

A Thesis

by

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ABSTRACT

Cartilaginous fish are located at a pivotal point in phylogeny where the adaptive immune system begins to resemble that of other, more-derived jawed vertebrates, including mammals. For this reason, sharks and other cartilaginous fish are ideal models for studying the natural history of immunity. The cartilaginous skeleton of sharks lacks bone marrow, also absent in bony fish despite calcified bone, but cartilaginous fish have other organs that function to provide hematopoiesis. Conserved across all vertebrate phylogeny in some form is gut-associated lymphoid tissues (GALT) which is seen from agnathans to mammals. Though it takes many forms, from typhlosole in lamprey to Peyer's patches in mammals. Though more complex lymphoid organs are not present in agnathans, they have several primitive tissues that appear to serve their variable lymphocyte receptor-based adaptive immune system. There are several similarities between the adaptive immune structures in cartilaginous and bony fish, such as the thymus and spleen, but there are mechanisms employed in bony fish that in some instances bridge their adaptive immune systems to that of tetrapods. Cattle has a restricted repository of antibody gene segments usable for antibody formation. Among these segments are a V and D that, together, form an antibody with an ultralong CDR3 region that manifests as a "knob" and "stalk" domain of which the knob has antigen binding capability. This antibody has boundless therapeutic potential, including for treatment of HIV and other antigens with veiled epitopes. The ultralong antibody appears in more species than just *Bos taurus*; it expands throughout the bovine subfamily as evidence by the presence of a V motif "TTVHQ", an 8 base-pair (bp) duplication, and a, comparatively, long D segment. The evolutionary event which led to the appearance of the ultralong antibody can only be speculated at this time. What we know for sure is that the cattle ultralong antibody is an invaluable tool in the bovine immunity toolbox.

DEDICATION

I'd like to dedicate this thesis to my parents, Connie and Clay Mitchell, as well as my fiancé, Morgan Brown. Your continuous support and love were essential in achieving this milestone.

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Contributors

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NOMENCLATURE

GALT	Gut-Associated Lymphoid Tissue
MHC	Major Histocompatibility Complex
APC	Antigen Presenting Cell
CD	Cluster of Differentiation
T_h	Helper T Cell
MALT	Mucosal Associated Lymphoid Tissue
CSR	Class-Switch Recombination
SHM	Somatic Hypermutation
Ig	Immunoglobulin
AID	Activation-Induced Cytidine Deaminase
RAG	Recombination-Activating Gene
TCR	T Cell Receptor
VLR	Variable Lymphoid Receptor
PCR	Polymerase Chain Reaction
CDR	Complementarity-Determining Region
BCR	B Cell Receptor
IgL/IgH	Immunoglobulin Light Chain/ Immunoglobulin Heavy Chain
FW	Framework
HIV	Human Immunodeficiency Virus
RSS	Recombination Signal Sequence
CDA	Cytoside Deaminase
CDRH	Complementarity Determining Region of the Heavy Chain
RAG	Recombination-Activating Genes
TEM	Transmission Electron Microscope

TEC	Thymic Epithelial Cells
TdT	Terminal Deoxynucleotidyl Transferase
PALS	Periarteriolar Lymphoid Sheath
NAR	New/Novel Antigen Receptor
APOBEC	Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-Like
SALT	Skin-Associated Lymphoid Tissue
VLR	Variable Lymphoid Receptor
SB	Supraneural Body
IgHV	Variable Segment of the Immunoglobulin Heavy Chain
IgHD	Diversity Segment of the Immunoglobulin Heavy Chain
IgHJ	Joining Segment of the Immunoglobulin Heavy Chain

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I: INTRODUCTION

1.1 Immunity

Immunity is the process of identifying and eliminating potential threats to the body. Whether this threat is a bacterium, a parasite, or an allergen, the immune system is equipped to protect the function of all systems. Within immunity, there are two major branches, innate and adaptive immunity [1]. Innate immunity is the first line of defense. It includes physical barriers, such as the skin, innate cells called granulocytes, a series of serum proteins called the complement system, as well as physical changes in the body such as fever and swelling [1]. The primary goal of the innate system is to destroy the pathogen, even if that means risking our own body cells to do so. The adaptive immune system is more strategic. It uses specialized cells to mark pathogens as well as other cells to destroy pathogens more specifically [2]. These specialized cells also have memory, so they may attack the pathogen quicker and more efficiently upon secondary exposure [3].

The adaptive immune system is further divided into two arms, humoral and cell-mediated immunity [2]. Humoral immunity centers around antibody and B cell processes for indirect elimination of microbes by marking them for apoptosis, initiating the complement cascade targeting the microbe, and by the neutralization of toxins released by the microbe [2]. Cell-mediated immunity revolves around T cells and their effector functions including cytotoxicity, cytokine secretion for opsonization and macrophage activation, as well as regulation of future T cell responses [2]. These two arms often overlap or interact with one another throughout the immune response. Both arms utilize the phagocytic capabilities of macrophages and dendritic cells as well as the signaling properties of cytokines [2]. As mentioned before, an effector function of B cells is to activate the complement cascade of the innate immune system showing an intermingling of the two arms of adaptive immunity [2].

1.2 Lymphocytes: B and T cells

Lymphocytes are the cells utilized in adaptive immunity and are divided into two categories, B and T cells. These cell types differ in several ways from their receptor organization to their effector function. B cell receptors, also known as immunoglobulins (Ig) or antibodies if secreted, are composed of

two identical heavy and two identical light chains [4]. Each chain is divided into regions, variable (V) and constant (C), together these two regions form the characteristic Y-shaped structure (Figure 1.1) [4]. The variable region contains the antigen binding domain where the constant region defines the isotype of the immunoglobulin. There are five isotypes (IgM, IgD, IgG, IgE, and IgA) each with their own specific target and function [5]. Immunoglobulins can be presented on the surface of a B cell or secreted by an activated B cell, known as a plasma cell [6, 7]. T cells, also known as thymocytes, are divided into two major subgroups, helper and cytotoxic T cells, based on which surface marker they display [8]. Helper T cells (T_h) display CD4 and cytotoxic, killer, T cells display CD8. These surface markers interact directly with MHC but not with the antigen [2]. The T cell receptor (TCR) has contact with antigen directly, but only when the antigen is bound to MHC on an antigen presenting cell [9]. The TCR is composed of two, membrane bound chains [10, 11]. There are four different chains used to assemble the TCR, α, β, γ, and δ, that only combine as αβ and γδ (Figure 1.1) [10, 11]. TCRs cannot be secreted by T cells, although, T cells do secrete a molecule called a cytokine to perform effector functions such as opsonization, macrophage activation, inflammatory triggers, and so on.

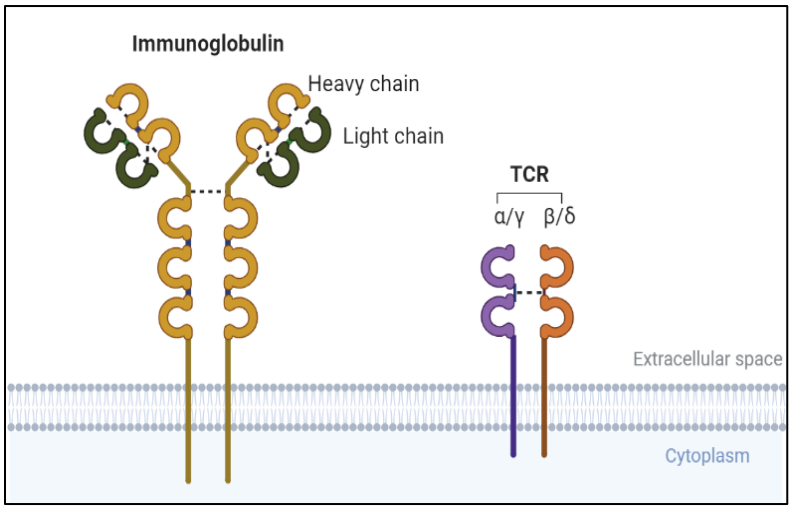

Figure 1.1 Immunoglobulin and TCR structure. The two identical heavy chains (yellow) and two identical light chains (green) form the immunoglobulin. The α/γ chain (purple) combines with a β/δ chain (orange)to give rise to the TCR (figure created in Biorender).

1.3 Lymphoid tissues

Lymphoid tissues are divided into two groups, primary and secondary, based on function. Primary, or central, lymphoid tissues are sites of immune cell development and maturation. The exact organ or tissue varies between phylogenetic groups, but the thymus is generally the site of T cell development and bone marrow for B cell development [2]. It is in the thymus that T cells undergo selection, driven by thymic epithelial cells (TEC), to ensure recognition and adequate affinity for self-MHC and non-reactivity to self-antigen [2, 12]. B cells in the bone marrow are selected by bone marrow stromal cells to define central tolerance which eliminates self-reactive B cells. Secondary, or peripheral, lymphoid tissues for example the spleen, lymph nodes, and gut-associated lymphoid tissue (GALT), are sites of antigen exposure and sites of lymphocyte migration to fight infection [2]. Here, naïve lymphocytes encounter antigen and are either activated or inactivated depending upon the affinity to the antigen [2]. Activated cells clonally expand, making copies of itself to fight the pathogen, or, in B cells, become antibody secreting plasma cells [6]. Cells that do not bind antigen become inactive and enter a state of clonal anergy, where they unresponsive but not dead [13]. Cells created by clonal expansion may also become memory cells for future exposure of the same antigen [6].

1.4 Receptor formation

In adaptive immunity, specificity to antigen is of key importance. This specificity is due to a vast diversity of antigen binding receptors. A series of processes including recombination, rearrangements, and mutations, create a diverse repertoire of receptors. In receptor formation, the first step is recombination where gene segments, variable (V), diversity (D) and joining (J), come together to form a complete antigen binding domain (Figure 1.2) [14]. Somatic recombination is catalyzed by two recombination-activating genes (RAG) which cleave the recombination signal sequences (RSS) on either side of the V, D, and J segments, then terminal deoxynucleotidyl transferase (TdT) and DNA ligase fuse the ends of the segments together for a complete gene [14, 15]. This process itself creates diversity, called combinatorial diversity, as there are a variety of V, D, and J segments encoded in the genome and they

can be grouped together in a number of different combinations [14]. There is also junctional diversity induced by the insertion of random nucleotides, P- and N-nucleotides, added to the end of each gene segment. Activation-induced cytidine deaminase (AID) catalyzed somatic hypermutation (SHM) induces mutations that can modify anywhere from a single base to a few amino acids to further diversify the antibody repertoire [16, 17]. The goal of the random mutations is to alter the antibody enough to improve antigen biding affinity but this is not always the case as sometimes the mutations render the receptor ineffective [16]. These mechanisms create a diverse repertoire of antigen binding receptors prepared to defend the body against a variety of pathogens.

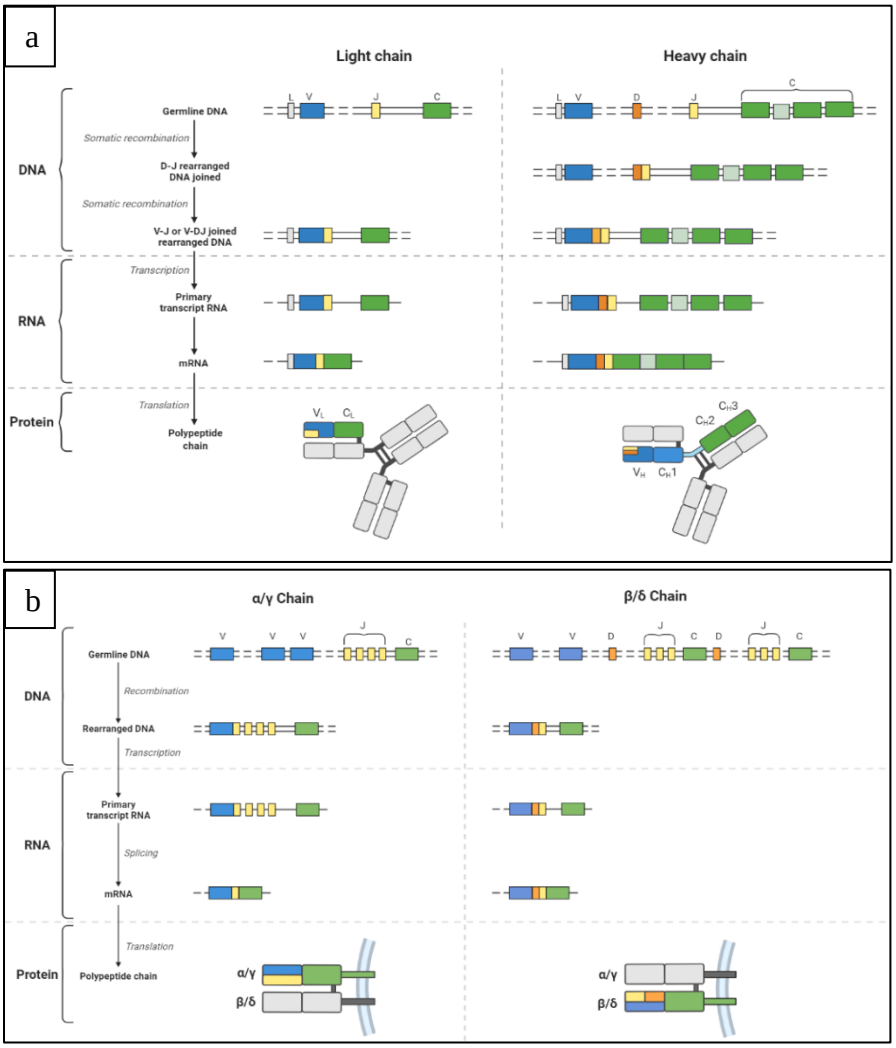

Figure 1.2 Somatic recombination in T and B cell receptors. (a) TCR recombination with V (blue), D (orange), J (yellow) and C (green) segments. The D segment is only present in the β and δ chains. (b) Immunoglobulin recombination is similar to TCR recombination with only the heavy chain portion of the antibody containing a D segment (figure created in Biorender).

1.5 Comparative vertebrate immunology

Comparative vertebrate immunology investigates the immune systems of vertebrate species other than common model organisms, such as mice and humans. This umbrella includes every species from jawless fish, lamprey and hagfish to mammalian species, such as cattle and sheep [18]. The more primitive species, jawless and cartilaginous fish, display immune characteristics that mirror those of more evolved groups but in a more simplistic form [19]. Cartilaginous fish, a group that includes sharks, rays, skates, sawfish, and chimeras, has the same basic components to their immune system as mammals [19]. Their lymphocytes, lymphocyte receptors, thymus structure, and MHC all strongly resemble that of mammals, and, therefore, cartilaginous fish mark the phylogenetic emergence of the adaptive immune system as seen in humans.

1.6 Gut-associated lymphoid tissue (GALT) species

Gut-associated lymphoid tissue (GALT) is considered a secondary lymphoid organ meaning it is a site of antigen exposure and antibody activation. In certain species, called GALT species, the lymphoid tissues of the gut have a more prominent role in B cell development than in other species. These species include rabbits, cattle, pigs, sheep, and chickens [20, 21]. Studies in rabbits show that gene conversion and somatic hypermutation as well as positive and negative selection of B cells occur in the appendix [22]. The appendix of young rabbits is the primary site of further antibody diversification forming a primary naïve repertoire, much like in the bursa of Fabricius in chickens. The microflora of the intestines is thought to play a major role in the diversification of antibodies and the formation of a functional repertoire in the GALT [23, 24, 25]. Studies in rabbits show that select members of the microflora naturally present in the intestine are essential, not only for GALT development but also for driving VDJ rearrangement [26].

Though rabbits are the most commonly studied GALT species, cattle also have several of these characteristics in the development of antibodies, including the role of microflora in diversification and the involvement of the GALT in antigen exposure [27].

1.7 Bovine antibodies

Cattle immune systems are functionally similar to other mammals. They have the same general cellular components, receptors, and diversification mechanisms, but they have a much more limited repertoire of gene segments available for V(D)J somatic recombination compared to other mammals. This restricted repertoire could be the trigger for the expression of a unique antibody called an ultralong CDR3H antibody [28, 29], but the exact trigger is not known. This ultralong antibody is unique in structure and function. It gets its name from an extended CDR3 region of the heavy chain creating a "stalk and knob" structure for the antigen binding domain [28]. The structure of the knob is utilized to bind the antigen itself where the stalk provides length and flexibility to the domain to allow the knob to reach epitopes that are unreachable by canonical antibodies [30]. It is this characteristic that has shown the promise of this antibody in therapeutics. Studies show that the ultralong antibody is effective in binding a portion of the human immunodeficiency virus (HIV) retroviral envelope directly [31]. Canonical antibodies do not have this capability and cannot bind the HIV retrovirus at any site other than the frequently mutating envelope proteins [31]. Studying bovine ultralong antibodies could lead directly to an extremely effective treatment for one of the world's deadliest diseases.

CHAPTER II

COMPARATIVE STUDY OF CARTILAGINOUS FISH DIVULGES INSIGHTS INTO THE EARLY EVOLUTION OF PRIMARY, SECONDARY AND MUCOSAL LYMPHOID TISSUE ARCHITECTURE*

2.1 Introduction: sharks and the evolution of immunity

2.1.1 Adaptive immunity

An adaptive immune response is initiated by one or both of two signals: the innate immune system being overpowered by the accumulation of antigen and lymphocyte antigen receptor signaling pathways. There are some pathogens that can be conquered by the innate immune system, but many pathogens require the intervention of the adaptive immune system in order to be cleared. Adaptive immune responses are activated in peripheral lymphoid tissues, such as spleen, lymph node, and GALT where naïve lymphocytes first encounter antigen and clonally proliferate. The mitotic lymphocyte proliferation maintains specificity for antigen and creates memory cells against that antigen, providing the two hallmark characteristics of adaptive immunity.

Adaptive immunity has two major arms, cell-mediated immunity and humoral immunity. Cell-mediated immunity begins with naïve T cells, lymphocytes that have not been exposed to the antigen that is specific to their antigen receptor [1, 2]. These naïve T cells will circulate through the lymphatics, blood, and peripheral lymphoid tissues in search of antigen until the specific antigen is found [32]. If the naïve lymphocyte does not find antigen, it will undergo apoptosis or programmed cell death. Once the naïve T cell has found specific peptide antigen presented by major histocompatibility complex (MHC) on a dendritic cell, or another activated antigen presenting cell (APC) expressing costimulatory signals, it no longer circulates through the vasculature and lymphatics and sustains a long proliferative phase, called clonal expansion, which includes differentiation into effector and memory T cells [2, 9]. These clonal

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descendants have the same antigen specificity as the original naïve T cell. The effector T cells enter the blood stream and migrate to the site of infection to perform their effector functions [2]. There are two major classes of adaptive (generally αβ T cell receptor-bearing) T cells: cytotoxic T cells, which don the coreceptor CD8, and helper T cells, which have the CD4 coreceptor [1, 2]. Cytotoxic T cells target and kill cells infected by intracellular pathogens such as viruses or intracellular bacteria. These target cells present peptide antigen on MHC class I on the cell surface to cytotoxic T cells which are MHC class I restricted [1, 2, 12]. Helper, or CD4, T cells have several further subsets that carry out different functions via cytokines [1, 2]. T_h1 cells help to control bacteria that persist in macrophages by evading traditional killing mechanisms of the phagocyte. The helper T cells activate infected macrophage microbicidal functions by recognizing peptide bacterial antigen being presented by MHC class II [9, 12]. T_h2 cells operate to control infections by parasites by activating and promoting eosinophil responses and instructing B cells to class-switch to the IgE isotype of antibody [1, 2, 12, 33]. T_h17 cells function to stimulate neutrophil responses and inflammation to protect against extracellular pathogens such as bacteria and fungi and can stimulate maintenance of epithelial barriers in the gut [1, 2]. Antigen presented to helper T cells generally must be presented by professional APCs (macrophages, dendritic cells, and B cells) using class II MHC, because CD4 T cells are MHC class II restricted [12]. MHC class II is not ubiquitously expressed, but MHC class I is [9].

2.1.2 Lymphoid tissues in mammals

Adaptive immunity requires lymphocytes to have interactions with antigen presenting cells and other lymphocytes in order to perform with regulated specificity and memory. These interactions occur in specialized sites in the body termed lymphoid tissues [2]. Lymphoid tissues are broadly defined as any tissue in the body where an immune reaction or lymphocyte maturation takes place, largely based on studies in mammals [1, 2]. Lymphoid organs, first seen in jawed vertebrates, are thought to have evolved for the purpose of facilitating antigen-receptor gene assembly [34]. They are split into two main types, central (primary, or generative) and peripheral (secondary) lymphoid tissues [1, 2].

Primary lymphoid tissues facilitate maturation of lymphocytes, bone marrow for B lymphocytes and thymus for T lymphocytes in mouse and man, as well as generating and shaping the repertoire of receptors for each cell type [1, 2, 12]. All lymphocytes originate from hematopoietic stem cells in the adult mammalian bone marrow [35]. Early thymocyte progenitors then migrate to the thymus and B lymphocyte precursors stay in the bone marrow for maturation. For both B and T cells, tests for autoreactivity occur before migration out of the primary lymphoid tissue and again in secondary tissues as mature naïve cells post-migration [1, 2, 36, 37]. B cells that do not react to self-antigen in the bone marrow survive and are said to have passed central tolerance [36]. Upon leaving the bone marrow and migrating to the secondary tissues, such as the spleen, transitional B cells, which are not fully mature but have intact receptors, are exposed to self-antigen again [1, 2, 36]. If the cells have too strong or too weak receptor signaling upon second exposure to antigen, they have not established peripheral tolerance and will not fully mature. Should the cells fail, they will either die or enter clonal anergy [13].

Mammalian T cell progenitors travel to the thymus from the bone marrow to mature. The thymus contains two anatomically distinct areas, the densely packed outer cortex and the exiguous inner medulla [1, 2, 38]. This organization is conserved throughout jawed vertebrate evolution. The thymus contains many cell types from thymocytes (developing T cells) to stromal cells (epithelial cells of the thymus) to intrathymic dendritic cells and macrophages [38, 39, 40]. The cortex contains immature thymocytes and sparse macrophages, whereas the medulla has more mature thymocytes and larger amounts of macrophages and dendritic cells [39, 40]. Thymocyte maturation mirrors that of B cells in sequentially testing the antigen receptor for functional chains, signal transduction capacity and self-tolerance [12]. In the T cell maturation process, positive selection additionally selects αβ lymphocytes that recognize self-allelic forms of MHC, instructing cytotoxic or helper subset differentiation based on class I or class II recognition, respectively [12, 41]. If the cell reacts weakly to presented self-antigen in the context of self MHC, it is positively selected to survive in the cortex [12, 41]. Lymphocytes that react strongly to self-antigen are negatively selected and

eliminated by programmed cell death (apoptosis) in the medulla [12, 41]. Approximately, only about 2% of T cells are successful in the maturation process and go on to differentiate into mature naive subsets [2].

Once the lymphocytes have completed maturation and passed selection, they migrate to the secondary lymphoid tissues such as the spleen, lymph nodes and mucosal-associated lymphoid tissues (MALT). In these tissues, lymphocytes become activated upon exposure to antigen specific for their antigen receptor paratope.

Peripheral lymphoid tissues have distinct areas of T and B cell residency as well as besprinkled macrophages, dendritic cells, and stromal cells. The spleen contains specific lymphoid areas called white pulp that contain areas concentrated with T cells (periarteriolar lymphoid sheaths) and B cells (follicles) [42, 43]. Germinal centers can develop in these B cell follicles, where follicular dendritic cells present native antigen complexes to B cells. Marginal zones of the spleen, locations between red and white pulp, also contain APCs such as dendritic cells and macrophages [42, 44, 45]. Germinal centers are the location of genetic alterations such as class-switch recombination (CSR) and affinity maturation by somatic hypermutation (SHM) [44]. If the IgM made by the B cell is not the most effective functional class for a particular threat, CSR replaces the heavy chain constant region of that Ig to another Ig isotype, so the receptor may go from IgM to IgG, IgA or IgE [16]. Affinity maturation via SHM occurs when the rearranged V-region genes of the heavy and light chains of an Ig are bombarded with point mutations to produce a higher affinity antibody [17]. This is not always the result; SHM can give rise to a higher, lower, or identical affinity [17], but competition for antigen and survival signals selects for the higher affinity in the iterative germinal center reaction. Lymph nodes are small, lumpy peripheral lymphoid tissues for antigen concentration [46]. They have multiple lobes and are enclosed by a tissue capsule [46]. The body has a network of lymphatic vessels that carry lymph, a liquid containing antigenic material as well as immune cells, to the lymph nodes from all areas of the body [47]. Lymph nodes are packed with lymphocytes, macrophages, and other APCs [48]. It is here where the APCs will present antigen to naïve antigen specific lymphocytes which will clonally expand to increase numbers of functional lymphocytes [49].

Mucosal associated lymphoid tissues (MALTs), such as respiratory epithelium, tonsils, and Peyer's patches of the intestine, provide physical barriers, points of entry, and lymphoid functions to combat pathogens [50, 51]. MALTs are arranged in a lymph node like structure with B cell follicles and T cell zones. Peyer's patches have specialized epithelial tissue, called follicle-associated epithelium (FAE), that lines the domes of the patch and houses T lymphocytes near the microfold (M) cells which transport antigen across the epithelial barrier [52]. A specific antibody, IgA, is restricted to mucosal immune tissues and plays a major role in mucosal immunity [51, 53]. IgA can be transported across the mucosal epithelium and secreted into the lumen in the form of secretory-IgA (sIgA) where it, then, binds antigen in the lumen or on the luminal surface of the mucosal tissue [51, 53].

2.1.3 Sharks

"Cartilaginous fish" is an umbrella term for the Chondrichthyes, that encompasses sharks, rays, skates, sawfish, and chimeras. All of these vertebrates have common characteristics of jaws, paired fins, gills, and a skeleton made of cartilage instead of bone.

Cartilaginous fish are the oldest group of living jawed vertebrates that have adaptive immune characteristics and lymphocyte antigen receptors similar to mammals, and, therefore, lie at a pivotal point in the evolution of the immune system [19]. They are the oldest group to have a polymorphic, polygenic MHC [54]. Cartilaginous fish are also the first group, phylogenetically, to have a true thymus whose structure was maintained, for the most part, throughout vertebrate evolution [34]. All of these indicate that cartilaginous fish mark the emergence of adaptive immunity as it is recognized throughout jawed vertebrate evolution, making their immune characteristics potential representatives of the ancestral building blocks of the system.

There are some peculiar immune characteristics seen in cartilaginous fish. For example, the genes for immunoglobulin (Ig) heavy and light chains are in many multicluster loci with single V, D, J, and C segments [55] which is seen in bony fish light chain loci as well [56], as opposed to the single large translocons containing many V(D)J segments in recent vertebrates. Without the multicluster organization

of cartilaginous fish Ig loci the evolution of unique new antigen receptors (IgNAR and NARTCR) [57, 58, 59] as well as interactions between immunoglobulin genes with T cell receptor (TCR) genes, potentially, could not have occurred [60]. Classical MHC class I genes (UAA) are observed in many cartilaginous fish as well as some unique class I MHC genes such as UBA in nurse shark and horn shark [61, 62, 63] and UCA in dogfish [61, 64]. Recent studies in nurse shark and horned shark show another nonclassical MHC class I gene, UDA present, to a lesser extent than the other nonclassical MHCs, in most organs [65].

Cartilaginous fish do not have bones or bone marrow but have other organs responsible for the development of blood cells and the maturation of B cells. The head kidney (anterior kidney, pronephros) is responsible for red blood cell production in addition to the spleen, which is also a site for antigen concentration [66]. The organs responsible for B cell maturation are the epigonal organ attached to the gonads [66] and Leydig's organ in the mid-dorsal area of the shark's body, associated with the esophagus [66]. Similar to other taxa of vertebrates, the T cells of sharks and rays mature in the thymus. The thymus originates from the pharyngeal pouches above the gills and, unique to fish, stays near each gill for the entirety of the fish's life. In humans, the thymus originates from the pharyngeal pouches but migrates to the anterior superior mediastinum and forms a single organ directly behind the sternum and in front of the heart.

Sharks are the most phylogenetically distant group from mammals to have immunoglobulins, long-term antibody-driven immunological memory [67], activation-induced cytidine deaminase (AID)-mediated somatic hypermutation (SHM), and recombination-activating gene (RAG) mediated somatic recombination. Recombination via RAG is the mechanism used in primary lymphoid tissues to form lymphocyte antigen receptors (e.g., TCRs and immunoglobulins) and this process is first seen in cartilaginous fish. AID catalyzes somatic hypermutation for affinity maturation and related APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) family members diversify variable lymphocyte receptors (VLR) in lamprey [68]. The AID gene and its immunogenetic activity is first seen in sharks, indicating its origin in cartilaginous fish.

2.2 Cartilaginous fish lymphoid tissues

2.2.1 Primary tissues in shark (and skate/ray)

The primary lymphoid organs seen first, in a primitive form, in jawless fish [69], gained complexity in cartilaginous fish and continued throughout the radiation of vertebrate evolution. As the name implies, cartilaginous fish have a cartilage frame as opposed to all more derived vertebrates which have bones. Several tissues contribute to B lymphopoiesis in cartilaginous fish, and different species employ them to varying degrees.

2.2.1a) B cells in cartilaginous fish

Shark B cells are similar in appearance and antibody production to those of more evolutionarily recent vertebrates such as fish and mammals. In fact, sharks are the oldest group, phylogenetically, to have these B cells [70].

Sharks possess a variety of Igs smaller than that of recent vertebrates, some of which are the same as or similar to those of mammals. IgM is an isotype seen in sharks as well as mammals, but the other two, IgW and IgNAR, are not like the immunoglobulins in mammals, although they may serve similar functions [70, 71]. Primitive vertebrates, cartilaginous fish and lungfish, have IgW which resembles IgD in recent vertebrates with similarity in constant domain sequence and transmembrane portion suggesting that these heavy chain isotypes originate from the same ancestor [72]. IgNAR is an isotype that has two disulfide bonded heavy chains not associated with light chains, forming a homodimer that is exclusive to cartilaginous fish [57, 73, 74]. Though IgNAR is unique to cartilaginous fish and has no simple orthology to mammalian isotypes, IgNAR is functionally analogous to IgG, due to its late appearance in serum during development and affinity maturation [75, 76, 77, 78]. Immunoglobulin isotypes are defined by the heavy chain constant region domains they employ, but sharks also use four different light chains: κ, λ, σ, and σ-2 [79].

The epigonal organ is attached to the gonads of the shark and is composed of sinuses similar to those observed in bone marrow; it is considered the closest to a bone marrow equivalent in sharks [76, 80]. Essential steps in B lymphocyte maturation such as V(D)J rearrangement of immunoglobulins occurs in

the epigonal organ [77, 81]. The epigonal organ contains antibody secreting cells such as plasma cells and B1-a cells, similar to bone marrow in mammals [76, 82, 83]. Studies in clearnose skates (*Raja eglanteria*) show that expression of PU.1, an essential transcription factor in the development of lymphocytes, is restricted to the epigonal and Leydig's organs [84, 85, 86]. The Leydig's organ, the second of the specialized replacement lymphopoiesis organs in shark, is characterized as white, spongy masses on the dorsal and ventral sides of the esophagus beneath the epithelium, containing sinuses similar to those seen in bone marrow and the epigonal organs [87]. Not all cartilaginous fish have Leydig's organ. Some species, such as the nurse shark (*Ginglymostoma cirratum*), have only an epigonal organ [88]. Presence of a Leydig's organ will often result in the epigonal organ being dwarfed, suggesting they may serve similar functions and that the Leydig organ is a form of compensation of an overworked epigonal organ [89, 90, 91]. As in mammalian bone marrow, the epigonal and Leydig's organ both contain sporadic granulocytes [86], consistent as granulocytes mature in bone marrow and these organs appear to be the hematopoietic equivalent in sharks. Finally, there is some evidence that hematopoiesis occurs in the epigonal and Leydig's organ, as well as kidney and gut, in cartilaginous fish [92].

2.2.1b) T cells in cartilaginous fish- Thymus

The thymus is said to be the most ancient of lymphoid organs [93], as it is the first primary lymphoid organ present in phylogeny that has remained consistent throughout jawed vertebrate evolution. The thymus a spongy organ located in the pharynx region of recent vertebrates, as it originates from one or more pharyngeal arches in development. Since sharks are fish and have gills for means of respiration one would find the thymus located above the gills bilaterally [94, 95]. In sharks, some but not all pharyngeal arches in each gill basket provide the basis of development of the thymus

Shark thymus can be single or multilobed depending upon species and stage of development [96, 97, 98]. For example, nurse shark thymus complexity increases as it develops, but this is not necessarily the case in all species of cartilaginous fish [99]. The thymus is covered in a connective tissue capsule, with extensions of the capsule, called trabeculae, dividing the lobes [100].

Sharks have several different T cell receptor (TCR) chains created using V(D)J somatic gene recombination thought to heterodimerize to give a fully functional TCR. There is TCRα predicted to dimerize with TCRβ, TCRγ predicted to dimerize with TCRδ, and NARTCR. NARTCR is unique in that it is produced by doubly-rearranging V domain encoding exons to make a TCRδ chain with a free variable without a pairing domain on TCRγ [58, 101]. This protruding variable domain has high identity with IgNAR V, which also lacks a heterodimerization partner. *In situ* hybridization experiments in 2010 on nurse shark thymus localized the mRNA of these different TCR chains showing that TCRα and TCRβ are located in the central cortex together with weak signal in the medulla, TCRγ is concentrated in the subcapsular region of the cortex as well as the central cortex, and TCRδ expression is highest in the subcapsular region of the cortex but has the most signal in the medulla of all TCRs [96]. These same experiments showed that MHC class I has a higher concentration in the medulla than the cortex, which is consistent with the idea of selection still occurring as the lymphocytes complete maturation[96].

Somatic hypermutation (SHM) is a process primarily used in B cell affinity maturation that nurse sharks also employ to diversify their T cell repertoire which is not seen in any other vertebrates, with the exception of camelids [102] thus far [96, 103]. Ott *et.al.* demonstrated SHM occurring in TCRγ, TCRα and TCRδ V segments with analysis of mutation data and following rearrangement lineages of nontemplate (N) and palindromic (P) nucleotides in sequences of nurse shark TCRs. Alongside this data, they confirmed SHM in the thymus by colorimetric in situ hybridization data showing expression of AID in the cortex. Fluorescent *in situ* hybridization on nurse shark thymus tissue for activation-induced cytidine deaminase (AID), the catalyst of SHM, showing sporadic signal throughout the cortex and increasing near strong foci of TCRα signal at the cortico-medullary junction provided further evidence of SHM via AID acting on TCRs in the thymic cortex [104].

2.2.2 Secondary tissues in shark (and skate/ray)

Secondary lymphoid tissues function as sites of antigen encounter allowing lymphocytes to interact with antigen, APCs, and one another to mount an immune response. The secondary lymphoid

tissues of sharks are spleen and mucosal associated lymphoid tissues (MALT), including those associated with the gut, but lymph nodes are absent [105]. These organs each have characteristics unique to them that will be discussed in the sections to come.

2.2.2a) Spleen

The spleen is considered the first peripheral lymphoid organ to be maintained throughout evolution, and sharks are the primordial living group to have a spleen [106, 107]. Though there are other organs that provide a secondary function as lymphoid tissue, the spleen is the main hematopoietic organ and only bona fide secondary lymphoid organ in sharks [107]. It is suspected that the shark spleen could be the site of red blood cell formation as well as a potential site of plasma cell differentiation [76].

The lymphocyte zones, termed white pulp, are the areas composed of lymphocytes as well as developing plasma cells, active antibody secreting B lymphocytes [77]. Older nurse shark immunohistochemistry (IHC) studies show that the white pulp of shark spleen is divided into B and presumptive T cell zones with no defined border but a concentration of B cell markers in the outer ring of the white pulp and T cell markers in the inner core [66, 77]. Recent research suggests that the white pulp is composed of strictly B lymphocytes with no T cell zones [76]. Expression of RAG in the spleen of nurse sharks and clearnose skates (*Raja eglanteria*) indicates it may have a role in B lymphocyte development as seen in Leydig and epigonal organs [108, 109]. In the Aleutian skate (*Bathyraja aleutica*), secretory immunoglobulins are first seen in the spleen hinting it may be the primary site of B lymphocyte generation, but this has not been confirmed [110, 111].

A characteristic of spleen seen in recent vertebrates is the germinal center, which appears post antigen exposure and is the location of affinity maturation of lymphocytes, proliferation of B lymphocytes into differentiated plasma cells, and the generation of memory B lymphocytes [112, 113]. Periarteriolar lymphoid (PALS) sheaths are collections of T cells in the white pulp of spleen surrounding the central arterioles, also commonly seen in recent vertebrates. There is no evidence of germinal centers or PALS in

sharks suggesting that these structures evolved later, perhaps in endotherms as frogs also appear to lack germinal centers [66, 114, 115].

2.2.2b) Mucosal Associated Lymphoid Tissues (MALT)

Mucosal associated lymphoid tissues, also known as MALTs, are any secondary immune tissue serving the mucosal sites, either in the digestive tract, respiratory tract, reproductive tract or even skin in some species. MALTs, predominantly in digestive organs, are present in all vertebrates from lamprey to human [106]. The most common MALT, called GALT or gut-associated lymphoid tissue, can be found in the digestive tract, specifically the lower intestine. GALT can take many forms over many species; poikilothermic vertebrates lack organized MALT, whereas birds and mammals have organized MALT such as Peyer's patches and lymph nodes [116, 117]. Studies in the Iberian ribbed newt (*Pleurodeles waltlii)* demonstrate that amphibians appear to have lymphoid aggregates in the gastrointestinal tract, but no organized tissue was observed [105].

As reviewed by Hart 1988, several species of cartilaginous fish, stingray (*Dasyatis skajei*), horned shark (*Heterodontus francisci*), and eagle ray (*Aetobatus narinari*), share common observations of GALT in the mid to low intestine [118] as well as dogfish (*Scyliorhinus canicula*) [119]. Sharks do not have Peyer's patches, but they do have GALT located in the spiral valve, a region of the intestine whose function is to increase the surface area of the intestinal wall with extra folds and twists [109, 116]. The spiral valve contains lymphoid aggregates beneath the epithelial surface but is not considered a lymphoid organ as the lymphoid aggregates are not encapsulated similar to the Peyer's patches in mammals; though it is thought that the GALT in spiral valve could be a primitive ancestor of the architecture in Peyer's patches [105, 111]. In these lymphoid aggregates, one can expect to see a variety of cellular components including macrophages, lymphocytes, and granular leukocytes such as neutrophils, all of which are characteristic of sites of antigen encounter [119, 120].

There is some evidence of B lymphopoiesis in the spiral valve indicated by the expression of RAG, seen in skate as well as AID, seen in nurse shark [92, 104, 109] Microscopy studies in the small-spotted

catshark, *Scyliorhinus canicular*, [120] and dogfish, *Scyliorhinus canicula*,[119] showed a presence of lymphocytes and macrophages in the spiral valve.

Along the theme of GALT as a secondary lymphoid structure, studies in cartilaginous fish, *Scyliorhinus canicula L*., show that development of primary immune organs, such as the thymus, occurs before that of secondary immune organs, such as spleen and GALT [120]. Dogfish from different stages in development were collected and GALT tissue was embedded and imaged using light microscopy. Each specimen was dissected and inspected for the presence or absence of the thymus, kidney, spleen, GALT, epigonal and Leydig's organs [120].

The gut is not the sole location of mucosal immune tissues in sharks. There is evidence of some immune aggregates in shark skin as well as gills in the presence of lymphocytes and granular cells [121, 122]. Meyer et.al. showed transmission electron microscopy (TEM) images of *Scyliorhinus canicular* skin as well as light microscopy images of toluidine blue stained nurse shark skin both with embedded granular cells in the epidermis [121]. Little else has been said on the potential for skin associated lymphoid tissue (SALT) in cartilaginous fish. Another potential site of shark MALT is the respiratory tract which is filled with mucus and a prime site of antigen entry. Sequence analysis and quantitative-PCR studies in the White-spotted bamboo shark (*Chiloscyllium plagiosu*) reveal MHC class II signal in the gill, suggesting it is a site of antigen presentation by professional APCs, and, therefore, a potential secondary lymphoid tissue [123]. Results from northern blot observing the expression of lymphocyte receptors in various tissues of nurse shark showed high expression of TCRγ and δ, in the gill [96] which indicates the T cell defense present at mucosal sites, such as the gill, could have an early emergence in evolution.

2.3 Comparison of lymphoid tissue architecture in early vertebrate lineages

2.3.1 Agnathan to gnathostomes

Agnatha is the first group to demonstrate primitive adaptive immune characteristics, although they are much simpler than the characteristics of mammals or even sharks [107]. Lamprey have an elementary body plan which does carry over to their immune organs. Thymus, spleen, bone marrow, and

other lymphoid tissues are lacking in lamprey (Figure 1), but they do have analogous structures seeming to be the starting point for the immune structures we are familiar with in recent vertebrates (Figure 2). Lymphoid-like cells do aggregate in certain areas of the lamprey such as the kidney, gill basket, and gut [69]. Where the definition of primary and secondary lymphoid organs is more rigid in recent vertebrates, primary is the site of maturation and differentiation and secondary is antigen exposure, those of jawless fish are far more fluid. Primary lymphoid organs in hagfish and lamprey can serve a multitude of functions as well as a site of lymphocyte-like cell maturation, a hematopoietic site, or even a site of antigen exposure [124]. Though some organs, such as the thymoid and typhlosole, are more restricted in their functions as primary lymphoid organs, several others, such as the kidney, gill and supraneural body, are lymphoid tissue with other purposes.

Hagfish and lamprey have different lineages of lymphocyte-like cells with receptors called VLRs that are analogous to receptors seen in adaptive immune cells of recent vertebrates. VLRA is an αβ T lymphocyte analogue whereas VLRB is B cell and VLRC resembles γδ T cells in location and putative function, based largely on transcriptional profiles [125, 126, 127]. VLRB bearing cells are much more numerous than their counterparts in every tissue except the gill region where VLRA are dominant, but both are detectable in primary lymphoid organs such as blood and kidney [128].

		Primary				Secondary		Mucosal	
		Thymus	Bone Marrow	Leydig's organ	Epigonal organ	Spleen	Lymph Node	GALT	Spiral Valve
	Jawless Fish	✓★						✓	
	Cartilaginous Fish	✓		✓	✓	✓		✓	✓
	Bony Fish	✓				✓		✓	
	Amphibians	✓	✓			✓		✓	
	Reptiles	✓	✓			✓		✓	
	Birds	✓	✓			✓	✓	✓	
	Mammals	✓	✓			✓	✓	✓	

Figure 2.1: Comparison of classes of vertebrates and the lymphoid tissues present in each where checks indicate the presence of a tissue. Each tissue has its point of emergence, like the thymus in cartilaginous fish, the lymph node in birds, bone marrow in amphibians, and GALT as far back as jawless fish. The star indicates that lamprey have a thymoid region potentially with similar function to thymus, but it is not a true thymus.

The thymoid is a thymus-like structure in lamprey located in the gill basket and is considered the primary lymphoid organ of agnathans (Figure 3). The thymoid is packed with lymphocyte-like cells, predominantly VLRAs, and epithelial cells [129]. The thymoid, much like the thymus, originates from the pharyngeal arches of the lamprey, but, unlike the thymus which develops from the third arch in tetrapods [32], it is not restricted to a particular pharyngeal arch from which to develop [129]. Upon antigen exposure, lymphoid cells of secondary organs proliferate whereas cells of primary lymphoid organs do not. In lamprey, the kidney and typhlosole lymphocyte-like cells proliferate, but those of the thymoid do not indicating that the thymoid is the primary lymphoid tissue [129]. The thymoid is also home to the assembly of VLRA and C which is also where the highest expression of these (potentially TCR analogous) genes is seen [129].

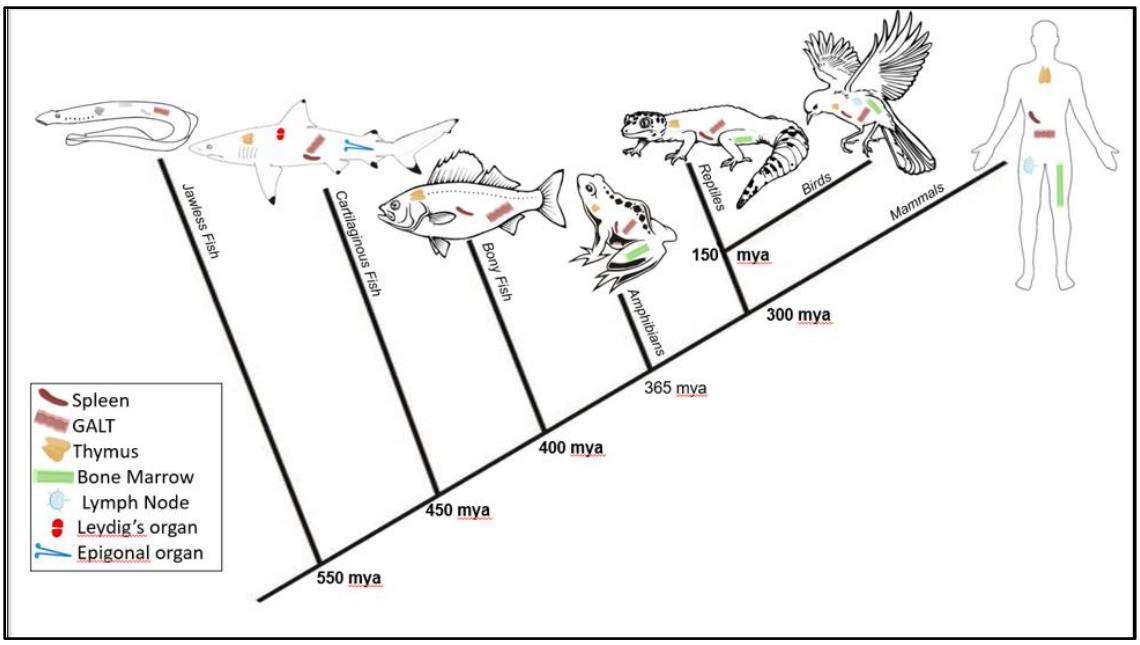


Figure 2.2: Phylogenetic tree portraying the emergence of key organs and tissues. As stated in Figure 1, bone marrow is not present until amphibians, lymph nodes do not appear until birds, true thymus tissue is first seen in cartilaginous fish, and GALT is ubiquitous across all groups. The gray colored tissues in the jawless fish indicate the analogous primary lymphoid function of thymus and bone marrow and secondary function of spleen are present, but in other tissue unique to agnathans.

Hematopoietic activity in lamprey is seen concentrated in the intestine, kidney, gill region, and typhlosole (Figure 3) [130]. The typhlosole is located in the gut of agnathans and is packed with lymphocyte-like cells and stromal cells; it is the main lymphopoietic and hematopoietic organ in developing lamprey larvae [69, 128, 131]. The typhlosole has higher expression of VLRB cells as opposed to VLRAs, and contain the lamprey equivalent to plasma cells, activated VLRB cells [128]. The kidney is considered another VLRB lymphopoietic organ in agnathans and is full of lymphocyte-like cells, which is similar to teleost fish who show hematopoietic activity in the anterior kidney [106, 128, 129]. These suggest physiology analogous to primary and secondary B lymphopoiesis in agnathan kidney and typhlosole, respectively, if VLRB and VLRB-expressing lymphocytes are indeed the humoral arm of agnathan adaptive immunity.

The supraneural body (SB), also called fat body, is placed dorsal to the agnathan vertebra and has been compared to bone marrow in recent vertebrates, because it is packed with a variety of blood cells in all stages of development as well as blood cell precursors [5, 69, 128]. Though there is some VLR rearrangement occurring in the SB, it is considered a secondary lymphoid structure (Figure 3), because the lymphocytes present in the SB proliferate upon antigen exposure, a key characteristic of secondary lymphoid tissues [69, 128]. In adult sea lamprey, the SB is the most important blood forming organ, but hematopoietic activity begins before metamorphosis from larvae to adult lamprey replacing the functions of the typhlosole and kidney [5, 69]. As the sea lamprey develops from larvae to adult, the blood cell forming activity of the typhlosole, nephric fold, and pharyngeal region decreases until it diminishes entirely just before metamorphosis where SB activity increases starting at metamorphosis through adulthood [69].

The expression of cytosine deaminase (CDA) 1 and 2 directly correlates with the presence of certain VLRs. As AID is to TCR and BCRs in recent vertebrates, CDA catalyzed receptor editing of VLRs. CDA1 expression is associated with VLRA where CDA2 is with VLRB; the expression of CDA can be used to assume locations of VLRs in tissues [129]. The expression of CDA1 fills the entire gill basket, which contains the thymoid, and suggests the thymoid has no true point of origin within the pharyngeal arches, as well as the thymoid is, most likely, the primary lymphopoietic tissue for VLRA lymphocyte-like cells [129].

2.3.2 Gnathostomes to bony fishes

The branch after cartilaginous fish in vertebrate phylogeny contains the bony fish, also known as Osteichthyes. This superclass is split into two classes: Sarcopterygii (lobe-finned fish) and Actinopterygii (ray-finned fish). Teleost fish, a group within bony fish, are the most studied group of Osteichthyes in comparative immunology. Even within this group, there is great variation between species regarding which organs they use for hematopoiesis and lymphocyte differentiation.

The term bony fish may be confusing from an immunological standpoint, because, though they do have bones, Osteichthyes do not have bone marrow as seen in recent vertebrates such as birds and

mammals (Figures 1 & 2) [34, 70, 105]. Instead, they have analogous structures in the head kidney, also called the anterior kidney or pronephros, and spleen that are responsible for hematopoiesis and B cell differentiation [92, 117, 132, 133, 134]. Expression of RAG [135, 136] TdT [137], and Ikaros, a transcription factor essential to lymphocyte maturation and lineage commitment for both T and B lymphocytes [92, 137], in Atlantic cod (*Gadus morhua*) and rainbow trout (*Oncorhynchus mykiss*) indicate the head kidney must be a site of immune cell production and differentiation. Fish express an AID-homologue, for somatic hypermutation, but fish do not perform class-switch recombination [57, 73, 138, 139]. The AID enzyme characterized in catfish (*Ictalurus punctatus*) [140] and zebrafish (*Danio rerio)* [138] show a longer cytidine deaminase motif than that of mammals and chickens as well as ample substitutions in the carboxy-terminal region, both of which are required for CSR.

Salmonid studies in Atlantic salmon (*Salmo salar*) and rainbow trout reveal mature B lymphocytes in the head kidney that travel to the spleen for activation [141]. Studies in zebrafish show RAG1 expression in kidney demonstrating there is gene recombination occurring in the kidney, indicating it is a site of lymphocyte differentiation (Figure 3) [142]. The pancreas of zebrafish, as well as the head kidney, serve as sites for B cell development [8], which is interesting due to the primary function of the pancreas being endocrine in the secretion of hormones, such as insulin and glucagon, and exocrine in the secretion of digestive enzymes, such as proteases and lipases.

Similar to those that came before them, bony fish use the thymus as a site of T lymphocyte differentiation and maturation. The thymus of bony fish is located dorsal to each set of gills and is said to have originated from the third pharyngeal pouch in development [105, 143]. Studies in zebrafish and medaka (*Oryzias latipes*) show the teleost thymus only has one thymic lobule, but there is a range of single lobed to multilobed thymus structures in other teleost fish [70, 144].

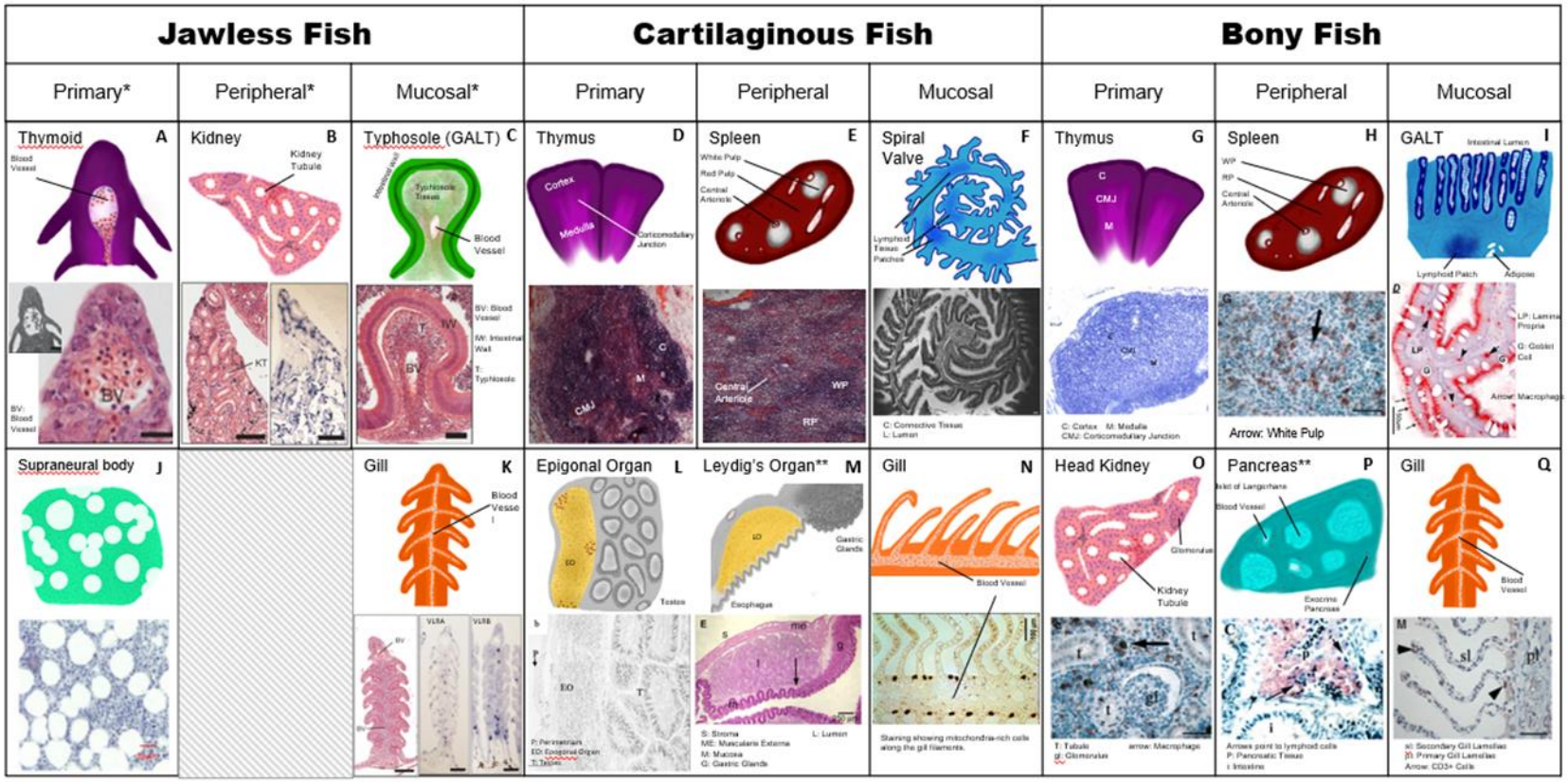


Figure 2.3: Diagram illustrating the structure and microarchitecture of each lymphoid organ or tissue present in agnathans, cartilaginous fish, and bony fish. Beneath the diagram are photomicrographs of the organ or tissue represented in the diagram. (*A,B,C and K*) [76] (*D and E*) personal images *(F)* [67] (*G*) [100] (*H,O, and Q*) [90] (*I*) [104] (*J*) [107] (*L*) [108] (*M)* [109] (*N*) [110] *(P*) [92] Copyright (2002) National Academy of Sciences, U.S.A

Histological findings in zebrafish [145], turbot (*Scopthalmus maximus L.*) [146], rainbow trout [147], salmon [148], carp (*Cyprinus carpio L.*) [132], sea bass (*Dicentrarchus labrax L.)* [149] and Atlantic halibut (*Hippoglossus hippoglossus L.*) [150], indicate there is distinction between the cortex and the medulla of thymus, even if there is no clear corticomedullary junction [151]. Genomic studies in zebrafish show expression of RAG in the thymus at exponentially higher concentrations than in kidney, implying it is the most productive primary lymphoid organ in zebrafish [142].

Teleost fish have similar secondary lymphoid organs, spleen, and GALT, to the sharks that predate them as well as not yet evolving lymph nodes seen in mammals. Teleost spleen does resemble that of sharks in that it lacks germinal centers but still contains white and red pulp. Studies in trout and medaka indicate there is distinction between B and T cell zones in the white pulp of spleen, but in zebrafish there is no clear resolution between the two [107]. Teleost GALT is more dispersed than we see in earlier phylogeny as studies suggest the second portion of the gut is an immunogenic area high in cell-mediated response [152, 153]. The GALT of teleost fish lacks defined organization such as a Peyer's patch, but they do have cells called intestinal epithelial lymphocytes (IEL) which function as epithelial cells with immunogenetic qualities similar to those seen in recent vertebrates [105, 117, 154].

Teleost fish have other secondary lymphoid tissue that is not as commonly seen in their cartilaginous counterparts, such as non-gut associated MALTs. The gills of fish are highly immunogenic due to the opportunity for pathogen entry [155, 156] and are known as gill-associated lymphoid tissue (GIALT) or intrabronchial lymphoid tissue (ILT) [141, 157]. The skin also contains diffuse lymphoid tissue, referred to as SALT, that contain T cells, B cells expressing IgT (a mucosal antibody), and microbiota [157]. Also associated with the respiratory system of teleost fish, diffuse lymphoid aggregates in the nasopharynx, NALT, is located in the olfactory organ and has a very high percentage of B cells and T cell markers but no definite evidence of T cells [157]. Nasopharynx-associated lymphoid tissue (NALT) is not considered a secondary lymphoid organ, but a lymphoid aggregate [157, 158].

2.4 Conclusions

Not enough studies look beyond anatomic, histologic, and genetic characterization into function of these proteins, cells, and tissues. More mechanistic studies of peripheral lymphoid tissues (including MALT) in animals immunized via distinct routes and infected with distinct classes of pathogen will be important for understanding the role of the cartilaginous fish peripheral immune tissues, and that early step in the evolution of our own. Understanding the journey of our immune system through evolutionary time is essential in fully understanding the physiology of the system. Sharks are at a pivotal point in evolution where they display so many of the characteristics of mammalian immunity, but often in its most basic form. More studies should be conducted in primitive vertebrates to better connect the dots in early adaptive immune evolution.

CHAPTER III

EVOLUTION OF IMMUNOGENETIC COMPONENTS USED TO FORM ULTRALONG VH COMPLEMENTARITY DETERMINING REGION 3 ANTIBODIES IN BOVIDAE

3.1 Introduction

The humoral branch of the adaptive immune system is mediated by membrane-bound receptors on B cells (BCR) and secreted immunoglobulins (Ig), also referred to as antibodies. Antibodies, of which there are 5 isotypes, IgM, IgD, IgG, IgE, and IgA, are composed of four chains, two light chains (IgL) and two heavy chains (IgH), linked together by disulfide bonds [1, 2, 36]. Together, the four chains form the Y-shaped structure commonly used to denote antibodies [1, 2, 36]. IgH is composed of a constant region (C), which determines the isotype, and a variable region (V), which contains the antigen binding site [159]. The heavy chain V region (IgHV) is encoded by three gene segments, variable (V), diversity (D), and joining (J) segments [160] that form functional genes through a process known as somatic recombination [161]. The assembled IgHV encodes a primary amino acid sequence that is divided into four framework (FW) and three complementarity determining regions (CDR) which alternate along its length [37]. The antigen binding paratope is comprised of the three CDR loops each from IgH and IgL, making a total of six antigen binding loops. In order to increase diversity in the antigen binding region, somatic hypermutation (SHM), catalyzed by activation-induced cytidine deaminase (AID), creates point mutations in the nucleotide sequence preferentially altering C and G nucleotides within targeted motifs of CDR loops to affinity mature an antibody for its antigen. [159, 162].

A novel antibody structure discovered in *Bos taurus* expands the antibody repertoire beyond what is seen in other vertebrates (Wang et al., 2013). In general, cattle immunoglobulins contain a longer third CDR of the heavy chain (CDRH3) on average compared to antibodies of mouse or human. A small subset of this repertoire consists of remarkably ultralong CDRH3 antibodies (Koti et al., 2010). The elongated CDRH3 loop extends up to 70 amino acids, more than three times the length of CDRH3 in canonical

antibodies, which average 13 amino acids [163]. Of the six CDR, this CDRH3 loop is usually the component of the paratope most responsible for epitope binding and specificity. The structure of cattle ultralong CDRH3 antibodies contains two somewhat conserved micro-domains, a knob and a stalk, both of which are formed by the elongated CDRH3 (Figure 1). This "stalk and knob" structure protrudes from the typical paratope surface, allowing the distal end of the knob to reach into concave epitopes (reviewed in [30]). In *Bos taurus* ultralong CDRH3 antibodies, the other CDRs have evolved to support the heavy chain CDR3 knob as the primary antigen binding site and have lost their original epitope binding function.

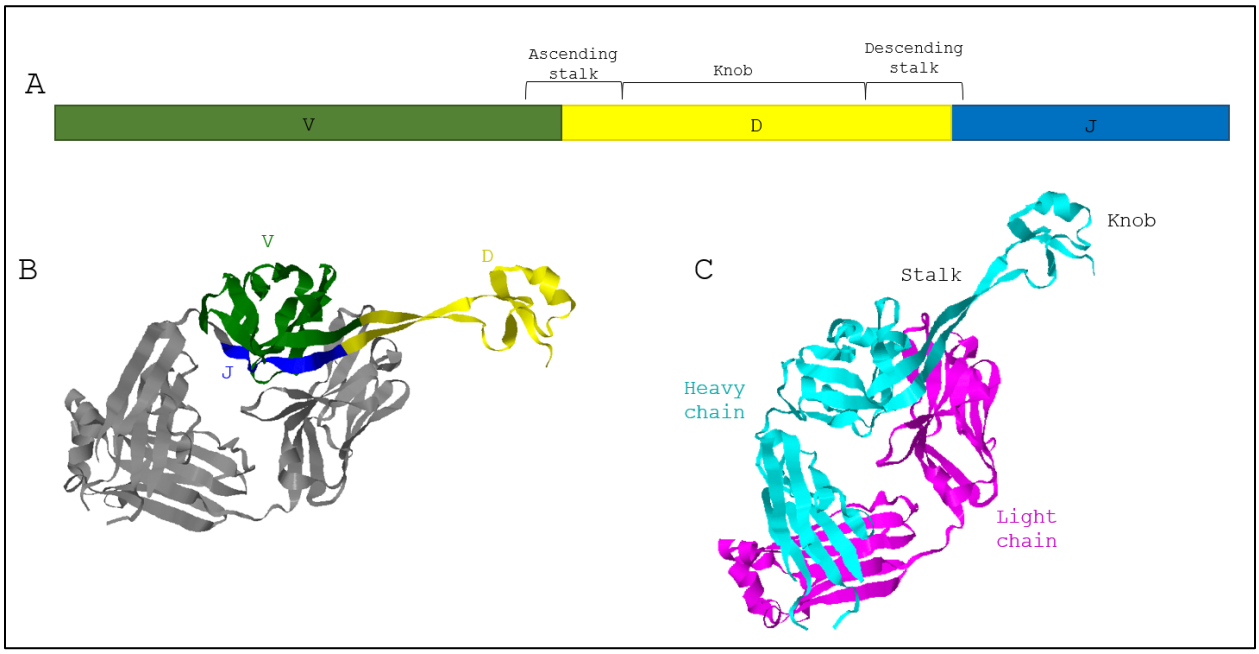


Figure 3.1: Assembly of the knob and stalk structure characteristic of ultralong antibodies in cattle. (A) The linear representation of the assembled gene encoding the variable region of an ultralong antibody with the regions color coded (variable is green, diversity is yellow, and joining is blue). Above these segments is denoted the portions of the knob and stalk that correspond to the segment. (B) A crystal structure of the bovine ultralong antibody ([164] PDB:6E9H) with the segments shaded in the same scheme as seen in (A). (C) is the same crystal structure with annotations of the knob and stalk domain as well as a color code of the heavy and light chains (heavy chain is cyan and the light chain is magenta).

Ultralong antibodies found in cattle almost always use the same germline V (IgHV1-7), D (IgHD8-2), and J (IgHJ2-4) gene segments. Despite all cattle Vs belonging to a closely related cluster of gene segments, there are several motifs which distinguish IgHV1-7 from other V segments [165]. Notably, IgV1-7 contains an 8bp duplication that extends the end of the V segment to form a YYCTTVHQ motif, compared to the YYCAR/K motif of canonical antibodies. The TTVHQ motif is a critical ascending structural component of the β-ribbon stalk (Figure 1) and is composed of eight or twelve hydrogen-bonded amino acid pairs that support the diverse knob of ultralong CDRH3 antibodies [166]. The IgHD8-2 segment encodes an additional distinguishing pattern of alternating aromatic tyrosine residues (YxYxY) within the descending stalk that provides added stability to the structure of the stalk [28, 167]. These aromatic residues are critical for supporting the knob and providing integrity to the stalk structure. IgHD8-2 is large and packed with hotspots for AID activity, with 48 codons primed to mutate to cysteines in addition to the 4 cysteines present in the germline. Compared to the features of IgHV1-7 and IgHD8-2, the IgHJ2-4 segment seems unremarkable in its contribution to the ultralong CDRH3.

The knob functions as the antigen binding domain, and, therefore, is more variable in sequence and structure than other portions of the antibody. Most of the variation within the knob results from differing patterns of disulfide bonds formed by the variable numbers of paired cysteines within the knob, primarily within the affinity-matured D segment [28, 167]. As more structures are solved, it is clear that diverse cysteine pairings can result in great structural diversity within the knob domain [164, 168]. Based on rearrangements observed from peripheral blood, the pattern of cysteines in the D following this first cysteine residue, in the 5' CPDG motif, appears randomly distributed. Thus, somatic hypermutation and possibly other AID-mediated mechanisms, supported by various disulfide bond combinations, seem important to diversifying the knob structure. Compared to the wide variation within the sequence and structure of the knob, the stalk structure is generally conserved, with only slight variations in length and flexibility. This overall structure is critical to the function of the ultralong CDRH3 antibody as a probe that fits into clefts on the antigen [164].

The structure of the bovine ultralong antibody allows for antigen binding that canonical antibodies are not capable of performing. The flexibility of the stalk as well as the diversity of the knob gives the ultralong antibody the ability to bind epitopes concealed within an antigen [164]. One example is the structure of human immunodeficiency virus (HIV), where the planar structure of canonical antibodies limits their binding to the gp120 protein on the envelope of HIV [31]. These surface proteins are frequently bound and, therefore, mutate regularly as a mechanism to evade immune activity. The flexibility of the stalk as well as the binding capabilities of the knob of the ultralong antibody allow for binding beyond the gp120 surface protein and to the envelope directly, which mutates very rarely [31]. A second related example of a structure functionally similar to bovine ultralong antibodies exists in the form on rare human cross-neutralizing anti-HIV antibodies which also have protruding CDR3 regions that bind the envelope of HIV [169].

Since it has only been identified in the cattle *Bos taurus*, our objectives were to discover whether other bovid species contain ultralong-enabling V and D regions, if these ultralong antibodies are used in the repertoire, and not simply encoded, by a broader taxonomic clade, and to suggest a natural history for the immunogenetic components required to rearrange a mature ultralong CDR3-encoding immunoglobulin heavy chain gene. We hypothesized that the D segment extension and the 8bp duplication at the 3' end of a V encoding the ascending stalk will be found in other extant relatives of the cow.

3.2 Methods

3.2.1 Selection of species

Species included in this study were selected based on two criteria: 1) taxonomic relatedness and 2) availability of full genome sequence data. The species tree used for reference relatedness was based on a Bayesian approach to analyzing mitochondrial DNA [170, 171]. Species that are very closely related to *Bos taurus* (taurine cattle) were selected preferentially to species that are more distantly related. We selected four species from the *Bos* genus (*Bos indicus*, *Bos frontalis*, *Bos mutus*, and *Bos grunniens*). Only one

representative species was selected from each of seven other closely related genera: *Bison bison* (American bison), *Bubalus bubalis* (domestic river buffalo), *Syncerus caffer* (African buffalo), *Tragelaphus eurycerus* (bongo), *Capra hircus* (domestic goat), *Ovis aries* (domestic sheep), and *Ammotragus lervia* (barbary sheep) (Figure 2). For most of these species, genome sequences from only one individual were available, and many assemblies still have unresolved regions and unplaced contigs, which could limit the completeness of our results. These 12 species spanning 8 genera all belong to the family *Bovidae.*

3.2.2 Searching for IgHV1-7 orthologs in other species

A tBLASTn nucleotide megaBLAST was performed using the full IgHV1-7 consensus sequence from *Bos taurus* as the query sequence. *Bos taurus* IgHV1-7 was BLASTed against the genome assemblies of the other 11 species and the top hits were recorded. Top matches across species were all within 91% similarity to the *Bos taurus* sequence with introns included. The translated sequences from NCBI were used to identify and remove introns, and the conserved heptamer and nonamer sequences of the recombination signal sequences (RSS) were used to identify the end of each sequence, YYC motif, and leading methionine residue were used to aid in manual alignment. Sequences then were examined for the presence of the 8bp duplication and signature TTVHQ motif of IgHV1-7.

3.2.3 Searching for IGHD8-2 orthologs in other species

BLAST searches using IgHD8-2 as the query sequence did not result in matches in the assayed genomic datasets. The conserved heptamer and nonamer from RSS were used to identify and align multiple D segments in regions between V and J segments or within scaffolds. Retrieved D segments were searched for the conserved CPDG motif encoded near the beginning of the segment. The sequences were arranged by the length of amino acids, and the longest D segment containing alternating tyrosine residues retrieved from each species' genomic assembly was selected for analysis.

3.2.4 Building a phylogenetic tree to visualize emergence and loss of ultralong antibody gene segments

The topology of a ruminant phylogenic tree assembled utilizing the Bayesian approach [170, 172, 173, 174, 175, 176] was annotated for both the IgHV1-7 and IgHD8-2 ortholog data. This tree depicts a clade of species containing the 8bp duplication as well as the pattern of increasing IgHD8-2 length. This tree provided us with the necessary information to pinpoint two potential events in evolutionary history pertaining to the emergence or loss of the bovine ultralong antibody.

3.2.4 Test for expressed ultralong CDRH3 rearrangements in bison

Peripheral blood of American bison was generously provided by Brush Meat Processors (Brush, CO). Bison peripheral blood mononuclear cells were extracted, and RNA was stabilized using the Leukolock total RNA isolation system (Invitrogen) and carried through to total RNA following the manufacturer's instructions. Superscript III (Invitrogen) reverse transcriptase was used to produce cDNA as template for PCR amplification. Primers MFC1141 and MFC1113 were used to amplify the ultralong antibody (Figure 5). Another set of primers, MFC1179/MFC1180 and MFC1112/MFC1113 (Supplemental Figure 2) were used to bind more specifically to the ultralong antibody by targeting the CDR1 region of the V segment which is unique to ultralong antibodies post transcription. Candidate bands of 800-1200bp were cloned into plasmids and Sanger sequenced as previously described [177].

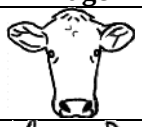
Genus	***species***	**Common name**	**Image**
Bos	*taurus*	Taurine Cattle	
Bos	*indicus*	Zebu Cattle	
Bos	*frontalis*	Gayal	
Bos	*grunniens*	Domestic Yak	
Bos	*mutus*	Wild Yak	
Bison	*bison*	American Bison	
Bubalus	*bubalis*	Domestic Water Buffalo	
Syncerus	*caffer*	African Buffalo	
Tragelaphus	*eurycerus*	Bongo	
Capra	*hircus*	Domestic Goat	
Ovis	*aries*	Domestic Sheep	
Ammotragus	*lervia*	Barbary Sheep	

Figure 3.2: List of genus and species names accompanied by their common names and images. These images will be used in the phylogenetic tree (Figure 5).

3.3 Results

3.3.1 IgHV1-7 found in four species closely related to Bos taurus

For each species, we aligned variable gene segment sequences with the highest nucleotide identity to the *Bos taurus* ultralong CDR3 enabling V segment IgHV1-7. We found the 8bp duplication and TTVHQ motif in the V segments from five species (*Bos taurus, Bos mutus, Bos indicus, Bos grunniens,* and *Bison bison*) (Figure 3). We found no evidence of the duplication in species beyond the *Bos and Bison* genera. Surprisingly, we also did not find a variable segment with the 8bp duplication and TTVHQ motif in *Bos frontalis*. This could be an artifact of the limited data available for this species, however, if accurate, it shows evidence of multiple evolutionary events.

3.3.2 D segment sequences do not show concerted maintenance of IgHV1-7 and IgHD8-2 orthologs

The presence of IgHV1-7-like V segments did not predict the presence of IgHD8-2 orthologs. In *Bos mutus,* which contains an IgHV1-7 ortholog, we found no IgHD8-2 ortholog (Figure 4). With its longest D segment encoding only 13 amino acids in length, the *Bos mutus* genome encoded one of the shortest D segments among all bovids analyzed. Species relatedness to *Bos taurus* also does not correlate with D segment length, with more distantly related species (e.g., *Bubalis bubalis*) actually showing greater similarity to IgHD8-2 than some of the closest relatives (Figure 4).

All the longest D segment sequences found in other species are notably shorter than the IgHD8-2 sequence from *Bos taurus* (12-28 amino acids compared to 48 amino acids in length). There was more variation in D segment sequences than seen in V segment sequences. None of the sequences from other species contained the conserved CPDG motif distinctive of many hypermutated ultralong variable domains in *Bos taurus.* Most sequences encoded zero or, at most, two cysteine residues, casting doubt upon the ability that the resulting structures would form the disulfide bonds necessary for the knob structure prior to SHM.

3.3.3 Evidence for ultralong antibody transcript has not been found in Bison bison serum

Sanger sequencing of cloned immunoglobulin from bison serum was identified as bison IgM, but with a canonical V segment (Figure 5). The YYCAK motif at the 3' end of the V segment was the indicator of a canonical IgM over the ultralong IgM. This V segment could either be IgHV1-11 or IgHV1-6 which displayed the closest resemblance with only 21 out of 348 nucleotides being mismatched. The D segment contained 22 amino acids, which compares to IgHD8-2, but aligns with IgHD7-1, which was not the ultralong D segment. The expressed D segment sequence also displayed a single cysteine residue instead of the multiple required to form the characteristic knob structure. The J segment, though not a significant contributor to the formation of the knob and stalk structure, was specific to IgHJ2-4 in the bovine ultralong antibody. The amplified sequence mapped to IgHJ10 not with IgHJ2-4.

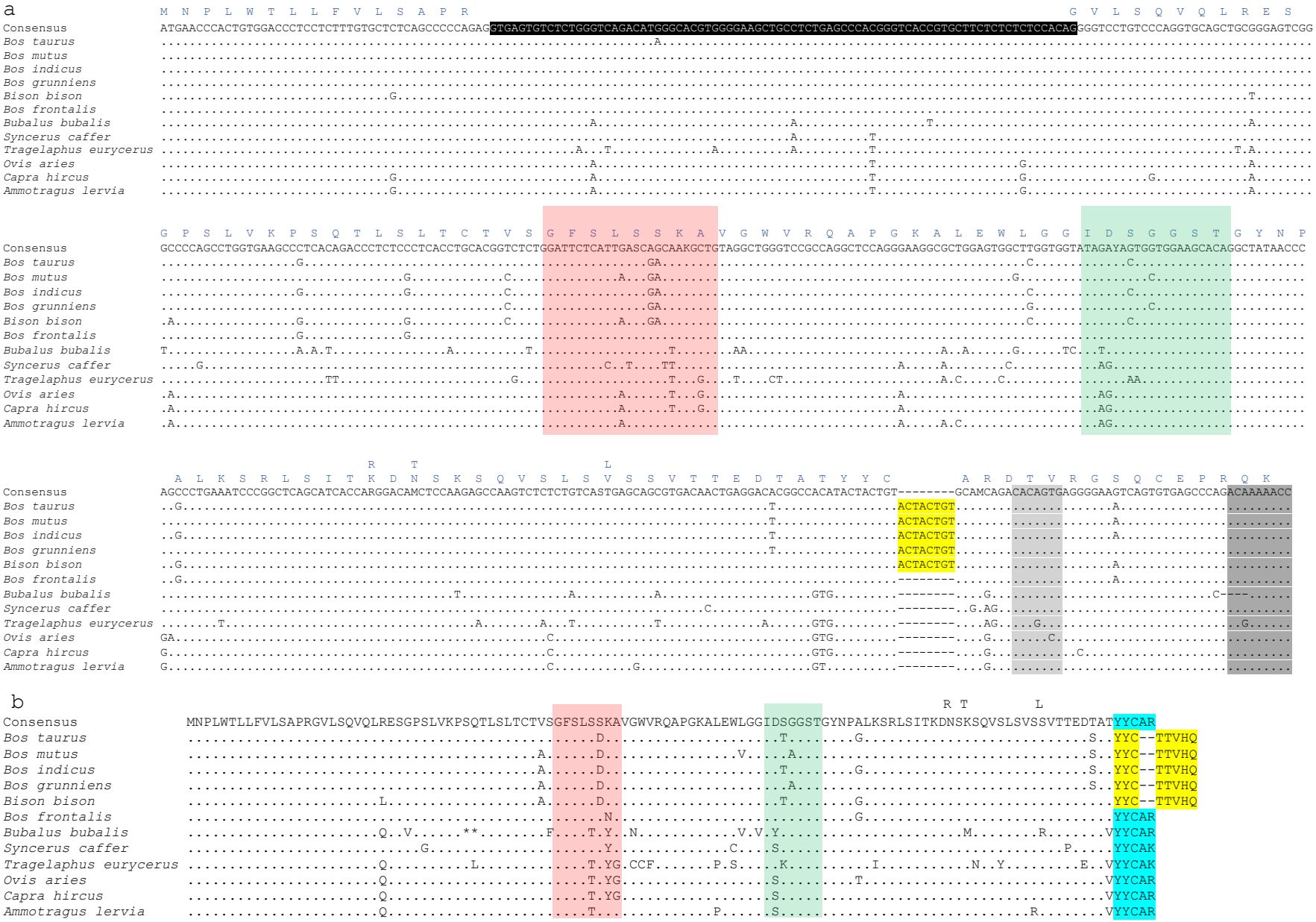

a
b
Consensus
Bos taurus
Bos mutus
Bos indicus
Bos grunniens
Bison bison
Bos frontalis
Bubalus bubalis
Syncerus caffer
Tragelaphus eurycerus
Ovis aries
Capra hircus
Ammotragus lervia

Figure 3: Alignment of germline V segment genes. Bases and amino acids that correspond to the consensus sequence are represented by a dot, gaps are represented by dashes, and dissimilar bases or amino acids to the consensus sequence are represented by the single letter code in place of the consensus. CDR1 is highlighted in red and CDR2 is highlighted in green. (a) Nucleotide alignment of bovine species and phylogenetically similar species. The bases highlighted in black represent the intron of the germline V segment that is not transcribed. The duplicated bases forming the ultralong V motif are highlighted in yellow. The bases highlighted in grey represent the heptamer (light grey) and nonamer (dark grey) that make up the recombination signal sequence (RSS). The amino acids are above the nucleotide sequence at the center base of the codon. (b) Peptide alignment of ultralong antibodies where the ultralong motif (TTVHQ) is highlighted in yellow and the consensus motif (YYCAR) is in light blue.

a

```
                                                     S  C  P  D  G  Y  S  Y  G  Y  G  C  G  Y  G  Y  G  C  S  G  Y  D  C  Y  G  Y
Bos taurus             GGTTTCTGATGCCGGCTGTGTCACGGTGGTAGTTGTCCTGATGGTTATAGTTATGGTTATGGTTGTGGTTATGGTTATGGTTGTAGTGGTTATGATTGTTATGGTTA
Bubalus bubalis        ....................................---------------------------------------------------------------------..
Bison bison            .....T...........................------------------------------------------------------------------------..
Syncerus caffer        ..................................A.---------------------------------------------------------------------..
Bos grunniens          ....................................---------------------------------------------------------------------..
Tragelaphus eurycerus  ..............................T...A.---------------------------------------------------------------------..
Capra hircus           ..................CA..T.............---------------------------------------------------------------------..
Bos mutus              ......................T..........------------------------------------------------------------------------..
Ovis aries             ..................CA..T...........A.---------------------------------------------------------------------..

                         G  G  Y  G  G  Y  G  G  Y  G  Y  S  S  Y  S  Y  S  Y  T  Y  E  Y
Bos taurus             TGGTGGTTATGGTGGTTATGGTGGTTATGGTTATAGTAGTTATAGTTATAGTTATACTTACGAATAT---------ACCACAGTGATACTCTCTGGGACAAAAACC
Bubalus bubalis        .A...........TA.GG.TA..A........G.GA.G.....G.....G.....GG...TAGT...GATTATACC.........G.................... 57.0%
Bison bison            .............---................G.TA.G.....G.....G.....GG...TA---------------............................. 57.5%
Syncerus caffer        .A...........---------T...........G..---...G.....G.....GG...TG.T...---------...........C.....A............. 57.4%
Bos grunniens          .A...........------TA...........G.---G.....G.....G.....GA...TA---------------..........C................... 57.0%
Tragelaphus eurycerus  .A.C.........---------------TA..G.TA.G.....G.....G.....GG...T.GTA-------------.........C.......C..C........ 49.8%
Capra hircus           .A...........---------------------TA.G.....G.....G.....G---------------------..........C................... 48.9%
Bos mutus              .A...........---------------------TA.G...........G......---------------------..........C...T............... 48.9%
Ovis aries             .A................C...------......-------------------------------------------..........C................... 48.9%
```

b

```
Bos taurus             SCPDGYSYGYGCGYGYGCSGYDCYGYGGYGGYGGYGYSSYSYSYTYEY---  48
Bubalus bubalis        SC-----------------------YSGYGYGYDYGCDGYGYGYGYSYDYT  28
Bison bison            S------------------------YGGYG-YGGYGCYGYGYGYGY-----  21
Syncerus caffer        SY-----------------------YSGYG---CYGYG-YGYGYGYGY---  21
Bos grunniens          SC-----------------------YSGYG--YGYGC-GYGYGYDY-----  20
Tragelaphus eurycerus  CY-----------------------YSGYG-----YCYGYGYGYGYG----  19
Capra hircus           SC-----------------------YSGYG-------YGYGYGY-------  14
Bos mutus              S------------------------YSGYG-------YGYSYGY-------  13
Ovis aries             SY-----------------------YSGYGYSY--GY--------------  12
```

Figure 4: Alignment of germline D segment genes. Gaps in the sequences are denoted by a dash. (a) Nucleotide alignment of the D segments of bovine species as well as neighboring species in phylogeny. The grey highlights represent the heptamer (light grey) and the nonamer (dark grey) of the RSS . Dots denote agreements to the *Bos taurus* sequence, and disagreements are denoted by the single letter nucleotide code. The corresponding amino acid sequence is above second nucleotide in the codon. The percent identities of each sequence to the reference, *Bos taurus*, is in red at the end of the nonamer. (b) Peptide alignment of the D segment of the same species. The peptide length is in red.

a

b

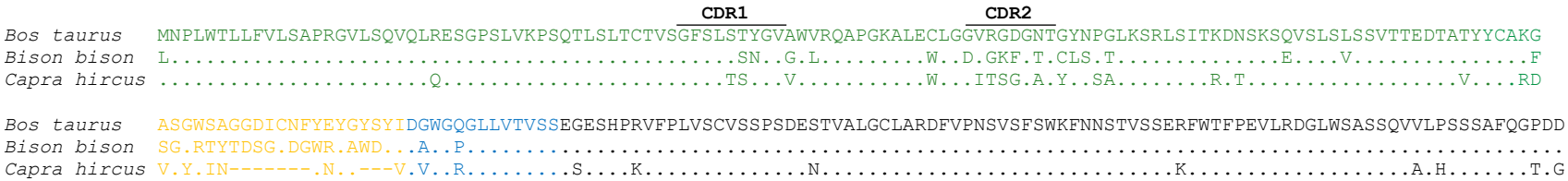


Figure 5: Alignment of sequences including expressed IgM in *Bos taurus, Bison bison,* and *Capra hircus*. The sequences are annotated by segment with the V (green), D (+ N/P, yellow), and J (blue) segments from the variable region as well as the constant region (black). (a) Nucleotide alignment of IgM between *Bos taurus*, *Bison bison*, and *Capra hircus* in order to observe the variability between species. The percent identity of the sequences to *Bos taurus* is at the end of the sequence in red. (b) Amino acid alignment of the same sequences visualizing the variability of the D segment.

3.4 Discussion

We found the 8bp duplication and TTVHQ motif in genomic V segments from five species and found no evidence of the duplication in species beyond the *Bos and Bison* genera (Figure 3). All five species containing the 8bp duplication are isolated in a monophyletic clade of six species containing *Bos* and *Bison* genera (Figure 5), though surprisingly, we did not find the 8bp duplication and TTVHQ motif in *Bos frontalis*, which is more closely related to *Bos taurus* than *Bison bison*. This absence could be due to the limited data available for this species as bison do not have a complete published genome or suggests that multiple evolutionary events directed its emergence. Species relatedness to *Bos taurus* does not correlate with length of D segment, with more distantly related species actually showing greater identity to IgHD8-2 than some of the closest relatives (Figure 4). If the clade is monophyletic for the ultralong antibody, then we can say the 8bp duplication is a more consistent and reliable indicator than the length of the D segment.

These findings are consistent with our hypothesis that genomes from close relatives of *Bos taurus* contain the immunogenetic components of ultralong CDRH3 antibodies. However, because we did not find the ultralong V segment in *Bos frontalis*, the presence of these components forms a paraphyletic group within the *Bos* genus. If *Bos frontalis* truly does not contain the ultralong IgHV1-7 and this is not an artifact due to an incomplete genome assembly, then there are two evolutionary possibilities that may explain this pattern. The first possibility is that a single mutation event in the common ancestor of the *Bos* and *Bison* genera led to the emergence of ultralong antibodies in both clades, and an additional mutation event in *Bos frontalis* led to the loss in this one species. The other possibility is that two separate mutation events occurred in the common ancestors of 1) *Bos taurus* and *Bos indicus* and 2) *Bison bison, Bos grunniens,* and *Bos mutus* that led to the emergence of ultralong antibodies in both clades.

The PCR and sequencing of expressed IgM from bison blood samples yielded evidence of canonical antibody using forward primers situated in the leader sequence, but only non-immune genes were cloned using forward primers situated in the CDR1 region more specific to variable predicted to

produce ultralong antibodies. This could mean several things 1) the ultralong IgM antibody is not expressed by bison and only encoded in the genome or 2) the ultralong IgM antibody is not expressed, to a large enough extent, in bison blood but may be present in other tissues and/or 3) our attempts to sequence expressed ultralong IgM from bison serum were unsuccessful. Further experiments are needed to clarify which is the case.

The discovery of other species within the bovine subfamily that could express ultralong antibodies has led to speculation on the events leading to its evolution. It has been hypothesized that the bovine ultralong antibody emerged in response to a particular bovine pathogen uniquely affecting bovids [167], as we have discovered segments encoding ultralong motifs only within the bovine subfamily. It is interesting that all species containing ultralong antibody sequences in their germline are ruminants, as ruminants are known for their restricted repertoire of functional V, D, and J gene segments [165, 166, 178].The restricted functional repertoire may have trigged the evolution of a specialized antibody that evolved in response. It is important to note that not all ruminant genomes encode the ultralong antibody in the germline, so we cannot assume the limited repertoire is the only contributing factor in the emergence of the ultralong antibody. Another important characteristic of ruminants is their diverse population of flora. Ruminants have multiple chambers within their digestive system used to break down and ferment plant-based food with the help of a wide variety of microorganisms including bacteria and fungi. The community of flora unique to ruminants could have provided a unique pathogen that triggered the emergence of the ultralong antibody as well. Should this be the case, we could speculate on the presence of the ultralong antibody being restricted to site in the digestive system with minimal expression in other immune tissues or the blood. Now that we have an idea of the species whose genomes code for this unique antibody, we can better define the evolutionary point where the ultralong antibody made its debut, as well as predict the triggering event.

The bovine heavy chain gene locus contains a clustered organization of D segments. One of these clusters, cluster 2, contains the ultralong D segment, IgHD8-2, as well as 4 other segments [179].

Investigation into the organization of the bovine genome showed that the length of the IgHD8-2 segment is attributed to a "deletion and fusion event" of segments within cluster 2 [179]. This deletion in cluster 2 (compared to clusters 3 and 4) is associated with a rearrangement event [179]. The fusion of two segments, IgHD6-3 and IgHD7-3, or the gene conversion of IgHD3-3 from IgHD6-3 and subsequent fusion to IgHD7-3 likely built IgHD8-2 [179].

The unique structure of the bovine ultralong knob and stalk allows for binding of epitopes that are hidden or unreachable by canonical antibodies. An example of one of these hidden epitopes lies within the envelope of the HIV retrovirus. Studies where cows were immunized with the BG505 SOSIP trimer, an antigen that mimics the HIV envelope glycoprotein gp120, show that ultralong antibodies are responsible for viral neutralization in the serum [31, 180]. The knob and stalk of the ultralong antibody can reach into the space containing the CD4 binding site creating a new binding location for immunoglobulin on the BG505 SOSIP trimer, where the CD4 binding site on the trimer is inaccessible to canonical antibodies [31]. These qualities of the bovine ultralong antibody are suited for therapeutic utilization, especially in regard to antigens like the HIV retrovirus.

Bovine ultralong antibodies are not the only antibodies with unique structures and motifs important for binding complex epitopes. Camelids construct an antibody containing only a heavy chain binding region, meaning the antigen binding domain is drastically smaller than a standard antibody (reviewed in [181]). This antibody, referred to as a nanobody, also has a longer third antigen binding loop increasing its flexibility and surface area for antigen binding [182, 183]. These nanobodies are used in therapeutics as imaging tools, enzyme inhibitors, and catalytic modulators of antigenic enzymes as well as use in research to investigate enzymatic mechanisms (reviewed in [181]).

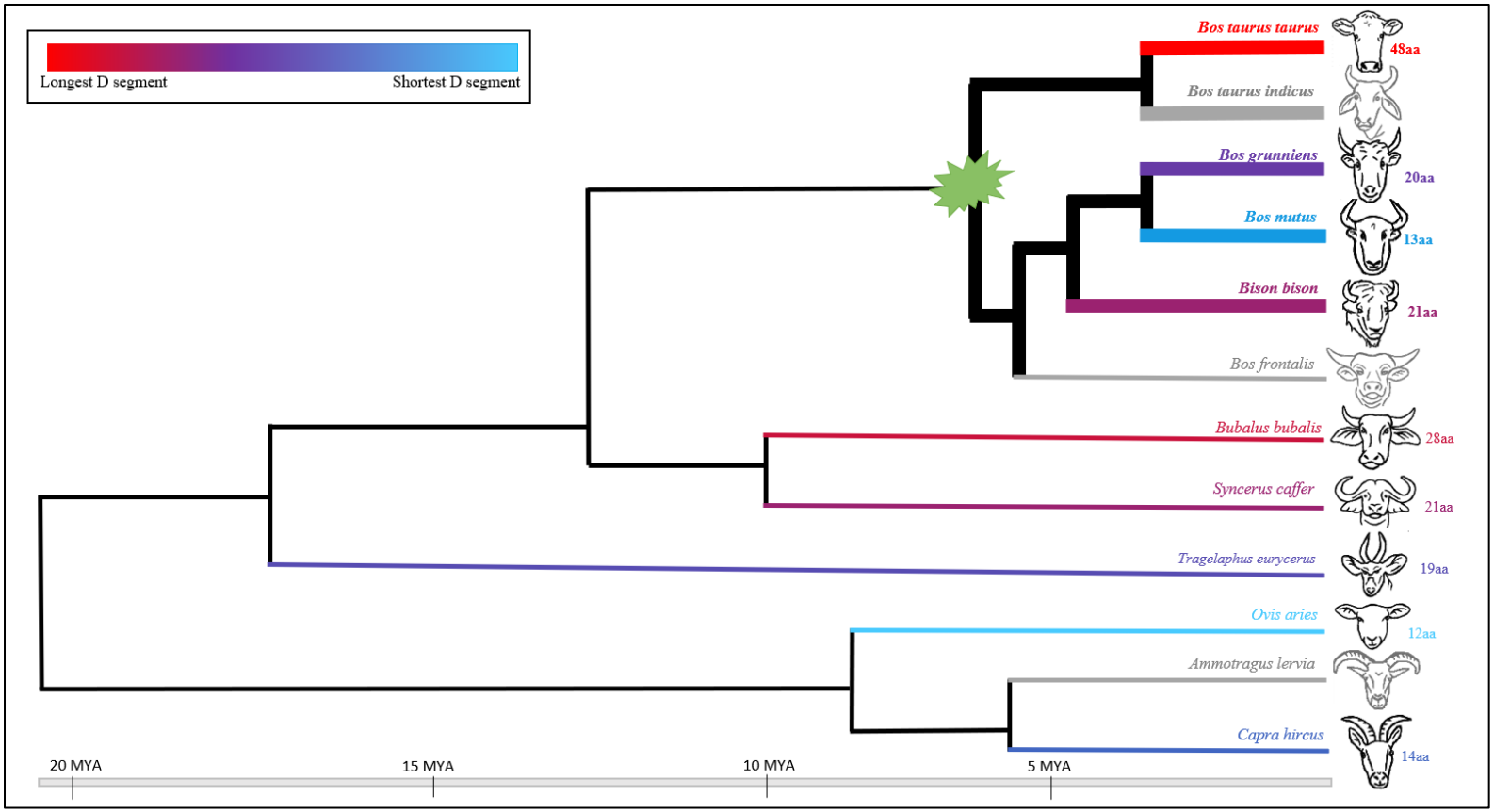


Figure 3.6: Phylogenetic tree of the characteristics of the ultralong antibody sequence used in order to identify the evolutionary event leading to its expression. The relative length of the D segment is represented by a gradient from red to blue where the brightest red is the longest D segment, and the brightest blue is the shortest. The actual length of the D segment is shown next to the species picture (aa means number of amino acids). Bold lines on the tree represent the species with the ultralong V motif present. The green star on the tree is representative of a time in evolutionary history where an event caused the emergence of the ultralong antibody. The species with no D segment data are shaded in grey. The timeline approximation of the emergence of the species in along the bottom of the tree (MYA means million years ago).

CHAPTER IV: CONCLUSIONS

The two previous chapters are examples of the utility of the field of comparative immunology, one of the conservation of characteristics throughout evolution and the other of the diversity within that conservation. Though many of the characteristics of the adaptive immune system, such as receptor formation and cellular components, have been conserved from cartilaginous fish, responses to different pathogens and physiological necessity have modified the adaptive immune system to best fit the needs of each group. Organs seen in cartilaginous fish for the development of B cells that are not seen in any other group[66], single domain antibodies in camelids [184], fish and amphibians that utilize skin as a site of antigen exposure in the form of SALT[157] , ultralong antibodies in bovine [28], and many other examples of unique presentations of immunity demonstrate the adaptive nature of the adaptive immune system.

The preceding chapters add to the body of knowledge of comparative immunology which is essential to understanding the origins and functionality of the immune system. The examination of lymphoid tissues in ancient species and the genomic phylogeny of cattle provided new and testable ideas and hypotheses. Undeniably, comparative immunology has led to some very important discoveries that are useful in the fields of therapeutics, such as HIV neutralizing cattle ultralong antibodies, and functional physiology, such as studies in thymectomized clawed frogs as well as jawless and cartilaginous fish that provide insight into the most primitive version of the immune system [31, 177, 185].

Further studies in cattle ultralong antibodies are needed to gain a greater understanding of their potential as a human therapeutic. Thus far, there are no extensive studies into the flexibility and utility of the stalk domain. Understanding the stalk structure is essential, because it is the stalk that allows the antigen-binding knob domain to reach restricted epitopes on a pathogen [164]. Fluorescence resonance energy transfer (FRET) experiments would be useful in the investigation of the flexibility of the stalk. FRET measures the distance between points on a protein using fluorophores that emit and excite one another at certain wavelengths, and it has been used to analyze shape changes in proteins [186]. FRET could

provide quantitative measures of the flexibility and stability of the stalk as well as shape and conformation changes in the knob.

Understanding the evolutionary emergence of the bovine ultralong antibody is reliant upon knowing, not only when the ultralong gene segments were first in the genome, but when the expression and utilization of this antibody first occurred. An expression study was attempted in bison (chapter 3) using genomic information isolated from peripheral blood leukocytes. This study was successful in showing the expression of canonical IgM but unsuccessful in the expression of ultralong IgM. One of several potential explanations for this is that ultralong IgM is not expressed in peripheral blood leukocytes and would be more likely found in a lymphoid tissue. Another explanation could be the lack of expression of the ultralong antibody in the form of IgM compared to other isotypes.

Expression studies in *Bison bison* need to be repeated with varying parameters such as changing the isotype used to design reverse primers to either IgG, IgA, IgD, and/or IgE for the ultralong antibody as well as the sample location in the animal from peripheral blood to either lymph nodes, GALT, and/or spleen. Using the IgA constant region for reverse primer design with RNA samples from the GALT of a bison would be a favorable combination as bovine species are GALT species, and IgA is concentrated in mucosal tissues. Should the ultralong antibody not be expressed in bison, the next step would be to attempt the same experiments with samples from other species within the monophyletic clade seen in Figure 3.6, such as *Bos mutus*, *Bos grunniens*, and *Bos indicus*. If there is no evidence of expression in bison, that does not mean the other species do not utilize the ultralong antibody in their antibody repertoire.

The first chapter compared different groups to one another and noted the conservation and divergence of the immune system within these chronologically evolved organisms. The second chapter explains just one of many examples of the diversity seen in evolutionary immunity where the immune system had to change and fit a specific need. These two chapters, together, describe the true adaptability of the adaptive immune system.

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APPENDIX

Primer Name	**Sequence**	**Amino Acids Bound**	**Direction**	**Location**
MFC 1112	CCGAGAGAGGACACAGGAGA	SPVSSLG	Reverse	IgM constant region
MFC 1113	CTTTGAAGGGCCGGATGACT	DDPGEFA	Reverse	IgM constant region
MFC 1141	GTGCTTTCCCAGGTCCAACT	VLSQVQL	Forward	Ultralong V segment leader
MFC 1179	TTAAGCGACAAGGCTGTAGGCTG	LSDKAVGW	Forward	Ultralong V segment CDR1
MFC 1180	TTGAGCGACAAGGCTGTAGGCTG	LSSKAVGW	Forward	Ultralong V segment CDR1

Supplemental Table 3.1: Primer details. All primers were designed using the genomic sequence for *Bison bison* ultralong IgM.

Species name	Accession number	V Segment	Type	Start Base Number	Stop Base Number	D segment	Type	Start Base Number	Stop Base Number
Bos taurus	KT723008.1	KT723008	Locus	254,411	254,849	KT723008	Locus	372,312	372,456
Bos indicus	GCA_002933975.1	CM009511	Chromosome	71,589,334	71,589,773				
Bos grunniens	GCA_005887515.2	VBZB01000018	Chromosome	76,089,982	76,090,421	VBZB01000018	Chromosome	76,051,395	76,051,460
Bos mutus	GCA_007646595.3	VBQZ03000564	Scaffold	15,628	16,067	VBQZ03000564	Scaffold	48,676	48,723
Bos frontalis	GCA_007844835.1	RBVW01003239	Scaffold	220,651	221,081				
Bison bison	GCF_000754665.1	JPYT01274964	Contig	754	1,193	XM_010833706	Locus	1,884	1,955
Bubalus bubalis	GCA_004794615.1	ML229204	Scaffold	3,152	3,575	XR_003107045	Locus	295	384
Syncerus caffer	GCA_006408785.1	SJXX01007725	Scaffold	42,054	42,477	CADEAB010000126	Conitg	246,618	246,687
Tragelaphus eurycerus	GCA_006410755.1	SJYI010005906	Scaffold	1,514	1,943	SJYI011518982	Scaffold	1,245	1,325
Ovis aries	GCF_002742125.1	NC_040269	Chromosome	70,744,450	70,744,873	NC_040269	Chromosome	70,711,134	70,711,173
Capra hircus	GCA_004361675.1	NC_030828	Chromosome	68,974,701	68,975,124	NC_030828	Chromosome	69,008,123	69,008,200
Ammotragus lervia	GCA_002201775.1	NIVO01051093	Scaffold	9,102	9,525				

Supplemental Table 3.2: Details of sequences used in each species. The shaded boxes represent data not found for those species.