EVOLUTION OF MUCOSAL IMMUNOGLOBULINS:

XENOPUS LAEVIS IGX AND *THUNNUS ORIENTALIS* IGZ/T

A Dissertation

by

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ABSTRACT

Despite a large number of studies during the last decade that investigated mucosal immunity in humans, very few works have been done on this immune compartment in lower vertebrates. In the following two studies we focused on the mucosal immunoglobulins in two important species of two classes of ectothermic vertebrates: amphibians and bony fishes. Many studies address the influence of the gut microbiome on the immune system, but few dissect the effect of T cells on gut microbiota and mucosal responses. We have employed larval thymectomy in *Xenopus* to study the gut microbiota with and without the influence of T lymphocytes. Pyrosequencing of 16S ribosomal RNA genes was used to assess the relative abundance of bacterial groups present in the stomach, and the small and large intestine. Clostridiaceae were the most abundant family throughout the gut, while Bacteroidaceae, Enterobacteriaceae, and Flavobacteriaceae also were well represented. Unifrac analysis revealed no differences in microbiota distribution between thymectomized and unoperated frogs. This is consistent with immunization data showing that levels of the mucosal immunoglobulin IgX are not altered significantly by thymectomy. This study in *Xenopus* represents the oldest organisms that exhibit class switch to a mucosal isotype and is relevant to mammalian immunology, as IgA appears to have evolved from IgX based upon phylogeny, genomic synteny, and function.

It is now appreciated that in addition to the immunoglobulin (Ig)M and D isotypes fish also make the mucosal IgT. In this study we sequenced the full length of Ig tau as well as mu in the commercially important *Thunnus orientalis* (Pacific bluefin tuna), the first

analysis of both of these Ig isotypes in a member of the order Perciformes. Tuna IgM and IgT are each composed of four constant (CH) domains. We cloned and sequenced 48 different variable (VH) domain rearrangements of tuna immunoglobulins and grouped the VH gene sequences to four VH gene segment families based on 70% nucleotide identity. Three VH gene families were used by both IgM and IgT but one group was only found to be used by IgM. Most interestingly, both Ig mu and Ig tau clones appear to use the same diversity (DH) segment, unlike what has been described in other species, although they have dedicated IgT and IgM joining (JH) gene segments. We complemented this repertoire study with phylogenetic and tissue expression analysis. In addition to supporting the development of humoral vaccines in this important aquaculture species, these data suggest that the DH-JH recombination rather than the VH-DH recombination may be instructive for IgT versus IgM/D bearing lymphocyte lineages in some fish.

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1. INTRODUCTION AND LITERATURE REVIEW

1.1 Immunology and the immune system

Immunology is the study of how the body defends itself against different infectious microorganisms that cause disease. The origin of experimental immunology is often cited as 1796 when Edward Jenner discovered protection against small pox through inoculation with cowpox [1].

The immune system defends the body against pathogens through immune responses. There are two major forms of immune responses: innate and adaptive, each of which employs its own cells and molecules.

Innate immunity includes the rapid reaction to an infectious organism generally within hours. Although innate immunity can recognize a wide range of pathogens it is not specific for an individual pathogen and the response which is provided does not generally last for a long time. In contrast to innate immunity, adaptive immune responses take days to develop but will usually produce more effective and longer lasting immunity with fine molecular specificity recognizing individual antigens and providing memory of those antigens for years and decades to come.

Major components of the innate immune system include: epithelial surfaces of the body, different phagocytes and other leukocytes (such as macrophages, dendritic cells,

neutrophils, eosinophils, basophils, and mast cells), specialized lymphocytes called natural killer cells, and immunological proteins like defensins, lysozyme, cytokines, chemokines, and components of the complement cascade. Macrophages, neutrophils, and dendritic cells recognize pathogen-associated molecular patterns (PAMPs) using their pattern recognition receptors (PRRs), toll-like receptors being perhaps the best understood of these. The PAMP recognized by a PRR is usually a simple repetitive molecular structure that is present on many microorganisms of a pathogen class such as components of bacterial cell walls or double stranded RNA of viruses.

In contrast to the innate immune system, the adaptive immune system recognizes particular antigenic components of pathogens through its two major types of lymphocytes: B (B cells) and T (T cells). B lymphocytes mediate humoral immunity using antibody production and T lymphocytes provide cell mediated immunity primarily through cytokine secretion and direct cellular cytotoxicity (Figure 1-1). Antibodies belong to the immunoglobulin superfamily of molecules and can be produced in secreted and membrane-bound forms. The membrane form is attached to the surface of the B cell and is also known as surface immunoglobulin or B cell receptor. Surface immunoglobulins recognize specific antigens and this binding is crucial for B cell activation, proliferation and differentiation in response to those particular antigens.

T lymphocytes are divided into three different groups of cytotoxic T cells, helper T cells, and regulatory T cells. Cytotoxic T cells kill neoplastically transformed cells and those infected with viruses or intracellular parasites. Helper T cells help other immune

cells to become activated against pathogens, and regulatory T cells suppress and control specific adaptive immune responses [2].

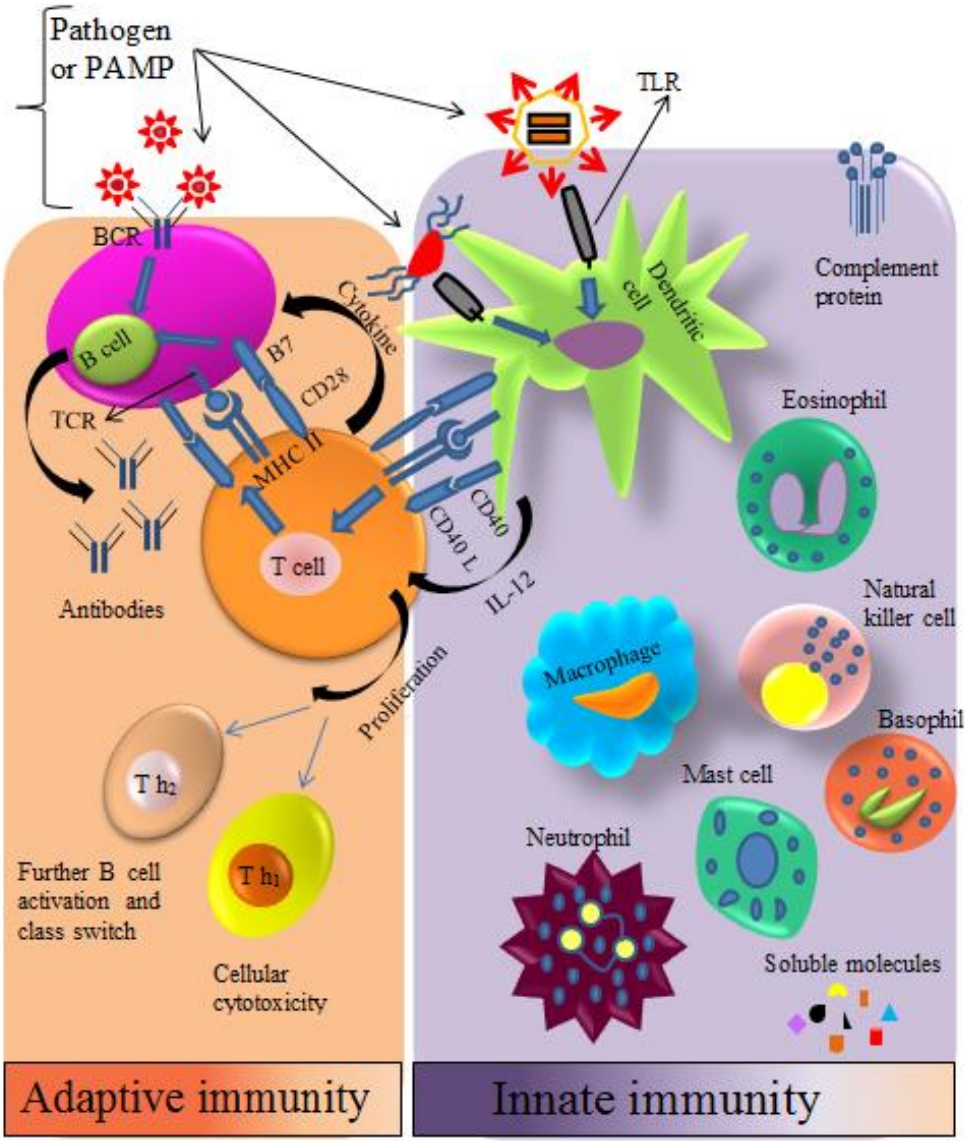


Figure 1- 1**.** Simplified overview of major innate and adaptive immune components. BCR: B cell receptor. TCR: T cell receptor. TLR: toll like receptor

1.2 Immunoglobulins

The lymphocyte based adaptive immune system evolved in jawless fish (Cyclostomata) [3]. One of the important characteristics of this system is the ability to produce antigen specific antibodies or immunoglobulins. Jawless vertebrates such as lamprey and hagfish do not express immunoglobulins but instead recognize antigens using variable lymphocyte receptors (VLRs) [4]. The immunoglobulins of jawed vertebrates were discovered more than a century ago [5].

These immunoglobulin superfamily antibodies emerged in the cartilaginous fish nearly one half billion years ago [5]. Different isotypes of immunoglobulins with specific functions have developed during their subsequent evolution. The two main partitions of the immune system in all jawed vertebrates are the systemic and mucosal immune compartments [6]. At least in all bony vertebrates immunoglobulin isotypes appear to have specialized for these systemic or mucosal compartments [7].

Antibodies are protein molecules composed of four chains: two identical heavy chains and two identical light chains. Each chain consists of two regions called variable and constant. The constant regions are nearly identical amongst all antibodies of a given isotype in the organism, but the variable regions are extremely diverse and create the repertoire of antigenic specificity within the animal. The light chains of all isotypes of antibodies are composed of a single variable domain and one constant domain. Heavy chains of all isotypes also are comprised of a single variable domain, but the number of constant domains differs from one isotype to another. Additionally, this number of

constant domains may be different in the same isotype from different vertebrate species. The constant domains of immunoglobulin heavy chains determine antibody function [8].

Each different major class of vertebrates (cartilaginous fish, bony fish, amphibians, reptiles, birds, and mammals) express multiple Ig isotypes [9].The isotype of the immunoglobulin determines effector functions such as complement activation, multimerization and Fc receptor binding which depends on the nature of motifs in the constant region of the antibody heavy chain [8]. In mammals five Ig (immunoglobulin) isotypes are recognized including IgM, IgD, IgA, IgG, and IgE [8] (Figure 1- 2). IgM is the oldest and the most conserved isotype present in all vertebrates except the coelacanth with slight structural variations in different species [9-13] (Figure 1- 3).

IgD and IgA (or its functional analogs IgX and IgZ/T) also exist in non-mammalian vertebrates. It has been shown that IgG and IgE arose from IgY which is expressed in birds, reptiles, and amphibians. Beside the canonical mammalian isotypes there are reports of other unique Ig classes such as IgO in the platypus, IgF in *Xenopus* and IgY in ducks [14], although these often are merely differential splice products of established isotypes.

Among these isotypes, IgM and IgG (or IgY in birds and reptiles and amphibians) play prominent roles in systemic responses and IgA (and ortholog of IgX and the functional analog of IgZ/T of fish) are most associated with mucosal immune responses [15, 16].

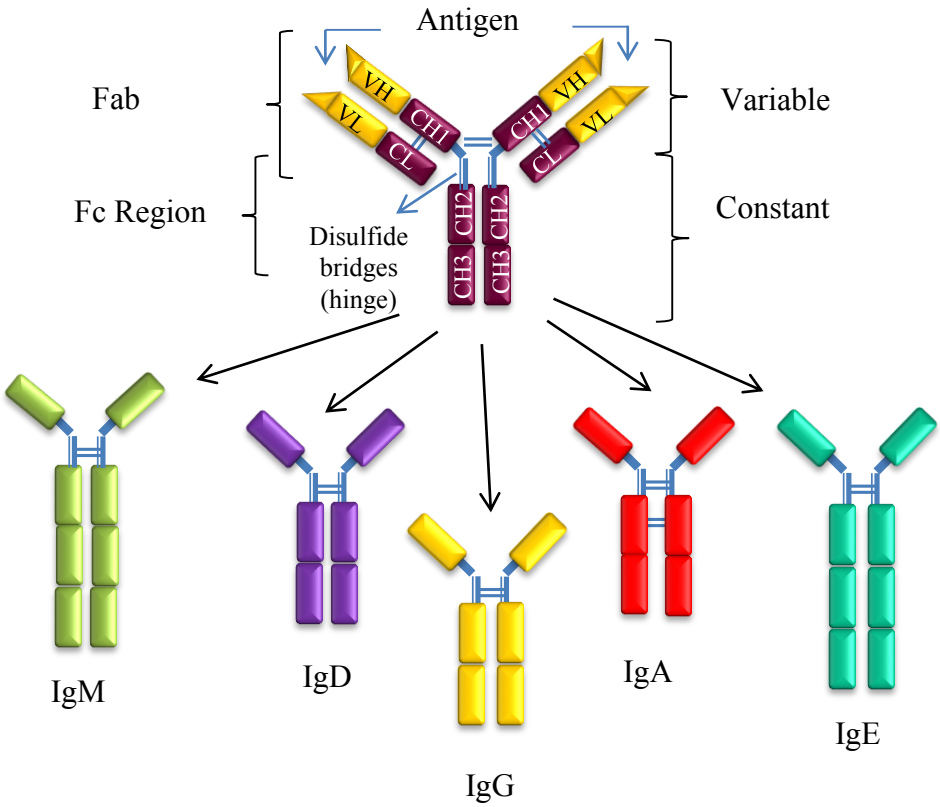


Figure 1- 2. Human immunoglobulin isotypes. The differences between different antibody classes are in their constant region. VH: variable domain heavy chain.CH: constant domain heavy chain. VL:variable domain light chain. CL: constant light chain

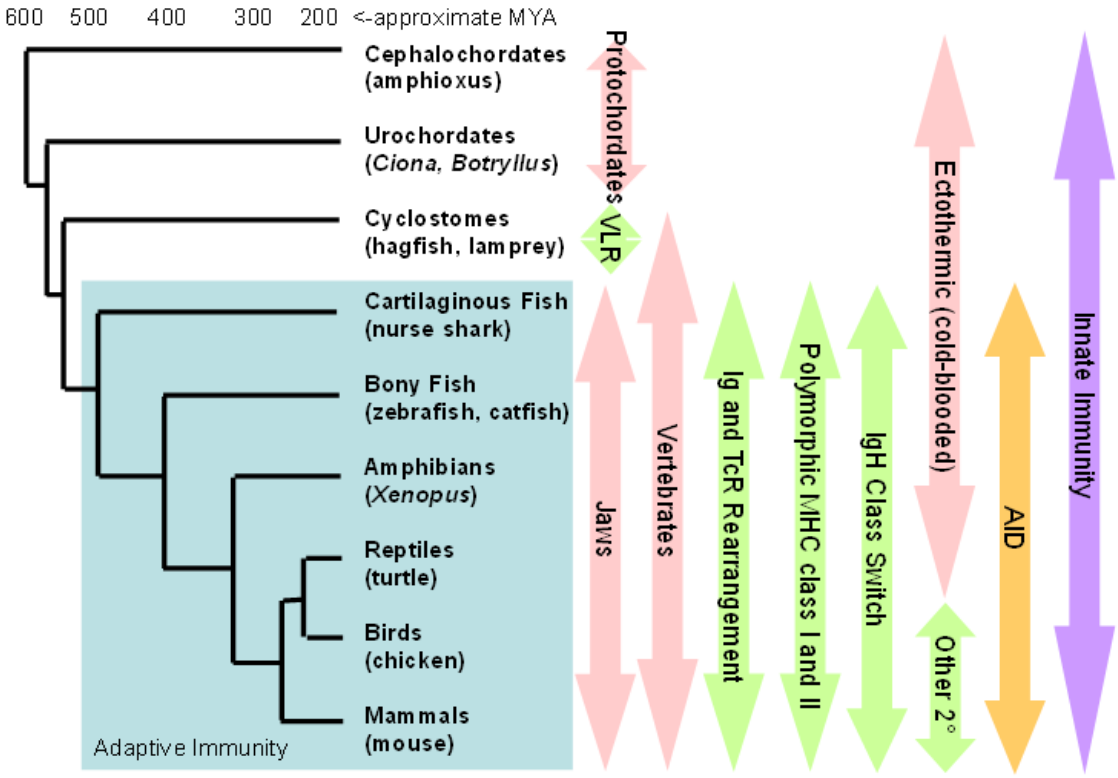


Figure 1- 3. Phylogeny of adaptive immunity

1.3 Mucosal immunity

The mucosal immune system is the first line of defense against most pathogens and forms the largest immune compartment in terms of total cells and total immunoglobulin. Mucosal immunity is composed of three major districts: gastrointestinal tract, respiratory tract, and urogenital tract.

The gastrointestinal tract is one of the most conserved parts of the adaptive immune system in jawed vertebrates [17]. The gut is populated with innate and adaptive lymphocytes as both scattered cells and more organized aggregates [2]. Dendritic cells, macrophages, T cells and B cells populate both the outer lumen and inner layer (lamina propria) of the intestine which is separated by a single layer of epithelial cells. The organized lymphoid tissues of the gut are called gut associated lymphoiud tissue (GALT) and include: Peyer's patches andisolated lymphoid folicules. B cells and T cells of the gut usually encounter their specific antigens in the GALT that is expressed to them by dendritic cells in these tissues. Activated T and B cells are also scattered in the lumen and lamina propria. Plasma cells of the mucosal immune system mostly produce secretory IgA,therefore this class of immunoglobulin is the major isotype that protects mucosal surfaces [2] (Figure 1.4)

1.3.1 IgA

IgA was discovered by Williams and Grabar in 1954 [18]. Then later in1957 and 1960 IgA was also described by Burtin and Heremans respectively but under different names [18]. Hermans was the first to isolate and characterize serum IgA [18]. Despite the other four classes of mammalian immunoglobulin (IgM, IgD, IgG, and IgE) all being associated prominently with serum, IgA was determined to be the major isotype in exocrine secretions [19, 20].

Sequence identity and functional analyses show that all mammals and birds studied have IgA. IgA accounts for 70% of antibody production in the human body [21]. The IgA molecule is composed of two identical heavy chains (α- chains for IgA) and two similar light chains which in mammals can be either λ or κ. The IgA heavy chain contains one variable domain (VH) and three constant domains (Cα1, Cα2, and Cα3). The heavy and light chains are attached with disulfide bridges. Inter-chain disulfide bridges between the Cα2 domains of two heavy chains stabilize the IgA molecule. Disulfide bridges between the Fc regions of two IgA monomers and the joining (J) chain polymerize to form IgA dimers (Figure1.2). Dimeric IgA is transferred to mucosal surfaces and secretions by the polymeric Ig receptor (pIgR) which is expressed by mucosal epithelial cells. The pIgR binds to dimeric IgA with disulfide bridges and transports it across epithelial cells before releasing it into the gut lumen [22]. The J chain is essential for this transportation [23]. There are two subclasses for IgA (IgA1 and IgA2) in some species of mammals such as primates, but in most other mammalian species IgA just has one subclass [24]. Vyas and Fudenberg discovered the first genetic marker representing IgA in 1969 and called it Am

1 [25]. The same genetic marker was described by Kunkel et al. In 1969 and they named it Am 2 [26]. Both belong to the IgA2 subclass [20, 25].

1.3.2 IgA producing B cells and class switch recombination

IgA1 is mostly found in human serum. It is chiefly monomeric in serum but it is also able to form dimers through disulfide linkages using J chain. Secretory IgA (S-IgA) belonging to the IgA2 subclass dominates in the gut and is dimeric. The human IgA2 subclass is more appropriate for the gut as it is more resistant to proteases due to the lack of 13 amino acids in the hinge region present in IgA1 [21, 22].IgA producing B cells and class switch recombination

IgA is produced by Peyer's patch plasma cells, isolated lymphoid follicle (ILF) plasma cells, peritoneal B1 cells, and spleen marginal zone B cells. Mucosal IgA-secreting cells are absent in neonates and are considerably decreased in germ free mice [27]. Although the IgA production in intestinal mucous drastically decreased in germ free mice, about half of normal serum IgA production is maintained [27-29]. It should be mentioned that there are independent IgA production sources in serum and mucosa [21].

B cells undergo class switch recombination (CSR) to be able to produce IgA. CSR may occur either independently of or dependent upon T cells. Theoretically CSR needs a total of two signals: first cytokines which activate transcription of switch regions, and second ligation of CD40 (on B cells) by CD40L (on T cells). But as mentioned CSR can also occur independently of T cell help, so there should be a third signal replacing the CD40-CD40L signal [30, 31]. Two members of the tumor necrosis factor (TNF) family comprise this third signal: BAFF (B-cell activating factor) expressed by monocytes and

dendritic cells, and APRIL (a proliferation-inducing ligand) expressed by monocytes, macrophages, dendritic cells and activated B cells. This third group of signals can induce local IgA production from human colonic epithelial cells in response to innate immune activation from Toll-like receptors [21, 32]. B cells in lamina propria and B1 lineage cells in the peritoneal cavity can produce IgA2 independently from T cells which is of a low affinity. High affinity IgA1 subclass is usually produced by B cells in germinal centers of Peyer's patches, mesenteric lymph nodes, and isolated lymphoid follicles and in a T cell dependent manner [33-35].

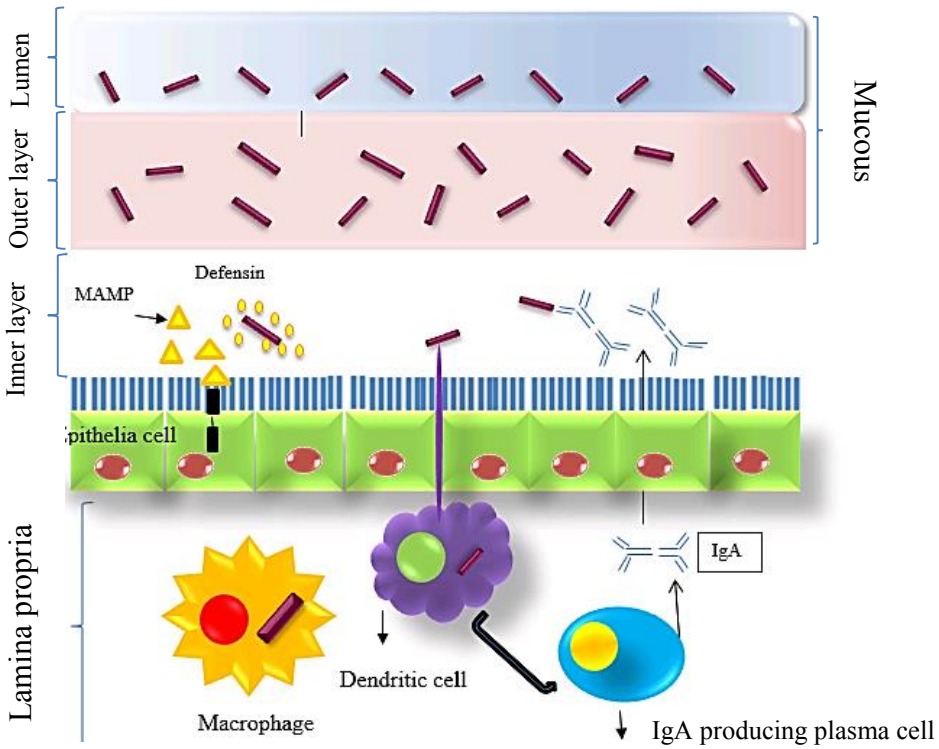


Figure 1- 4. Human gut immune system.

1.3.3 Evolutionary origins of immunoglobulins

Antibody molecules with immunoglobulin superfamily structure are only observed in jawed vertebrate species. It is proposed by some scientists that immunoglobulin genes originated from a common ancestor through mechanism of gene duplications followed by divergence in to variable and constant region gene families [36]. Generally gene duplication and point mutations are considered as fundamental mechanisms as far as creating diversification in antibody gene structure during evolution [20, 37].

1.3.4 Gut flora

Soon after birth the sterile body of an infant encounters environmental microorganisms and the mucosal surfaces and skin are colonized by vast numbers of these symbionts [38]. This polymicrobial community consists of archaea, fungi, viruses, protozoans, multicellular helminthes and most abundantly bacteria [39]. The large population of microbes resides in skin, upper and lower respiratory passages, the reproductive mucosa and gastrointestinal tract. The greatest density and diversity of microbial flora is associated with the lower gastrointestinal tract [40]. In a human the number of microorganisms living as normal flora exceeds the number of cells of the human body tenfold and this microbiota contains more than 1000 species in the human population, yet each person harbors around 160 species. So there was individual variations from person to person depending on many factors, including diet, environmental differences and host genetics [40, 41].

The relationship between the microflora and host is normally mutualistic as both sides benefit from each other. The host body is a permanent habitat and a rich source of energy uptake for the bacteria, and on the other side the host takes advantage of the bacteria for digestion of otherwise indigestible food components such as cellulose, synthesis of K1 and B12 vitamins, forming a barrier against pathogen invasion by niche occupancy, and fostering the development of the host immune system through the stimulation of Peyer's patch formation and antibody production [38, 42].

Studies on germ-free mouse models show symptoms of imbalances in mucosal immune system development such as: smaller Peyer's patches, considerably less IgA producing plasma cells, and less lamina propria $CD4^+$ T cells [38].The content of gut microflora has previously been analyzed with culture based methods but a considerable limitation of this method is that it can only identify easily cultivable microorganisms, which account for a minor population of the microbial species of gut flora. The discovery and application of molecular methods such as 16S rRNA gene analysis has facilitated recognition and classification of many more diverse species of bacterial flora [43].

The majority of research on gut flora employing current, molecularly based methods focuses on human and mouse microbiota and very few studies have been done with other species. Recently we investigated the gut flora content of the frog *Xenopus laevis* using 16S rRNA, a first in an amphibian.

Despite a lot of good work performed on human and mouse mucosal immunity there is still very little known about this important and the ancient immune compartment in lower vertebrates. There is a need for understanding this part of the immune system in

species from more ancestral groups of vertebrates, which can effectively help us gain a better understanding of the origins and evolution of our own adaptive and mucosal immunity. This may also provide us new tools for developing more effective treatment methods and designing novel vaccines against diseases that involve mucosal and specifically gut associated immunity such as inflammatory bowel disease (IBD), Crohn's disease, AIDS and others.Two studies were performed on the mucosal specific immunoglobulins in two species of lower vertebrates: *Xenopus laevis* and *Thunnus orientalis.*

Class switch recombination at a single IgH locus evolved in amphibians, therefore *Xenopus laevis* is a strong model to study the evolution of the adaptive immune system and physiology of different classes of immunoglobulins. We took advantage of this ancient model of IgH class switch to investigate the effect of T cell help on systemic and mucosal IgA/X production. As other studies have shown human IgA2 subclass can be produced without T cell help in the gut, we were curious about the effect of T cell help on a mucosal Ig production in lower vertebrates to address the evolutionary origins of T cell help.

Another important gap in our knowledge of the mucosal adaptive immune system in lower vertebrates is whether all fish have mucosal specific antibody or not. IgZ/T seems to be a mucosal isotype of several bony fish but seems to be absent in others [44]. Tuna comprise a group of bony fish that we have special interest in since some are endothermic (warm-blooded) species although via different mechanisms than those used to maintain mammalian body temperature. Unfortunately very little information is available about the

immune system in the large Perciforme group of fish, including whether they express any sort of antibody associated with the mucosal surfaces or not. The focus of my second study is to answer the aformentioned question in one of the most economically important fish: *Thunnus orientalis.*

2. ANCIENT T-INDEPENDENCE OF MUCOSAL IGX/A: GUT MICROBIOTA UNAFFECTED BY LARVAL THYMECTOMY IN *XENOPUS LAEVIS*[*]

2.1 Introduction

The thymus is the primary T lymphoid organ of vertebrates from shark to man [45, 46]. In humans, a small deletion on chromosome 22 in DiGeorge syndrome often results in an absent or hypoplastic thymus, with resulting loss of T mediated responses (reviewed in [47]). The hairless "nude" strain of mouse has an absent or greatly degenerated thymus due to a mutation in the Foxn1 gene [48]. These mice do have B cells but T cells are very few. Due to the lack of both cytotoxic and helper T cells, nude or thymectomized mice no longer have allograft and mixed leucocyte reactions, proliferative responses to classical T cell mitogens, and antibody responses against T-dependent antigens [49].

The mucosal immune system forms the largest immune compartment and is mediated by specialized cells and immunoglobulins, such as plasma cells producing

[*] Reprinted with permission from "Ancient T-independence of mucosal IgX/A: gut microbiota unaffected by larval thymectomy in Xenopus laevis" by S Mashoof, A Goodroe, C C Du, J O Eubanks, N Jacobs, J M Steiner, I Tizard, J S Suchodolski and M F Criscitiello,2013. Mucosal Immunology, 6, 358-368, Copyright © 2012, Rights Managed by Nature Publishing Group

secretory IgA. IgA secretion in the gut is not constitutive as shown by its absence and the lack of lamina propria plasma cells producing it in germ-free mice [50]. Experiments investigating the humoral mucosal immune responses of mammals lacking a T cell compartment have yielded mixed results [51, 52], but a picture is emerging of a significant T-cell independent mechanism of gut IgA management of mutualistic flora [53, 54]. Gut IgA producing plasma cell in mammals employ tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) usually associated with innate phagocytes [55]. Interestingly, B cells of lower vertebrates have been found to have strong phagocytic activity [56], continuing a theme of primitive, innate, T-independent B cells producing IgA in the gut. These findings prompted the present assessment of the T-dependence of humoral mucosal immunology in a phylogenetically relevant model species.

The African clawed frog *Xenopus laevis* belongs to the tongue-less frog family Pipidae. It is a choice model for ontogeny and phylogeny of both humoral and cell mediated immunity, sharing a common ancestor with mammals 350 million years ago and linking them to the more ancient vertebrates where the adaptive immune system arose (reviewed in [57]). The ability to perform thymectomy on transparent early stage *Xenopus* tadpoles made this frog an ideal model species to query the thymic dependence of management of gut microbiota and mucosal antibody responses from a fundamental point in vertebrate humoral immunity (reviewed in [58]).

This present study is the first investigation determining the gut bacterial population of *Xenopus laevis* using massive parallel 16S rRNA gene pyrosequencing. In addition, we examined the flora and the ability of thymectomized frogs produce mucosal antibody

responses. IgX has been functionally associated with mucosal responses and (in contrast to IgY) found in thymectomized frogs [59] [60] but evolutionarily has been thought to be closer to IgM [61]. T-independent responses are known from *Xenopus* serum [62], but here we aimed to determine the effect of thymectomy upon the gut flora, mucosal and systemic IgX response. Lastly we evaluated the relationship of amphibian IgX to mammalian IgA, in hopes of resolving ambiguity as to the origins and natural history of the class of antibody that manages vast numbers of mutualistic microbes and is the first defense of the barriers breached by most pathogens.

2.2 Methods

2.2.1 Animals

Xenopus laevis was used as a model for the tetrapod vertebrate immune system. Outbred frogs were initially purchased from *Xenopus* Express (Brooksville FL). Subsequent generations were bred in-house using human chorionic gonadotropin to prime for egg and sperm maturation (Sigma-Aldrich, St. Louis MO). Frogs were maintained at the Texas A&M Comparative Medicine Program facility. They were housed in two separate but similar recirculating rack systems (Techniplast, Buguggiate Italy) on a 12-hour light cycle. Frogs were moved from an antigen free system to a "DNP-KLH exposed" system upon first immunization. Adults were fed a sinking pellet and tadpoles a powder diet (*Xenopus* Express). All husbandry, surgery, and immunization protocols were approved by the Texas A&M Institutional Animal Care and Use Committee (AUP 2008-

33). Post-metamorphosis frogs were micro-chipped (Avid, Norco CA) and assigned to gut microbiota harvest or immunization protocols.

2.2.2 Thymectomy

Frogs were thymectomized nine days post-fertilization through microscopic cauterization, adapting the protocol devised by Horton [63]. The surgery was performed under a dissecting microscope using a micro-cautery apparatus originally designed for insect stylectomy (http://aphidzapper.com), delivering a VHF pulse of 10 millisecond duration and power of 10 watts via an abraded tungsten wire to the target tissue. Tadpoles were anesthetized using a 300mg/L MS222 (tricaine methanesulfonate, Argent, Redmond WA) bath for 2-10 minutes, before placing on wet cool cheesecloth over a grounded metal plate stage. The thymus was then burned with one pulse on each side. The thymus is located bilaterally caudally and medially to the eyes and lateral by the dark central nervous system (Figure 2 - 1). The tadpole was transferred to an ice bath immediately after thymic ablation to cool the animal for three seconds before being allowed to recover in an aerated tank with but 80mg/L carbenicillin (and no MS-222) to prevent superficial infections. After 24 hours tadpoles were transferred back to the primary recirculating Xenoplus systems (without antibiotic). Three days after surgery tadpoles were monitored for any re-growth of the thymus by visual inspection for the melanized organ under the microscope (approximately 20% of frogs that recover from surgery). The monitoring continued every 4-6 days for one month post-surgery. Tadpoles with thymic regrowth due to incomplete thymectomy were euthanized.

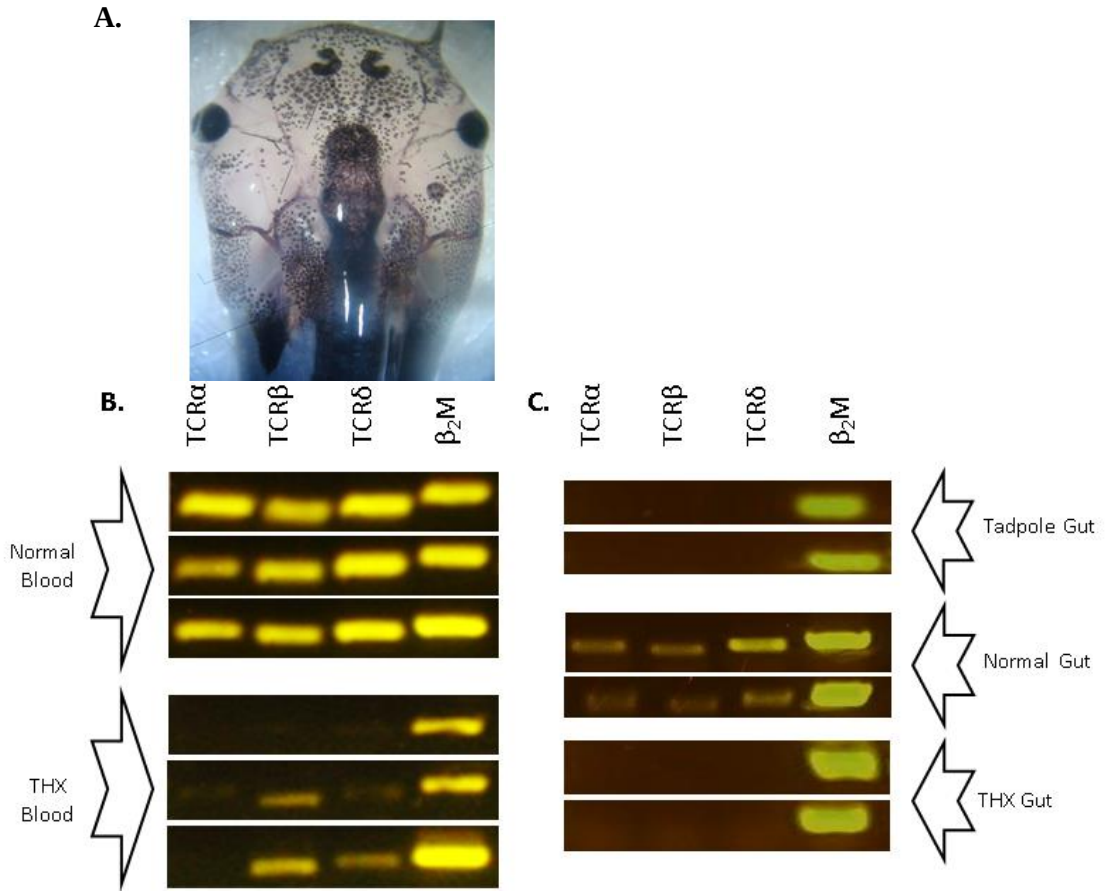


Figure 2 - 1. Larval thymectomy greatly depletes TCRα and δ message from the adult frogs. A. unilateral thymectomy at day 9 shows absence of naturally melanized thymus on the left compared with intact organ on the right. Experimental frogs were bilaterally thymectomized. Image captured at day 20 at original magnification x 3. B. PCR contrasting levels of TCR α, β, and δ to β_2-microglobulin in peripheral blood of three intact post-metamorphic frogs and three thymectomized post-metamorphic frogs. Results of 40 cycles of amplification. C. reverse transcriptase PCR comparing the same amphibians from intestine of 9-day tadpoles (the age of thymectomy, gut of 10 animals pooled per row) and two intact post-metamorphic frogs and two thymectomized post-metamorphic frogs.

Unilateral thymectomy at day 9 shows absence of naturally melanized thymus on the left compared to intact organ on the right. Image captured at day 20 at 3X magnification. Experimental frogs were bilaterally thymectomized. **B.** PCR contrasting levels of TCRα, β and δ to β_2-microglobulin in peripheral blood of three intact post-

metamorphic frogs and three thymectomized post metamorphic frogs. Results of 40 cycles of amplification. **C.** RT-PCR comparing the same amplicons from intestine of nine-day tadpoles (the age of thymectomy, gut of 10 animals pooled per row) and two intact post-metamorphic frogs and two thymectomized post-metamorphic frogs.

2.2.3 PCR validation of thymectomy

Frogs thymectomized as tadpoles were checked for the presence of TCR α, β, and δ mRNA using PCR at least six months post-surgery (they undergo metamorphosis in the second month, (Table 2 - 1). The more exposed tarsal veins associated with digits two and three were bled for 100-500 μL with 1 mL syringes and 28 gauge needles. Additional checks for TCR expression were performed on the gut of nine-day tadpoles, the gut of thymectomized adult, and the gut of normal adult. PCR was performed on cDNA prepared from RNA prepared from whole peripheral blood, due to the small volumes of blood collected using RNAeasy preparations (Qiagen, Valencia CA) with on-column genomic DNA digestion. First strand cDNA was synthesized using random hexamer priming with Superscript III (Invitrogen, Carlsbad CA) according to the manufacturer's protocol. PCR amplification was performed using oligonucleotide primers designed for constant domain genes of TCR α, β, δ and β2M (Table 2 - 2). Gotaq polymerase (Promega, Madison, WI) and 50 ng template were used during these PCR reactions for 35 cycles for blood, 2 μg template for 39 cycles for intestine. Primer sets for TCR α and β2m annealed at 52 °C and β and δ at 58 °C.

Frog ID	Spawn	Thx	Metamorphosis	Bleed	Immunizations	Sacrifice/ Harvest
056.262.321	8/10/09	-	9/10/09 to 10/10/09	7/28/11	-	10/7/11
056.263.571	2/10/09	-	3/10/09 to 4/10/09	8/25/11	-	10/7/11
056.271.011	8/10/09	-	9/10/09 to 10/10/09	8/25/11	-	10/7/11
056.282.258	10/2/10	10/13/10	11/2/10 to 12/2/10	5/26/11	-	10/7/11
056.280.606	10/2/10	10/13/10	11/2/10 to 12/2/10	5/26/11	-	10/7/11
056.277.339	5/6/10	5/17/10	6/6/10 to 7/6/10	5/26/11	-	10/7/11
056.106.606	8/10/09	-	9/10/09 to 10/10/10	6/6/11	5/3/11, 5/10/11, 5/17/11	6/7/11
056.259.055	6/3/09	-	7/3/09 to 8/3/09	6/6/11	5/3/11, 5/10/11, 5/17/11	6/7/11
040.080.063	9/8/09	9/18/09	10/8/09 to 11/8/09	6/6/11	5/3/11, 5/10/11, 5/17/11	6/7/11
040.080.105	9/8/09	9/18/09	10/8/09 to 11/8/09	6/6/11	5/3/11, 5/10/11, 5/17/11	6/7/11
040.593.882	8/10/09	-	9/10/09 to 10/10/10	6/6/11	5/3/11, 5/10/11, 5/17/11	6/7/11
056.105.865	8/10/09	-	9/10/09 to 10/10/10	6/6/11	5/3/11, 5/10/11, 5/17/11	6/7/11
040.065.593	9/8/09	9/18/09	9/10/09 to 10/10/10	6/6/11	5/3/11, 5/10/11, 5/17/11	6/7/11
048.531.048	9/8/09	9/18/09	9/10/09 to 10/10/10	6/6/11	5/3/11, 5/10/11, 5/17/11	6/7/11

Table 2 - 1. Timeline of frog manipulations. After sacrifice, nucleic acid was prepared from non-immunized frog gut contents and lymphocytes were harvested for culture from immunized frogs. Only the last three digits of frog microchip numbers are used in other figures and text.

Primer Name	For/Rev	Domain	Sequence	Priming Site	Anneal Temp.
Xl TCRaCF2	F	TCRαC	5′-TCAATGTATCGCCTCAAAG-3′	SMYRLK	53
Xl TCRaCR2	R	TCRαC	5′-CATTCCACAGGACTGAAC-3′	SSVLWN	54
Xl TCRdCF	F	TCRδC	5′-CACTGAAGCCTCAACAAGGTG-3′	ALKPQQG	61
Xl TCRdCR	R	TCRδC	5′-GATGCTTGGCTAGGCACTG-3′	QCLAKH	60
XlTCRbF1	F	TCRβC	5′-CGAACTGACCATGGCTACT-3′	DPWLL	58
XlTCRbR1	R	TCRβC	5′-CTCATTCTTGGTGAGGCTGA-3′	QPHQEV	58
XlB2MF1	F	β_2M	5′-AACATTAGTCCCCCGGTGG-3′	NISPPVV	60
XlB2MR1	R	β_2M	5′-GGGAGACCACACATTCCACT-3′	VECVVSH	60
530F	F	rRNAV4	5′-GTGCCAGCMGCNGCGG-3′		60
1100R	R	rRNAV6	5′-GGGTTNCGNTCGTTR-3′		60

Table 2 - 2. Primers used in PCR.

2.2.4 DNA preparation and 16S rRNA gene sequencing

Gut contents were sampled from normal and thymectomized frogs at three anatomical sites: stomach, small intestine and large intestine (Figure 2 - 2). Bolus and chyme were scraped from longitudinally opened organs and collected as ~300 μL samples for DNA isolation via phenol-chloroform-isoamyl alcohol extraction [64]. Bacterial tag-encoded FLX-titanium amplicon pyrosequencing (bTEFAP) was performed similarly as described previously at the Research and Testing Laboratory,

Lubbock, TX, USA [42], but based on the V4-V6 region (E. coli position 530-1100) of the 16S rRNA gene, with primers forward 530F and reverse 1100R (Table 2 - 2). Briefly, the DNA concentration was determined using a Nanodrop spectrophotometer (Nyxor Biotech, Paris France). A 100 ng (1 μl) aliquot of each DNA sample was used for a 50 μl PCR reaction. HotStarTaq Plus Master Mix Kit (Qiagen, Valencia CA) was used for PCR under the following conditions: 94°C for 3 min followed by 32 cycles of 94°C for 30 sec; 60°C for 40 sec and 72°C for 1 min; and a final elongation step at 72°C for 5 min. A secondary PCR was performed for FLX (Roche, Nutley NJ) amplicon sequencing under the same conditions by using designed special fusion primers with different tag sequences as: LinkerA-Tags-530F and LinkerB-1100R. The use of a secondary PCR prevents amplification of any potential bias that might be caused by inclusion of tag and linkers during initial template amplification reactions. After secondary PCR, all amplicon products from the different samples were mixed in equal volumes, and purified using Agencourt Ampure beads (Agencourt, Danvers MA).

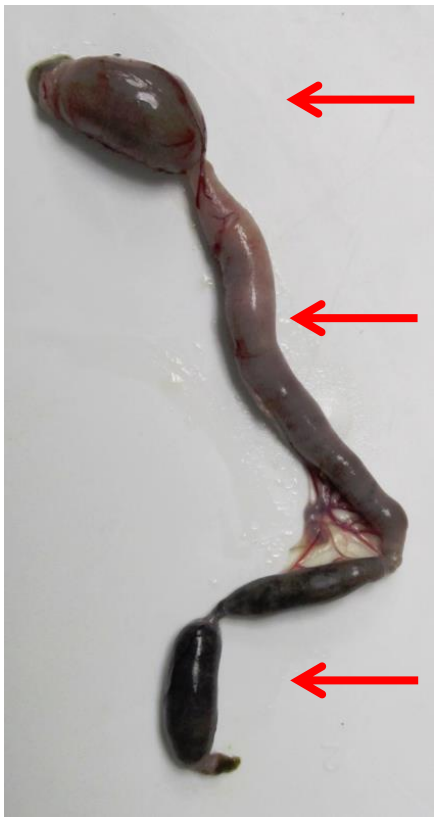


Figure 2 - 2**.** Site of flora sampling from *Xenopus laevis* gastrointestinal tract.
Resected alimentary canal from adult *X. laevis* fed one day before tissue harvest. Red arrows show the three sites where luminal contents were harvested**.**

2.2.5 Gut flora analysis

Raw sequence data were screened, trimmed, filtered, denoised, and chimera depleted with default settings using the QIIME pipeline version 1.4.0 (http://qiime.sourceforge.net) [65] and with USEARCH using the OTU pipeline (www.drive5.com). Operational taxonomic units (OTUs) were defined as sequences with at least 97% similarity using QIIME. For classification of sequences on a genus level the naïve Bayesian classifier within the Ribosomal Database Project (RDP, v10.28) was used. The compiled data were used to determine the relative percentages of bacteria for each individual sample, six frogs by three samples each for a total of eighteen. To account for

unequal sequencing depth across samples subsequent analysis was performed on a randomly selected subset of 1800 sequences per sample. This number was chosen to avoid exclusion of samples with lower number of sequence reads from further analysis. Alpha diversity (i.e., rarefaction) and beta diversity measures were calculated and plotted using QIIME. Differences in microbial communities between different groups were investigated using the phylogeny-based unweighted Unifrac distance metric. This analysis measures the phylogenetic distance among bacterial communities in a phylogenetic tree, and thereby provides a measure of similarity among microbial communities present in different biological samples.

2.2.6 IgX phylogenetics

Amino acid sequences of tetrapod immunoglobulin heavy chain constant regions were compiled and aligned in BioEdit with ClustalW employing gap opening penalties of 10 and gap extension penalties of 0.1 for pairwise alignments and then 0.2 for multiple alignments with the protein-weighting matrix of Gonnett or Blossum [66, 67]. These alignments were then heavily modified by hand. MEGA was used to infer the phylogenetic relationships of the immunoglobulin heavy chain constant genes. Evolutionary distances were computed using the Dayhoff matrix [68] and 509 column positions in the 56 selected sequences. Several tree-building algorithms were employed, including a consensus neighbor-joining tree made from 1000 bootstrap replicates.

2.2.7 Immunizations

For immunization, two routes of administration were used. Four frogs received intracoelomic (IC, frogs have no peritoneal cavity) and four oral (PO) immunizations. We used an oral gavage needle to deliver conjugated dinitrophenol- keyhole limpet hemocyanin (DNP-KLH, Calbiochem, San Diego CA) for mucosal immunization as previously described for X. laevis [60]. There were two normal and two thymectomized frogs in each group for a total of eight frogs. Frogs orally immunized received 2.5mg DNP/KLH with 10µg cholera toxin as adjuvant three times, each at weekly intervals. Animals in the intracoelomic group received 200µg of antigen with equal volume (200µl) of Freund's complete adjuvant once and Freund's incomplete adjuvant twice, at weekly intervals.

2.2.8 Lymphocyte isolation and culture

Three weeks after the last boost, we euthanized frogs with an MS-222 overdose and decapitation. Spleens were removed and cells dissociated by scraping the organs over a wire mesh inundated with amphibian-adjusted PBS. The intestine was excised below the stomach and above the rectum. We removed mucous, chime and fecal matter by physically scraping and flushing the organ with PBS. The intestine was cut into smaller pieces (approximately 0.5 cm2) and placed in PBS. We then added 2% collagenase (Calbiochem, San Diego CA) in amphibian PBS to aid in isolation of the intestinal lymphocytes. We allowed the intestine to be digested for 120 minutes at room temperature. It was vortexed initially and then once every thirty minutes. The intestine was strained through a 100µm

nylon cell strainer (BD Falcon, San Jose CA) at the end of the 120 minutes. Lymphocytes were isolated from the washed supernatant of the intestine and from the cells isolated from the spleen with Lymphocyte Separation Medium (Mediatech, Manassas VA). All intestine and spleen cells isolated were counted Figure 2 - 3) and cultured in twenty-four well plates containing 250µl of L-15 media with 10% fetal calf serum at a density of 2.3x106 cells/ml for the spleen cells and 1.0x106 cells/ml for the intestinal cells. Cultures were incubated at humidified 28°C and 5% CO2. On the third day, supernatant was collected and new media was added to maintain a volume of 250µL per well. On the sixth day, supernatant was once again collected and pooled with the day three collections.

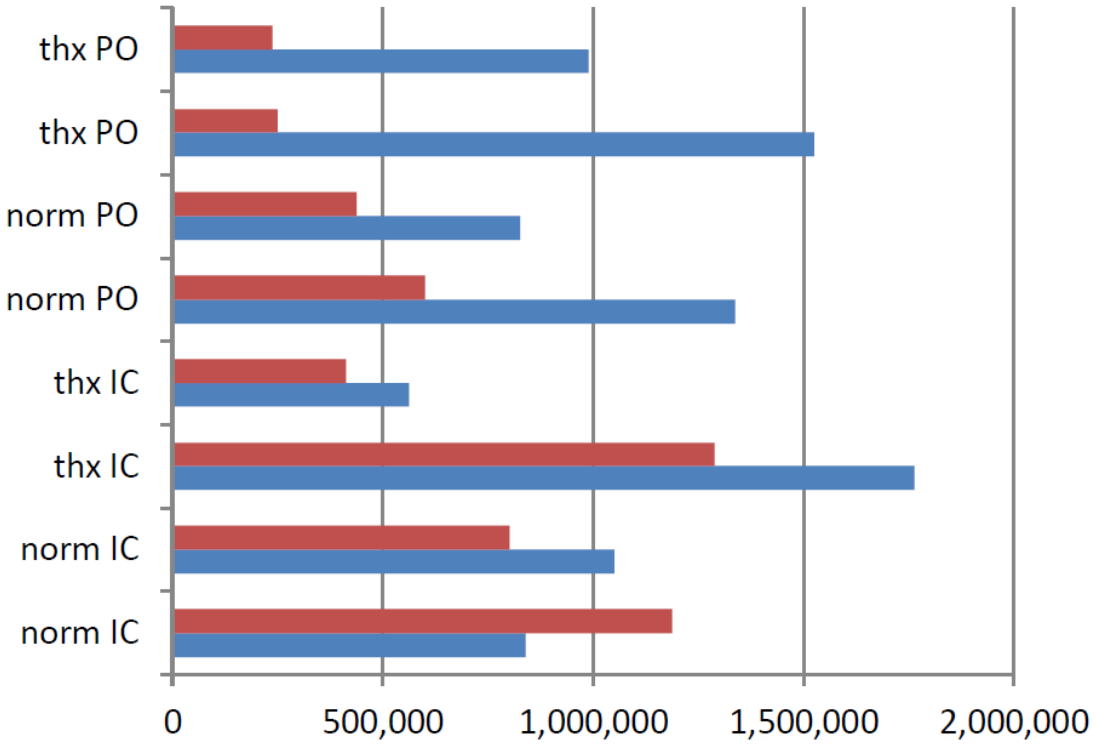


Figure 2 - 3**.** Total cell counts from tissue harvests. After sacrifice, lymphocytes were isolated from spleen and intestine as described in Methods. Total cells isolated from each immunized frog are shown, identified by surgery status and immunization route.

2.2.9 Enzyme-linked Immunosorbent Assay (ELISA)

We used ELISAs to determine the total (non-antigen specific) and antigen-specific levels of amphibian mucosal antibody isotype IgX produced in response to DNP-KLH. Serial dilutions from 1/10 to 1/1011 were made from the supernatants of the spleen and intestinal cultures. For the total immunoglobulin ELISAs, we added 100µL of each dilution to the well of a plate and incubated for one hour at 37°C. The plate was then washed 2X with 200µL PBS and 200µL of 2% casein in PBS was added as blocking solution. The plate was allowed to sit overnight at 4°C, and was then washed 3X with

200µL PBS. We added mouse anti-*Xenopus* IgX monoclonal 4110B3 (kind gifts of Martin Flajnik, University of Maryland at Baltimore and Louis Du Pasquier, University of Basel), to each well to obtain a volume of 100µl [69]. The plate was allowed to incubate at room temperature for one hour and then was washed 4X with 200µl PBS with 0.05% Tween-20 (PBS-T, Sigma, St. Louis MO). The wells received 100µl of anti-mouse IgG peroxidase conjugated secondary antibody (Sigma) and were incubated for one hour at room temperature. We then washed the plate 4X with 200µl PBS-T and 1X with PBS. A 3, 3', 5, 5'-tetramethylbenzidine substrate solution was then added to each well and the reaction was allowed to take place for three minutes before being stopped with 2M H2SO4. Plates were read at an optical density of 450nm in a BioRadiMark Microplate Reader (Hercules CA). We used the 104 dilution of supernatant for all trials. The antigen-specific ELISAs used a similar protocol, except 100 µl of 10 µg/ml DNP-KLH was added to each well for initial coating. After blocking overnight, serial dilutions of the sample supernatant were added and allowed to incubate for two hours at 37°C. The remaining protocol was the same as that for total IgX. We assayed wells in triplicate, showed the standard error of the mean and employed a student's t-test.

2.3 Results

2.3.1 Larval thymectomy depletes T cells in the adult

To study the role of T cells in the management of gut bacterial communities and mucosal humoral immunity from a fundamental standpoint in vertebrate evolution, we

used the frog larval thymectomy model. We performed thymectomies largely executed as in the original studies [63], but two days later in development than in the original protocol due to slightly slower development of the larvae in our system. No frogs used in the thymectomized group showed any thymic regrowth after cauterization (using microscopic inspection). Thymectomy greatly diminished the detectable number of TCRα transcripts using constant region PCR in the adult frog. TCR β and δ amplicons could be detected in some frogs, but at a much lower frequency than in frogs that had not undergone surgery. Regardless, larval bilateral thymectomy almost completely ablated expression of TCRα, showing that the classic αβ T cell compartment had been removed from the experimental gut model.

2.3.2 Pyrosequencing reveals diverse gut bacterial flora in amphibian

DNA was prepared from frog luminal contents at three distinct locations in the gastrointestinal tract, which is relatively simple in poikilothermic frogs and reptiles as they have lower metabolic rates compared to most mammals and birds (Figure 2 - 2). Pyrosequencing of the 16S rRNA gene resulted in a total of 51,992 quality sequencing tags (mean, range: 2888, 1870-5314).

Figure 2 - 4 illustrates the rarefaction curves for all samples at 1800 sequences, suggesting sufficient coverage. Although rare OTUs might have been identified with a higher sequencing depth, the number of sequence tags used here was deemed adequate to allow comparison of the beta diversity between samples [70]. Samples taken from Large intestine showed the richest diversity of flora. Individual frogs showed distinct microbiota composition (Figure 2 - 5). Four frogs yielded similar rarefaction curves, whereas one

non-thymectomized frog (N321) showed more diversity and one non-thymectomized (N571) less. Averaged thymectomized samples gave a very similar rarefaction plot as those from normal animals. These data allowed us to gain an initial molecular description of the base amphibian gastrointestinal microbiota and look for differences between anatomic sites and surgical groups.

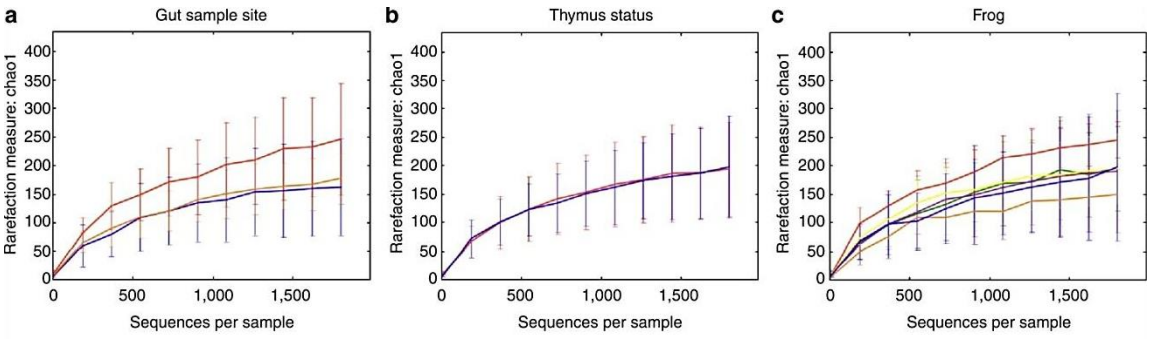


Figure 2 - 4**.** Rarefaction analysis of 16S rRNA gene sequences obtained from frog gastrointestinal content. The analysis was performed on a randomly selected subset of 1800 sequences per sample.Lines represent the average of each sample type. A. stomach = orange, large intestine = red,and small intestine = blue. B. normal (no surgery) = blue and thymectomized = red. C. frog N011 = red, N321 = blue, T258 = green, T606 = yellow, T339 = brown, and N571 = orange.

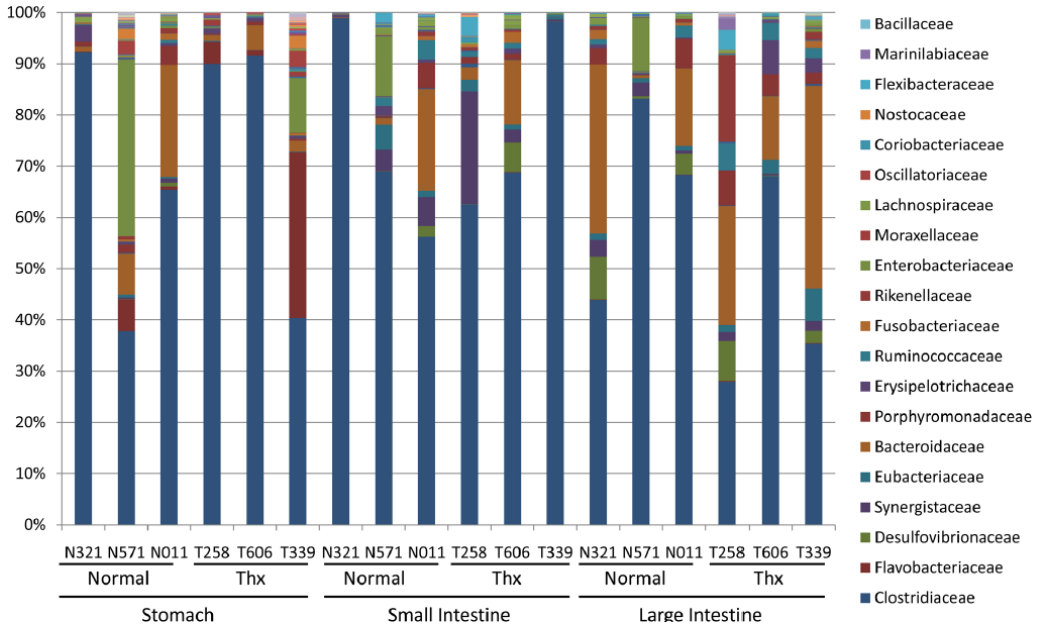


Figure 2 - 5**.** Bacterial families in the gastrointestinal tract of *Xenopus laevis.* Familial distribution is shown with different colors from individual samples, grouped by thymus status from each of the three sampled positions in the gastrointestinal tract. Families with at least 1% representation in any sample are listed at the right. Complete taxonomic data is in Table 2 - 3. Clostridiaceae were the predominant family, and no differences in bacterial groups between normal and thymectomized frogs were observed.

2.3.3 Frog gut flora is anatomically distinct but not altered by T cells

Many of the prokaryotic groups that dominate the human flora are also major components in the frog flora. Clostridiaceae dominated this amphibian community, and Bacteroidaceae and Enterobacteriaceae were abundant in our sequencing (Figure 2 - 5). In contrast to terrestrial mammalian flora, the environmental Flavobacteriaceae constituted nearly a third (32.34%) of the stomach flora of one frog and comprised 6.20% and 4.20% in two other individuals. The Synergistaceae, Desulfovibrionaceae, Erysipelotrichaceae, Ruminococcaceae, Rikenellaceae and Porphyromonadaceae also were substantial contributors (over 5% in at least one sample) to the X. laevis microbiota.

The PCoA plots based on the unweighted UniFrac distance metric indicated that the X. laevis stomach is composed of distinct microbial communities compared to the small and large intestine (Figure 2 - 6). This was most pronounced in the greater representation of Flavobacteriaceae in the stomach compared to the more distal sites. Oscillatoriaceae cyanobacteria and Enterobacteriaceae were in greater abundance in the stomach. This latter group includes the common gram negative sometimes pathogenic *Salmonella, Escherichia coli, Klebsiella, Shigella and Yersinia pestis* more commonly associated with the mammalian intestine. Synergistaceae were found in the intestines more than the stomach, particularly in the small intestine of thymectomized frog #258. However, PCoA plots based on the unweighted UniFrac did not reveal differences between small and large intestinal microbiota and, importantly, between normal and thymectomized frogs (Figure 2 - 6). The similar microbiological findings in the guts of

normal and T cell depleted frogs prompted investigations of the mucosal humoral immune compartment in this model.

2.3.4 Amphibian IgX is likely orthologous to mammalian IgA

In order to assess the T-dependence of humoral immunity in the frog alimentary canal, we wanted to be more confident of the mucosal immunoglobulin in this amphibian. As more immunogenetic data have recently become available from reptiles, birds, and ancestral groups of mammals, we revisited the phylogenetic relationships of tetrapod antibody classes to see if there was now convergence of expression and functional data suggesting IgX as the mucosal isotype. Entire immunoglobulin heavy chain C region sequences from diverse vertebrates were used to determine the relationship between amphibian IgX and mammalian IgA (Figure 2 - 7). Sequences were aligned and manually adjusted to maintain domain alignment between isotypes having either three or four constant domains (Figure 2 - 8). The resulting trees showed that IgX did not cluster closest to IgM. Unlike any past phylogenetic analyses, these data show that IgX and IgA share a common ancestor earlier than either IgX or IgA does with IgM with high statistical support (91% of 1000 bootstrap replications). This finding provided confidence in assaying IgX in the frog as an ortholog as well as a functional analog of mammalian IgA in mucosal immunity.

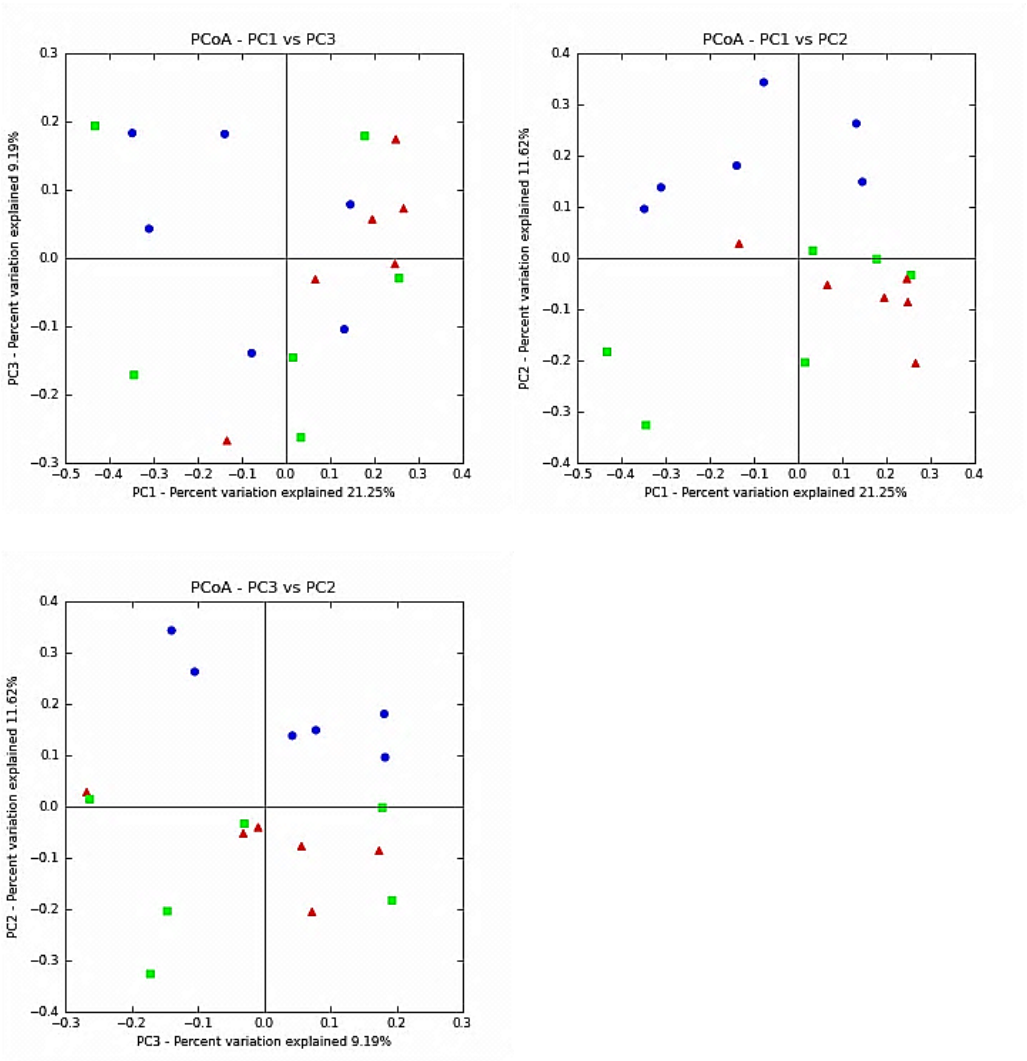


Figure 2 - 6**.** Principal Coordinates Analysis (PCoA) of unweighted UniFrac distances of 16S rRNA gene sequencing. The analysis was performed on a randomly selected subset of 1800 sequences per sample. Stomach (blue circles) samples separated from small (green square) and large (red triangle) intestine, indicative of distinct flora.

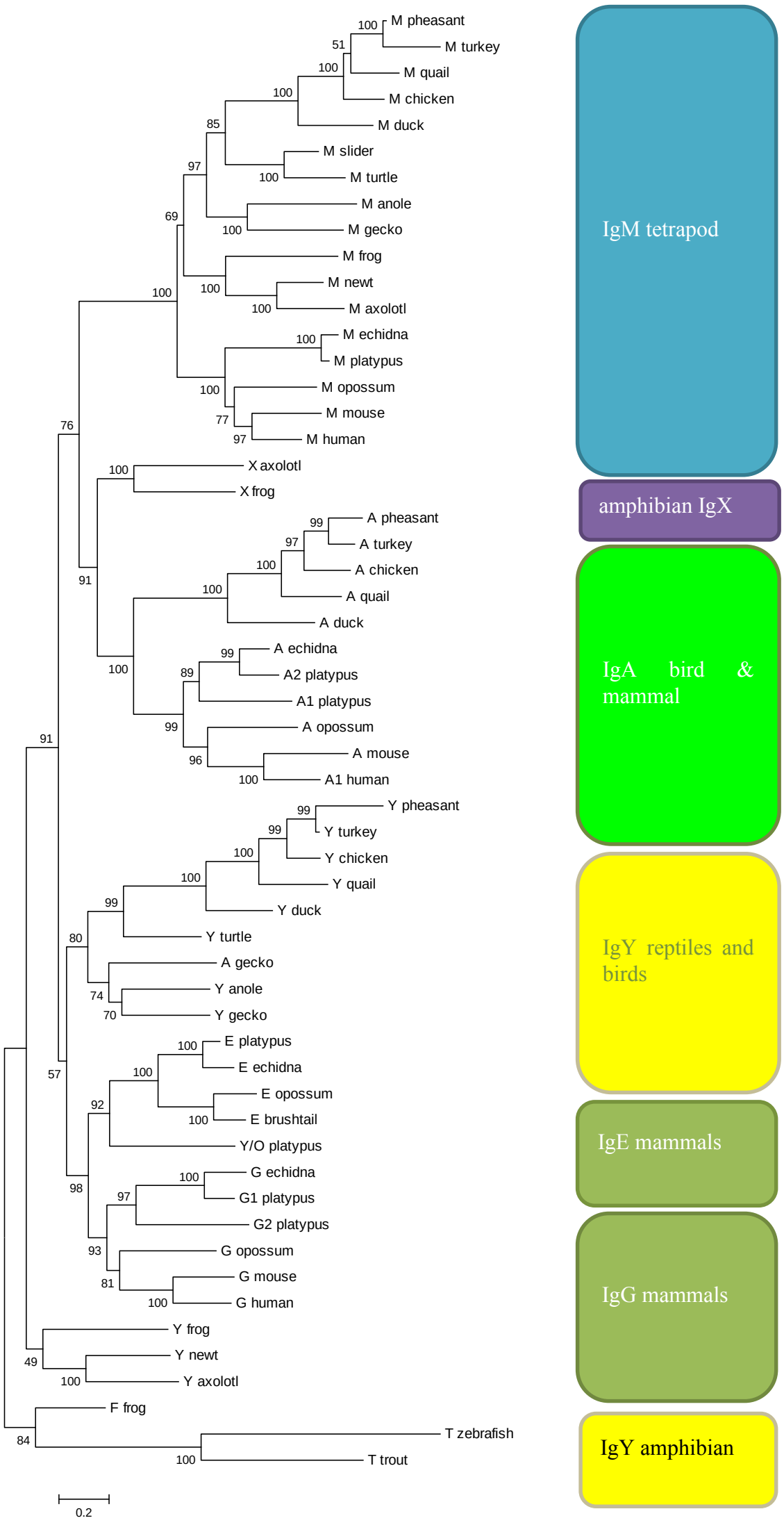


Figure 2 - 7. Amphibian IgX is orthologous to IgA of birds and mammals.
Neighbor joining phylogenetic tree of the constant regions of diverse tetrapod immunoglobulin heavy chains, with the fish mucosal isotype IgZ/T included as an outgroup. Numbers at nodes show bootstrap support for each bifurcation after 1000 replications.

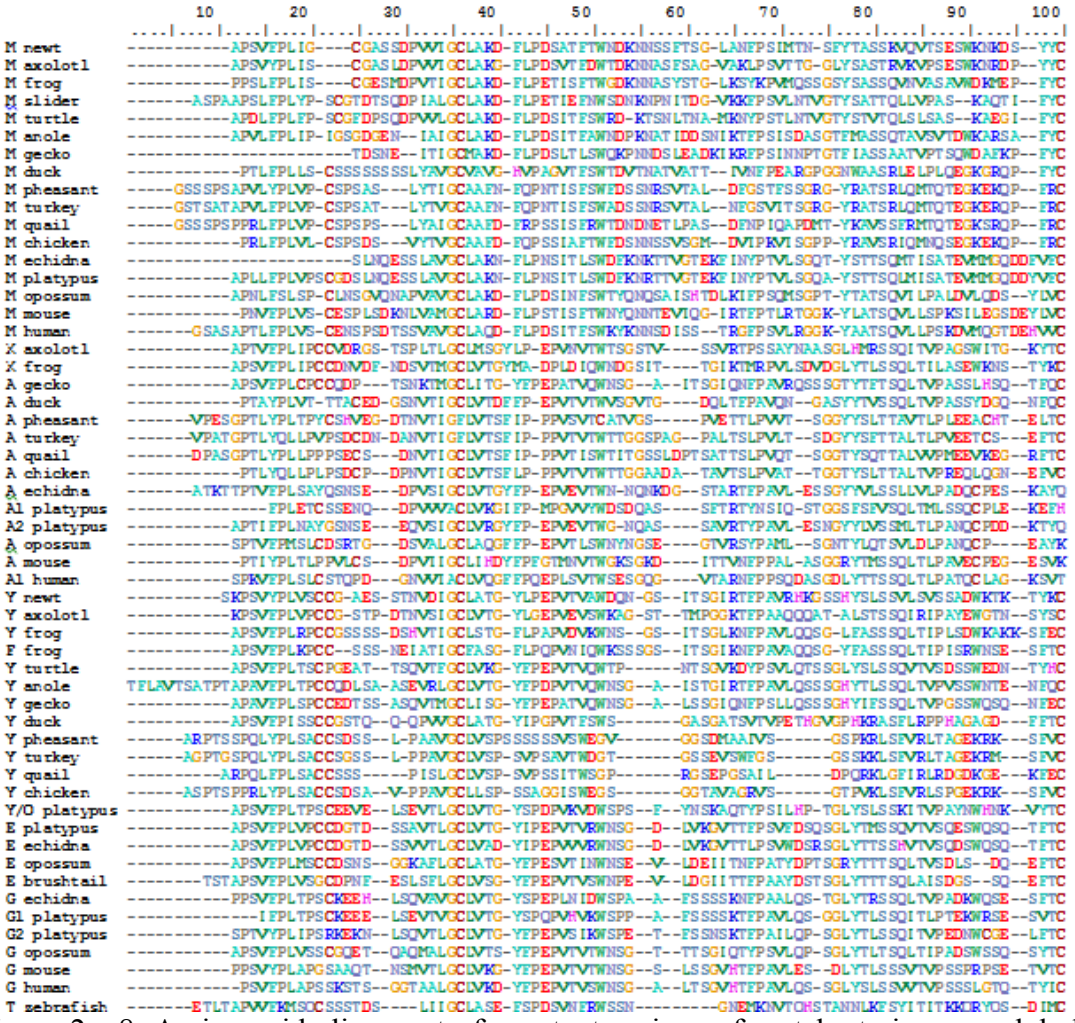
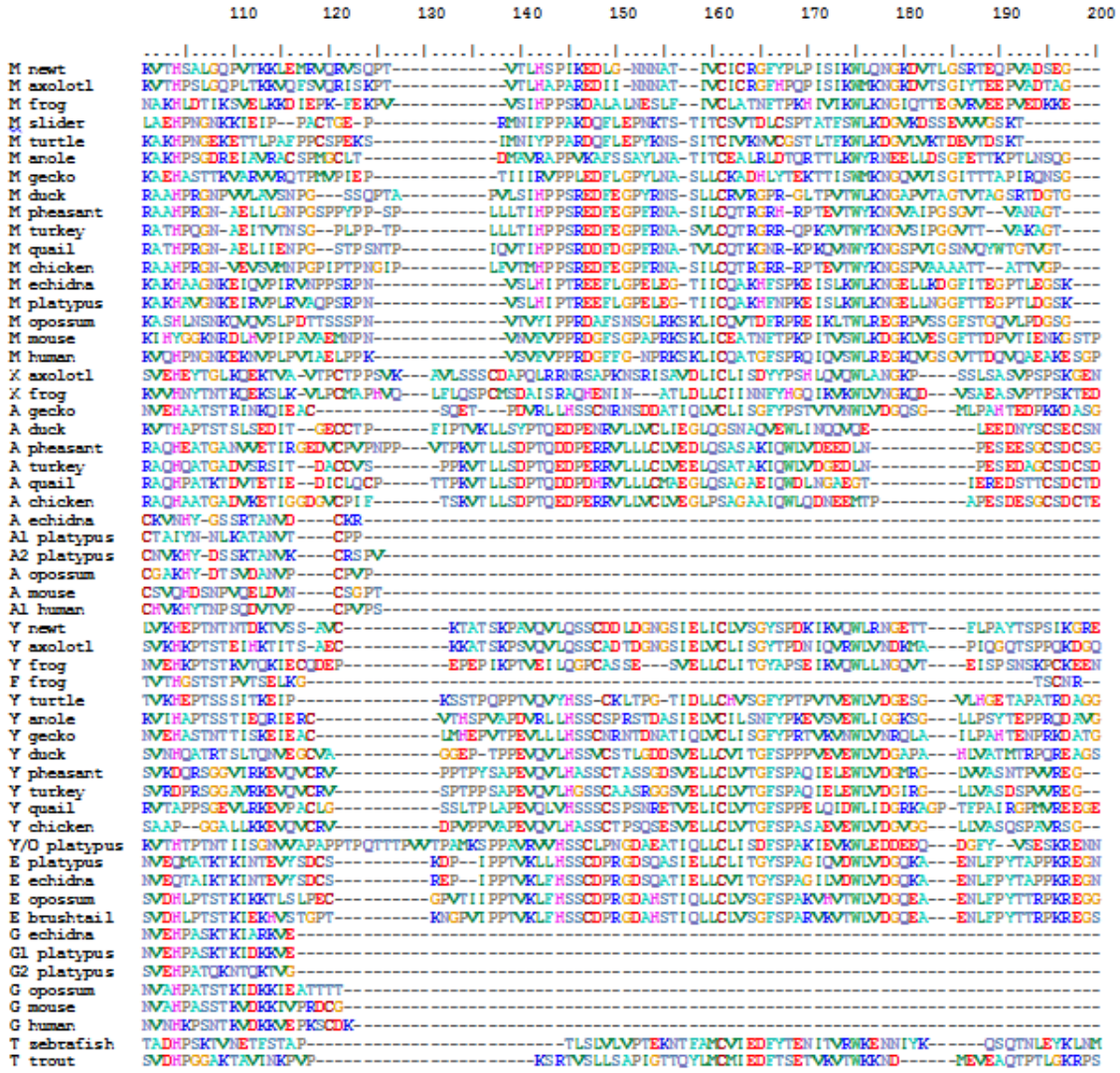
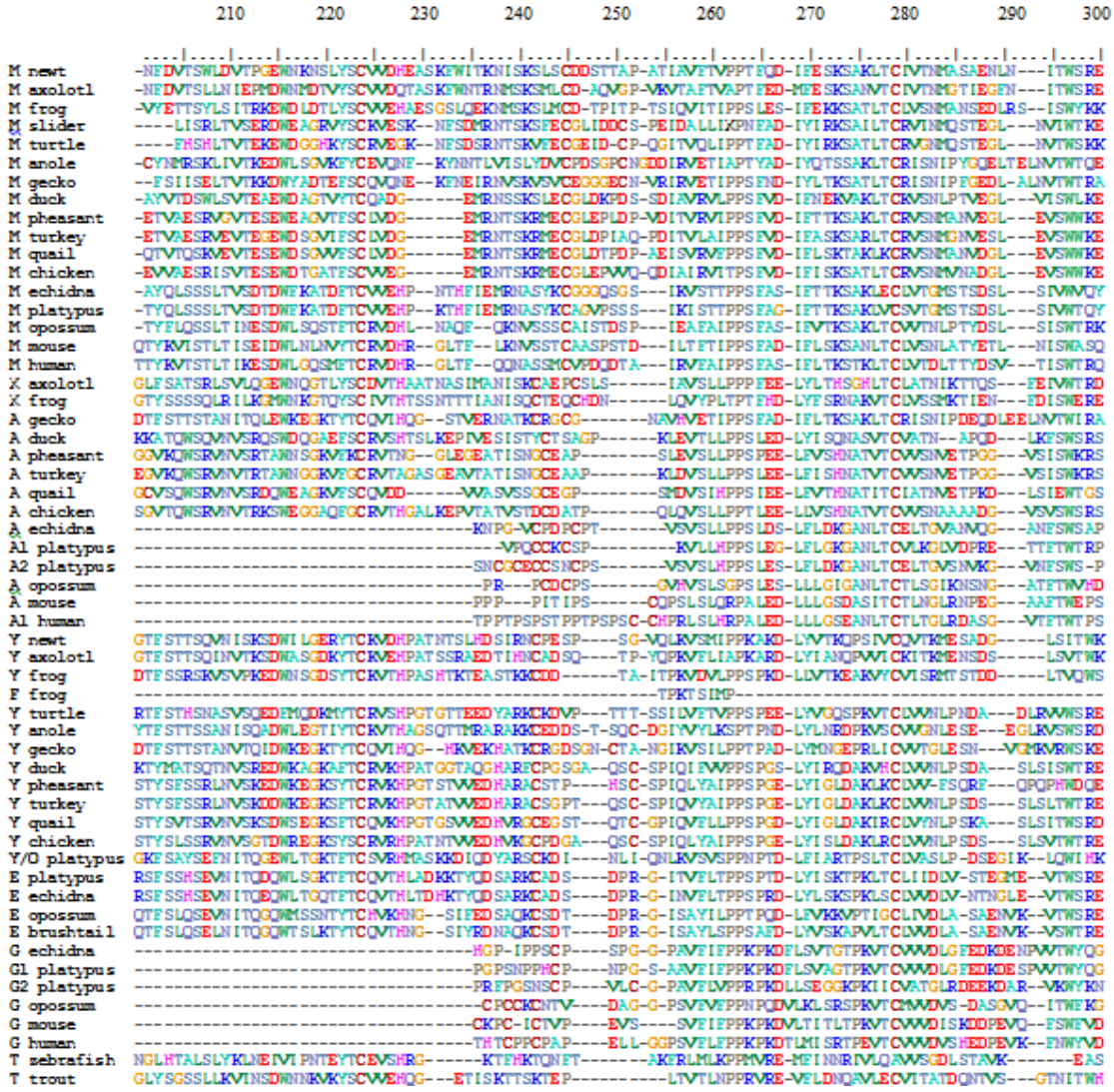


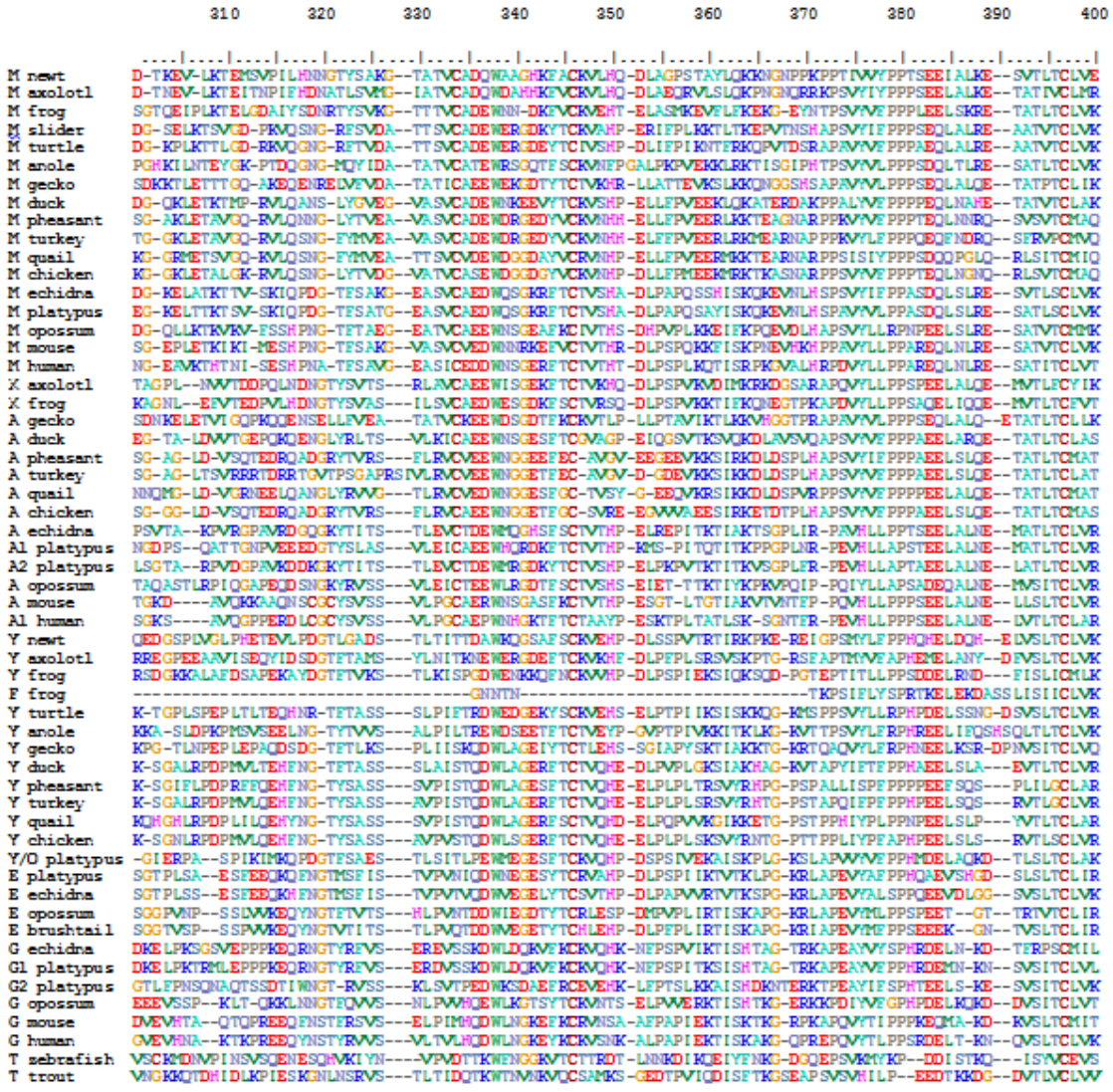
Figure 2 - 8**.** Amino acid alignment of constant regions of vertebrate immunoglobulins used in Figure 2 - 7 phylogeny**.** Sequences were aligned using Clustal in MEGA then manually adjusted based on cysteine residues in paralogous domains. That alignment was used for the dendrogram and exported to Bioedit for creation of this figure.



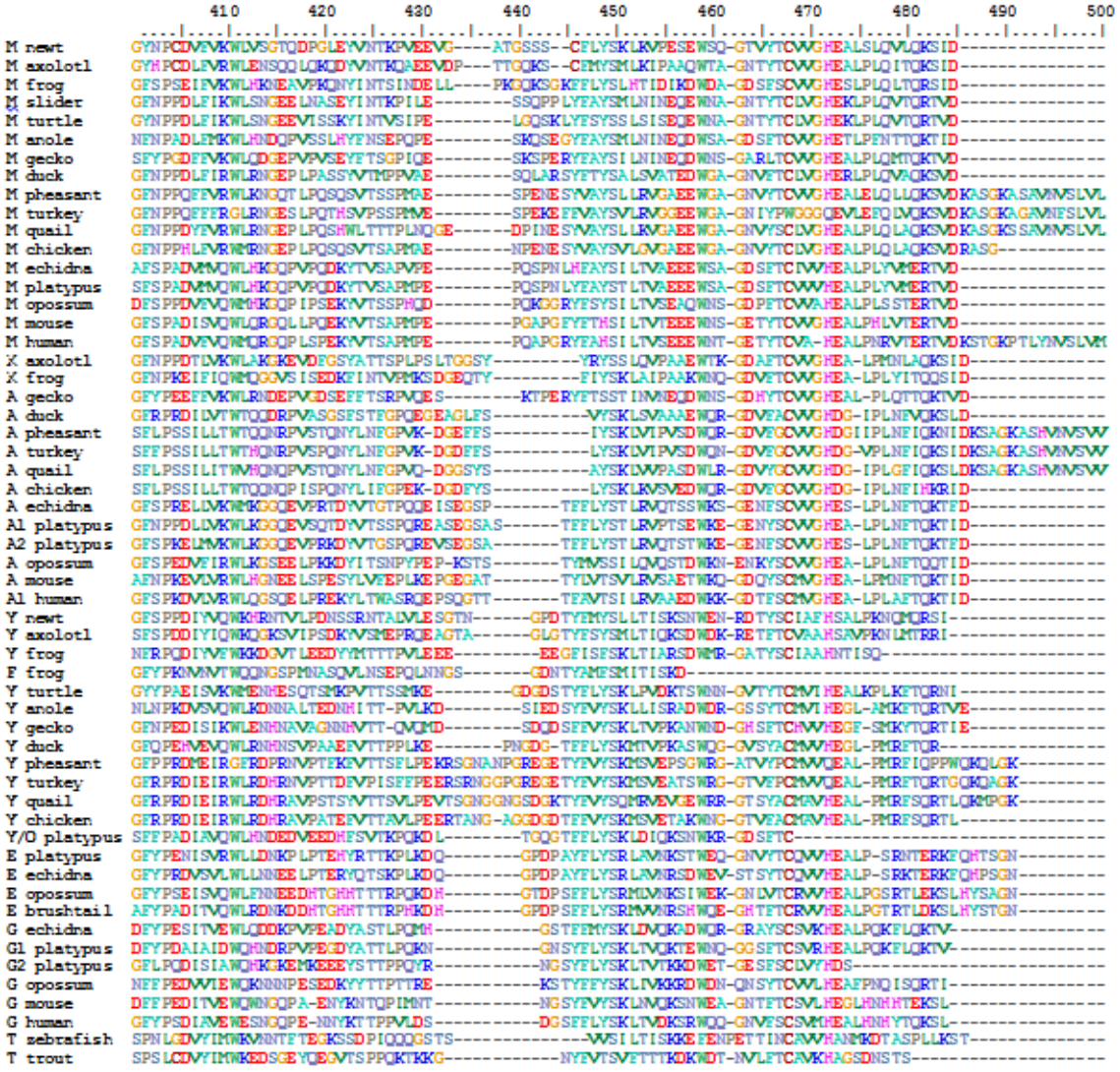
(Figure 2 - 8 continued).



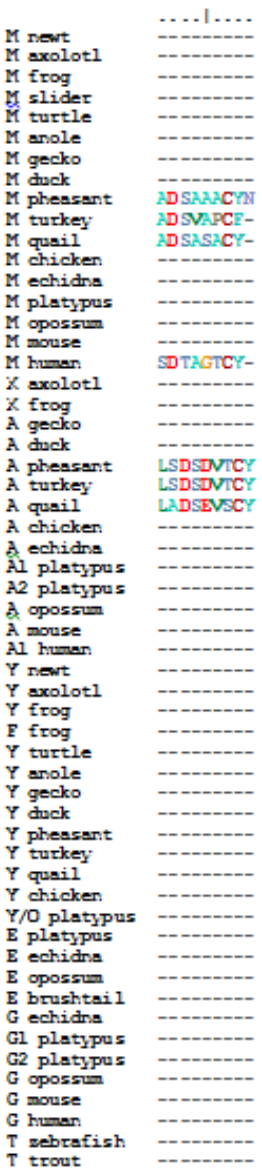
(Figure 2 - 8 continued).



(Figure 2 - 8 continued).



(Figure 2 - 8 continued).

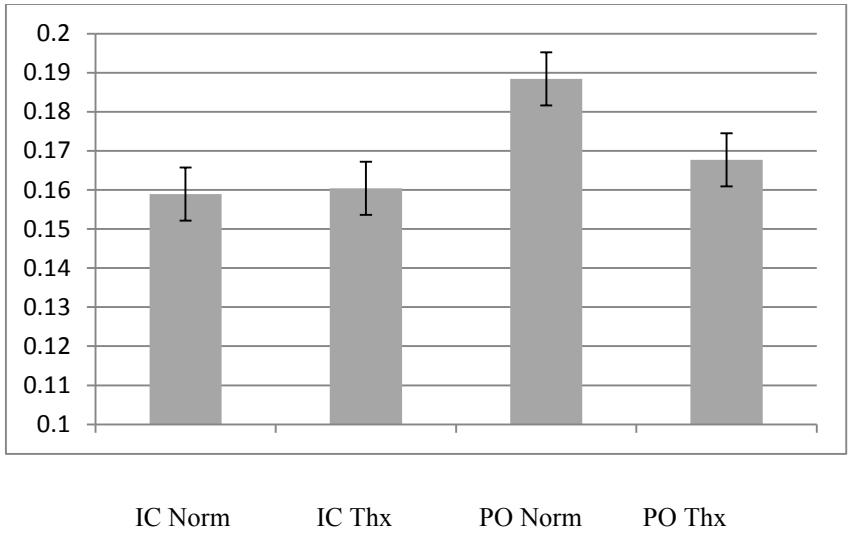


(Figure 2 - 8 continued).

2.3.5 Thymectomy does not impede mucosal antibody production

Normal and thymectomized frogs distinct from those assayed for gut flora were immunized with DNP-KLH either intracoelomically or orally (Figure 2 - 9). B cells were harvested and cultured from spleen and gut of these animals, and ELISAs were performed for both total IgX and antigen-specific IgX on the supernatant. Oral immunization elicited significantly ($p=0.025$) more total IgX from intestinal B cells than intra-coelomic delivery, but no significant difference was seen from the spleen cells or between B cells from normal and thymectomized animals. When DNP-KLH specific IgX was assayed, the only significant ($p=0.013$) difference seen was an increase in specific IgX from orally immunized splenocytes from thymectomized frogs versus orally immunized spleen cells from normal frogs. Thus larval thymectomy does not appear to retard the frogs' ability to make total IgX, or IgX specific to this hapten-carrier conjugate.

A: Gut Total IgX



B: Gut Ag-Specific IgX

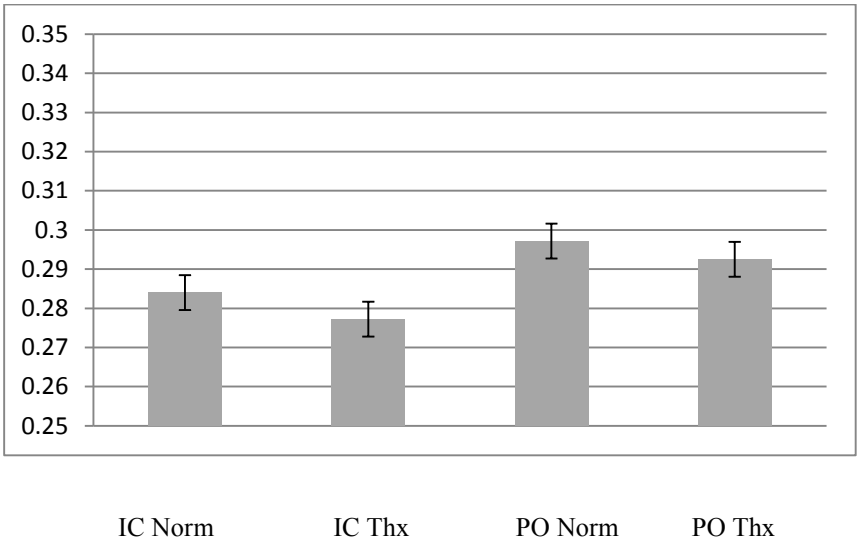
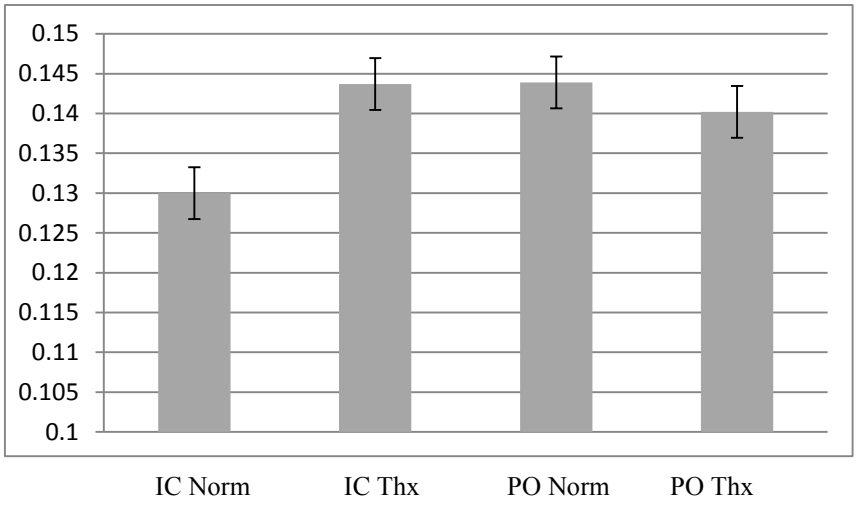
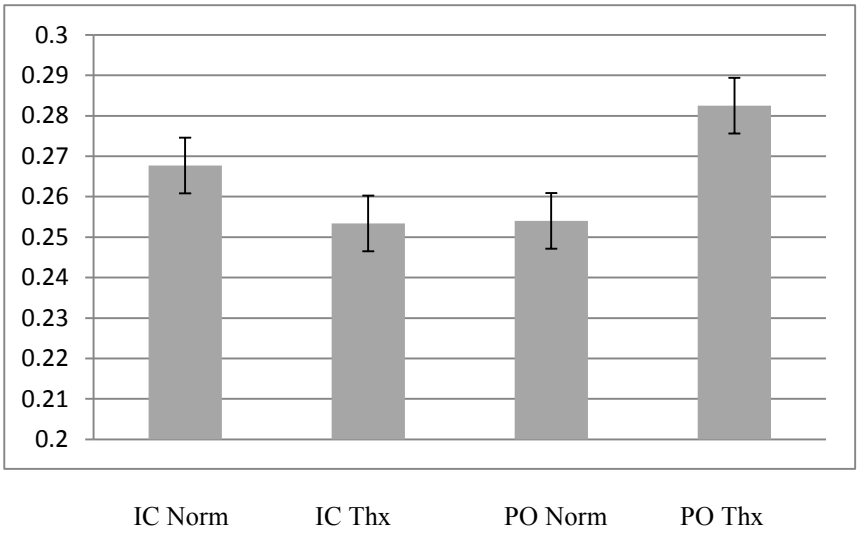


Figure 2 - 9**.** Thymectomy does not retard induction of mucosal IgX response
ELISA for the IgX isotype on supernatant of lymphocytes cultured from spleen or intestine of frogs immunized to DNP-KLH. Units of the Y axes are absorbance at 450nm. A. Oral (PO) gives a significantly greater total IgX response in the gut than intracoelomic (IC) immunization ($p=0.025$, marked by *), but no significant difference was seen with thymectomized frogs. B. No significance was seen monitoring antigen-specific IgX in the gut. C. no significant difference was seen with total IgX in the spleen, D. Antigen specific IgX actually increased from spleen cells after thymectomy ($p=0.013$, marked by*).

C: Spleen Total IgX



D: Spleen Ag-Specific IgX



(Figure 2 - 9 continued)

2.4 Discussion

2.4.1 Frog gut flora

Molecular sequencing techniques have surpassed culture methods of investigating gastrointestinal microbiota due to their increased sensitivity (and the majority of unculturable genera present there) [71]. The amplification and subsequent sequencing of the 16S rRNA gene allows the identification of bacterial groups present in the GI tract of humans and other animal species [72-74].

High throughput sequencing techniques had not been applied to the gut microbiota in any amphibian model, and *X. laevis*'s use in developmental, cell- and immuno-biology made it an obvious first candidate [57]. High-throughput 16S rRNA sequencing has been used to analyze the anti-fungal cutaneous bacterial populations in a salamander [75]. The most comprehensive culture based studies of amphibian gut flora have been performed in the leopard frog (*Rana pipiens*) [76]. Similar to the present work in *Xenopus*, *Rana* was found to have many Clostridiaceae, Eubacteriaceae, and Bacteroidaceae, and hibernating frogs at lower temperatures had a significant shift of flora to dominant Pseudomonodaceae [77]. Using the pyrosequencing based approach described here we were able to identify >60 families of bacteria in the *Xenopus* gut that to our knowledge have not been described previously in amphibians. These include the known decomposers of plant polymers Marinilabiaceae [78], the potential pathogens in the guts of humans and fish Porphyromonadaceae [79], insect endosymbiont Sphingobacteriaceae [80] and the known fermenters of fish microbiota Verrucomicrobiaceae [81].

The frogs in our gut flora analysis all spent their lives in the same aquatic animal room descended from the same outbred founders just one generation before. They all received the same prepared diet. Although not sterilized, this homogenous, consistent feed is certainly in stark contrast to the varied, inconsistent, and microbe rich diet wild *Xenopus* would consume in Africa. Yet we saw great individual variance in their gastrointestinal flora, as has been described in humans [82] and dogs [64]. These communities are undoubtedly temporally dynamic as well within the individual animals. This work in X. laevis provides a reference for anatomically discrete gut microbial communities in an omnivorous amphibian. This is the Class of vertebrates that gave rise to the amniotic reptiles, birds and mammals, and first employed immunoglobulin heavy chain isotype switching to a mucosal isotype.

2.4.2 T cell influence on mucosal immunity

Formative studies in nude (athymic) mice found that the poorly developed Peyer's patches lacking substantial germinal centers and low IgA levels of this rodent model could be largely restored by thymic grafts and the resulting T cell population [83]. Yet loss of T cell function was not found to dramatically alter the cultivable gastrointestinal microbiota in these mice [84]. As our understanding of T cell help, class switch recombination and mucosal immunity have improved in the decades since this work; much energy has focused on the relationship between the adaptive immune system and the gut flora. This has extended to hypotheses of the two coevolving and even rationale for the original genesis of the adaptive system [85, 86].

We turned to the major biological model "between" *Danio rerio* and mammals to ask questions about the influence of T cells in the mucosal immune compartment. This choice affords a comparative view of what the first immune system with both MHC-restricted T cells and a humoral response capable of class switch to a mucosal isotype was like in the ancestral tetrapod 300 million years ago. Thymectomy in this animal is an established model system for removal of the T cell compartment [58, 87-90], but this study is the first to rigorously test the adults for T cell receptor expression by PCR. We assayed TCRβ, δ, and α, as TCRγ has been shown to be expressed early in the X. laevis thymus [91]. Initially we were dismayed by some constant region (not necessarily indicative of functional rearrangement) TCR mRNA expression from peripheral blood of adults in which we visually scored the thymectomies to be perfectly clean. But perhaps this is to be expected, as TCRβ constant region message has been shown from bone marrow derived lymphocytes [92] and even functional TCRδ and TCRγ transcripts have been found in nude mice [93]. Despite these observed low levels of constant domain nucleic acid expression, we are confident that the scrupulous culling of tadpoles with incomplete surgeries and molecular diagnostics of mature animals yielded frogs with no functional αβ T cell compartment (and γδ as well). Monoclonal antibodies have confirmed this absence at the cellular level of receptor expression in this model [94].

The possibility of extra-thymic T cell development was explored in *Xenopus* by including PCR controls of gut tissue at the time of thymectomy in addition to adult gut from normal and thymectomized frogs (Figure 2 - 1). Extra-thymic T cell development has been described in mouse gut [95] and may even be stimulated by thymic ablation [96].

Highly organized mucosal lymphoid structures such as Peyer's patches do not exist in poikilothermic vertebrates such as *Xenopus* [97], yet a suggestion of gut T lymphopoiesis has been described in bony fish [98]. Tadpoles showed no TCR expression in gut at the age of thymectomy, nor was TCR expression seen in adult gut of larval thymectomized frogs. While extrathymic routes of T cell development are possible in frog our data suggest that these are at best minor relative to thymic for gut seeding, and that thymectomy does not force extrathymic developmental programs.

Some "natural" gut IgA in mouse has been thought to be from T-independent B-1 cells [99]. The specificity of gut IgA was later shown to be less "natural" and actually driven by specific antigens of the gut microbial symbionts and food [53]. Moreover, use of TCRβ/δ double knock-out transgenic mice ensured that T cells from thymus or elsewhere were not responsible for this phenomenon, nor was any "bystander" contribution of their lymphokines [100]. They further showed that this response was at least in part due to B1 peritoneal cells in mice [53]. These findings seem consistent with the lack of effect that thymectomy has on mucosal IgX in the present study. Yet in mammals there is plenty of evidence for T-dependence in gut IgA too. Most human IgA-switched plasma cells in the lamina propria show evidence of somatic hypermutation, presumably from a germinal center event with T cell help [101, 102]. Moreover, most (~80%) plasma cells in the gut were found to be antigen specific and not poly-reactive [103]. AID transgenic mice defective somatic hypermutation but still competent to make IgA exhibited greater colony counts of small intestinal flora, germinal center hyperplasia and susceptibility to *Yersinia enterocolitica* [104]. This suggests T-dependent, germinal

center processes do shape the gut flora through the specific IgA generated against it in mice. Deep sequencing of IgA rearrangements in CD3-/- mice showed evidence for T-dependent somatic hypermutation in aged mice, thus the relatively young age of the frogs in this study could be a factor [51]. We did not find evidence corroborating such T dependences in the amphibian, but recognize that this is one relatively small study in a captive population.

2.4.3 Evolution of mucosal antibody isotypes and IgA

Recent data now allow more rigorous studies of the natural history of tetrapod immunoglobulin genes. Sequence similarities and predicted structural resemblance to IgM, originally suggested that IgX might be the functional analog, but not the ortholog of IgA [59, 61]. The IgA of Aves appears to be a mucosal functional analog of mammalian IgA [105] and there is high sequence identity that suggests orthology [106]. However, there are four C domains in avian IgA suggesting a deletion occurred to yield the mammalian IgA of three [107]. The incomplete evolutionary loss of the Cα2 domain present in IgX and avian IgA could have given rise to the hinge region in mammalian IgA before the divergence of monotremes and the therian marsupial and placental mammals [108].

Although IgX has been hypothesized to be an ortholog of IgA [60, 109] the phylogenetic analysis described here is the first to lend experimental support to the notion. The availability of more diverse tetrapod immunoglobulin sequences allowed us to make this analysis. The CH1 and CH2 encoding exons of the IgX gene may have been derived from the IgY encoding locus and the CH3 and CH4 from the IgM encoding gene [110,

111]. These relationships will need to be retested as more amphibian, reptile, bird and non-placental mammal genomes are sequenced. However, this scenario is consistent with the known synteny of the C region encoding genes in the immunoglobulin heavy chain loci of known tetrapod genomes (Figure 2 - 10), where IgX/A is in between the genes encoding the IgM/D and IgY encoding loci that birthed it. The locus in mammals has undergone duplicative expansions giving rise to subfunctionalization of IgG and IgE from IgY and a proliferation of sub-isotypes [112]. Thus, the resulting IgY/IgM chimera IgX gave rise to (or perhaps should be synonymous with) IgA in endothermic vertebrates. This appears to be the second time vertebrate evolution has produced a dedicated mucosal immunoglobulin isotype (Figure 2 - 11), the first being IgZ/T that is unique to some teleost fish [113-115]. IgX/A is the first mucosal isotype whose expression is controlled via AID mediated class switch recombination, as IgZ/T is produced via deletional RAG-mediated V(D)J recombination similar to the rearrangement at the α/δ T cell receptor locus [9, 116].

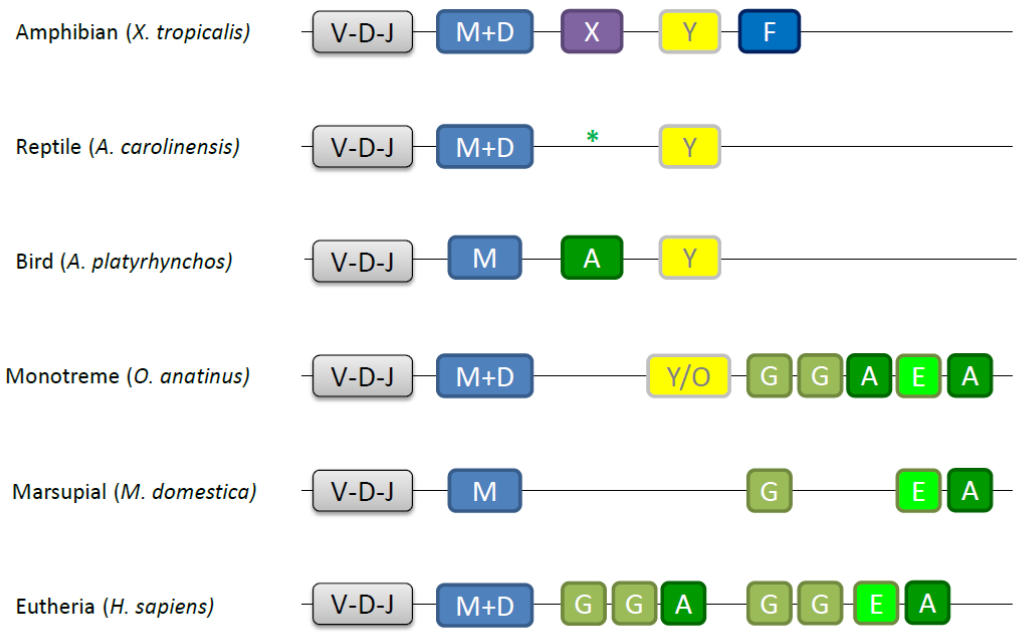


Figure 2 - 10**.** Genomic synteny of IgX is consistent with its giving rise to IgA.
Genomic assemblies and published genomic studies of the IgH locus were used to compare the position of the constant genes of IgX of amphibians with IgA of birds and mammals. The asterisk denotes that IgA is present in some reptiles such as the leopard gecko though genomic data are lacking.

A

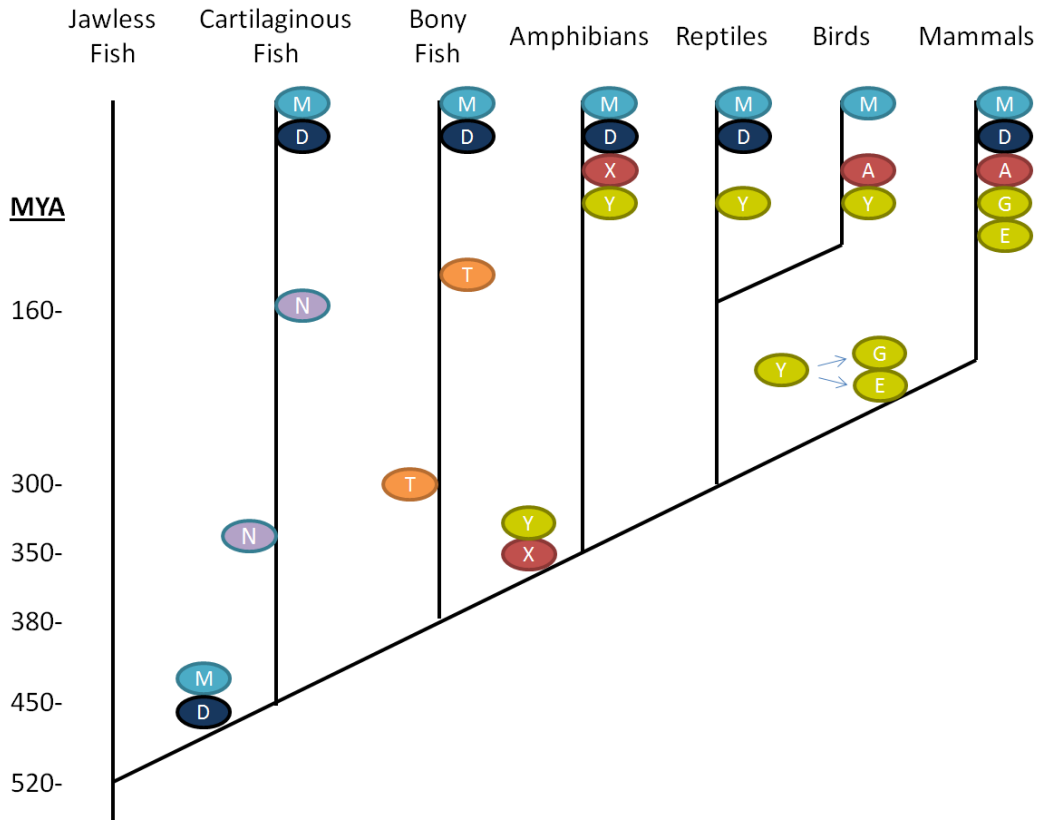
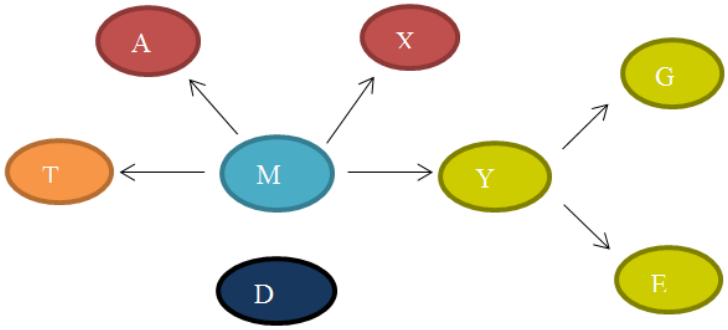
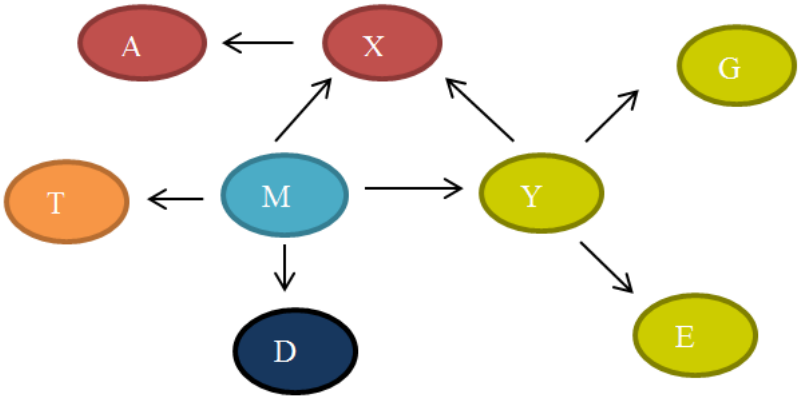


Figure 2 - 11**.** Model of immunoglobulin natural history with mucosal IgX/A emerging in early tetrapods. A. Simplified phylogeny of vertebrates showing approximate emergence times of heavy chain isotypes. B. This analysis suggests that the isotype previously described as IgX in amphibians may be orthologous to IgA of warm blooded vertebrates (model adapted from M. Flajnik's chapter of Fundamental Immunology, W. Paul editor). In addition to the isotypes extant in man, the immunoglobulin light chain-less IgNAR of cartilaginous fish and the mucosal IgZ/T of bony fish is also shown.

B



Previous Consensus



Revised Model

(Figure 2 - 11 continued).

2.5 Conclusions

In the frog we find evidence for an ancient, T-independent, humoral mucosal response. We defined the gut flora of the model amphibian X. laevis but found the bacterial communities of the stomach, small and large intestine to be unaffected by thymectomy. More representative phylogenetic analysis of the relationship between amphibian IgX and IgA of amniotic tetrapods shows their orthology, explaining the origin of human's most abundant immunoglobulin. Therefore we conclude that the T cell independent IgA pathway is likely an ancient mechanism to manage the microbial symbionts of the gut and other mucosal surfaces. More comparative studies must resolve the (convergent?) functional relationship between the two vertebrate mucosal isotypes: IgZ/T and IgX/A.

Families	STO	+/-	SMI	+/-	LIN	+/-	NOR	+/-	THX	+/-
P. ACTINOBACTERIA										
C. ACTINOBACTERIA										
O. ACTINOMYCETALES										
Actinomycetaceae	0.00	±0.00	0.00	±0.00	0.00	±0.01	0.00	±0.00	0.00	±0.01
Corynebacteriaceae	0.00	±0.00	0.00	±0.01	0.00	±0.00	0.00	±0.00	0.00	±0.01
Microbacteriaceae	0.00	±0.00	0.00	±0.01	0.00	±0.00	0.00	±0.01	0.00	±0.00
Nocardioidaceae	0.00	±0.00	0.01	±0.03	0.00	±0.00	0.00	±0.00	0.01	±0.02
Scytonemataceae	0.01	±0.02	0.00	±0.00	0.00	±0.00	0.01	±0.02	0.00	±0.00
O. LENTISPHAERALES										
Victivallaceae	0.01	±0.01	0.01	±0.01	0.00	±0.00	0.00	±0.01	0.00	±0.01
P. ARMATIMONADETES										
C. ARMATIMONADALES										
Armatimonadaceae (OP10)	0.04	±0.10	0.00	±0.00	0.00	±0.00	0.03	±0.09	0.00	±0.00
P. BACTEROIDETES										
C. BACTEROIDIA										
O. BACTEROIDALES										
Marinilabiaceae	0.03	±0.05	0.00	±0.00	0.38	±0.90	0.02	±0.03	0.26	±0.73
Noctuoidea	0.00	±0.01	0.01	±0.02	0.01	±0.02	0.01	±0.02	0.01	±0.01
Porphyromonadaceae	1.22	±1.27	1.30	±1.91	3.71	±2.43	2.29	±2.20	1.87	±2.24

Table 2 - 3**.** Percentage of 16S rRNA gene clones belonging to bacterial families.

Averages +/- standard deviation of samples from stomach, small intestine, large intestine, normal (thymus intact) and thymectomized frogs.

Deferribacteraceae	0.04	±0.06	0.27	±0.35	0.17	±0.19	0.20	±0.21	0.12	±0.27
P. FIRMICUTES										
C. BACILLI										
O. BACILLALES										
Bacillaceae	0.53	±0.42	0.46	±0.45	0.26	±0.15	0.48	±0.45	0.36	±0.25
Sphingomonadaceae	0.09	±0.13	0.00	±0.00	0.00	±0.00	0.03	±0.08	0.03	±0.08
Thermoactinomycetaceae	0.00	±0.00	0.01	±0.03	0.00	±0.00	0.00	±0.00	0.01	±0.02
O. LACTOBACILLALES										
Aerococcaceae	0.00	±0.00	0.00	±0.01	0.00	±0.00	0.00	±0.00	0.00	±0.01
Carnobacteriaceae	0.15	±0.16	0.07	±0.09	0.06	±0.08	0.11	±0.15	0.07	±0.07
Enterococcaceae	0.02	±0.04	0.03	±0.04	0.00	±0.01	0.02	±0.03	0.01	±0.03
Lactobacillaceae	0.06	±0.14	0.01	±0.01	0.00	±0.00	0.00	±0.01	0.04	±0.12
Paenibacillaceae	0.00	±0.01	0.02	±0.02	0.01	±0.02	0.01	±0.01	0.02	±0.02
Staphylococcaceae	0.05	±0.06	0.03	±0.03	0.00	±0.00	0.04	±0.05	0.02	±0.02
Streptococcaceae	0.03	±0.05	0.10	±0.10	0.06	±0.06	0.06	±0.08	0.07	±0.07
C. CLOSTRIDIA										
O. CLOSTRIDIALES										
Catabacteriaceae	0.01	±0.02	0.08	±0.11	0.15	±0.14	0.04	±0.05	0.11	±0.15
Clostridiaceae	69.57	±25.71	75.63	±20.75	54.45	±22.38	68.33	±20.65	64.76	±25.81
Eubacteriaceae	0.20	±0.21	1.68	±1.79	2.26	±2.10	1.14	±1.51	1.61	±2.02
Graciibacteraceae	0.00	±0.00	0.00	±0.01	0.00	±0.00	0.00	±0.01	0.00	±0.00
Lachnospiraceae	0.12	±0.11	0.36	±0.42	0.37	±0.38	0.38	±0.40	0.18	±0.23

(Table 2 - 3 continued)

Prevotellaceae	0.01	±0.02	0.00	±0.00	0.05	±0.07	0.01	±0.02	0.03	±0.06
Rikenellaceae	0.28	±0.40	0.28	±0.32	3.31	±6.65	0.42	±0.40	2.17	±5.52
C. CYTOPHAGIA										
O. CYTOPHAGALES										
Cytophagaceae	0.09	±0.14	0.00	±0.01	0.01	±0.02	0.01	±0.03	0.05	±0.11
Flexibacteraceae	0.05	±0.05	1.00	±1.54	0.82	±1.49	0.29	±0.62	0.95	±1.62
O. FLAVOBACTERIALES										
Flavobacteriaceae	7.44	±12.42	0.01	±0.03	0.08	±0.07	0.80	±2.03	4.22	±10.63
C. FLAVOBACTERIIA										
O. FLAVOBACTERIALES										
Cryomorphaceae	0.00	±0.00	0.00	±0.00	0.01	±0.01	0.00	±0.00	0.01	±0.01
P. CYANOBACTERIA										
O. NOSTOCALES										
Nostocaceae	0.77	±1.14	0.00	±0.00	0.00	±0.00	0.24	±0.66	0.27	±0.82
O. OSCILLATORIALES										
Oscillatoriaceae	1.02	±1.49	0.01	±0.02	0.00	±0.00	0.32	±0.86	0.36	±1.07
Pseudanabaena	0.00	±0.01	0.00	±0.00	0.00	±0.00	0.00	±0.01	0.00	±0.00
O. SYNECHOCOCCALES										
Synechococcaceae	0.00	±0.00	0.01	±0.02	0.00	±0.00	0.00	±0.01	0.00	±0.00
P. DEFERRIBACTERES										
C. DEFERRIBACTERES										
O. DEFERRIBACTERALES										

(Table 2 - 3 continued)

Lachnospiraceae	0.12	±0.11	0.36	±0.42	0.37	±0.38	0.38	±0.40	0.18	±0.23
Oscillospiraceae	0.01	±0.01	0.02	±0.05	0.04	±0.07	0.02	±0.04	0.03	±0.06
Ruminococcaceae	0.19	±0.24	1.35	±1.37	2.36	±1.75	1.12	±1.31	1.48	±1.77
Peptococcaceae	0.01	±0.02	0.11	±0.18	0.07	±0.09	0.03	±0.05	0.09	±0.16
Peptostreptococcaceae	0.03	±0.03	0.01	±0.01	0.01	±0.02	0.02	±0.02	0.02	±0.02
Syntrophomonadaceae	0.00	±0.00	0.04	±0.10	0.00	±0.00	0.00	±0.00	0.03	±0.09
O. THERMOANAEROBACTERALES										
Thermoanaerobacteraceae	0.00	±0.00	0.00	±0.00	0.01	±0.02	0.00	±0.01	0.00	±0.01
C. ERYSIPELOTRICHI										
O. ERYSIPELOTRICHALES										
Erysipelotrichaceae	1.07	±1.09	0.66	±0.69	1.78	±2.63	0.92	±1.00	1.42	±2.16
C. NEGATIVICUTES										
O. SELENOMONADALES										
Veillonellaceae	0.08	±0.14	0.05	±0.07	0.14	±0.12	0.09	±0.12	0.09	±0.10
P. PROTEOBACTERIA										
C. ALPHAPROTEOBACTERIA	0.37	±0.50	0.52	±0.89	0.64	±0.75	0.57	±0.62	0.45	±0.80
O. CAULOBACTERALES										
Caulobacteraceae	0.01	±0.02	0.00	±0.00	0.01	±0.02	0.01	±0.02	0.00	±0.00
O. RHIZOBIALES										
Bradyrhizobiaceae	0.05	±0.10	0.06	±0.13	0.05	±0.13	0.10	±0.15	0.01	±0.01
Hyphomicrobiaceae	0.00	±0.01	0.00	±0.01	0.00	±0.01	0.00	±0.00	0.01	±0.01

(Table 2 - 3 continued)

Rhizobiaceae	0.16	±0.25	0.00	±0.00	0.00	±0.00	0.04	±0.12	0.07	±0.19
O. RICKETTSIALES										
Anaplasmataceae	0.00	±0.01	0.00	±0.01	0.00	±0.00	0.00	±0.01	0.00	±0.01
O. RHODOBACTERALES										
Rhodobacteraceae	0.01	±0.02	0.00	±0.00	0.00	±0.00	0.00	±0.00	0.01	±0.02
O. RHODOSPIRILLALES										
Fusobacteriaceae										
O. Sphingomonadales										
Planococcaceae	0.02	±0.02	0.00	±0.00	0.00	±0.00	0.00	±0.01	0.01	±0.02
C. BETAPROTEOBACTERIA										
O. BURKHOLDERIALES										
Alcaligenaceae	0.01	±0.02	0.00	±0.00	0.00	±0.00	0.01	±0.02	0.00	±0.00
Burkholderiaceae	0.13	±0.16	0.00	±0.00	0.00	±0.00	0.03	±0.07	0.06	±0.14
Comamonadaceae	0.18	±0.17	0.02	±0.04	0.00	±0.00	0.02	±0.03	0.12	±0.16
Oxalobacteraceae	0.04	±0.09	0.00	±0.01	0.01	±0.02	0.01	±0.02	0.03	±0.07
O. GALLIONELLALES										
Gallionellaceae	0.00	±0.00	0.00	±0.00	0.00	±0.01	0.00	±0.00	0.00	±0.01
O. HYDROGENOPHILALES										
Hydrogenophilaceae	0.01	±0.01	0.00	±0.00	0.01	±0.03	0.00	±0.01	0.01	±0.02
O. NEISSERIALES										
Neisseriaceae	0.09	±0.16	0.02	±0.05	0.00	±0.00	0.02	±0.04	0.06	±0.14
O. NITROSOMONADALES										

(Table 2 - 3 continued)

Nitrosomonadaceae	0.00	±0.00	0.00	±0.01	0.01	±0.02	0.01	±0.01	0.00	±0.00
C. DELTAPROTEOBACTERIA										
O. BDELLOVIBRIONALES										
Bdellovibrionaceae	0.00	±0.01	0.02	±0.04	0.01	±0.02	0.02	±0.03	0.01	±0.02
O. DESULFOVIBRIONALES										
Desulfovibrionaceae	0.12	±0.25	1.34	±2.32	3.88	±3.56	1.74	±2.81	1.82	±2.99
Geobacteraceae	0.00	±0.00	0.01	±0.02	0.00	±0.01	0.00	±0.00	0.01	±0.02
O. DESULFUROMONADALES										
Pelobacteraceae	0.00	±0.00	0.00	±0.00	0.00	±0.01	0.00	±0.00	0.00	±0.01
C. GAMMAPROTEOBACTERIA										
O. AEROMONADALES										
Aeromonadaceae	0.18	±0.20	0.01	±0.01	0.00	±0.00	0.05	±0.14	0.07	±0.14
O. ALTEROMONADALES										
Shewanellaceae	0.03	±0.04	0.00	±0.00	0.00	±0.00	0.01	±0.03	0.00	±0.01
O. CHROMATIALES										
Chromatiaceae	0.12	±0.26	0.00	±0.00	0.00	±0.00	0.01	±0.02	0.07	±0.22
O. ENTEROBACTERIALES										
Enterobacteriaceae	7.67	±13.77	1.98	±4.75	1.86	±4.21	6.36	±11.54	1.31	±3.55
O. PSEUDOMONADALES										
Moraxellaceae	0.38	±0.56	0.01	±0.01	0.00	±0.01	0.01	±0.03	0.25	±0.48
Pseudomonadaceae	0.09	±0.09	0.01	±0.02	0.01	±0.02	0.04	±0.07	0.04	±0.07

(Table 2 - 3 continued)

O. VIBRIONALES										
Vibrionaceae	0.04	±0.06	0.00	±0.00	0.00	±0.00	0.01	±0.03	0.02	±0.05
O. XANTHOMONADALES										
Xanthomonadaceae	0.05	±0.10	0.01	±0.02	0.00	±0.00	0.01	±0.02	0.03	±0.08
P. SPIROCHAETES										
C. SPIROCHAETIA										
O. SPIROCHAETALES										
Spirochaetaceae	0.00	±0.00	0.00	±0.01	0.05	±0.09	0.01	±0.01	0.03	±0.08
P. SYNERGISTETES										
C. SYNERGISTIA										
O. SYNERGISTALES										
Synergistaceae	0.22	±0.32	5.77	±8.19	1.74	±1.14	1.95	±2.06	3.21	±7.07
P. TENERICUTES										
C. MOLLICUTES										
O. ACHOLEPLASMATALES										
Acholeplasmataceae	0.03	±0.06	0.08	±0.09	0.13	±0.19	0.07	±0.08	0.09	±0.16
O. ENTOMOPLASMATALES										
Spiroplasmataceae	0.04	±0.09	0.19	±0.29	0.12	±0.14	0.07	±0.09	0.17	±0.26
O. MYCOPLASMATALES										
Mycoplasmataceae	0.01	±0.01	0.00	±0.00	0.05	±0.13	0.00	±0.01	0.04	±0.10
P. VERRUCOMICROBIA										
C. OPITUTAE										

P. VERRUCOMICROBIA										
C. OPITUTAE										
Opitutaceae	0.01	±0.02	0.00	±0.00	0.00	±0.00	0.00	±0.01	0.00	±0.00
O. PUNICEICOCCALES										
Puniceicoccaceae	0.00	±0.00	0.01	±0.02	0.00	±0.00	0.00	±0.01	0.00	±0.00
C. VERRUCOMICROBIAE										
O. VERRUCOMICROBIALES										
Verrucomicrobiaceae	0.00	±0.01	0.01	±0.02	0.08	±0.16	0.01	±0.02	0.05	±0.13

(Table 2 - 3 continued).

3. EXPRESSED IGH M AND T TRANSCRIPTS SHARE DIVERSITY SEGMENT IN RANCHED *THUNNUS ORIENTALIS*[*]

3.1 Introduction

The immunoglobulin (Ig) superfamily-based adaptive immune system evolved in cartilaginous fish (Chondriechtyes), including sharks and skates, is maintained in all jawed vertebrates [117]. One of the major characteristics of this adaptive immune system is the production of a repertoire of antibodies through somatic V(D)J recombination of the loci that encode them. While mammals possess five functionally distinct Ig isotypes (IgM, IgD, IgG, IgA and IgE), teleost fish have only three: IgM, IgD and IgT [114, 115, 118, 119].

IgT was concomitantly discovered in trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*, where it was given the appellative IgZ) and IgT or forms of Ig with IgT domains have since been described in fugu (*Fugu rubripes*) [120], carp (*Cyprinus carpio*) [121], and stickleback (*Gasterosteus aculeatus*) [122]. IgT perhaps exists in most teleost groups, although it has yet to be found in catfish [116, 123] and medaka [124]. So far IgT

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[*] Reprinted with permission from "Expressed IgH μ and τ transcripts share diversity segment in ranched Thunnus orientalis." by Sara Mashoof, Camilo Pohlenz, Patricia L. Chena, Thaddeus C. Deiss Delbert Gatlin III, Alejandro Buentello, Michael F. Criscitiello, 2014. Developmental and Comparative Immunology, 43(1), 76-86, Copyright © 2014, Elsevier

is an isotype restricted to bony fish (Osteichtyes), and sequence characteristics [114], gut localization and functional work [113] have suggested that it is a dedicated mucosal isotype [125], functionally analogous but not orthologous with IgX/A of tetrapods [126]. IgT was found to be expressed in gill of Chinese perch (*Siniperca chuatsi*) [127], IgT positive cells were identified in the epithelium of *Oncorhynchus mykiss* gill lamellae [128], and clonal IgT responses were induced to *Oncorhynchus mykiss* viral pathogens [129], all further supporting the idea of this isotype filling a mucosal role in teleost humoral adaptive immunity. The IgT encoding DH-JH-CH elements are located 5' of the μ and δ DH-JH-CH regions in the fish genomes in which it has been studied, with most or all VH genes 5' to the τ block [115, 120, 122]. Although class switch recombination has been described in shark [130] and fish activation-induced cytidine deaminase (AID) is competent to induce somatic hypermutation and class switch in mammalian cells [131], it does not appear that teleosts employ this for Ig heavy (H) chain isotype switching, instead they use deletional VH(DH)JH rearrangement to remove τ in IgM and IgD expressing cells and differential RNA splicing to control expression of IgM and IgD [132], the τ/μ rearrangement appearing to have influence on lineage commitment similarly to the mechanism operating at the T cell receptor αδ locus.

We recently turned our attention to the expressed IgH transcripts of the Pacific bluefin tuna (*Thunnus orientalis*). *Thunnus* species are the most valuable global aquaculture product [133], yet infections from several groups of parasites plague high intensity tuna mariculture ranches [134], impeding the industry from optimal relief of fishing pressures upon wild adult stocks. In addition to their economic importance, the

extreme physiological specializations of these migratory apex predators made their Ig of interest to us. Tuna are among the fastest fish and have countercurrent heat exchangers that minimize convective heat loss to maintain a form of endothermy distinct from that of birds and mammals [135, 136]. Specifically, we were curious whether tuna Ig harbored any special adaptations evident in their primary amino acid sequence to this rare form of fish endothermy.

Here we report the first full-length μ and τ sequences from a tuna species. We have analyzed representative clones of the expressed variable domain repertoire of these isotypes, performed phylogenetic analysis of the IgH genes of this modern teleost, and analyzed their relative expression in *T. orientalis* primary and secondary lymphoid tissues, including the mucosal gill. Our results demonstrate that these fish employ the same Ig VH gene families as other teleosts, can use the same VH genes in both IgM and IgT heavy chains, make diverse IgH complementarity determining region (CDR)3 regions, and surprisingly employ the same DH segment in both τ and μ rearrangements in what appears to be a previously undescribed mechanism of B cell isotype determination.

3.2 Methods

3.2.1 Animals and collection of tissues

Sample tissues of spleen, gill and kidney from ranched *T. orientalis* were collected during the regular slaughter process from two different commercial tuna facilities located off the coast of Ensenada, Baja California, Mexico. At the time of harvest, fish weight and

fork length were 16.2 ± 6.5 kg and 96.3 ± 14.3 cm, respectively. Samples were placed in RNAlater (Qiagen, Valencia CA), frozen in liquid nitrogen, shipped to Texas A&M on dry ice and stored at -80◦C until further use.

3.2.2 Total RNA isolation and cDNA synthesis

Total RNA was purified from spleen, gill and head kidney (pronephros, or anterior kidney) (35 mg from each tissue) using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instruction. The quantity and quality of the RNA samples were assessed by NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent, Santa Clara CA) respectively. Message representation of RNA was assessed by PCR of common (β-actin) and less common transcripts (TNF-α, IL1-β), using previously published primer sets [137]. The GeneRacer kit (Life Technologies, Grand Island NY) with GeneRacer oligo dT and gene specific primers was used to produce 5' rapid amplification of cDNA ends (RACE) PCR products. Pools of 3' RACE products were synthesized by Superscript III First-Strand Synthesis SuperMix kit (Life Technologies) using the oligo dT primer.

3.2.3 IgH RACE PCR, cloning, and sequencing

5' and 3' RACE products were amplified by standard PCR using various combinations of 5' GeneRacer (as forward primer in 5'RACE), Oligo dT (as reverse primer in 3'RACE), and specifically designed primers for the conserved regions encoding the C domains of *T. orientalis* IgM and IgT (as forward or reverse for 3' RACE or 5' RACE, respectively). Primers are listed in Table 3 - 1. The PCR conditions were as

follows: one cycle of 95°C for 2 minutes, 35 cycles of 95°C for 30 seconds, 50-53°C for 30 seconds, 72°C for 2 minutes, followed by one cycle of 72°C for 7 minutes. The amplicons were purified from a 0.8% agarose gel after electrophoresis in Tris/acetic

Primer ID	Primer name	Sequence (5'-3')	Designed to clone	Position	Amino acid
MFC 261	TolgMCF1	TGGATCMGACAGVMWCAGG	#6 KF713344	286-306	NGQNVQP
MFC 244	TolgMCR1	GCACARTAAAHACAGCDCTGTC	#6 KF713344	310-333	DTAVYYCA
MFC 260	TolgMCR	GCACARTAATAHACAGC	#6 KF713344	316-333	AVYYCA
MFC 340	MCF1	CCCTATTTCGCACAGACGCAACAA	#6 KF713344	1279-1302	GIKRVCVV
MFC 350	TolgMGR3F4	CTCATGGTACCCACCAGTGACTTGAGA	#6 KF713344	919- 945	LMVPTSDLR
MFC 351	TolgMGR3F5	ACTGTGACCTACGGAGGGTCATGT	#6 KF713344	1009-1032	TVTYGGSC
MFC 352	TolgMGR3F6	TTCTCTGTAGCTCTGCTGCTGCTGTTG	#6 KF713344	4- 30	FSVALLLLL
MFC 365	TolgZCR1	ACTTGGAGGGTTCAGTGTCACTGT	#59 KF713336	975- 996	TVTLNPPS
MFC 367	TolgZCR2	TGTGTTGACTTGCAGCCACTCAGT	#59 KF713336	1185-1206	TEWLQNQVNT
MFC 369	TolgZCR3	GAATGTGGACTGTCACTGTTGTCTTGCT	#59 KF713336	1278-1302	SKTTVTVHI
MFC 381	TolgMC1R4	TGTAAACTCGGTGGCGAGGCA	#6 KF713344	475- 495	CLATEFT
MFC 382	TolgMC1R5	TGGAGGATACTGGATGAAGTC	#6 KF713344	544-564	DFIQYPP
MFC 384	TolgZRC1R4	ACTGTTGGCTGCCTTGCACGTGAC	#59 KF713336	468- 489	CVGCLARD
MFC 386	TolgZRC1R6	TGGGAGTTTCACTGTCACAGCCATGGT	#59 KF713336	663- 687	TMAVTVKLP
MFC 387	TolgZRC1R7	ACCAAGATGATCGACGGA	#59 KF713336	642-657	SVDHLG

Primer name*	Sequence (5'-3')
βactin	ATCGTGGGGCGCCCCAGGCACC
βactin	GTCATCTTCTCYCTGTTGGC
TNF-α	CCAGGCRGCCATCCATTTAGAAG
TNF-α	CCGACCTCACCGCGCT
IL-1β	GGRSAGCGACATGGYRCGATTTCT
IL-1β	GGTGCTGATGTACCAGTTG

Table 3 - 1. Primers**.** Mladineo, I., Block,B.A., 2009. Expression of Hsp70, Na+/K+ ATP-ase, HIF-1 alpha, IL-1 beta and TNF-alpha in captive Pacific bluefin tuna (*Thunnus orientalis*) after chronic warm and cold exposure. J Exp Mar Biol Ecol 374, 51-57.

acid/EDTA (TAE) buffer, cloned into pCR II vector with the TOPO TA cloning kit (Life Technologies), and transformed into chemically competent TOP10 Escherichia coli cells (Invitrogen). Colonies were picked based on blue/white screening produced by X-Gal (Sigma-Aldrich, Saint Louis MO). The plasmid DNA was purified using Zyppy Plasmid Miniprep kit (Zymo Research Corporation, Irvine CA) and was digested with EcoRI (Promega, Madison, WI) to identify clones with inserts. Products for sequencing were amplified using either M13 forward or reverse primers, purified using ABI BigDye X terminator purification kit (Life Technologies), and sequenced by the DNA Technologies Core lab of the Department of Veterinary Pathobiology at Texas A&M University.

3.2.4 Sequence analysis of μ and τ gene rearrangements in pacific bluefin tuna

BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and visual inspection were used to identify the Ig isotype as well as VH, JH and CH sequences of *T. orientalis* amplicons based upon homology to those from representative fish and other vertebrates. The amino acid sequences were blasted to discriminate the VH segments and CH domains. SignalP 4.1 was used to determine the leader peptides [138]. Three prediction methods concurred upon the cleavage site of the representative μVH (after the 18th residue) and were in less agreement for τVH (after the 20th residue) as shown in Figure 3 - 1 and Figure 3 - 2 Sequences were translated with Expasy translate tool (http://web.expasy.org/translate/), and the Clustal W program in Bioedit was employed to align amino acid sequences (http://www.mbio.ncsu.edu/bioedit/bioedit.html) for figures. Sequences were managed

and assembled in Bioedit and have been deposited in Genbank under accession numbers (pending final numbers, submitted 8/30/2013). CDR3 length was calculated using the "CDR3 length = exclusive number of amino acids from C (of VH segment YxC) to F (of JH segment FGxG)" IMGT formula [139].

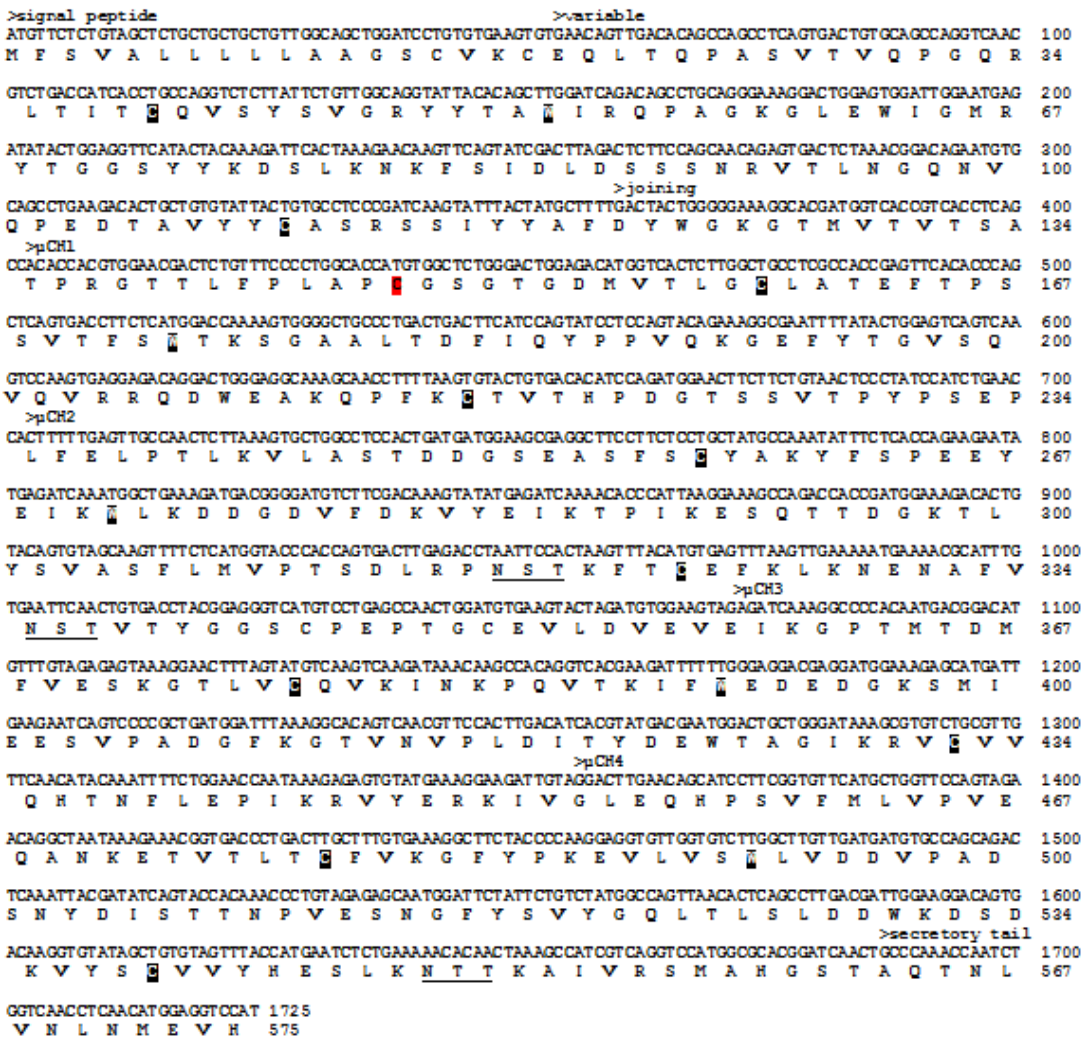


Figure 3 - 1. Nucleic acid and deduced amino acid sequence of *T. orientalis* IgHμ full length clone 6. The start of the predicted signal peptide, VH, JH, CH domains and secretory tail are marked above the sequence. Potential N-linked glycosylation sites are underlined. Cysteines and tryptophans necessary for the Ig superfamily fold are highlighted in black, the cysteine that forms the disulfide bond to the Ig light chain is highlighted in red.

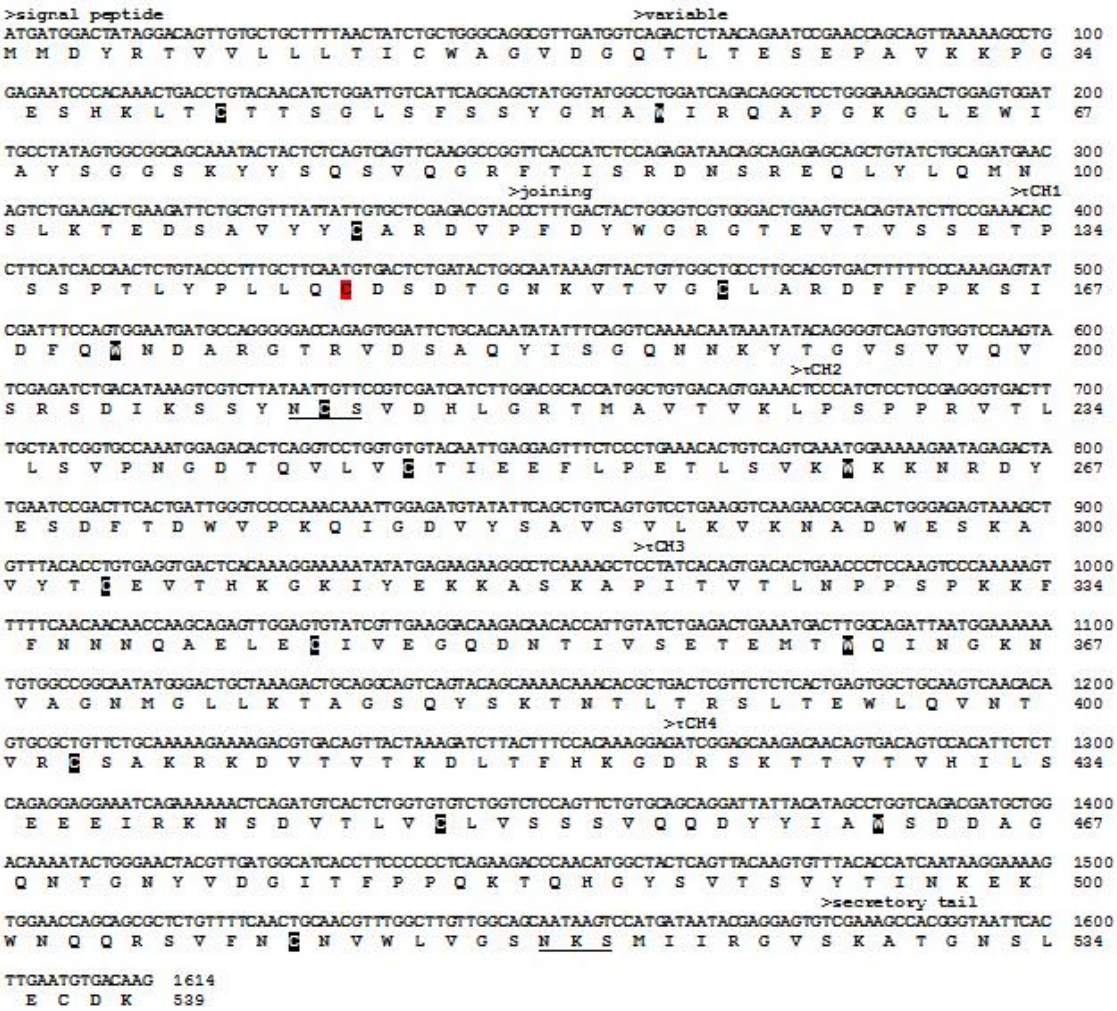


Figure 3 - 2**.** Nucleic acid and deduced amino acid sequence of *T. orientalis* IgHτ full length clone 59. The start of the predicted signal peptide, VH, JH, CH domains and secretory tail are marked above the sequence.

3.2.5 Phylogenetic studies

Amino acid alignments were made with Clustal W employing gap opening penalties of 10 and gap extension penalties of 0.1 for pairwise alignments, then 0.2 for multiple alignments using a Dayhoff matrix based method [68]. Phylogenetic trees were constructed using MEGA 5 software [140]. Neighbor joining trees using the substitution

method of Jones, Taylor and Thornton [141] and pairwise deletion of empty positions were constructed from alignments of VH and CH domain sequences.. Trees were bootstrapped 1000 times [142] and were viewed and adjusted using the TreeView Software [143].

3.2.6 Real time quantitative PCR

Oligo-dT transcribed cDNA samples from spleen, gill and anterior kidney were assayed for levels of μ and τ message using β-actin as a constitutively expressed control. Real-time PCR reactions were performed using 25 and 50ng of cDNA with SYBR Advantage qPCR Premix (Clontech, Mountain View, CA) per the manufacturer's instructions. Primers were designed to span across introns. Using a Roche LightCycler 480 a three-step thermal cycling program was followed: 1 cycle at 95°C for 5 minutes, then 45 cycles of 95°C for 10 seconds, then 60°C for 5 seconds, then 72°C for 5 seconds. The Roche LightCycler software was utilized for raw data acquisition and calculation of Ct (threshold cycle) values. Changes in gene expression were estimated using the $2-\Delta\Delta Ct$ method (Livak and Schmittgen, 2001), with β-actin utilized as the stable reference gene for all experimental situations. The fold changes in gene expression were calculated with respect to the expression level of the genes in the anterior kidney (the primary B lymphopoietic tissue of bony fish).

3.3 Results

3.3.1 Characterization of μ cDNA of T. orientalis

The initial full length *T. orientalis* μ was cloned and sequenced using a cDNA RACE library that was obtained from RNA pooled from several *T. orientalis* anterior kidney, spleen and gill samples. The secretory *T. orientalis* μ sequence shown in Figure 3 - 1 is an 1827 bp open reading frame which encodes a 609 amino acid protein containing a leader peptide of 18 residues, one Ig VH and four CH domains. The primary amino acid sequence showed two cysteine residues (and intervening tryptophan) conserved for intra-domain disulfide bond formation present in each of the Ig domains with the cysteines being spaced by approximately 70 residues in the VH domain and 60 in the CH domains. The amino-terminal cysteine in the CH1 domain forms an interdomain disulfide bond between the IgH chain to the IgL chain. The potential N-linked glycosylation site near the carboxyl terminus of the IgM chain was found at this position of the *T. orientalis* IgM [144].

3.3.2 Characterization of tuna IgT

While sequencing 3' RACE PCR products employing VH primers designed from μ clones we found other clones with Ig CH region amino acid sequences distinct from IgM, although they often shared a VH domain highly homologous with μ clones. The CH1 domain of these clones shares 56% amino acid identity with the CH1 of *Siniperca chuatsi*. More primers allowed the complete cloning of the IgT encoding cDNAs, with CH3 proving to be even more definitively of the isotype (60% identical amino acids to S. chuatsi).

The secretory *T. orientalis* τ cDNA is composed of 1614 base pairs translating to 539 amino acids forming a leader peptide, VH domain and four CH domains. As in *T. orientalis* IgM, two conserved cysteine residues and one tryptophan were identified in the VH and each CH domain which are important for folding of the β-sandwich Ig domains. There is also one conserved cysteine in the CH1 domain which forms a disulfide covalent linkage between the IgH chain to the IgL chain. The secretory tail of *T. orientalis* IgT is composed of 12 amino acids.

3.3.3 IgHμ and τ VH, DH and JH segments

The same cDNA pools were used to examine *T. orientalis* IgH μ and τ VH (DH) JH rearrangement diversity. In total 50 different sequences encoding VH domains (Figure 3 - 3) that possessed full or partial unique VH regions were cloned, 36 spliced to μ CH regions and 11 with τ (three contained complete VH regions but were incompletely rearranged or did not splice to a CH). Based on percent identity the VH segment sequences were divided into four separate families of IgH V genes. Members of each family were more than 70% identical in their nucleotide sequences [145, 146] (Figure 3 - 4).

Analysis of the carboxy-terminal portion of the VH domains gave insight into the DH and JH gene segments used to rearrange mature VH exons. We predicted 11 different JH segments used in these clones and one DH segment (TATACGGGGGGGGTACTGGG) could be identified in the 48 unique CDR3 encoding rearrangements analyzed (Figure 3 - 5) The one DH segment apparently was employed by both isotypes, as various stretches of the sequence (including portions at each end) are found in both μ and τ clones. The predicted DH germline nucleotide contribution to the

final expressed CDR3 encoding sequence ranges from 3 to 10 with a mean of 5.5 base pairs. All three reading frames of the D segment were used (Figure 3 - 6). The τ clones all used a dedicated JH segment (J9).

IgH CDR3 is the crucial loop in the paratope of most antibody-antigen interactions. This sample of the *T. orientalis* Ig heavy chains expressed at the mRNA level allowed an initial analysis of the length of IgH CDR3 of μ and τ. Figure 3 - 3 shows that *T. orientalis* μ clones display a broader range of CDR3 lengths (from 9 to 18aa) as well as an average of one amino acid longer CDR3 length than those found in *T. orientalis* τ.

3.3.4 IgM and IgT CH regions of tuna

The *T. orientalis* IgM CH region amino acid sequence showed the most identity to the mandarin fish (*S. chuatsi*, also known as the Chinese perch and also a member of the Order *Perciformes*) with 53.6% and then to the *Oncorhynchus mykiss* with 39.8% identity and presented the least with chicken (23.8% identity) amongst the sequences we included in our analysis (Figure 3 - 7). The *T. orientalis* IgT CH region has the highest identity also to that of the mandarin fish with 52.5% and the least to grass carp with 20.4% (Figure 3 - 8). Unlike the cyprinid grass carp and *Danio rerio*, the CH3 domain of *T. orientalis* IgT conforms to the canonical immunoglobulin domain fold with cysteines and tryptophans in positions common for β-sandwich tertiary structure.

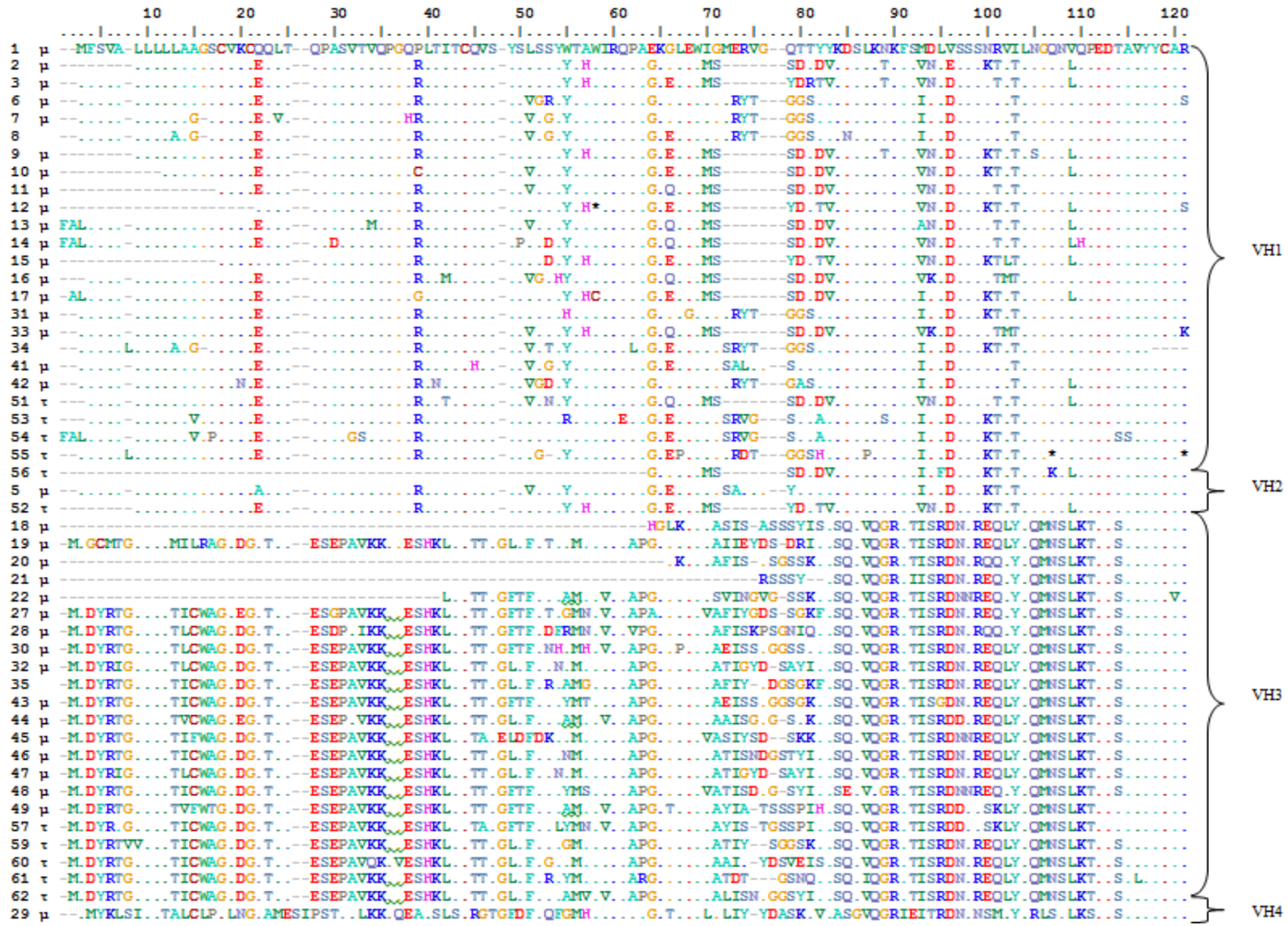


Figure 3 - 3**.** Four VH families used by *T. orientalis* IgH.
Amino acid alignment of VH segment encoded sequences found within *T. orientalis* μ and τ cDNAs. Clone numbers are shown to the left and VH family designations are shown to the right. VH gene segment families were ascribed based upon 70% nucleotide identity in a pairwise matrix (Figure 3 – 4). Gaps introduced into the alignments are indicated by dashes ("-") and identity to the first sequence is indicated by a period (".") in the column. CDR1 and CDR2 are indicated below the alignment. If clone contained CH region encoding region, μ or τ is indicated at left of sequence after the clone name.

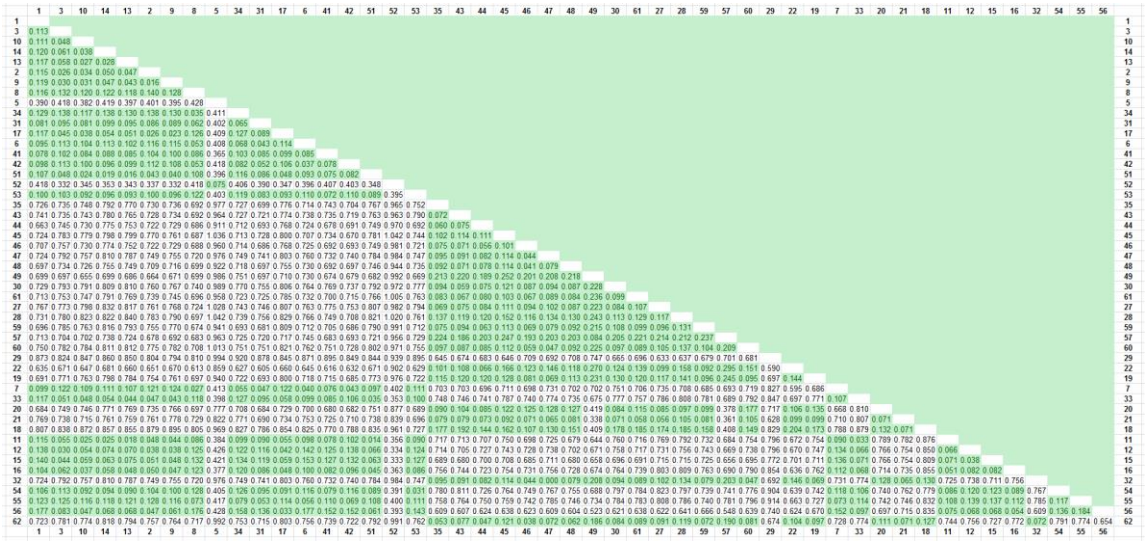


Figure 3 - 4**.** Nucleotide identity matrix of VH region coding sequences to identity VH families. Clone numbers are in bold the left and right of rows and top and bottom of columns. Pairwise differences of less than 30% using nucleotide version of alignment in Figure 3 - 6 are highlighted in green and indicate inclusion in same VH family (by 70% nucleotide identity).

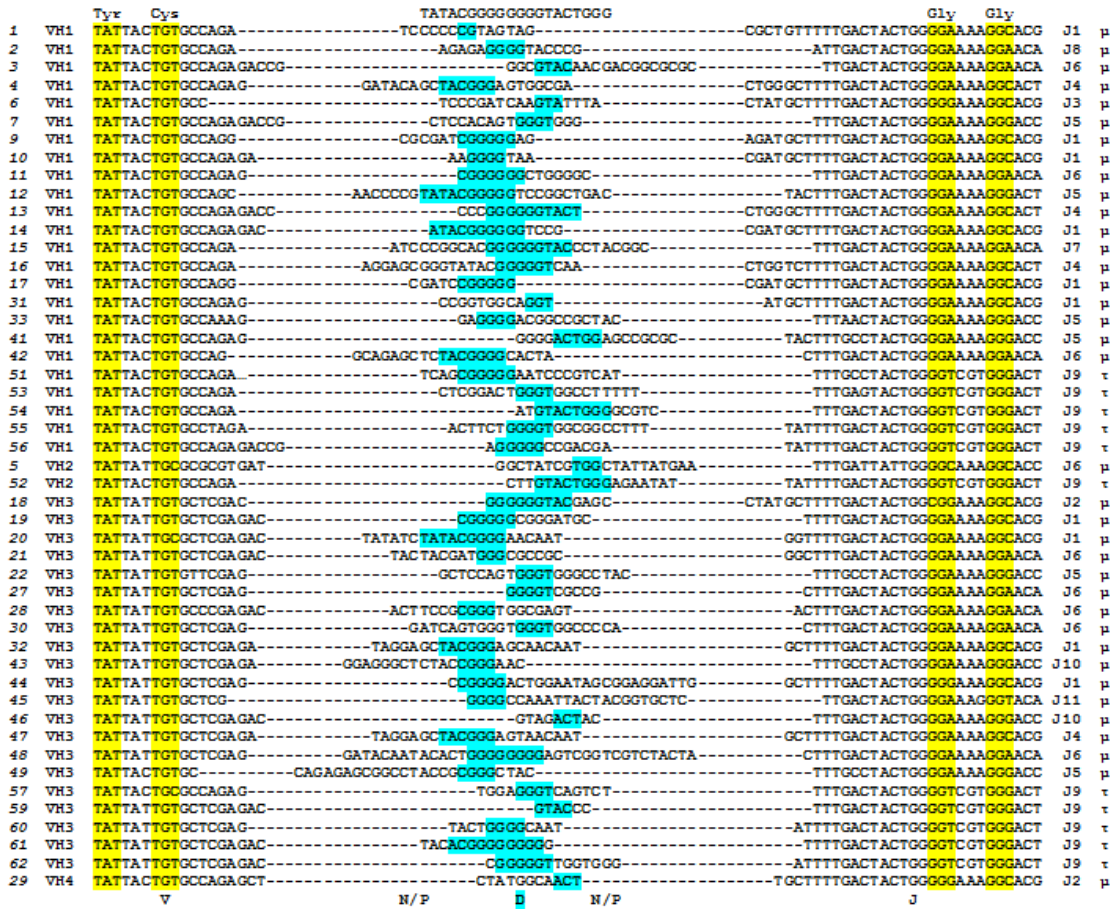


Figure 3 - 5**.** *T. orientalis* VH (DH) JH junctional diversity.
Nucleotide alignment arranged by VH family VH(DH)JH junctional region. Clone names and VH family are denoted on the left, JH gene and CH region is given to the right. Conserved tyrosine and cysteine codons of YxC motif of VH segment as well as GxG glycines of JH gene are highlighted in yellow. Predicted DH segment is highlighted in blue.

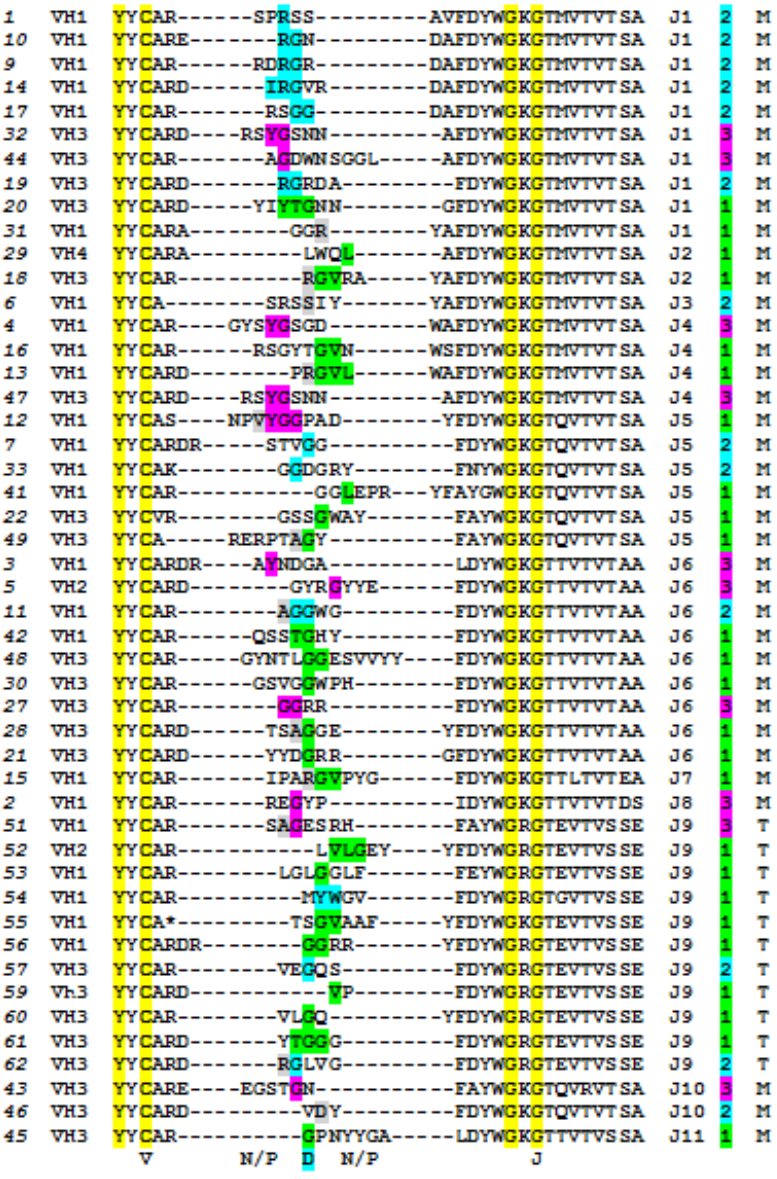


Figure 3 - 6**.** Translated complementarity determining region (CDR) 3 repertoire sampling of *T. orientalis* IgH. Amino acid alignment arranged by JH gene of the VH (DH) JH junctional region. Clone names and VH family are denoted on the left, JH gene, reading frame of DH used and CH region is given to the right. Conserved tyrosine and cysteine of YxC motif of VH segment as well as GxG glycines of JH gene are highlighted in yellow. Predicted DH segment is highlighted in green, blue or magenta depending on the use of reading frame one, two or three, respectively, in panel B. Amino acids were assigned to VH, DH or JH based on at least two bases of codon matching consensus, grey highlighting indicates a residue partially encoded by D consensus that does not encode consensus amino acid.

3.3.5 Phylogenetic analysis

To assess the phylogenetic relationship of the *T. orientalis* Ig VH gene segments with those of other teleosts we created dendrograms with their pairwise genetic distances (Figure 3 - 9).

The four *T. orientalis* IgH V gene families interleaved amongst those VH segment sequences used by the other fish, indicating that they are using members of the same ancient VH families that have been conserved by trans-species maintenance since at least the common ancestor of these divergent fish. However *T. orientalis* families VH1 and VH2 appear to have arisen from a more recent duplication in the order Perciformes.

We also explored the relationship of these new *T. orientalis* IgH C regions to those of other fish and other vertebrates (Figure 3 - 10). As expected, the *T. orientalis* IgM grouped with that isotype from other fish, most closely the Perciforme trumpeter fish (*Latris lineata*). Within the IgM, teleosts group together as a sister group to all of the other vertebrates, including the shark which shares a more ancient common ancestor and would be expected to branch outside of teleosts and tetrapods. However this incongruence with the organisms' natural history is not unusual for phylogenetic analyses of teleost antigen receptors, unless balancing numbers of operational taxonomic units fill the other vertebrate classes [147]. IgT of *T. orientalis* clusters with that isotype from other representative fish.

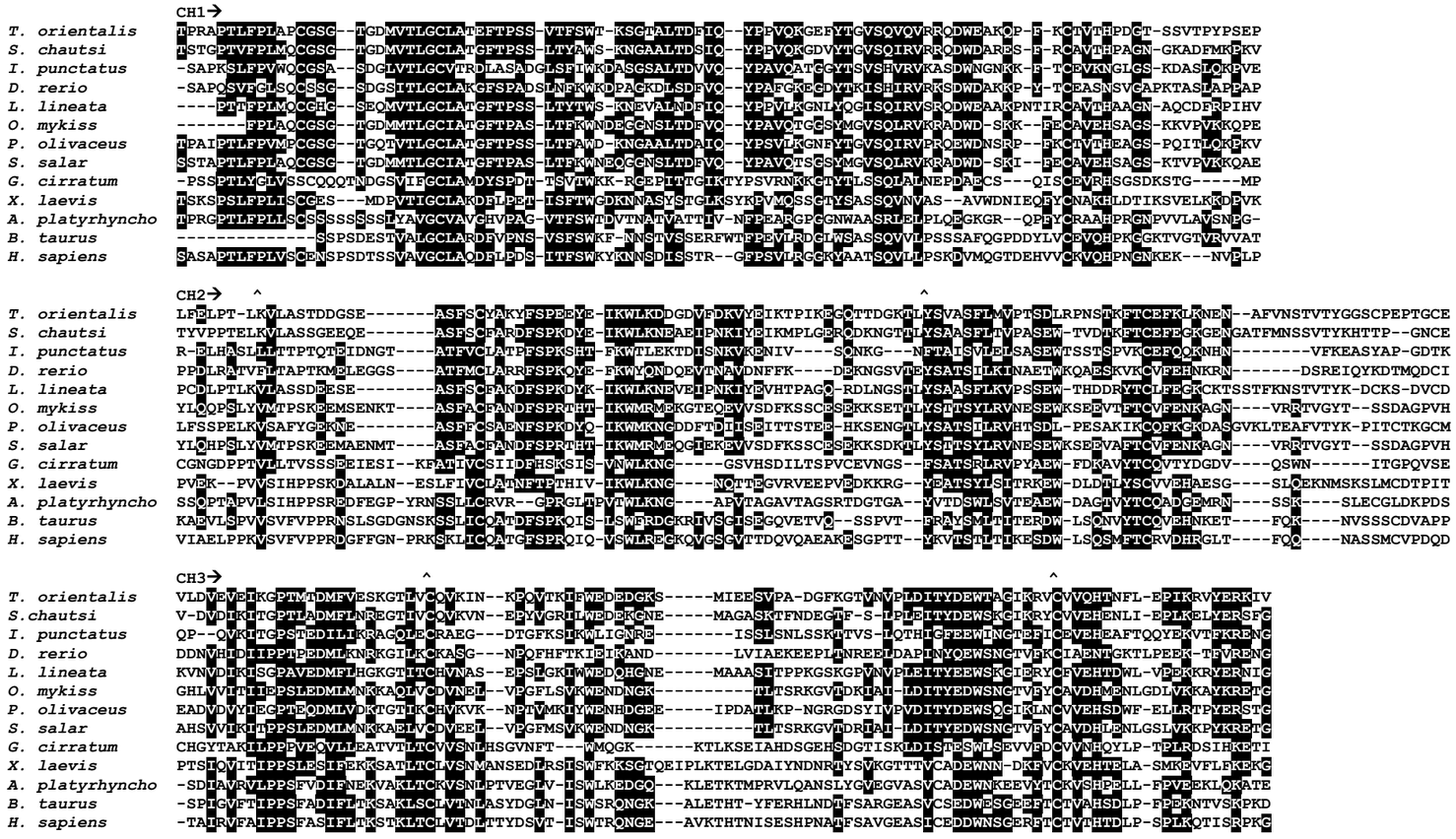
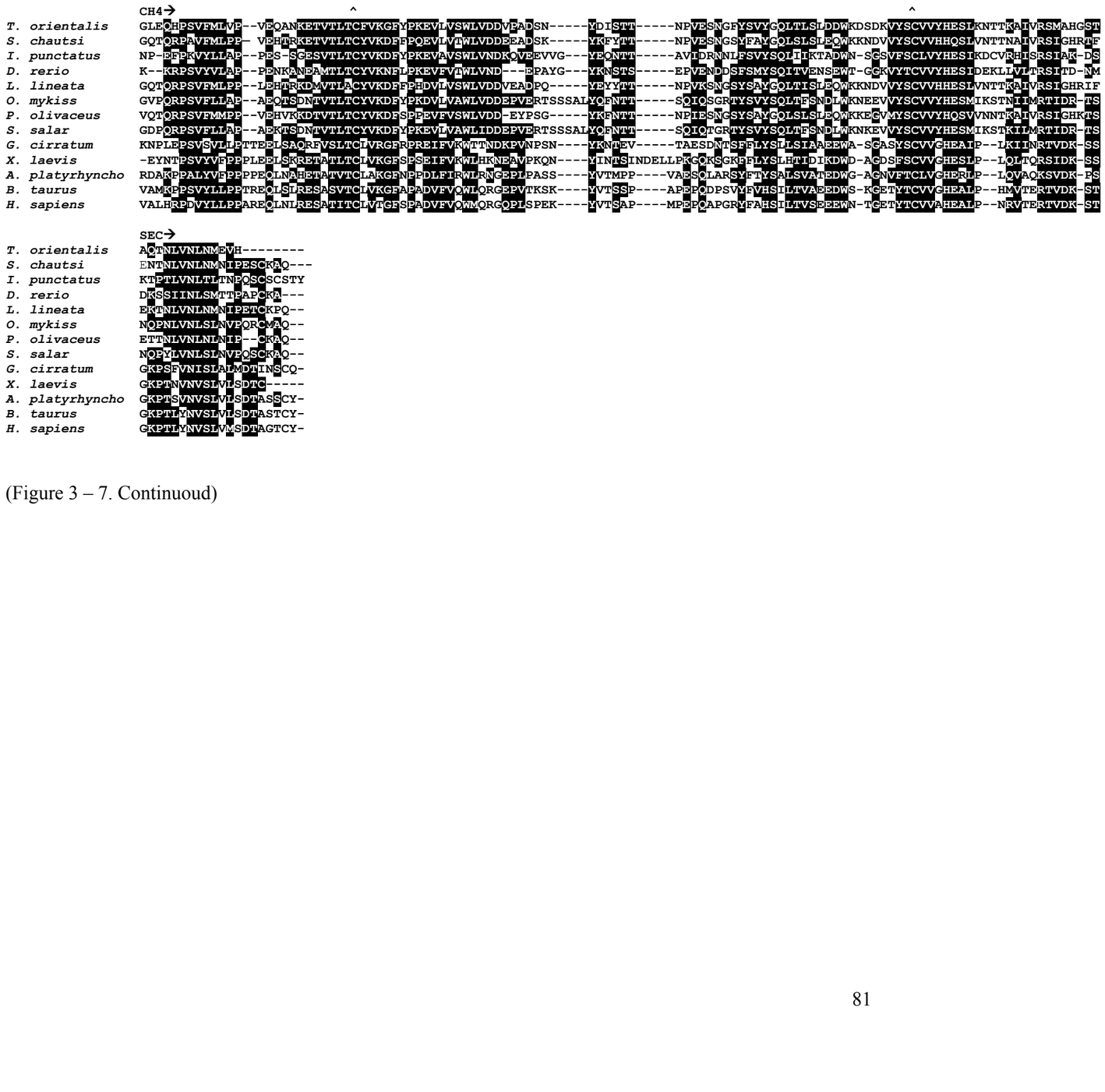


Figure 3 - 7**.** Amino acid sequence alignment of the heavy chain of IgM in different vertebrate species. The conserved (identical and similar) residues are highlighted in black. Arrows indicate CH1-CH4 and the secretory tail. An asterisk (*) is above the conserved cysteine that forms a disulfide bond with the light chain, a carrot (^) is above conserved cysteines that form intra-domain disulfide bonds. Gaps are indicated by dashes. Genbank accession numbers are: AAQ14846.1 *Siniperca chuatsi* (Chinese perch), A45804 *Ictalurus punctatus* (channel catfish), AF281480_1 *Danio rerio* (zebrafish), ADC45388.1 *Latris lineata* (striped trumpeter), AAW66973.1 *Oncorhynchu mykiss* (rainbow trout), AF226284_1 *Paralichthys olivaceus* (flounder), AAB24064.1 *Salmo salar* (salmon), AAU04507.1 *Ginglymostoma cirratum* (nurse shark), AAA49774.1 *Xenopus laevis* (African clawed frog), CAC43280.1 *Anas platyrhyncho* (duck),AAN60017.1 *Bos taurus* (cattle), and AAS01769.1 *Homo sapiens* (human).



(Figure 3 – 7. Continuoud)

Figure 3 - 8**.** Amino acid sequence alignment of the heavy chain of IgT CH domain.
The conserved (identical and similar) residues are marked in black. Arrows indicate CH1-CH4 and the secretory tail. An asterisk (*) is above the conserved cysteine that forms a disulfide bond with the light chain, a carrot (^) is above conserved cysteine of intra-domain bonds. Gaps are indicated by dashes. Genbank accession numbers are: ACZ54909.1 *Epinephelus coioides* (grouper), ABF19723.1 *Ctenopharyngodon idella* (grass carp), AAY42141.1 *S. chuatsi*, ACX50291. *S. salar*, AAW66981.1 *O. mykiss*, and CAI20890.1 *D. rerio*.

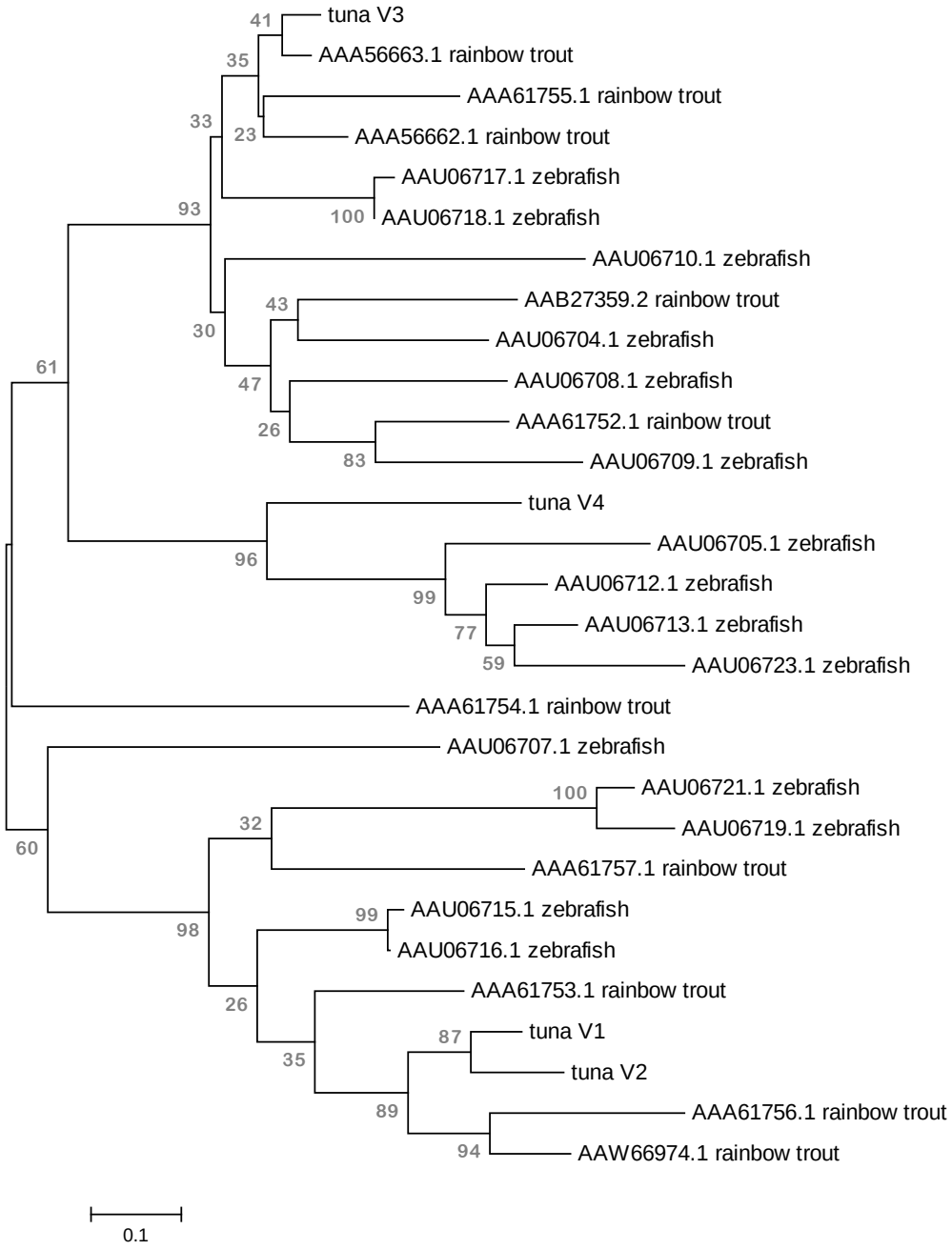


Figure 3 - 9. *T. orientalis* IgH V genes shared with 1 other fish. Phylogenetic analysis of representatives of the four *T. orientalis* IgH V families (clone 3 for VH1, 5 for VH2, 57 for VH3 and 29 for VH4) with VH genes from two of the better studied teleost models, rainbow trout and *Danio rerio*. *Oncorhynchus mykiss* and *Danio rerio* accession numbers are labeled at each branch terminus.

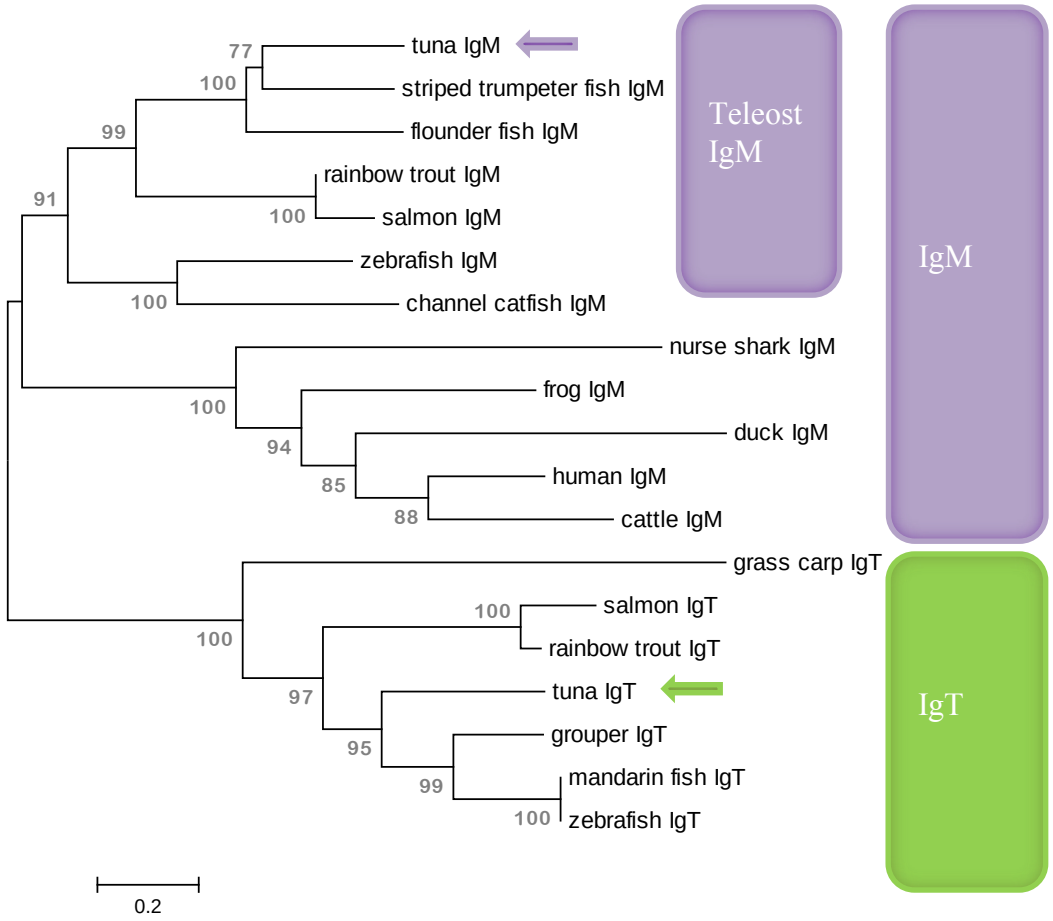


Figure 3 - 10**.** Tuna IgM and IgT CH regions group with those isotypes of other teleosts. Neighbor joining phylogeny using Dayhoff matrix and 1000 bootstrap replications.

3.3.6 *IgH μ and τ relative tissue expression*

Quantitative real-time PCR (Figure 3 - 11) was used to assess the expression of these isotypes at the mRNA level in secondary lymphoid tissues relative to the anterior kidney (the chief primary lymphoid tissue of fish [119, 148, 149]). Relative levels of μ were higher than τ in both spleen and gill, but μ did not predominate τ to as great an extent in gill as it did in spleen. The averaged ratio of HCμ to HCτ in *T. orientalis* spleen was 7.35 compared to 2.89 in the gill.

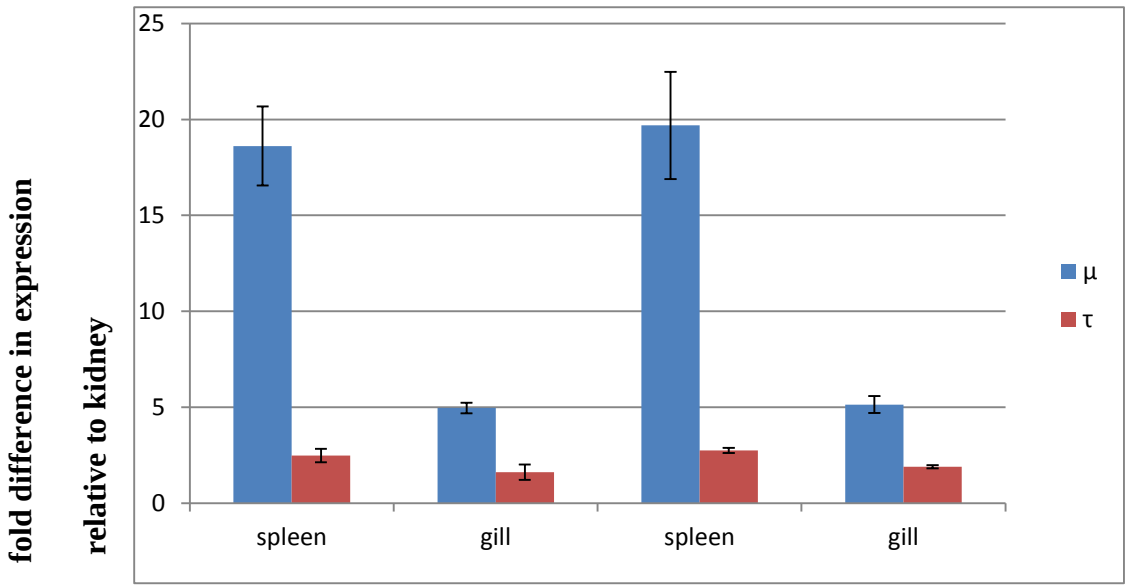


Figure 3 - 11**.** IgH μ and τ expression in systemic and mucosal lymphoid tissues. Quantitative real-time PCR of secondary lymphoid tissue μ and τ C region mRNA expression relative to that in anterior kidney, standardized to β-actin. Two template concentrations were analyzed. Experiment performed in triplicate, error bars indicate standard deviation.

3.4 Discussion

3.4.1 Repertoire

The 20-30% sequence disparity between some VH family members in tuna suggests either ample somatic hypermutation for affinity maturation of these fish antibodies or an older divergence date of VH family members than has been seen in some other teleosts such as stickleback [122]. Families VH1 and VH2 share between 52% and 64% nucleotide identity (Figure 3 - 4) and appear recently diverged (Figure 3 - 9), perhaps within a Perciformes branch including tuna.

Despite the initial report that found shorter CDR3 in *Oncorhynchus mykiss* IgM than IgT [114], in *T. orientalis* we found a small skewing towards shorter IgT CDR3 (Table 3 - 2). We predict that this may be an effect of a different immunogenetic rearrangement mechanism involving a single shared DH gene segment that governs τ versus μ/δ in a clade including tuna and other fish (more below). IgH CDR3 often dominates antigen recognition properties of the six CDRs comprising the Fab paratope [150, 151]. The three reading frames usually supplied by DH gene segments therefore contribute significantly to the eventual translated repertoire of antigenic specificities. Additionally, extended length of IgH CDR3 has been crucial in many clinically important antibodies against viral scourges [152, 153], and the loop has evolved into an entirely new domain in some antibodies of cattle [154]. Thus, restricting the entire repertoire to rearrangements based on a single DH would be expected to place constraints on antigen recognition.

As Perciformes, tuna belong to the largest order of vertebrates that accounts for approximately 40% of all bony fishes. As *T. orientalis* is the first Perciforme member to have either their IgM and T repertoire or IgH locus analyzed immunogenetically, there may be a great many fish that employ this system for Ig isotype control and B lineage commitment. As successful as the Perciformes have been in radiating to occupy most fresh and saltwater niches on Earth, the potential restriction in CDR3 length variability must not have taken too great a toll on the fitness of these fish.

	IgM	IgT
Maximum	18	13
Minimum	9	8
Range	9	5
Median	12	11
Mean	12.49	11.00
Variance	3.31	2.00

Table 3 - 2. CDR3 Lengths in amino acids

3.4.2 Genomic organization

The generalized translocon configuration of the teleost IgH locus with a set of VH genes and downstream μ and δ CH regions has been confirmed in many studies [155-157], but many deviations fromthe theme are present as catfish and medaka appear to lack τ and many fish have duplications of blocks of the locus [119]. Although reported in shark [130],

class switch recombination (CSR) has not been described in a teleost. However, one study showed that teleost AID could induce CSR in mouse [158].

The IgH τ gene together with its dedicated DH and JH gene segments are located between the VH gene segment block and the (DH-JH-CH) μ cluster in *Danio rerio*, fugu (*Takifugu rubripes*) and three-spined stickleback (*Gasterosteus aculeatus*) [114, 115, 122, 159], or it is inserted within the VH gene segment array as in *Oncorhynchus mykiss* [160]. Thus, in these fish the RAG mediated joining of a VH gene segment to either DH of τ or DH of μ/δ will determine whether the developing pro-B lymphocyte (using mammalian convention) becomes an IgT or IgM/D producer. Experiments in *Danio rerio* [161] and *Oncorhynchus mykiss* [113] have demonstrated heavy chain isotype exclusion at the cellular level in fish.

The repertoire data presented here suggest that something different may be occurring in tuna. Like in other fish, VH genes appear to be shared between both τ and μ/δ. Three of the four families we found expressed in these fish clearly were used in both μ and τ, although a fourth was only found with μ. This could easily be a case of low sampling depth as VH4 appeared as a singular use in the described clones. Since this is a more parsimonious explanation than a dedicated μ VH rearranging to a shared DH segment that rearranges to dedicated JH segments, Figure 3 - 12 depicts an array of VH gene segments that can be used in either primary transcript type.

However, unlike in other fish, both τ and μ rearrangements of *T. orientalis* appear to employ the same DH gene segment. As all the *T. orientalis* JH genes appear with only μ or τ (none seem to be shared), this points to an arrangement where a single shared DH

can rearrange with JH segments upstream of either μ or τ to determine isotypic fate of the cell, and this DH's rearrangement to several shared VH's is not the event that stochastically determines isotype. So at least two possibilities of IgT vs. IgM/D lineage fate are now supported by data, one in which τ and μ/δ share VH genes from one block (as in *Danio rerio*) or more than one array 5' and 3' to the τ elements (as in *Oncorhynchus mykiss*) but DH and JH are dedicated to isotype, and now the tuna paradigm where VH and DH are shared and JH is dedicated to isotype. In one instance (tuna) the DH-JH join would instruct lineage and in the other the VH-DH join would. Importantly, we note that genomic sequencing of the locus has not yet confirmed this organization in the *T. orientalis* or the absence of additional DH that we did not sample. Interestingly, this hypothesized organization could also explain why in *Oncorhynchus mykiss* a significant difference was seen in CDR3 length and repertoire between τ and μ clones (each using dedicated DH and JH gene segments [129], while we do not see a great difference in *T. orientalis* (sharing VH and DH and only having dedicated JH (Table 3 - 2). Future work must determine if this is truly stochastic in lymphocyte development or if there are more complex control mechanisms instructing this important juncture determining the B cell's fate.

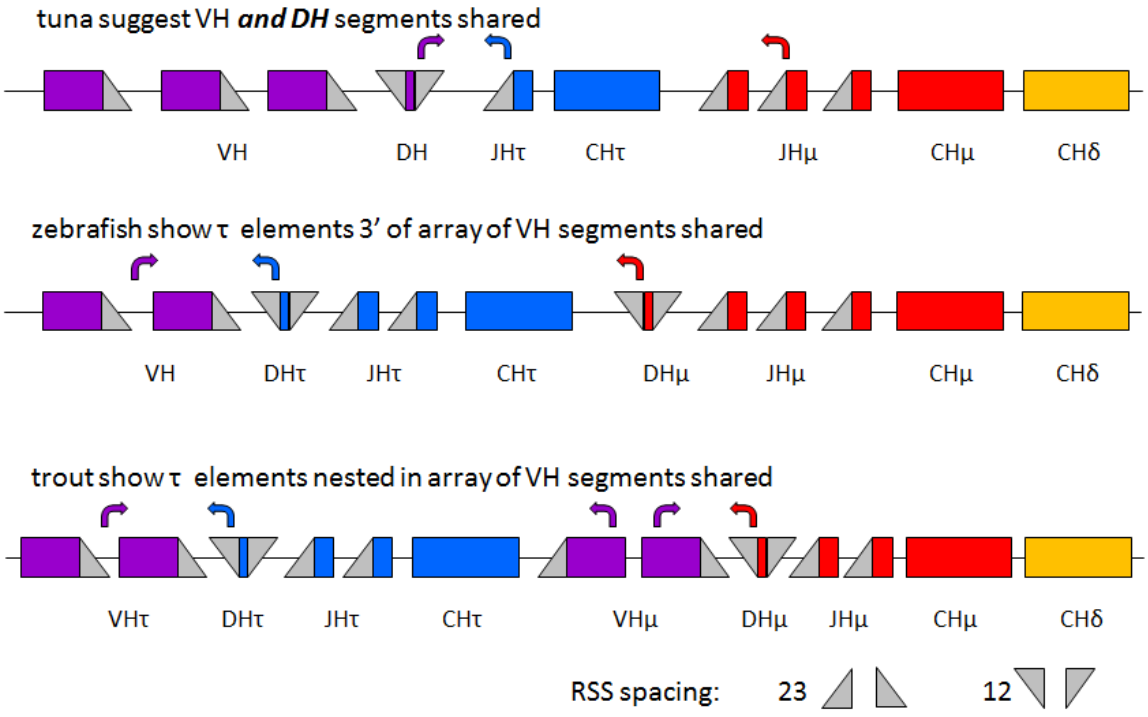


Figure 3 - 12**.** Hypothetical organization of elements in the tuna IgH locus suggests a novel method of lineage determination at the fish IgH locus. Simplified cartoon showing three paradigms in the locus organization and immunogenetic control of IgH τ vs μ/δ rearrangement.

3.4.3 CH regions

IgM is the most conserved isotype in jawed vertebrates (Gnathostomata) and was thought to be omnipresent until the discovery of its absence in coelacanth [162]. The tuna IgM CH region seems very consistent with its orthologs in other fish.

As also noted in other IgT sequences [114], there are many prolines in the region of the tuna IgT CH1/CH2 juncture which may be indicative of hinge-like flexibility. Tuna IgT CH3 seems to conform to the classical Ig superfamily β-sandwich with canonical cysteines and tryptophan positions seen in the domain of the salmonids and grouper that are important in the folding of this domain (Figure 3 - 8) [163]. The tryptophan to cysteine replacement seen in *Danio rerio* and grass carp appears to be a cyprinid characteristic, and has been suggested by modeling to still allow an immunoglobulin superfamily domain fold [164].

In this limited sampling, we found no evidence of the IgT hybrid molecule with two CH domains identified in the common carp Cyprinus carpio [121], the IgM/D hybrids (with or without VH domains) found in catfish [165], the IgMCH1/IgTCH4 variant IgT2 in carp [166], nor the run-on transcription secreted IgD form of *Oncorhynchus mykiss* [167].

In mammalian IgM a carboxyl terminal glycosylation site in the secretory tail is important in J chain polymerization [168, 169], but may have distinct physiology in teleost such as catfish and *Danio rerio* that have it [170]. This conserved N-linked glycosylation site is part of a larger sequence motif enabling polymerization of IgM and IgA of mammals but is not present in the secretory tail of tuna IgM or IgT, although there is a conserved

cysteine in IgT shared with other teleosts. *Oncorhynchus mykiss* IgT was found as a monomer in serum but a multimer in mucus [113]; however these IgT multimers did not appear to be covalently linked as they are known to be for *Oncorhynchus mykiss* IgM [171]. More biochemical studies are necessary to resolve the stoichiometry and functional avidity of IgT.

3.4.4 Expression

Isotype expression studies in *T. orientalis* echo what has been determined in other fish species: IgT and IgM both are present in primary and secondary lymphoid tissues, yet more IgM than IgT, however the gap closes at mucosal sites [114, 120, 166, 172]. IgT1 in adult *Danio rerio* deviated from this pattern in being primarily in the head-kidney and thymus [173]. The molecular data presented here could serve as a springboard for revisiting immunoglobulin studies in tuna at the protein level that were initiated in the southern bluefin (*Thunnus maccoyii*) [174]. The work also opens gates to explorations of B lineage development and commitment, where molecular markers might could be adapted from fish species such as *Danio rerio* [175] and *Oncorhynchus mykiss* [176, 177] where more work has been performed.

3.5 Conclusions

Endothermic birds and mammals employ immunoglobulin isotypes IgM, IgY, IgE and IgG in systemic immunity but have specialized IgA for mucosal immunity. Poikilothermic vertebrates lack IgA, although amphibians do have an orthologous

mucosal isotype in their IgX. IgM had long been the primary functional immunoglobulin isotype recognized in teleost until the recent discovery of the mucosal specialization of IgT. The mucosal epithelium is the barrier breached or exploited by most internal pathogens of vertebrates, and also ectoparasites of fish [178]. This penetration of mucosal defense is also true of many pathogens of concern in the tuna ranching industry, including sea lice [179], betanodaviruses [180] and gill platyhelminths [181]. It is hoped that this basic molecular characterization of humoral immunity in these economically important endothermic fish will enable more studies of host-pathogen interactions and the feasibility of vaccine development for offshore ranches. Increasing the productivity of these operations by reducing infectious disease mortality will reduce pressures on wild tuna stocks and the fish species used to feed ranched tuna.

Moreover, the apparent shift of isotype determination from VH-DH recombination to DH-JH recombination at the tuna IgH locus is interesting from a fundamental standpoint of lymphocyte antigen receptor immunogenetics, and begs many questions that must be verified and queried with new algorithms [182] at the levels of the tuna genome, the immunoglobulin proteins, tuna B cells, development in the pronephros, and the fish's response to pathogen. If the single tuna DH gene is verified at the genome, it will be interesting to know whether this IgH locus orientation is found only within this clade of endothermic fish or a broader set of Perciformes. These studies should provide insight into the natural history and fundamental physiology of antibodies while providing much needed tools for managing the health of ranched, and thereby wild, tuna stocks.

4. CONCLUSION

The major focus of these two studies was on two mucosal immunoglobulins in two groups of non-mammalian vertebrates: IgX in an amphibian *Xenopus laevis* (African clawed frog) and IgT in a teleost fish *Thunnus orientalis* (Pacific bluefin tuna).

In the first study we showed that IgX, which was known as a mucosal immunoglobulin and functional analog of mammalian IgA, is also the ortholog of IgA. The entire immunoglobulin heavy chain C region sequences from different vertebrates were used to revise the phylogenetic relationship of tetrapod antibody classes and the specific relationship between amphibian IgX and mammalian IgA. Unlike previous phylogenetic analysis, the resulting tree showed that IgX and IgA shared a common ancestor and branched closer together than either of them does with IgM.

Thymectomy did not affect IgX production neither in systemic nor in mucosal secretions. The same IgX expression in our larval-thymectomized post-metamorphosis frog model as the normal non-thymectomized model indicates that IgX expression is not dependent upon T cells, corroborating IgX identity to IgA. The significant higher IgX production after oral immunization in cultured gut B cells rather than intra-coelomically immunized frogs supports other studies showing that IgX is the specific isotype of the gut.

Thymectomy did not influence the gut microbial flora either. For the first time our study investigated an amphibian gut microbial population using 16S rRNA gene pyrosequencing based on distinct anatomical sites. The microbial population in the normal

non-thymectomized and thymectomized frogs showed no significant difference but a significant difference was detected based upon the anatomical district sampled. The large intestine microbial samples presented the richest diversity in the whole gut and samples obtained from stomach composed of distinct microbial families compared with the small and large intestine as confirmed by principle component analysis. Generally the predominant groups of gut microbial flora in *Xenopus* resemble major microbial components in human flora such as *Clostridiaceae*, *Bacteroidaceae*, and *Enterobacteriaceae*. In contrast to small and large intestine the most abundant bacterial family in the stomach was Flavobactriaceae, followed by Oscillatoriaceae, cyanobacteria, and Enterobacteriaceae.

IgT was discovered in 2005 and subsequently has been identified in several teleost fish classes. In the second study we described isolation and characterization of tuna IgT, the first from Perciformes (the largest order of vertebrates). Tuna is one of a few endothermic fishes. The full length of IgM was also sequenced for the first time in this species of tuna.

The sequencing analysis of *Thunnus orientalis* secretory IgT showed that it is composed of four constant domains as in other teleosts' IgZ/T except for the IgH in fugu and the chimeric IgM-IgZ in common carp which has two constant domains. Each domain contains two conserved cysteines except for the third domain with just one conserved Cys. These conserved Cys residues play an important role in forming intra- domain disulfide bridges. There is an additional Cys within the first domain (Cys-13) that shows this molecule can attach to the light chain. Despite the lack of J chain IgZ/T in tuna associated

with N-glycosylation site like IgZ and IgT respectively in *Danio rerio* and *Oncorhynchus mykiss* but it shares little similarity to the motif required for J chain similarity.

The phylogenetic analysis of the full length of C domain in tuna and other teleost IgZ/T showed that the IgT in tuna branched closest to mandarin fish or Chinese perch. Also the phylogenetic analysis of the full C domain of varies vertebrates immunoglobulin showed that IgZ/T is phylogenetically distinct from mammalian IgA but is functionally identical to mammalian IgA.

Figure 4 - 1 summarizes the evolution of different isotypes of antibody in main classes of jawed vertebrates. As you can see in this figure IgM is the most ancient and conserved class of antibody with four constant domains in almost all groups of vertebrates [13]. IgW in the earliest vertebrate is believed to be related to IgD [183]. IgD is lost in birds but it is expressed with high plasticity in other species and the function poorly understood. IgY is believed to be the ancestor of mammalian IgE and IgG and it first appeared in amphibians[184]. IgA, an accepted isotype of mucosal immunity, first was found in reptiles and then later in other vertebrates. IgX in frog was believed to be functionally a mucosal antibody but genetically not related to mammalian IgA until our recent phylogenetic studies on *Xenopus laevis* that revealed IgX as an evolutionary ancestor of IgA. The recently discovered IgZ/T in, lower group of vertebrate, teleost is shown to provide mucosal immunity but phylogenetically originated from IgM so IgA and all IgA analogues serve the same function in all animals and it is yet to be discovered in jawless vertebrates (Figure 4 - 1).

There are a lot of open questions in the evolution of mucosal immunity and the antibodies that mediate it. Some potential work to be continued by our study are: investigating the presence or absence of IgZ/T in other species of tuna and finding out the structure of Ig genes, and performing phylogenetic analysis with IgA/X in other vertebrates which may find a novel structure of Ig gene and even some characteristics in common with other warm-blooded animals. Also performing oral and systemic immunization of different species of vertebrate especially lower cartilaginous vertebrates and analysis of their gut flora will result in acquiring a better understanding of the evolution, function, and development of this immune compartment.

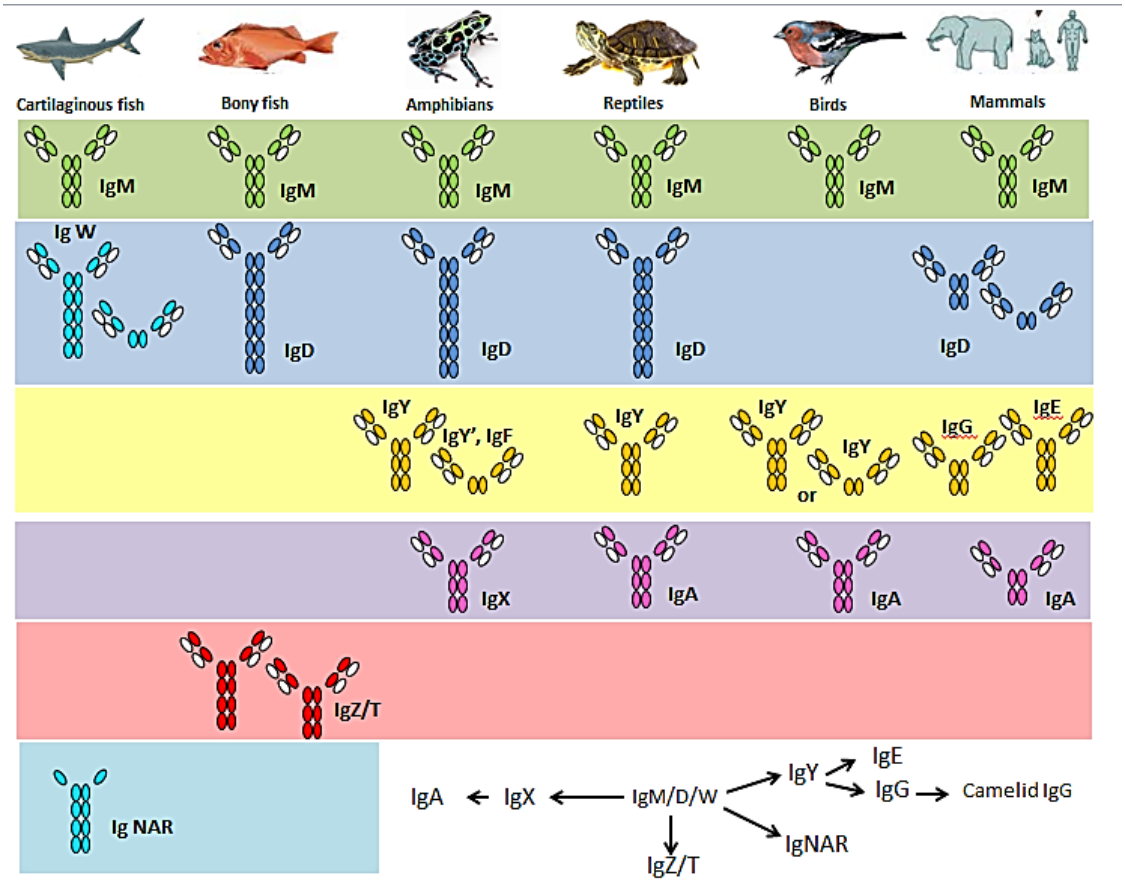


Figure 4 - 1. Evolution of immunoglobulin isotypes .

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