Systematic Fish Pathology

Part 7. Immunology:

a) Evolution & b) Practical Aspects

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The Australian Animal Pathology Standards (AAPSP) program

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Before Entering Training Program

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- This series of training modules has been prepared for The Australian Animal Pathology Standards (AAPSP) program as part of the Australian Veterinary Pathology training and further education resources, with financial and in-kind support from Animal Health Australia and from the Tasmanian Department of Primary Industries and Water.
- The major aim of this course is to convey an approach to diagnosis rather than to cover all fish diseases, through Power-Point presentations based on a histopathology teaching slide set representative of the pathology found in in this (Tasmanian) fish laboratory.
- This presentation diverges from that format, but is designed to complement those presentations through an understanding of what you cannot see (but need to understand) about fish responses. To do that we firstly (part a) review the development of immunology in vertebrates, then look specifically at immunology of fish (part b).

Acknowledgments.

- Photos to illustrate fish pathology for the Power Point series were generated by multiple contributors within the Tasmanian industries and the Tasmanian Department of Primary Industries & Environment Fish Health Unit. Contributors of cases from other laboratories have been acknowledged wherever possible and specific material and photographs used with permission. Any inadvertent omissions in this regard are unintended.

- References quoted for this review presentation are listed at the end.

- Photographs of animals and illustrations outlining the general principals are also included from other sources such as web-sites and acknowledged whenever the source was known.

Course Outline
A. Systematic Fish Pathology

1. Consider the Fish": An evolutionary perspective on comparative anatomy and physiology *(this presentation)*
2. Pathology of the kidney I – interstitial tissue Part A
3. Pathology of the kidney II – interstitial tissue Part B
4. Pathology of the kidney III – the nephron
5. Pathophysiology of the spleen
6. Fish haematology
7. **Fish immunology – evolutionary & practical aspects**
8. Pathology of the digestive system I – the oesophagus, stomach, and intestines.
9. Pathology of the digestive system II – the liver and pancreas.
10. Pathology of fish skin
11. Pathology and diseases of circulatory / respiratory system – heart, gills and vessels
12. Pathology of the musculoskeletal system and nervous systems
13. Pathology of gonads and fry

*(*Course *B: Presentations 14-16 - mollusc pathology)*

Introduction : *relevance*

- In this presentation, we cover evolution of vertebrate immunity (as Part 7A), and the fish immune system (as Part 7B), in more detail that in the introductory Presentation 1, in part to explain the diversity of fish responses to infection (remember that that there are more fish species than all the other vertebrates together, but immunity has been studied in very few).
- Members may find it better to view these as 2 units.
- Understanding fish immunity is important for the assessment of susceptibility (and therefore for disease control), because sub-clinical pathogen carriage is common in fish, and **vaccines** are becoming key control measures.
- As Part 7A has greater relevance to other vertebrate species, some repetition of PPT1 is included to make Part 7A "stand-alone".

Understanding of the evolution of the vertebrate immune system is evolving rapidly: *expect updated information to follow & please forward relevant new findings to the program manager, for updated versions.*

Presentation outline

Part 7A . EVOLUTION OF VERTEBRATE IMMUNITY

Section I. Evolution of adaptive immunity

- Recap of vertebrate adaptive immunity, compared with innate immune system
- How , why, & when did adaptive immunity evolve?
- Why is the system so complex?
- What started the evolution of adaptive immunity?
- Major steps in evolution of adaptive immunity

Section 2. Evolution of organs of the immune system

- Organs of the immune system of various chordate classes.
- The jaw hypothesis & origin of the thymus.
- Phylogeny & ontogeny of thymus

- Functions of the thymus
  - The thymus as MALT?
  - Thymus selection functions

Part 7B . FISH IMMUNOLOGY

SECTION 3. Fish Immunoglobulins

- Surface Immunity
- Allergic Responses

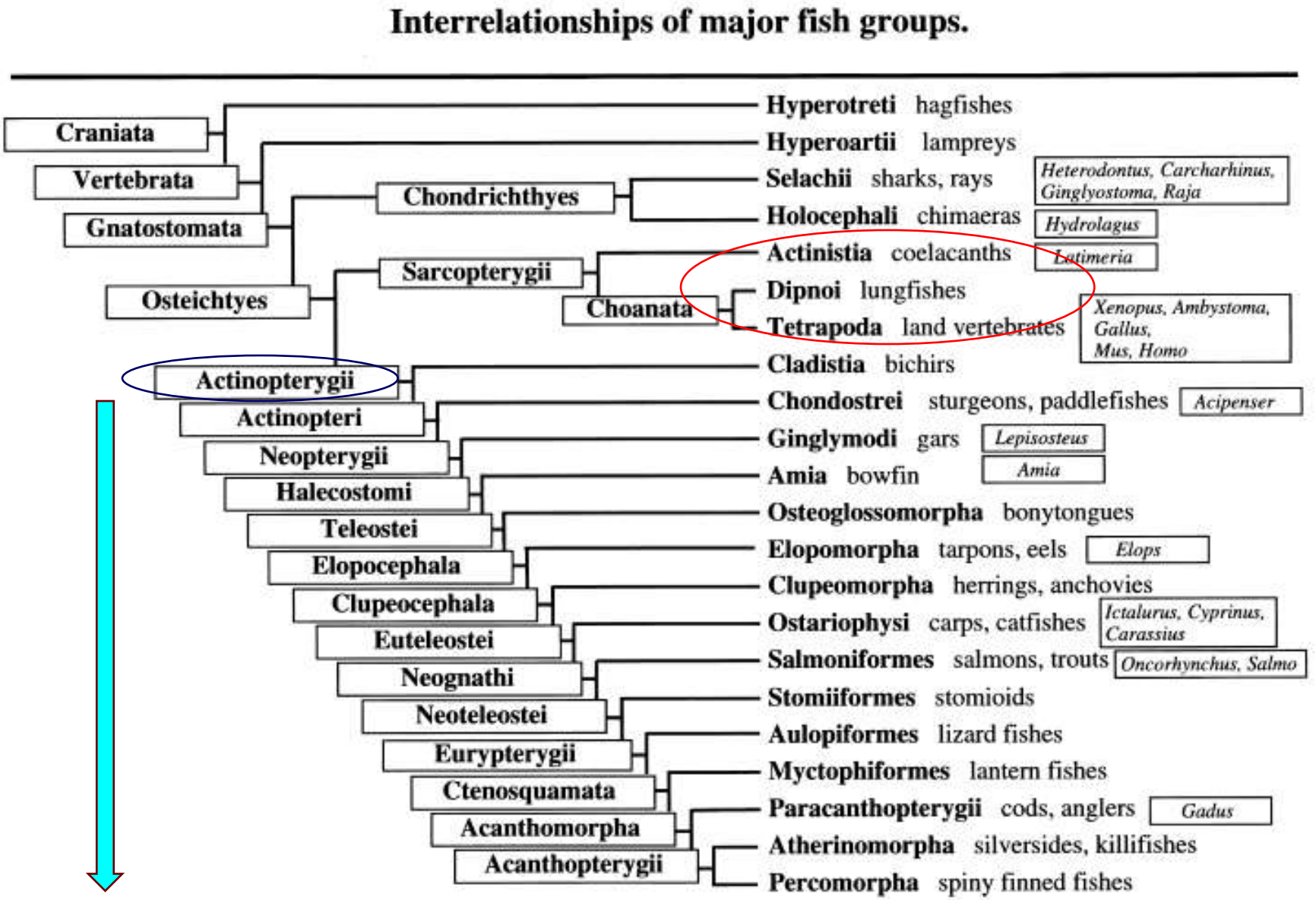
SECTION 4. Fish immunity – practical aspects .

PART 7A .

SECTION 1.
Evolution of adaptive immunity:

As this is about understanding commonalities and divergence (between fish species as well as between fish and higher vertebrates), we start with a reminder of vertebrate evolutionary divergence points and the extent of fish diversification after the divergence of terrestrial lines, and the major aspects of fish immunity & diversity.

Reminder : fish diversification, before & after the divergence of terrestrial lines



From Pilstrom and Bengten, 1996.(Boxed at right = species with immunoglobulin data available)

So:

- Land vertebrates evolved from an early branch of bony fish (the lobe-fin group, including lung-fish): we inherited and then modified their immune and anatomical heritage.

- Ray-fin bony fish (Actinopterygii) later diversified into multiple fish classes, particularly within the major teleost group (infraclass Teleostei): some will have features not present in our ancestors.

- The cartilagenous fish (sharks and rays), and the jawless lampreys and hagfish are progressively further away from the evolutionary point of the fish:terrestrial divergence.

Fish Revision – **Immune system** (simplified) Mammal

Invertebrates show neither an adaptive response nor immune memory:
and were regarded as having no immune system – *rather surprising, considering their long & successful history.*

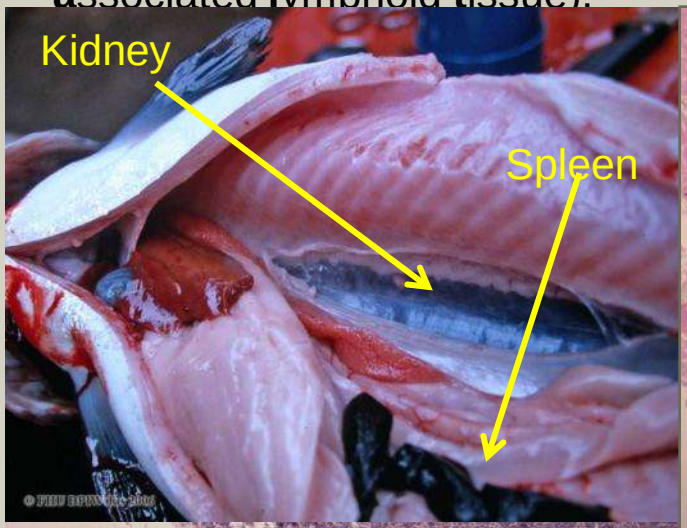
- **Fish:** have an adaptive antibody body-based immune system, with **B and T-cells**, a **thymus**, and **immune memory.**
- This combination arose relatively suddenly (in evolutionary terms) in jawed fish.
- This followed the evolution of recognisable lymphoid organs in primitive fish groups.
- The main fish immunoglobulin is of IgM type
- All fish groups also have innate immunity, similar to invertebrates.

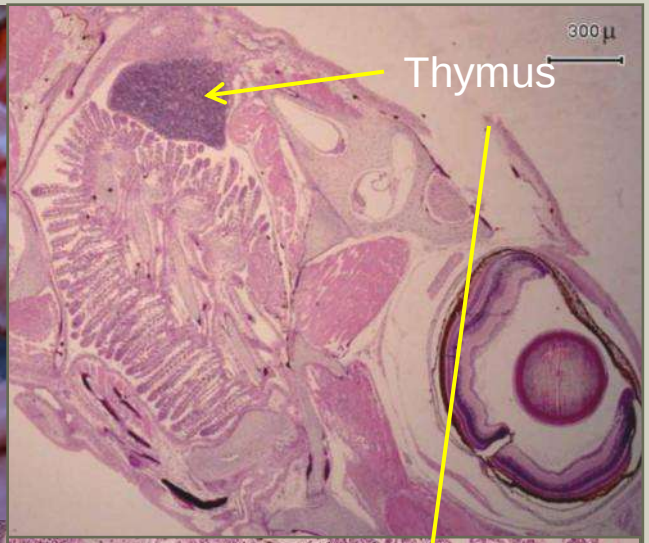
- **Higher vertebrates:** have a similar adaptive antibody-based immune system, with the main difference from fish being more "highly evolved", varied, and generally smaller immunoglobulins.
- The larger IgM is still retained as the first antibody produced.
- The Ig range includes specialized surface immunoglobulin (IgA), and an allergic form (IgE).
- Innate immunity is also present.

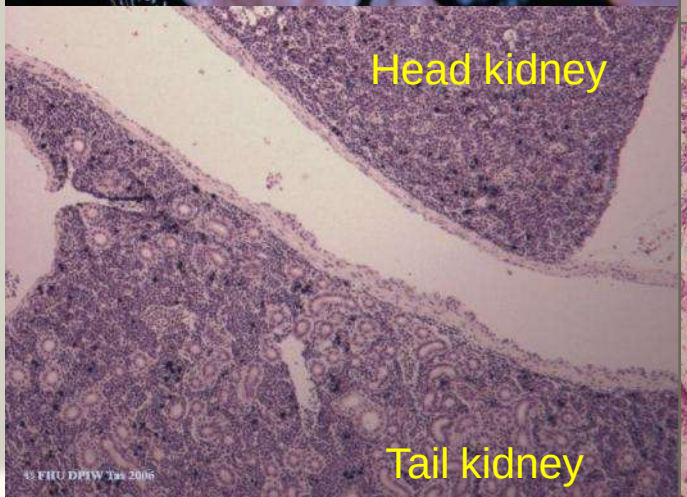
Thus **vertebrates** share an **adaptive immune system** with a common ancestry.

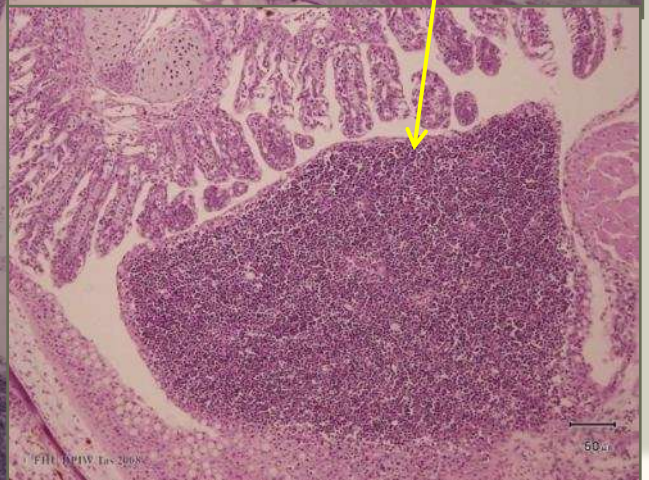
Fish Revision - Haematopoietic & Immune Organs Mammal

- Major sites of haematopoiesis in teleosts are the head kidney (pronephros - with no nephrons); its extension as the interstitium of the tail kidney (mesonephros) in most fish; and the spleen.
- Lymphoid organs include the thymus, spleen, kidney and GALT (**g**ut **a**ssociated **l**ymphoid **t**issue).









- Major site of haematopoiesis is the bone marrow
- Specialized lymphoid organs include the thymus, spleen, lymph notes.
- Smaller lymphoid aggregates occur as the GALT, more broadly expressed as part of the MALT (**m**ucosa-**a**ssociated **l**ymphoid **t**issue).
- Of these, Peyer's patches are large enough to be visible grossly.

Where did adaptive immunity come from?

Current theories of immune system evolution

- The adaptive antibody-based or "specific" immune system, capable of responding to almost infinitively variable recognition sites, arose in jawed fish, but has precedents in the invertebrates and was not quite the sudden event previously described.
- This system arose by re-use and recombination of older elements of the innate immune system.
  - Key components were modified by gene duplication and somatic recombination to generate receptor diversity.
  - Other parts were adapted to activation & regulation of the new "specific" adaptive immune system.
- The major new component was the generation of highly variable antigen receptors by irreversible somatic genetic recombination of antigen receptor gene segments (*i.e.* affecting that cell and all its descendants).
- Recent findings suggests invertebrates/lower chordates also generate expanded receptor diversity, but they use other mechanisms to do so. Thus diversity was already well developed before the antibody system arose.

The next section explores these theories, & how they relate to fish.

Therefore to understand the steps in the evolution adaptive immunity in vertebrates:

- As well as a basic knowledge of vertebrate evolution and the nature of the adaptive (antibody) immune system, we need knowledge of:
  - the nature of the innate immune system, and the interactions with the adaptive antibody system.
  - the evolutionary processes needed to achieve this.

Invertebrates & innate immunity

"the major system of host defense against pathogens in nearly all living things" (Wikipedia).

Effector mechanisms ***linked*** ***to*** **Receptors**

A diverse range of effector molecules, some conserved from plants to mammals:

- C-type lectin,
- (Haem)agglutinins
- (Haemo)lysins,
- perforin proteins
- & many others

plus

- phagocytes, other granulocytes

- Leucine-rich repeats (LRR) of Toll and Toll-like receptors
- Immunoglobulin superfamily members (Igsf)
- Thio-ester bond-forming proteins (TEP) of the complement family
- Lectins
- Peptidoglycan-recognizing proteins
- Scavenger receptors such as "cysteine rich" receptors (SRCR)

These are used with **highly conserved signaling cascades.**

Diversity is generated by **combinations** of these, and at times by **additional mechanisms** such as alternate splicing of RNA (which is very common across plants & animals, for expressed products in general)

How did adaptive immunity evolve?

Du Pasquier (2005) reviewed how the demands for an immune system were met, and concluded that the antibody-based adaptive immune system of vertebrates – with lymphocytes and their specific receptors of the immunoglobulin superfamily (the major histocompatibility complex MHC) – developed from innate immunity evolutionary lines that can be traced back in earlier deuterostomes.

The major **additional** component is a set of **gene segments to be assembled during the ontogeny of a lymphocyte** that randomly generates receptors, so that a large number of possible receptors are generated from a small number of genes. This creates an irreversible change in the DNA of each cell, so that all progeny of that cell will inherit genes for the same specific receptor, including the memory cells that are the keys to long-lived specific immunity (*e.g* clones).

This process (the somatic mutation of lymphocytes, which generates large number of possible receptors from a small number of genes), fulfills what Du Pasquier considered to be the criteria of an ideal immune system, *which must*:
"generate diversity and flexibility of its recognition (and effector) functions, **without using either too many genes or too many cells**".

Why did adaptive immunity evolve?

Du Pasquier addressed the question of **why** vertebrates bothered to develop an adaptive immune system with another question: why did (most) invertebrates **not** do so?

He concluded that there is greater evolutionary advantage for such a system in longer-lived late-maturing animals that produce fewer progeny, than in shorter lived prolific animals which benefit more from greater germ-line (population) diversity.

Hence a link with the gradually developing parental nurture by vertebrates, culminating in placental nutrition as well as parental care.

Why is the system so complex?

Once diversity is large (essentially unrestricted initially, given the way diversity is generated), it **must** be put under control and restricted in expression to avoid autoimmunity and to maintain specificity.

- The key to this control is **the major histocompatibility complex (MHC)** and the gene selection process that occurs in the thymus (and elsewhere), as a way to avoid clones which give responses against MHC genes.

- We will see more examples of how this "specific" immune system is activated and regulated, often by the "non-specific" and evolutionarily older innate immune system components (expect more to be found).

So the vertebrate adaptive immune system retains elements of the innate immune system

- both in their original functional context (some still poorly understood)
- & adapted as control elements of the antibody system.

Major steps in evolution of adaptive immunity

Adaptive immunity evolved quite rapidly, with some sudden steps, adapting older molecules to new uses (the following is from Sima. 2000).

A. Molecules of the immunoglobulin superfamily probably arose in protosome invertebrates (ie. *pre*-deuterostomes) from cell adhesion molecules of plasma membranes known as **cadherins**. These arose as mediators of cell interactions.

- They are more related to neural cell adhesion molecules than to immunoglobulins.
- The immunoglobulin **superfamily molecule Thy-1** (a T-cell marker, also found on brain tissues of some mammals), is likely to be closely related to the primordial gene for imummoglobulins and MHC in vertebrates. It is more primitive than immunoglobulin and MHC molecules, with homologous molecules found in annelids, molluscs, and tunicates.

B. It is probable that the **genetic mechanism for gene duplication** came from horizontal transfer of **RAG** (**r**ecombination **a**ctivating **g**enes), which were **originally microbial genes** that were then incorporated into genomes of the predecessors of jawed vertebrates.

- This step Sima describes as the “big bang” part of immunoglobulin evolution.

and then Evolution of Antibodies

C. The next big step was the **formation of immunoglobulin**, using gene duplication.

- Theory suggests this was by duplication of a primordial gene coding for about 100 amino acids forming a single Ig-domain.
- A candidate relative of this primordial immunoglobulin molecule is the ß2-microglobulin consisting of 99 amino acid residues in a single chain with one intra-chain disulphide bond, which is ubiquitous on all mammalian cells except erythrocytes. A molecule with high homology to this has been also found in many invertebrates.
- It is suggested that ancestral ß2-microglobulin gene diversified into a “primitive gene“ in protostomes and into “primordial gene“ in deuterostomes.

The end result is T and B cells with specific antigen-binding receptors, the T-cell antigen-binding receptor (**TCR**) being found on the cell surface and B-cell receptors being initially membrane-anchored immunoglobulin that is subsequently released as a circulating antibody.

When did adaptive immunity evolve? (it probably still is)

- While evolution of the major components of the antibody immune system was relatively sudden, refinements continued, both in the lobe-fin/land vertebrate stream, and in teleosts (and quite probably also within the cartilagenous fish and the jawless lampreys and hagfish that diverged during this process).

- As pathologists we can't "see" the diversity of the immune mechanism, but the following slides provide hints about how the organs of the immune system developed to cater for these refinements.

- *So...*

SECTION 2.
The histologocial context:
Evolution of organs of the immune system in the phylum chordata.

Here we trace the co-evolution of the immune system & and the major organs housing it.

Evolution & the immune system - *Phylum chordata*

- To look at the evolution of these organs in vertebrates, we need to cover at least the **phylum chordata.**
  - This has **three subphyla: Urochordata** (tunicates), **Cephalochordata** (lancelets or amphioxus), and **Vertebrata** (vertebrates).
- **Reminder: all chordates, at some time in their lives, have four distinctive features:**
  - A notochord which is a long rod of stiffened tissue that supports the body. Later in development, it changes to bony units in vertebrates.
  - A dorsal, tubular nerve cord lies above the notochord and gut.
  - A muscular pharynx with gill slits at the entrance to the digestive tract (at least in the embryo).
  - A tail, or rudiment thereof, exists near the anus (at least in the embryo).

Evolution & organs of the immune system
1. Pre-chordates

- Chordates, hemichordates, echinoderms (starfish, sea urchins etc) and a couple of other worm-like animals make up the **deuterostomes** (first embryonic opening becomes the anus.

- Gill slits evolved relatively early though were not always retained – signs of gill slits are seen in some primitive fossil echinoderms.

- Hemichordates ("acorn worms") are apparently an intermediate stage between echinoderms and chordates, as they have pharyngeal gill slits and dorsal tubular nerve cords but not the other features.

Acorn worm

Organs of the immune system 1 *continued.*
Diversity & early deuterostome defenses - echinoderms

- **Echinoderms** contain some unique factors (such as sea star factor, involved in inflammation) that also inhibit macrophages and suppress T-cell-dependent mammalian immune responses
- They also possess vertebrate-like interleukins – IL-1-lik, IL-2-like, IL-6-like, TNF-like, IFN-γR and C3-like homologue.
- Plus receptors for Ig superfamily Il-IR, IL-6R .
- Alternate splicing is used by sea-urchins for increasing expressions of cystine-rich scavenger receptors (SCR), giving thousands of types from only 150 genes
  - Overall, the purple sea-urchin has a vastly expanded receptor repertoire, compared to other invertebrates: 222 toll-like receptors (TLRs), 203 NOD/NALP-like receptors (NLRs), and 218 scavenger receptors (SRs).

This supports the speculation that before vertebrates evolved somatic diversity-based adaptive immunity, the germline-encoded diversity of innate immunity was well developed.

Organs of the immune system
2. Primitive chordates - tunicates

- **Urochordates** (tunicates – "sea squirts", the most primitive present-living Chordates) only have a notochord in the larval stage when they look like tadpoles (probably we evolved by just keeping the larval stage).

Photo from
http://trc.ucdavis.edu/biosci10v/bis10v/week9/08tunicates.html

- Anatomically, tunicates show the **first distinct mesodermal-derived haematopoietic structures.** These are present as accumulations of stem haemoblasts, which may be diffuse or structured into "lymph nodules" along the digestive tract.

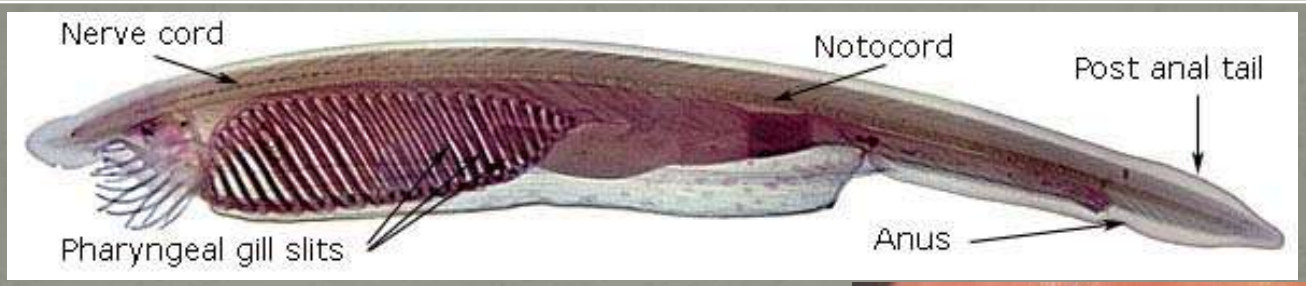
  • These "nodes" include pharyngeal wall accumulations where interactions of ectodermal epithelium, mesenchymal tissue, and endoderm take place: this may be the origin of the thymus.

- Immunologically: their immune repertoire includes invertebrate type factors: immune factors unique to tunicates; and vertebrate-like factors such as:

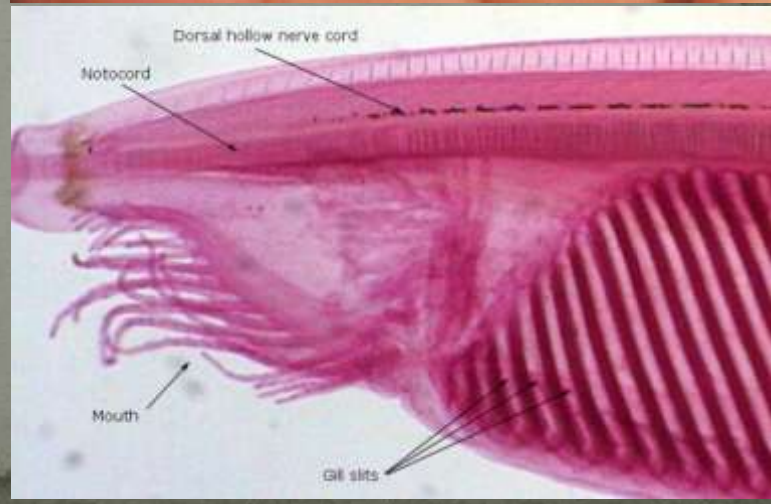
  • IL-1β, (C-type lectins), involved in stimulation of cell proliferation

  •IG superfamily members - Thy-1 & Lyt-2/3 homologues

Organs of the immune system
3. Primitive chordates – lancelets (*Amphioxus*)



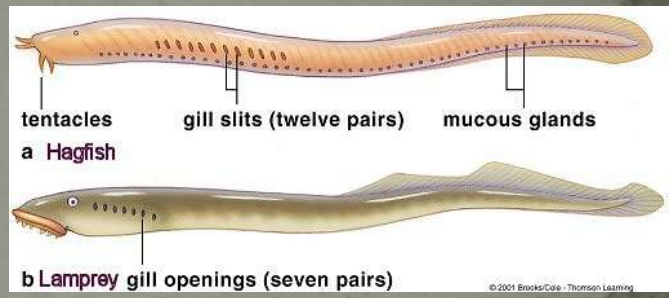
- Lancelets (*Amphioxus*) are small fish-like animals with tapered bodies, segmental fish-like myomeres, a closed circulation (but no red cells, just a few amoebocytes), a dorsal nerve cord but no brain or jaws.
- They do not have vertebrate type adaptive immunity, but Huang et al (2008) have recently shown they have an **extraordinarily complex innate immune system**, with gene expansion of several receptor families, including 1205 C-type lectins, hundreds of models containing complement-related domains, and a sophisticated TNF system. Domain combinations of immune proteins are also increased.
  - They do have **complement component C3** (involved in killing of *Vibrio* species, with a response that differentiates between *Vibrio* species.)
  - They show **chronic (but not acute) graft rejection**.
  - They retain an invertebrate prophenoloxidase system.



Organs of the immune system

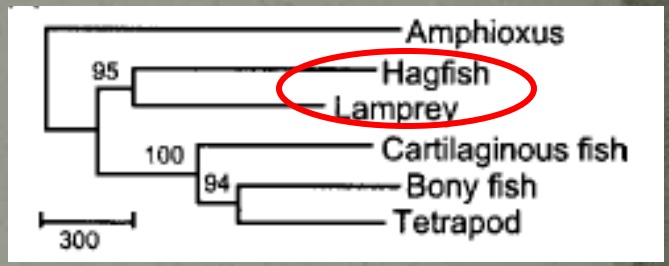
4. Jawless fish - the agnatha. A: Anatomy

- The jawless cyclostomes or agnatha, most primitive of true vertebrates, look eel-like with cartilaginous skeletons but have no jaws derived from gill arches, no ribs, no shoulder or pelvic girdles and no paired appendages. The gill passages are expanded into pouches connecting to the exterior through > 5 external openings (7 in lampreys, more in hagfish). Modern species are secondarily adapted for parasitic life.



http://universe-review.ca/option2.htm#L

- First to show **fish-like haematopoietic tissue** in the pronephros and supraneural body, as well as haematopoietic aggregations along veins of the gut with a function analogous to GALT.



- **Hagfishes** have poorly developed haematopoietic tissues, compared to lampreys, but this may reflect loss of function with adaptation to parasitism, as they are closely related.

- Better developed **lampreys** may be more representative (especially in the prolonged larval stage). Their organs include a protospleen that develops in an infolding of the alimentary canal called the typhosole. This (and the kidney) are sites of production of **lymphocyte-like cells***, erythrocytes and granulocytes, but no cells showing differentiation as monocytes or thrombocytes.

Hagfish mouth

Lamprey mouth

www.english-nature.org.uk/.../lamprey.html
Hagfish mouth – multiple sites, source unknown.

- At metamorphosis, haematopoietic activity is taken over by the supraneural body (called = provertebral arch by some authors): this is fatty (a bit like bone marrow).

- ***Lymphoid like cells** accumulate in the pharynx region but this is **not yet a thymus**, and these are **not** true lymphocytes – don't yield T or B cells.

Organs of the immune system (*continued*)
4. Jawless fish – the agnatha B: Immunology

- The agnathan immune repertoire includes haemagglutinins and haemolysins and **several components of the vertebrate complement system (C3, C4, C5)** resembling an **alternate complement pathway** which aids phagocytosis.
- They do not have immunoglobulins or T or B cells, or genes for T-cell antigen binding receptor (TCR), MHC or RAG.
- Their fish-like haematopoietic tissue in the pronephros and along gut show show no differentiation into primary and secondary lymphoid organs.
- Although the **lymphoid cells in the branchial** (gill) **region are not a thymus, they specialise in trapping particles**
  - These branchial lymphoid accumulations do **involute with age.**

- **They <u>are</u> capable of adaptive immune responses**, but have **a <u>different</u> type of variable lymphocyte receptor.**
  - This is composed of highly diverse **leucine-rich repeats (LRR),** sandwiched between amino- and carboxy-terminal LRRs, (plus a invariant tether to the cell surface). A single locus with a large bank of diverse LRR cassettes can generate highly diverse lymphocyte receptors through LRR modules rearrangement (Pacer et al, 2004)
- **Recent findings** (Nature June 2009, Gou et al, Litman & Cannon) indicate that like antigen receptors on T-cells (TCR) and on B-cells, these **V**ariable **L**ymphocyte Receptors (VLR) of lampreys divide into 2 classes, with VLRA being expressed only on the cell surface, VLRB expressed initially on the cell surface and later also excreted as a humoral response. **Apparent convergent evolution**

Organs of the immune system
5. The jaw hypothesis & origin of the thymus

- **A thymus** is present in all jawed vertebrates (gnathostomes), apparently arising near the gills, in conjunction with **GALT** (gut associated lymphoid tissue).
- There is speculation that this could reflect greater exposure of the digestive system to insults with **the development of jaws, which arose from the gill arches** (**the jaw hypothesis of adaptive immunity**).
- **The first gill arch became the upper and lower jaws, the second gill arch moved forward to brace the jaw.**
    - In this context it is interesting that in seahorses, which feed with a sucking motion and effectively have a secondary loss of true jaws, there is an absence of GALT, though there is a thymus.
- The origins of the ears, larynx, throat, and some bones, muscles, nerves and arteries of the head can all be found in the gill arches.

Organs of the immune system
6. Cartilagenous fish (sharks & rays):
true vertebrate type adaptive immunity

- **Anatomy: Sharks and their relatives** have a **thymus**: paired lobed masses dorsal to the first 2 gill arches. This has a clear cortex and medulla that is often (but not always) lost in higher fish.
- The thymus typically involutes after a few weeks.
- Also other lymphoid organs, including:
  - A well defined spleen with red & white pulp
  - Organs specific to this group (not present in higher fish) - the Leydig and epigonal organs located in oesophagus and gonads, & smaller accumulations in other organs including heart.

- **Immunology**: They show **T-cell receptors, MHC class I and II, and RAG-1 genes,** and produce IgM (**mainly as a pentamer**, also dimer and monomer molecule) as mucus and serum antibody, and show true adaptive immunity.
  - Sharks actually possess three classes of L-chains, including 1 type restricted to them, suggesting that IgM may **not** have been the primordial antibody class.

Organs of the immune system 7.
7 Bony fish (e.g teleosts) A. Anatomy *(revision)*

- The spleen and thymus are the major lymphoid organs of fish, although kidney and a usually well-developed GALT are also important.
  - The spleen is generally more important for antibody production (but this may vary with species.)
  - No lymph nodes (mammals only?) or germinal centres (birds, mammals -?reptiles)

- Generally the thymus shows no differentiation into cortex and medulla, but may show structures similar to Hassall's corpuscles.

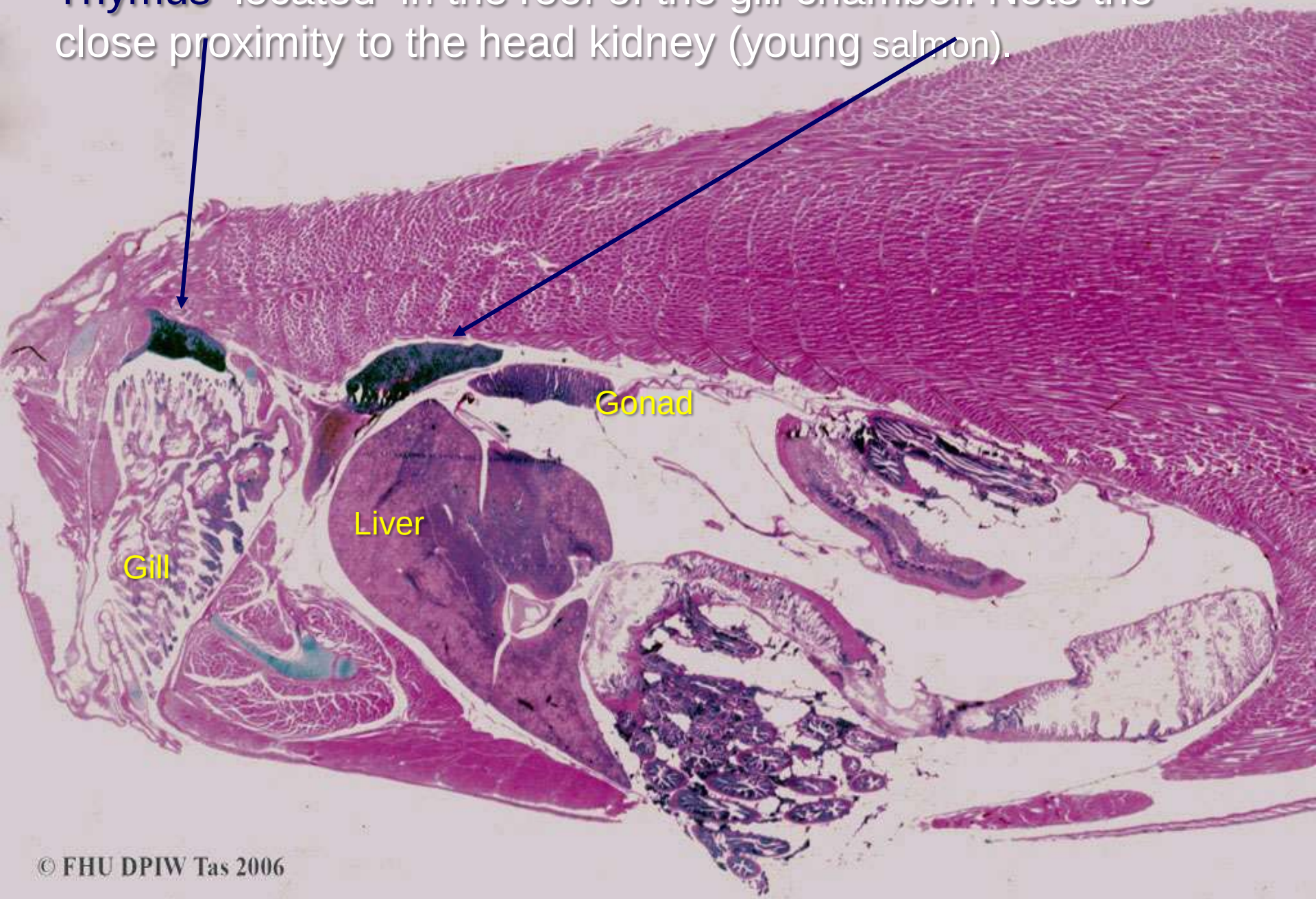
- We have seen that the thymus, located in the dorsal margin of the gill cavity in close association with the surface epithelium, **evolved as a site of antigen trapping** in association with jaw development.

- The direct contact of these trapping cells with the environment is shown by the fine epithelial pores demonstrated by scanning electron micrography of rainbow trout thymus (following slides).

Thymus in upper anterior corner of the gill cavity, salmon smolt.

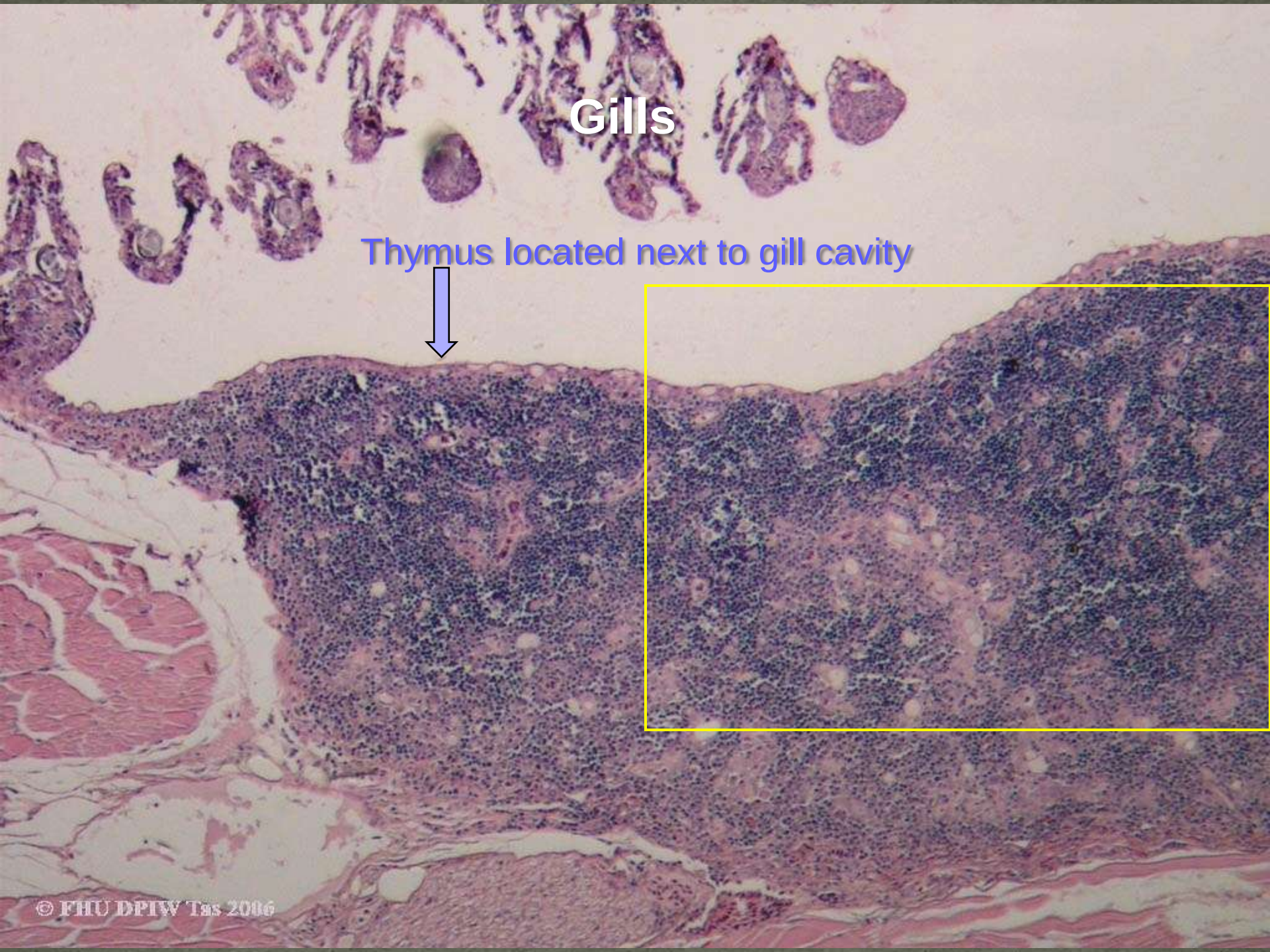
Note relationship to the gill and the vascular plexus-rich pseudobranch (P).

Thymus located in the roof of the gill chamber. Note the close proximity to the head kidney (young salmon).

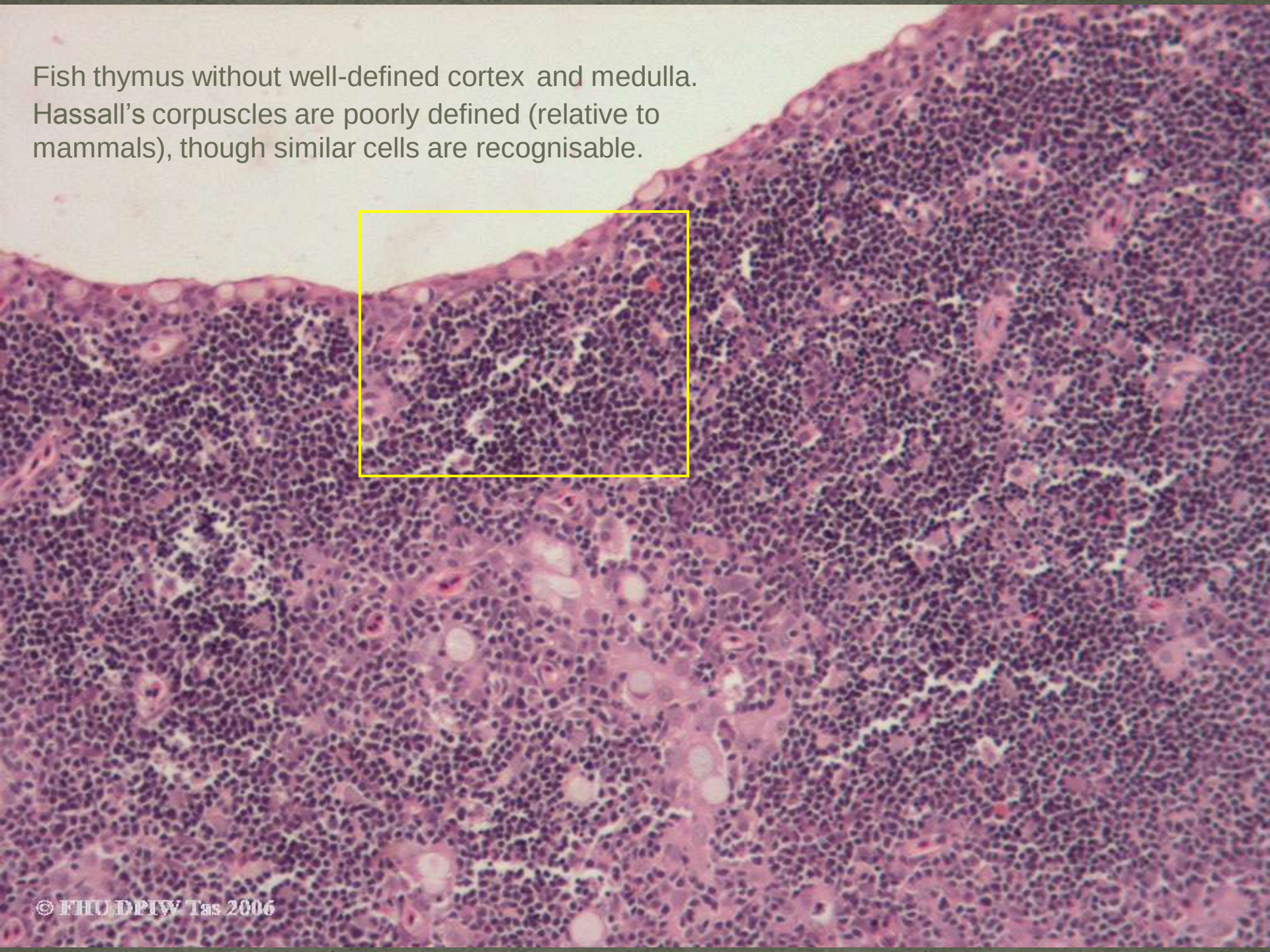


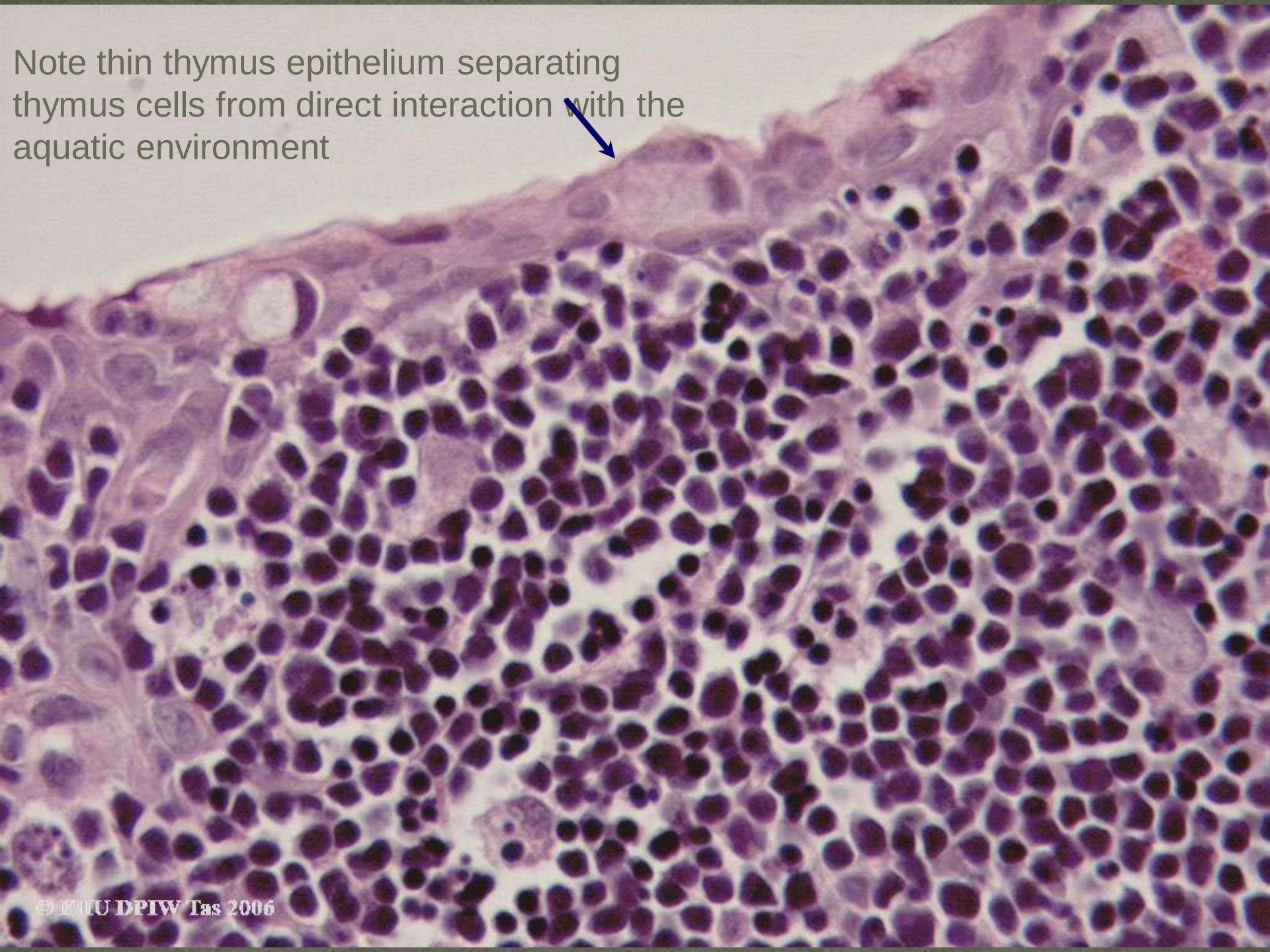
Gills

Thymus located next to gill cavity



Fish thymus without well-defined cortex and medulla.
Hassall's corpuscles are poorly defined (relative to mammals), though similar cells are recognisable.




Note thin thymus epithelium separating thymus cells from direct interaction with the aquatic environment
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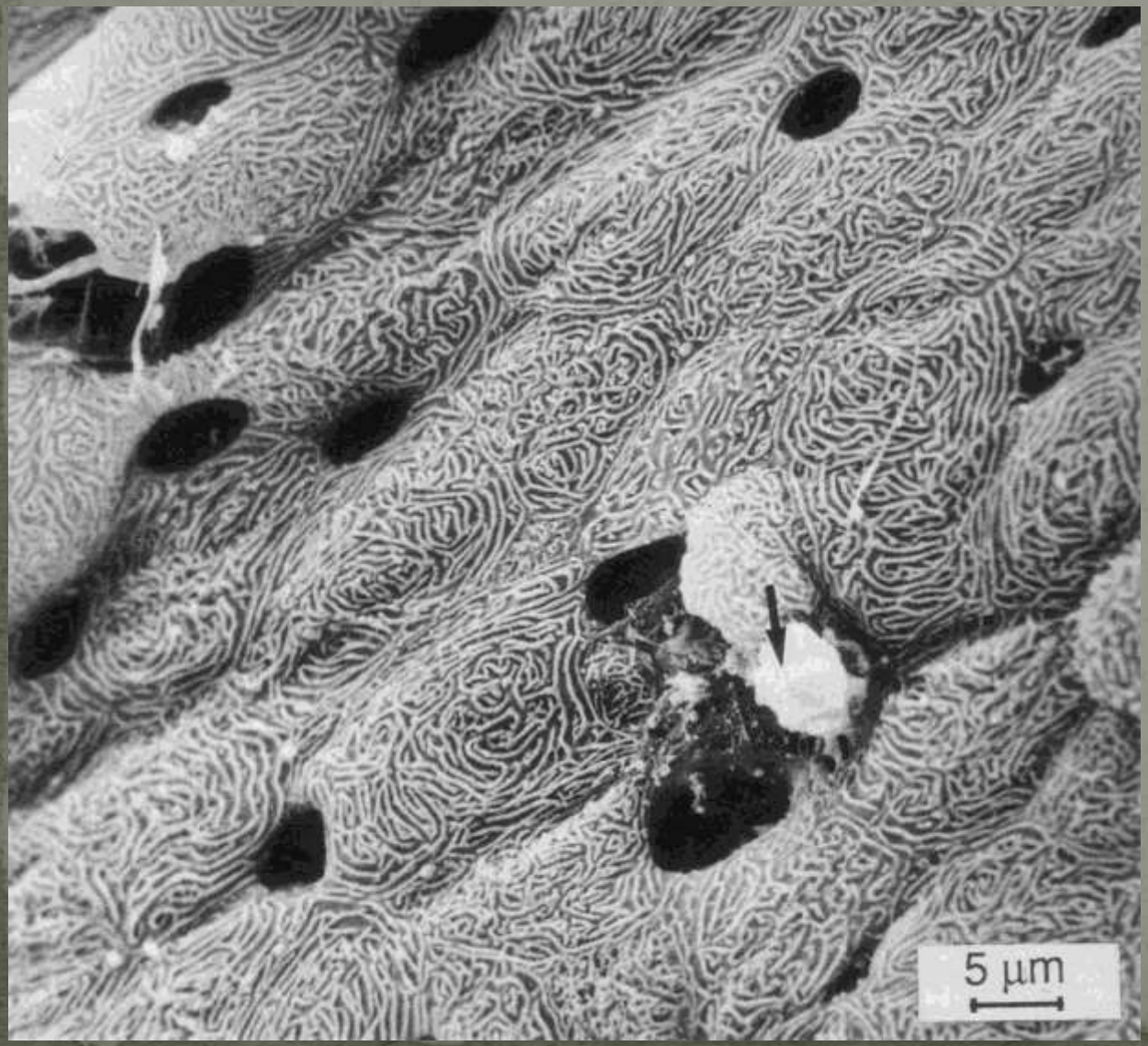
The surface of the thymus in salmonids is superficial, with pores up to 20 μm in diameter, which close over in older fish.

In contrast, the thymus of carp is initially superficial but quickly becomes embedded in deeper tissues (as it does in higher vertebrates)



Scanning electron micrograph of thymic region surface, fingerling rainbow trout, showing the pores. (Arrow = mucous secretion).

From
Chilmonczyk, S. (1992) *Annual Rev of Fish Dis. 2: 181-200.*, with permission.

Organs of the immune system
7. Bony fish (teleosts) B: Immunology

- Fish show true adaptive immunity, with **IgM** as the major fish immunoglobulin, **but not the only one (see later slides).**
- **Teleost IgM is generally tetrameric** (in contrast to mammals) without J-chain.
- Fish L-chains are variable, with uncertain homology to mammalian L-chains.
- The GALT is often abundant but is diffuse, not organized into Peyer's patches.

More on thymus evolution: Phylogeny & ontogeny of thymus

1. **E**volution of the **pharyngeal pouches** was driven by the shift from filter feeding to oral feeding and then development of respiratory function by the pharyngeal region (Sima)
2. Some of the pharyngeal arches were modified into other organs, including the development of **jaws from the first arch** and of the **thymus from some of the more posterior arches**.

- These modifications largely resulted from the **interaction between the incoming neural crest cells and the pharyngeal endoderm.**
  - In the case of the mammalian thymus, the neural crest cells are derived from rhombomere 6 of the hindbrain and the region of the neural tube posterior to it.
- **Which pharyngeal pouches develop into which organs** is regulated by a set of genes (PAX1 and PAX9, plus the HOXA3 genes). These also differentiate the thymic rudiment onto two domains that develop into the **parathyroid** and the **thymus**.
  - In humans, only the **third** pharyngeal pouch develops into the thymus, the **second** into the palatine tonsil (which is mucosa associated lymphoid tissue).
  - The second gill pouch may develop into the thymus is some lower vertebrates.
  - In some fish species, **every** pharyngeal pouch may develop into thymus – giving paired lobes in the dorso-lateral gill region.
- Further development of the thymic rudiment depends on the **arrival of lymphocyte progenitor cells**.
  - Ultimately, the endodermal cells develop into the thymic epithelium and the neural crest cells condense into the capsular elements.

The thymus as MALT?

- Matsunaga & A. Rahman (2001) reviewed evidence that **mucosal immunity at the body surface is more primitive than the systemic immunity** driven by the thymus and other lymphopoietic tissues, and suggested that:
  - As is outlined above, the thymus evolved as an expansion or sub-set of **MALT** (mucosa-associated lymphoid tissue).
  - This followed evolution of gut-associated lymphoid tissue (GALT), driven by vulnerability of the gut with the development of jaws.
- The closeness of this relationship has been reinforced by the recent finding that **generation of local T cells also occurs in gut-associated lymphoid tissues (GALT)** in mammals (at least in the mouse & fetal humans).
- The distribution of the 2 types of T-cell **antigen** receptors (TCR) - as **TCR αβ** or **TCR γδ** molecules, plus 2 classes of MHC molecules (**class I** & **class II**), helps to clarify this relationship *(see following slides)*

The thymus as MALT
2. Thymus selection functions

- The thymus undertakes **both positive and negative selection** of the repertoire of T-cell antigen receptors (TCR):
  - This is primarily selection of **αβ TCR** lymphocytes.
  - Both +ve and –ve selection involves antigen sites presented by MHC (major histocompatibility complex) molecules
- **Positive selection** expands the repertoire **& depends on interacting with epithelial cells:** the extent of +ve selection *in vitro* depends on the accessibility of thymocytes to the thymus epithelium.
  - Positively selected αβ TCR lymphocytes are fit to recognize foreign peptides presented by MHC molecules.
- **Negative selection** involves the deletion of T-cells that bind too strongly to ligands of **self-peptides** presented by the MHC.
- In the thymus, two classes of MHC molecules select 2 classes of T-cells in thymus: **CD8** & **CD4** T-cells selected by **class I** & **class II MHC molecules**, respectively.

The thymus as MALT
3. GALT lymphocyte types.

- T-cells with the other class of antigen receptor, **TCR γδ** lymphocytes, are predominantly located in the **mucosal epithelium** of the gastrointestinal tract, uterus, lungs, etc.
  - Ontogeny, structure & antigen specificity suggest **TCR γδ** lymphocytes evolved earlier that **TCR αβ** lymphocytes
- **Intraepithelial lymphocytes** (IEL) (that make up a large % of mouse lymphocytes) contain both **TCR γδ** & **TCR αβ** lymphocytes, both of which are generated mostly **extrathymically** from cryptopatches of intestinal lamina propria (*cryptopatches = small numerous lymphoid aggregates*).
  - But only one **class** of αβ TCR [CD4 TCR αβ IEL, selecting for class II MHC], is found in gut epithelium.
- **Lamina propria T-cells** are **largely thymus derived** (ie, with both CD4 TCR αβ lymphocytes & CD8 TCR αβ populations)**.**
- **Thus GALT T-cells are supplied from two different sources, the thymus and GALT itself, but those of the epithelium are more restricted.**

The thymus as MALT
4. GALT selection functions

- Positive selection has **not** been demonstrated within the GALT:
  - As this is epithelial dependent, it could only occur if T-cells produced in the cryptopatches of the lamina propria cross the basal membrane to reach the gut epithelium.

- There is evidence for negative selection in GALT, which does not require the epithelium.

The thymus as MALT?
Overall function & summary

- Cytokines such as interleukin (IL)-1, IL-6, IL-7, IL-8, transforming growth factor (TGF)-β, T-cell chemokine (TECK), are produced by both gut epithelium and the thymus epithelium.
- In GALT, the antigen-specific immune responses as well as the T-cell production occur in parallel.
  - GALT is therefore a complete self-sustained immune tissue – a key expectation of a primordial system.
  - Thymus needs external effector mechanisms.

- Overall, this suggests that the thymus evolved from the mucosa-associated immune tissues as a mechanism to provide a 3-D framework for **better interaction of developing T-cells with the epithelium,** to enlarge the receptor repertoire size and the overall production of T cells for **systemic** immunity.
  - Note the bursa of Fabricius in birds, located in the cloaca, increases **the B-cell repertoire generation**, & is also rich in reticular epithelium derived from intestinal epithelium.

PART 7B .

FISH-SPECIFIC ASPECTS OF IMMUNITY

SECTION 3 .
Fish immunoglobulins

This section gets back to the specific immune responses of fish to pathogens, and the factors known to influence immunology in fish, such as age, temperature and stress that influence susceptibility to infection and the success of vaccination.

We start by looking in more detail at fish immunoglobulins and their functions.

Fish immunoglobulins

1. Immunoglobulin structure - IgM

- Immunoglobulins of all gnathostome (jawed) vertebrates are composed of 2 heavy chains and 2 light chains. These molecules may combine to form larger units.

- **The major fish immunoglobulin is of IgM type**. It is the first Ig to appear in phylogeny, ontogeny & as antibody in immune responses in higher vertebrates.

- In most typical (teleost) fish, this has a **tetrameric structure**, with 8 light and 8 heavy chains and 8 binding sites (occasionally smaller amounts of dimeric and monomeric forms of IgM are also found in fish).

- In contrast, the IgM of all other gnathostome vertebrate taxa is typically a pentamer of the basic (2 heavy- +2 light-chain) unit.

- Elasmobranchs (sharks and rays) also have a pentamer IgM form.

Fish immunoglobulins
2. Vertebrate receptor diversity

- Fish antibodies are of lower affinity and diversity than those of mammals and birds (Du Pasquier,1982, Pilström & Bengtén,1996) .
  - Thus fish lymphocytes may not detect as many antigen sites on a pathogen as mammals (i.e. for some pathogens, passive immunity using mammal antisera gave greater protection than vaccination, due to greater range of antigens recognized).

Vertebrate mechanisms for creating receptor diversity:

- We have seen that lampreys and hagfish solved the receptor diversification problem by the recombinatorial assembly of leucine-rich-repeat genetic modules to encode variable lymphocyte receptors – ie diversity without antibodies.
- Birds show a different type of receptor gene organization to mammals
- The mechanism may also be different in sharks and rays (Pilstrom & Bengten)
- There may be another in the coelacanth.

Variable ways to create **receptor diversity** in vertebrates should not be surprising, given the multiple mechanisms for creating receptor diversity in invertebrate phyla, well before this was linked to soluble antibody production (Du Pasquier, 2005)

Fish immunoglobulins

3. Fish & antibody diversity

- **Antibody diversity:**

  Though IgM is the major component, 5 types of teleost fish immunoglobulin are now known: IgM, IgD, IgZ, IgT and IgH (Vesely ett al, 2006).

  - Thus **smaller molecules are known in fish,** but their exact role is poorly understood.
  - The major known functions are mediated by antibody of IgM type.

The evolution of the smaller IgG as the major mediator of systemic antibody responses may be an adaption to the vascular resistance offered to the large immunoglobulins by the thicker vessels required by the terrestrial circulatory system, as IgM appears to traverse the thin vessel walls of fish with relative ease.

Fish immunoglobulins

4. Surface immunity

- Fish IgM penetrates through vessels into the tissues but epithelia pose a greater physical barrier to the passage of these large IgM molecules.
  - The smaller Ig molecules of fish do not correlate with those of mammals.
- Fish (teleosts) do have surface Ig activity – but this is mediated by IgM indistinguishable from the systemic antibody: systemic immunization can result in local surface immunity.
  - For example, intraperitoneal injection of purified immobilizing antigens of the large ciliate *Ichthyophthirius mulifiliis* (commonly known as "Ich") results in immunity through immobilization via antibody binding to these receptors on the parasite (Maki & Dickerson, 2003)
- The cutaneous antibodies do not arrive there by passive diffusion from the blood.
  - Hamura et al, 2007, showed that **fish tetrameric IgM is transported to the skin mucus by the same transport system as in mammalian intestine** – using a polymeric receptor or pIgR (this particular receptor unique to fish – Fugu).

Fish immunoglobulins

Fish 5. Allergic responses Mammal

- Fish have large numbers of mast-cell type effector cells, the eosinophilic granular cells (**EGC**), now confirmed as mast-cell related.
- Most (as name implies) are eosinophilic, though basophilic/metachromatic forms are seen in some species.
- Staining may reflect the wider range of mediators that may be released, compared to mammalian mast cells (e.g. piscidins)
- Do contain serotonin.
- Many fish (& amphibian) mast cells are devoid of histamine, but Perciformes do have histamine (it regulates respiratory burst of phagocytes). [Mulero et al, 2007.]
- **No IgE – they just do it with IgM!**

- Produce specialized immunoglobulin (**IgE**) that activates mast cells.
- Mast cells release vasoactive amines such as histamine and 5-hydroxytryptamine from granules that stain metachromatically with Geimsa.
- Mast cells interact with other effector cells, especially eosinophils, to release cytokines and other effector molecules.

Fish innate immunity - overview

- The **range** of effector functions is similar to that of other vertebrates:
  - Innate immunity is important to fish, since antibody reactions are slow and highly influenced by temperature.
  - Marine fish develop antibodies later – **innate v. important**
  - Innate parameters relatively temperature **independent.**
  - High spontaneous activity of the alternative C'. pathway – with multiple isoforms of C3.
  - "Natural antibodies" are important.
  - Effector mechanisms includes a range of antibacterial factors, some homologous to mammals, some fish-specific e.g. the piscidins which are present in mast cells (EGCs).

Reminder: innate immunity effector components.

- **Phagocytes**: kill with O_2 and nitrogen radicals
- **Natural antibodies:** are produced by long-lived lymphocytes without gene re-arrangement, especially during early embryonic life.
  - Important to clear apoptotic & tumor cells without immune response, & in early life.
  - Shown to be important in fish, but high levels inhibit a specific antibody response.
  - Active against non-self molecules like LPS, viral & parasite products.
- **Leukocyte derived proteins / peptides:** Extensive & variable group - most are "membrane active", a few enzyme active

Innate immunity effector components: 2

Leukocyte derived proteins/peptides – "membrane active", a few are enzyme active

- **A. Complement molecules**
- **B. Proteins** such as:
  - **lysozyme** (bactericidal, especially for Gram+ve bacteria, also an opsonin that activates phagocytosis)
  - **lactoferrins**, **peptidoglycan recognition proteins**, **phospholipase**, Serine protease homologues (**serprocidins**), calcium binding protein (**calprotectin**) & others.
  - **transferrin** (acts as bacterial growth inhibitor by chelating available iron needed by the bacteria).
  - **interferon** induces expression of Mx & other antiviral proteins (has been found several fish species).
- **C. Antimicrobial peptides** small, positively-charged (cationic); adopt amphipathic structure (a hydrophobic & a hydrophilic side, good for attaching to/penetrating cell membranes), present from plants to humans. **4 groups**:
  - α-helical peptides
  - cyclic and open-ended cyclic peptides with 1-4 disulphide bridges.
  - peptides with a dominance of particular amino-acids.
  - peptides from partial hydrolysis of large molecules with no antimicrobial activity (eg haemoglobin, histones)

Over 800 found so far - not surprising that some fish ones are different to mammals.

Many are homologous.

Overall, a very diverse and complex interactive system, to complement antibodies.

SECTION 4 .
Fish immunity – practical aspects

This section covers the known factors that influence the specific immune responses of fish, such as age, temperature and stress, and how these determine susceptibility and strategies for successful vaccination.

Fish immunity – practical aspects
1. Ontogeny

- **Embryo protection:** Embryo and fry of some species may show humoral activity (Ig) of maternal origin (but generally not salmonids).
  - Innate factors – including some activities of vitellin, also have protective action.
- **In fry of freshwater fish (& anadromous fish that breed in freshwater)** *i.e.* most major aquaculture species, lymphocytes are initially seen in thymus, then in blood and kidney at the same time, and spleen slightly later (the kidney may contain haematopoietic progenitors earlier than this, but not lymphocytes.)
  - In rainbow trout, many lymphocytes are present in thymus from ~ 1- 4 mo age.
  - In rainbow trout the thymus:body wt ratio starts declining ~ 2 months, reaches low levels & slower decline after 4 mo.
  - The earliest lymphocytes are seen in the kidney at a similar time, but peak proportion to body wt is ~ 2-5 mo with slower decline (eventually to about half peak levels).
- **In marine teleosts,** lymphocytes seen first in kidney, then spleen, then thymus (though spleen initially more erythropoietic than lymphopoietic)
- Antibody production to thymus-independent antigens (eg bacterial lipopolysaccharides) preceded thymus dependent response (eg soluble protein antigens)

Reference: Ellis, 1988. Zaputa etc al, 2006

Fish immunity – practical aspects
2. Age & initiation of immunity

- **Initiation of immunity:**

- In rainbow trout vaccinated against *Aeromonas salmonocida:*
  - showed an antibody response (but no immune memory) when vaccinated at 4 wks (0.13 g)
  - immune memory development not seen until vaccinated ~ 8 weeks (0.26 g).

- Indicates that B cells and T suppressor cells may be mature at 4 weeks, but T-helpers not until about 8 weeks.

- Injection of HCG @ ~ 4 weeks produced tolerance in both carp and rainbow trout, but not if immersed in the antigen.

- For salmonids, size rather than age seems to be the best indicator of immune maturity, as fish develop more slowly at low temperature.
  - At 10^{o}C protection was achieved at about 10 weeks (0.5 g) – started feeding at 4 weeks post-hatch. Correlated with maximum relative size of lymphoid organs.

Fish immunity – practical aspects
3. Duration of immunity

- Protection of rainbow trout against enteric redmouth bacteria (*Yersina ruckeri* Hagerman strain):
  - lasted 120 days for fish vaccinated at 1g BW,
  - 180 days for fish vaccinated at 2g,
  - 1 year for 4 g fish (and adults).
  - *i.e.* immune system fully mature at about 4g BW.

- Antibody levels also vary with physiological state
  - Studies in Atlantic salmon looked at antibody levels before, during and after smolting and found that they fell during the smolt window

    ("smolting": juvenile moves to salt water; increased sodium regulatory enzyme activity, etc)

- Temperature has a major effect on the expression of immunity. B-cell function is more resistant to temperature-mediated membrane effects than that of primary T-cells (Kaattari, 1992), though circulating antibody may not be produced at low temperature, even if the fish has memory cells established.

Implications - Fish Vaccination

- Age of immune competence has been a major factor for first-generation fish vaccines, as these were bacterins (*e.g.* killed bacteria suspensions) usually applied by bath or dip, so fish were vaccinated when as small as possible, to keep volumes practical.
  - Vaccination was also required as early as it would be effective, to protect the fry since passive maternal immunity is low or absent (only innate immunity present).
- Second generation vaccine types were injectable, often multivalent, usually with adjuvant (so fish needed to be of sufficient size for machine injection to be practicable).
- The next generations are peptide and gene vaccines, also given by injection.
- Stimulation of innate immunity (eg with ß-glucans) has also been used, but with variable & often indifferent success.

How much of this do you actually need?

For an experienced animal pathologist, an evolutionary perspective enables **judgements on the validity of extrapolation** from better studies of mammalian work.

- The above demonstrates the limitations on serology as a fish diagnostic tool (slow-developing immune responses; unreliable test of past exposure). Therefore direct evidence of the pathogen is preferable.

- *What about other tests for demonstrating pathogens?*
  - Culture of bacteria & viruses require correct fish-specific media & temperature.
  - Many viruses (& some bacteria) still not grown – no suitable cell lines or media
  - PCR tests widely used for direct demonstration of pathogen genes – but very few have been fully validated, so usually need to use as an adjunct test.

As well as direct evidence of a (potential) pathogen, there is a need to know:

- if this is causing disease;
- if the disease is typical of the pathogen that is reportedly present;
- how well the animal / or population is responding.

Thus histopathology remains an important part of diagnosis, & knowledge of fish immune responses is important for assessing histopathology findings.

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The end.

Back to systematic pathology - see slide 4 for presentations to follow.