The ontogeny of the common carp (*Cyprinus carpio* L.) immune system

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The ontogeny of the common carp (*Cyprinus carpio* L.) immune system

Heidi B.T. Huttenhuis

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The ontogeny of the common carp (*Cyprinus carpio* L.) immune system

De ontwikkeling van het immuunsysteem bij de karper (*Cyprinus carpio* L.)

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Contents

General Introduction

Heidi B.T. Huttenhuis

The study on the ontogeny of the immune system in fish is relevant for two reasons. First, the comparison of the ontogeny of the fish immune system with that of 'higher' vertebrates offers new insights into developmental biology in general and the generation of a functional immune system in particular. Second, knowledge of the developmental sequence of immune function is imperative to design preventive measures against problems concerning infectious diseases in aquaculture.

Preventive measures against infectious disease in aquaculture

Over the past 15 years production of farmed fish more than doubled[1]. A major problem accompanying this vast increase is the occurrence of infectious diseases, which causes considerable losses in cultured fish, especially larvae and juveniles. Problems in larvae usually occur by opportunistic bacteria that are part of the environmental flora[2,3]. Prophylaxis based on sanitary isolation is difficult to achieve, especially in open water systems, due to the presence of other fish species, invertebrates or the water itself. Antibiotic therapy is a frequently used strategy in intensive rearing of fish larvae, but can result in enhanced microbial resistance[4], the accumulation of residues in tissues[4] and immuno-suppression[5]. Vaccination, although highly effective in many instances is time consuming, costly and protection is often pathogen specific. Moreover, the acquired immune system is not functional in fish up to a certain age[5-8]. In contrast, the administration of immuno-stimulants would activate the immune system in a non-specific way, thus providing resistance against a variety of pathogens, and can be applied at young ages. Especially the administration of immuno-stimulants via the food offers a sustainable approach because of its uncomplicated use, fast delivery, and less stressful entry route.

The application of immuno-stimulants in aquaculture

Immuno-stimulants, such as yeast glucans, levamisole and LPS, increase resistance against infectious disease, primarily by enhancing non-specific defence mechanisms[9]. Therefore, there is no memory component and the response is likely to be of short duration. Use of immuno-stimulants, in addition to chemotherapeutic agents and vaccines, has been widely accepted by fish farmers, but the efficacy remains elusive. Immuno-

stimulants have been reported to induce increased survival, growth, cytokine expression and acute phase protein expression *in vitro*, proliferation of B cells and the secretion of immunoglobulins (Ig), increased lysozyme levels, and activation of the complement system and macrophages[9].

Oral administration of immuno-stimulants to fish (especially larval/juvenile fish) is rather unprecedented, although the application of poly-mannuronic acids (from algae) was reported to increase survival in Atlantic halibut (*Hippoglossus hippoglossus* L.) and turbot (*Scophthalmus maximus* L.)[10,11], and increase lysozyme levels and complement activity in sea bass (*Dicentrarchus labrax*)[12].

The teleost immune system

The fish immune system is surprisingly similar to the mammalian immune system, consisting of both an innate and an acquired component[15]. Although T cells are generated in the thymus[16], fish do not possess bone marrow. Instead, hematopoiesis in adult fish takes place in the kidney and spleen. The main hematopoietic organs are displayed in Figure 1.

In addition, the main immunoglobulin class is a tetrameric IgM, although IgD was also identified[17], while other classes (IgG, IgA, IgE) were not convincingly recognised yet. Fish do not have lymph nodes, Peyers Patches or germinal centres, which indicates that antigen presentation might be different. Fish secondary humoral immune responses are not as high as in mammals, while minor affinity maturation[18] and no Ig class switching were observed. However, fish also display characteristics of the immune system that are unknown to mammals: multiple isoforms of innate immune factors were identified, suggesting a differentiated function to enlarge the capacity of the immune system against pathogens[19].

Carp as a model system for comparative immunology

Cyprinid fish (carp species) are quantitatively the most important cultured fish in the world[1]. Carp are normally cultivated on a semi-intensive scale in natural ponds that harbour fish from one year-class only. In these systems, especially carp fry are highly

vulnerable to diseases and predators and a mortality rate of 40-65% in the first weeks to an additional 20-40% in the first months is considered standard[20,21]. The common carp (*Cyprinus carpio* L.) can be regarded as a representative for modern bony fish and is well characterised with respect to the immune system. Over the past few years, antibodies against different types of leucocytes were characterised[22-27]. In addition, several studies have been attributed to the identification of genes involved in immune responses[28-33], and tests were developed to quantify immune function[28,34].

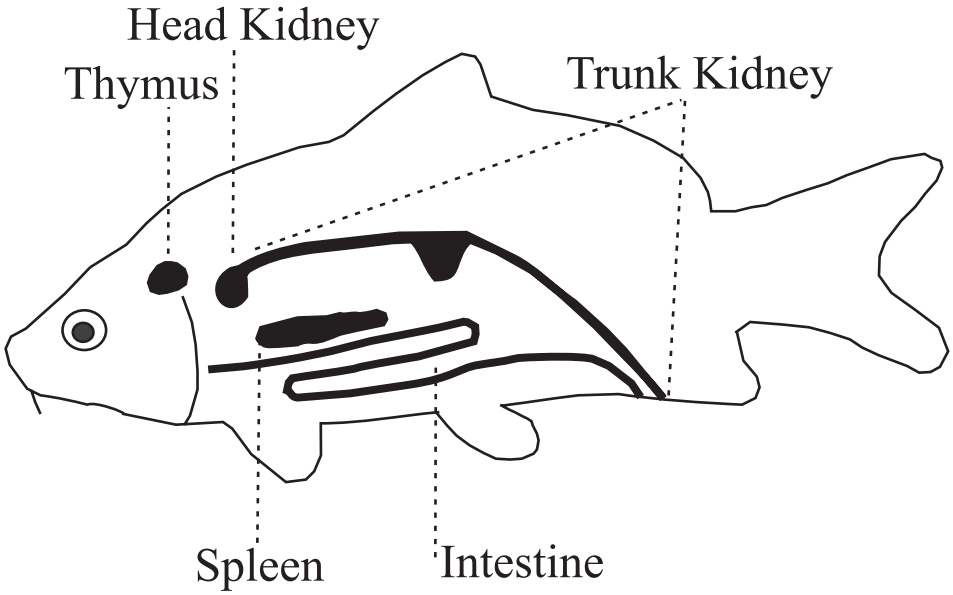

Figure 1. The main hematopoietic organs in teleost fish. The head kidney (pronephros) and trunk kidney (mesonephros) produce myeloid cells, erythrocytes and B cells, the spleen erythrocytes and thrombocytes and the thymus is the main source for T cells. The intestine supposedly also produces lymphoid cells because recombination activating gene is expressed[13,14].

Fish models for developmental immunology

Over the past few years, a considerable amount of work was performed on the ontogeny of the zebrafish (*Danio rerio*) innate immune system. Potential embryonic and

larval myelopoietic sites were identified with mainly gene expression studies and histo-morphological work[35-41]. Zebrafish is an extensively used fish model for the study of embryonic development because of the relative ease to study the transparent embryos, the powerful genetics, which can be applied for the generation of mutants and transgenic animals, the availability of many molecular markers, and on short notice the genomic sequence[42-45]. However, it is well known that early development[46] and organogenesis[47] differ considerably between species. Studying the development of the carp immune system in addition to zebrafish offers several new possibilities: firstly the larger size facilitates research on a wide variety of immune functions like cell population kinetics and cell culture, secondly a panel of antibodies is available and thirdly carp represent a major commercial value in aquaculture[1]. In addition, sequences obtained in zebrafish have high similarity to carp sequences because of close evolutionary relationship, and can often be used in carp as well. However, the extensive molecular techniques used in zebrafish can never be as efficient in carp, because carp are tetraploid and the generation time is relatively long (1-2 years).

The development of carp

The development of carp at 25ºC is displayed in Figure 2. Carp embryos hatch at 2-3 dpf (days post fertilisation) and start feeding at 4-5 dpf. In this thesis, the embryonic stage is considered to be from 0-5 dpf, because at 5 dpf fish have absorbed the yolk sac, have formed all major organs and started feeding on *Artemia salina* nauplii. At hatching, the fish measure (head-tail) approximately 5 mm and at 5 dpf approximately 6 mm. From 5 dpf up to 2-3 weeks (1-1.5 cm) the fish are considered 'larvae', while older fish are considered 'juveniles' because they are no longer transparent and have acquired the shape of adult carp. From the age of 3-5 weeks the fish are kept at 23ºC and on a restricted feeding schedule of commercial pelleted food. After approximately 6 months, the fish measure 20-25 cm and are considered adult, although they do not reproduce until the second year, when they measure approximately 30-35 cm.

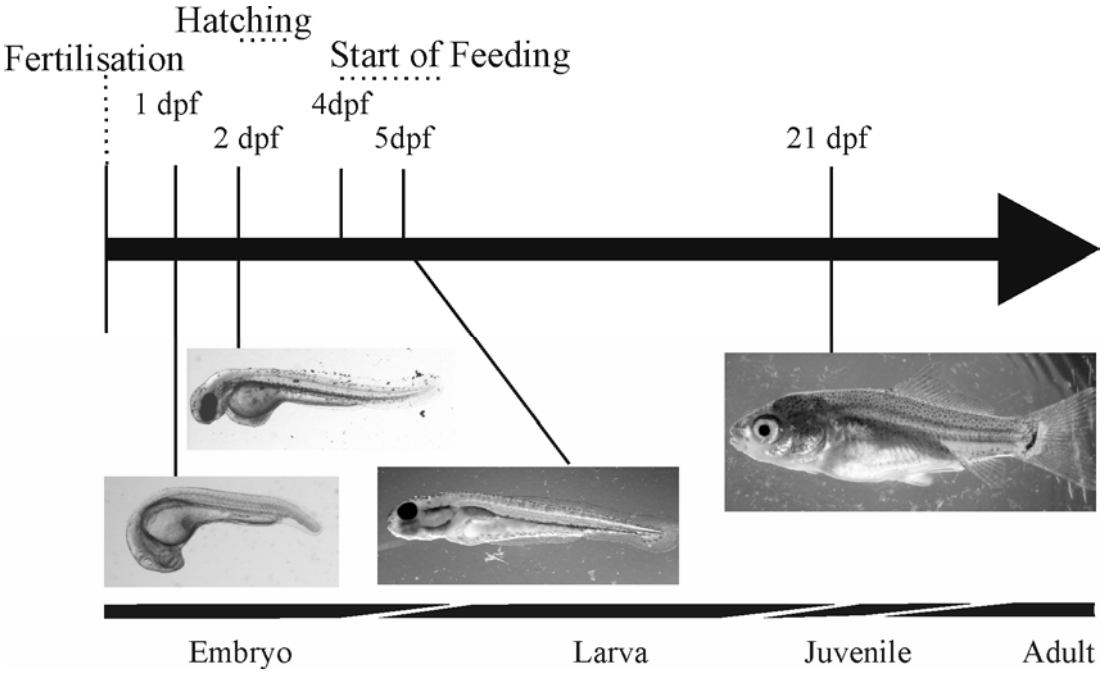

Figure 2. A schematic presentation of carp development at 25ºC. dpf = days post fertilisation.

Aim and Scope of this thesis

Ontogenesis is a sequence of molecular and cellular events regulated by time and space, leading to the development of a fully functional organism. Literature clearly shows that young fish use innate mechanisms during the first weeks/months (dependent on the species) of their development[39,40,47,48]. With respect to this, ontogenetic studies are important to understand evolutionary pathways and to rationally design effective immune defence strategies in order to defend farmed fish against pathogens at early age.

This thesis investigated the ontogeny of the immune system of the common carp. To this end, the different pathways of the immune system were studied in separate chapters. The first components of the immune system to appear are humoral factors of the immune system (Chapter 2). Second, cells of the innate immune system are generated (Chapter 3), followed by cells of the acquired immune system (Chapter 4). In addition to these mainstream pathways, the appearance of leukocytes in mucosal organs was studied (Chapter 5). Furthermore, a number of data and tools obtained in carp developmental

immunology were used to test the effect of oral immuno-stimulation in juvenile carp (Chapter 6). Finally, the development of the teleost immune system, especially based on zebrafish and carp data, was addressed in the general discussion (Chapter 7).

Carp (*Cyprinus carpio* L.) innate immune factors are present before hatching

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Abstract

Expression of the innate immune factors complement factor 3 (*C3*), α_2-macroglobulin (*α_2M*), serum amyloid A (*SAA*) and a complement factor 1 r/s - mannose binding lectin associated serine protease-like molecule (*C1/MASP2*) was determined with real time quantitative PCR in carp (*Cyprinus carpio* L.) ontogeny around hatching. Furthermore, the expression of *C3* mRNA and the presence of C3 protein were studied in carp embryos and larvae using *in situ* hybridisation, western blotting and immunohistochemistry. *C3*, *α_2M*, *SAA* and *C1/MASP2* mRNA was produced by embryos from 12 hours post fertilisation, which is relatively long before hatching (2 days post fertilisation; dpf), indicating either involvement of these factors in development itself or more probably a preparation of the immune system for the post-hatching period. In addition, maternal mRNA of the aforementioned innate immune factors and maternal C3- and immunoglobulin protein was present in unfertilised eggs. Furthermore, *C3* mRNA production was situated in the yolk syncytial layer in embryos from 24 hours post fertilisation to 5 dpf, followed by the liver in larvae, providing a new series of C3 production in teleost development.

Introduction

Over the past 15 years production of farmed fish more than doubled, of which carp species are quantitatively the most important[1]. A major problem accompanying this vast increase is the occurrence of infectious diseases, which causes high losses in cultured fish, especially larvae and juveniles. Knowledge on the ontogeny of the immune system is imperative to design preventive measures against this problem.

Carp (*Cyprinus carpio* L.) kept at 25˚C hatch already from 2 days post fertilisation (dpf; embryonic stage), and they start feeding at 4-5 dpf (larval stage). Mucus and epithelium from skin, gills and intestine can be regarded as a first line of defence. In addition, innate immune cells (including macrophages and granulocytes), of which macrophages are present at hatching[26], and innate immune factors, which have not been investigated before in carp ontogeny, constitute a second barrier against invading pathogens. Moreover, the adaptive immune system can respond specifically and with memory to 'non' self cells and molecules. The thymus is the first organ of the adaptive immune system in carp and starts developing from 3-4 dpf (Chapter 4)[50], while systemic antibody responses appear at 1-2 months, although juvenile tolerance is evoked before this age[5,7,51].

The innate immune factors complement factor 3 (C3), α_2-macroglobulin (α_2M), serum amyloid A (SAA) and a complement factor 1 r/s - mannose binding lectin associated serine protease-like molecule (*C1/MASP2*) were studied during carp development. Cleavage of C3, the key protein of the complement system, can result in lysis of foreign cells by the lytical pathway, chemotaxis of myeloid cells, and opsonisation of pathogens or antibody-antigen complexes. C3 was implicated in *in vitro* and *in vivo* immune responses in carp[28,52]. C3 is predominantly synthesised in liver in both mammals and fish, but can also be produced by other cell types[28,53]. Although the liver starts developing before hatching, it is extremely small and immature at this time. A *C1/MASP2* was cloned from carp hepato-pancreas[54] and is supposedly homologue to (part of) the initiation complex of the classical pathway (activated by immune complexes) and/or the lectin pathway in mammals. α_2M is an abundant protein in the plasma of vertebrates. It is

synthesised in the liver and functions as a broad-spectrum protease-binding protein[55], although it can also bind a variety of cytokines[56]. SAA is suggested to detoxify endotoxins, mediate inflammatory responses, inhibit lymphocyte and endothelial cell proliferation and induce leukocyte extravasation[57], and expression of both *SAA* and *α_2M* was induced after *Trypanoplasma* infection in adult carp[28]. α_2M, SAA and C1/MASP2 were never studied in fish ontogeny before.

In this study, the expression of the innate immune factors *C3*, *α_2M*, *SAA* and *C1/MASP2* was determined with real time quantitative polymerase chain reaction (RQ-PCR) in carp ontogeny. Furthermore, the temporal and *in situ* presence of *C3* mRNA and C3 protein were studied in carp embryos and larvae using *in situ* hybridisation, western blotting and immunohistochemistry providing a new sequence of C3-producing tissues.

Animals, Materials and Methods

Animals

Common carp (*Cyprinus carpio* L.) of the R3R8 strain were bred and kept in the facilities of 'de Haar Vissen', department of Animal Sciences of Wageningen University (The Netherlands). They were kept at 25 ± 0.5°C for the first 5-6 weeks and subsequently at 23°C in circulating, filtered, UV-treated water. Carp hatch from 2 dpf (embryonic stage) and were fed with *Artemia salina* nauplii starting at 4-5 dpf (larval stage) for 3 weeks followed by Trouvit K30 pellets (juvenile stage; Trouw & Co., Putten, The Netherlands). Fish were anaesthetised with 0.03 % tricaine methane sulphonate (Crescent Research Chemicals, Phoenix, AZ) buffered with 0.06 % sodium bicarbonate to pH 7.2 in aquarium water.

Monoclonal antibodies

A rabbit polyclonal anti carp C3 antibody was produced previously[28]. WCI12 is reactive with the IgM heavy chain and subsequently B cells of carp[22].

Immunohistochemistry

Carp embryos and larvae were embedded in 1.5 % agarose with 15 % sucrose and subsequently snap-frozen in liquid nitrogen. Frozen samples were stored at -80ºC. Serial transverse or longitudinal 7 µm sections were mounted on poly-L-lysine-coated microscope slides (BDH Laboratory Supplies, Poole, UK) and fixed in cold acetone for 5 minutes (min). Then, sections were washed twice (first on ice, then at room temperature) in phosphate buffered saline (PBS) and incubated with an anti carp C3 polyclonal antibody (1:200) for 1 hr. After washing twice with PBS, sections were incubated with Swine-anti-Rabbit-FITC (SwAR-FITC, Dako, Glostrup, Denmark) for 1 hr. Afterwards, sections were washed twice in PBS, embedded in Vecta-Shield Mounting Medium with propidium-iodide (Vector Laboratories, Burlingame, USA) and examined with a laser-scanning microscope (LSM 510, Carl Zeiss, Germany). All necessary controls, including pre-absorption, were performed and did not result in any staining.

Protein electrophoresis and western blotting

Samples (0-2 dpf embryos, normal carp serum and purified C3, kindly provided by dr. M. Nakao, Kyushu University, Fukuoka, Japan) were reduced and analysed by SDS-PAGE and transferred onto nitro-cellulose (Protran: Schleicher & Schuell, Dassel, Germany). Transfers were saturated in TTBS (0.02 M Tris-HCL pH 7.4-7.6, 0.05 M NaCl, 0.05 % Tween 20) with 1 % Bovine Serum Albumin for 1 hr and incubated with polyclonal anti-C3 antibody or WCI12 (1:50) for 1 hr. After washing in TTBS, transfers were incubated for 1 hr with GAR-HRP (polyclonal anti-C3) or GAM-HRP (WCI12;1:2000; Bio-Rad, Hercules, USA), washed in TTBS, and incubated with ECL™ Western Blotting Detection Reagents (Amersham Biosciences UK limited, Little Chalfunt, England) according to the manufacturers' instructions. For the estimation of molecular weights, Bio-Rad molecular weight standards were used.

Probe synthesis

Primers were designed on a conserved part of five C3 sequences (AB016210-5). Sequencing (performed as described previously Chapter 4) confirmed that the sequence was carp C3, isoform Q2 (nucleotides 1115-1713; AB016215). A digoxygenine (DIG) labelled probe was synthesised from a PGEM-Teasy (Promega, Madison, USA) vector containing the 598 basepair insert using a DIG RNA labelling kit according to the manufacturers' instructions (Roche Applied Science, Basel, Switzerland). Probe labelling was ascertained by Northern Blotting.

In situ hybridisation on whole mounts and sections

Embryos and larvae (12, 24, 36, 48 hours post fertilisation (hpf) and 3,4,5 dpf; 4 per age) were fixed for 4-24 hours (dependent on size) in 4% paraformaldehyde (PFA) in PBS and stored at 4ºC in 1% PFA in PBS. Sections of adult liver were obtained as described under *immunohistochemistry*. In *situ* hybridisation was performed as described before (Chapter 4) at a hybridisation temperature of 68˚C (65˚C for whole mounts). Sections were prepared from Whole Mounts as described under *immunohistochemistry*, and examined with light microscopy using Normarsky optics.

RNA extraction and cDNA synthesis

3-8 Single unfertilised eggs (referred to as 0 hpf for practical reasons) and embryos from 4 hpf up to 5 dpf were snap frozen in liquid nitrogen and stored at -80˚C. RNA was isolated using the method of Chomzynski[58] as described before (Chapter 4).

Real time quantitative-polymerase chain reaction (RQ-PCR)

RQ-PCR was performed as described before (Chapter 4). *β-actin* and *40S* were used as house keeping genes and *C3*, α_2M, *SAA* and *C1/MASP2* as genes of interest. Primer sequences are described in Table 1. Expression was calculated as a ratio of gene of interest versus house keeping gene according to the following equation: ratio = $((E_{\text{house keeping gene}})^{\text{Ct house keeping gene}})/((E_{\text{gene of interest}})^{\text{Ct gene of interest}})$, where E is the amplification

efficiency and Ct is the number of PCR cycles needed for the signal (generated by Sybr-green I that attaches to double stranded DNA) to exceed a predetermined threshold. Efficiency and threshold values used for each primer set were: *β-actin,* 2.1, 0.0329; *40S*, 2.11, 0.0442; *SAA*, 2.0, 0.0393; *α_2M*, 1.99, 0.0193; *C3*, 2.06, 0.0957; *C1r/C1s/MASP2*, 1.96, 0.0298. Ratios calculated with either *β-actin* or *40S* were comparable if not identical, consequently only values calculated with *β-actin* are shown. Samples were considered positive when the signal exceeds the threshold. Non template controls were always included. For every sample, non-RT controls were also tested with all primer-combinations of the genes of interest. Both controls consistently were negative or had higher Ct values than samples.

Table 1. Primers used for amplification of specific gene products with RQ-PCR. C3: complement factor 3, SAA: serum amyloid A, α_2M: alpha$_2$-macroglobulin, C1/MASP2: a complement factor 1 r/s - mannose binding lectin associated serine protease-like molecule.

Name		Sequence	Product size (basepairs)	Genbank Accession Numbers
40S	FW	ccgtgggtgacatcgttaca	69	AB012087
	RV	tcaggacattgaacctcactgtct		
β-Actin	FW	gctatgtggctcttgacttcga	89	M24113
	RV	ccgtcaggcagctcatagct		
C3	FW	ccctggacagcattatcactc	154	AB016210-5
	RV	gatggtcgcctgtgtggt		
SAA	FW	gcagatgggcagccaaagta	70	AB016524
	RV	gaattaccgcggcgagaga		
α_2M	FW	ttccaggacagacagtgaactttaga	107	AB026129
	RV	tctgttaccctgactgtcctcaag		
C1/MASP2	FW	aacaccctcggctcctacct	89	AB042609-10
	RV	ccacctccacactccaagac		

Results

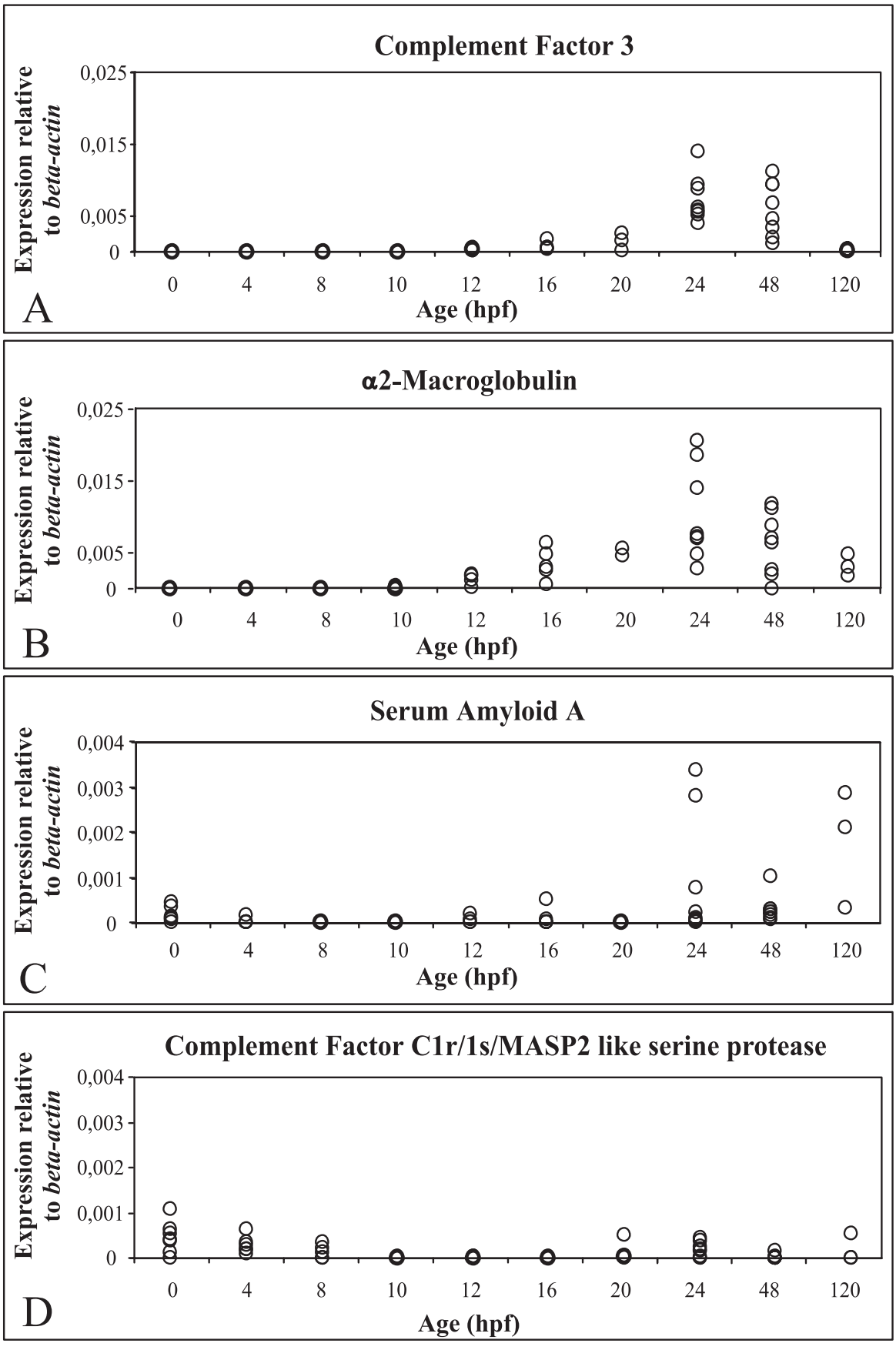
Expression of C3, α_2M, SAA and C1/MASP2 in carp ontogeny

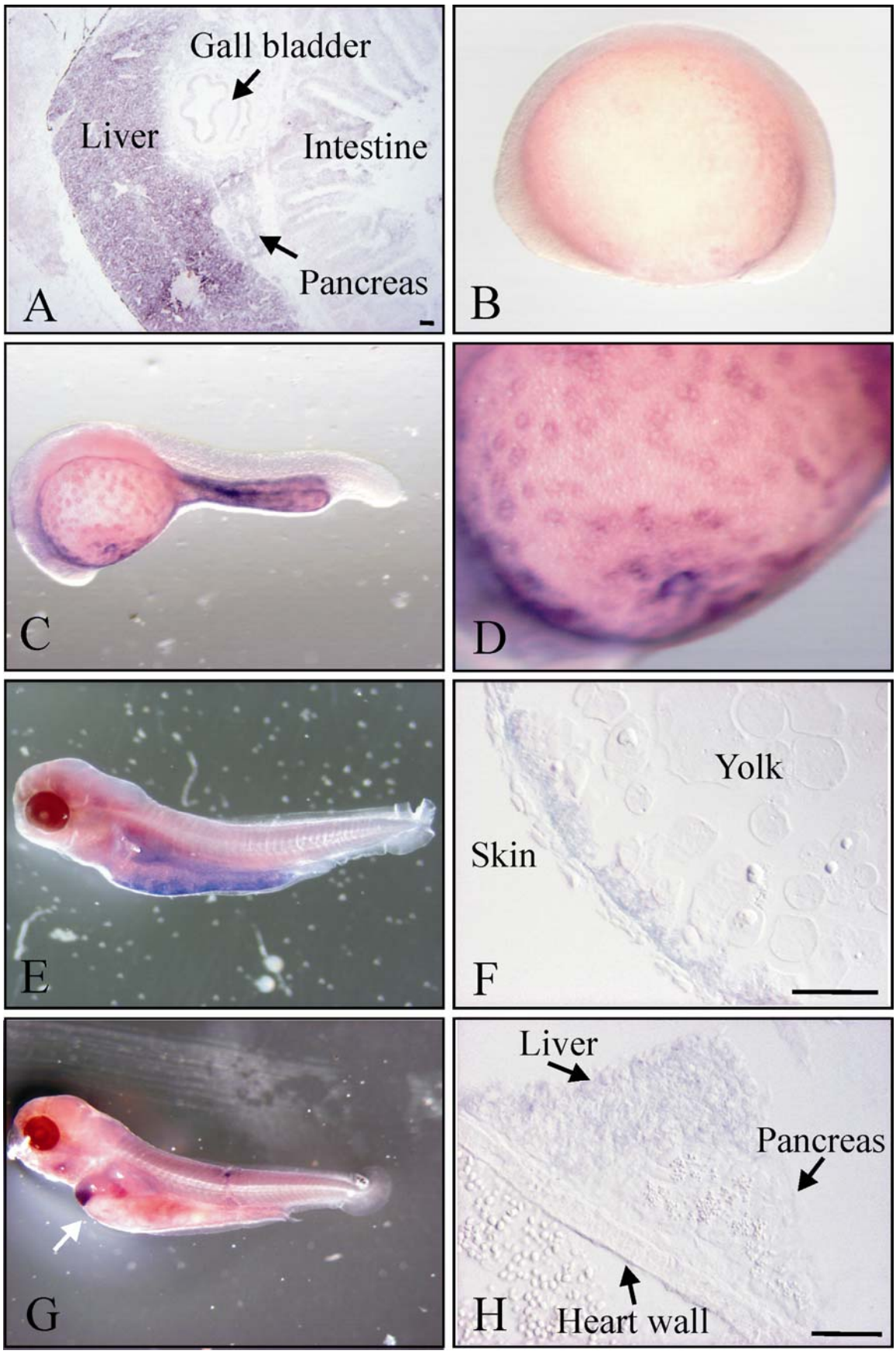
C3, *α_2M*, *SAA* and *C1/MASP2* were expressed at all ages examined (except for *SAA* at 8-10 hpf and *α_2M* at 4 hpf), although not in all embryos (Table 2). *C3* and *α_2M* expression at the early stages (0-10 hpf) was very low compared to later stages and is therefore not visible in the charts (Figure 1A, B). From 12 hpf, expression of *C3*, *α_2M* and *SAA* increased (*C1/MASP2* from 20 hpf; Figure 1). *C3* and *α_2M* expression were considerably higher then *SAA* and *C1/MASP2* expression (note that *C3* and *α_2M* expression are displayed with a different scale compared to *SAA* and *C1/MASP2* expression). *C3* and *α_2M* expression reached the highest level at 24/48 hpf.

Table 2. Expression of *C3*, *α_2M*, *SAA* and *C1/MASP2* in carp ontogeny. The number of embryos expressing the gene of interest/ total number of embryos tested per age is given.

Age (hpf)/Gene of interest	C3	α_2M	SAA	C1/MASP2
0	7/7	2/7	5/7	6/7
4	5/7	0/7	2/7	6/7
8	3/7	4/7	0/7	5/7
10	3/5	5/5	0/5	2/5
12	4/4	4/4	2/4	4/4
16	5/5	5/5	3/5	2/5
20	3/5	2/4	1/5	2/5
24	8/8	8/8	7/8	6/8
48	8/8	8/8	8/8	6/8
120	3/3	3/3	3/3	2/3

Figure 1 (following page). Expression of *C3* (A), *α_2M* (B), *SAA* (C) and *C1/MASP2* (D) in carp ontogeny determined with RQ-PCR. All data were normalised against *β-actin* levels, expression relative to *β-actin* is displayed on the vertical axis. On the horizontal axis age is displayed in hours post fertilisation (hpf). Individual values (per embryo) are displayed as circles.


Complement Factor 3
Expression relative to beta-actin
0,025
0,015
0,005
0
0 4 8 10 12 16 20 24 48 120
Age (hpf)
A
α2-Macroglobulin
Expression relative to beta-actin
0,025
0,015
0,005
0
0 4 8 10 12 16 20 24 48 120
Age (hpf)
B
Serum Amyloid A
Expression relative to beta-actin
0,004
0,003
0,002
0,001
0
0 4 8 10 12 16 20 24 48 120
Age (hpf)
C
Complement Factor C1r/1s/MASP2 like serine protease
Expression relative to beta-actin
0,004
0,003
0,002
0,001
0
0 4 8 10 12 16 20 24 48 120
Age (hpf)
D


Gall bladder
Liver
Intestine
Pancreas
A
B
C
D
E
Yolk
Skin
F
G
Liver
Pancreas
H
Heart wall

A carp *C3* probe was synthesised to determine the source of *C3* production. At 3 months, *C3* mRNA was expressed in the liver, but not in other tissues like intestine, gall bladder and pancreas (Figure 2A). In ontogeny, *C3* expression was not visible at 12 hpf (Figure 2B) using *in situ* hybridisation, but expression was observed from 24 hpf onwards (Figure 2C, D) in a pattern of large circles on the yolk sac. In older embryos (Figure 2E), *C3* was expressed diffusely on the yolk sac. In sections of these embryos, the expression was situated between the yolk material and the skin, in the periblast or yolk syncytial layer (Figure 2F). Staining was not observed in other tissues. At 5 dpf, *C3* remained expressed on the yolk sac as long as it was present (results not shown), but was expressed in the liver of embryos that had already lost their yolk sac (Figure 2G, H). Also at this stage, *C3* was not expressed in other tissues like heart, erythrocytes or pancreas (Figure 2H). All sense controls showed negligible staining.

The appearance of C3 protein in carp ontogeny

Using western blotting (Figure 3A), purified carp C3 reacted with the anti C3 antibody in several bands. The bands over 120 kilodalton (kDa) in size are consistent with unreduced C3 protein, the two bands at approximately 115 kDa correspond to the α chain and the band at approximately 60 kDa corresponds to the β chain of the carp C3 protein[59]. C3 protein (only the β chain) was already present in unfertilised eggs. At 2 dpf, in addition to the β chain another smaller band (approximately 40 kDa) was detectable.

At 2 dpf (Figure 4A), C3 immuno-reactivity was present in the yolk syncytial layer, in the large blood vessels (aorta, vena cardinalis, the vessels lateral of the spinal marrow) and in the cerebrospinal canal, but not in the intestine, embryonic liver and muscle tissue.

Figure 2 (previous page). Expression of carp complement factor 3 (C3) in ontogeny determined with *in situ* hybridisation. C3 is expressed in liver sections of 3-months-old carp (A). C3 was not expressed at 12 hpf (B), but expression was present on the yolk sac at 24 hpf (C, D) and 4 dpf (E, F), and in the liver (arrow) at 5 dpf (G, H). Panel D is a magnification of panel C, panels F and H are sections of the embryos in panels E and G respectively. Bar is 20 μm.

At 7 dpf (Figure 4B), C3 immuno-reactivity was present in the liver (like in adult liver: Figure 4C) and blood (heart), but not in the intestine. In adult carp (Figure 4C), C3 immuno-reactivity was situated in the liver and blood vessels, but not in the pancreas.

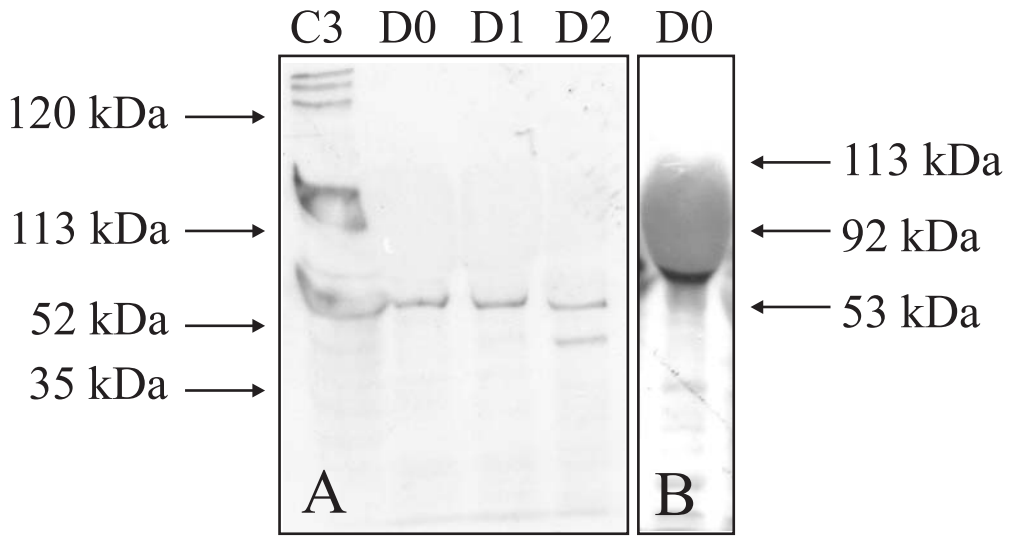


Figure 3. The presence of complement factor 3 (C3) and immunoglobulin (Ig) protein in carp development determined by western blotting. C3 (panel A) is present in 0-2 dpf eggs (first lane is purified C3) and Ig in unfertilised eggs (0 dpf; panel B). Molecular weights are displayed in kilodalton (kDa) and were determined with molecular weight markers. C3 = purified carp C3; D0, D1 etc. = 0 dpf, 1 dpf, etc.

The presence of immunoglobulin (Ig) protein in carp eggs

Using western blotting with the mAb against carp IgM (WCI12), a 70 kDa band (consistent with the carp Ig heavy chain) was present in unfertilised carp eggs (Figure 3B).

Discussion

This is the first paper to present data on expression of the innate immune factors α_2M, *SAA* and a *C1/MASP2* in teleost development. Furthermore, this study characterises the presence of carp *C3* mRNA and protein in early development, providing the first description of *C3* expression in the yolk syncytial layer of teleost embryonic fish.

The carp innate immune factors *C3*, *α2M*, *SAA* and *C1/MASP2* were all produced from about 12 hpf (*C1/MASP2* from 20 hpf), which is relatively long before hatching,indicating very similar expression kinetics and a significant role in carp embryos. *C3* and α_2M expression peaked at 1-2 dpf, ensuring sufficient levels around hatching time. C3, α2M and SAA were convincingly implicated in adult carp innate immunity in previous studies: C3 mediated β-1,3-glucan elicited rosette formation of carp head kidney granulocytes and macrophages[52] and also killing of *Trypanoplasma borreli*[28]. *C3*, α_2M and *SAA* expression were induced by *Trypanoplasma* infection *in vivo*[28], and SAA expression was induced after stimulation *in vitro*[33]. Taken together, these results indicate that these factors play an important part in carp innate immunity and must therefore be crucial for embryos that come in contact with micro-organisms in their environment after hatching. *C3* and α_2M were expressed at higher levels compared to *SAA* and *C1/MASP2*, reflecting the relative abundance of C3 and α_2M proteins later in life[28]. The expression of a *C1/MASP2*, together with the presence of maternal Ig in eggs, suggests both the classical- (with Ig) and lectin pathway can be activated, although expression levels were very low.

In addition, maternal mRNA of all four factors (although at low expression levels) and maternal C3 and Ig protein were present in unfertilised eggs and young embryos. Maternal C3 protein was also detected in spotted wolffish (*Anarhichas minor* Olafsen)[60], but was found later in development (though before hatching) in cod (*Gadus morhua* L.)[61]. Maternal Ig was detected previously in carp with an anti-pike (*Esox lucius*) Ig antibody[62] and in many other teleosts[63-65], although not in cod[66]. In addition, maternal Ig mRNA was detected in sea bass (*Dicentrarchus labrax*) eggs[65]. The presence of only the C3 β-chain on western blot is probably due to instability of the C3 α-chain[67], because the *C3* RQ-PCR primers and the *C3* probe were 7both designed based on the C3 α-chain. In addition, the C3 α-chain was also not (consistently) detected in other teleost species[60,61]. The presence of smaller products (found in 2 dpf carp embryos) reacting with anti-C3 antibodies was attributed to degraded products of the α-chain in a previous study[60]. Maternally derived immune factors might play an important role in defending the egg,

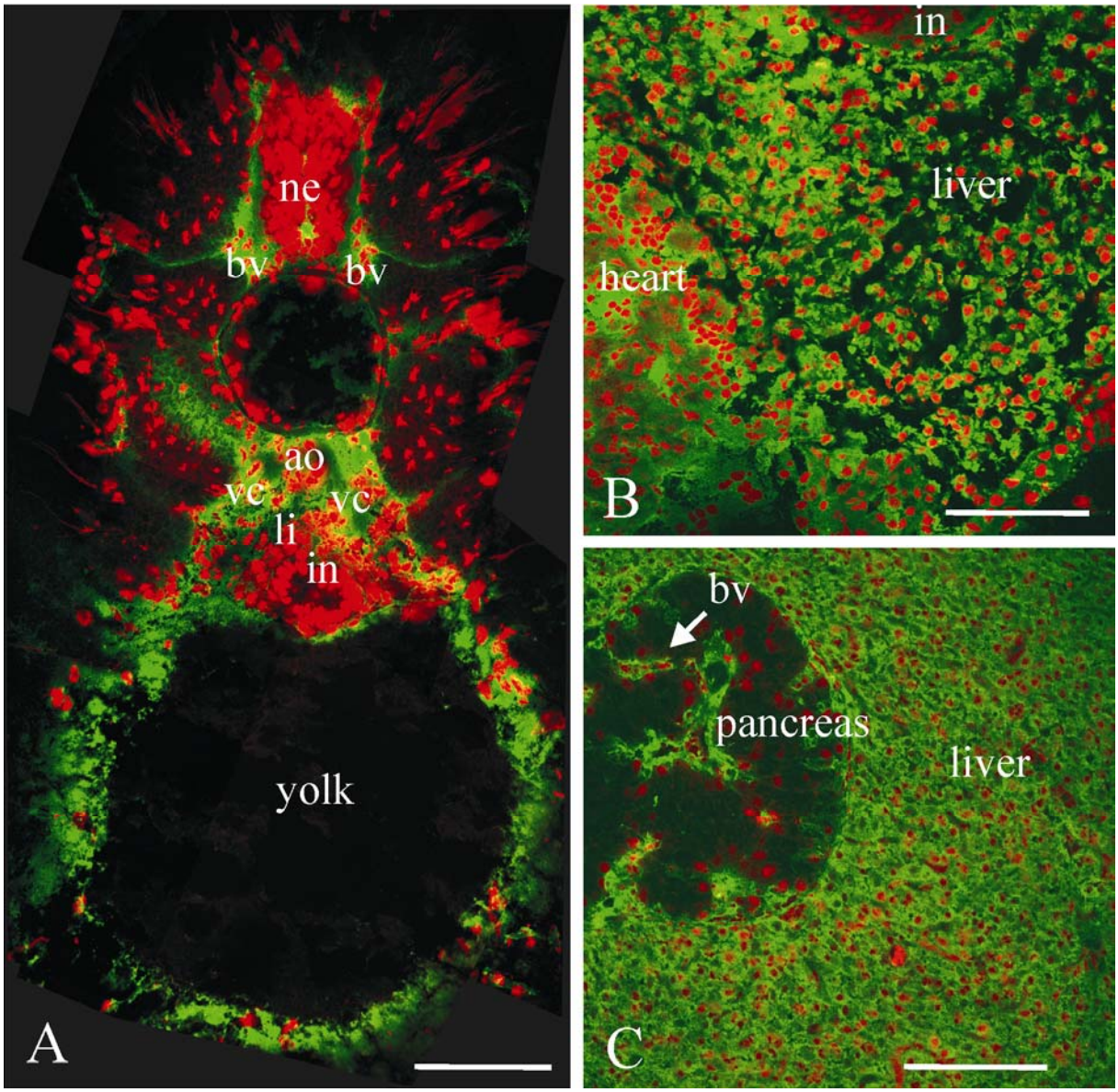


Figure 4. The presence of complement factor 3 (C3) protein determined by immunohistochemistry in carp ontogeny. C3 protein was present at 2 dpf (cross section: A) and in the liver at 7 dpf (sagittal section: B). Panel C represents C3 in the liver of a 3-months-old carp. ao = aorta, bv = blood vessel, in = intestine, li = liver, ne = neural tissue, vc = vena cardinalis, Bar is 100 μm.

because bacterial growth was shown on fish eggs from 2 hpf[68]. Ig attaches to micro-organisms, blocking their function, α_2M scavenges both endogenous and exogenous proteases, and *SAA* can be important for tissue regeneration in development. C3 attracts innate immune cells for phagocytosis, though not present before hatching[26], or activates

the complement factors C5-C9 for lysis. The presence of these factors remains to be investigated when appropriate tools are available.

Furthermore, complement components are also suggested to be relevant in developmental processes like bone formation, tissue and organ regeneration, hematopoiesis and vascular development[69]. In addition, α_2M is implicated in the regulation of cytokine distribution and activity[56]. Therefore, the early production of these innate immune factors possibly is also important for development itself, as has also been suggested in C3 studies in halibut (*Hippoglossus hippoglossus*;)[66] and cod[70] development. However, the increase of expression at 1-2 dpf indicated that these proteins are needed shortly after hatching, and thus are predominantly important for innate immune function.

C3 is produced in the yolk syncytial layer of carp embryos, which was not shown before in teleost fish. C3 production started in a circular pattern, around the nuclei of the yolk syncytial layer, while production was located all over the yolk sac in older embryos. The yolk syncytical layer is a structure unique to fish and is not found in other vertebrate embryos. It is involved in the absorption of the yolk material[71-73], and a number of genes (e.g. transferrin) that are first expressed in the zebrafish (*Danio rerio*) yolk syncytial layer are later expressed in liver or intestine[74-76]. This suggests that *α_2M* and *C1/MASP2*, that are both predominantly expressed in the liver of adult carp[28,54], may also be produced by the yolk syncytial layer in embryos. Interestingly, complement factor 7 was expressed in the yolk syncytial layer of zebrafish embryos[76].

After the absorption of the yolk material C3 is produced in the liver, which is consistent with data in other fish species[60,66,70], although the liver rudiment is present earlier in carp (this study) and spotted wolffish[60]. The differential expression of C3 in embryonic and larval fish might impose consequences for eliciting an immune response, because when injecting carp embryos with LPS, *C3* and *α_2M* expression decreased (Chapter 3), whereas these proteins are generally induced in inflammatory responses in adult fish[28,77].

C3 protein was present in the cerebrospinal canal in carp embryos. The brain and spinal cord are effectively shielded from plasma components by the blood brain barrier,

which implicates these components are synthesised in the brain itself as was shown in mammals[53].

This study presents expression of the innate immune factors *C3*, α_2M, *SAA* and *C1/MASP2* and the presence of C3 protein of both maternal and embryonic origin before hatching, indicating either involvement of these factors in development itself, or more probably a preparation of the innate immune system for the post-hatching period. In addition, C3 production was situated in the yolk syncytial layer in embryos, followed by the liver in larvae, providing a new sequence of C3-producing tissues in teleost development.

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Ontogeny of the common carp (*Cyprinus carpio* L.) innate immune system

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Abstract

The ontogeny of the teleost innate immune system was studied in carp using cellular, histological and quantitative molecular techniques. Carp myeloid cells first appeared ventro-lateral of the aorta at 2 days post fertilisation (the start of hatching), and subsequently around the sinuses of the vena cardinalis (or posterior blood islet), head kidney and trunk kidney. In addition, the hematopoietic tissue around the sinuses of the vena cardinalis transformed into that of the trunk kidney, which is the first description of the ontogeny of the trunk kidney hematopoietic tissue in teleosts. The mAb's used in this study reacted with carp myeloid surface molecules that are already transcribed and processed from the first appearance of myeloid cells, and thus serve a significant role in unravelling ontogenetic processes of teleost immunology. Finally, this study associated the first appearance of myeloid cells with an immune response on the molecular level: 2 days post fertilisation embryos responded to LPS injection with upregulation of interleukin 1-β, inducible nitric oxide synthase and serum amyloid A, and down-regulation of complement factor 3 and alpha$_2$-macroglobulin, implying a functional embryonic innate defence system.

Introduction

Carp (*Cyprinus carpio* L.) embryos hatch already at 2-3 days post fertilisation (dpf), whereupon they are confronted with micro-organisms in their environment. Skin and mucus form a first line of defence. Secondly, cells (including macrophages and granulocytes) and humoral factors of the innate immune system would logically form a second barrier against invading pathogens, although knowledge on this subject is limited in fish. Thirdly, the adaptive immune system can respond specifically and with memory to 'non' self cells. The adaptive immune system in carp however starts developing from 4 dpf (Chapter 4) and reaches functionality between 1 and 2 months[7].

Over the past few years, a considerable amount of work was performed on the ontogeny of the zebrafish (*Danio rerio*) innate immune system. Potential embryonic and larval myelopoietic sites were identified with mainly gene expression studies and histo-morphological work[35-41]. Common carp and zebrafish are both cyprinid fish, and probably have a comparable immune system. Studying the development of the carp immune system besides zebrafish offers several advantages: firstly the larger size facilitates research on a wide variety of immune functions like cell population kinetics and cell culture, secondly a panel of antibodies is available and thirdly carp represent a major commercial value in aqua culture[1].

Recently, monoclonal antibodies (mAb's) were raised against carp macrophages (WCL15[25,26] and granulocytes (TCL-BE8[27]). A limited number of studies were published on the ontogeny of the carp innate immune system using WCL15[26,78], but these were mainly focused on organs involved in hematopoiesis in adults and not the myelopoietic sites that were recently demonstrated in zebrafish ontogeny. To the author's best knowledge no data were published on the early development of carp granulocytes.

In adult carp, several studies have been attributed to the identification of genes involved in immune responses. Carp cytokines (interleukin 1 beta (IL1-β), and tumour necrosis factor alpha (TNF-α)), inducible nitric oxide synthase (iNOS), and serum proteins (complement factor 3 (C3), serum amyloid A (SAA) and alpha$_2$-macroglobulin (α_2M)) were all implicated in the carp innate immune system in *in vitro* or *in vivo*

studies[28-33]. Recently, also a complement factor C1r/C1s/MASP2 like serine protease (C1rs)[54], and the anti-inflammatory cytokines interleukin 10 (IL-10[79]) and transforming growth factor beta (TGF-β[80]) were identified in carp. None of the aforementioned innate immune system factors were examined in fish ontogeny until now, with the exception of C3[66,70].

In this study, the mAb's WCL15 and TCL-BE8 were used to qualify and quantify innate immune cell kinetics in carp ontogeny. In addition, the development of myelopoietic tissues was characterised, including the intermediate cell mass (ICM), the tissue ventro-lateral of the aorta, the tissue around the sinuses of the vena cardinalis (or posterior blood islet), the head kidney and the trunk kidney. Finally, in order to examine the functionality of the innate immune system around hatching, the expression patterns of a panel of genes involved in carp innate immune responses were measured following bacterial lipopolysaccharide (LPS) injection in embryos.

Methods

Animals

Common carp (*Cyprinus carpio* L.) of the R3R8 strain were bred and kept in the facilities of 'de Haar Vissen', department of Animal Sciences of Wageningen University (The Netherlands). They were kept at 25 ± 0.5°C for the first 5-6 weeks and subsequently at 23°C in circulating, filtered, UV-treated water. The embryos hatch at 2-3 dpf and were fed with *Artemia salina* nauplii starting at 4 dpf for 3 weeks followed by Trouvit K30 pellets (Trouw & Co., Putten, The Netherlands). Fish were anaesthetised with 0.03 % tricaine methane sulphonate (Crescent Research Chemicals, Phoenix, AZ) buffered with 0.06 % sodium bicarbonate to pH 7.2 in aquarium water.

Monoclonal antibodies

TCL-BE8 is reactive with carp neutrophilic granulocytes (strong affinity), monocytes (low affinity)[27], and basophilic granulocytes (intermediate affinity:

unpublished results). WCL15 reacts (on tissues) with the cytoplasm of macrophages and monocytes[25,26].

Flowcytometry

All incubation steps were performed at 4ºC, all washing steps of cells consisted of centrifuging twice at 1800 rpm for 10 min. Between 1 and 10 dpf, whole fish were macerated. From 7 dpf onwards, blood, thymus, gills, gut, spleen and head kidney were sampled. Blood was collected in RPMI medium with 10 % extra water (c-RPMI) and heparin (50 IU/ml) upon cutting the tail until 6 weeks, in older animals it was sampled from the caudal vein using a syringe containing c-RPMI with heparin. Leucocytes from blood and organs from 3-50 individuals (number decreasing from 50 to 3 with age) were pooled in order to obtain a sufficient number of cells. The procedure was performed on 2 offspring. Cell suspensions were prepared in c-RPMI containing 0.1 % sodium azide (c-RPMI^{+}) by teasing the tissues through a nylon gauze filter (50 µm mesh). Cell suspensions and blood were washed once in c-RPMI^{+}, layered over a discontinuous gradient of Percoll (Pharmacia AB, Uppsala, Sweden), diluted in c-RPMI^{+} to yield densities of 1.060, 1.070, and 1.080 g ml^{-1}. The 1.070-1.080 g ml^{-1} density usually contains numerous granulocytes in adult animals[34], but both densities were used in this study because at the 1.060-1.070 g ml^{-1} density firstly (small) macrophages are most abundant[34], and secondly TCL-BE8^{+} cells appeared earlier in ontogeny in this fraction. After centrifugation (30 min at 2000 rpm, brakes disengaged) the 2 interfaces were removed and washed once in c-RPMI^{+} with 1 % BSA (c-RPMI^{++}). Leucocytes were incubated with TCL-BE8 (1:50) for 1 hour (h), washed, and incubated with fluorescein isothiocyanate-conjugated Goat-anti-Mouse Ig (GAM-FITC: Dako, Glostrup, Denmark) diluted 1:100 in c-RPMI^{++}. After washing, 10^4 cells were measured with a flow cytometer (FACStar, Becton Dickinson Immunocytometry Systems, Mountain View, USA) and analysed using the DataMate analysis package. Until 6 weeks of age peripheral blood leucocytes (PBL) of adult fish were used as positive controls. The percentage of mAb positive cells was calculated by subtracting the percentage of cells labelled with only the secondary antibody (GAM-FITC).

Immunohistochemistry

Whole carp up to 14 dpf were snap-frozen in liquid nitrogen; small embryos had previously been embedded in 1.5 % agarose with 15 % sucrose. Frozen samples were stored at -80ºC. Serial transverse or sagittal 7 µm sections were mounted on poly-L-lysine-coated microscope slides (BDH Laboratory Supplies, Poole, UK) and fixed in cold acetone for 7 min. Then, sections were washed twice (first on ice, then at room temperature) in phosphate buffered saline (PBS) and incubated with TCL-BE8 (1:50) or WCL15 (1:50) in PBS for 1 h. After washing twice with PBS, sections were incubated with horseradish peroxydase-labelled Goat-anti-Mouse immunoglobulins (GAM-HRP, Bio-Rad laboratories, Hercules, USA) for 45 min. Sections were washed in 0.05 M, pH 5 sodium acetate buffer. 3-Amino-9-ethyl-carbazole (AEC; Sigma-Aldrich, St-Louis, USA) in sodium acetate buffer with 0.03 % H_20_2 was added and after staining (2-6 min), rinsed 4 times with distilled water. Sections were counter stained in 1:1 haemalum (BDH Laboratory Supplies) for 20 sec, rinsed in running tap water, embedded in Kaiser's glycerine gelatine (Merck, Darmstadt, Germany) and examined with a Nikon Microphot-FXA microscope (Nikon, Japan). Pictures were taken using the Analysis Extended Pro 3.1 software (Soft Imaging System GmbH, Olympus, Japan), and an Olympus DP50 camera. Sections treated with other mouse antibodies (of several isotypes) or without first antibody showed no staining.

Whole mount immunohistochemistry

Whole mount immunohistochemistry was not performed with TCL-BE8, because this mAb does not react with paraformaldehyde-fixed tissues (unpublished results). Carp aged 2-10 dpf were fixed in 4 % paraformaldehyde in PBS for 12 hr and stored in 1 % paraformaldehyde in PBS at 4ºC. Samples were washed 3 times in PBS containing 15 % sucrose, and incubated in PBS with 0.2 % Triton X-100 and 10 % foetal calf serum for 8 hr at room temperature. After 3 rinses the embryos/larvae were incubated with WCL15 (1:10) for 72 hr at 4ºC. After 3 washes, samples were incubated with GAM-FITC (1:50; DAKO) for 6 hr at 4ºC, rinsed in water and mounted on slides with Vecta-Shield

Mounting Medium with propidium-iodide (Vector Laboratories, Burlingame, USA). Samples were examined with a laser-scanning microscope (LSM 510, Carl Zeiss, Germany).

Preparation of cell suspensions and consecutive semi-thin and ultra-thin sections for electron microscopy

Cell suspensions of the 1.060-1.070 g ml^{-1} density were obtained from 2 and 3 dpf embryos. The cells were washed, suspended in 1 ml of c-$RPMI^{++}$ and centrifuged (1000 g) to yield a compact pellet. The pellet and 1-5 dpf carp embryos and larvae were fixed in 1 % (w/v) $K_2Cr_2O_7$, 2 % (v/v) glutaraldehyde and 1 % (w/v) OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 hr at 4ºC, and subsequently washed in double distilled water, dehydrated in alcohol and propylene oxide, and embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, USA). Consecutive semi thin (1 μm) sections and ultra-thin sections (of the complete embryo without the head region) were cut on a Reichert Ultracut S (Leica, Rijswijk, the Netherlands), and stained with toluidin blue or uranyl acetate and lead citrate, respectively. Semi thin sections were examined with light microscopy and ultra-thin sections with a Philips EM208 electron microscope (Philips, Eindhoven, the Netherlands).

LPS injection in embryos

First, 24 and 48 hours post fertilisation (hpf) eggs were incubated with 0.6 % trypsin in Holfreter solution (60 mM NaCl, 0.7 mM KCl, 0.7 mM $CaCl_2$ and 2.4 mM $NaHCO_3$ in Double distilled water water of 25ºC) for 15 min. After 3 washes in Holfreter the eggs were decapsulated with gentle pressure. Embryos were kept in Holfreter solution at 25ºC until they were frozen.

After injection 2 different protocols were followed for measuring gene expression, resulting in quantification with Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) or Real Time Quantitative Polymerase Chain Reaction (RQ-PCR; which was used additionally because this technique is more sensitive and can detect smaller differences).

Differences in protocols will from here on refer to the quantification method: 'RT-PCR' or 'RQ-PCR'

Before injection or freezing the embryos were anaesthetised with 0.026 % tricaine methane sulphonate and 0.038 % sodium bicarbonate in aquarium water. Embryos were situated on solidified agarose 3 % and injected in the yolk sac with 0.08 ± 0.04 µl of a 2.3 %, 0.2 µm sterile cellulose-acetate filtered solution of *Aeromonas salmonicida* LPS (obtained from dr. Ian Bricknell, Marine Laboratory, Aberdeen, United Kingdom) or with PBS (LPS free for RQ-PCR) as a control. Injection was performed using glass needles in a micromanipulator (Narishige, Tokyo, Japan) connected to an air pump (Narishige, Greenvale, U.S.A.). For RT-PCR or RQ-PCR, 1x3 or 6x1 embryos respectively were snap frozen at 1, 2, 4, 8, and 24 hours post injection (hpi) in liquid nitrogen and stored at -80ºC until further analysis. Two-four offspring were examined with RT-PCR and one offspring was examined using RQ-PCR.

RNA extraction and cDNA synthesis

Before RT-PCR, RNA was extracted following the protocol for preparation of lysates from small tissue samples (≤30 mg) and RNA purification by centrifugation of the SV Total RNA Isolation System (Promega, Madison, USA), without synthesis of cDNA. For RQ-PCR, RNA was isolated using the following method[58]: tissues were homogenised in lysis buffer (4 M guanidium thiocyanide, 25 mM sodium citrate pH 7.0, 0.5 % sarkosyl, 0.1 M 2-mercaptoethanol) followed by phenol/chloroform extraction. Total RNA was precipitated in ethanol, washed and dissolved in water. Concentrations were measured by spectrophotometry (Genequant, Pharmacia Biotech, Uppsala, Sweden). 0.1 – 1 µg RNA was DNase treated (Deoxyribonuclease I, Amplification Grade: Invitrogen, Carlsbad, U.S.A.) and subsequently cDNA was synthesised using RT Superscript™ (Invitrogen) according to the manufacturer's protocol. RNA was also treated identically but without RT Superscript™, yielding non-RT controls.

Reverse transcriptase-polymerase chain reaction

RT-PCR was performed with the Superscript One-Step RT-PCR with Platinum TAQ System (Invitrogen). In short: 0.5 µl RT-Platinum Taq Mix, 12.5 µl 2x Reaction Mix, 0.2 µl RNAse Inhibitor (40 U/µl), 0.4 µM forward primer and 0.4 µM reverse primer were mixed and added to 10 µl template. Reverse transcription was performed at 50ºC for 35 min. The mixture was then denatured at 94ºC for 2 min and subjected to 25-40 cycles of 94ºC for 30 sec, 55ºC for 30 sec and 72ºC for 1 min. The products were visualised by separation on a 1.5 % agarose gel. A 259 base pair β-actin fragment, amplified from 5 µl RNA using RT-PCR conditions for 25 cycles with primers based on a carp β-actin sequence was used as a positive control for RT-PCR. Primer combinations of the following genes were used (between brackets is the number of cycles needed to amplify a detectable gene product under non-saturating conditions): IL1-β (40), TNF-α (40), iNOS (40), C3 (25), SAA (32), and α_2M (32). The primer sequences are described in previous studies: TNF-α, C3, SAA and α_2M[28], iNOS[31] and IL1-β (1.f3/ 1.r1[81]).

PCR products were separated on a 1.2 % agarose gel containing 0.5 µg/ml ethidium bromide at 120 V. mRNA levels were determined semi-quantitatively by calculating the intensity (luminescence) of each band of the PCR with the Gel Doc 1000 (Bio-Rad laboratories) using the Multi-Analyst 1.1 software volume analysis option (Bio-Rad Laboratories). Values were expressed as arbitrary units being the ratio of mRNA level and the corresponding β-actin mRNA level after subtraction of background luminescence[value = {intensity gene of interest – intensity background}/{intensity corresponding β-actin – intensity background}].

Real time quantitative-polymerase chain reaction

RQ-PCR was performed as follows: cDNA was added to 2x QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany), 1.5 µl 5 µM forward and reverse primers and additional water to a total volume of 25 µl. The following PCR protocol was run on a Rotorgene (Corbett, Sydney, Australia) according to the manufacturer: an initial activation step at 94ºC for 15 min was followed by 45 cycles with a denaturation step at 94ºC for 30

sec, an annealing step at 60ºC for 30 sec and an extension step at 72ºC for 30 sec, and subsequently a melting curve running from 60º to 90º with 1 min intervals. The Ct value of a reaction mixture is defined as the point when fluorescence (generated by Sybr-green I attaching to double stranded DNA) reaches the threshold which gives the most efficient

Table 1. Primers used for amplification of specific gene products with RQ-PCR. Primers are designed to amplify all known carp isoforms. IL1-β: interleukin 1 beta, iNOS: inducible nitric oxyde synthase, TNF-α: tumour necrosis factor alpha, C3: complement factor 3, SAA: serum amyloid A, α_2M: alpha 2-macroglobulin, C1rs: complement factor 1r/1s/MASP2 like serine protease, IL-10: interleukin 10, TGF-β: transforming growth factor beta.

Name		Sequence	Product size (basepairs)	Genbank Accession Numbers
40S	FW	ccgtgggtgacatcgttaca	69	AB012087
	RV	tcaggacattgaacctcactgtct		
β-Actin	FW	gctatgtggctcttgacttcga	89	M24113
	RV	ccgtcaggcagctcatagct		
IL1-β	FW	aaggaggccagtggctctgt	69	AB010701
	RV	cctgaagaagaggaggctgtca		
iNOS	FW	aacaggtctgaaagggaatcca	101	AJ242906
	RV	cattatctctcatgtccagagtctcttct		
TNF-α	FW	gctgtctgcttcacgctcaa	106	AJ311800
	RV	ccttggaagtgacatttgctttt		
C3	FW	ccctggacagcattatcactc	154	AB016210-5
	RV	gatggtcgcctgtgtggt		
SAA	FW	gcagatgggcagccaaagta	70	AB016524
	RV	gaattaccgcggcgagaga		
α_2M	FW	ttccaggacagacagtgaactttaga	107	AB026129
	RV	tctgttaccctgactgtcctcaag		
C1rs	FW	aacaccctcggctcctacct	89	AB042609-10
	RV	ccacctccacactccaagac		
IL-10	FW	gctgtcacgtcatgaacgagat	132	AB110780
	RV	cccgcttgagatcctgaaatat		
TGF-β	FW	acgctttattcccaaccaaa	97	AF136947
	RV	gaaatccttgctctgcctca		

amplification for the pertinent primer combination. *β-actin* and *40S* were used as house keeping genes. Primer sequences were specifically designed for RQ-PCR (and are therefore different than primers used for RT-PCR), and are listed in Table 1. R-values were calculated with Pfaffl's method ($E^{GOI\wedge}(Ct^{reference}-Ct^{sample}))/(E^{HKG\wedge}(Ct^{reference}-Ct^{sample}))$[82], using the average uninjected control Ct values as a reference (E= efficiency of primer combination, GOI = gene of interest, reference = sample at 24 hpf or 48 hpf without injection, HKG = house keeping gene). R-values calculated with either *β-actin* or *40S* as house keeping gene were comparable, consequently only values calculated with *β-actin* are presented. Non template controls were always included.

Statistical Analysis

Significance of differences was determined by Wilcoxon signed ranks test (RT-PCR) or Man-Whitney test (RQ-PCR). $P< 0.05$ was accepted as significant.

Results

The appearance of TCL-BE8$^+$ and WCL15$^+$ cells in carp ontogeny

In separate organs from approximately 4 wpf, TCL-BE8$^+$ cells with both densities were clearly divided (using flowcytometry) into 2 populations with distinct FSC/SSC characteristics (Figure 1A) which were characterised as granulocytes and monocytes[34]. Spleen TCL-BE8$^+$ cells of the 1.06-1.07 g ml^{-1} density mainly consisted of monocyte-like cells (results not shown), while head kidney TCL-BE8$^+$ cells of the 1.07-1.08 g ml^{-1} density displayed granulocyte characteristics in the FSC/SSC blot (Figure 1B). TCL-BE8$^+$ cell fractions in spleen and head kidney of the other density and in other organs (thymus, PBL, gut and gills) of the two densities consisted of both cell populations (like in Figure 1A). TCL-BE8$^+$ cells from 4 dpf whole fish macerate (Figure 1C) did not distribute into the FSC/SSC populations as in separate organs. Therefore a gate which only excludes cell

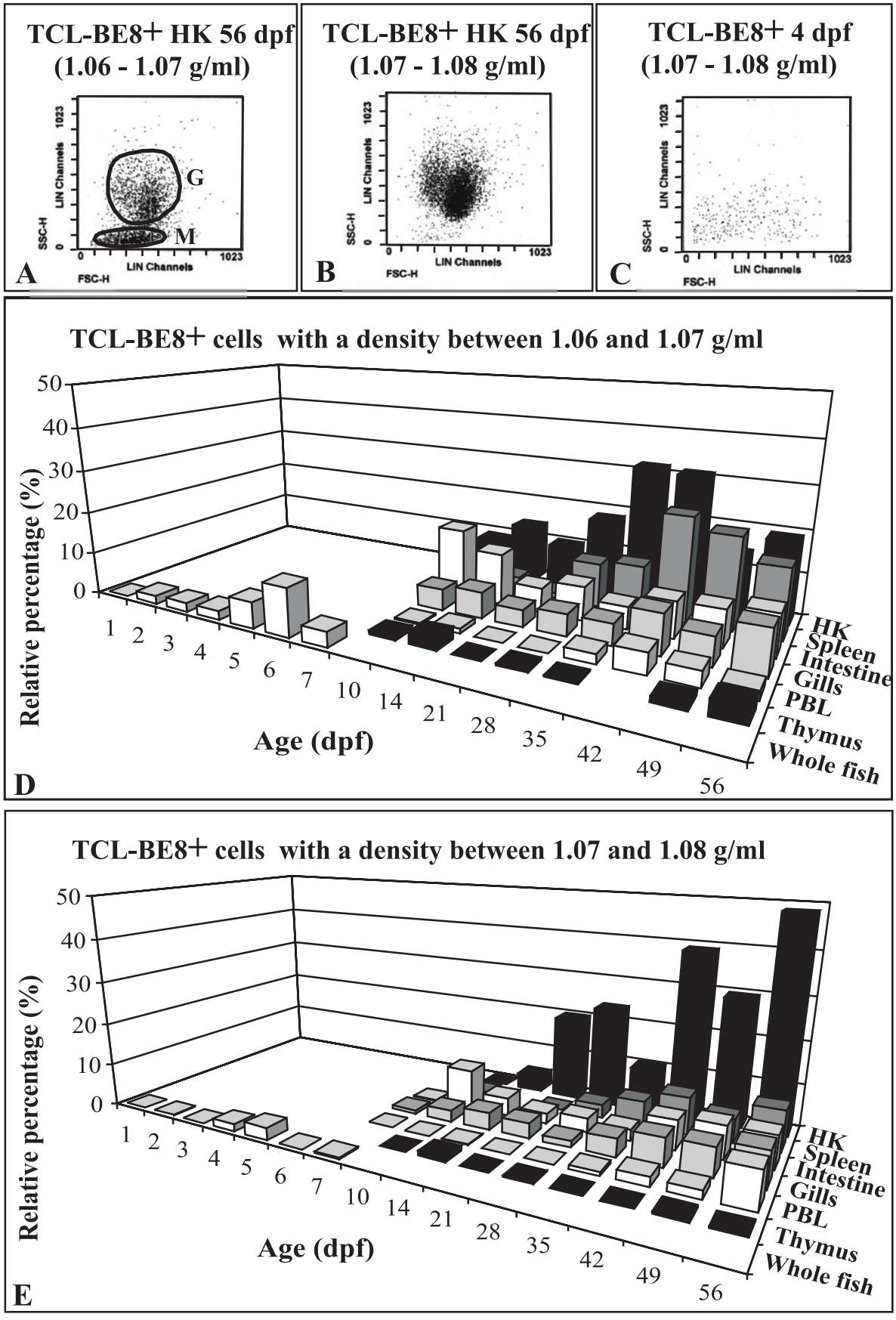
TCL-BE8+ HK 56 dpf
(1.06 - 1.07 g/ml)
G
M
SSC-H
LIN Channels
FSC-H
A
TCL-BE8+ HK 56 dpf
(1.07 - 1.08 g/ml)
B
TCL-BE8+ 4 dpf
(1.07 - 1.08 g/ml)
C
TCL-BE8+ cells with a density between 1.06 and 1.07 g/ml
Relative percentage (%)
Age (dpf)
HK
Spleen
Intestine
Gills
PBL
Thymus
Whole fish
D
TCL-BE8+ cells with a density between 1.07 and 1.08 g/ml
E

Figure 1 (previous page). FSC/SSC profiles and relative percentages of TCL-BE8$^+$ cells in distinct organs during carp development determined by flowcytometry. Panels A-C represent FSC/SSC profiles of TCL-BE8$^+$ cells from the following fractions: head kidney (HK) cells from 56 dpf carp with a density between 1.06 and 1.07 g ml^{-1} (A), and between 1.07 and 1.08 g ml^{-1} (B), and whole fish macerate cells from 4 dpf carp embryos with a density between 1.07 and 1.08 g ml^{-1} (C). In panel A the typical FSC/SSC characteristics of monocytes (M) and neutrophilic granulocytes (G) are displayed. Cells with a density between 1.06 and 1.07 g ml^{-1} (D), and between 1.07 and 1.08 g ml^{-1} (E) were measured by flow cytometry in whole fish macerate (Whole fish), thymus, peripheral blood leukocytes (PBL), gills, intestine, spleen, and head kidney (HK) of carp aged between 1 and 56 dpf.

particles and very large cells or cell agglomerates was used to calculate the relative percentage of TCL-BE8$^+$ cells (not shown).

The first TCL-BE8$^+$ cells (granulocytes/monocytes) appeared in whole fish macerate cell fractions with a density between 1.06 and 1.07 g ml^{-1} at 2 dpf (Figure 1E). From 5 dpf the relative percentage increased, and from 10 dpf TCL-BE8$^+$ cells were most abundant in head kidney and spleen, followed by intestine and gills, while only a small percentage resided in PBL and thymus.

Using immunohistochemistry, TCL-BE8$^+$ cells were consistently present ventro-lateral of the aorta at 2 dpf (results not shown), and 3 dpf (Figure 2A-C), whilst several cells were also detected in the tissue surrounding the sinuses of the vena cardinalis (Figure 2B). Occasionally, TCL-BE8$^+$ cells were located in other locations, for instance ventral of the intestine in Figure 2A. At 7 dpf, numerous TCL-BE8$^+$ cells were present in head kidney (Figure 2D) and along the major vessels in the trunk (Figure 2E), with also a considerable number ventro-lateral of the aorta and around the vena cardinalis sinuses in the tail (Figure 2F, G). At 14 dpf the vena cardinalis sinuses were no longer prominent, while most TCL-BE8$^+$ cells were present in head kidney and kidney (Figure 2I).

WCL15$^+$ cells (macrophages/monocytes) displayed cytoplasmic staining and were shaped rounded or with extensions (Figure 2H). Like TCL-BE8$^+$cells, WCL15$^+$ cells were present in comparable numbers ventro-lateral of the aorta and surrounding the vena cardinalis sinuses at 3 dpf (results not shown) and 5-7 dpf (Figure 2H, J, K).

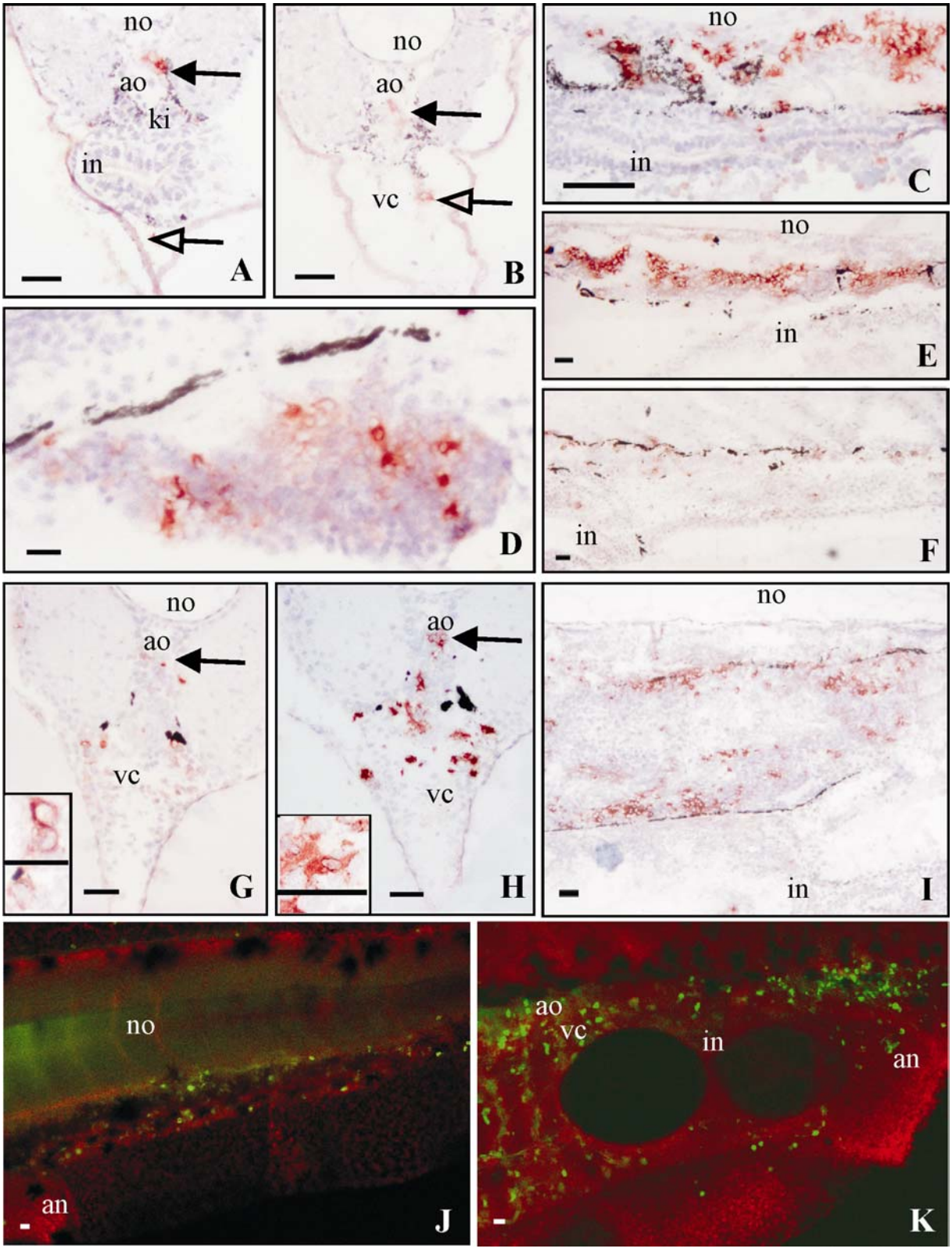
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Figure 2 (previous page). Immunohistochemistry using the mAb's TCL-BE8 (granulocytes/monocytes) and WCL15 (macrophages/monocytes) on 3-14 dpf carp tissue. Throughout panels A-I, immunohistochemistry was performed on sections, with immuno-reactive cells stained reddish-brown and tissue purple (haemalum). In panels J-K, immunohistochemistry was performed on whole larvae and examined with laser scanning microscopy, with immuno-reactive cells stained green fluorescent and tissue red fluorescent (propidium-iodide). Black arrows point to cells ventro-lateral of the aorta, open arrows point to cells at other locations. A: cross section just caudal to the yolk sac at 3 dpf (TCL-BE8); B: cross section of the tail at 3dpf (TCL-BE8); C: sagittal section of the middle part at 3 dpf (TCL-BE8); D: sagittal section of the head kidney at 7 dpf (TCL-BE8); E: sagittal section of the middle part at 7 dpf (TCL-BE8); F: sagittal section of the tail at 7 dpf (TCL-BE8); G: cross section of the tail at 7 dpf (TCL-BE8; note the presence of weak membrane^{+} cells (bottom) and strong cytoplasmic^{+} cells (top) in the inset); H: cross section of the tail at 7 dpf (WCL15; note the characteristic macrophage-like morphology of WCL15^{+} cells in the inset); I: sagittal section of the middle part at 14 dpf (TCL-BE8); J: the tail at 5 dpf (WCL15); K: the posterior part of the intestine and major vessels at 7 dpf (WCL15; in the intestine *Artemia* eggs are present as non-fluorescent circles). The cells with the intensely black granules are melanocytes. An = anus, ao = aorta, in = intestine, ki = kidney tubule, no = notochord, vc = vena cardinalis. Bar is 20 μm.

Histo-morphological identification of myelopoietic tissues in embryonic and larval carp

Granulocytes or macrophages were not present at 24 hpf. 2 Groups of cells were located between aorta, yolk sac and the kidney tubules, especially along the posterior extension of the yolk sac (Figure 3). Using electron microscopy, this group of cells consisted of progenitor cells (Figure 3D). In some sections, the 2 groups had merged and endothelial cells were present amongst the cells (Figure 3C).

At 30 hpf (results not shown), the groups of progenitor cells found at 24 hpf were no longer present as such. On the same location, the vena cardinalis had formed, and blood circulation filled the vessels with mainly erythrocytes. The vena cardinalis had formed sinuses in the tail part of the embryo. A significant number of progenitor cells were present ventro-lateral of the aorta and around the vena cardinalis sinuses.

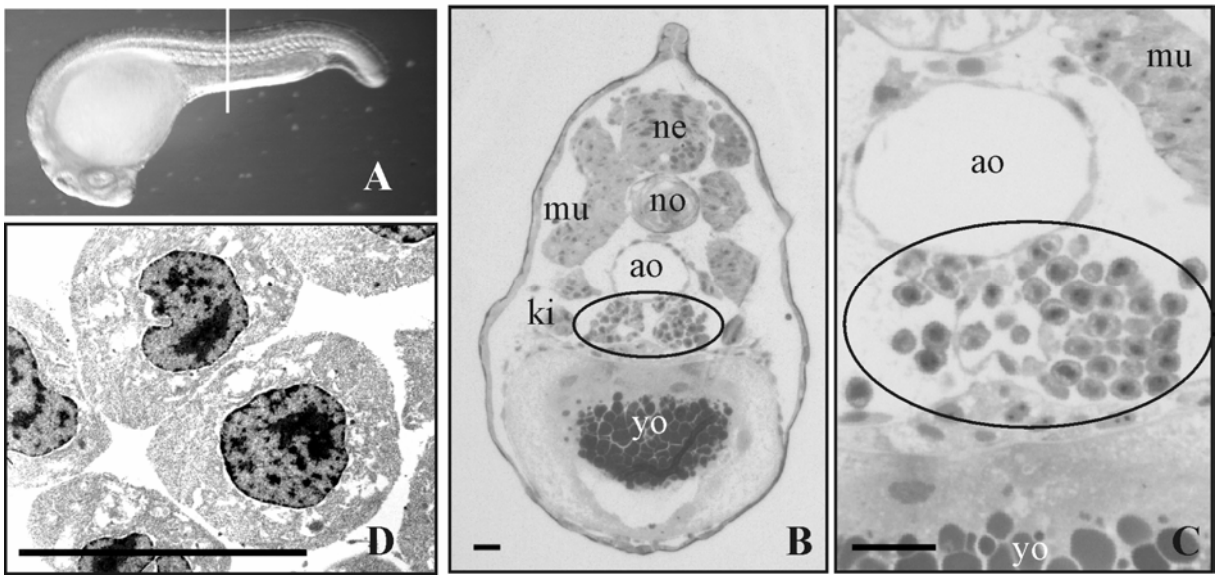
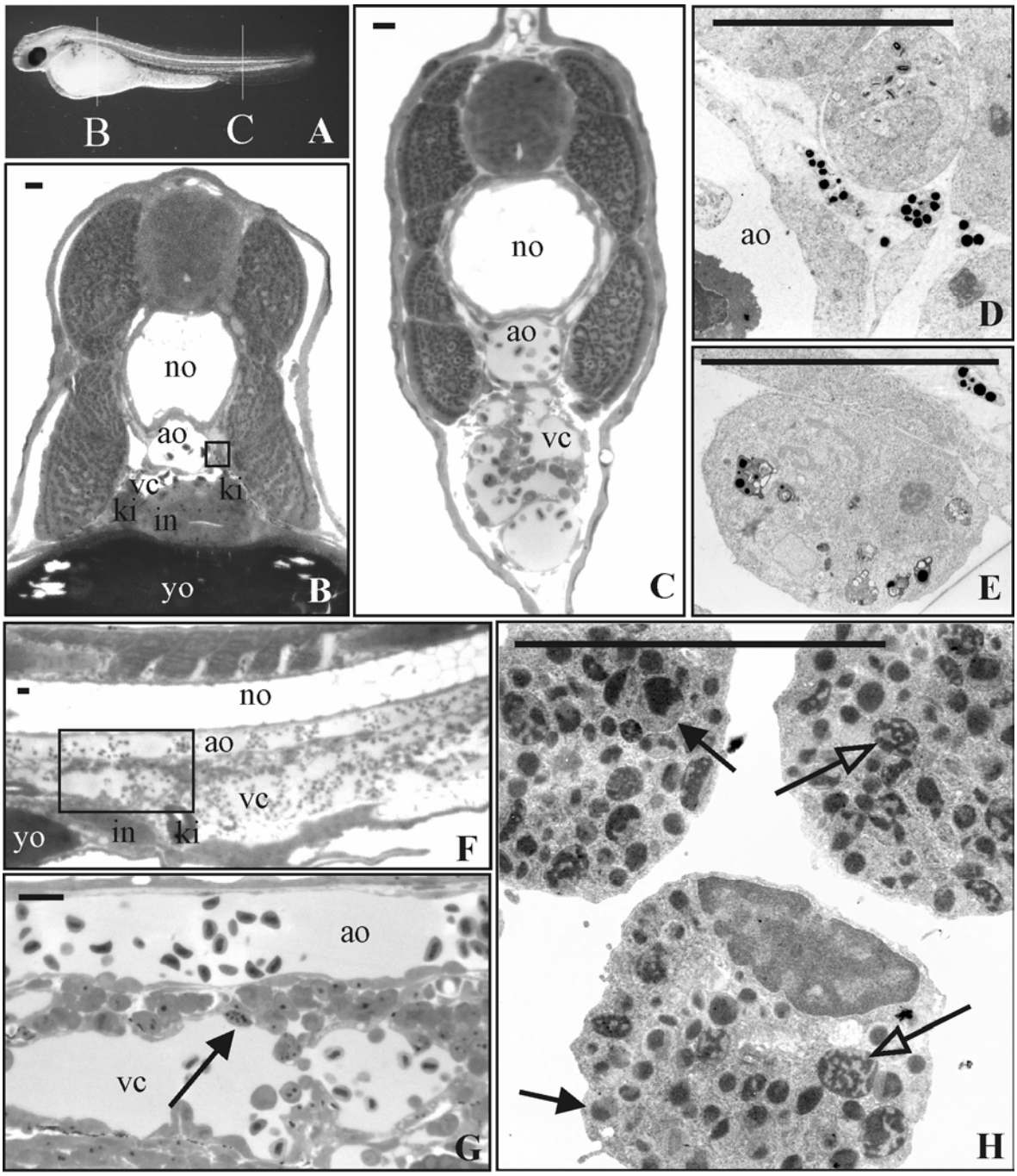


Figure 3. Early morphology of hematopoietic tissue in 24 hpf carp embryos. Panels B and C represent pictures of osmium fixed, toluidine blue stained, 1 µm cross sections from the location indicated in panel A. Just caudal of the broad part of the yolk sac, a cluster of progenitor cells (encircled) was located between aorta, yolk sac and the two kidney tubules: the intermediate cell mass (ICM). In panel C endothelial cells are present in the ICM. Panel D displays an electron-microscopical picture of progenitor cells in the ICM. Ao = aorta, ki = kidney, mu = muscle, ne = neural tissue, no = notochord, yo = yolk sac. Bar is 10 µm.

Figure 4 (following page). Hematopoietic tissues and myeloid cells in 2-3 dpf (hatching) carp embryos. Panels A-G show 2 dpf embryos, panel H depicts cells from 3 dpf embryos. Panels B and C represent pictures of osmium fixed, toluidine blue stained 1 µm cross sections taken from the respective locations indicated in panel A. Using electron-microscopy, neutrophilic granulocytes (D) and macrophages (E) were present ventro-lateral of the aorta (boxed area in panel B). In the tail of the embryo (panel C), the vena cardinalis consisted of several sinuses that were surrounded by numerous progenitor cells. The extensiveness of this tissue is depicted in sagittal sections (panel F). Panel G is a magnification of the boxed area in panel F and shows a solitary granulocyte (closed arrow). Panel H represents basophilic granulocytes from a cell suspension of 3 dpf embryos, with closed arrows pointing to granules with globular electron dense material and open arrows pointing to granules with a patchy (mixed electron dense and electron lucent) content. The cells with the intensely black round granules are melanocytes (panels D-E, G). Ao = aorta, in = intestine, ki = kidney tubule, no = notochord, vc = vena cardinalis, yo = yolk sac. Bar is 10 µm.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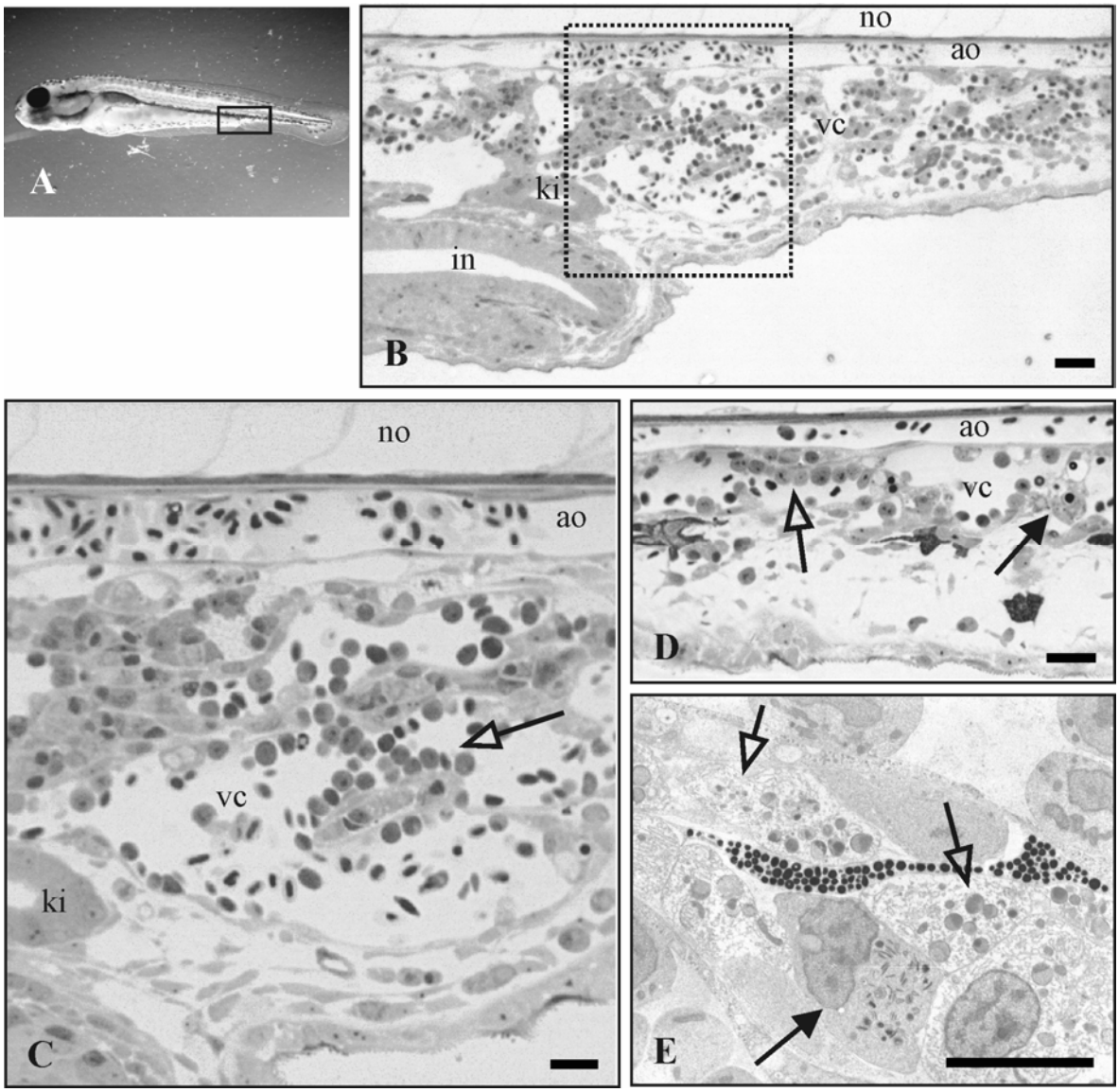

Figure 5. Hematopoietic tissue in the tail of 5 dpf carp larvae. Panel B represents a picture of an osmium fixed, toluidine blue stained, 1 µm sagittal section from the boxed area in panel A, panel C is a magnification of the boxed area in panel B, and panel D is a picture taken caudal from panel B. The vena cardinalis consisted of sinuses intermingled with tissue strands, which were covered with blood cells (panels C and D: open arrows). Macrophages were present (closed arrow, panel D), and using electron-microscopy numerous neutrophilic (closed arrow) and basophilic (open arrows) granulocytes were situated here (panel E). The cells with the intensely black round granules are melanocytes. Ao = aorta, in = intestine, ki = kidney tubule, no = notochord, vc = vena cardinalis. Bar is 10 µm.

Neutrophilic granulocytes and macrophages first appeared at 48 hpf (Figure 4D, E). Neutrophilic granulocytes are easily recognised because of their granules with electron-dense rods[85]. The number of granules in 48 hpf neutrophils was decreased compared to adult neutrophils. Macrophages were distinguished because of their vacuoles with phagocytozed material. Both macrophages and neutrophilic granulocytes were consistently present ventro-lateral of the aorta throughout the embryo (Figure 4B), although they occasionally appeared between the vena cardinalis sinuses (Figure 4C, G; identical to the location of $WCL15^{+}$ and $TCL\text{-}BE8^{+}$ cells).

The vena cardinalis sinuses extended throughout the tail at this age, harbouring numerous progenitor cells (Figure 4F, G). Basophilic granulocytes, identified by irregular or patchy electrondense structures in their granules[85], were present in whole fish macerates at 3 dpf (Figure 4H), although they were not localised *in situ* up to 5 dpf.

At 5 dpf, the sinuses of the caudal vena cardinalis had expanded considerably (Figure 5), completely filling the ventral part of the tail as well as the area between aortaand posterior part of intestine and kidney tubules. Numerous, large rounded cells in the blood stream were attached to the sinus tissue (Figure 5C, D). The tissue around the sinuses contained macrophages (Figure 5D) and extensive amounts of neutrophilic and basophilic granulocytes (Figure 5E).

The head kidney hematopoietic tissue was first present as strands of tissue between a network of blood vessels (sinuses; Figure 6). Hematopoietic tissue was clearly present at 7 dpf (Figure 6A, B), although small numbers of cells were localised at earlier ages. This tissue was localised just caudal to the glomerulus, and expanded among the kidney tubules in cranial and caudal direction from 7 dpf.

The hematopoietic tissue around the sinuses of the vena cardinalis in the tail extended in cranial direction towards the level of the 2nd segment of the intestine from 6 dpf. In the most cranial part the sinuses disappeared, with hematopoietic tissue remaining in the dorsal wall of the vena cardinalis (Figure 6D, E). At 7 dpf, this hematopoietic tissue extended further in cranial direction up to the caudal part of the swim bladder, after which it gradually expanded until it nearly reached the hematopoietic tissue of the head kidney at 3 weeks (results not shown). From 10-14 dpf, the hematopoietic tissue of the dorsal wall

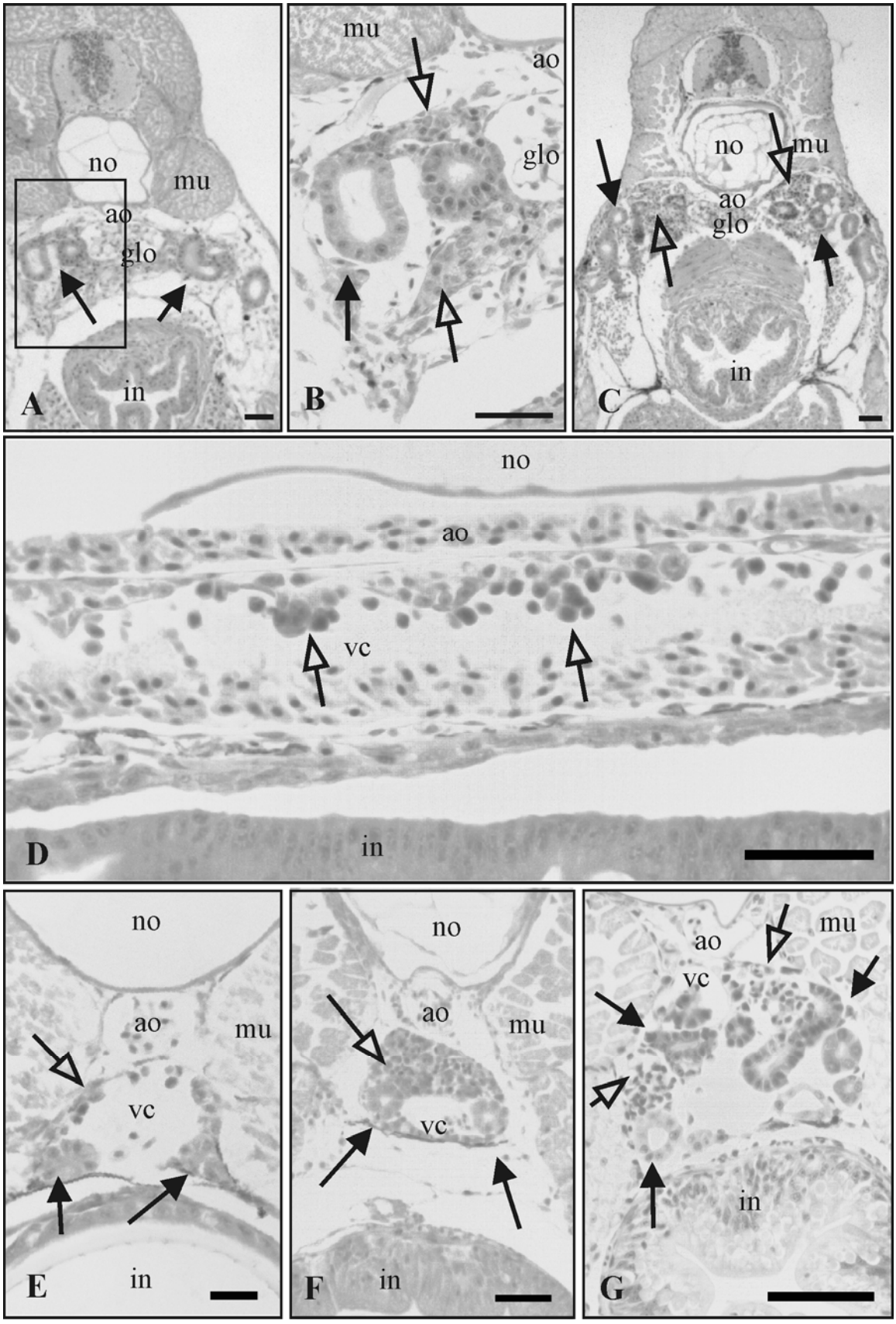
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Figure 6 (previous page). Development of the kidney hematopoietic tissue in juvenile carp. Pictures represent paraformaldehyde fixed, paraffin embedded, haemalum/eosin stained sections. Closed arrows point to kidney tubules, open arrows point to hematopoietic tissue. Panels A-C display head kidney development, panels D-G display trunk kidney development. Panel A (magnified in panel B) represents the kidney tubules with the glomerulus, surrounded by numerous blood vessels and strands of hematopoietic tissue at 7 dpf. Panel C shows the head kidney at 5 weeks. Panel D represents a sagittal section of the caudal part of a 7 dpf carp larva with hematopoietic progenitor cells in the dorsal wall of the vena cardinalis (which contains numerous erythrocytes in the ventral part). Panels E, F, and G represent cross sections caudal to the swim bladder of 7, 10 dpf , and 5 weeks-old carp. At 7 dpf (panel E), progenitor cells were present in the dorsal wall of the vena cardinalis, at 10 dpf (panel F), the number of progenitor cells in this location increased considerably and grew around the kidney tubules, and at 5 weeks (panel G) the caudal part of the kidney tubules were intermingled with hematopoietic progenitor cells and the vena cardinalis sinuses. Ao = aorta, glo = glomerulus, in = intestine, mu = muscle, no = notochord, vc = vena cardinalis. Bar is 20 μm.

of the vena cardinalis gradually enveloped the kidney tubules (Figure 6F), forming the trunk kidney hematopoietic tissue with the embedded vena cardinalis (Figure 6G). At the same time, the sinuses of the vena cardinalis in the tail disappeared.

Modulation of immune gene expression in embryonic carp following LPS injection

IL1-β, *iNOS*, *C3*, *C1rs*, *SAA*, and *α_2M* were already expressed at 24 hpf (although at lower levels compared to 48 hpf; results not shown). *IL-10* and *TGF-β* were not expressed at 24 hpf or 48 hpf, while *TNF-α* was expressed with RT-PCR but not with RQ-PCR (results not shown).

After LPS injection in 48 hpf embryos, expression of a number of immune genes was increased or decreased (significantly using RQ-PCR but not using RT-PCR) compared to not injected - or PBS injected embryos (Figure 7). 1 Offspring examined with RT-PCR presented low and unresponsive *IL1-β* and *iNOS* expression (results not

shown). *TNF-α* expression increased after LPS injection at 1-2 hpi in only 1 offspring (results not shown).

IL1-β expression (panels A-B) was increased after LPS injection, although in some embryos also after PBS injection (1 offspring in panel A at 1 hpi and 1 embryo out of 6 in panel B at 2 hpi).

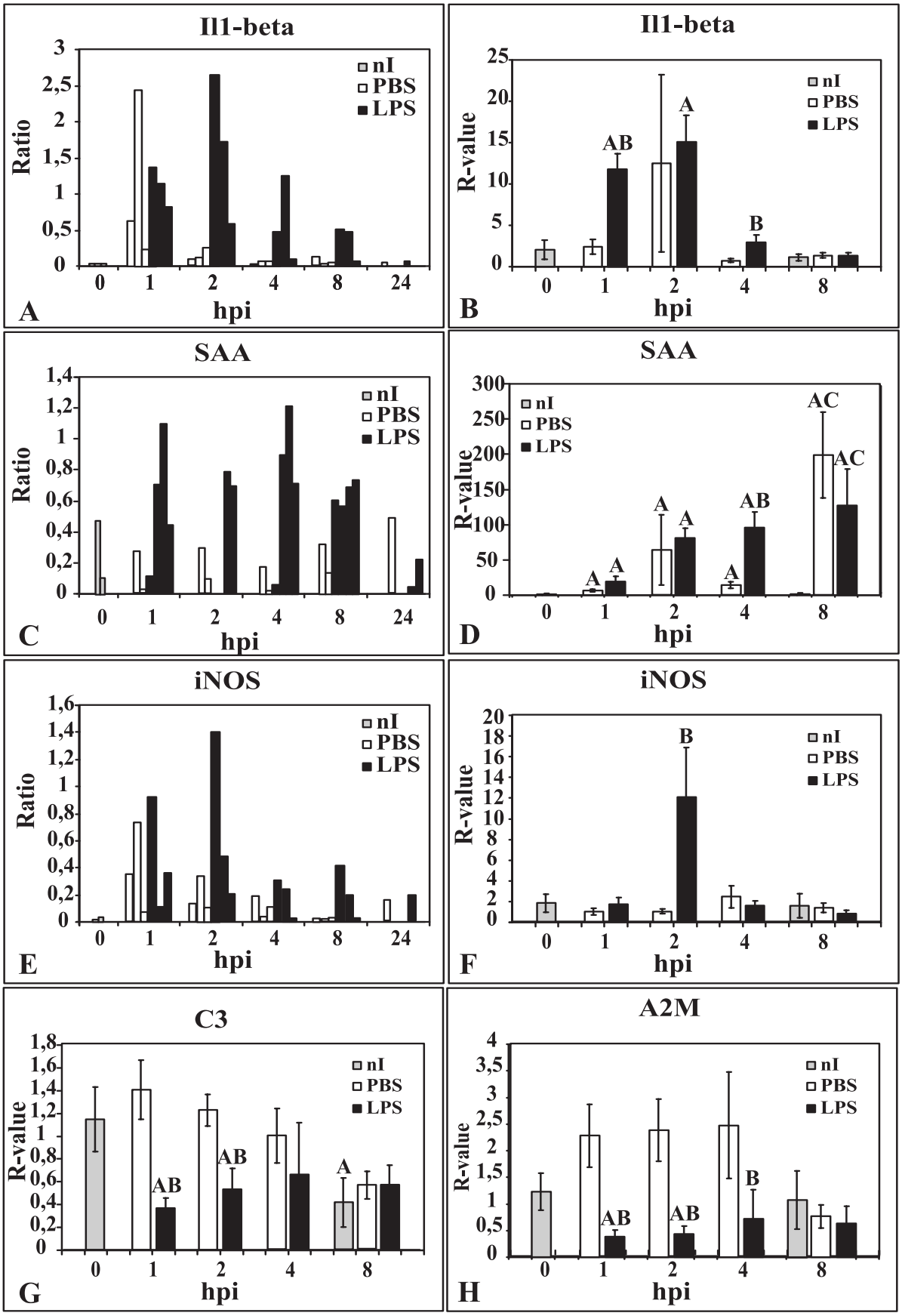
SAA expression (panels C-D) was increased after LPS injection compared to PBS injection at 1-8 hpi using RT-PCR (panel C), and was increased significantly at 4 hpi using RQ-PCR (panel D), while both injected groups at all times had an increased *SAA* expression compared to not injected embryos using RQ-PCR (panel D).

iNOS expression (panels E-F) was moderately increased after LPS injection using RT-PCR (panel E), although PBS-injected embryos also displayed higher expression at 1-2 hpi. *iNOS* expression was significantly higher after LPS injection at 2 hpi using RQ-PCR (panel F).

Using RQ-PCR (using RT-PCR no differences were observed), both *C3* and *α_2M* expression decreased after LPS injection at 1-2 hpi (*α_2M* also at 4 hpi) compared to not injected - and PBS injected embryos (panels G and H respectively). *C1rs* expression increased after both LPS and PBS injection at all time points (results not shown).

With respect to LPS injection at 24 hpf (results not shown), *SAA*, *α_2M* and *C1rs* expression patterns were similar to injection at 48 hpf, but *IL1-β*, *iNOS*, and *C3* expression were unresponsive to LPS injection.

Figure 7 (following page). Expression of innate immune system factors in 48 hpf carp embryos after LPS injection. Panels A, C, and E represent ratios following RT-PCR: bars represent measurements of 2-4 different offspring, panels B, D, F-H represent R values following RQ-PCR (bars represent average of 6 embryos of 1 offspring: error bars depict standard error of the mean). IL1-β = interleukin 1 β, SAA = serum amyloid A, iNOS = inducible nitric oxide synthase, C3 = complement factor 3, α_2M = alpha 2-macroglobulin. Legend: nI = not injected, PBS/LPS: injected with PBS or LPS. Statistical significance levels were set at $p < 0.05$ and are indicated with capital A (compared to nI at 0 hpi), capital B (compared to PBS at the same hpi), or capital C (compared to nI at 8 hpi).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Discussion

This study offers three major additions to the already considerable amount of data obtained in zebrafish on the ontogeny of the teleost innate immune system. Firstly, the development and histology of myelopoietic tissues were identified throughout carp development using monoclonal antibodies and combining extensive histology and electron microscopy studies. The obtained data enabled the construction of a timeline displaying the temporal development of carp myelopoietic tissues in development (Figure 8), which is a valuable addition to the study of the development of the teleost innate immune system. Secondly, this paper provides the first description of the ontogeny of trunk kidney hematopoietic tissue in teleosts. Thirdly, the first appearance of myeloid cells was associated with an LPS generated upregulation of cytokines and acute phase proteins in carp embryos.

The first TCL-BE8^{+} - and WCL15^{+} cells (monocytes/granulocytes and monocytes/ macrophages, respectively, as confirmed with electron microscopy) were consistently present ventro-lateral of the aorta in carp embryos from 2 dpf onwards. At 24 hpf, myeloid cells were not present, but 2 strands of progenitor cells were localised in the axis of the embryo similar to the ICM described in zebrafish[36,37]. Consequently, neutrophilic granulocytes and macrophages appear around the time of hatching, ready to defend the embryo against micro-organisms in the environment. The appearance of carp myeloid cells corresponds to an increase in immune chemokine expression in carp embryos of the same age[86]. The presence of myeloid cells and progenitor cells ventro-lateral of the aorta, and the assumption that especially granulocytes are relatively scarce in non-myeloid tissues because of their short life span implies that this area is the first myelopoietic site in carp development. This conclusion is supported by zebrafish studies, in which myeloid cells were situated at the same location[36] and genes implicated in hematopoiesis were expressed dorsal to the caudal yolk sac extension with whole mount in situ hybridisation[35,38,40,41]. Also in 2 dpf zebrafish, granulocytes migrated to inflicted trauma[39], and macrophages migrated to and phagocytozed bacteria[40]. The 'dorsal aorta' was proposed the zebrafish equivalent[35,36,87] of the aorta-gonad-mesonephros region, the first

site of definitive hematopoiesis in tetrapods. This is the first study to show more specifically that the region ventro-lateral of the aorta is the first major provider of granulocytes (and possibly macrophages) in embryonic cyprinid fish around hatching. In addition, the carp specific mAb's TCL-BE8 and WCL15 proved to react with myeloid surface molecules that are already transcribed and processed from the first appearance of myeloid cells, and thus offers a valuable addition to the study of the development of the teleost innate immune system. Moreover, these mAb's are useful tools to study myeloid cell population kinetics after experimental treatments, of which data are already available from adult animals[88].

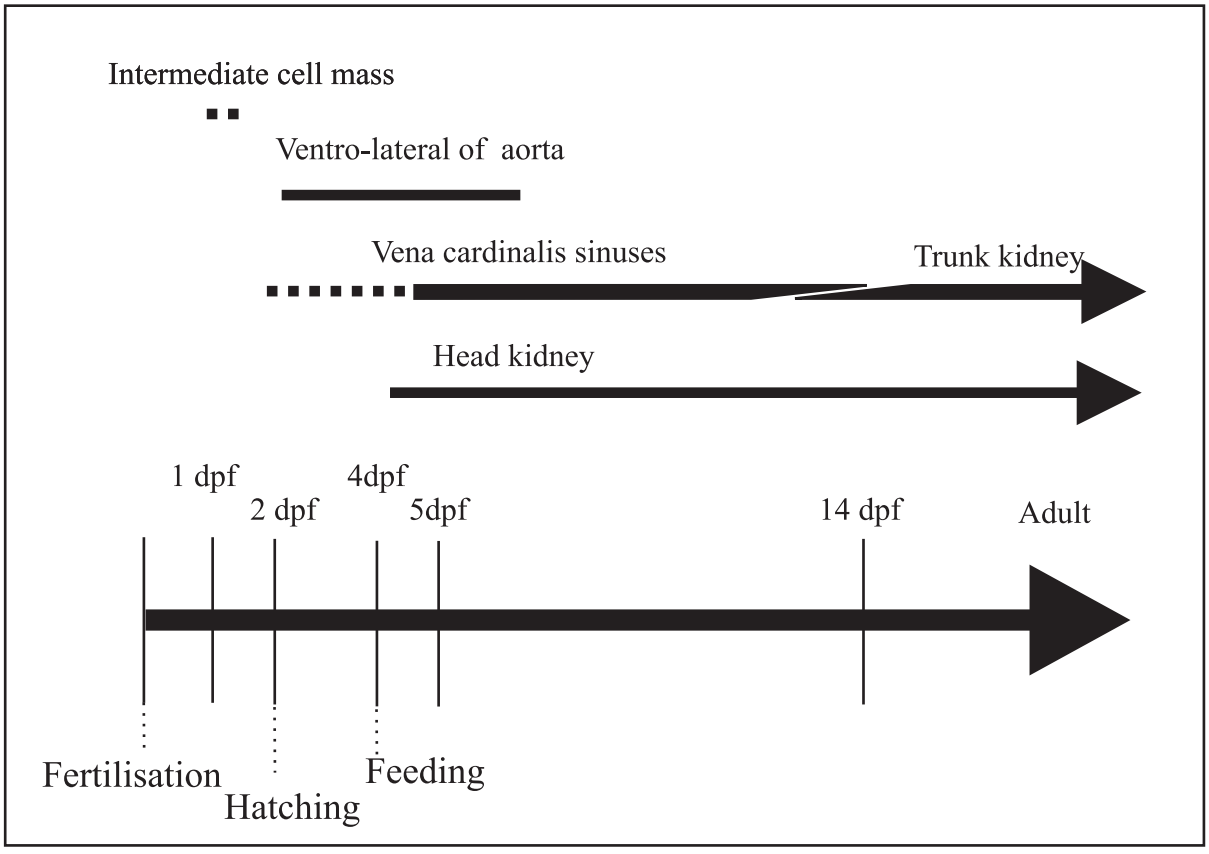

Figure 8. Myelopoietic tissues in carp ontogeny. Dotted lines indicate that the tissue contains myeloid progenitors, but mature cells were very rare, while solid lines indicate that mature myeloid cells were present. Data were obtained from this study, another carp study[26], and several zebrafish studies[35-41], assuming that carp and zebrafish have a comparable immune system. Herbomel et al.[83,84] traced macrophages to the anterior-most lateral mesoderm of the head, which was not included in this study.

Subsequently in development, a significant number of myeloid cells were located between the sinuses of the vena cardinalis, implicating this tissue as the second major myelopoietic site in carp larvae. This conclusion is corroborated by studies in zebrafish, in which expression of genes relevant for hematopoiesis[35,40,41,84,89], and myeloid cells[36,39,41] were present in the ventral tail region (posterior blood islet) of whole embryos and larvae, although the histology of this tissue was not described extensively. Taken together, the generation of myeloid cells around the sinuses of the vena cardinalis bridges myelopoiesis ventro-lateral of the aorta in embryos and in the kidney of older animals, ensuring a continuous supply of myeloid cells throughout cyprinid development.

Basophilic granulocytes were also present surrounding the sinuses of the vena cardinalis from 5 dpf, and although they appeared in whole embryo cell suspensions from 3 dpf they were not located *in situ*. Possibly these cells originate from the head region, a part not examined in this study but where embryonic macrophages were traced in zebrafish[40,84].

The sinuses and dorsal wall of the vena cardinalis (which develops into trunk kidney hematopoietic tissue at 10-14 dpf) exceeded the size of the head kidney to a large extent at all ages examined, and can therefore be considered as a quantitatively more important hematopoietic organ at this age. Both the head kidney and the trunk kidney originate from hematopoietic tissue surrounding sinuses of the vena cardinalis.

In concordance with the first appearance of myeloid cells, 2 dpf carp embryos presented increased cytokine and acute phase protein expression, in contrast to 1 dpf embryos. It must be stressed that expression patterns were measured in complete embryos, while the changes in expression most probably are caused by a small population of cells, signifying the magnitude of the generated response. *IL1-β* expression in 2 dpf embryos was comparable to *in vitro* LPS stimulation[28,32], and to *in vivo* infection experiments in adult carp[28]. *iNOS* expression was increased in the LPS injected group earlier then after *in vitro* LPS administration to adult carp head kidney phagocytic cells[31]. iNOS produces nitric oxide, which has inhibitory effects of bacterial fish pathogens[90]. It must be noted that in all studies of adult carp *Escherichia coli* LPS was used, while in this study *Aeromonas salmonicida* LPS was injected.

In addition, *SAA* expression was up-regulated after LPS injection, although earlier then in *in vivo* infection experiments[28]. *SAA* expression increased after up regulation of *IL1-β*, suggesting *IL1-β* induced *SAA* expression. *C3* (the central protein of the complement system) and *α2M* (proteinase-binding protein[55]) expression were suppressed after LPS injection, which is in contrast to *in vivo* infections in adult carp[28]. In other fish, SAA and C3 are acute phase proteins that are up regulated after trauma or infection, but α_2M is not[77,91]. It is striking however that *α2M* expression in the PBS-injected group is moderately upregulated, while expression in the LPS-injected group is downregulated, implicating opposite effects from the injection procedure and LPS administration. It remains to be investigated whether the unexpected suppression of *α2M* and *C3* expression is related to the developmental stage, the type of LPS injected or other unknown factors. The acute phase proteins examined in this study were all expressed from 24 hpf onwards (1 day before hatching), indicating a crucial role in defence and tissue repair already early in development, but also a possible role in development itself as indicated by studies with C3[66,70].

PBS-injected embryos also displayed a significant increase in *SAA* expression and a moderate (inconsistent) increase in *IL1-β*, *iNOS*, and *α2M* expression (not in all embryos or offspring) compared to not-injected embryos. This increase is most probably due to wound healing processes after the injection procedure, which may vary considerably between animals. These variations probably also account for the differences between data obtained with RT-PCR and RQ-PCR, although these generally support each other.

In conclusion, carp myeloid cells first appeared ventro-lateral of the aorta at 2 dpf (the start of hatching), and subsequently around the sinuses of the vena cardinalis, head kidney and trunk kidney. The data obtained in this study implicate the aforementioned tissues as the myelopoietic sites in cyprinid ontogeny. In addition, the mAb's TCL-BE8 and WCL15 reacted with carp myeloid surface molecules that are already transcribed and processed from the first appearance of myeloid cells, and therefore offer a valuable addition to the study of the development of the teleost innate immune system. Furthermore, the hematopoietic tissue around the caudal sinuses of the vena cardinalis transformed into that of the trunk kidney, supplying the first specific description of the

ontogeny of the trunk kidney hematopoietic tissue in teleosts. Finally, this study associated the first appearance of myeloid cells with increased cytokine and acute phase protein expression, thus presenting the first record of immune gene expression kinetics in fish ontogeny: 48 hpf (hatching) embryos responded to LPS injection with upregulation of *IL1-β*, *iNOS*, and *SAA*, and downregulation of *C3* and α_2M, implying a functional embryonic innate defence system.

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Rag expression identifies B and T cell lymphopoietic tissues during the development of common carp (*Cyprinus carpio)*

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Developmental and Comparative Immunology, in press

Abstract

The generation of lymphoid cells during carp development was studied by analysing expression of the recombination activating genes (*rag*) using *in situ* hybridisation and real time quantitative PCR. These data were combined with immunohistochemistry using the mAb's WCL9 (cortical thymocytes) and WCI12 (B cells). Carp *rag-1* and *rag-2* showed 90% and 89% amino acid identity respectively to the corresponding zebrafish sequences. *Rag-1* was first expressed in the thymus at 4 days post fertilisation (dpf), while both *rag-1*$^{+}$/WCL9^{+} and *rag-1*$^{-}$/WCL9^{-} areas were distinguished from 1 week post fertilisation (wpf), suggesting early cortex/medulla differentiation. From 6 dpf, *rag-1*$^{+}$ cells were also present cranio-lateral of the head kidney. From 1 wpf, *rag-1*/*rag-2* was expressed in kidney (together with immunoglobulin heavy chain expression) but not in spleen, while WCI12^{+} cells appeared 1 week later in both organs, suggesting B cell recombination in kidney but not in spleen. *Rag-1* expression exceeded *rag-2* levels in thymus and in head- and trunk kidney of juveniles, but this ratio was reversed in head- and trunk kidney from approximately 16 wpf onwards. *Rag-1*/*rag-2* expression was detected in thymi of animals over 1 year old, but in kidney only at low levels, indicating life-long new formation of putative T cells but severely reduced formation of B cells in older fish.

Introduction

The common carp (*Cyprinus carpio* L.) is an important fish species for fish culture, which faces significant mortality especially in the early stages of life. Knowledge of the immune system in ontogeny can offer new protective strategies and indicate optimal vaccination points. The development of the carp acquired immune system was previously investigated using histology and monoclonal antibodies (mAbs)[50,78,92] or immunisation studies[6,7]. However, the lack of a pan-T cell marker in carp severely hampered the study of T cell development in this species. In addition, the limited number of Ig^+ cells found at early ages hinders the determination of the primary B cell organ(s) in juvenile carp.

Mature T- or B cells have experienced V(D)J recombination of their B cell receptor (BCR) or T cell receptor (TCR) genes. It is convincingly established that this recombination coincides with the expression of recombination activating gene-1 (*rag-1*) and *rag-2*[93,94], and can be performed exclusively by these proteins[95,96]. The *rag* sequence is widely conserved throughout vertebrates[97-101], and was demonstrated indispensable for V(D)J joining in a teleost fish[102]. Studying *rag-1/2* expression can therefore point out the temporal and spatial patterns of T- and B cell generation in the common carp.

In addition, thymocytes in mammals experience V(D)J recombination in the cortex, where after TCR cross linking with major histocompatibility complex (MHC) molecules results in positive selection as well as down-regulation of *rag-1* and *rag-2* expression in mature T cells[103]. Subsequently, thymocytes eventually undergo negative selection in the rag^- thymic medulla. The appearance of a rag^+ cortex and rag^- medulla in the carp thymus indicates the differentiation of mature T cells and will further contribute to determining the onset of T cell generation in carp.

Recently, a zebrafish (*Danio rerio*) study reported the development of B cells in the pancreas[13], although *rag-1* expression was also observed in the zebrafish kidney[13,45,104,105], which appears analogue to the bone marrow of mammals with respect to hematopoietic function. However, *rag* expression was not reported in the pancreas in other studies of zebrafish[43,45,105,106] or other fish[107]. The collection of more data concerning this subject will certainly shed light on the generation of teleost B cells.

In this study, we describe *rag* and Ig heavy chain (*IgH*) expression patterns in combination with immuno-histochemistry (IHC), using two lymphoid-specific mAb's (WCI12 for B cells and WCL9 for cortical thymocytes), from early development far into adulthood, allowing us to identify B and T cell lymphopoietic tissues in carp. In addition we will present an overview of carp lymphoid development based on previous data and this study.

Animals, Materials and Methods

Animals

Common carp (*Cyprinus carpio* L.) of the R3R8 strain were bred and kept in the facilities of 'de Haar Vissen', department of Animal Sciences of Wageningen University (The Netherlands). They were kept at 25°C for the first 5-6 weeks and subsequently at 23°C in circulating, filtered, UV-sterilised water. The embryos hatch at 2-3 days post fertilisation (dpf) and were fed with *Artemia salina* nauplii starting at 4 dpf for 3 weeks followed by Trouvit K30 pellets (Trouw & Co., Putten, The Netherlands). Animals over 1 year old were either of the R3 or R8 strain. Fish were anaesthetised with 0.03 % tricaine methane sulphonate (Crescent Research Chemicals, Phoenix, AZ) buffered with 0.06 % sodium bicarbonate to pH 7.2 in aquarium water.

Monoclonal antibodies

WCI12 is reactive with the IgM heavy chain and subsequently B cells of carp[22]. WCL9 reacts with a 155 and 200 kilo-Dalton membrane antigen on cortical thymocytes[24]. Both are mouse antibodies of the IgG_1 isotype.

Immunohistochemistry

Whole carp up to 29 dpf and organs were snap-frozen in liquid nitrogen; small embryos or organs had previously been embedded in 1.5 % agarose with 15 % sucrose or in gelatine. Frozen samples were stored at -80ºC. Serial transverse or longitudinal sections

(5-7 µm depending on the developmental stage of the embryo) were mounted on poly-L-lysine-coated microscope slides (BDH Laboratory Supplies, Poole, UK) and fixed in 4% paraformaldehyde (PFA) in pH 7.5 phosphate-buffered saline (PBS; WCI12 procedure) or cold acetone (WCL9 procedure) for 5 minutes (min). Then, sections were washed in PBS and incubated with WCI12 (1:100 in PBS) or WCL9 (1:100 in PBST-BL: see ISH protocol) for 1 h. After washing twice with PBS (PBST for WCL9), sections were incubated with Goat-anti-Mouse-FITC (GAM-FITC, Dako, Glostrup, Denmark; WCI12) for 1 h or horseradish peroxydase-labelled Goat-anti-Mouse (GAM-HRP, Bio-Rad laboratories, Hercules, USA; WCL9) for 45 min. Afterwards, sections with WCI12 were washed twice in PBS, embedded in Vecta-Shield Mounting Medium with propidium-iodide (Vector Laboratories, Burlingame, USA) and examined with a laser scanning microscope (Carl Zeiss laser scanning system LSM 510). Sections with WCL9 were incubated with 3-3'-diaminobenzidin (DAB; Sigma, Spruce St Louis, USA), washed in double distilled water and embedded in Aqua Mount Improved (BDH Laboratory Supplies).

Rag-1 and Rag-2 sequencing

Rag-1 and *rag-2* primers were designed on conserved parts of the corresponding zebrafish sequences (accession numbers U71093 and U71094 respectively) and employed in a homology cloning approach. PCR was performed on a carp thymus cDNA library[108]. The 5' and 3' ends were amplified with anchored PCR using λGT10 specific primers. PCR products were ligated and cloned in JM-109 cells using the pGEM-Teasy kit (Promega, Leiden, the Netherlands) according to the manufacturer's protocol. Plasmid DNA was isolated from single colonies using the QIAprep spin miniprep kit (QIAGEN, Leusden, The Netherlands) following the manufacturers' protocol. From each product, both strands of at least 8 clones were sequenced using the ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, USA) on an ABI 377 sequencer.

Probe synthesis

A digoxygenine (DIG) labelled carp *rag-1* probe was synthesised from a PGEM-Teasy (Promega) vector containing a 584 basepair insert using a DIG RNA labelling kit according to the manufacturers' instructions (Roche Applied Science, Basel, Switzerland). The probe was constructed from a conserved part of the *rag-1* sequence (nucleotides 1228-1807 of the carp *rag-1* sequence). Probe labelling was ascertained by Northern blotting. A second *rag-1* probe was constructed starting from a pBluescript KS9- vector (Stratagene, LaJolla, USA) containing a 638 basepair zebrafish *rag-1* insert (kindly provided by Dr. L. Steiner[97]), from another conserved part of the *rag-1* sequence.

In situ hybridisation on whole mounts and sections

Embryos and larvae were fixed several hours (4-24 dependent on size) in 4% PFA in PBS and stored at 4ºC in 1% PFA in PBS. They were washed for 2x5 min with PBS containing 0.1% Tween 20 (PBST), incubated in 0.1 M glycine in double distilled water water for 10 min, washed, and bleached with 3% H_2O_2, and washed. Cryosections (obtained as described under IHC) were fixed for 10 min in 4 % PFA in PBS and washed 3 times in PBST. Whole mounts were incubated in Pre-Hybridisation Mixture: 50% formamide, 2x SSC (Sodium chloride Sodium citrate: 0.3 M NaCl, 0.03M Na-citrate.$2H_20$ in double distilled water, pH 7), 2% Blocking Reagent (Roche Applied Science) and 0.1% Tween-20 in double distilled water for 7 min. Samples were incubated in hybridisation buffer (pre-hybridisation mixture + 0.5% Torula-yeast and 0.005% heparin for 10 min (2 h for whole mounts) at 68ºC and 70ºC for whole mounts (63ºC at all steps for the zebrafish probe), where after samples were incubated at 68ºC/70ºC with a pre-heated DIG-labelled *rag-1* probe in hybridisation buffer at a concentration of 0.05-0.5 ng/µl overnight. Subsequently, a number of stringent washing steps at 68ºC/70ºC were applied with decreasing concentrations of formamide and SSC containing 0.01% Tween (SSCT): 2x15 min (1x for whole mounts) 50% formamid/2x SSCT, 15 min 25% formamid/2xSSCT, 15 min (2x for whole mounts) 2x SSCT, 2x15 min 0.2x SSCT, 15 min 0.2x SSCT. During the last washing step samples were allowed to cool to room

temperature, followed by 2x5 min washing (10 min for whole mounts) in PBST. Then, sections were blocked with PBST containing 1 % Blocking Reagent (PBST-BL) for 30 min (4 h for whole mounts). Sections were incubated with anti-DIG/alkaline phosphatase (AP) 1:1000 (Roche Applied Science) for 1 h (1:2000 overnight for whole mounts). After washing 2x10 min in PBST-BL (6x25 min for whole mounts), 2x10 min PBST and pre-incubation with AP buffer (0.05 M Tris, 0.05 M NaCl, 0.025 M $MgCl_2.6H_20$, pH 9.5), samples were incubated in the dark with nitrobluetetrazolium (NBT; 0.09 µl/ml; Roche Applied Science) and 5'-bromo-4'-chloro-3'-indolyl phosphatase (BCIP; 0.035 µl/ml; Roche Applied Science) according to the manufacturers' protocol for approximately 45 min. Sections were washed in PBST and embedded in Aqua Mount Improved (BDH Laboratory Supplies). Sections of adult thymus were stained in Nuclear fast red for 5 min before embedding. Whole mounts were fixed in 4% PFA, washed and photographed. A carp complement factor 3 probe of equal length stained the liver in whole mount ISH, showing that probes in this ISH procedure penetrated into deep lying tissues.

RNA extraction and cDNA synthesis

In early development it is not feasible to isolate separate organs, therefore from 2 different offspring complete heads (containing thymus) and trunks (containing head- and trunk kidney, spleen and hepato-pancreas) from 3 animals from 2 dpf up to 7 dpf were pooled, snap frozen in liquid nitrogen and stored at -80˚C. Tissues (thymus, head kidney, trunk kidney, spleen, intestine and hepato-pancreas) were pooled from 1-30 animals (dependent on size) at 1,2,4,8, and 16 weeks post fertilisation (wpf) and ½, 2, 3, and 6 years. At the early stages, complete heads (1 wpf: containing thymus) and dorsal trunk regions (1-4 wpf: containing muscle, spine, dorsal skin and trunk kidney, but not head kidney, intestine, spleen, and hepato-pancreas) were frozen. The carp pancreas (with intertwined exocrine and endocrine tissue) is connected to the liver by the mesentery at 1 wpf, and from 2 wpf the two tissues are connected to each other (own unpublished observations based on consecutive sections of at least 6 juvenile carp). For RNA extraction, tissues were homogenised in lysis buffer (4 M guanidium thiocyanide, 25 mM sodium citrate pH 7.0, 0.5 % sarkosyl, 0.1 M 2-mercaptoethanol) followed by

phenol/chloroform extraction. Total RNA was precipitated in ethanol, washed and dissolved in water. Concentrations were measured by spectrophotometry (Genequant, Pharmacia Biotech, Uppsala, Sweden). 0.1 – 1 μg RNA was DNase treated (Deoxyribonuclease I, Amplification Grade, Invitrogen, Carlsbad, U.S.A.) and subsequently cDNA was synthesised using RT Superscript™ (Invitrogen) according to the manufacturer's protocol. RNA was also treated identically but without RT Superscript™, yielding non-RT controls.

Real time quantitative-polymerase chain reaction (RQ-PCR)

cDNA was added to 2x QuantiTect SYBR Green PCR kit (Qiagen, Leusden, the Netherlands), 1.5 μl 5 μM forward and reverse primers and additional water to a total volume of 25 μl. The following PCR protocol was run on a Rotorgene (Corbett, Sydney, Australia) according to the manufacturer: an initial denaturation step at 94˚C for 15 min was followed by 45 cycles with a denaturation step at 94˚C for 30 sec, an annealing step at 60˚C for 30 sec and an extension step at 72˚C for 30 sec, and subsequently a melting curve running from 60˚ to 90˚ with 1 min intervals. The Ct value of a reaction mixture defines the point when fluorescence (generated by Sybr-green I that attaches to double stranded DNA) reaches the threshold, which was set at the same point for all primer combinations in the exponential phase of the reaction.

β-actin and *40S* were used as house keeping genes (HKG) and *rag-1* and *rag-2* as genes of interest (GOI). Primer sequences were as follows: *β-actin* FW 5'-GCTATGTGGCTCTTGACTTCGA-3'; RV 5'-CCGTCAGGCAGCTCATAGCT-3'; *40S* FW 5'-CCGTGGGTGACATCGTTACA-3' and RV 5'-TCAGGACATTGAACCTCACTG TCT-3'; *rag-1* FW 5'-CATGGTGCTACATTCCATCACA-3' and RV 5'-TCACGCAGTT

CATCAGCAGACT-3'; and *rag-2* FW 5'-TTGGCGGCAGGTCCTACA-3' and RV 5'-AGTCCACCACGCTGTTCCA-3'. The efficiencies (E) of the different primer sets were determined with a dilution series of adult thymus cDNA: 2.09 (*β-actin*), 1.99 (*40S*), 1.91 (*rag-1*) and 2.04 (*rag-2*). R-values were calculated with Pfaffl's method $(E^{GOI\wedge}(Ct^{reference}\text{-}Ct^{sample})/E^{HKG\wedge}(Ct^{reference}\text{-}Ct^{sample})$[82], using a sample with insignificant *rag-1*

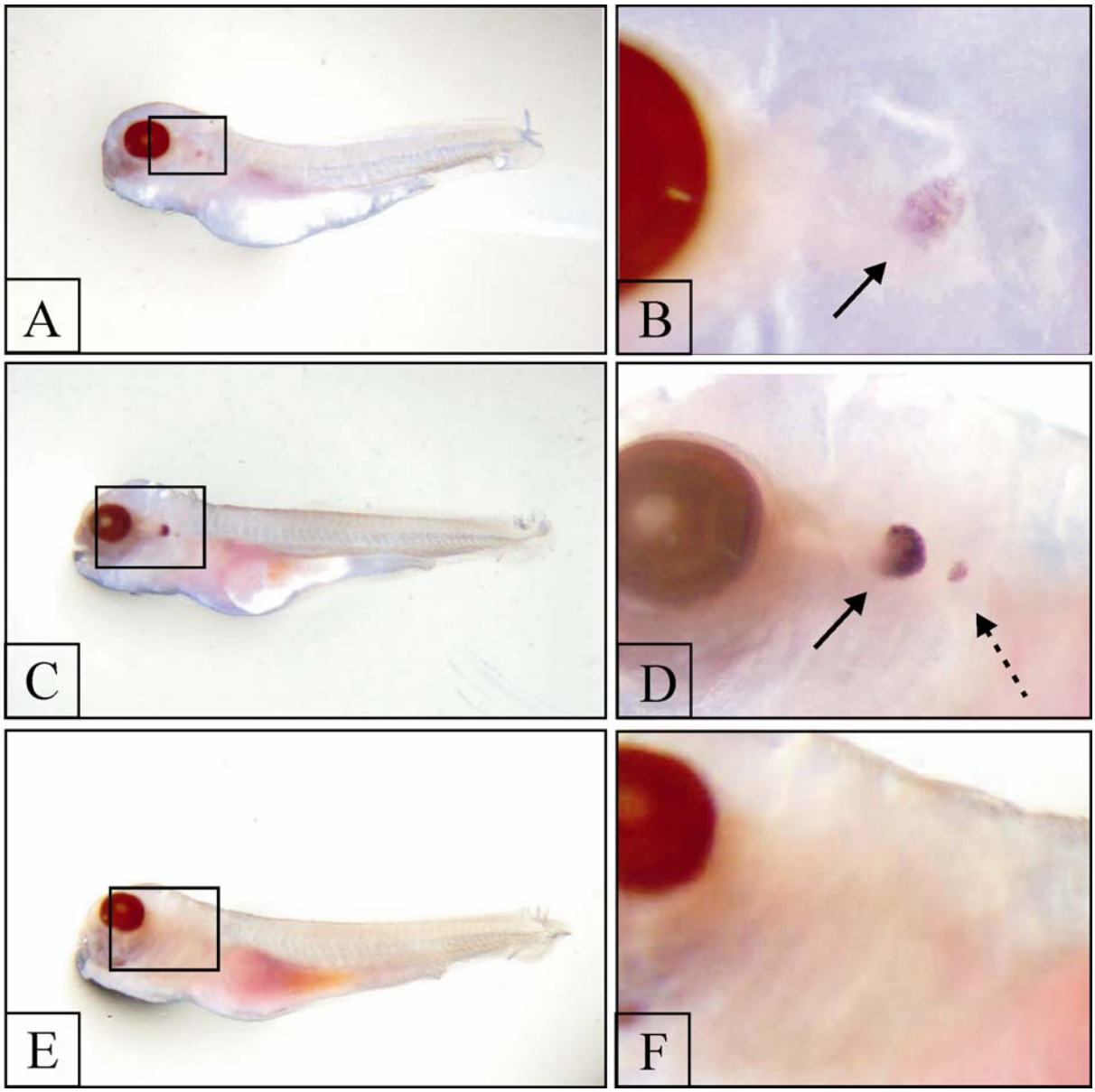


Figure 1. *Rag-1* expression in larval carp. Following whole mount *ISH* with a carp *rag-1* probe, a distinct bilateral group of cells situated just caudal of the eye and dorsal to the gills appeared from 4 dpf onwards (Panels A-D: closed arrows). This area coincides with the location of the thymus. From 6 dpf, a second but smaller, also bilateral group of was present (Panels C and D: dashed arrow). Panels B, D, and F are magnifications of the boxed areas in panels A, C, and E respectively. Panel E and F depict a negative control performed with a *rag-1* sense probe at 6 dpf.

and *rag-2* expression as a reference. R-values calculated with either *β-actin* or *40S* as HKG were comparable if not identical, consequently only values calculated with *β-actin* are shown. Non template controls (NTC's) were always included. For every sample, non-RT controls were also tested with *rag-1* and *rag-2* primers. Both controls consistently had high (> 30) Ct values. Samples were considered 'detectable' when Ct values were at least 3.2 cycles lower compared to the corresponding NTC or non-RT controls. All data points are averages of duplicate measurements.

Semi-quantitative polymerase chain reaction

Detection of immunoglobulin heavy chain transcripts was performed by PCR on head kidney cDNA at 1,2,4 and 8 wpf with the following conditions: denaturation at 95ºC for 5 min; 35 cycles of 30 sec at 94ºC, 30 sec at 55ºC, 30 sec at 72ºC and post extension for 7 min at 72ºC. The forward primer for the immunoglobulin heavy chain was constructed on the 3' side of the zebrafish variable part FR3 (5'-CWGCYGTNTATTAYTGTGC-3') [109] and the reverse primer on the carp constant region (5'-AGCGAGTCYGCAGGTGAGAAACCT-3')[110]. *β-actin* was used as a HKG. The products were visualised by separation on a 1.5 % agarose gel containing 0.5 % ethidium bromide. The luminescence of the PCR product was measured with the Multi-Analyst Gel-Doc 1000 software (Bio-Rad Laboratories). Values were expressed as the ratio of *IgH* expression level and the corresponding *β-actin* level after subtraction of background luminescence. PCR product identity was confirmed by sequencing.

Results

Characteristics of carp rag-1 and rag-2 sequences

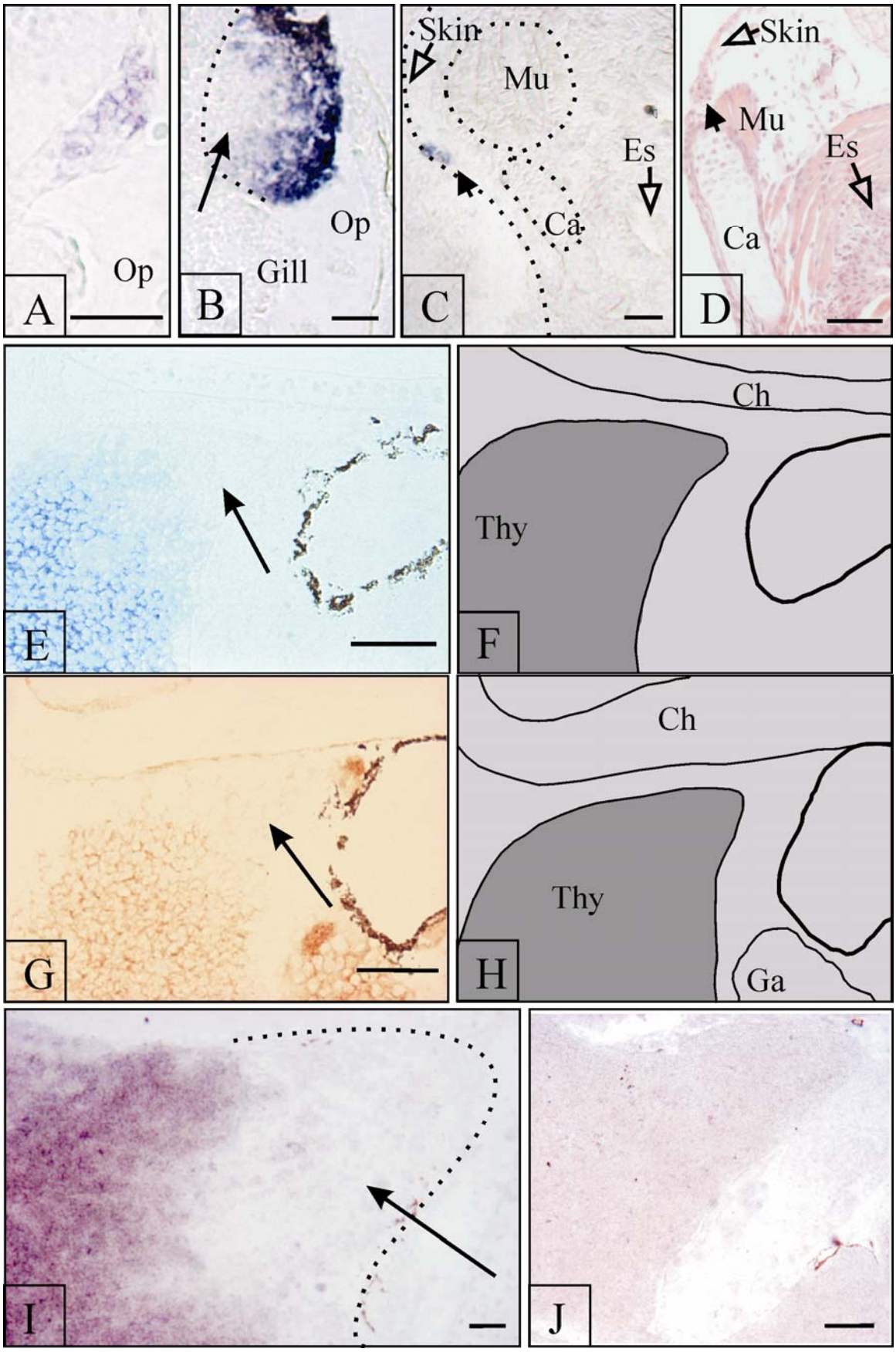
The cDNA sequences for carp *rag-1* and *rag-2* can be accessed with the numbers AY787040 and AY787041. The carp *rag-1* sequence consisted of 3186 nucleotides and encoded a 1062-amino acid protein. It displayed an 89 % nucleotide identity and 90 % amino acid identity with zebrafish *rag-1*. The *rag-2* sequence consisted of 1593

nucleotides and encoded a 531-amino acid protein, which displayed an 87 % nucleotide identity and 89 % amino acid identity with zebrafish *rag-2*.

Rag-1 expression and WCL9$^+$ cells in the thymus of carp larvae

Whole mount *in situ* hybridisation (ISH) was performed with a *rag-1* probe on carp larvae (Figure 1). At 4 dpf initial *rag-1* expression was detected in an area located caudal to the eye and dorso-lateral to the gills (Figure 1A and B). This expression coincides with the location of the carp thymus at later ages. At 6 dpf (Figure 1C and D), *rag-1* expression in the thymus was more intense and also limited to the most superficial part of the organ. In addition, a second, more caudal focus of *rag-1* expression was observed. This second area was significantly smaller than the presumed thymus, although the staining had a comparable intensity. The area was generally bilateral, although occasionally unilateral, and had a superficial location. This second focus of *rag-1* expression was observed in two different offspring in all larvae (> 20) examined, using both the carp *rag-1* probe and the zebrafish *rag-1* probe from 6 dpf at least up to 12 dpf (results not shown). *Rag-1* expression was not detected in any other part of the larvae, and reactions with the corresponding *rag-1* sense probes on carp larvae did not result in any staining (Figure 1E and F).

To investigate *rag-1* expression in larvae and juveniles in more detail, cross sections of the head region were used from the first stage of thymus development onwards (Figure 2). *Rag-1* expression was detected throughout the thymus at 4 dpf (Figure 2A). From 7 dpf, the lateral/superficial part of the thymus expressed *rag-1*, but the medial part did not (Figure 2B, E). In addition, the mAb WCL9 reacted with thymocytes in the *rag-1*$^+$ part of the thymus, whereas the medial part was both *rag-1*$^-$ and WCL9$^-$ (Figure 2E-H). The second *rag-1*$^+$ focus from Figure 1 was situated immediately under the skin, cranial to the renal tubes and the renal hematopoietic tissue (Figure 2C), indicating that these *rag-1*$^+$ cells were not situated in the head kidney. In corresponding haemalum/eosin stained paraffin sections of several (6 out of 6 examined) 7 dpf larvae, a generally bilateral consolidated group of cells was detected with a similar size and location as the second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rag-1^{+} group (Figure 2D). Kidney tubules were not detected in any of these sections, but consistently appeared more caudal, with no histological connection to the *rag-1*$^{+}$ group at least up to 14 dpf. In pre-adult (Figure 2I) and adult carp (18 months old, results not shown) *rag-1*$^{+}$ and *rag-1*$^{-}$ areas were still distinguished in the thymus, although they were irregularly distributed.

We consistently obtained comparable results using both the carp *rag-1* probe and the zebrafish *rag-1* probe. Reactions with the corresponding *rag-1* sense probes on carp thymus sections did not result in any staining (Figure 2J). With sections of other tissues (intestinal epithelium, kidney hematopoietic tissue, spleen, and pancreas, but not muscle, lamina propria of intestine, brain) we obtained scattered unspecific staining with both *rag-1* probes but also with other unrelated probes (*complement factor 3* antisense and sense), so that we could not draw any conclusions about *rag* expression in these organs. For this reason we performed RQ-PCR to study *rag* expression in other tissues.

Figure 2 (previous page). *Rag-1* expression and WCL9 immuno-reactivity in the thymus of carp. Both a carp *rag-1* probe (panels A, B, C, I, and J) and a zebrafish *rag-1* probe (panel E) were used. The first *rag-1* expression was detected in the presumed thymus at 4 dpf (A). At 7 dpf (B) the thymus consisted of a *rag-1*$^{+}$ lateral part and a *rag-1*$^{-}$ medial part (arrow). The dashed line represents the boundary of the thymus. Panel C shows the second, most caudal group of *rag-1*$^{+}$ cells (closed arrowhead) at 7 dpf, with a corresponding haemalum/eosin stained section in panel D. At 9 dpf *rag-1* (E, F) and WCL9-ligand (G, H) were both expressed on identical locations in the largest, most superficial part of the thymus (cortex), leaving a small medial part negative (medulla; arrow). Panels E and G are serial sections. WCL9 cross-reacts with neural tissue (G, H). Panel I depicts *rag-1* expression in the thymus of adult carp: the dashed line represents the thymic boundary and the arrow the *rag-1*$^{-}$ medulla of the thymus, panel J shows a thymus processed with a sense *rag-1* probe (sections were counter stained with Nuclear Fast Red). Panels A, B, and C represent pictures taken from sections of whole mount ISH reactions. Panels F and H show pictures taken of sections that are represented schematically in panels E and G respectively. Dark grey areas in panels F and H indicate the thymus, whereas light gray areas represent all other tissue, and the black circle represents pigment cells. Ca = cartilage, Ch = chondrocranium, Es = Oesophagus, Ga = ganglion, Gill = gill arches, Mu = muscle, Op = operculum, Thy = thymus. Bar is 50 μm.

Rag expression during development measured with RQ-PCR

In carp development, *rag-1* expression was first detected in the head region at 3 dpf (Figure 3), this expression increased substantially at 4 dpf and then levelled of from 5 dpf onwards. *Rag-1* expression in the trunk was observed at 6 and 7 dpf, although at a significantly lower level.

From 7 dpf it is possible to isolate separate organs from the larvae. We tested thymus, head kidney, trunk kidney, spleen and hepato-pancreas from 7 dpf up to 6 years for *rag-1* and *rag-2* expression (Figure 4). In the thymus, *rag-1* and *rag-2* were detected from 1 wpf, although the expression level at 1 wpf was very low (Figure 4A). *Rag-1*

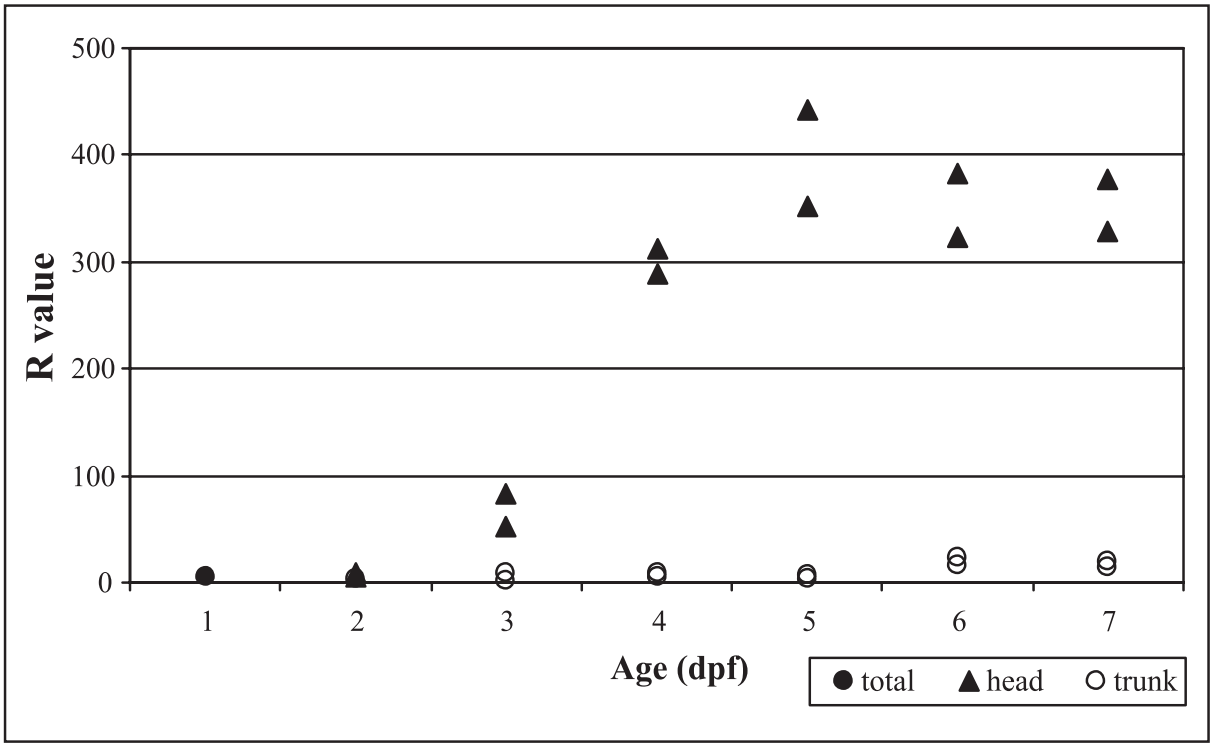

Figure 3. *Rag-1* expression in embryos and larvae from 1 dpf up to 7 dpf. Total embryos (total: only 1 dpf: closed circle), head regions (head: closed triangles) and trunk regions (trunk: open circles) of carp embryos and larvae were tested for the presence of *rag-1* with RQ-PCR. All data were normalised against *β-actin* levels and compared to a sample with insignificant *rag-1* expression (muscle of adult carp) using Pfaffl's method: R values are displayed on the vertical axis. Age is displayed on the horizontal axis.

expression exceeded *rag-2* expression by far at all ages. *Rag-1* and *rag-2* were still expressed in the thymus of animals over 1 year old, although thymi of these fish were considerably smaller when compared to ½-year-old fish.

In head kidney and trunk kidney, *rag-1* and *rag-2* were expressed at considerably lower levels compared to the thymus (Figure 4B and C). Until 4 wpf, similar to the pattern in the thymus, *rag-1* expression consistently exceeded *rag-2* expression (although the difference was not as large as in thymus), but from 8-16 wpf onwards *rag-2* levels were higher than *rag-1* levels. *Rag-1* and *rag-2* were expressed at high levels in both head kidney and trunk kidney at ½ years, but expression was low in animals over 1 year old although 1 animal of 6 years old displayed moderate *rag-2* expression.

Rag-1 and *rag-2* expression was not detectable in the spleen, while in the hepato-pancreas *rag-1* expression was not detectable and *rag-2* expression was very low (Ct > 30 cycles).

Detection of IgH transcripts and WCI12$^+$ cells

To ascertain first *IgH* expression, a semi-quantitative PCR was performed on cDNA of the trunk of embryos from 3 dpf until 1 wpf and of head kidney from 1 wpf up to 8 wpf (Figure 5). No *IgH* transcripts were detected in the trunk part of embryos up to 1 wpf (results not shown). In head kidney, low-level expression was detected at 1 wpf, whereupon levels increased considerably at later ages.

In previous studies low percentages of WCI12$^+$ cells (IgH containing cells) were found in the head kidney and spleen from 2-3 wpf[78]. To pinpoint the first appearance of WCI12+ cells, we performed IHC using WCI12 on head- and trunk kidney and spleen (Figure 6). We did not observe WCI12$^+$ cells at 10 dpf, but first detected them in spleen and head- and trunk kidney at 2 wpf (Figure 6A and B). At 4 wpf the number of WCI12$^+$ cells had increased substantially and also more intensely staining cells were discerned (Figure 6C). At 6 wpf the number of WCI12$^+$ cells had increased further in both head- and trunk kidney (Figure 6D) and spleen (results not shown).

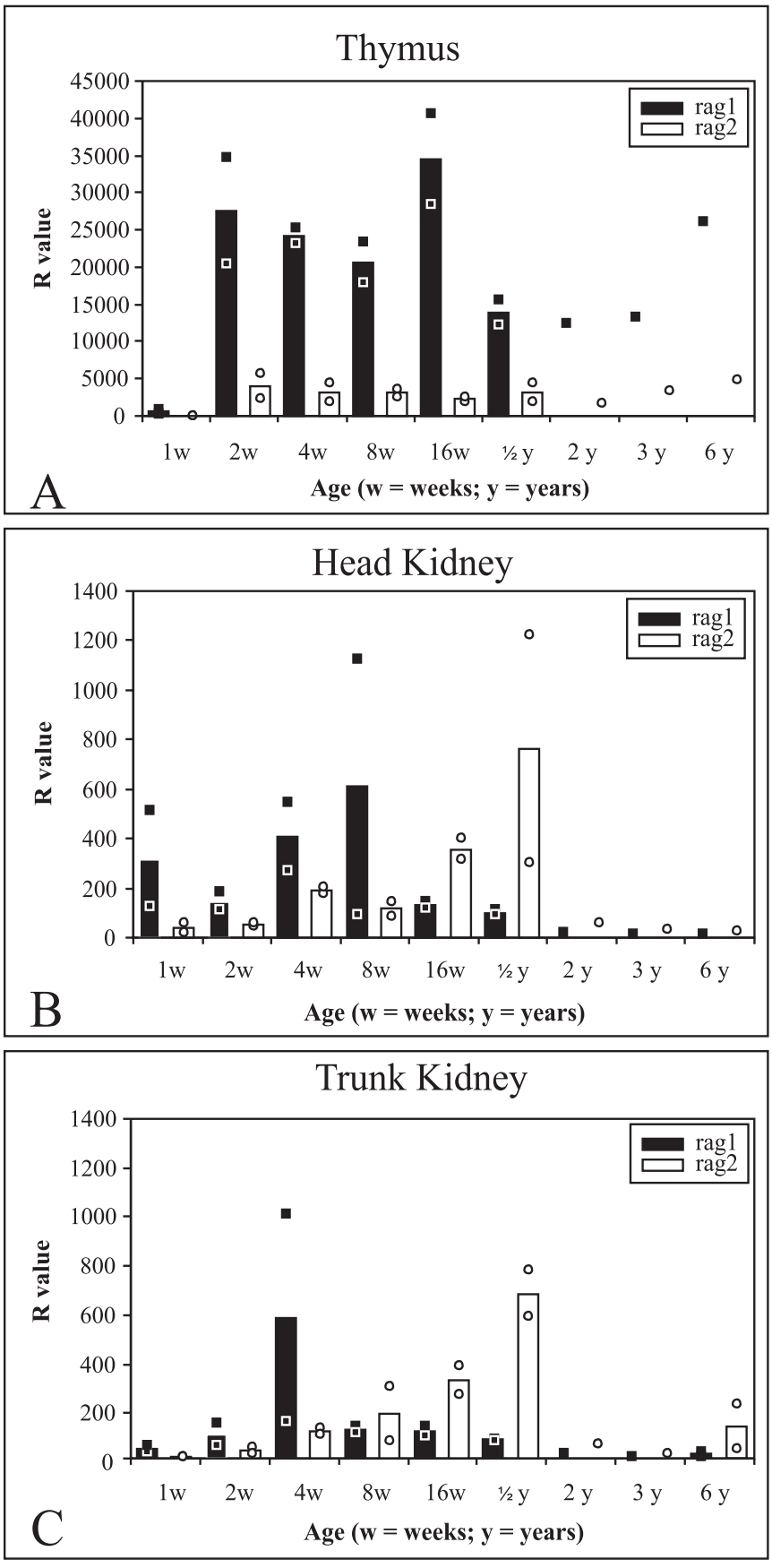
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Figure 4 (previous page). *Rag-1* and *rag-2* expression in carp thymus (A), head kidney (B) and trunk kidney (C) detected with RQ-PCR from 1 wpf up to 6 years. All data were normalised against *β-actin* levels and compared to a sample with insignificant *rag-1*/*rag-2* expression (muscle of adult carp) with Pfaffl's method: R-values are displayed on the vertical axis. On the horizontal axis age is displayed in weeks (w) or years (y). For all time points the average of two offspring is given (bar), with the individual measurements displayed as squares (*rag-1*) or circles (*rag-2*).

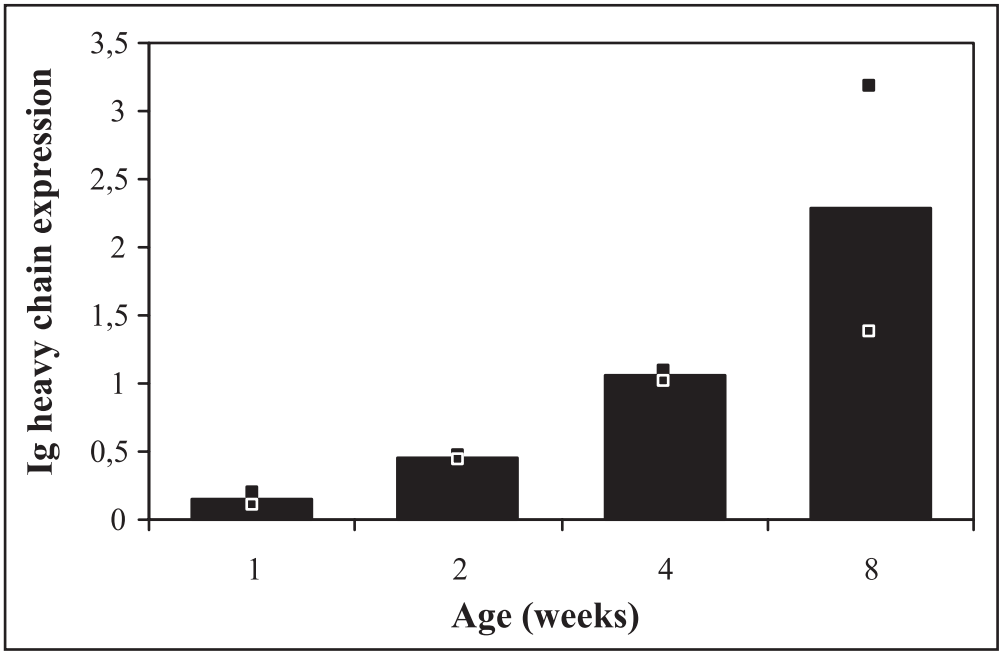

Figure 5. Detection of *IgH* transcripts in carp head kidney during development with RT-PCR. The ratio of *IgH* levels/ *β-actin* levels is displayed on the vertical axis, while age is displayed on the horizontal axis. For all time points the average of two offspring is given (bar), with the individual measurements displayed as squares.

Discussion

To investigate the generation of T and B cells in carp development, *rag* expression was studied in combination with the appearance of $WCL9^+$ cells (cortical thymocytes), $WCI12^+$ cells (B cells) and *IgH* expression. Major events of lymphopoiesis in carp are summarised in Figure 7 (data are from this work and previous studies).

Rag-1 expression was first detected with RQ-PCR in heads of embryos at 3 dpf, and with ISH in the thymus at 4 dpf. The *rag-1*- and *2* genes perform V(D)J recombination in mammals[93-96] and in fish[102], which implies that V(D)J recombination starts as soon as the carp thymus is colonised with lymphoid cells[50]. In zebrafish (cyprinidae) initial *rag-1* expression in the thymus was found at a similar age using ISH when kept at 28.5 C°[42,105], although the thymic primordium can be identified earlier than in carp (54 hpf)[105]. The discrepancy between results of the two different techniques used in this study can be explained by higher sensitivity of RQ-PCR.

However, the expression of *rag-1* in the thymus (and presumably the recombination process of TCR) does not mean that functional T cells have been formed at that time. It was shown that approximately 10 days later, at 16 days post hatch (18-19 dpf), carp can reject skin allografts[6,111]. This rejection can be attributed to either T cells or non-specific cytotoxic cells. Other studies showed that immunised carp can produce antibodies when injected with thymus-dependent antigens like human gamma globulin (HGG) at 9-10 wpf[7], while they were tolerant when injected with HGG[7], sheep red blood cells[5], or when orally vaccinated[8] at 4 wpf. When taking into account the scale graft rejection at an earlier age, initial T cell generation possibly gives rise to predominantly cytotoxic T cells rather than T helper cells. However, this is not described in mammalian literature, although in contrast to adult mice, around birth the number of CD8+ cells exceeds the number of CD4+ cells[112]. Another possibility is that non-specific cytotoxic cells performed the observed scale graft rejection. Unfortunately no information is available on the ontogeny of these cells in fish. Consequently, the gap between first *rag-1* expression and the demonstration of T cell functionality is considerable.

A *rag-1*$^+$/WCL9$^+$ area was distinguished from a *rag-1*$^-$/WCL9$^-$ area in the thymus from approximately 7 dpf, which indicates that a cortex/medulla organisation is present from this stage. As a consequence, cortex and medulla exist earlier in carp thymus than was described using histology and the distribution of WCL9$^+$ cells in previous studies[24,50]. Consistent with our results, a previous study concluded that MHCI does not play a role in ontogeny until 7 dpf[113]. Another confirmation of this organisation and hence commencing positive/negative selection at 7-9 dpf is the fact that apoptosis was not found in carp

thymus using *in situ* end-labelling of fragmented DNA up to 1 wpf[50]. In zebrafish a cortex/medulla demarcation was found in a recent study with *rag-1* expression at 1 wpf and histologically at 3 wpf[114]. With respect to the functionality of the zebrafish thymus in ontogeny, TCRα transcripts were detected in the thymus at 4 dpf[16], and an antibody response towards a thymus-dependent antigen (HGG) was observed after immunisation at 6 wpf[45].

Rag-1 and also *rag-2* were expressed in the thymus of animals over 1 year old, which indicates a life-long generation of T cells. In addition, we found that the carp thymus continuously grows to its maximum size at approximately 6-12 months, after which it reduces over the following years. This is in accordance with decreasing *rag-1* expression and thymic involution in the adult zebrafish[114], and with morphological studies in carp[115] as well as in cichlid fish[116]. In 17-months-old channel catfish however, the thymus cannot be distinguished macroscopically and does not contain any lymphocytes[117]. Indeed the age-related regression, that characterises the thymus of higher vertebrates, occurs in some fish species but not in others. In addition, thymus involution in fish can also be affected by sexual maturity, stress or seasonal changes[118]. In mammals it was shown that bone marrow cells of 'old' mice still retain their potential to give rise to new T cells, but that this development is determined by the thymic stroma[119]. Apparently both lymphoid precursor cells and thymic stroma of adult carp retain the capacity to generate new T cells, although the volume of thymus tissue was decreased. Indeed the thymus remains functional in 'older' animals of several fish species: thymectomy affected immune responses in ½-year-old rainbow trout (*Oncorhynchus mykiss*)[120], and adult rock fish (*Sebasticus marmoratus*)[121]. The absence in fish of T cell proliferating areas, like in the lymph nodes and spleen of mammals, might explain the difference in thymic involution between mammals and fish, although this remains to be investigated for fish species showing complete involution of the thymus.

A second *rag-1*$^{+}$ focus was observed with whole mount ISH from 6 dpf, which was confirmed with RQ-PCR on embryo trunks, until at least 12 dpf. The *rag-1*$^{+}$ cells were located superficially under the skin and cranio-lateral of the head kidney, and were therefore clearly not part of the hematopoietic tissue of the head kidney. To the author's

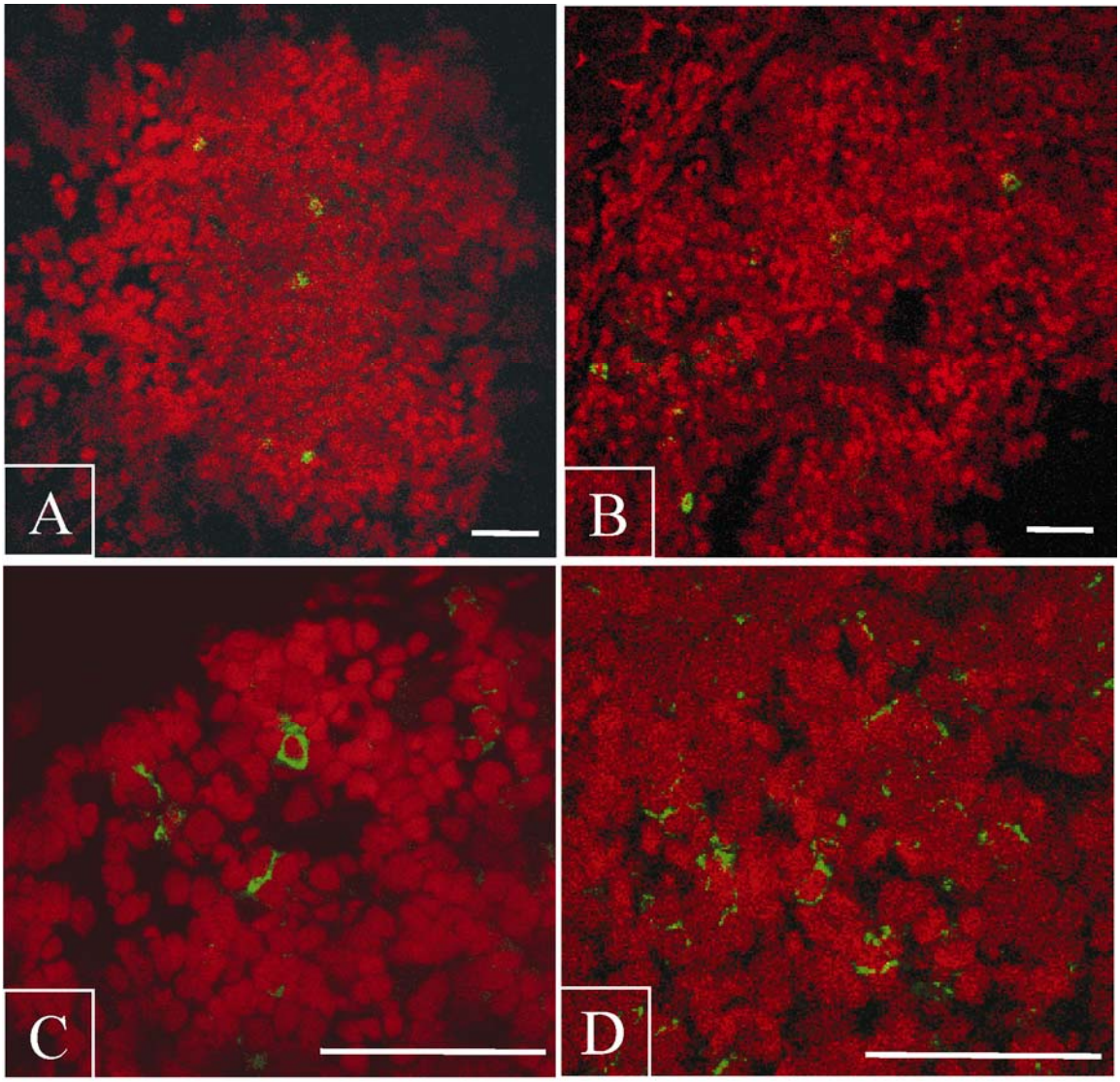

Figure 6. B cells (WCI12$^+$ cells) detected with immunohistochemistry in head kidney and spleen during the first weeks of carp development. WCI12$^+$ cells are green fluorescent and all nuclei are red fluorescent after treatment with propidium-iodide. Isolated positive cells appeared in spleen (A) and head kidney (B) from 2 wpf. At 4 wpf, the number of positive cells in head kidney had increased and more intensely stained cells were found (C). The number of cells had substantially increased at 6 wpf in both head kidney (D) and spleen (not shown). Bar is 50 μm.

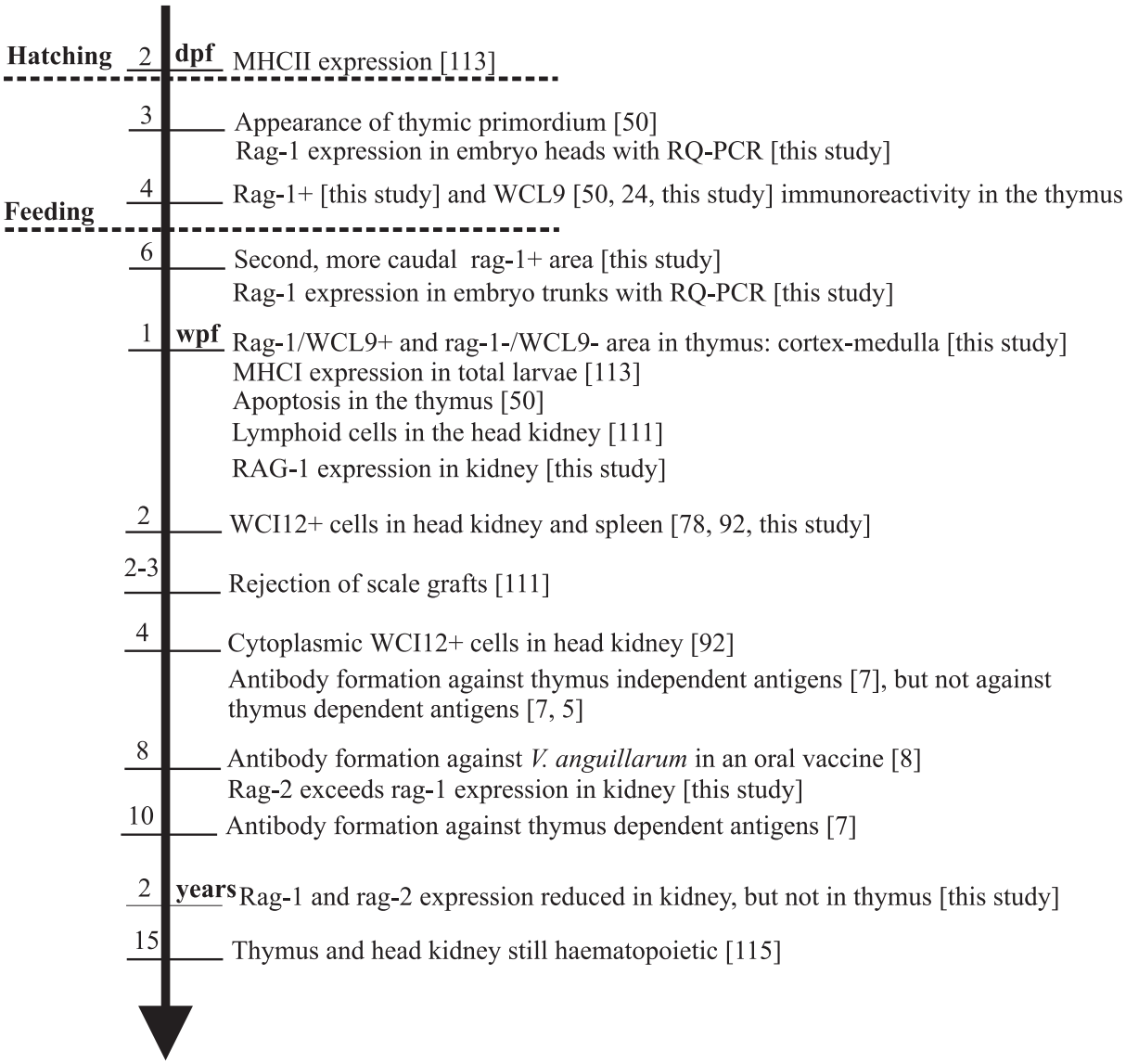

Figure 7. Events in the ontogeny of the carp acquired immune system. Data are based on previous papers (described in the text) and present study.

best knowledge, no tissue structures with similar characteristics were described in other teleosts including zebrafish[43,105,106,122], or cartilaginous fish, although comparable ages and techniques were used only in zebrafish. This phenomenon may therefore be carp-specific. *Rag* expression has been found in neural tissues: olfactory placodes of zebrafish[106] and the central nervous system of mice[123]. At present, it cannot be excluded that these cells are also of neural origin. The *rag-1* expression in this small group of lymphoid cells

implies that recombination occurs in this region. The superficial location bares similarity to the sub-epithelial location of the thymus. The second *rag-1*$^+$ focus might produce T cells or B cells or contain neural cells, however further research is necessary to characterise this group of recombining cells.

Rag-1 and *rag-2* were first detected in head- and trunk kidney at 1 wpf, which, similar to the thymus, implies that V(D)J recombination starts as soon as lymphoid cells are present in the kidney[6]. Together with expression of *IgH* and Ig positive cells at 2 wpf, this indicates the onset of B cell generation in the kidney at that stage. In mammals, *rag-1*, *rag-2*, and *IgH* transcription is followed by expression of a pre B cell receptor complex (which also contains IgH) on the surface, and eventually the BCR[124]. The initial WCI12$^+$ cells at 2 wpf might still represent immature B cells. *IgH* was transcribed more extensively at 4 wpf, coinciding with a higher abundance and the appearance of intensely stained WCI12$^+$ cells. This corroborates previous studies in carp, which demonstrated an abrupt increase of the percentage of WCI12$^+$ cells at 4 wpf[78] as well as the occurrence of cells containing cytoplasmic Ig in predominantly head and trunk kidney[92]. These findings all consistently correlate with the fact that neutralising antibodies were produced after *Aeromonas salmonicida* vaccination of 4-weeks old carp[7,125]. With respect to the generation of lymphoid cells in zebrafish, rearrangements of the membrane form of *Igμ* were detected with PCR on cDNA as soon as 7 dpf[13], which is in line with our observations. However, the detection of *rag-1* in the head- kidney with ISH only from 2-3 wpf onwards[13,45], is considerably later then found for carp, especially when taking into account that zebrafish develop faster than carp. This can however be attributed to the fact that ISH is less sensitive than RQ-PCR.

WCI12$^+$ cells appeared in the spleen around the same time as in head- and trunk kidney, but in contrast to these organs *rag-1*/*rag-2* expression was not detectable in spleen, and consequently it does not play an intensive role in the recombination of lymphoid cells in carp. Also in zebrafish, *rag-1* was not detected in the adult spleen[13], but in rainbow trout both *rag-1* and terminal deoxynucleotidyl transferase were expressed at moderate levels in adult spleen[14]. In mammals, B cells complete their recombination process in the bone marrow before migrating to the spleen for further maturation. Studies

with transgenic mice emphasised that only a small number of *rag*-expressing immature B cells are actually located in the spleen[126]. Consequently, *rag-1* expression in the spleen of zebrafish and carp resembles that in mammals, while it is different in rainbow trout.

In zebrafish, *rag-1* and *Igμ* transcripts were reported in the pancreas from 4 dpf and 10 dpf respectively[13]. The authors concluded that B cells develop in the zebrafish pancreas. In our study of the common carp, *rag-1* expression was not detectable in the hepato-pancreas with whole mount ISH or RQ-PCR, while a very low level of *rag-2* expression was detected with RQ-PCR. Throughout development we never detected lymphoid cells in carp hepato-pancreas (using only histology). In addition, *rag-1*/*rag-2* expression in pancreas was not reported in other zebrafish studies[43,45,105,106] nor adult pufferfish[107]. The low level of *rag-2* expression detected in the hepato-pancreas in our study can possibly be attributed to small numbers of peripheral B cells or T cells containing *rag-2*, as was shown for peripheral B cells in mice[127] and T cells in human liver[128], or to a recombination process similar to zebrafish[13]. In conclusion, in contrast to the findings in zebrafish we only found minimal *rag* expression in the hepato-pancreas, which might be explained by species differences.

In head kidney and trunk kidney, *rag-1* and *rag-2* were expressed up to ½-year-old carp, while expression was generally low in older animals. This suggests that formation of lymphoid cells in the head- and trunk kidney is diminished somewhere between ½ and 2 years of age. In zebrafish[13,106], rainbow trout[14], and fugu[107] *rag-1* expression was still detected in the kidney of adult animals, although a clear definition of 'adult' lacks in these studies. The kinetics of *rag-1*/*rag-2* expression in the carp head kidney and trunk kidney is consistent with the *rag-1* expression in mouse bone marrow, which has a declining *rag-1* expression with age[129].

Rag-2 expression in head- and trunk kidney exceeded the level of *rag-1* expression from 16 wpf onwards. In general *rag-1* expression exceeds *rag-2* expression[103,105]. Literature about *rag-1*/*rag-2* ratios is scarce, although on the cellular level B cells were found with either *rag-1* or *rag-2* expression in human tonsils and peripheral blood[130]. It is possible that the regions outside the core of the *rag-1/rag-2* proteins help in the proper regulation of recombination, with for instance distinct roles for these proteins in T- or B

cell lineages[131]. A clear explanation, however, for the change of *rag-1*/*rag-2* ratios in carp kidney cannot be given so far with the data obtained on the carp immune system.

In summary, the cortex/medulla distinction in carp thymus was estimated at 7 dpf using *rag-1* expression patterns. *Rag* expression and thus recombination starts as soon as the thymus and the head kidney contain lymphoid cells and continues throughout carp life, although severely reduced in the head kidney and trunk kidney of adults. *Rag* expression in head- and trunk kidney can be detected from 1 wpf, which together with the presence of Ig transcripts suggests recombination of B cell receptors. *Rag* expression was not found in spleen and at very low levels (only *rag-2)* in hepato-pancreas. The latter finding does not convincingly show that carp B cells originate from the pancreas as in zebrafish.

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The ontogeny of mucosal immune cells in common carp (*Cyprinus carpio* L.)

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Abstract

The ontogeny of carp (*Cyprinus carpio* L.) immune cells was studied in mucosal organs (intestine, gills, and skin) using the monoclonal antibodies WCL38 (intraepithelial lymphocytes), WCL15 (monocytes/macrophages), and WCI12 (B cells). In addition, recombination activating gene 1 expression was examined in the intestine with real time quantitative PCR and *in situ* hybridisation to investigate extrathymic generation of lymphocytes. $WCL38^{+}$ intraepithelial lymphocytes (putative T cells) appeared in the intestine at 3 days post fertilisation (dpf), which is shortly after hatching but before feeding, implying an important function at early age. These lymphoid cells appear in the intestine before the observation of the first thymocytes at 3-4 dpf, and together with the expression of recombination activating gene 1 in the intestine, suggests that alike mammals at least part of these cells are generated in the intestine. $WCL15^{+}$ monocytes/macrophages appeared in the lamina propria of the intestine at 7 dpf, but considerably later in the epithelium, while $WCI12^{+}$ (B) cells appeared in intestine and gills at 6-7 weeks. From these results it can be concluded that putative T cells occur much earlier than B cells, and that B cells appear much later in the mucosae than in other internal lymphoid organs (2 wpf).

Introduction

Over the past 15 years, production of farmed fish more than doubled, of which carp species are quantitatively the most important[1]. A major problem accompanying this vast increase is the occurrence of infectious disease, which causes high losses in cultured fish, especially larvae and juveniles. Vaccination or immuno-stimulation can restrict this problem, although the latter method is rather unprecedented. Vaccines are often administrated via injection, but administration via mucosal surfaces is less stressful and may be a cost effective and labour-extensive alternative[132,133]. When applying antigens or immuno-stimulants to mucosal surfaces however, knowledge of the ontogeny of the mucosal immune system is imperative, because fish become immuno-tolerant when vaccinated via the intestine at a too young age[8].

Carp (*Cyprinus carpio* L.) embryos hatch at 2 days post fertilisation (dpf) and start feeding at 5 dpf. Cells[26] and humoral factors (chapter 2, 3) of the innate immune system are already present at 2 dpf, while the thymus (the first organ of the adaptive immune system) starts developing from 3-4 dpf (Chapter 4)[50]. Systemic antibody responses appear considerably later at 1-2 months, while juvenile tolerance is evoked before this age[5,7,51]. Knowledge on the ontogeny of the mucosal immune system in carp however is limited.

In adult fish mucosal immune cells are diffusely distributed[134,135], and do not form localised accumulations of immune cells (e.g. Peyers Patches) like in mammals. The carp intestine contains many immunoglobulin positive (Ig^+) lymphoid cells (recognised with the mAb WCI12[22]) and granulocytes in the lamina propria, but also (Ig^+) macrophages (recognised with the mAb WCL15[25,26]) and even more Ig^- lymphoid cells in the epithelium[135]. Macrophages[8] have been implicated in the uptake of macromolecules in the 2nd segment of the intestine[136]. A considerable amount of data indicates that the mucosal immune system is separate from the systemic immune system[23,137,138]. It has been established that mucosal antibodies in adult carp differ from serum antibodies in composition[139] and response after route of administration and antigenic composition[138].

The monoclonal antibody (mAb) WCL38 is reactive with an Ig^- subpopulation of carp intraepithelial lymphocytes (IEL), which are present in large numbers in the

epithelium of intestine, gills, and skin, but not in thymus, head kidney (which is together with the trunk kidney homologue to the bone marrow of mammals regarding hematopoietic function), or peripheral blood leucocytes (PBL)[23]. Rainbow trout (*Oncorhynchus mykiss*) IEL exhibited non-specific cytotoxic activity[140], but further information on fish IEL is extremely limited. Mammalian IEL are almost exclusively T cells of the T Cell Receptor (TCR)$\gamma\delta^+$ or TCR$\alpha\beta^+$ category[141,142].

IEL of tetrapods can originate from the gut epithelium itself instead of from the thymus[143,144], although inefficient and mostly skewed to the TCR$\gamma\delta^+$ population[141]. Since fish appeared before other vertebrates, a considerable number of T cells may generate in the gut, although a thymus is present. Mature T- or B cells have experienced V(D)J recombination of their B cell receptor (BCR) or T cell receptor (TCR) genes. It is convincingly established that this recombination coincides with the expression of recombination activating gene-1 (*rag-1*) and *rag-2*[93,94], and can be performed exclusively by these proteins[95,96]. Recently, *rag-1* was sequenced in carp (Chapter 4), enabling us to investigate *rag-1* expression in the carp intestine.

In this paper, the appearance of WCL38$^+$- (IEL), WCI12$^+$- (B cells), and WCL15$^+$ cells (monocytes/macrophages) was studied with flowcytometry, immunohistochemistry and (immuno)electron-microscopy to provide an overview of cell kinetics in the ontogeny of carp mucosal organs. In addition, *rag-1* expression in the intestine was studied using real time quantitative PCR (RQ-PCR) and *in situ* hybridisation, and correlated with the appearance of lymphoid cells.

Animals, Materials and Methods

Animals

Common carp (*Cyprinus carpio* L.) of the R3R8 strain were bred and kept in the facilities of 'de Haar Vissen', department of Animal Sciences, Wageningen University (The Netherlands). They were kept at 25°C for the first 5-6 weeks and subsequently at 23°C in circulating, filtered, UV-treated water. The embryos hatch at 2-3 dpf and were fed

with *Artemia salina* nauplii starting at 4-5 dpf for 3 weeks followed by Trouvit K30 pellets (Trouw & Co., Putten, The Netherlands). The principles of laboratory animal care (NIH no. 86-23) and the Dutch Law on experiments on animals were followed. Fish were anaesthetised with 0.03 % tricaine methane sulphonate (Crescent Research Chemicals, Phoenix, AZ) buffered with 0.06 % sodium bicarbonate to pH 7.2 in aquarium water.

Monoclonal antibodies

WCI12 (mouse IgG_1) is reactive with the IgM heavy chain and subsequently B cells of carp[22]. WCL38 (mouse IgM) reacts with intraepithelial lymphocytes in mucosal organs (putative T cells)[23]. WCL15 (mouse IgM) reacts (on tissues) with the cytoplasm of macrophages and monocytes[25,26].

Flowcytometry

All steps were performed at 4ºC and all washing steps consisted of centrifuging twice at 1800 rotations per minute (rpm) for 10 minutes (min). Between 1 and 10 dpf, whole fish were sampled. From 10 dpf onwards it was possible to separately sample blood, thymus, gills, gut, spleen and head kidney. Blood was collected in RPMI medium after adding 10 % water (c-RPMI) with heparin (50 IU/ml) upon cutting the tail until 6 weeks post fertilisation (wpf). In older animals it was sampled from the caudal vein using a syringe containing c-RPMI with heparin. Leucocytes from blood and organs from 3-50 individuals (number decreasing from 50 to three with age) were pooled in order to obtain enough cells for analysis. The procedure was performed on two offspring. Cell suspensions were prepared in c-RPMI containing 0.1% sodium azide (c-$RPMI^+$) by teasing the tissues through a nylon gauze filter (50 µm mesh). Cell suspensions and blood were washed once in c-$RPMI^+$, layered over a discontinuous gradient of Percoll (Pharmacia AB, Uppsala, Sweden), and diluted in c-$RPMI^+$ to yield densities of 1.060 and 1.070 g/ml^{-1}. Skin cells (that come of during the sampling procedure) with a density between 1.020 and 1.060 g/ml^{-1} mainly contain $WCL38^+$ skin epithelial cells, whereas skin cells with a density between 1.060 and 1.070 g/ml^{-1} predominantly contain lymphoid cells[23]. After centrifugation (30 min at 2000 rpm) both interfaces were removed and

washed once in c-RPMI^{+} with 1% Bovine Serum Albumin (c-RPMI^{++}). Leucocytes were incubated with WCL38 (1:50) or WCI12 (1:100) for 1 hour (hr), washed, and incubated with fluorescein-conjugated Goat-anti-Mouse Ig (GAM-FITC: Dako, Glostrup, Denmark) diluted 1:100 in c-RPMI^{++}. After washing, 10^4 cells were measured with a flow cytometer (FACStar, Becton Dickinson Immunocytometry System, Mountain View, USA) and analysed using the DataMate analysis package. Until 6 wpf peripheral blood leucocytes (PBL) of adult fish were used as positive controls. The percentage of mAb positive cells was calculated by subtracting the percentage of cells labelled with only the secondary antibody (GAM-FITC).

Immunohistochemistry

Immunohistochemistry was performed as described before (Chapter 4). Note that animals were bled completely before the procedure. Sections to be labelled with WCI12 (1:100) were fixed in 4% paraformaldehyde (PFA) in pH 7.5 phosphate-buffered saline (PBS), whereas sections to be labelled with WCL38 (1:50) or WCL15 (1:50) were fixed in ice-cold acetone for 5 min. Sections labelled with WCI12 were incubated with Goat-anti-Mouse-FITC (GAM-FITC, Dako, Glostrup, Denmark), and sections labelled with WCL38 or WCL15 with horseradish peroxydase-labelled Goat-anti-Mouse immunoglobulins (GAM-HRP, Bio-Rad Laboratories, Hercules, USA). Afterwards, sections labelled with WCI12 were washed twice in PBS, embedded in Vecta-Shield Mounting Medium with propidium-iodide (Vector Laboratories, Burlingame, USA) and examined with a laser scanning microscope (Carl Zeiss laser scanning system LSM 510). Sections labelled with WCL38 or WCL15 were washed in 0.05 M, pH 5 sodium acetate buffer. 3-Amino-9ethyl-carbazole (AEC: Sigma-Aldrich, St-Louis, USA) in sodium acetate buffer with 0.03 % H_20_2 was added and after staining (2-6 min), rinsed 3 times with double distilled water. Sections were stained in haematoxylin (BDH Laboratory Supplies, Poole, England) diluted 1:1 with distilled water for 20 seconds (sec), rinsed in running tap water, embedded in Kaiser's glycerin gelatine (Merck, Darmstadt, Germany) and examined by light microscopy. Sections treated without first antibody showed no staining.

Whole mount immunohistochemistry

Carp aged 2-10 dpf were fixed in 4 % PFA in PBS for 12 hr and stored in 1 % PFA in PBS at 4ºC. Samples were washed 3 times in PBS containing 15 % sucrose, and incubated in PBS with 0.2 % Triton X-100 and 10 % foetal calf serum for 8 hr at room temperature. After 3 rinses (10 min) the embryos/larvae were incubated with WCL15 (1:10) for 72 hr at 4ºC. After 3 washes, samples were incubated with GAM-FITC (1:50: DAKO) for 16 hr at 4ºC, rinsed in water and mounted on slides with Vecta-Shield Mounting Medium with propidium-iodide (Vector Laboratories, Burlingame, USA). Samples were examined with a laser-scanning microscope (LSM 510, Carl Zeiss, Germany). Samples treated without first antibody showed no staining.

Electron microscopy

Cell suspensions of 2 and 3 dpf animals were obtained from the 1.060-1.070 g ml^{-1} interface and labelled with WCL38 as described before (*Flowcytometry*). WCL38-labelled cells were washed in c-$RPMI^{++}$ and incubated (30 min at 4°C) with 25-nm gold-conjugated goat anti-mouse Ig (Aurion, Wageningen, the Netherlands) diluted 1:5. The cells were washed, suspended in 1 ml of c-$RPMI^{++}$ and centrifuged (1000 g) to yield a compact pellet. The pellet and 3-9 dpf carp larvae were fixed in 1% (w/v) $K_2Cr_2O_7$, 2% (v/v) glutaraldehyde, 1 % (w/v) OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 hr at 4ºC, washed in double-distilled water, dehydrated in ethanol and propylene oxide, and embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, USA). Ultra-thin sections were cut on a Reichert Ultracut S (Leica, Rijswijk, the Netherlands), and stained with uranyl acetate and lead citrate. Sections were examined with a Philips 208 electron microscope (Philips, Eindhoven, the Netherlands).

Whole mount in situ hybridisation

Intestines of 1 wpf and 2 wpf carp were fixed overnight in 4% PFA in PBS and stored at 4ºC in 1% PFA in PBS. Unfortunately it was not possible to examine intestines of older fish with *in situ* hybridisation, because then background reaction was to high

(Chapter 4). Whole mount *in situ* hybridisation was performed as described previously with a carp *rag-1* probe (Chapter 4). For the staining reaction, samples were incubated 3 hr at room temperature and then overnight at 4°C in the dark with nitrobluetetrazolium (NBT; 0.09 µl/ml; Roche Applied Science) and 5'-bromo-4'-chloro-3'-indolyl phosphatase (BCIP; 0.035 µl/ml; Roche Applied Science) according to the manufacturers' protocol. Samples were fixed in 4% PFA, washed, frozen, sectioned, embedded in Aqua Mount Improved (BDH Laboratory Supplies) and examined (see *immunohistochemistry*).

RNA extraction and cDNA synthesis

Intestines from 1-30 animals (dependent on size) from 4 dpf up to 0.5 year were pooled (10-1 dependent on age), snap frozen in liquid nitrogen and stored at -80˚C. RNA was isolated and cDNA was synthesised as described previously (Chapter 4). The stages 4-7 dpf and 4-26 wpf were obtained from different offspring.

Real time quantitative-Polymerase Chain Reaction (RQ-PCR)

RQ-PCR was performed as described previously (Chapter 4). *β-actin* and *40S* were used as house keeping genes and *rag-1* as gene of interest.

Results

Ontogeny of WCL38$^+$ cells

Figure 1A and C show the gate (that was previously shown to contain lymphoid cells[34]) used to calculate the relative percentage of WCL38$^+$ cells. In whole fish macerates of 4 dpf (panel A), the percentage of gated lymphoid cells was 42.6 %. At the same age, a number of WCL38$^+$ cells (panel B; not gated) displayed characteristics of lymphoid cells on the FSC/SSC (forward scatter/ sideward scatter) dot plot, while others clearly did not. At 8 wpf however (panel C), the intestine contained a high percentage of gated lymphoid cells (87.2 %). The majority of the WCL38$^+$ cells in the intestine at the same age (Panel D) presented lymphoid characteristics on the FSC/SSC dot plot.

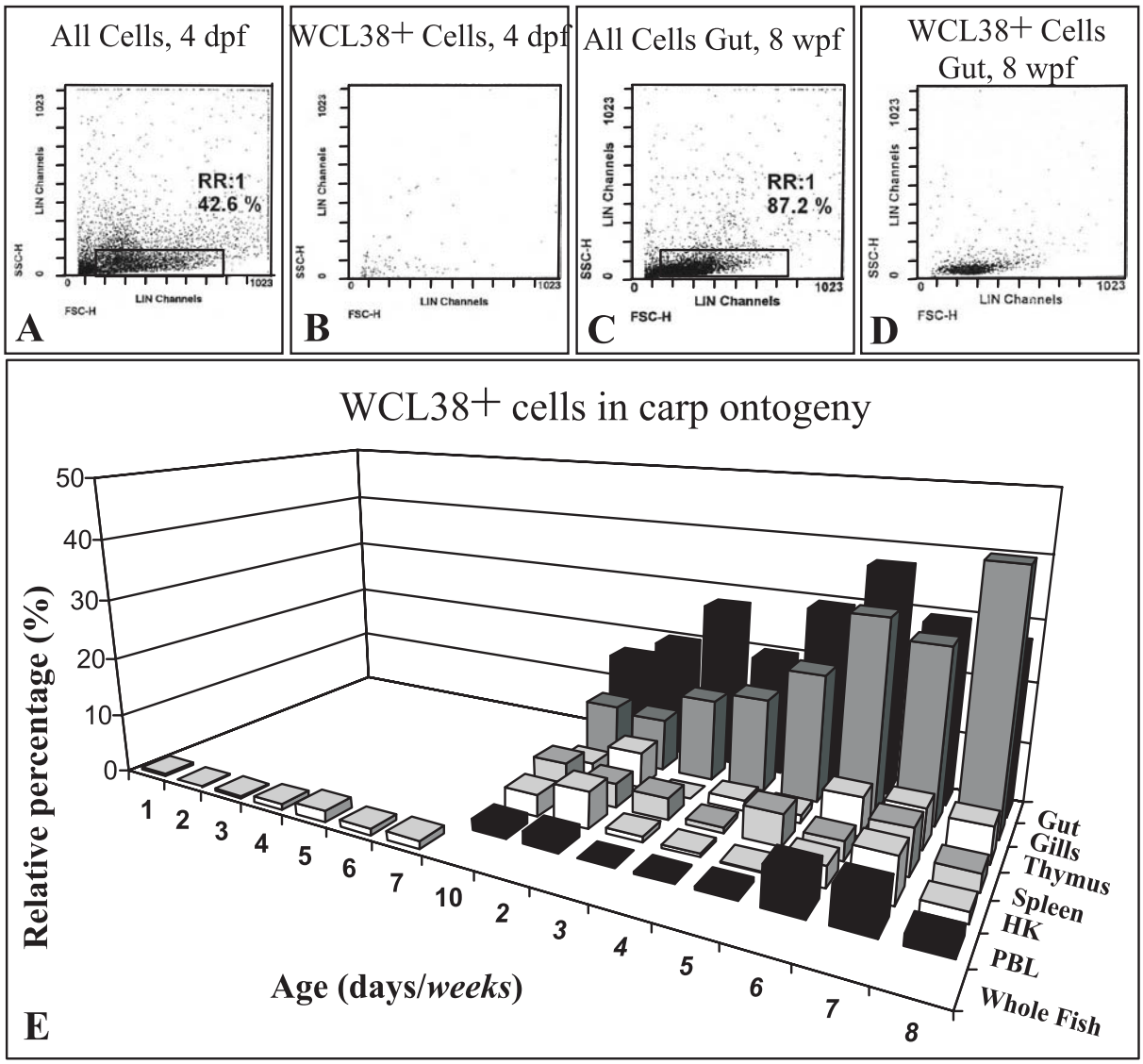

Figure 1. FSC/SSC profiles and relative percentages of WCL38^{+} cells in distinct organs during carp development determined with flowcytometry. Panels A-D represent FSC/SSC profiles from the following fractions: whole fish macerate at 4 dpf (A, B) and intestine at 8 wpf (C, D) showing all cells (A and C) and WCL38^{+} cells only (B and D). Panels A and C display the gate used (RR: 1) with the percentage of gated cells. The percentage of WCL38^{+} cells relative to all gated lymphoid cells with a density between 1.06 and 1.07 g/ml^{-1} was measured by flowcytometry in whole fish macerate (Whole Fish), peripheral blood leukocytes (PBL), head kidney (HK), spleen, thymus, gills and gut of carp (panel E).

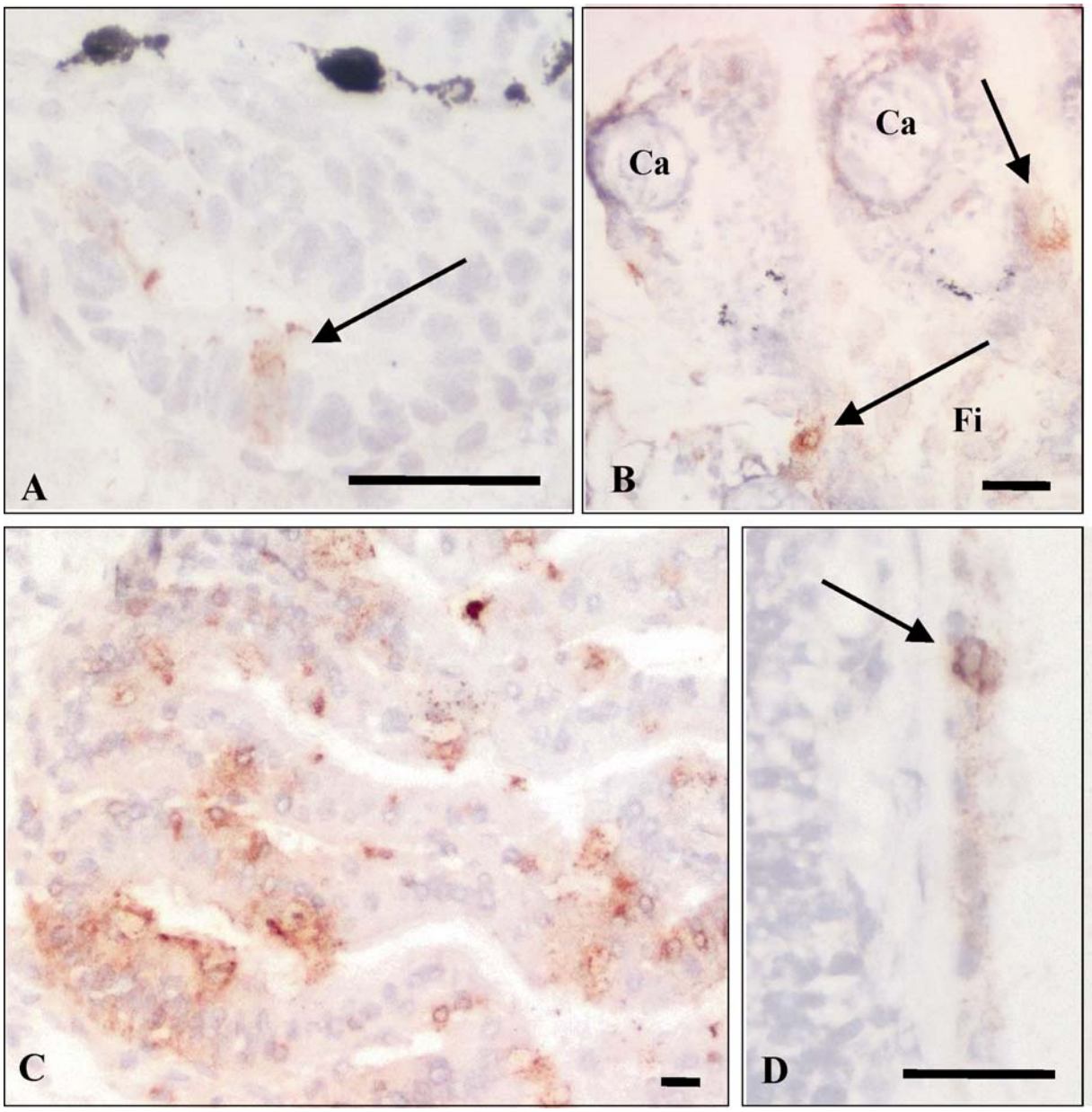

Figure 2. Immunohistochemistry using WCL38 on carp tissue sections. WCL38$^+$ cells first appeared in the intestine at 3 dpf (A), and in the gills (B) and skin (D) at 7 dpf (arrows). The number of positive cells in the intestine had increased considerably by 10 dpf (panel C). The cells with the intensely black granules (panels A and B) are melanocytes. Ca = cartilage of large gill lamella, Fi = small gill filament. Arrows point to WCL38$^+$ cells. Bar is 20 μm.

WCL38$^+$ cells first appeared in whole fish macerates at 4-5 dpf using flowcytometry (Figure 1E). From 10 dpf onwards, WCL38$^+$ cells mainly resided in gut and gills (also considering these organs are the largest investigated). Positive cells were also present in thymus, spleen, head kidney and PBL, though at a considerably lower frequency.

Using immunohistochemistry (Figure 2), WCL38$^+$ cells first appeared at 3 dpf in the epithelium of the intestine (panel A), although they were rare and only weakly reactive. From 5-6 dpf the number of WCL38$^+$ cells in the intestine (predominantly the epithelium) increased substantially (results not shown), until they were numerous by 10 dpf (panel C). From 7 dpf, WCL38$^+$ were also present in the epithelium of the gills (panel B) and the skin (panel D). Positive cells were rare in other tissues, such as muscle, liver and neural tissue.

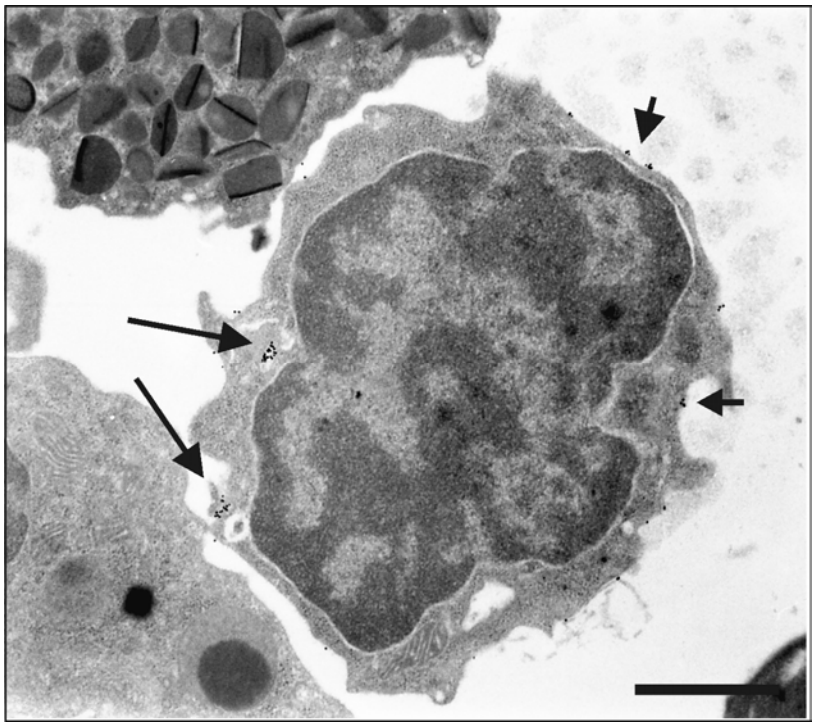

Figure 3. Electron microscopy picture of a WCL38$^+$ lymphoid cell from a 3-dpf Whole Fish macerate cell suspension. Arrows point to gold particles representing WCL38 immuno-reactive molecules. Note the WCL38$^-$ neutrophilic granulocyte (top) and basophilic granulocyte (left bottom). Bar is 1 μm.

Using immuno electron microscopy, lymphoid WCL38$^+$ cells were detected in whole fish macerate cell suspensions already at 3 dpf (Figure 3). In addition, putative intraepithelial lymphoid cells (based on the almost straight cut from base to apex of the surrounding epithelial cells, and the fact that intestinal epithelial cells divide apically and

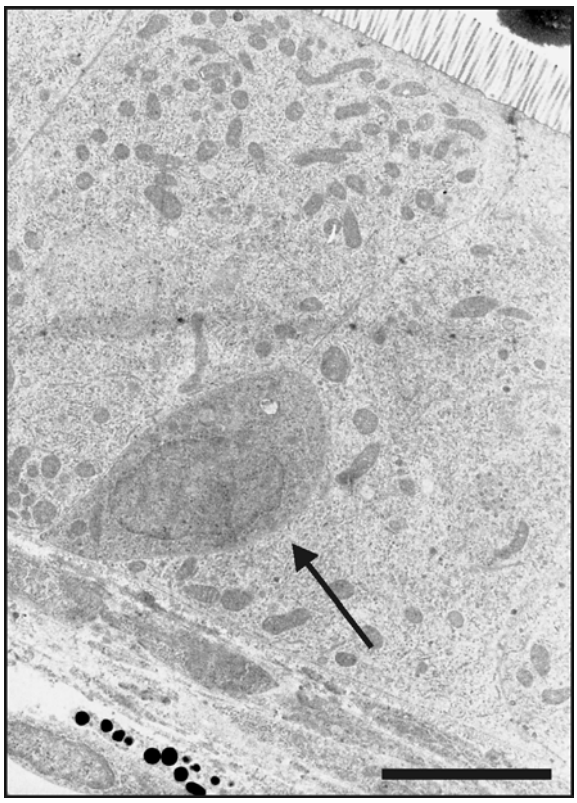

Figure 4. Electron microscopy picture of a putative intraepithelial lymphocyte (arrow) in the intestine at 5 dpf. Bar is 5 μm.

not basally) were located near the basal layer in the intestinal epithelium at 5 dpf (Figure 4). IEL sometimes contained a high cytoplasm/nucleus ratio.

Ontogeny of WCL15$^+$ cells in mucosal organs

WCL15$^+$ cells appeared in the lamina propria of the intestine at 7 dpf (results not shown), and were more numerous at 9 dpf (Figure 5 A-B). Numerous WCL15$^+$ cells were also present dorsal of the intestine, in the hematopoietic tissue along the vena cardinalis (Chapter 3). Intestinal intraepithelial WCL15$^+$ cells were not present at least up to 2 wpf. WCL15$^+$ cells appeared in the dermis of the skin at 9-10 dpf (Figure 5 C-D), with numerous cells with extensions present at 14 dpf (Figure 5 E).

Ontogeny of WCI12$^+$ cells (B cells)

Using flowcytometry, WCI12$^+$ cells first appeared in head kidney and spleen from 3 wpf, and in PBL from 5 wpf (Figure 6). WCI12$^+$ cells were present in mucosal organs (gut

and gills) from 6-7 wpf, and in thymus at 8 wpf. WCI12$^+$ cells were never abundant in thymus and intestine.

Using immunohistochemistry (Figure 7), WCI12$^+$ appeared in the intestine from 5 wpf onwards (6 wpf is shown in Figure 7A), in the gills from 6 wpf (Figure 7B), and in the skin at 3 months (Figure 7C: at 2 months positive cells were not present). WCI12$^+$ cells in the intestine were located in the lamina propria. Large WCI12$^+$ cells in the epithelium of the 2nd segment, representing Ig binding intraepithelial macrophages, appeared from 6 wpf (results not shown). In the skin, however, positive cells were not present in the dermis but in the epidermis (epithelium), while the dermis reacted diffusely with WCI12.

Rag-1 expression in the intestine

Rag-1 was expressed in carp intestine (Figure 8). Using RQ-PCR (panel A), expression increased from 4 dpf to 5 dpf in both offspring tested. In juvenile and adult carp (4 and 26 wpf), *rag-1* expression was lower then in larvae from 5 and 7 dpf.

Using *in situ* hybridisation (panel B), *rag-1*$^+$ cells were present in the intestine at 1 wpf and 2 wpf. Positive cells were located in the lamina propria (panel B), but it cannot be excluded that some positive cells were present in the epithelium (results not shown). The number of positive cells was very low (approximately 1 cell per 20 sections).

Discussion

This study for the first time describes the development of the mucosal immune system of fish. WCL38$^+$ IEL first appeared in the intestine at 3 dpf, which is after hatching but before feeding, while their numbers increased substantially at the time of first food ingestion (5 dpf). The distribution and abundance of these cells in carp larvae is very similar to that in adult animals[23], implying that WCL38$^+$ IEL play a significant role in mucosal organs already early in development. According to a previous study[23], WCL38$^+$ cells are not B cells or natural killer cells. Knowledge on the IEL function in teleost fish is unfortunately very limited, although non-specific cytotoxic activity was observed in

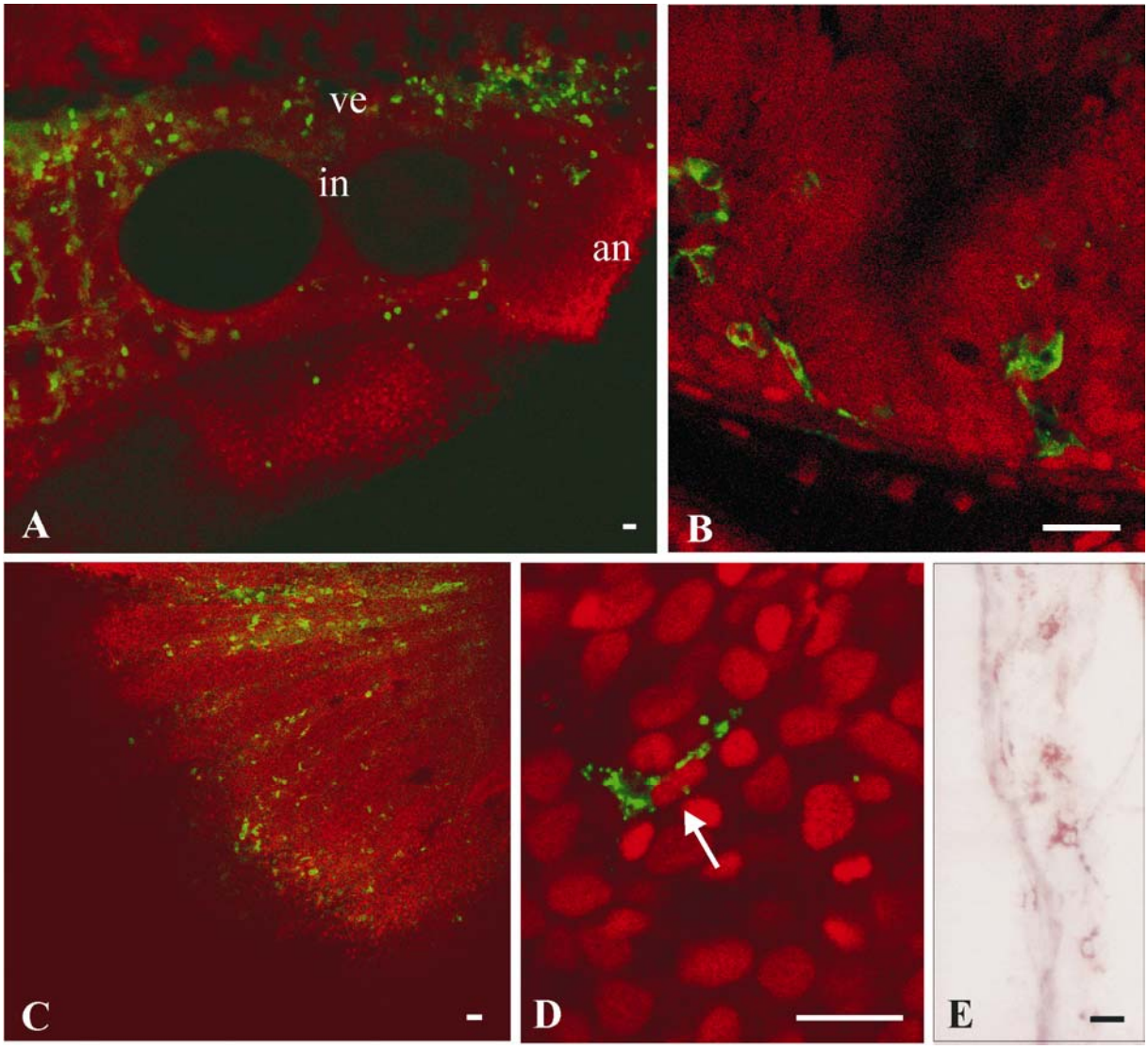

Figure 5. Monocytes/macrophages (WCL15$^+$ cells) detected with immunohistochemistry in mucosal organs during the first two weeks of carp development. WCL15$^+$ cells are green fluorescent and tissue is red fluorescent after treatment with propidium-iodide (panels A-D), or WCL15$^+$ cells are reddish-brown and tissue is purple after counter staining with diluted haemalum (panel E). WCL15$^+$ cells were present in intestine (A-B), and skin from 9 dpf. Panels C-D and E represent the skin at 10 and 14 dpf respectively. In panel A *Artemia* egg shells are present as non-fluorescent circles. Panel C represents the skin in the tail of the larva, showing the abundance of WCL15$^+$ cells. The arrow in panel D points to the nucleus of the WCL15$^+$ cell. Ve = blood vessel, in = intestine, an = anus. Bar is 20 μm.

rainbow trout[140]. In carp, WCL38$^+$ cells exhibit no specific or non-specific cytotoxic responses (JHWM Rombout, unpublished results). Mammalian IEL are T cells that are implicated in the conventional role of mounting specific immune responses, but also in the preservation of the mucosal epithelial layer[142], gut metabolism[145], and the prevention of undesirable immune responses[141]. TCR$\alpha\beta^+$ IEL bear the hallmarks of conventional T cells, whereas TCR$\gamma\delta^+$ IEL and TCR$\alpha\beta^+$CD8$\alpha\alpha$ IEL also have characteristics of innate immune cells: they respond to antigens not restricted by the conventional Major Histocompatibility Complex[142] and recognise conserved antigens[146,147]. Consequently, the early appearance of WCL38$^+$ cells, which are similar to mammalian IEL regarding

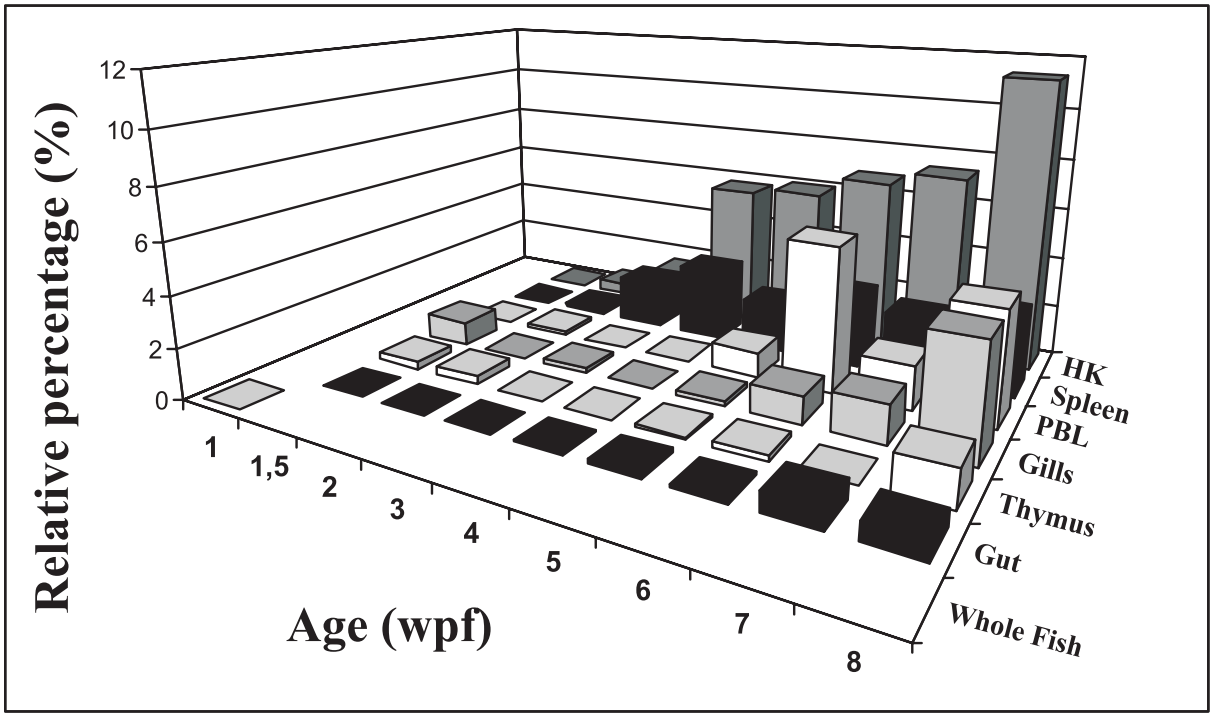

Figure 6. Relative percentages of WCI12$^+$ cells in distinct organs during carp development determined with flowcytometry. The percentage of WCI12$^+$ cells relative to all gated lymphoid cells with a density between 1.06 and 1.07 g/ml^{-1} was measured by flowcytometry in whole fish macerate (Whole Fish), thymus, gut, gills, peripheral blood leukocytes (PBL), spleen and head kidney (HK) of carp. The gate used is shown in figure 1 (RR: 1).

location and morphology, suggests the presence of T cells with at this age most probably innate characteristics, although their function remains to be investigated.

WCL38$^+$ cells appeared before the development of a cortex and medulla in the thymus (Chapter 4)[50], and also before the onset of *rag-1* expression in the thymus (Chapter 4). Assuming WCL38$^+$ IEL are T cells, these data strongly suggest that at least part of these cells originate from the intestine itself. This is corroborated by the expression of *rag-1* in the intestine around 1 wpf, when the number of WCL38$^+$ cells is increasing considerably. However, the level of expression and the number of *rag-1* positive cells is very low compared to the thymus or even kidney (Chapter 4), and the number of WCL38$^+$ cells only increases substantially after the appearance of the thymus, suggesting the thymus is still the main provider of these cells. This is consistent with data obtained in mammals, in which *rag-1* expression in IEL suspensions was considerably lower than in thymic cells[127], and a large amount of evidence shows a thymic origin of all or most IEL[141,143]. The limited *rag-1* expression in carp can also be explained by the fact that part of the WCL38$^+$ IEL originate from the thymus but via a short partway without negative selection as is suggested for mammals[141], or by clonal expansion of a limited number of differentiated IEL.

IEL that are generated in the mammalian gut are mostly the TCR$\gamma\delta^+$ population[141], and this subpopulation is also the most abundant in young mammals[148]. With respect to these data it is interesting that γ and δ TCR genes were recently identified in teleost fish (Chapter 4)[1]. TCR$\gamma\delta^+$ cells are suggested to have evolved before $\alpha\beta$TCR$^+$ cells and to represent the first step in the development of adaptive immunity[146].

Rag-1 expression was located in the lamina propria of the intestine, but it cannot be excluded that *rag-1* positive cells were also present in the epithelium. The first location is consistent with data in mammals where cryptopatches in the lamina propria are associated with the generation of IEL[141,146]. In contrast to our data, *rag-1* was abundantly expressed in both intestinal epithelium and lamina propria in zebrafish (cyprinidae)[13], suggesting the extent of extrathymic T cell production might differ among fish species.

Because WCL38$^+$ cells are present in the intestine before the appearance of the thymus, they possibly are the progeny of precursors from tissue homologue to foetal liver or bone marrow in mammals[142]. Progenitors of WCL38$^+$ cells tentatively originate from the intermediate cell mass, a tissue described in zebrafish[36,37] and carp embryos (Chapter 3), which expresses many genes implicated in hematopoiesis including *ikaros*[150], which is a transcription factor specifically of the lymphoid lineage. The intermediate cell mass transforms into the 'dorsal aorta' in zebrafish[37], which is the equivalent of the aorta-gonad-mesonephros region, the first site of definitive hematopoiesis in tetrapods.

WCL15$^+$ cells (monocytes/macrophages) first appeared in the lamina propria of the intestine at 7 dpf, which is about the same age as in other studies[26], while intraepithelial macrophages (IEM) appear considerably later around 4 wpf[8]. These intraepithelial macrophages have been shown to ingest antigens, and are proposed to have a role in antigen presentation[8,134]. Ig binding IEM are first detectable at 6 wpf, which corresponds to the appearance of plasma-cells at 4 wpf[92]. The delayed appearance of IEM indicates an immature mucosal immune system in juvenile carp. WCL15$^+$ cells were also present in the dermis of the skin. The long extensions and network formation are similar to mammalian Langerhans cells (antigen presenting cells), although these cells are present in the epidermis of the skin. Whether these cells also function as Langerhans cells remains to be investigated.

WCI12$^+$ cells (B cells) first appeared in head kidney and spleen and then in PBL, which is consistent with earlier studies[78,92], and suggests that B cells, like in mammals home to the spleen for maturation. Subsequently, B cells were situated in intestine and gills at 5-6 wpf, suggesting B cells are now mature because this is also the first appearance of B cells outside kidney and spleen. The ‘lag’ period between appearance of B cells in head kidney and spleen compared to mucosal organs corresponds with data obtained for sea bass (*Dicentrarchus labrax*) and spotted wolffish (*Anarhichas minor*)[151,152]. The appearance of 'mature' B cells in peripheral organs (intestine and gills) at 5-6 wpf corresponds to the appearance of plasma cells[92], and the mounting of antibody responses against *Aeromonas salmonicida*[7]. B cells appeared in the skin considerably later than in intestine, which is consistent with data in spotted wolffish[152]. In contrast to

intestine, B cells are situated in the epithelium (epidermis), similar to spotted wolffish[152], with the dermis reacting diffusely with WCI12 like in adult carp[23].

The induction of tolerance in juvenile carp after oral vaccination with *Vibrio anguillarum* up to 4 wpf and the development of an increased antibody response at 2

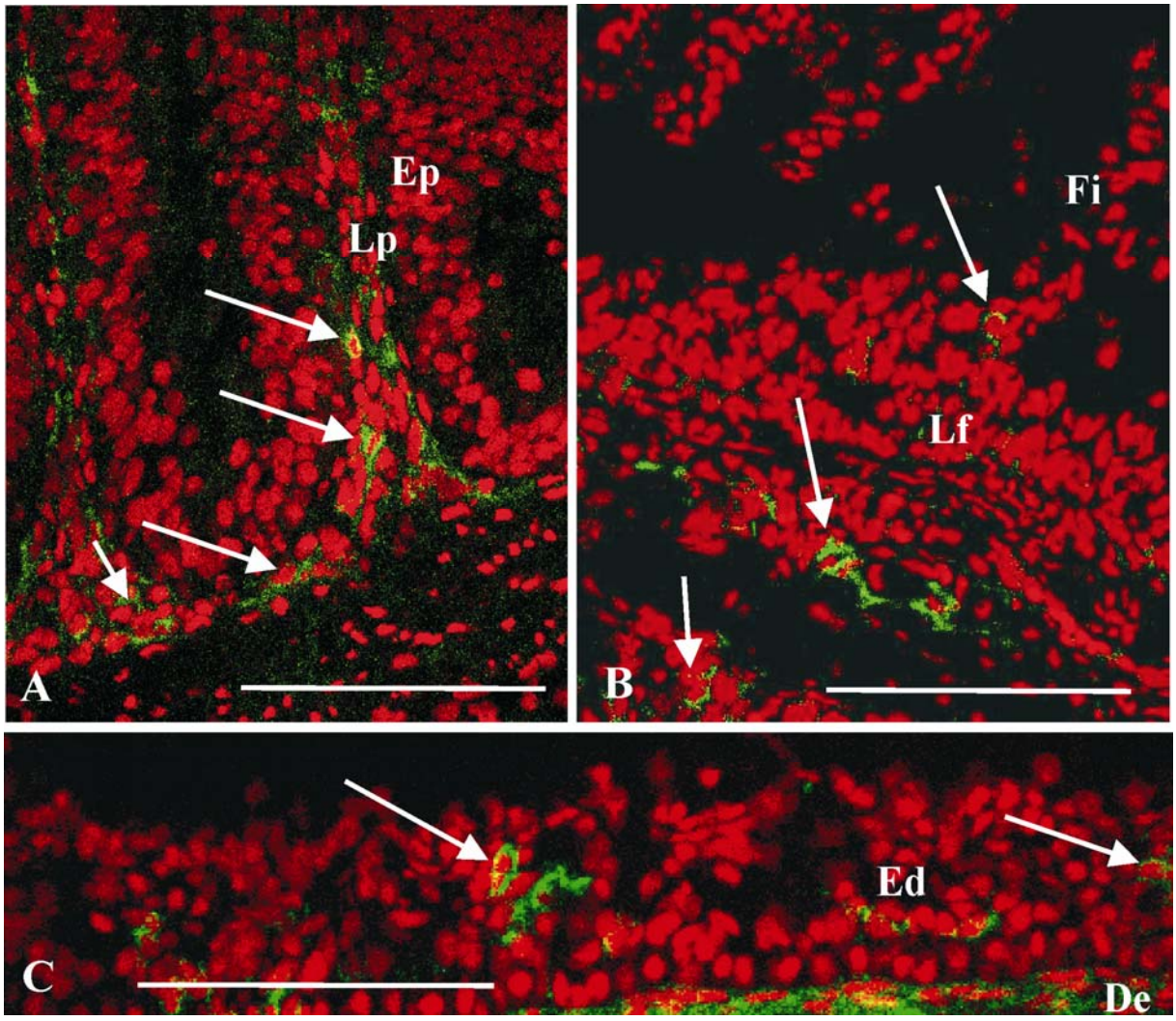

Figure 7. B cells (WCI12$^+$ cells) detected with immunohistochemistry in mucosal organs during the first weeks of carp development. WCI12$^+$ cells are green fluorescent and tissue is red fluorescent after treatment with propidium-iodide. Positive cells were present in the lamina propria of the intestine (A) and in gills (B) at 6 wpf, and in the epithelium of the skin at 3 months (C). De = Dermis, Ed = epidermis, ep = epithelium, Fi = small gill filament, Lf = large gill filament, lp = lamina propria, Arrows point to positive cells. Bar is 100 μm.

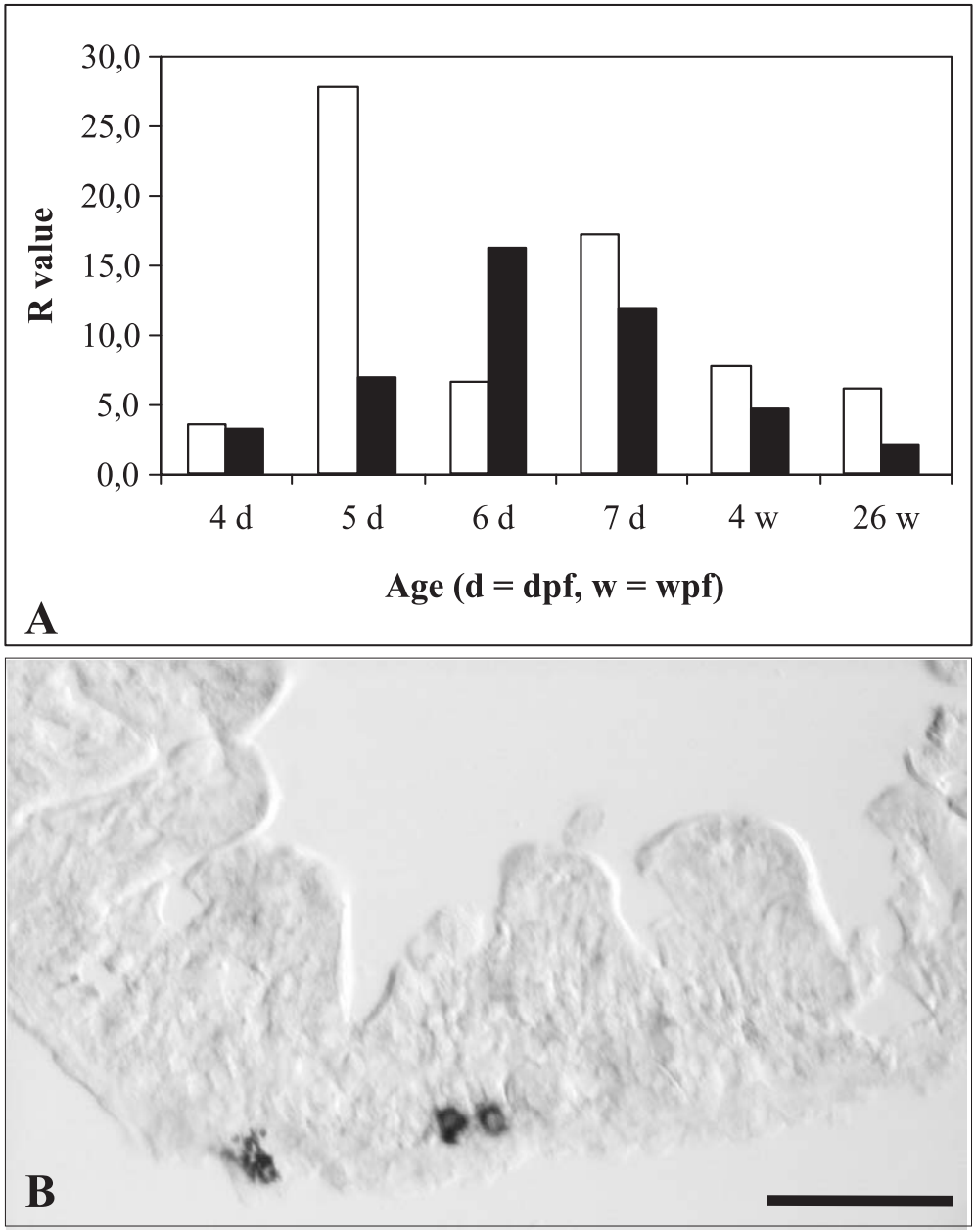

Figure 8. ***Rag-1*** **expression in carp intestine. Panel A displays** ***rag-1*** **expression detected with RQ-PCR in two different offspring (depicted with white and black bars) from 4 dpf up to 26 weeks. All data were normalised against** ***β-actin*** **levels and compared to a sample with insignificant** ***rag-1*** **expression (muscle of adult carp) with Pfaffl's method: R-values are displayed on the vertical axis. On the horizontal axis age is displayed in dpf (d) or wpf (w). Panel B shows** ***rag-1*** **positive cells (with** ***in situ*** **hybridisation) in the intestine at 1 wpf. Bar is 50 μm.**

months[8], corresponds to the appearance of Ig binding intraepithelial macrophages and B cells, but not to the earlier presence of $WCL38^+$ IEL or Ig^- intraepithelial macrophages[8]. Consequently the ability to mount a systemic antibody response against oral vaccines is correlated to the presence of Ig binding IEM and B cells but not to $WCL38^+$ IEL.

In conclusion, this study provides the first description of IEL ontogeny in carp, and suggests that these cells are partly produced in the intestine besides the thymus because of their early appearance (before the thymus) and concurrent *rag-1* expression in the intestine. Furthermore, monocytes/macrophages and especially B cells appeared later in development, suggesting particularly acquired mucosal immunity is not functional up to 6-7 wpf, which is much later than the development of the systemic humoral immune system (2-3 wpf)

Acknowledgements

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The effect of oral immuno-stimulation in juvenile carp (*Cyprinus carpio* L.)

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Submitted

Abstract

The effect of a two-week period of oral immuno-stimulation from the age of 2 or 6 weeks post fertilisation (wpf; before and after reaching the ability to produce antibodies) onwards was investigated on various immune functions. The immuno-stimulants *Aeromonas salmonicida* lipopolysaccharide, Yeast DNA (containing unmethylated CpG motifs) or high-M alginate (an extract of algae containing poly-mannuronic acid) were used. The effect of this treatment was studied on the kinetics of B cells in head kidney and peripheral blood leucocytes using flowcytometry, on the total plasma IgM level using ELISA, and on cytokine and inducible nitric oxide synthase (iNOS) expression in the intestine and acute phase protein expression in the liver using real time quantitative PCR, and on exposure to *Vibrio anguillarum*.

Oral administration of immuno-stimulants from 6 wpf resulted in decreased $WCI12^+$ (B) cell percentages in PBL (only after administration of LPS) and head kidney (all test groups), and a decreased total IgM level in plasma, suggesting suppressive effects strongly indicative of oral or juvenile tolerance. After administration from 2 wpf, the effects on $WCI12^+$ (B) cell percentages were less pronounced: the group fed with yeast DNA showed higher percentages compared to the control group at 6 wpf, but lower percentages at 8 wpf. No changes were observed in the cytokine or iNOS expression levels in the intestine or acute phase protein expression in the liver. A challenge with *Vibrio anguillarum* resulted in an initially higher cumulative mortality in the group fed with LPS, but lower mortality in the groups fed with Yeast DNA or high-M alginate compared to the control group, providing a provisional warning for the use of especially pathogen-derived immuno-stimulants, such as *A. salmonicida* LPS, in larval and juvenile fish.

Introduction

The intensive culture of fish is often restricted by high mortality due to infectious diseases in especially juveniles. Problems in larvae mostly occur by opportunistic bacteria that are part of the aquatic flora[2,3]. Prophylaxis, based on sanitary isolation is difficult to achieve due to the presence of other fish species, invertebrates or the water itself. Antibiotic therapy is a frequently used strategy in intensive rearing of fish larvae, but can result in enhanced microbial resistance, the accumulation of residues in tissues[4] and immuno-suppression[5]. Vaccination, although highly effective in many instances is time consuming, costly and protection is usually pathogen specific. Moreover, the adaptive immune system is not functional in fish up to a certain age[5,7,8]. In contrast, the administration of immuno-stimulants activates the immune system in an aspecific way, thus providing resistance against a variety of pathogens, and can be applied at early ages[10]. In particular oral administration of immuno-stimulants is a potentially efficient method.

The innate immune system is activated by pathogen associated molecular patterns[153], which are recognised by amongst others Toll Like Receptors (TLR) that have also been described in various fish species[154,155]. Bacterial lipopolysaccharide (LPS) is recognised by TLR 4 in mammals[156], and convincingly induced expression of cytokines and acute phase proteins in fish cells *in vitro*[29,31,32,157-159]. Yeast DNA contains, like bacterial DNA, unmethylated CpG motifs[160], that stimulate the immune system via TLR9 in mammals[161], and stimulate fish cells *in vitro* to produce antiviral supernatants[162] and activate non-specific cytotoxic cells in catfish (*Ictalurus punctatus*)[163]. High-M alginate is an extract of algae containing poly-mannuronic acids, that are recognised by TLR2 and TLR4 in mammals[164], and oral administration increases survival in several fish species[11,165].

The described effects of the aforementioned immuno-stimulants can be placed in different categories[9], of which 2 are relevant for this study. Firstly, elaborate studies showed that bacterial LPS[157-159], CpG motifs[28,166,167] and high-M alginate[168] induced cytokine expression and acute phase protein expression *in vitro*. These effects were not yet described after oral administration. Secondly, immuno-stimulation in mice results in

proliferation of B cells and the secretion of immunoglobulins (Ig), as was shown for bacterial DNA and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides *in vitro* after injection and *in vivo*[169]. In addition, oral administration of Ergosan (an extract from algae) induced a (non significant) increase in B cells in peripheral blood leucocytes (PBL) in sea bass (*Dicentrarchus labrax*)[12], and injection of oligodeoxynucleotides with CpG motifs increased specific antibody responses in goldfish (*Carassius auratus*)[170,171].

In this paper, the effect of oral immuno-stimulation with *Aeromonas salmonicida* LPS, Yeast DNA (containing unmethylated CpG motifs) and high-M alginate (an extract from algae containing poly-mannuronic acids) was studied on B cell kinetics in head kidney and PBL, total plasma IgM level, cytokine and inducible nitric oxide synthase (iNOS) expression in the intestine (the point of entry for food-derived immuno-stimulants, and containing numerous leucocytes), acute phase protein expression in the liver, and a challenge with *Vibrio anguillarum* in carp (*Cyprinus carpio* L.). A challenge with *Aeromonas salmonicida* was not performed because repeated oral administration of antigens induced tolerance in a previous study[8]. Oral immuno-stimulants were administered at two different ages from 2 and 6 weeks post fertilisation (wpf) respectively, which is before and after the onset of adaptive immunity. This study provides the first description in juvenile fish of immunological parameters after oral immuno-stimulation with the aforementioned substances.

Animals, Materials and Methods

Animals

Common carp (*Cyprinus carpio* L.) of the R3R8 strain were bred and kept in the facilities of 'de Haar Vissen', department of Animal Sciences of Wageningen University (The Netherlands). They were kept at 25 ± 0.5°C for the first 5-6 weeks and subsequently at 23°C in circulating, filtered, UV-treated water. The embryos hatch at 2-3 days post fertilisation and were fed with *Artemia salina* nauplii starting at 4-5 days post fertilisation

for 2-3 weeks followed by Trouvit K30 pellets (Trouw & Co., Putten, The Netherlands). Fish were anaesthetised with 0.03 % tricaine methane sulphonate (Crescent Research Chemicals, Phoenix, AZ) buffered with 0.06 % sodium bicarbonate to pH 7.2 in aquarium water before sampling.

Experimental design

Fish from 2 or 6 wpf were fed with food containing immuno-stimulants (yeast DNA, purified *Aeromonas salmonicida* LPS[172] (kindly provided by dr. I.R. Bricknell, Marine Laboratory, Aberdeen, UK), high-M alginate (an extract from algae, obtained from FMC Biopolymers, Drammen, Norway), or PBS (phosphate buffered saline; control)) during 2 weeks. The administration of food with immuno-stimulants from 2 wpf will be referred to as Exp 1, the administration from 6 wpf as Exp 2, and the challenge with *Vibrio anguillarum* as Exp 3. In Exp 1, fish were sampled to determine the length of the fish (8-25 fish per group per sampling point), and B cell population kinetics (using the mAb WCI12; 5 fish per group per sampling point) in head kidney and PBL. The measurement of the length of the fish and flowcytometry were performed in two different trials. Fish from Exp 2 were sampled (6 fish per group per sampling point) to determine total Ig level in serum, B cell population kinetics in head kidney and PBL (using the mAb WCI12), total weight of all sampled fish per group, and cytokine and acute phase protein expression in intestine and liver respectively (2 fish per group per sampling point: at 0, 2, 4, 8 h, and 1, 2, 3 and 15 days after the start of the experiment). All sampling points are displayed in Figure 1.

In Exp 3, 230 fish per tank (1 tank per food type) were fed pellets with immuno-stimulants from 17 dpf. At 26 dpf, the fish were exposed to 10^5 colony forming units *V. anguillarum* serotype 2 per ml for 4 hours with reduced flow, and they received pellets without immuno-stimulants for the rest of the experiment.

Preparation of the food

The commercial food (pellets) was ground, and a solution of the immuno-stimulant dissolved in PBS was added. The food mix was dried and ground again until the size of

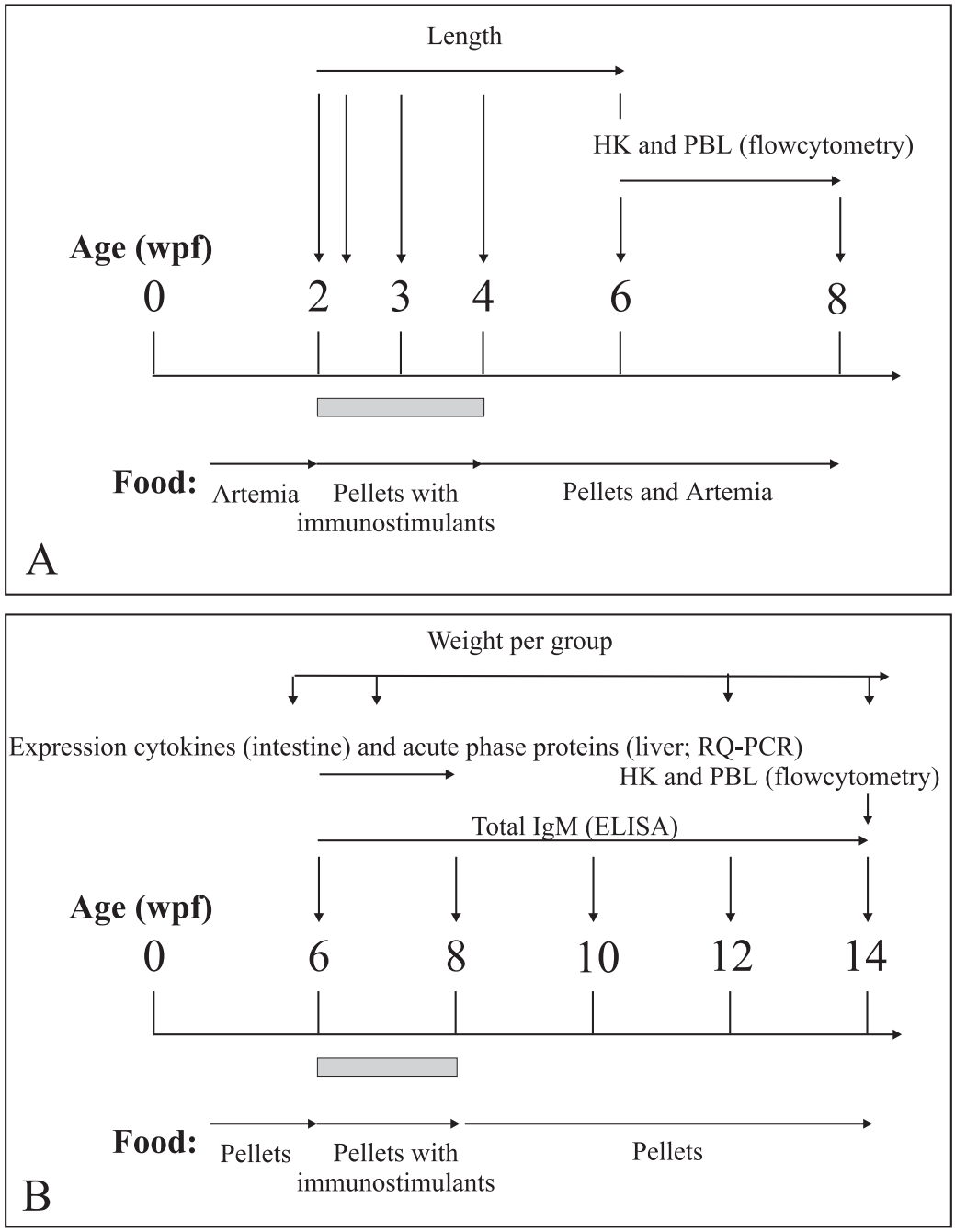

Figure 1. Experimental design displaying all sampling points in Exp 1 (A) and Exp 2 (B). The expression of cytokines in intestine and acute phase proteins in liver displayed in panel B was essayed at day 0 (the first day: 1, 2, 4 and 8 hours), 1, 2, 3 and 15 days after first feeding of immuno-stimulants. Oral administration of immuno-stimulants is indicated with a grey bar. HK = head kidney, PBL = peripheral blood leukocytes, RQ-PCR = real time quantitative PCR, wpf = weeks post fertilisation.

the pellets was appropriate. The final concentration of the immuno-stimulants in the food was 0.5% for high-M alginate and 0.1% for *A. salmonicida* LPS and yeast DNA (0.01% for flowcytometry in Exp 1).

Extraction of genomic DNA from yeast, purification of LPS and preparation of high-M alginate

Yeast cells were suspended in a hypertonous solution with 425-600 nm acid washed glass beads (Sigma-Aldrich, St-Louis, USA). Subsequently, phenol and chloroform were added, and the organic and aqueous phases were vigorously mixed. The highly enriched DNA was collected by standard ethanol precipitation and concentrations were measured by spectrophotometry (Genequant, Pharmacia Biotech, Uppsala, Sweden).

Aeromonas salmonicida LPS was purified as described before[172].

The alginate used in all experiments was isolated from the brown seaweed *Durvillea antarctica* (FMC Biopolymer, batch 103-215-01-TP). The fraction of β-D-mannuronic acid units was determined to 0.88 by using ^{1}H-NMR spectroscopy.

Monoclonal antibodies

WCI12 (IgG_1) is reactive with the IgM heavy chain and subsequently B cells of carp[22].

Flowcytometry

All incubation steps were performed at 4ºC, all washing steps consisted of centrifuging the cells twice at 1800 rpm for 10 min. Blood was collected in RPMI medium with 10 % extra distilled water (c-RPMI) and heparin (50 IU/ml) upon cutting the tail until 6 weeks of age. In older animals it was sampled from the caudal vein using a syringe containing c-RPMI with heparin. Cell suspensions were prepared in c-RPMI containing 0.1 % sodium azide (c-RPMI^{+}) by teasing the tissue through a nylon gauze filter (50 µm mesh). Cell suspensions and blood cells were washed once in c-RPMI^{+} and layered on Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden). After centrifugation (30 min at

2000 rpm, brakes disengaged) the interface was removed and washed once in c-RPMI^{+} with 1 % bovine serum albumin (c-RPMI^{++}). Leucocytes were incubated with WCI12 (1:100) for 1 hour (hr), washed, and incubated with fluorescein isothiocyanate-conjugated Goat-anti-Mouse Ig (GAM-FITC: Dako, Glostrup, Denmark) diluted 1:100 in c-RPMI^{++} for 30 min. In Exp 1, after washing propidium iodide (1:100; Dako) and a known amount of fluorescent beads (Fluoresbrite YG Carboxylate microsperes 10 μm, Polysciences, Warrington, PA) were added. 10^4 cells were measured with a flow cytometer (Coulter Epics XL-MCL, Beckman Coulter, Miami, USA) and analysed using the EXPO32 ADC Analysis software. To determine the percentage of positive cells, lymphocytes were gated (SSC^{low}) and beads and necrotic cells were excluded. The ratio between number of fluorescent beads measured and the number of beads added was used to calculate the total number of cells isolated from the sample. In Exp 2, the percentage of mAb positive cells was calculated by subtracting the percentage of cells labelled with only the secondary antibody (GAM-FITC), while for Exp 1 all controls (without primary antibody) had a relative percentage of positive cells under 1%.

Enzyme-linked immuno sorbent assay (ELISA) for measuring total IgM

All steps were performed with washing steps between, in PBS, shaking and at room temperature unless mentioned otherwise. ELISA plates were treated with 0.025 % glutaraldehyde at 57ºC for 2 h, and coated with 1 μg/ml WCI12 overnight. The plates were blocked with 0.5% BSA for 1 h. Dilutions of the serum samples and a standard series of purified Ig were incubated for 3 h, where after 0.5 μg/ml WCI12, labelled with biotin, in PBS-T was added. The plates were incubated for 1 h, and streptavidine poly HRP (Sanquin, Amsterdam, the Netherlands) was added (1:10000), and incubated for 1 h. TMB peroxidase substrate (KPL, Gaithersburg, USA) was added for 10 min. The reaction was stopped by adding 1M ortho-phosphoric acid (H_3PO_4), and the samples were measured at 492 nm in an ELISA plate reader (Anthos 2001, Austria: ref. 600 nm) using Mikrowin software.

RNA extraction, cDNA synthesis and real time quantitative-polymerase chain reaction

RNA was isolated, cDNA synthesised, and real time quantitative PCR (RQ-PCR) and calculations were performed as described previously[49]. *β-actin* and *40S* were used as house keeping genes. Interleukin 1-β (IL1-β), tumour necrosis factor α (TNFα), inducible nitric oxide synthase (iNOS), complement factor 3 (C3), serum amyloid A (SAA) and $\alpha_2$macroglobulin (α_2M) were used as genes of interest. Primer sequences were specifically designed for RQ-PCR and are described previously (Chapter 3). R-values were calculated using the average control Ct values as a reference. R-values calculated with either *β-actin* or *40S* as house keeping gene were comparable. Non template controls were always included.

Statistical analysis

All statistical analyses were performed using SPSS 10.1 software. Data were tested for normal distribution with Shapiro-Wilk's test and for homogeneous variance with Levene's test. In case of normal distribution, differences between groups and times were tested with One-Way ANOVA and Dunnett's T test (homogeneous variance; two-sided) or Dunnett's T3 test (unequal variance). In case of not normal distribution, Kruskall Wallis and Mann-Whitney U tests were performed. $P< 0.05$ was accepted as significant.

Results

Growth and mortality

In Exp 1, mortality occurred especially 2-7 days after the administration of immuno-stimulants was stopped (2-25 % per tank unrelated to group; results not shown), which is probably due to the transition to another batch of food after immuno-stimulation and the age of the fish. In Exp 1, differences between the average length of the fish in the different

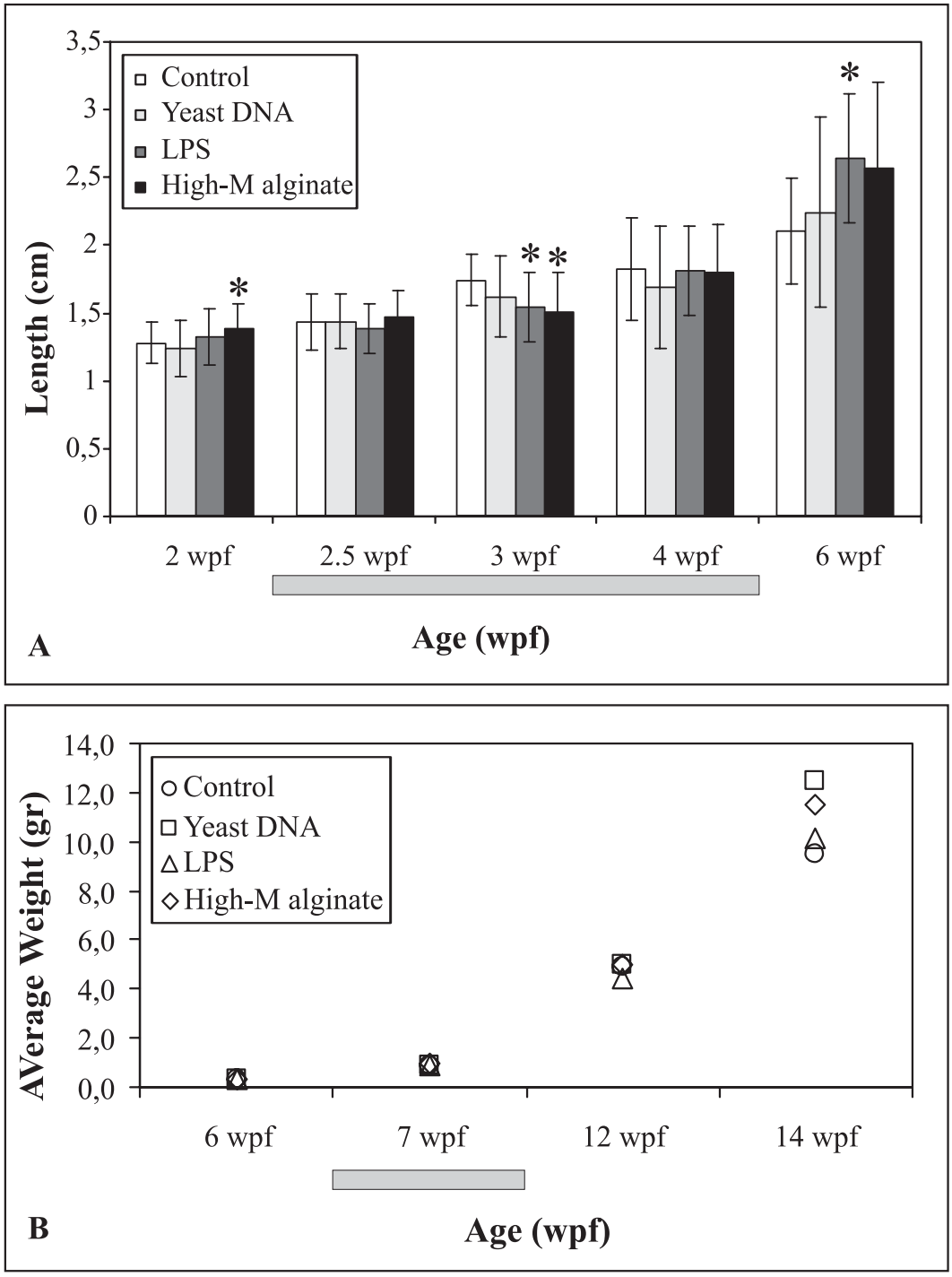

Figure 2. Average length (A) and weight (B) per group after feeding different immuno-stimulants during 2 weeks from the age of 2 (Exp 1) and 6 wpf (Exp 2) respectively. Significant differences ($p<0.05$) between the test groups and control group are depicted with asterisks. Oral administration of immuno-stimulants is indicated with a grey bar. Wpf = weeks post fertilisation.

groups were present during immuno-stimulation, and at 6 wpf the group fed with LPS had a significantly higher average length then the control group (Figure 2A).

In Exp 2, the average weight (Figure 2B) of the fish per group was initially similar, while at 14 wpf (8 weeks after the start of the experimental feeding) the groups that had received food with yeast-DNA and high-M alginate were heavier then the group fed with LPS or the control group. Of a total of 124 fish per group, 2 fish died in the group fed with Yeast-DNA and 1 in the control group.

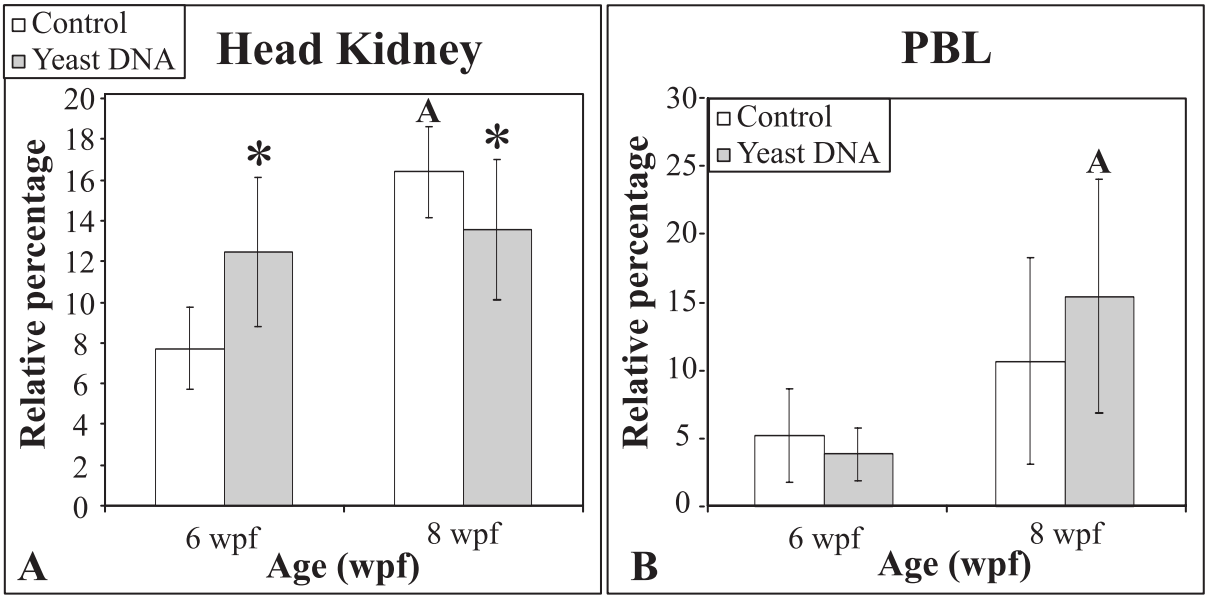

Figure 3. Relative percentages of WCI12^{+} cells in cell fractions of head kidney (A) and peripheral blood leukocytes (PBL; B) in Exp 1 (Immunostimulation from 2 wpf during 2 weeks). Significant differences ($p<0.05$) between the group fed with Yeast DNA and control group are depicted with an asterisk, while differences between ages are displayed with a capital A. For the groups fed with A. salmonicida LPS or high-M alginate no differences compared to the control group were observed. Absolute numbers of WCI12^{+} cells showed no significant differences between groups, although in head kidney the group fed with yeast DNA contained higher and lower (not significant) numbers of WCI12^{+} cells at 6 and 8 wpf respectively. Wpf = weeks post fertilisation.

Cell population kinetics of WCI12$^+$ (B) cells determined with flowcytometry

In Exp 1, the Yeast DNA group had increased and decreased relative percentages of WCI12$^+$ cells compared to the control group in head kidney at 6 and 8 wpf respectively (Figure 3A). The absolute number of WCI12$^+$ cells in the yeast DNA group was also decreased compared to the control group at 8 wpf, although not significantly (results not shown). In PBL no significant differences were detected between the groups (Figure 3B), although the percentage of WCI12$^+$ cells in the Yeast DNA group compared to the control group at 8 wpf was higher then at 6 wpf. In addition, no significant differences were present between the groups fed with LPS or high-M alginate and the control group, and between absolute WCI12$^+$ numbers or percentages of necrotic cells in the different groups (results not shown).

In Exp 2 (Figure 4), the relative percentage of WCI12$^+$ cells in head kidney was lower in the test groups compared to the control group at 14 wpf (significant only for the group fed with LPS; panel A), while the relative percentage of WCI12$^+$ cells in PBL was

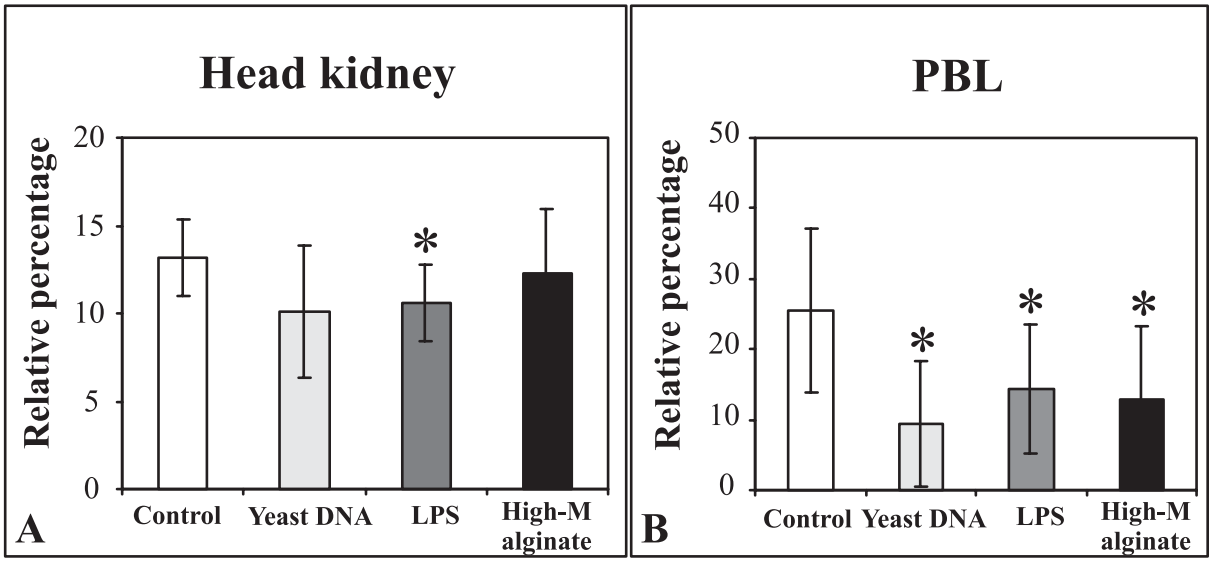

Figure 4. Relative percentages of WCI12$^+$ cells in cell fractions of head kidney (A) and peripheral blood leukocytes (PBL: B) in Exp 2 (Immunostimulation from 6 wpf during 2 weeks) at 14 wpf. Significant differences ($p<0.05$) between the test groups and control group are depicted with an asterisk. Wpf = weeks post fertilisation.

significantly decreased in all experimental groups compared to the control group at 14 wpf (panel B).

The total IgM level in plasma

The total IgM level in plasma was significantly lower in the group fed with yeast DNA compared to the control group at 12 wpf in Exp 2 (Figure 5).

The total IgM level was lowest at 8 wpf, increased significantly at 12 wpf and then slightly decreased again (non significant) at 14 wpf.

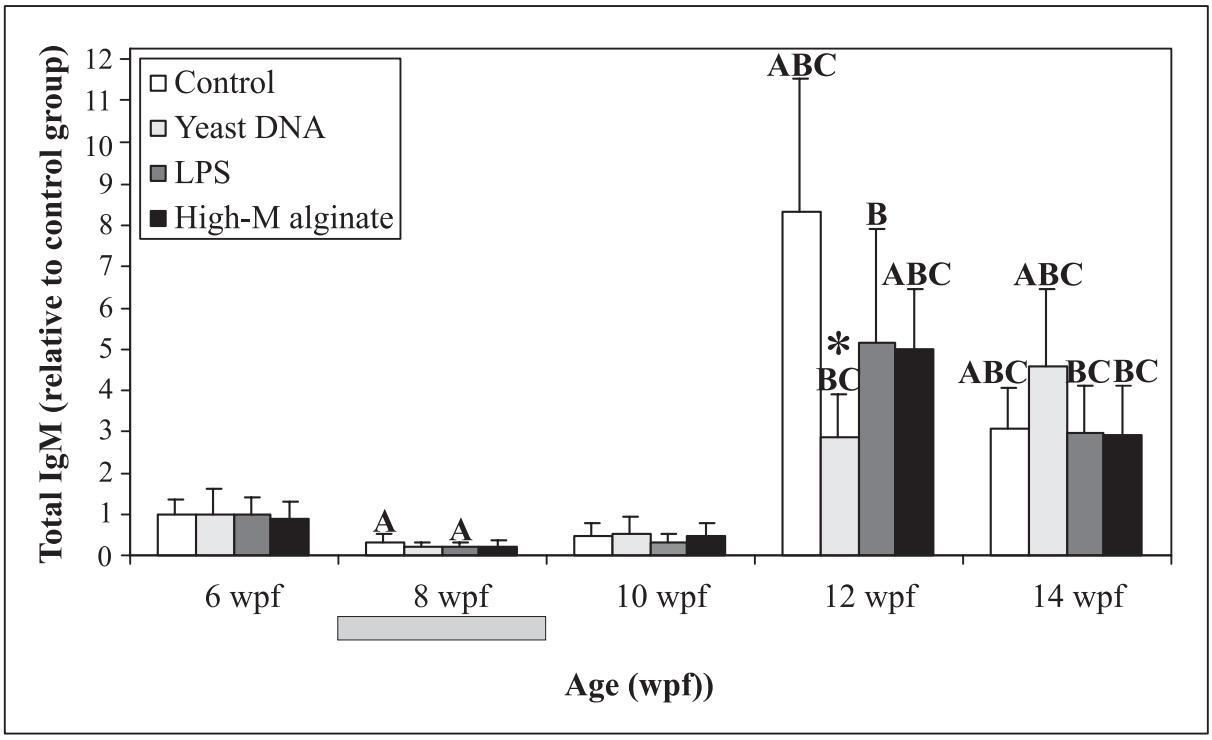

Figure 5. Total plasma IgM levels relative to the control group at 6 wpf in carp fed with normal food (control), yeast DNA, LPS and high-M alginate during 2 weeks (between 6 and 8 weeks post fertilisation (wpf)) in Exp 2 determined with ELISA. Significant differences ($p<0.05$) between the test groups and control group are depicted with an asterisk, while differences between ages are displayed with capital A (different from 6 wpf), B (different from 8 wpf) and C (different from 10 wpf). Oral administration of immuno-stimulants is indicated with a grey bar.

The expression of cytokines and iNOS in intestine and acute phase proteins in liver determined with RQ-PCR

Although *IL1-β*, *TNF-α*, and *iNOS* were expressed in the intestine, and *C3*, *SAA* and $\alpha_2 M$ in the liver, significant differences were not detected between the expression levels in the four groups of Exp2 (results not shown).

Challenge with Vibrio anguillarum

Cumulative mortality is displayed in Figure 6, and mean survival time is depicted in Table 1. Two days after adding bacteria, first mortality occurred. The challenge resulted in

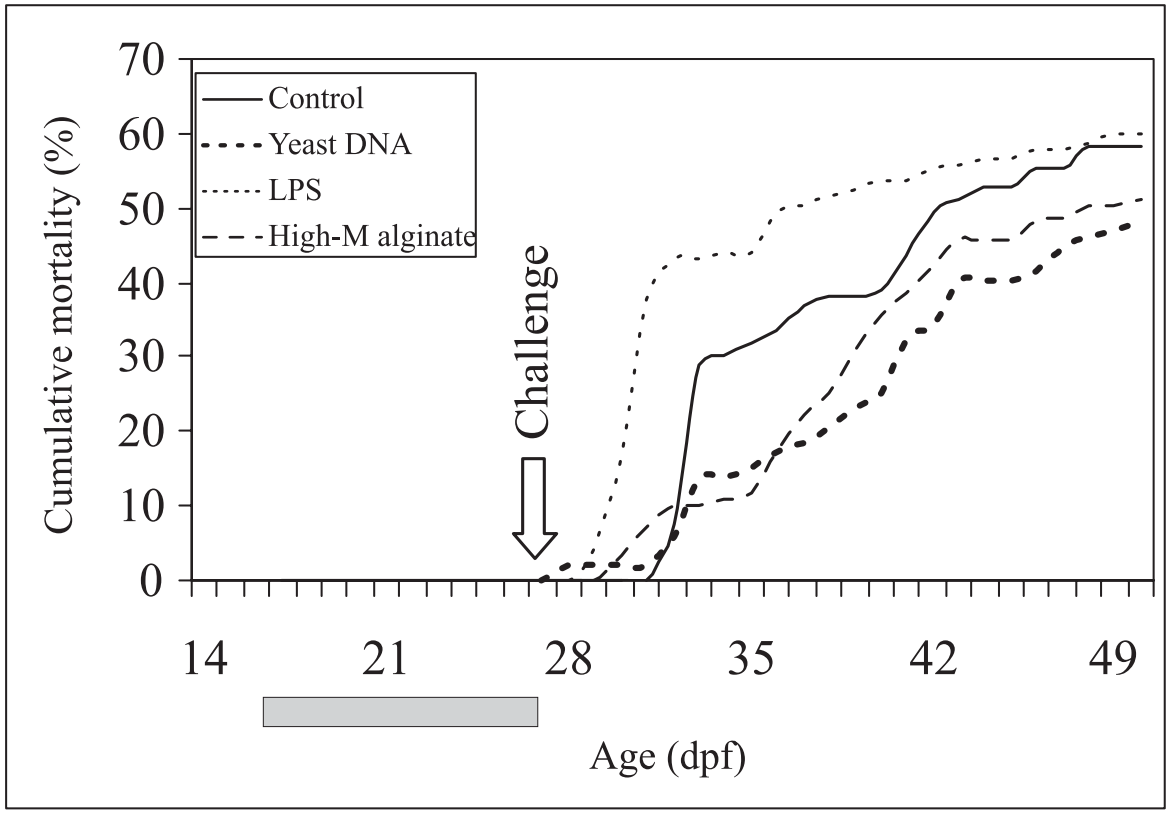

Figure 6. Cumulative mortality after challenge with *Vibrio anguillarum* following a 9 day period of oral administration of the immuno-stimulants yeast DNA, LPS and high-M alginate from the age of 17 days post fertilisation (dpf). Oral administration of immuno-stimulants is indicated with a grey bar.

approximately 60 % mortality in the control group after 24 days. Mortality in the group supplemented with LPS started earlier compared to the control group and remained higher for the first 15 days after challenge. The mean survival time (Table 1) was significantly shorter in the group fed with LPS compared to the control group. After 24 days, the mortality in the group fed with LPS equalled that in the control group. Cumulative mortality in the groups fed with Yeast DNA and high-M alginate was about 20 % lower at days 6-13 after challenge, and was approximately 50 % (10 % less then the control group) at the end of the experiment. The mean survival time was longer in the groups fed with high-M alginate (not significant) and Yeast DNA (significant) compared to the control group.

Table 1. Mean survival time (days) after challenge with *Vibrio anguillarum* at 26 dpf following a 9-day period of feeding immuno-stimulants. Mortality was recorded from 0-50 days after challenging. Asterisks indicate a significant difference ($p<0.05$) compared to control group.

Immuno-stimulant	Average ± SD
Control	16 ± 8.0
LPS	14 ± 9.5*
High-M alginate	18 ± 7.3
Yeast DNA	19 ± 7.2*

Discussion

This study presents the effects of oral administration of Yeast DNA, *A. salmonicida* LPS and high-M alginate to juvenile carp during 2 weeks from 2 and 6 wpf onwards.

Oral immuno-stimulation resulted in an ultimate decrease in the percentage of $WCI12^+$ (B) cells in head kidney (in Exp 1 only after administration of Yeast DNA) and PBL (only in Exp 2), and a decreased total IgM level several weeks after oral immuno-stimulation. In all groups the total IgM levels first decreased with age, and then increased, which was also observed in carp performing an ELISA with an anti-Pike Ig antibody (J. Van Loon, unpublished results; Chapter 7: Figure 5). The suppressive effect of the

immuno-stimulants is in contrast to findings in both mammals and fish, where bacterial DNA and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides induce murine B cells to proliferate and produce Ig *in vitro* and *in vivo* after injection[169], and oral administration of Ergosan (an algal extract) induced a (non significant) increase in B cells in PBL in sea bass[12]. It must be stressed however, that the aforementioned study in mammals was performed after parenteral administration, and the study in sea bass was not performed with juvenile animals. In adult sea bass, a significant stimulating effect after oral administration of alginate was observed on innate immune parameters[12]. Although subsequent studies are necessary to elucidate kinetics and dose-response relationships after oral administration of immuno-stimulants in fish, our data suggest an inhibiting rather then a stimulating effect on B cells after oral immuno-stimulation in juvenile carp. Even though suppressive responses can be explained by too high doses (high dose suppression) or by prolonged administration (long term reversion)[9], our data strongly indicate oral tolerance or juvenile tolerance, which was also observed after oral vaccination of carp at 2-4 wpf [8]. Unfortunately, literature is scarce on these subjects[138,173].

This conclusion is supported by the outcome of the challenge experiment, in which mean survival time in the group fed with *A. salmonicida* LPS was shortest. The supplemental feeding of LPS at young ages presumably suppressed B cells reacting to LPS, impairing the immune response to *Vibrio anguillarum* (assuming anti-LPS B cell clones cross-react with different LPS species). Another possibility is that the purified *A. salmonicida* LPS still contained components that have suppressive on effects the immune response[174,175]. On the other hand, the groups fed with Yeast DNA and high-M alginate demonstrated lower cumulative mortality, which can be explained with LPS-reactive B cell clones not being suppressed, or due to intrinsic properties of the immuno-stimulants. Despite the suppressing effect on B cells, cumulative mortality was even lower then the control group, probably because innate immune mechanisms still offer an increased protective effect. However, these conclusions are based on a single challenge experiment without data on B cell percentages or IgM levels during the challenge, which was technically not possible because the fish were too young. Nevertheless, if this hypothesis

will prove true with future experiments, it will provide a serious warning for the use of especially pathogen-derived immuno-stimulants in larval and juvenile fish.

Oral immuno-stimulation with high-M alginate resulted in increased growth at the end of both experiments, which is in concordance with results using turbot (*Scophthalmus maximus* L.)[176], but in contrast to results in Atlantic salmon (*Salmo salar*)[177]. At the end of Exp 1, LPS in food also resulted in increased (not significantly) length of the fish. This is in concordance with earlier results in which fish food was supplemented with fatty acids[178], which suggests the effects of increased growth are related to nutritional value.

Expression of *IL1-β*, *TNF-α* and *iNOS* in intestine or *C3*, *SAA* and *α2M* in liver was not affected by oral immuno-stimulation, providing the first record after non-invasive immuno-stimulation in fish. Future studies on oral immuno-stimulation must be focussed on cytokine expression in intestinal cell populations rather then complete organs.

In conclusion, oral administration of high-M alginate, LPS and particularly Yeast DNA induced changes in B cell kinetics in PBL and head kidney, suggesting suppressive effects strongly indicating oral or juvenile tolerance. This hypothesis is supported by challenging with *Vibrio anguillarum*, providing a warning for the use of especially pathogen-derived immuno-stimulants in larval and juvenile fish. However, because there is hardly any precedent on this application method or this very young age, additional data are required on dose-response relationships, kinetics, administration route and survival percentages at different ages. Increased knowledge on the application of oral immuno-stimulants in fish is highly relevant because of the considerable problems with infectious diseases and the potential efficiency of oral immuno-stimulation following certain conditions.

Acknowledgements

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General Discussion

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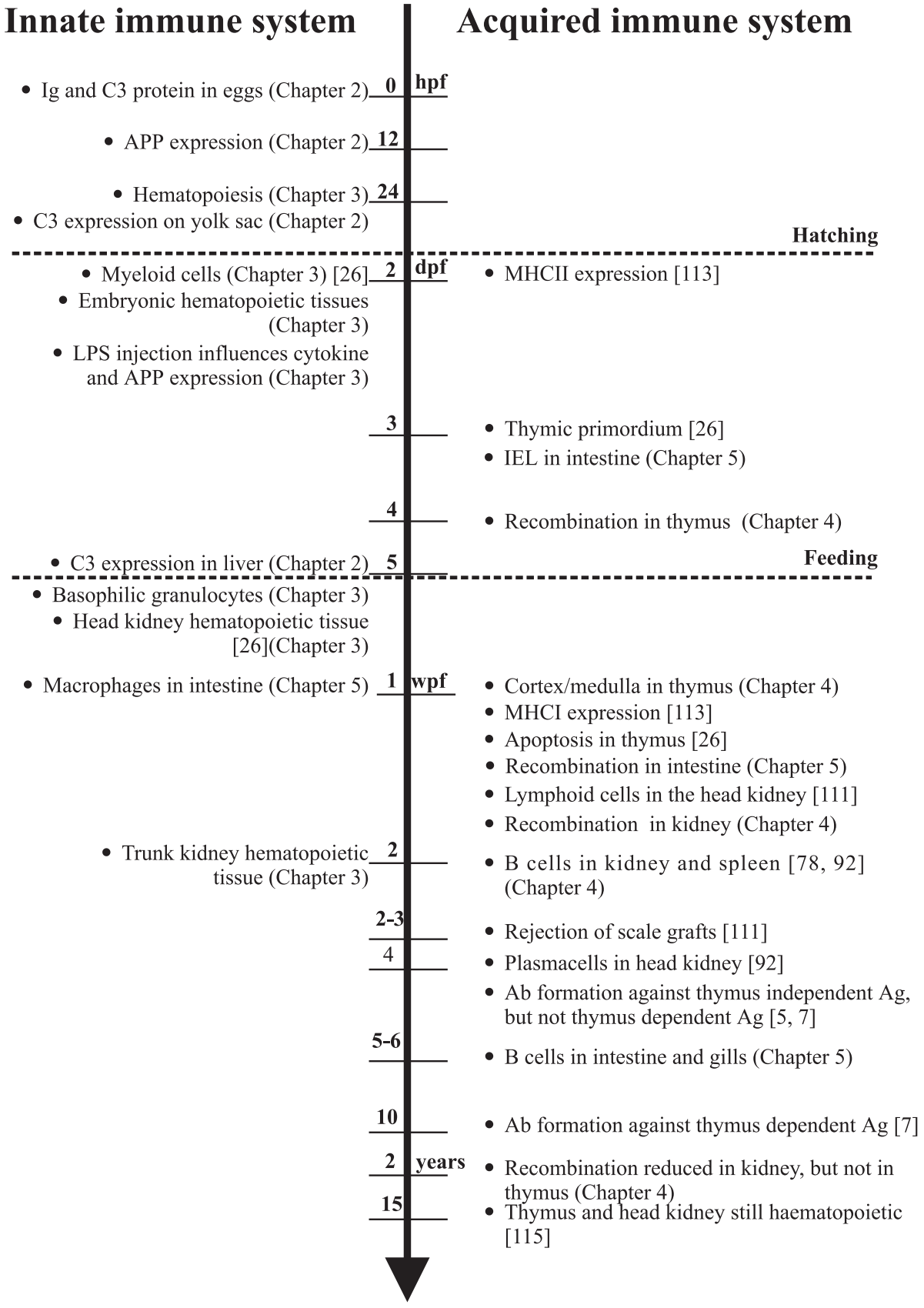
In contrast to higher vertebrates, most fish species are free-living organisms already at the embryonic stage of life. Living in an aquatic environment, they must posses defence mechanisms to protect themselves upon exposure to a variety of micro-organisms. It is widely accepted that acquired immunity is not yet functional at hatching time in fish. Consequently, during a rather long period in their early life fish are dependent on the innate immune system, and it is expected that this defence mechanism starts to develop already at a very early embryonic age. In this discussion the current status on the ontogeny of the immune system in teleosts will be presented, based predominantly on the available data of zebrafish (*Danio rerio*) and carp (*Cyprinus carpio* L.; both belonging to the family of cyprinidae). Subsequently, the oral administration of immunostimulants to protect young fish will be discussed.

Over the past few years, a considerable amount of work was performed on the ontogeny of the zebrafish innate immune system. Potential embryonic and larval myelopoietic sites were identified with mainly gene expression and histo-morphological studies. Zebrafish is used extensively as the prime fish model for the study of embryonic development because of the relative ease to study the transparent embryos, the straightforwardness of housing and breeding, the short generation time, the powerful genetics, which can be applied for the generation of mutants and transgenic animals, the availability of many molecular markers, and on short notice the genomic sequence. Studying the development of the carp immune system besides zebrafish offers several additional advantages: firstly the larger size facilitates research on a wide variety of immune functions like cell population kinetics and cell culture, which offer the possibility for functional studies *in vitro*, secondly a panel of antibodies is available and thirdly carp represent a major commercial value in aqua culture.

Figure 1 (following page). Landmarks of developmental immunology in the common carp. Ab = antibody, Ag = antigen, APP = acute phase protein, C3 = complement factor 3, IEL = intraepithelial lymphocyte, Ig = immunoglobulin, MHC = major histocompatibility complex.


Innate immune system
Acquired immune system
• Ig and C3 protein in eggs (Chapter 2)
0 hpf
• APP expression (Chapter 2)
12
• Hematopoiesis (Chapter 3)
24
• C3 expression on yolk sac (Chapter 2)
Hatching
• Myeloid cells (Chapter 3) [26]
2 dpf
• MHCII expression [113]
• Embryonic hematopoietic tissues (Chapter 3)
• LPS injection influences cytokine and APP expression (Chapter 3)
3
• Thymic primordium [26]
• IEL in intestine (Chapter 5)
4
• Recombination in thymus (Chapter 4)
• C3 expression in liver (Chapter 2)
5
Feeding
• Basophilic granulocytes (Chapter 3)
• Head kidney hematopoietic tissue [26](Chapter 3)
• Macrophages in intestine (Chapter 5)
1 wpf
• Cortex/medulla in thymus (Chapter 4)
• MHCI expression [113]
• Apoptosis in thymus [26]
• Recombination in intestine (Chapter 5)
• Lymphoid cells in the head kidney [111]
• Recombination in kidney (Chapter 4)
• Trunk kidney hematopoietic tissue (Chapter 3)
2
• B cells in kidney and spleen [78, 92] (Chapter 4)
2-3
• Rejection of scale grafts [111]
4
• Plasmacells in head kidney [92]
• Ab formation against thymus independent Ag, but not thymus dependent Ag [5, 7]
5-6
• B cells in intestine and gills (Chapter 5)
10
• Ab formation against thymus dependent Ag [7]
2 years
• Recombination reduced in kidney, but not in thymus (Chapter 4)
15
• Thymus and head kidney still haematopoietic [115]

With the data obtained in this thesis and from previous studies a schedule was constructed presenting landmarks of developmental immunology in the cyprinid common carp (Figure 1). More details of this schedule are given in the following paragraphs.

The ontogeny of the immune system in teleost fish

Innate immune factors in teleost development

Maternal fish Ig protein (which can be considered innate because it is transferred from the mother) was detected in the eggs of many fish species[179], including carp (Chapter 2)[62,180], but not in cod (*Gadus morhua* L.)[61]. The transfer of specific maternal antibodies however was not correlated with increased survival after infection in several fish species[61]. In addition, maternal complement factor 3 (C3) protein, which is the central component of the complement system, was detected in carp (Chapter 2) and spotted wolffish (*Anarhichas minor* Olafsen)[60], but not in cod[61], and lysozyme, lectins, pentraxins and proteinase inhibitors were present in the eggs of various fish species[179].

C3, α_2-macroglobulin (*α_2M*), serum amyloid A (*SAA*) and complement factor 1 r/s - mannose binding lectin associated serine protease-like molecule (*C1/MASP2*) mRNA's were produced by carp embryos from 12 hours post fertilisation (hpf; *C1/MASP2* from 20 hpf), which is relatively long before hatching (2 days post fertilisation (dpf)), indicating either involvement of these factors in development itself or more probably a provision of the immune system for the post-hatching period (Chapter 2). The abundant *C3* expression in carp embryos is indicative for the presence of other components of the complement system at that age. C3, α_2M and SAA have all been implicated in the adult carp immune system: C3 mediated killing of *Trypanoplasma borreli*[28], while *C3*, *α_2M* and *SAA* expression were induced by *T. borreli* infection *in vivo*[28], and *SAA* expression was induced after stimulation *in vitro*[33].

In addition, LPS injection in the yolk sac of carp at 2 dpf resulted in up-regulation of *SAA* expression, and down-regulation of *C3* and *α_2M* expression, implying a functional embryonic innate defence system (Chapter 3). The kinetics of this induction however, is

not consistent with responses in adult carp, where infection with *T. borreli* resulted in up-regulation of *C3* and α_2M expression[28], suggesting age-related differential regulation of transcription.

C3 mRNA production was situated in the yolk syncytial layer in carp embryos from 24 hpf to 5 dpf, followed by the liver in larvae, providing a new series of *C3* mRNA production in teleost development (Chapter 2). The yolk syncytical layer is a structure unique to fish and is not found in other vertebrate embryos. It is involved in the absorption of the yolk material[71-73]. A number of genes (e.g. *transferrin*) that are first expressed in the zebrafish yolk syncytial layer are later expressed in liver or intestine[74-76]. This suggests that α_2M and *C1/MASP2*, that are both predominantly expressed in the liver of adult carp[28,54], may also be expressed by the yolk syncytial layer in carp embryos (Chapter 2). Interestingly, complement factor 7 was also expressed in the yolk syncytial layer of zebrafish embryos[76]. The differential expression of *C3* in embryos and larvae can explain the aforementioned inconsistent *C3* expression patterns seen in carp embryos (down-regulation after LPS injection) and adults (up-regulation after infection with *T. borreli*) after an inflammatory stimulus, which is an interesting hypothesis to test in the future.

Throughout development, C3 protein (cod and halibut (*Hippoglossus hippoglossus* L.)) and *C3* mRNA (cod) were present in a wide variety of organs, suggesting C3 production in many different cell types and also a role for C3 in development itself [66,70,181]. In contrast to data in carp, *C3* expression was not observed in the yolk syncytial layer of these species, although C3 protein was detected here. In addition, the presence of carp *C3* mRNA was limited to yolk syncytial layer or liver, and C3 protein to yolk syncytial layer, liver and blood vessels, indicating different patterns of C3 distribution among fish species. Also, the increase of *C3* mRNA levels close before hatching at 1-2 dpf in carp signified these proteins are needed shortly after hatching, and thus are predominantly important for innate immune function (Chapter 2).

Teleost myelopoiesis in ontogeny

The lateral plate mesoderm and migration to the yolk sac

Embryonic and definitive (equal to adult) hematopoiesis are separate and different processes in zebrafish development, similar to the situation described in mammals[87]. The

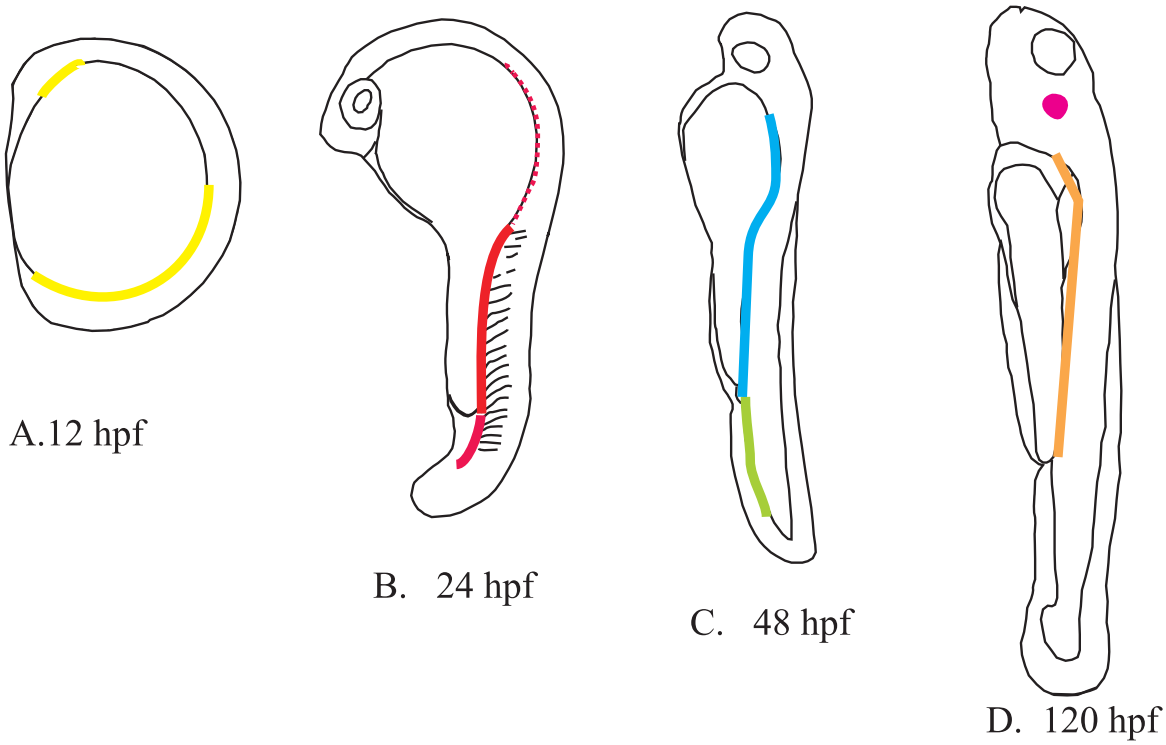


Figure 2. Hematopoietic tissues during zebrafish development, identified using cell morphology and expression of genes important for hematopoiesis. Anterior is up, dorsal is to the right. Lateral plate (ventral) mesoderm (LPM) (35); intermediate cell mass (ICM) (36), the dotted line represents mainly vasculogenic tissue (37); ventral part of dorsal aorta (35, 38); caudal (posterior) ventral vein region (plexus) or posterior blood islet (36); kidney; thymus. From 16 hpf, macrophages originate from the rostral LPM (40), and from 24 hpf erythrocytes and *mpx* positive cells appear in the ICM (35- 36, 39, 41). The ICM can be divided into separate areas based on expression patterns and developmental speed (35, 38). Between 24 and 30 hpf, the ICM disappears as cells are taken up by the nascent circulatory system (36). At 48 hpf hematopoiesis takes place in the ventral wall of the aorta (35, 38), and in the posterior blood islet (36). From approximately 4 dpf, hematopoiesis starts in thymus and head kidney (36). Possibly, hematopoiesis still continues in the posterior blood islet at 120 hpf, because a high number of *mpx* positive cells were detected (39).

hematopoietic tissues of zebrafish development are presented in Figure 2. In the lateral plate mesoderm (LPM), the first expression of genes relevant for hematopoiesis was observed around 12 hpf[35]. Macrophages, that were discerned on the anterior yolk in 22-29 hpf zebrafish, were traced to the anterior-most lateral mesoderm of the head[40].

The intermediate cell mass

During the migration of LPM cells, the intra-embryonic intermediate cell mass (ICM) is formed (Figure 2), which expresses genes relevant for hematopoiesis[35,182], containes proerythroblasts[36], and neutrophil-specific myelo-peroxidase (*mpx*)[39,41]. Zebrafish and carp embryos have a similar developmental speed, which is illustrated by the presence of the carp ICM at the same stage as in zebrafish (Chapter 3). At an early stage (20 hpf) the zebrafish ICM consisted of undifferentiated hemangioblasts, that still have the potential to form endothelial, erythroid, myeloid, and lymphoid precursors[37,44]. At 21 hpf, the zebrafish cranial ICM formed endothelia, in the middle endothelia and hematopoietic cells, and the most caudal part was undifferentiated at that age[37]. Between 24 and 30 hpf the zebrafish ICM disappeared as cells are taken up by the nascent circulatory system[36], a process similar to carp ICM (Chapter 3). The convergence of the zebrafish LPM into ICM ends just caudal of the future anus[36,37]. In other fish species, such as angelfish, initial hematopoiesis can also proceed on the yolk sac[47].

Embryonic myeloid cells

In zebrafish, extensive studies have been performed using *in situ* hybridisation (ISH) to reveal the expression sequence of genes relevant for hematopoiesis. These genes are given in Figure 3, which indicates the differentiation of the different hematopoietic cell lineages. Myeloid cells develop from a multipotent myeloid-erythroid progenitor (MMP), which also has the potential to differentiate into thrombocytes, as shown in Figure 3. The MMP is considered a myeloid lineage precursor when *spi.1* or *c/ebp1* is expressed[44]. Macrophages differentiate from this precursor as soon as they start to express *fms* and *L-plastin*[44]. In zebrafish, this process commences between 12 and 20 hpf in the aforementioned LPM-derived macrophages[40,84]. These cells, that bypassed the monocytic series, migrated onto the yolk sac around 24 hpf, where after many of them progressed to

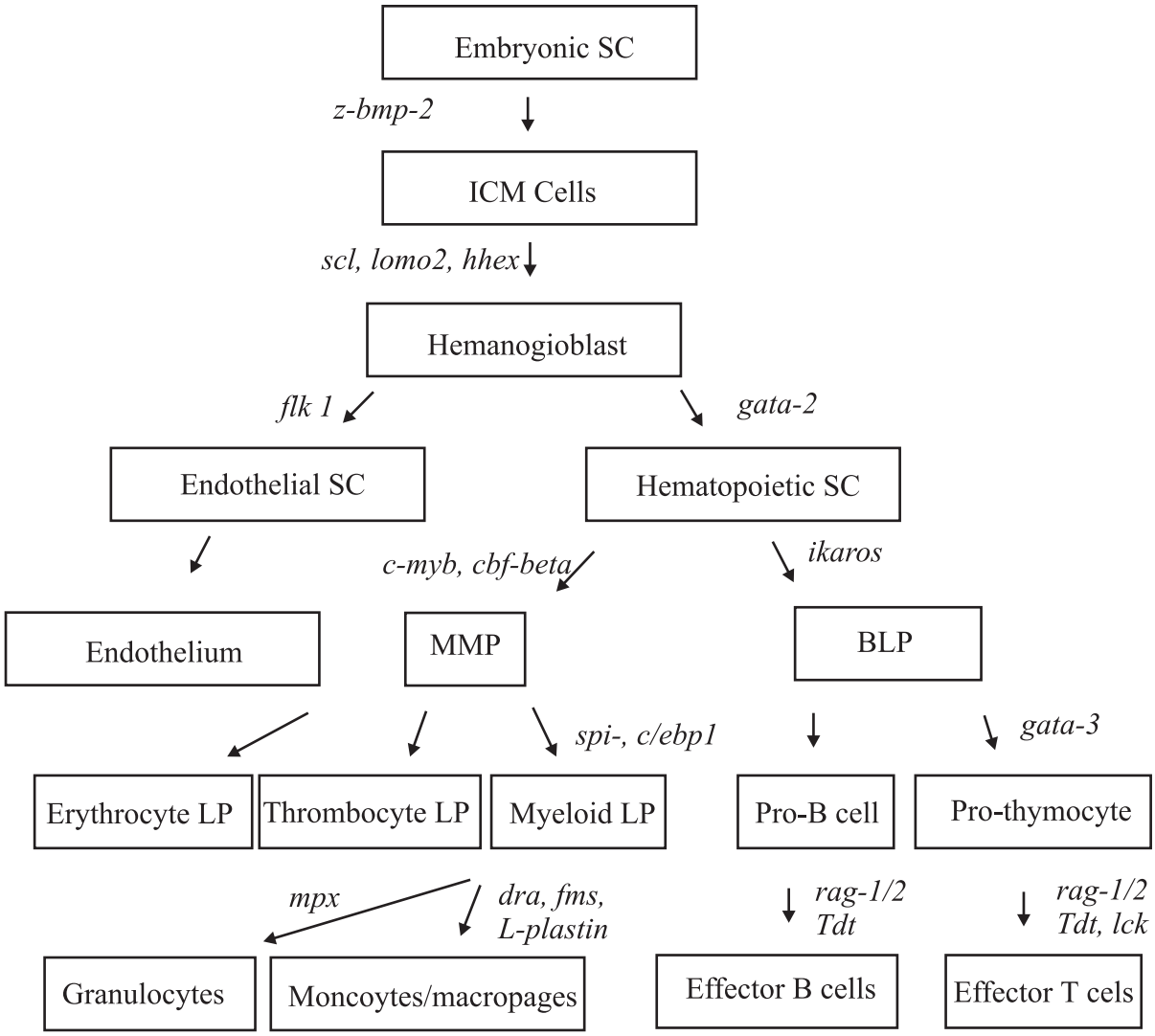


Figure 3. Schematic representation of the molecular pathways involved in zebrafish hematopoiesis. This scheme should be considered a simplified working model of the actual biological situation. SC: stem cell; ICM: intermediate cell mass; LP: lineage precursor; MMP: multipotent myelo-erythroid progenitor; BLP, bipotent lymphoid progenitor. Genes: *zbmp-2*: zebrafish bone morphogenic protein 2; *scl*: stem cell leukemia transcription factor; *lmo-2*: lim domain only-2; *hhex*: hematopoietically expressed homeobox; *flk-1*: foetal liver tyrosine kinase-1; *gata-2/3*: gata-binding protein-2 and -3 respectively; *c-myb*: myeloblastosis oncogene; *cbfß*: core binding factor β; *spi-1*: serine protease inhibitor-1 (= *pu.1*); *c/ebp1*: CCAAT/enhancer binding protein 1; *mpx*: myelo-peroxidase; *dra*: draculin; *L-plastin*: leucocyte-specific plastin; *fms*: receptor for macrophage colony-stimulating factor; *rag-1/2*: recombination activation gene-1 and -2, respectively; *TdT*: terminal deoxynucleotidyl transferase; *lck*: T cell-specific tyrosine kinase gene promotor. References:[14,35,44,184-187].

the mesenchyme of the head and into the blood circulation[40]. The presence of macrophages on the yolk sac corresponds to expression of genes relevant for hematopoiesis in zebrafish[35,39-41,84,89]. In addition, neutrophilic myelocytes and myeloblasts[36] were present on the yolk sac in prehatch zebrafish embryos. Also in carp, $WCL15^+$ monocytes/macrophages were present on the yolk sac at 48 hpf [26], but also earlier at 54 hpf (HBT Huttenhuis, unpublished results). The emergence of macrophages at 24-48 hpf corresponds to an increase of immune chemokine expression in carp embryos of the same age[86].

Before and during migration early macrophages initially expressed the *dra* (draculin) gene, which was subsequently replaced by *L-plastin*[40]. In addition to phagocytosing apoptotic cells, these macrophages also engulfed and destroyed intravenously injected $Gram^+$ and $Gram^-$ bacteria, and they showed activation and migration towards infected sites[40]. In addition, carbon particles were phagocytozed and granulocytes (although in low numbers) migrated to a trauma at 2 dpf [39]. These data indicate that zebrafish macrophages and neutrophilic granulocytes operate as a first line of defence at this early age.

In zebrafish, the macrophage colony stimulating factor was implicated in the migration of macrophages to the brain[83], but further knowledge on messenger molecules like chemokines and cytokines is extremely limited. In carp however, the cytokine interleukin 1-β and the enzyme inducible nitric oxide synthase were expressed in whole embryos at 1-2 dpf, and expression was up-regulated at already 2 dpf after LPS injection in the yolk sac, implying a functional embryonic innate immune system (Chapter 3). The presence of myeloid cells before hatching and the induction of cytokine expression in carp corresponds to the early expression of Toll Like Receptor genes in zebrafish embryos[183].

Larval and definitive myelopoietic tissues

When the vena cardinalis has formed, the ICM in the trunk transforms into hematopoietic tissue situated (according to zebrafish literature[35]) in the ventral part of the dorsal aorta, but was found (in carp) to be located more specifically ventro-lateral of the aorta (Chapter 3). Myelopoiesis was observed ventro-lateral of the cyprinid aorta from 48-

96 hpf, until hematopoiesis starts in the kidney (Chapter 3)[35,38]. In carp (Chapter 3), myeloid cells were demonstrated histologically and with the mAbs TCL-BE8 (monocytes/granulocytes)[27] and WCL15 (monocytes/macrophages)[25,26].

In the tail the cyprinid ICM transforms into hematopoietic tissue situated around the sinuses of the vena cardinalis at around 30-42 hpf in carp and zebrafish respectively (Chapter 3)[36], which is also named 'posterior blood islet' or 'ventral vein region' in zebrafish literature. From 24-72 hpf, *mpx*-expressing cells were mainly situated in the posterior blood islet[39,41,44]. This tissue remains hematopoietic in zebrafish up to at least 96 hpf[38], but possibly up to 6 dpf, because myeloid cells were still present at that stage[39]. In carp (Chapter 3), the posterior blood islet was observed histologically up to 2 weeks post fertilisation (wpf), where after the hematopoietic tissue around the sinuses of the vena cardinalis transformed into that of the trunk kidney, providing the first description of the ontogeny of the trunk kidney hematopoietic tissue in teleosts. From 2-4 dpf (at least) and 2-14 dpf in zebrafish and carp respectively, the posterior blood islet is considered the major producer of myeloid cells, because the kidney hematopoietic tissue has not developed yet. In carp, the posterior blood islet and later the trunk kidney exceed the size of the head kidney hematopoietic tissue at all stages (Chapter 3). From 4 dpf (zebrafish) and 5-7 dpf (carp) onwards the head kidney develops hematopoietic tissue (Chapter 3)[26,36].

Teleost T cell lymphopoiesis in development

T cells and the thymus

When embryonic developmental processes are completed, the various hematopoietic processes gradually shift towards the organs they are associated with in the juvenile and adult situation. In freshwater teleosts, the thymus is the first of these organs to become lymphoid, but in marine teleosts, such as Atlantic cod and flounder (*Platichthys flesus* L.), the thymus develops after kidney and spleen[47]. In zebrafish and carp the thymus anlage is lymphoid at 3 and 4 dpf, respectively[36,50]. These early thymocytes probably originated from the ICM as indicated by *Ikaros* gene expression[150]. Ikaros is a zinc finger DNA binding protein considered to be a master hematopoietic switch gene involved in the

earliest commitment of hematopoietic stem cells to the lymphoid lineage[188,189]. With RT-PCR (reverse transcriptase PCR; JHWM Rombout, unpublished results), carp and zebrafish both expressed at least 5-6 isoforms of *ikaros*, of which 4-5 were expressed at high levels (*ik-1, ik-2, ik-7* and *ik4/8*) and 1 (*ik-3)* at a low level. In rainbow trout (*Oncorhynchus mykiss*), 8 isoforms were described[188]. Figure 4 displays the expression of these isotypes in early zebrafish development. The production of lymphoid progenitor starts when all isotypes are present, which is from 17 hpf. *Ikaros* expression was first observed in the lateral mesoderm of zebrafish at 16 hpf, followed by the ICM from 19 hpf and was detected in thymus at 72 hpf[150]. In trout, developing considerably slower at lower temperatures, *ikaros* was also expressed early in ontogeny: at 3-4 days in the yolk sac and

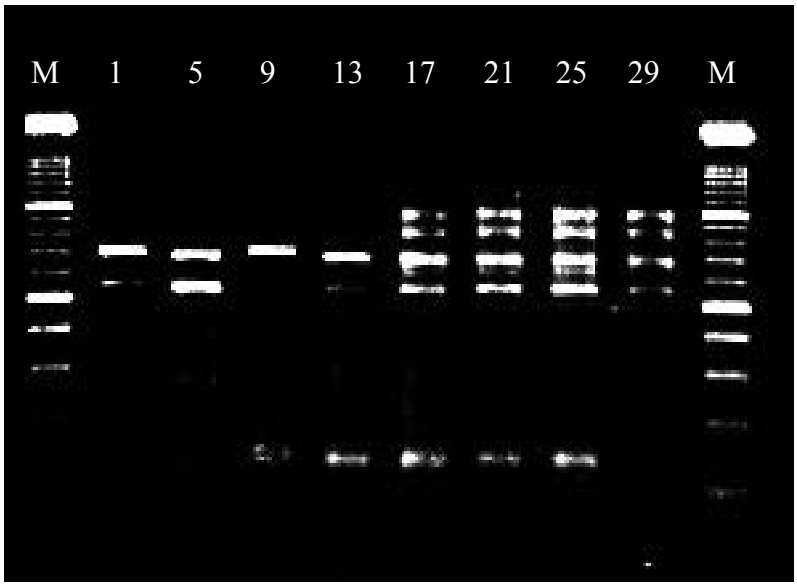


Figure 4. *Ikaros* expression during the first 29 hours of zebrafish development. RT-PCR with *ikaros* primers was performed on RNA isolated from pools of 10 embryos of equal age, which is indicated above each lane (in hours post fertilisation). All embryos were grown from the same clutch of eggs. A molecular weight marker was loaded on the gel on the far left and right sides. Note the presence of the 5 isoforms in zebrafish (4 strongly and 1 weakly expressed) from 17 hours post fertilisation (hpf) onwards, before that age only 2-3 bands were detected. Embryos were monitored until 61 hpf, but showed the same 5- 6 isotype pattern (JHWM Rombout, unpublished results, with thanks to M.O. Huising and T. Van Der Meulen; MSc thesis, 1999).

at 5-6 days in the embryo proper[188]. T cell progenitors originate from the kidney marrow later in life[190].

Mature T- or B cells have experienced V(D)J recombination of their B cell receptor (BCR) or T cell receptor (TCR) genes. It is convincingly established that this recombination coincides with the expression of recombination activating gene-1 (*rag-1*) and *rag-2*[93,94], and can be performed exclusively by these proteins[95,96]. The *rag* sequence is widely conserved throughout vertebrates[97-101], and was demonstrated indispensable for V(D)J joining in a teleost fish[102]. At 84 hpf *rag-1, gata-3* and *lck* expression was detected in zebrafish thymus[43,45], and in carp, *rag-1* expression was detected from 4 dpf in the thymus (Chapter 4). In trout *rag-1/2* expression was found at 10 dpf and *TdT* expression at 20 dpf (6 days before hatching), when thymus and head kidney appear[188].

From 1 wpf, both *rag-1*$^+$/WCL9$^+$ (mAb reactive with cortical thymocytes[24]) and *rag-1*$^-$/WCL9$^-$ areas were distinguished, suggesting early cortex/medulla differentiation. In zebrafish a cortex/medulla demarcation was found in a recent study with *rag-1* expression at 1 wpf and histologically at 3 wpf[114]. Interestingly, WCL9$^+$ lymphoid cells in carp were detected in blood, spleen, head kidney and gut in the first weeks, although in significantly lower proportions compared to thymus. However, after 5 wpf their presence was restricted to the thymus[78]. The significance of this feature and the distribution of WCL9$^+$ cells in embryos still have to be investigated, but is considerably hampered by the fact that the function of the WCL9 immuno-reactive molecule is not known yet.

Major histocompatibility complex (*MHC*) expression is intimately related to T cell function. In carp, *MHC-Iα* expression (using RT-PCR) was detected from 1 dpf, although β-2 Microglobulin (*β2M*) was first expressed at 7 dpf, indicating that MHC-I molecules are functional from 1 wpf [113]. *MHC-II* was expressed from 2 dpf [113]. With antibodies against both components of MHC-I, expression on thymocytes was observed from 3 wpf onwards, which may be earlier because younger stages were not investigated. However, β2M was expressed on the surface of a higher number of thymocytes than MHC-Iα, suggesting an association of β2M to another molecule, possibly a non-classical class I molecule[113]. In spleen and head kidney the expression of both molecules was similar, but

from 8 wpf onwards all cells expressed both molecules, while in thymus the difference in immuno-reactivity remained.

Another clear indication for effective selection of thymocytes is the presence of apoptotic cells. The first apoptotic cells in carp thymus were described at 7 dpf and their number increased with age, especially in the cortical area[50]. At 4 wpf high numbers of apoptotic cells were found, indicating an active selection process. This is supported by the appearance (electron microscope) of reticular epithelial cells and nurse-like cells between 1 and 4 wpf [50].

The first *TCRα* gene expression was detected in zebrafish at 3 dpf using RT-PCR, and the expression steadily increased until adult expression levels were reached between 4 and 6 wpf[45]. In another zebrafish study, using ISH, the first *TCRα* expression was found in thymus at 4 dpf and adult levels were reported at 3 wpf [16]. In the same report the first TCRα-positive cells outside the thymus were found in the region of intestine and gills at 9 dpf, followed by head kidney and subsequently spleen.

T cells (using the mAb DLT15) were detected in the thymus of sea bass (*Dicentrarchus labrax* L.) at about 28 dph (Table 1)[191,192]. DLT15$^+$ cells expressed the *TCRβ* receptor[193]. The first DLT15$^+$ cells were detected between 5 and 12 days post hatching (dph)[194] using flowcytometry on macerated embryos. However, this early appearance is likely due to cross-reaction with neural cells located in the olfactory bulbs (N. Romano, unpublished results). A similar reaction has also been observed for carp anti-T cell population antibodies (WCL9, WCL38) and *rag* expression in zebrafish[106] and mice[123], indicating that molecules involved in the acquired immune system also play a role in the nervous system. DLT15$^+$ T cells were detected in gut and thymus before spleen and head kidney.

The ontogeny of mucosal 'putative' T cells

The monoclonal antibody (mAb) WCL38 is reactive with an Ig$^-$ subpopulation of carp intraepithelial lymphocytes (IEL), which are present in large numbers in the epithelium of intestine, gills, and skin, but not in thymus, head kidney, or peripheral blood

leucocytes (PBL)[23]. WCL38$^+$ IEL expressed IL-1β, IL10 and TNFα (JHWM Rombout, unpublished results).

In addition, preliminary results showed negligible cytotoxic activity of these cells (JHWM Rombout, unpublished results) and hardly any reaction with the non-specific cytotoxic cell (NCC) marker 5C6[23], suggesting mainly regulatory T cell function. Mammalian IEL are almost exclusively T cells of the T Cell Receptor (TCR)γδ$^+$ or TCRαβ$^+$ category[141,142] . WCL38$^+$ IEL (putative T cells) appeared in the intestine at 3 dpf, which is shortly after hatching but before feeding, implying an important function at early age (Table 1; Chapter 5). Mammalian IEL are T cells that are implicated in the conventional role of mounting specific immune responses, but also in the preservation of the mucosal epithelial layer[142], gut metabolism[145], and the prevention of undesirable immune responses[141]. TCRαβ$^+$ IEL bear the hallmarks of conventional T cells, whereas TCRγδ$^+$ IEL and TCRαβ$^+$CD8αα IEL also have characteristics of innate immune cells: they respond to antigens not restricted by the conventional Major Histocompatibility Complex[142] and recognize conserved antigens[146,147]. Consequently, the early appearance of

Table 1. Immunocytochemical appearance of B and T cells in lymphoid organs of sea bass (in days post hatching (dph) ± SD; at 16°C; data from G. Scapigliati) and carp (in days post fertilisation at 25°C; hatching at 2 dpf). The sea bass data are based on ± 100 sections/organ, and in carp on immuno-histochemistry and flowcytometry. *) Based on mAb WCL9 (cortical thymocytes in adult carp). **) Based on mAb WCL38 (putative mucosal T cells).

Organ	T cells (DLT15) seabass	B cells (DLIg3) seabass		Ig$^-$ lymphoid cells carp	B cells (WCI12) carp
Thymus	28 ± 2	90 ± 5		4*	negligible
Head kidney	35 ± 5	45 ± 5		7*	14
Spleen	45 ± 3	45 ± 3		7*	14
Intestine	28 ± 2	90 ± 5		3**	35

WCL38$^+$ cells, which are similar to mammalian IEL regarding location and morphology, suggests the presence of T cells with at this age most probably innate characteristics, although their function remains to be investigated. In addition, *rag-1* expression was present in the carp intestine (although at low levels), which together with the appearance of WCL38$^+$ IEL before the thymus suggests that part of these cells, like in mammals[141], are generated in the intestine besides the thymus (Chapter 5). Also in sea bass, T cells (DLT15$^+$cells) were detected in both intestine and thymus at about the same age (Table 1)[151]. In zebrafish, IEL were detected by electron microscopy at 8 dpf [47], while TCR α positive cells were detected in intestine from 9 dpf [16].

Teleost B cell lymphopoiesis in development

Next to thymus as primary T cell organ, kidney is considered the primary B cell organ in teleosts. Expression of genes specific for the lymphoid cell lineage, such as *rag-1, rag-2* and *ikaros* were described in thymus and head kidney[43,45].

Using RT-PCR, first *Igμ* expression in carp was found in head kidney from 7 dpf (Chapter 4), which agrees with the first expression of the membrane form of *Igμ* in zebrafish[13]. In the latter study the secretory *Igμ* was expressed from 2 wpf. However, another zebrafish report described the first *IgLc* expression in zebrafish at 3 dpf [45].

At 4 dpf, first extrathymic *rag-1* expression in zebrafish was observed outside the thymus in the endocrine part of the pancreas, suggesting zebrafish B cells develop in the pancreas, which is supported by the first detection of *Igμ* expression at the same location at 10 dpf [13]. In carp, neither *rag-1* expression nor WCI12$^+$ cells were observed in the hepato-pancreas (Chapter 4), indicating this organ does not play a role in carp B cell production. Another recent and elegant zebrafish study also indicated the kidney as primary organ for B cells[122]. In this report transgenic zebrafish were used in which *rag-2* or *lck* was coupled to GFP expression. Fluorescence was observed in the thymus and kidney, but not in the pancreas. Within zebrafish further experiments are indicated to explain this discrepancy.

Rag-1 and *rag-2* were first detected in head- and trunk kidney using real time quantitative PCR (RQ-PCR) at 1 wpf, which, similar to the thymus, implies that V(D)J

recombination starts as soon as lymphoid cells are present in the kidney[6]. Together with expression of *IgH* and Ig positive cells at 2 wpf, this indicates the onset of B cell generation in the kidney at that stage. With respect to the generation of lymphoid cells in zebrafish, *rag-1* was detected in the head kidney with ISH from 2-3 wpf onwards[13,45,47], which is considerably later then found for carp. This can however be attributed to the fact that ISH is less sensitive than RQ-PCR. Indeed the construction of *rag-2*^{GFP} zebrafish indicated first *rag* expression at 8 dpf in the head kidney[195].

Surface Ig$^+$ cells were first detected in carp head kidney at 2 wpf (Table 1; Chapter 4)[78] using WCI12, a mAb directed against serum IgM heavy (H) chain. In an earlier study[22], heterogeneity in carp IgM was detected using a second mAb against the carp IgM H chain (WCI4). With WCI4 an additional population of B cells was detected, which reacted with 50% of all B cells detected in carp head kidney at 2 wpf, but the proportion decreased till around 20% in head kidney, spleen and blood from 4 weeks onwards. These data indicate that WCI4$^+$12$^-$ B cells play a more important role in ontogeny than WCI4$^+$12$^+$and WCI4$^-$12$^+$cells, of which the latter is the main B cell population in older animals. Although IgD was sequenced in several fish species[17,196,197], it is unlikely that WCI4 detects IgD, because the molecular weight of the immuno-reactive molecule is of the IgM H chain size. A separate IgM isotype is more logical, because the use of a distinct IgM isotype is also suggested for mucosal immunity[139].

In sea bass, B cells also appeared considerably later than T cells (45 days post hatching (dph) vs 28 dph: Table 1). The first IgM$^+$ cells in sea bass were cytoplasmic-positive, followed by surface IgM$^+$ cells 1-2 weeks later[194]. This sequence of IgM appearance is also reported for trout[198], and comparable with the first μ-chain appearance in amphibians[199,200] and mammals[201]. The first cytoplamic Ig$^+$ plasma cells were detected in carp head kidney from 4 wpf (Chapter 4)[92], although immunoglobulin production starts approximately 2 weeks earlier (Figure 5). In zebrafish secretory *Igμ* expression started at 2 wpf [13], which fits more or less with the first plasma cell detection in carp. In any case the first appearance of plasma cells in carp and zebrafish is not correlated to the first feeding (at 4-5 dpf), which is contrast to some marine species[202].

Carp B cells depart from kidney and spleen from 5 wpf onwards to populate peripheral organs, amongst which the intestine (Chapter 5). Consequently, B cells appear considerably later in the intestine then carp WCL38$^+$ IEL, indicating that mucosal immunity is not complete before this stage. Similar conclusions were drawn for spotted wolffish, because ISH using a probe complementary to the secretory Igμ chain revealed also a delayed appearance of plasma cells in the intestine[152].

The appearance of a functional acquired immune system

The appearance of carp plasma cells at 4 wpf is consistent with the induction of an antibody response against the thymus independent antigen *Aeromonas salmonicida* at 4

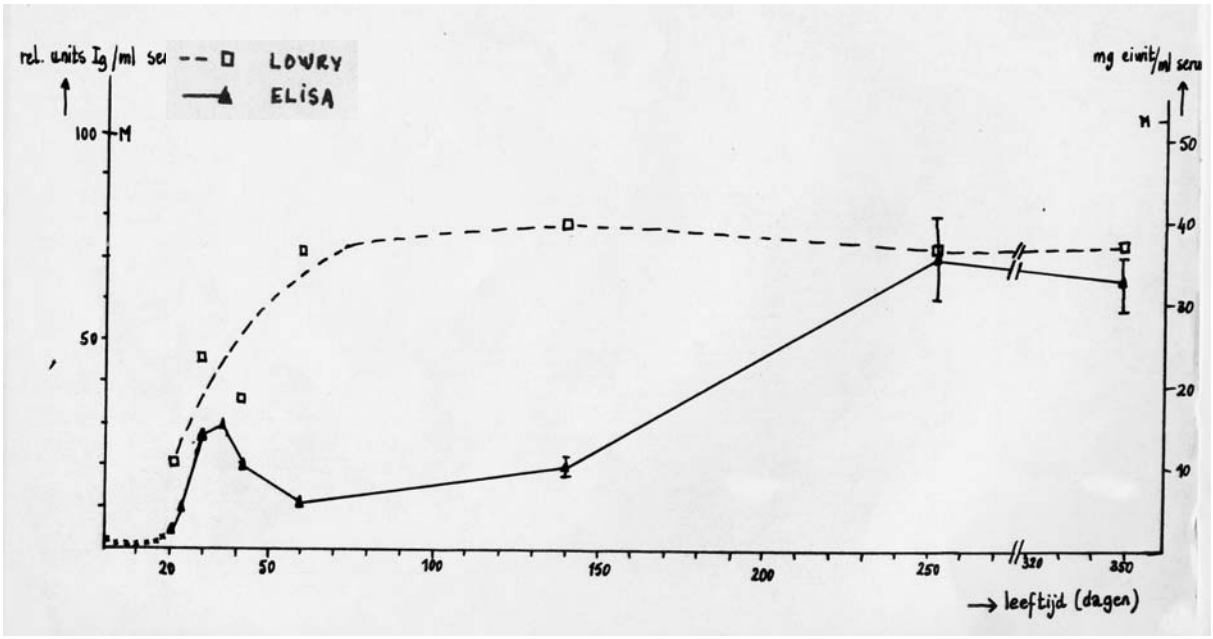


Figure 5. Serum immunoglobulin (Ig) levels throughout development of the common carp. Ig levels were determined with an anti-Pike Ig antibody using ELISA. Ig levels are depicted with triangles (closed line), and described as relative units Ig/ml serum (left axis), while the total protein content was determined with the Lowry method (open squares with dotted line) described as mg protein/ml serum (right axis). The horizontal axis depicts age in days post fertilisation. This picture was reproduced from a MSc thesis (J. van Loon, 1980), and was partly described before[62].

wpf [51,125]. In contrast, the injection with thymus-dependent antigens at 4 wpf like human gamma globulin[7], sheep red blood cells[5], or an orally administered bacterin[8] induced tolerance, while immunisation at 9-10 wpf produced an increased antibody response[7,8]. Similar results were obtained for rainbow trout[7,203] and recently also for zebrafish[45], presenting T cell independent antibody responses from 4 wpf and T cell dependent antibody responses at 6 wpf. In conclusion, T helper cell function appears somewhere between 6 and 9 wpf in both zebrafish and carp, which is considerably later then the appearance of a cortex/medulla organisation in the thymus. Another explanation for the disparity between thymus-independent and thymus-dependent antibody response would be the earlier presence of B1 cells, that produce natural (thymus-independent) antibodies as was shown in mice[204].

The first cytotoxic responses in carp were described at 19 dpf (at 22˚C) based on scale graft rejection[6,111]. Following the mammalian paradigm, this immunological response was caused by either specific cytotoxic/helper T cells, or by non-specific cytotoxic cells. The presence of specific T cells at this age was not detected in the immunisation experiments mentioned in the preceding paragraph, so that the latter option gains credibility. Unfortunately knowledge about the ontogeny of non-specific cytotoxic cells is limited.

Oral immuno-stimulation of fish

The production of farmed fish (in particular carp species) has increased considerably over the past few years, and is now providing a significant section in the food market. Similar to intensive farming of domestic animals, the high density of animals is accompanied by a high incidence of infectious diseases, especially in young fish. Prophylactic measures like sanitary isolation, antibiotic therapy, vaccination and the administration of immuno-stimulants are all applied, although they all have their drawbacks. The administration of immuno-stimulants via the food would be a very sustainable approach especially in young animals because of its uncomplicated use, fast delivery, less stressful entry route and broad-spectrum effect.

The oral administration of poly-mannuronic acids (present in extracts of algae) induced increased survival in Atlantic halibut and turbot (*Scophthalmus maximus* L.)[10,11], growth in turbot[176,177], and complement, lysozyme and liver heat shock protein activity in sea bass[12]. The effect of oral administration of bacterial LPS and DNA with unmethylated CpG motifs was to the author's best knowledge not studied before. In addition, in juvenile fish oral immuno-stimulation with the aforementioned substances was never investigated before with immunological parameters. In carp (Chapter 6), oral administration of immuno-stimulants from 6 wpf resulted in decreased $WCI12^+$ (B) cell percentages in PBL (only after administration of *A. salmonicida* LPS) and head kidney (all test groups), and a decreased total IgM level in plasma. Although suppressive effects can result from too high doses (high dose suppression) or prolonged administration (long term reversion)[9], the data are strongly indicative for oral or juvenile tolerance, which was also observed after oral vaccination of carp at 2-4 wpf [8]. No changes were observed in cytokine or iNOS expression levels in the intestine or acute phase protein expression in the liver. After oral administration from 2 wpf, the effects on $WCI12^+$ (B) cell percentages were less pronounced. A challenge with *Vibrio anguillarum* resulted in an initially higher cumulative mortality in the group fed with LPS, but lower mortality in the groups fed with Yeast DNA or high-M alginate compared to the control group, providing a provisional warning for the use of especially pathogen-derived immuno-stimulants, such as *A. salmonicida* LPS, in larval and juvenile fish.

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Summary

The production of farmed fish (in particular carp species) has increased considerably over the past few years, and is now providing a significant section in the food market. Similar to intensive farming of domestic animals, the high density of animals in aquaculture is accompanied by a high incidence of infectious diseases, especially in young fish. Prophylactic measures like sanitary isolation, antibiotic therapy, vaccination and the administration of immunostimulants are applied, although they all have their drawbacks. The administration of immuno-stimulants via the food would be a very sustainable approach especially in young animals because of its uncomplicated use, fast delivery, less stressful entry route and broad-spectrum effect. Extensive knowledge on the ontogeny of the fish immune system is required to facilitate the rational design and selection of immuno-stimulants which are effective in young fish. Knowledge on the ontogeny of the immune system in teleost fish has progressed considerably in zebrafish (*Danio rerio*) over the past few years due to the relative ease to study the transparent embryos, the straightforwardness of housing and breeding, the short generation time, the powerful genetics, which can be applied for the generation of mutants and transgenic animals, the availability of many molecular markers, and on short notice the genomic sequence. Potential embryonic and larval myelopoietic sites were identified with mainly gene expression studies and histo-morphological work. This thesis will address the ontogeny of the immune system in carp (*Cyprinus carpio* L.), because first the larger size facilitates research on a wide variety of immune functions like cell population kinetics and cell culture, second a panel of antibodies is available, third carp represent a major commercial value in aquaculture, and fourth zebrafish DNA sequences often bear great similarity to carp sequences and facilitate studies on the molecular level in carp (**Chapter 1**). Subsequently the study of the ontogeny of the carp immune system offers a functional approach to study the generation of effective immune responses as an additional advantage.

In carp ontogeny, innate immune factors were the first components of the immune system to appear (**Chapter 2**). Complement factor 3 (C3) and immunoglobulin protein were transferred from mother to egg, together with mRNA's of complement factor 3, α_2-macroglobulin, serum amyloid A and a complement factor 1 r/s - mannose binding lectin

associated serine protease-like molecule, while these mRNA's were produced by the embryo starting from 12-20 hours post fertilisation (hpf). This is relatively long before hatching (2 days post fertilisation; dpf), indicating either involvement of these factors in development itself or more probably a preparation of the immune system for the post-hatching period. In addition, C3 mRNA production was situated in the yolk syncytial layer in embryos from 24 hpf to 5 dpf, followed by the liver in larvae, providing a new series of C3 producing tissues in teleost development.

In **chapter 3**, the second group of components of the immune system to appear, the cells and myelopoietic tissues of the innate immune system, are described. Carp myeloid cells first appeared ventro-lateral of the aorta at 2 dpf (the start of hatching), and subsequently around the sinuses of the vena cardinalis (or posterior blood islet), head kidney and trunk kidney. In addition, the hematopoietic tissue around the sinuses of the vena cardinalis transformed into that of the trunk kidney, which is the first description of the ontogeny of the trunk kidney hematopoietic tissue in teleosts. The monoclonal antibodies used in this study reacted with carp myeloid surface molecules that are already transcribed and processed from the first appearance of myeloid cells, and thus serve a significant role in unravelling ontogenetic processes of teleost immunology. Finally, this study associated the first appearance of myeloid cells with an immune response on the molecular level: 2 dpf embryos responded to lipopolysaccharide (LPS) injection with upregulation of interleukin 1-β, inducible nitric oxide synthase and serum amyloid A, and down-regulation of complement factor 3 and alpha$_2$-macroglobulin, implying a functional embryonic innate defence system.

Mature T- or B cells have experienced V(D)J recombination of their T cell receptor or B cell receptor genes. It is convincingly established that this recombination coincides with the expression of recombination activating gene-1 (*rag-1*) and *rag-2*, and can be performed exclusively by these proteins. The *rag* sequences are widely conserved throughout vertebrates, and was demonstrated indispensable for V(D)J joining in a teleost fish. The generation of lymphoid cells during carp development was studied by analysing expression of the *rag* genes (**Chapter 4**). *Rag-1* was first expressed in the thymus at 4 dpf, while both *rag-1*$^+$/WCL9$^+$ (cortical thymocytes) and *rag-1*$^-$/WCL9$^-$ areas were

distinguished from 1 week post fertilisation (wpf), suggesting early cortex/medulla differentiation. From 1 wpf, *rag-1*/*rag-2* was expressed in kidney (together with immunoglobulin heavy chain expression) but not in spleen, while WCI12$^+$ (B) cells appeared 1 week later in both organs, suggesting B cell recombination in kidney but not in spleen. *Rag-1* expression exceeded *rag-2* levels in thymus and in head- and trunk kidney of juveniles, but this ratio was reversed in head- and trunk kidney from approximately 16 wpf onwards, for which no explanation can be given. *Rag-1*/*rag-2* expression was detected in thymi of animals over 1 year old, but in kidney only at low levels, indicating life-long new formation of putative T cells but severely reduced formation of B cells in older fish.

The ontogeny of carp immune cells was studied in mucosal organs (intestine, gills, and skin; **Chapter 5**). In addition, *rag-1* expression was examined in the intestine to investigate extrathymic generation of lymphocytes. WCL38$^+$ intraepithelial lymphocytes (putative T cells) appeared in the intestine at 3 dpf, which is shortly after hatching but before feeding, implying an important function at early age. Together with the appearance of these cells before the thymus and *rag-1* expression in the intestine, this suggests that alike mammals at least part of these cells are generated in the intestine besides the thymus. WCL15$^+$ monocytes/macrophages appeared in the lamina propria of the intestine at 7 dpf, but considerably later in the epithelium, while WCI12$^+$ (B) cells appeared in intestine and gills at 6-7 weeks, suggesting a functional mucosal immune system from this age.

In **chapter 6**, the effect of a two-week period of oral immuno-stimulation from the age of 2 or 6 weeks (before and after the first ability to produce antibodies) with *Aeromonas salmonicida* LPS, Yeast DNA (containing unmethylated CpG motifs) or high-M alginate (an extract of algae containing poly-mannuronic acid), was studied. Oral administration of immuno-stimulants from 6 wpf resulted in decreased WCI12$^+$ (B) cell percentages in PBL (only after administration of LPS) and head kidney (all test groups), and a decreased total IgM level in plasma, suggesting suppressive effects consistent with oral or juvenile tolerance. After administration from 2 wpf, the effects on WCI12$^+$ (B) cell percentages were less pronounced. No changes were observed in the cytokine expression levels in the intestine or acute phase protein expression in the liver. At 26 dpf (after a

period of oral immuno-stimulation), a challenge with *Vibrio anguillarum* resulted in an initially higher cumulative mortality in the group fed with LPS, but lower mortality in the groups fed with Yeast DNA or high-M alginate compared to the control group, providing a provisional warning for the use of especially pathogen-derived immuno-stimulants, such as *A. salmonicida* LPS, in larval and juvenile fish.

In conclusion, this thesis provides an overview of the appearance of the main components of the immune system in carp ontogeny, and adds to the knowledge of developmental immunology in teleost fish in general (**Chapter 7**). The availability of antibodies and the larger size facilitate cell kinetics studies in carp, and gives the study on the ontogeny of a functional immune system in this teleost additional value next to the extensively used zebrafish. Moreover, tools and data obtained in this thesis were applied for evaluation of oral immuno-stimulation, paving the way for more efficient production of this extensively cultured fish species.

Samenvatting

De produktie van gekweekte vis (o.a. karperachtigen) is aanzienlijk gestegen in de laatste jaren, en omvat nu een groot segment van de wereldvoedselmarkt. Net zoals bij het intensief houden van landbouwhuisdieren is het kweken van vissen in hoge dichtheden gerelateerd aan een toename van infectieziekten. Deze problemen zijn vaak het grootst bij jonge vissen. Preventieve maatregelen zoals het voorkomen van overdracht door isolatie, het toedienen van antibiotica, vaccinatie en het toedienen van weerstandsverhogende stoffen worden op grote schaal toegepast, hoewel ze allemaal hun specifieke nadelen hebben. Het toedienen van weerstandsverhogende stoffen in het voer is een benadering die op de lange termijn vol te houden is omdat het gemakkelijk, snel en diervriendelijk toe te dienen is en een breed-spectrum effect heeft. Uitgebreide kennis van de vroege ontwikkeling van het immuunsysteem bij vissen is echter vereist om het ontwerpen en selecteren van weerstandsverhogende middelen te vergemakkelijken. Kennis over de vroege ontwikkeling van het immuunsysteem bij vissen is de laatste jaren vooral opgedaan bij de zebravis dankzij de gemakkelijk te bestuderen transparante embryo's, het eenvoudige huisvesten en fokken, de korte generatietijd, de grote kennis en mogelijkheden op genetisch gebied, die toegepast kan worden voor het genereren van mutanten en transgene dieren, de beschikbaarheid van moleculaire markers, en binnenkort zelfs de DNA-sequentie van het genoom. Potentiële locaties voor het produceren van immuuncompetente cellen in embryo's en larven zijn geïdentificeerd met behulp van genexpressie studies en histo-morfologisch werk. Dit proefschrift zal de vroege ontwikkeling van het immuunsysteem van de karper beschrijven. Het door ons gekozen proefdier heeft een aantal voordelen: ten eerste vergemakkelijkt de grootte van deze vis het onderzoek van een groot scala aan immuunfuncties, zoals de kinetiek van celpopulaties en het kweken van cellen, ten tweede zijn er antilichamen beschikbaar, ten derde hebben karperachtigen een grote waarde voor de visteelt, en ten vierde lijken zebravissequenties veel op karpersequenties, waardoor het onderzoek op moleculair niveau in de karper wordt vergemakkelijkt (**Hoofdstuk 1**).

Tijdens de ontwikkeling van de karper zijn de 'zogenaamde' aangeboren factoren de eerste componenten van het immuunsysteem die verschijnen (**Hoofdstuk 2**). Complement factor 3 en immunoglobuline eiwitten werden doorgegeven van moederdier via de dooierzak

naar het embryo, samen met mRNA's van complement factor 3, α_2-macroglobuline, serum amyloid A en een complement factor 1 r/s - mannose binding lectin geassociëerd met serine protease - achtig molecuul. Deze mRNA's werden geproduceerd door het embryo zelf vanaf 12-20 uur na bevruchting (unb). Dit is relatief lang voordat de embryo's uit het ei komen (op 2 dagen na bevruchting; dnb), wat betekent dat deze factoren mogelijk betrokken zijn bij het ontwikkelingsproces zelf, maar het is nog waarschijnlijker dat deze factoren door het immuunsysteem worden gevormd als voorbereiding voor de periode na het uitkomen. Daarnaast was van 24 unb tot 5 dnb complement factor 3 productie in embryo's gelocaliseerd in de syncytiale laag om de dooierzak, gevolgd door de lever in larven, een volgorde die nog niet eerder beschreven is bij vissen.

In **hoofdstuk 3** wordt de tweede component van het immuunsysteem die verschijnt in de ontwikkeling, namelijk de cellen van het aangeboren immuunsysteem, beschreven. Myeloïde (immuuncompetente) cellen en weefsels verschenen rond 2 dnb ventro-lateraal (d.w.z. aan de buikzijde en aan de zijkant) van de aorta, vervolgens rondom de sinussen van de vena cardinalis (ook wel achterste bloed eilandje genoemd), en uiteindelijk in kopnier en rompnier. De kopnier en rompnier zijn de belangrijkste bloedvormende organen in volwassen karpers, terwijl de kopnier qua functie ook homoloog is aan de bijnier in zoogdieren). Daarnaast gaat het bloedvormende weefsel rondom de sinussen van de vena cardinalis over in dat van de rompnier. De monoklonale antilichamen die gebruikt zijn in deze studie reageren met myeloïde oppervlakte moleculen van de karper die al geproduceerd worden vanaf het eerste verschijnen van myeloïde cellen, en spelen dus een belangrijke rol in het ontrafelen van ontwikkelingsprocessen in het immuunsysteem van beenvissen. Tenslotte werd het eerste verschijnen van myeloïde cellen in verband gebracht met een immuunrespons op moleculair niveau: 2 dagen oude embryo's reageerden op een injectie met bacteriëel lipopolysaccharide (LPS) door meer interleukine 1-β, induceerbaar nitrietoxide synthetase en serum amyloid A tot expressie te brengen, en minder complement factor 3 en α_2-macroglobuline, wat betekent dat het immuunsysteem van deze embryo's op dat moment al functioneel is.

Uitgerijpte T of B cellen hebben V(D)J recombinatie van hun T cel receptor of B cel receptor genen doorgemaakt. Het is bij zoogdieren overtuigend vastgesteld dat deze

recombinatie samengaat met de expressie van recombinatie activerend gen 1 (*rag-1*) en *rag-2*, en kan uitgevoerd worden door uitsluitend deze eiwitten. De *rag* sequenties zijn zeer geconserveerd bij alle gewervelde dieren, en het is ook aangetoond dat ze onmisbaar zijn voor V(D)J recombinatie in beenvissen. Het genereren van lymphoïde cellen tijdens de ontwikkeling van de karper werd bestudeerd met behulp van expressie van deze *rag* genen (**Hoofdstuk 4**). *Rag-1* kwam op 4 dnb het eerst tot expressie in de thymus, terwijl *rag-1*$^+$/WCL9$^+$ (corticale thymocyten) en *rag-1*$^-$/WCL9$^-$ gebieden het eerst onderscheiden konden worden vanaf 1 week na bevruchting (wnb), hetgeen suggereert dat er sprake is van een vroege cortex-medulla differentiatie is. *Rag-1* en *rag-2* kwamen vanaf 1 wnb ook tot expressie in de nier (samen met immunoglobuline zware keten mRNA), maar niet in de milt, terwijl WCI12$^+$ (B) cellen 1 week later in beide organen verschenen, wat betekent dat B cellen in de nier recombineren, maar niet in de milt. *Rag-1* expressie was hoger dan *rag-2* expressie in de thymus en in de kop- en rompnier van jonge dieren, maar deze ratio was omgekeerd in kop- en rompnier vanaf ongeveer 16 wnb, waarvoor geen afdoende verklaring gegeven kan worden. *Rag-1*/*rag-2* expressie werd gevonden in de thymus van dieren die ouder waren dan 1 jaar, maar slechts op een laag niveau in de nier, wat aangeeft dat T cellen gedurende het hele leven opnieuw gevormd worden maar B cellen slechts in mindere mate.

De vroege ontwikkeling van karper immuuncellen werd bovendien bestudeerd in mucosale organen (darm, kieuwen en huid: **Hoofdstuk 5**). De expressie van *rag-1* werd onderzocht in de darm om te kijken of T cellen ook buiten de thymus geproduceerd kunnen worden zoals bij zoogdieren is aangetoond. WCL38$^+$ intraepitheliale lymfocyten (waarschijnlijk T cellen) verschenen in de darm vanaf 3 dnb, wat kort na het uitkomen is maar vóór de eerste voeding, wat impliceert dat deze cellen al op jonge leeftijd een belangrijke rol spelen. Samen met het verschijnen van deze cellen voor de ontwikkeling van de thymus en de expressie van *rag-1* in de darm, suggereert dit dat nèt zoals bij zoogdieren tenminste een gedeelte van deze cellen in de darm tot ontwikkeling komt. WCL15$^+$ monocyten/macrofagen verschenen in de lamina propria van de darm vanaf 1 wnb, maar pas veel later in het epitheel, terwijl WCI12$^+$ (B) cellen pas na 6-7 weken in darm en kieuwen voorkomen. Dit betekent dat pas vanaf deze leeftijd een volledig

functioneel mucosaal immuunsysteem aanwezig is, hetgeen later is dan het systemische B cel systeem (2-4 weken).

In **hoofdstuk 6** wordt het effect van het gedurende twee weken toedienen van weerstandsverhogende middelen bestudeerd vanaf 2 en 6 wnb (vóór en na de eerste productie van antilichamen). Als voedsel-additieven zijn daarbij gebruikt: *Aeromonas salmonicida* LPS, gist DNA (bevat ongemethyleerde CpG motieven), en hoog-M alginaat (een algenextract dat poly-mannuronzuren bevat). Orale toediening van weerstandsverhogende stoffen vanaf 6 wnb resulteerde in een afname van het percentage B cellen in het bloed (alleen na toediening van LPS) en kopnier (alle testgroepen), en een afname van het totale IgM niveau in het plasma, wat suggereert dat er suppressieve effecten zijn die overeen komen met orale of juveniele tolerantie. Na toediening vanaf 2 wnb waren de effecten op het percentage B cellen niet zo duidelijk. Er werden geen veranderingen gevonden in de cytokine expressie in de darm of acute fase eiwit expressie in de lever. Nadat de vissen (aan welke de weerstandsverhogende stoffen waren toegediend) op 26 dnb geconfronteerd werden met een infectie met *Vibrio anguillarum* werd vergeleken met de controle groep een initiëel hogere cumulatieve mortaliteit gevonden in de groep die LPS kreeg toegediend, maar een lagere mortaliteit in de groepen die gist DNA of hoog-M alginaat kregen. Deze resultaten kunnen als een waarschuwing uitgelegd worden voor het gebruik van pathogeen-gerelateerde 'weerstandsverhogende' middelen, zoals bacteriëel LPS, in larvale en juveniele vissen.

Dit proefschrift geeft een overzicht van het verschijnen van de belangrijkste componenten van het immuunsysteem tijdens de ontwikkeling van de karper, en heeft bovendien waarde voor het onderzoek naar de ontwikkeling van het immuunsysteem bij vissen in het algemeen (**Hoofdstuk 7**). De beschikbaarheid van antilichamen en de grootte van de karper vergemakkelijken het bestuderen van celkinetiek, en geeft zo het onderzoek naar de vroege ontwikkeling van een functioneel immuunsysteem in deze vissoort extra waarde naast de veel bestudeerde zebravis. Bovendien werden de resultaten die verkregen werden gedurende dit onderzoek gebruikt om de orale toediening van weerstandsverhogende middelen te bestuderen, wat een aanzet kan zijn voor een efficiëntere productie van deze op grote schaal gekweekte vissoort.

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Curriculum Vitae

Ik, Berendina Theresia (roepnaam Heidi) Huttenhuis werd geboren op 24 mei 1974 te Oldenzaal. In 1992 behaalde ik mijn VWO diploma aan het Twents Carmel Lyceum te Oldenzaal.

In datzelfde jaar ben ik begonnen met de studie Diergeneeskunde aan de universiteit van Utrecht. Tijdens de studie heb ik een extra jaar aan wetenschappelijk onderzoek (Excellent Tracée) doorlopen over het onderwerp 'Verminderde immuniteit bij melkkoeien met leververvetting'. Daarnaast heb ik een scriptie geschreven over het bepalen van de voedingsstatus van paarden en runderen in natuurgebieden in Nederland met behulp van metabolische parameters in het bloed. Ik ben in 2000 afgestudeerd met als specialisatie landbouwhuisdieren.

Op 1 januari 2001 ben ik gestart als Assistent In Opleiding (AIO) bij de leerstoelgroep Celbiologie en Immunologie aan de Universiteit van Wageningen. Het promotieonderzoek maakte gedurende de eerste drie jaar deel uit van een EU-projekt met deelnemers uit verschillende Europese landen, dat tot doel had de ontwikkeling van het immuunsysteem bij vissen nader te karakteriseren, en het toedienen van weerstandsverhogende middelen te testen.

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