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A comprehensive transcriptional body map of Atlantic salmon unveils the vital role of the intestine in the immune system and highlights functional specialization within its compartments

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ABSTRACT

The intestine is a barrier organ that plays an important role in the immune system of Atlantic salmon. The immune functions are distributed among the diffuse gut lymphoid tissue containing diverse immune cells, and other cell types. Comparison of intestinal transcriptomes with those of other organs and tissues offers an opportunity to elucidate the specific roles of the intestine and its relationship with other parts of the body. In this work, a meta-analysis was performed on a large volume of data obtained using a genome-wide DNA oligonucleotide microarray. The intestine ranks third by the expression level of immune genes after the spleen and head kidney. The activity of antigen presentation and innate antiviral immunity is higher in the intestine than in any other tissue. By comparing transcriptome profiles, intestine shows the greatest similarity with the gill, head kidney, spleen, epidermis, and olfactory rosette (descending order), which emphasizes the integrity of the peripheral mucosal system and its strong connections with the major lymphoid organs. T cells-specific genes dominate among the genes co-expressed in these tissues. The transcription signature of $CD8^+$ (86 genes, $r > 0.9$) includes a master gene of immune tolerance *foxp3* and other negative regulators. Different segments of the intestine were compared in a separate experiment, in which expression gradients along the intestine were found across several functional groups of genes. The expression of luminal and intracellular (lysosome) proteases is markedly higher in pyloric caeca and distal intestine respectively. Steroid metabolism and cytochromes P450 are highly expressed in pyloric caeca and mid intestine while the distal intestine harbors genes related to vitamin and iron metabolism. The expression of genes for antigen presenting proteins and immunoglobulins shows a gradual increase towards the distal intestine.

1. Introduction

In addition to digestion and absorption of nutrients, the intestine is involved in diverse physiological processes including osmoregulation and ion balance, neural and endocrine regulation, maintenance of microbiota, detoxification, and defense against pathogens [1–3]. The immune function of the intestine is associated with its barrier function meaning a permanent contact with microbes and other potentially harmful antigens. The intestinal tract, being the largest organ in the body, consists of a diverse array of cells derived from both non-hemopoietic sources (such as epithelial cells and goblet cells) and hemopoietic sources. Diffusely scattered macrophages, granulocytes including mast cells, dendritic cells, B and T-cells are collectively referred to as gut associated lymphoid tissue (GALT) by fish immunologists. This terminology has been challenged in a recent review regarding the anatomy of teleost fish immune structures and organs. The review contends that the term “diffuse tissue” is at odds with the conventional definition of tissue [4]. Despite this critique, it is important to note that the term GALT remains firmly entrenched in the scientific literature as it seems accepted that fish needs their separate anatomical nomenclature unhinged form that of mammals and, therefore, the term GALT will be used in the current study. Mucosa-associated lymphoid

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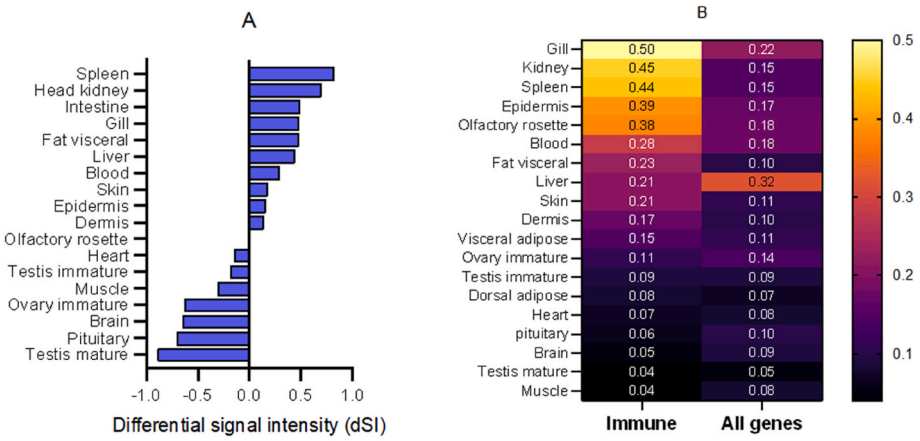

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	Intestine	Head kidney	Spleen	Gill	Epidermis	Olfactory	Fat visceral	Dermis	Skin	Blood	Liver
Antigen presentation - 102	1.05	0.67	0.72	0.92	0.46	0.19	0.05	0.04	0.07	0.16	0.15
Antiviral response - 235	0.92	0.55	0.69	0.69	0.62	0.41	-0.07	0.12	0.17	0.42	-0.27
Chemokine - 70	0.88	0.41	0.90	1.10	0.37	0.45	0.42	0.61	0.71	-0.07	-0.04
T cell - 254	0.75	1.12	1.29	0.83	0.33	0.25	0.42	0.20	0.20	0.53	0.27
Lymphocyte - 208	0.64	0.97	1.15	0.64	0.39	0.25	0.67	0.32	0.32	0.06	-0.07
Effector - 179	0.58	0.57	0.34	0.59	0.47	0.01	0.40	0.31	0.47	0.34	0.60
Lectin - 191	0.58	0.50	0.65	0.40	-0.36	-0.13	0.69	-0.09	0.06	0.15	1.06
Protease - 57	0.52	-0.10	0.06	0.04	0.06	-0.20	0.59	0.49	0.49	-0.05	0.54
B cell - 113	0.45	1.32	1.34	0.43	0.03	-0.05	0.59	0.07	0.01	0.49	-0.15
Immune receptor - 107	0.41	1.35	1.56	0.52	0.09	-0.33	0.77	0.15	0.23	0.84	-0.12
Cytokine receptor - 111	0.34	0.55	0.82	0.58	0.44	-0.02	0.44	0.36	0.35	0.40	0.36
Chemokine receptor - 57	0.32	1.17	1.32	0.63	0.02	-0.13	0.82	0.10	0.21	0.81	-0.19
TNF-related - 158	0.25	0.42	0.59	0.29	0.17	-0.21	0.14	0.07	0.03	0.58	0.10
Cytokine - 58	0.25	0.52	0.56	0.50	0.02	-0.17	0.27	0.21	0.15	0.38	0.02
Eicosanoid signaling - 110	0.23	0.41	0.01	0.63	0.58	0.03	0.27	0.35	0.41	-0.29	0.60
Immune regulator - 366	0.23	0.24	0.48	0.24	0.21	-0.07	0.13	0.10	0.12	0.35	0.12

Fig. 1. Transcriptome comparison of the intestine and other tissues and organs. A. Tissue are ranked by mean differential signal intensity (dSI) of 2795 immune genes. B: cooccurrence of highly expressed genes. Genes with high dSI in the intestine (>1.5 units above average) were selected and the number of genes that meet this criterion was determined in other tissues. The heat map presents ratios of gene counts in a tissue to gene counts in the intestine (all genes and immune genes). C: functional groups of genes (STARS annotation); average dSI. The numbers of genes are indicated. The groups are sorted from highest to lowest values in the intestine.

tissues are also present in gills, skin and nasopharynx. However, not much is known about contact between these compartments and the existence of an integral mucosal immunity in fish is debated [5]. Relations between the peripheral immune system and systemic primary and secondary lymphoid organs (head kidney and spleen) are also unclear. For instance, it remains to be found if B and T lymphocytes differentiate locally from progenitor cells or migrate at advanced stages of differentiation [5]. In addition to the immune cells of GALT, other intestinal cell types may take part in immune responses. The intestinal tract serves as a habitat for a vast population of microorganisms [1,6]. The intestinal immune system critically requires a precise balance between proactive defence against pathogens and immune tolerance, that is suppression of responses to commensal bacteria and repeatedly ingested dietary antigens, allergens, and other alien components. Teleost mucosal immunity is believed to be characterised by tolerance rather than responsiveness [7], but the mechanisms remain unclear. Based on knowledge from mammals, these may include the induction of regulatory T cells and anergy or deletion of T cells; the transcription factor foxp3 is suggested as a candidate indicator of oral tolerance [8].

The teleost intestine can be subdivided longitudinally into different compartments based on macro- or microscopical anatomy, or functional characteristics [9,10]. The anatomical terminology has not been standardized and can be confusing [11]. Here, we adhere to the division of the intestine into three compartments: the pyloric intestine with it adjected pyloric caeca, mid, and distal intestine. Several studies in salmon have indicated a relatively higher immunological activity of the distal intestine as compared to more proximal segments, based on elevated expression levels of selected immune gene transcripts [12–14]. Furthermore, adverse immune reactions appear to be more pronounced in this specific portion when compared to other segments of the intestine. A notable example is the well-documented inflammatory changes induced by soybean meal and certain other legume feed ingredients, which are observed more frequently in the distal intestine compared to other segments [15–19].

Transcriptome analysis provides a comprehensive overview of the pathways and functional groups of genes expressed in biological material. Microarrays and RNA sequencing have been widely used in studies of gut responses to pathogens and dietary disorders in teleosts (reviewed in Refs. [20,21]). The purpose of this study was to compare transcriptional profiles of Atlantic salmon tissues and organs under basal conditions in order to assess the specific role of the intestine in the immune system of Atlantic salmon both quantitatively and qualitatively. To do this, we constructed a transcriptional body map using a large volume of meta-data from the Nofima transcriptome database. A special experiment was conducted to compare different parts of the digestive system of the Atlantic salmon.

2. Materials and methods

2.1. Meta-analysis of transcriptome data

The meta-analyses were performed on data stored in Nofima's DNA oligonucleotide microarray database, which has been built as a part of the STARS bioinformatic system [22]; the database currently contains

Gene	Locus	Function	SI	dSI
MHC class II beta-chain	LOC106579407	Antigen presentation	10.9	6.6
IFIT-2	LOC106560512	Antiviral	11.8	9.5
Receptor-type tyrosine phosphatase	LOC106569656	Antiviral	11.2	7.5
B-cell linker protein-like	LOC106580424	B cell	12.5	5.7
C-X-C chemokine 9	LOC106608877	Chemokine	10.5	7.7
Interleukin-6	ccl21	Chemokine	12.5	7.6
TNFa LPS-induced	LOC106564308	Cytokine	14.0	7.9
Antimicrobial peptide 2 (LEAP2)	LOC101448022	Effector	11.1	7.7
C1q-like protein 2	LOC106571137	Effector	14.4	8.9
Chitinase, acidic	LOC106565309	Effector	14.1	10.9
Chitinase, acidic.3	LOC106567267	Effector	16.8	13.4
Perforin-1-like	LOC106570884	Effector	12.2	5.6
Cytochrome P450 2J2-like	LOC106584291	Eicosanoid	12.0	6.5
C-type lectin M4, cd209l	LOC106578890	Lectin	16.4	6.4
Fucolectin-3-like	LOC106581127	Lectin	12.3	9.5
Galectin-2-like	LOC106564424	Lectin	14.3	8.5
Ladderlictin-like	LOC106576105	Lectin	10.5	7.5
Lectin precursor	LOC106575578	Lectin	13.6	7.9
Mannose-binding protein C-1	mbl2	Lectin	15.9	4.5
Mannose-binding protein C-2	LOC106602034	Lectin	15.9	4.5
Nattectin	LOC106576106	Lectin	12.7	9.4
Type-2 ice-structuring protein	isp2	Lectin	13.5	10.2
Stat1	LOC106604676	Regulator	12.9	5.1
Butyrophilin subfamily 3 member A1	LOC106578435	T cell	10.8	6.9

Fig. 2. Immune genes with intestine-specific expression were ranked by a combination of SI and dSI.

8765 samples (arrays) from 177 experiments. Most studies have used two platforms: either 44 k Salgeno (GPL28080) containing probes to all known Atlantic salmon protein coding genes or 15 k SIQ-6 (GPL16555) with probes to genes selected by expression profiles and functional roles. The genes are annotated using public resources (GO and KEGG) and custom vocabulary adapted to the objectives of aquaculture research. To build the body map, data from fish not exposed to any stressful treatments (pathogen challenge, handling, exhaustive exercise, enteritis causing diet etc.) were used. Eighteen tissues were chosen for meta-analysis: blood, brain and pituitary, epidermis, dermis and whole skin, dorsal and visceral fat, gill, heart, head kidney, intestine, liver, olfactory rosette, ovary (immature), skeletal muscle, spleen, and testis (immature and mature). The meta-analysis assumed that the intensity of the hybridisation signal with subtracted background (SI) is proportional to the number of transcripts. Normalization was carried out in two stages: in tissues and between tissues. In tissues, the correction factor was calculated for each array as the ratio of the average SI of all arrays in the tissue to the average SI of the array. Each spot was multiplied by a correction factor so that the normalized mean SI of all arrays in the tissue was equal. This calculation was then performed to equalize the average SI across tissues. The average SI and standard deviation (σ) across all arrays are 5.9 and 3.; SI < 2.8 and >9 (mean SI $\pm$ σ) can be considered as low and high expression, respectively. Data were centered to assess the tissue specificity of expression. Average values were calculated for each gene and subtracted from each value of the gene, thereby obtaining dSI – differential or relative signal intensity. Enrichment analysis was performed using GO, KEGG, and STARS annotations by comparing the proportions of term-related genes in a selected gene set and in the entire transcriptome; significance was assessed by Yates' adjusted chi-square. The terms were ranked by p-values, enrichment, and number of genes and finally by the sum of these ranks.

2.2. Comparison of pyloric caeca, mid and distant intestine

Fish material, sampling and analyses using 15 k microarray SIQ-6 are described in detail in Ref. [23]. In brief, pyloric caeca, mid and distal intestine (PC, MI and DI) were sampled from five fish fed with a salmon diet high in fish meal (35 %). Slides, reagents, and equipment were fabricated by Agilent Technologies (Santa Clara, CA, USA) and a standard hybridisation and scanning protocol was applied. Data were processed with Nofima's bioinformatics pipeline STARS [22]. Differentially expressed genes (DEG) were selected at cut-off $\log_2$-Expression Ratios (ER) > 0.8 (1.75-fold) and $p < 0.05$ (*t*-test). The functional groups of genes (STARS annotation) were compared by mean $\log_2$-ER. Results were submitted to GEO Omnibus (GSE221800).

3. Results and discussion

3.1. The place and role of the intestine in the immune system of Atlantic salmon

Salgeno microarray platform includes DNA oligonucleotide probes for 2795 genes that are known or can be involved in immune responses. The overall immune activity in tissues was evaluated and compared by average dSI. This metric was highest in the spleen and closely followed by the head kidney (Fig. 1A). The intestine was ranked third, and its

Table 1
Enrichment of functional categories (GO, STARS) and pathways in genes with preferential expression in mucosal and lymphatic tissues of Atlantic salmon.

Term	Genes	Enrichment[a]	Rank[b]	Vocabulary
T cell	41	10.3	1	STARS
Lymphocyte	28	8.4	2	STARS
T cell activation	14	5.5	20	GO
T cell receptor signaling pathway	33	4.0	6	GO
B cell receptor signaling pathway	15	6.6	12	GO
Cell surface receptor signaling pathway	36	3.1	14	GO
CCR chemokine receptor binding	8	17.9	15	GO
Cytokine-cytokine receptor interaction	22	3.8	17	KEGG
Antigen presentation	21	13.5	3	STARS
Antigen processing and presentation	17	8.5	5	GO
MHC class II protein complex	7	18.9	19	GO
Immune effector	20	7.2	4	STARS
Innate antiviral response	18	5.0	10	STARS
Lymphocyte chemotaxis	8	16.2	18	GO
Neutrophil chemotaxis	17	6.8	8	GO
Monocyte chemotaxis	11	8.8	13	GO
Neutrophil activation	7	25.2	16	GO
Leukocyte migration	29	3.4	11	GO
Glycosphingolipid biosynthesis	13	9.1	9	KEGG
Epidermis development	19	5.8	7	GO

[a] Share in 654 selected probes divided to share in 44 k probes on Salgeno platform.
[b] The terms were ranked by p-values (Yates' corrected chi – square), enrichment and number of probes and finally, by the sum of these ranks.

immune activity was almost equal to that of the gill, visceral fat, and liver. The lowest expression of immune genes was observed in the brain, pituitary, and maturing male gonad. To assess similarity between tissues, we selected genes with high expression in the intestine (dSI >1.5, in total 3754 genes, including 741 immune genes) and counted the number of genes that met this criterion in other tissues (Fig. 1B). For example, 0.5 in column "immune" means that half of immune genes highly expressed in the intestine were also above the threshold in the gill. The gill was closest to the intestine followed by two lymphoid (spleen and head kidney) and two mucosal (epidermis and olfactory rosette) tissues. Co-occurrence of immune genes in these tissues was markedly greater than in the complete set of genes. Of note was an opposite ratio in the liver: this tissue was most similar to the intestine by the entire transcriptome profile, but their immune properties did not have much in common. The functional annotations revealed the distribution of immune functions among organs and tissues (Fig. 1C). The intestine was in the first place by antigen presentation and innate antiviral responses (102 and 235 probes), which was higher than in next ranked gill and systemic lymphoid organs. The activity of chemokines (70 probes) was also high but lower than in the gills. Preferential expression of T cells and lymphocyte-specific genes (254 and 208 probes) was observed in the spleen and to a slightly lower extent in the head kidney followed by the gills and intestine. In this respect, cellular and humoral arms of adaptive immunity were markedly different: expression of B cell-specific genes and especially immunoglobulins was much higher in the lymphoid organs.

Fig. 2 presents probes with the highest dSI in the intestine and all these genes were highly expressed (SI = 10.5–16,8). The transcripts of one of two *acidic chitinases* were most abundant. *Chitinase* LOC106565309 was the only gene with highly specific intestinal expression. These genes may encode enzymes required for digestion of arthropods [24] that comprise a large part of the Atlantic salmon diet, especially during early life stages. Previous studies have, however, shown that salmon chitinase activity is unaffected by dietary chitin, and that the Atlantic salmon seems to be unable to utilize dietary chitin to any major extent [25,26]. Salmon chitinases have not been characterized at the protein level, and it is known that some of animal chitinases have lost enzymatic activity and acquired properties of lectins [27]. Mammalian chitinases may take part in Type 2 helper T (Th2)-mediated inflammation and enhance innate and adaptive immunity to pathogen invasion [28]. They can also inhibit chitin induced inflammation [29]. Additional evidence for the presence of chitinous structures surrounding the intestinal mucosal barrier of ray-finned fish [30] may indicate that chitinases participate in the remodeling of these structures. Other probes included in Fig. 2 have shown high expression in at least one more tissue in addition to the intestine. Elevated expression has been observed in a suite of *lectins*. The functional groups shown in Fig. 1C are represented by one or more intestine-specific genes. *C1q-like protein 2* is a member of a large multi-gene family that includes many Atlantic salmon genes. Given that expression of the complement components in the intestine is relatively low, this gene most likely does not belong to the classical complement pathway. Antimicrobial peptide *leap2* can be involved in control of food intake as an antagonist of ghrelin receptor [31].

To investigate the common characteristics of mucosal tissues and their relationship with the systemic lymphoid organs, 654 probes with dSI >1 were selected in at least four of the six tissues (intestine, gills, epidermis, olfactory rosette, head kidney, and spleen) were selected. Immune genes were prevalent (47.3 %) in 378 probes with high expression in all six tissues and the number of immune genes was relatively small among mucosal-specific genes with low expression in the spleen and head kidney. The enrichment analysis confirmed the prevalence of immune genes: only two of 20 highly ranked functional categories are not directly related to the immune system (Table 1); T cells and lymphocytes are on the top, followed by antigen presentation. The list also includes signaling from T and B cell receptors and chemokines, innate antiviral responses, and immune effectors. These results indicate a shared pool of T cells as the main or one of the most important factors determining the integrity of the mucosal and lymphatic immune systems

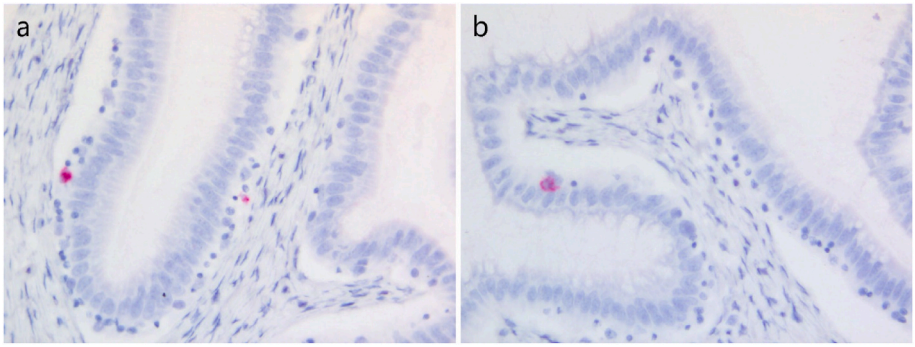

Fig. 3. *In situ* hybridisation targeting T cells expressing γδ T-cell receptor mRNA. a) Positive cells (red) in the lamina propria of the distal intestine. b) A single intraepithelial positive cell (red) in the periphery of the fold in the distal intestine. Samples were obtained from a healthy, unvaccinated Atlantic salmon and processed as described previously [37,38].

A

Gene	Symbol	SI	dSI
CD8 alpha-1	LOC100136450	7.90	2.99
CD8 alpha-2	LOC100136450	8.14	3.14
CD8 beta	LOC100136451	8.12	2.28
CD2-1	LOC106575540	8.00	2.06
CD2-2	LOC106575562	11.12	2.27
CD3 epsilon	cd3e	7.67	2.52
CD5-like	LOC106602649	8.70	2.59
CD6-like	LOC106605368	7.02	2.15
T cell receptor alpha		7.21	2.23
T-cell receptor beta chain	NP565024	8.40	2.56
Tyrosine-protein kinase LCK	lck	8.65	2.92
Tyrosine-protein kinase ZAP-70	LOC106584369	8.89	3.07
IKAROS family zinc finger 1	LOC106564952	5.61	1.92
IL-2 inducible T cell kinase BTK	LOC106604351	7.15	2.66
FYN-binding protein-like	LOC106573888	7.51	2.48
VAV2	LOC106594609	11.89	2.08
Protein THEMIS2-like	LOC106605107	6.79	1.80
Protein unc-13 homolog D	un13d	6.98	1.84
Proto-oncogene vav	LOC106593543	5.26	1.67
SLAM family member 8 precursor	LOC106609026	5.76	1.70
SLAM family member 8-like	LOC106609011	6.19	1.87
SLAM family member 8-like	LOC106609012	6.50	1.95
SLAM family member 9-like	LOC106608989	8.92	2.85
Forkhead box P3	foxp3	6.59	2.39
B- and T-lymphocyte attenuator	LOC106575803	5.30	1.65
CMRF35-like-1	LOC106598866	6.13	2.26
CMRF35-like-2	LOC106598872	6.04	2.07
Leupaxin-like	LOC106613835	8.68	2.63
NLR family, CARD domain containing 3	nlrc3	9.92	2.37

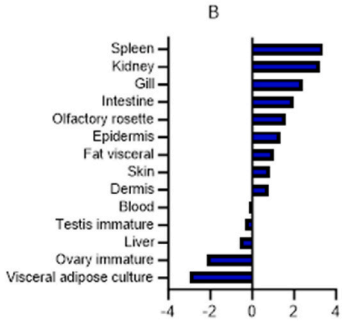

Fig. 4. Genes coexpressed with *cd8*: transcription signature of CD8$^+$. A. Three genes that encode *cd8* and immune genes with highly correlated expression profiles (Pearson r > 0.9). Negative regulators are highlighted with italics. B. Atlantic salmon tissues ranked by the expression levels of T cell-specific genes (average dSI of CD8$^+$ transcription signature).

of Atlantic salmon. The presence of T cells in the Atlantic salmon intestine is validated with *in situ* hybridization (Fig. 3).

The expression profiles of three *cd8*-like genes in the tissues of Atlantic salmon are almost identical, and the search of the body map found 86 probes with very similar patterns (Pearson r > 0.9); their intestinal expression ranged from moderate to high. This set of genes can probably be considered as the transcriptional signature (TS) of CD8$^+$ lymphocytes, although close coordination of two or more cell populations cannot be ruled out. TS contains a suite of representative T cell markers (but not *cd4*) and the key regulators of lymphocyte and T cells differentiation and activity (Fig. 4A). An important feature of CD8$^+$ TS is the presence of several negative immune regulators. *Foxp3* plays the key role in intestinal tolerance to antigens of food and commensal microbiota; in mammals, this function is performed by CD4(+)Foxp3(+) T cells [32,33]. Foxp3+ regulatory T cells with high regenerative capacity have been identified in zebrafish [34,35]. The apparent coexpression of *cd8* and *foxp3* in Atlantic salmon may be a novel finding. In addition, The CD8$^+$ TS includes five genes encoding immune suppressive proteins: *b- and t-lymphocyte attenuator*, two *cmrf35*-like genes, *leupaxin* and *nlr family, card domain containing 3*. This suggests tight control of cytotoxic activity of salmon lymphocytes in mucosal and lymphoid tissues, which can be involved in oral tolerance. The transcription signatures can be used for quantitative comparison of tissues [36]. By the expression of CD8$^+$ TS, the intestine ranks fourth after skin, spleen, and gills (Fig. 4B). Relatively high expression of this set of genes has also been observed in olfactory rosette, epidermis, and visceral fat. Co-expression of T cell-specific genes sharply contrasts with innate antiviral immunity. Of 98 antiviral gene probes upregulated in the intestine, only 17 probes were included in the gene set with high mucosal and lymphatic expression. Antiviral defense is most likely distributed among immune cells and intestine-specific cell types, which are not present in the mucosal and lymphatic tissues.

3.2. Spatial gene expression along the intestinal tract

A smaller 15 k microarray platform was used to compare three

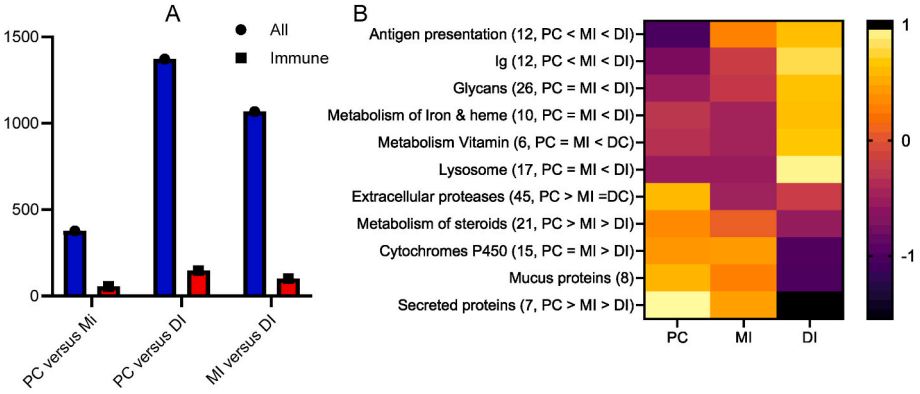

Fig. 5. Comparison of parts of the intestine. A. Numbers of genes with differential expression between the parts of intestine (PC – pyloric caeca, MI – mid intestine and DI – distal intestine). B: Functional groups (STARS annotation). Heat maps present differential expression (dSI), significant differences between intestine sections ($p < 0.05$) are indicated as > or <.

Table 2
Genes with greatest expression differences (folds) between parts of intestine.

Role	Gene	Locus	PC DI	MI DI
High expression in PC and MI				
Endocrine	Peptide YY	pyy	27.5	31.0
Absorption	Apolipoprotein A-II	LOC106570190	56.0	48.3
Absorption	Apolipoprotein C–I-1	apoc1	1685.0	113.1
Absorption	Apolipoprotein C–I-2	apoc1l	131.2	8.4
Absorption	Bile salt export pump	LOC106581787	71.6	15.0
Absorption	Oligopeptide transporter. solute carrier family 15-1	slc15a1	839.0	510.2
Bile	Cytochrome P450 7A1	LOC106569008	23.7	4.6
Detox	Thiosulfate sulfurtransferase KAT	kat	30.9	18.3
Digestion	Chymotrypsin B	ctrb	24.5	1.0
Digestion	Enteropeptidase		34.8	28.4
Digestion	Lactase (LCT)	S18154208	116.9	149.4
Digestion	Meprin A subunit alpha	LOC106571463	19.8	1.5
Digestion	Trypsin-1	trp-ia	35.3	1.1
Digestion	Trypsin-2		34.2	1.2
Ion	Chloride anion exchanger	LOC106609369	107.6	83.7
Lectin	Type-4 ice-structuring protein precursor		72.2	40.5
Lipid	Elongation of very long chain fatty acids 5	LOC100192340	29.7	5.5
Microbiome	Ethanolamine kinase 1	LOC106611931	34.0	19.1
Mucus	Alpha-tectorin	LOC106592971	7088.6	296.5
Other	Peroxisomal sarcosine oxidase	pipox	54.6	59.8
	High expression in DI		**DI PC**	**DI MI**
Antigen presentation	H-2 class I HC antigen, Q10 alpha chain-like	LOC106588377	27.7	1.3
Digestion	Phospholipase B1, membrane-associated	LOC106608185	1501.6	1.1
Endocrine	17-beta-hydroxysteroid dehydrogenase 14	LOC106606926	5.5	5.7
Lipid	Fatty-acid amide hydrolase 1	LOC100286622	12.7	6.2
Lysosome	Lysosomal protective protein	LOC106562245	12.0	9.4
Lysosome	Lysosomal alpha-mannosidase	LOC101448032	10.5	10.6
Lysosome	N-acetylated-alpha-linked acidic dipeptidase 1	naaladl1	9.3	2.2
Lysosome	Cathepsin Z	LOC106565426	7.6	4.8
Lysosome	N-acetylated alpha-linked acidic dipeptidase 2		7.5	2.1
Lysosome	Lysosomal protective protein	LOC106587401	6.4	4.7
Lysosome	Galactocerebrosidase	galc	6.4	6.1
Lysosome	ATPase, H+ transporting, V0 subunit c, b	LOC106578297	6.2	4.8
Lysosome	ATPase_ H+ transporting, V1 subunit E−11	LOC106576269	5.9	4.9
Lysosome	Acid ceramidase	LOC106602555	5.0	4.6
Lysosome	Pro-cathepsin H	LOC106561173	4.6	4.7
Lysosome	IFNg-inducible lysosomal thiol reductase-like	LOC106568950	22.9	17.2
Lysosome	Sphingomyelin phosphodiesterase	smpd1	9.0	8.5
Lysosome	Legumain	lgmn	7.5	6.0
Vitamin	25-hydroxyvitamin D-1 alpha hydroxylase	LOC106586130	140.5	118.2
Vitamin	Vitamin D3 hydroxylase-associated protein		14.3	6.8

segments of the Atlantic salmon intestine. The transcriptome difference between PC and MI assessed by numbers of differentially expressed genes (DEG) was moderate and increased 2.8 and 3.6-fold in contrasts between DI and respectively MI and PC (Fig. 5A). This observation is consistent with previous transcriptome studies in teleosts [10,39] and shows that the DI transcriptional signature differs substantially from those of the rest of the intestine. It should be noted that the proportion of immune genes among DEG was relatively small (4.0–14.8 %). An expression gradient was observed along the digestive tract for several functional groups of genes (Fig. 5B) and the genes with the greatest differences between the segments are shown in Table 2. A number of genes involved in macronutrient digestion and absorption showed declining expression gradient, anterior to posterior, in accordance with observations in other teleosts [10]. Proteases and peptidases (*trypsins, chymotrypsin, meprin, enteropeptidase)* were among the genes with highest expression in the proximal intestine, corresponding to their relatively higher enzymatic activity in this part of the salmon gut [40]. The key role of PC in lipid absorption is demonstrated by very high expression of *apolipoproteins*, in accordance with previous reports showing that the PC is the most important site for fatty acid absorption in the gastrointestinal tract of Atlantic salmon [41]. Steroid and bile metabolism also gradually decreased in the direction toward the distal intestine, in accordance with previous biochemical data [42]. The high proximal expression levels of the *peptide yy*, an intestinal hormone that regulates digestion and food intake [43,44] as well as the peptide transporter 1 (*slc15a1*) also correspond to earlier observations [10,39]. Most genes shown in the lower part of Table 2 (upregulated in DI) encode lysosomal proteins, which could be attributed to antigen processing and immune functions, as well as digestion. Similar observations have been reported in Ballan wrasse (*Labrus berggylta*) [39]. While overall metabolic activity was higher in PC and MI, the expression of genes involved in iron, heme, and vitamin metabolism was greater in DI. In this context, it is important to emphasize that the digestive apparatus of the intestine, including the expression of relevant genes, responds rapidly and reversibly to changes in dietary load and composition [1]. Thus, the relatively higher expression of macronutrient digestion and absorption genes observed in the proximal parts of the intestine most likely reflects the relatively higher substrate (i.e. ingested feed) levels in this part of the gut. In situations where the digestive capacity of the proximal intestine is exceeded and more substrates enter the posterior segments, an animal will likely respond by upregulating the digestive capacity, including the necessary genes, throughout the intestinal tract as an attempt to maximise the utilisation of nutrients from the ingested feed.

Similar numbers of immune genes showed higher levels of expression in PC and MI or DI, respectively (67 and 81 probes). A steady increase towards the distal part of the intestine was observed in two functional groups: antigen presentation (both *mhci* and *mhcii* presented with four and seven probes) and *immunoglobulins*. The latter could be due to a larger number of B cells retrieved for the lymphatic system in the DI or

Table 3
Immune genes. Positive and negative values (folds) mean higher expression in respectively PC and MI or DI. Differential expression is indicated with underlined italics.

Role	Gene	Locus	PC DI	MI DI
Antiviral	Gig2-4		*5.2*	*6.2*
Antiviral	Gig2-2		*4.7*	*6.2*
Antiviral	Gig2-7		*4.2*	*3.9*
Antiviral	Mx3	LOC100136587	*2.0*	*3.7*
Antiviral	STAT1a	stat1	1.4	*1.8*
Antiviral	ISG15-like	LOC100136541	*−3.5*	−1.0
Antiviral	RTP3	LOC106574173	*−4.4*	−1.6
Antiviral	VLIG1-2	LOC106607153	*−9.7*	*−4.8*
B cell	BANK1	bank1	*2.3*	*1.8*
B cell	STAP1	LOC106604676	*2.2*	*1.9*
B cell	DAPP1	dapp1	*2.0*	*1.9*
B cell	CD319 (SLAMF7)	slaf7	*−2.0*	−1.6
B cell	CD80-like protein		*−3.8*	*−3.3*
Cytokine	LPS-induced TNFa	LOC106564308	1.5	*1.9*
Cytokine	Interleukin 17D	il17d	1.5	*4.1*
Effector	Neutrophil cytosolic factor 2	LOC106579561	*1.9*	1.6
Effector	LBP (LPS binding protein)/ BPI		*−2.0*	−1.4
Effector	Leukocyte elastase inhibitor	ileu	−2.3	1.3
Effector	Neutrophil cytosolic factor 1	ncf1	*−2.5*	−1.3
Effector	Lysozyme g	LOC100136420	*−3.0*	*−2.3*
Effector	Serine protease 1-like	LOC106613755	*−3.3*	−1.6
Effector	Matrix metalloproteinase-9	mmp9	*−3.6*	−1.5
Effector	Myeloperoxidase (mpo)	LOC100380666	*−4.1*	*−2.0*
Lymphocyte	PTPN7	ptn7	*2.6*	*1.9*
Lymphocyte	Transcription factor MafB [Maf-B]	LOC106582887	*−2.8*	*−1.8*
Macrophage	Macrophage-expressed gene 1 protein	mpeg1	*2.6*	*2.3*

their differentiation toward antibody-producing plasma cells. The available results are not sufficient to draw conclusions, but some regulators of B cell development were present among DEG (Table 3). Three genes with higher expression in PC and MI (*bank1, stap* and *dapp1*) are involved in BCR signaling prior to switching from secreted to membrane immunoglobulin expression [45–47]. *Cd319 (slamf7)* and *cd80* are markers of plasma and memory cells [48,49]. Differences were also found in innate immunity. Interestingly, several genes with most active responses to viruses [36,50] showed preferential expression in either proximal or distal segments. Two cytokines, *il17d* and *lps-induced tnfa,* showed the highest expression in MI and a small set of immune effectors was the most active in DI.

4. Conclusions

The study demonstrated a high immune activity of the Atlantic salmon intestine, which is second only to specialized lymphatic organs, the head kidney and spleen, its specific roles, as well as the integration of the lymphatic and mucosal systems, especially T cells. Despite the large disparity in the transcriptome between the three segments of the intestine, no major differences have been found with respect to immune functions. However, a gradient in antigen presentation and immunoglobulin production has been shown suggesting heterogeneity of B cells. The composition of intestinal lymphocytes is waiting for investigation and significant progress is anticipated from combination of traditional transcriptomics with single cell and nuclear sequencing.

CRediT authorship contribution statement

Trond M. Kortner: Conceptualization, Project administration, Investigation, Validation, Writing – review & editing. **Sergey Afanasyev:** Methodology, Software, Data curation, Investigation, Formal analysis, Validation, Visualization, Writing – review & editing. **Erling Olaf Koppang:** Investigation, Writing – review & editing. **Håvard Bjørgen:** Methodology, Investigation, Writing – review & editing. **Åshild Krogdahl:** Project administration, Funding acquisition, Writing – review & editing. **Aleksei Krasnov:** Conceptualization, Data curation, Investigation, Validation, Visualization, Writing – original draft.

Declaration of competing interest

None of the co-authors or any of the others involved have conflicts of interest regarding the conductance and presentation of the experiment.

Data availability

Data will be made available on request.

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