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Anatomical distribution of scavenger endothelial cells in bony fishes (Osteichthyes)

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ABSTRACT

The scavenger endothelial cells (SECs) of vertebrates are an important class of endocytic cells responsible for clearance of foreign and physiological waste macromolecules, partitioning in the immune system, functioning as a cellular powerplant by producing high energy metabolites like lactate and acetate. All animal phyla possess SECs, but the tissue localization of SECs has only been investigated in a limited number of species. By using a specific ligand for scavenger receptors (formalin treated bovine serum albumin), the study revealed that in all tetrapod species (amphibia, reptiles, birds and mammals) the SECs were found lining the sinusoids of the liver. No SECs were found in the liver of any of the bony fishes (Osteichthyes) investigated. Interestingly, we found the SECs not only to be located in the heart of marine species but also in some freshwater species such as *Lota lota*, *Percichthys trucha* and *Perca fluviatilis*. In some fish species, the SECs were found both in the heart and/or kidney in a number of marine and freshwater fishes, whereas in some marine, diadromous and freshwater fishes the SECs were confined only to the kidney tissue. However, from these results it can be suggested that there is neither a clear phylogenetic trend when it came to anatomical localization of SECs nor any pattern in terms of habitat (salinity preferences).

1. Introduction

The body of normal adults is kept in a state of biological equilibrium often referred to as homeostasis, which is the result of highly dynamic, yet tightly regulated anabolic and catabolic processes. This key feature is reflected in a high turnover of macromolecules. Even apparently inert structures such as the extracellular matrix are subjected to vivid degradation and renewal. Thus, collagen, a protein that makes up as much as 30 % of all animal proteins and constitutes the major protein moiety of renewed bone-at a rate of several grams per day in humans. Hyaluronan, a major connective tissue polysaccharide, is also renewed at a rate of kilograms per year in humans [1,2]. Some of this is turned over locally where it is produced, some is degraded by the lymph nodes and the remainder (not metabolized in the lymphatic system) enters the blood stream. However, the serum concentration of these waste macromolecules is usually very low, which reflects an extremely rapid rate of clearance from the blood [2].

The most important site of elimination of collagen, hyaluronan and other soluble waste macromolecules in vertebrates is the scavenger endothelial cells (SECs) [3,4]. The SECs are a population of specialized endothelium that are specialized to scavenge circulating waste macromolecules. The SECs are pinocytic and expresses certain number of specific endocytosis receptors; i) the scavenger/hyaluronan receptor [5,31], ii) The collagen α-chain receptor [6], iii) the mannose receptor [7], and iv) – only in mammals – the Fc γ receptor [8]. With the use of these receptors, the cells are able to recognize waste macromolecules of protein, carbohydrate, lipid and nucleic acid nature.

The first SEC to be characterized was the liver endothelial cell (LEC) of mammals. Ultrastructural studies by Wisse [9], 50 years ago, demonstrated numerous "smooth walled macropinocytic vesicles" in the sinusoidal endothelial cells of rat liver. From this he hypothesized that the cells took up proteins from the blood circulation. Numerous studies have later shown that scavenger liver endothelial cells (SLECs) represent the most important site of uptake of colloids and soluble waste macromolecules. It is, however, important not to confuse this function with phagocytosis in macrophages.

Previous studies have shown that species from all extant vertebrate groups carry a specialized scavenger endothelium that is very active in receptor mediated endocytosis of colloids and waste macromolecules [3]. Interestingly, in all species studied to date, the population of

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scavenger endothelium is located in no more than two different organs. In land based tetrapods (reptiles, amphibians, birds and mammals) the SECs are located in the liver where they make up the sinusoidal wall. In a normal human adult, the total area covered by the population of SECs amounts to 323 square metres [10], providing an efficient system for the exchange of macromolecules between cells and blood. In the phylogenetically older vertebrate lineages such as the jawless (Agnatha), cartilaginous (Chondrichthyes) and bony fishes (Osteichthyes), the distribution of these cells are different. In jawless and cartilaginous fishes, the SECs are found in the gills. In the bony fishes, SECs are located either in the kidney sinusoid or in the heart where they make up the endothelium of the sinusoids or the endocardium [3,4].

The organ localization of SECs shows a high variance among the different vertebrate lineages. The bony fishes contain SECs in different organs which is peculiar and unlike other animal phyla. The aim of this study is to find out whether the anatomical localization of SECs is related to phylogeny, function or merely an adaption to chosen environment in the sense of salinity.

2. Materials and methods

Animals. A total of 3 individuals from 31 species and 12 orders (Table 1) was included in this study: The fishes were collected from fish farms or wild caught dependent on availability. Due to sampling procedure the weight of the fish varied between individuals and species. The wild caught fish were kept in a small tank some hours after injection.

Acipenseriformes; Siberian sturgeon (*Acipenser baerii*).

Anguilliformes; European eel (*Anguilla Anguilla*).

Cypriniformes; tench (*Tinca tinca*), white bream (*Blicca bjoerkna*), rudd (*Scardinius erythrophthalmus*), ide (*Leusicus idus*), bleak (*Alburnus alburnus*), roach (*Rutilus rutilus*).

Siluriformes; brown bullhead (*Ameiurus nebulosus*).

Salmoniformes; European whitefish (*Coregonus lavaretus*), grayling (*Thymallus thymallus*), rainbow trout (*Oncorhynchus mykiss*), Arctic char (*Salvelinus alpinus*), brook trout (*Salvelinus fontalis*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*).

Esociformes; northern pike (*Esox lucius*).

Gadiformes; burbot (*Lota lota*), pollack (*Pollachius pollachius*), saithe (*Pollachius virens*), whiting (*Merlangius merlangus*), Atlantic cod (*Gadus morhua*), poor cod (*Trisopterus minutus*).

Scombriformes; Atlantic mackerel (*Scomber scombrus*).

Pleuronectiformes; Atlantic halibut (*Hippoglossus hippoglossus*).

Labriformes; goldsinny-wrasse (*Ctenolabrus rupestris*).

Centrarchiformes; pumpkinseed (*Lepomis gibbosus*), creole perch (*Percichthys trucha*).

Perciformes; European perch (*Perca fluviatilis*), ruffe (*Gymnocephalus cernua*), lumpfish (*Cyclopterus lumpus*).

Experimental protocols were approved by the Norwegian Ethics Committee for Research on Animals (Ref 2005/31069).

2.1. Probe to study non-phagocytic endocytosis in scavenger endothelial cells

Bovine serum albumin (Sigma Chemical Company, USA) was treated with formaldehyde (FSA) as described in Mego et al. [11] to make it as a ligand for the scavenger receptors. Blomhoff et al. [12] was the first to demonstrate that denatured albumin was taken up via the scavenger receptor pathway in rat endothelial cells. They suggested that formaldehyde treatment increased the negative charge of the protein and thus made it a ligand for the scavenger receptors. Formaldehyde-treated bovine serum albumin (FSA) was labelled with fluorescein isothiocyanate (FITC) as described in Sørensen et al. [13], enabling tracing using fluorescence microscopy. In short FITC-FSA was prepared by incubating FSA (5 mg ml^{-1}) with FITC (5 mg ml^{-1}) in 0.2 mol^{-1} sodium bicarbonate buffer (pH9.5) for 20 h at 4 °C. Unreacted dye was removed by gel filtration on a PD-10 column (Prepacked Sephadex G-25, Pharmacia, Uppsala, Sweden). Fluorescein is an organic dye molecule based on the xanthene tricyclic structural motif, formally belonging to triarylmethane dyes family. Fluorescein isothiocyanate group, abbreviated FITC, reacts with the amine groups of many biologically relevant compounds including proteins to form a thiourea linkage. The specific fluorescence due to FITC-FSA was observed as bright green to yellow colour. Sections were examined using a Leica DM microscope equipped with incident-light fluorescence optics (Leica Microsystems GMBH, Wetzlar, Germany).

2.2. Administration of ligand

Fish were anaesthetized by immersion in 0.004 % benzocaine solution and injected intravenously through the caudal vein with FITC-FSA (5–10 mg kg^{-1} body weight) in a total injection volume of 500 μl phosphate buffered saline (PBS). Between 1 and 24 h after administration, the fish were killed by a blow on the head using a priest before tissues were placed in PBS with 4 % formaldehyde. The following tissues were investigated, heart (ventricle and atrium), head and trunk kidney, liver, spleen, gills and intestine.

2.3. Preparation of tissues and fluorescence microscopy

After fixation for at least 3 days at RT, tissues were dehydrated, embedded in paraffin and sectioned (<5 mm). After deparaffinization, standard contrast staining was omitted [14].

3. Results

3.1. Distribution of FITC-FSA in tissues of various fish species

A fluorescently labelled scavenger receptor ligand (FITC-FSA) was administered to assess the uptake and accumulation of the ligand and determine the cellular and anatomical localization in the different fish species. We looked for presence of specific green fluorescence in all investigated organs (ventricle and atrium of the heart, head and trunk kidney, liver, spleen, gills and intestine) in sections from three individuals per species sampled. The results are grouped as found below.

3.2. Heart, freshwater fishes

In Gadiformes (*Lota lota*) and Perciformes (*Perca fluviatilis*), the scavenger endothelial cells lining the heart muscle tissue of the ventricle and atrium were clearly stained by the ligand as shown in Fig. 1.

3.3. Heart, marine fishes

In Gadiformes, all species investigated showed staining of the scavenger endothelial cells lining the heart muscle tissue of the ventricle and atrium, and the same was evident for Perciformes (*Cyclopterus lumpus*), as shown in Fig. 2.

3.4. Heart and kidney

In Scombriformes (*Scomber scombrus*) and Pleuronectiformes (*Hippoglossus hippoglossus*), both the scavenger endothelial cells lining the heart muscle tissue of the ventricle and atrium and the sinusoidal endothelial cells of the head and trunk kidney contained the fluorescently labelled ligand. This was also the case in Centrarchiformes (*Lepomis gibbosus*) as shown in Fig. 3. In Acipenseriformes, (*Acipenser baerii*), we found large fluorescent cells scattered in the nodular lymphatic tissue covering the pericardium (Fig. 4). The cells do not look like endothelial cells lining blood vessels.

Table 1
Anatomical distribution of scavenger endothelial cells (SECs) in different fish species identified by uptake of the scavenger receptor ligand, of FITC-FSA. Phylogenetic order follows Betancur-R et al. (2017) but note that the position of the *Tinca tinca* is unclear. English vernacular names follow Fishbase (www.fishbase.org) except for *Salmo trutta*.

Order	Family	Species		Heart	Gills	Liver	Kidney
Acipenseriformes	Acipenseridae	*Acipenser baerii*	Siberian sturgeon	x*			x
Anguilliformes	Anguillidae	*Anguilla anguilla*	European eel				X
Cypriniformes	Cyprinidae(?)	*Tinca tinca*	Tench				X
Cypriniformes	Leuciscidae	*Blicca bjoerkna*	White bream				X
Cypriniformes	Leuciscidae	*Scardinius erythrophthalmus*	Rudd				X
Cypriniformes	Leuciscidae	*Leusicus idus*	Ide				X
Cypriniformes	Leuciscidae	*Alburnus alburnus*	Bleak				X
Cypriniformes	Leuciscidae	*Rutilus rutilus*	Roach				X
Siluriformes	Ictaluridae	*Ameiurus nebulosus*	Brown bullhead				X
Salmoniformes	Salmonidae	*Coregonus lavaretus*	European whitefish				X
Salmoniformes	Salmonidae	*Thymallus thymallus*	Grayling				X
Salmoniformes	Salmonidae	*Oncorhynchus mykiss*	Rainbow trout				X
Salmoniformes	Salmonidae	*Salvelinus alpinus*	Arctic Char				X
Salmoniformes	Salmonidae	*Salvelinus fontinalis*	Brook trout				X
Salmoniformes	Salmonidae	*Salmo trutta*	Brown trout				X
Salmoniformes	Salmonidae	*Salmo salar*	Atlantic salmon				X
Esociformes	Esocidae	*Esox lucius*	Northern pike				X
Gadiformes	Gadidae	*Lota lota*	Burbot	X			
Gadiformes	Gadidae	*Pollachius pollachius*	Pollack	X			
Gadiformes	Gadidae	*Pollachius virens*	Saithe	x			
Gadiformes	Gadidae	*Merlangius merlangus*	Whiting	X			
Gadiformes	Gadidae	*Gadus morhua*	Atlantic cod	X			
Gadiformes	Gadidae	*Trisopterus minutus*	Poor cod	X			
Scombriformes	Scombridae	*Scomber scombrus*	Atlantic mackerel	X			X
Pleuronectiformes	Pleuronectidae	*Hippoglossus hippoglossus*	Atlantic halibut	X			X
Labriformes	Labridae	*Ctenolabrus rupestris*	Goldsinny-wrasse				X
Centrarchiformes	Centrarchidae	*Lepomis gibbosus*	Pumpkinseed	X			X
Centrarchiformes	Percichthyidae	*Percichthys trucha*	Creole perch	X			
Perciformes	Percidae	*Perca fluviatilis*	European perch	X			
Perciformes	Percidae	*Gymnocephalus cernua*	Ruffe				X
Perciformes	Cyclopteridae	*Cyclopterus lumpus*	Lumpfish	x			

*Cells identified by ingestion of FITC-FSA in the sturgeon heart was found in lymphoid-like nodules on pericardial side.

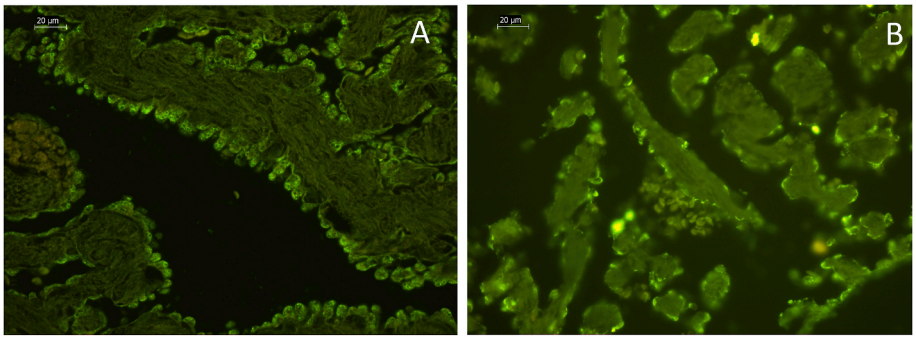

Fig. 1. Scavenger endothelial cells in the heart of a) Lota lota and b) Perca fluviatilis 24 h after intravenous injection of FITC-formaldehyde treated albumin (n = 3).

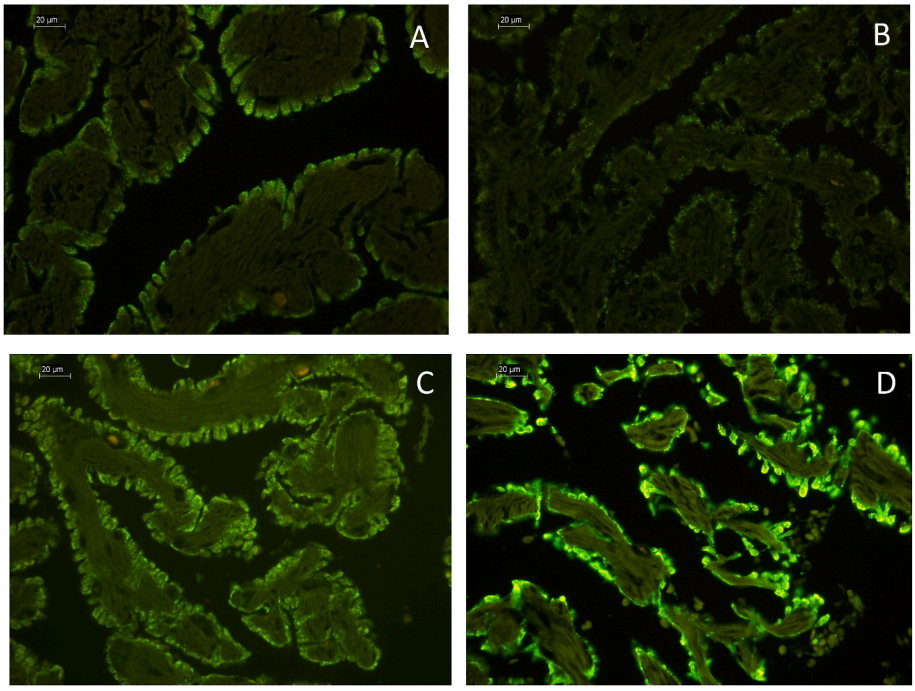

Fig. 2. Scavenger endothelial cell in the heart of a) *Pollachius pollachius*, b) *Pollachius virens*, c) *Merlangius merlangus* and d) *Cyclopterus lumpus* 24 h after intravenous injection of FITC-formaldehyde treated albumin (n = 3).

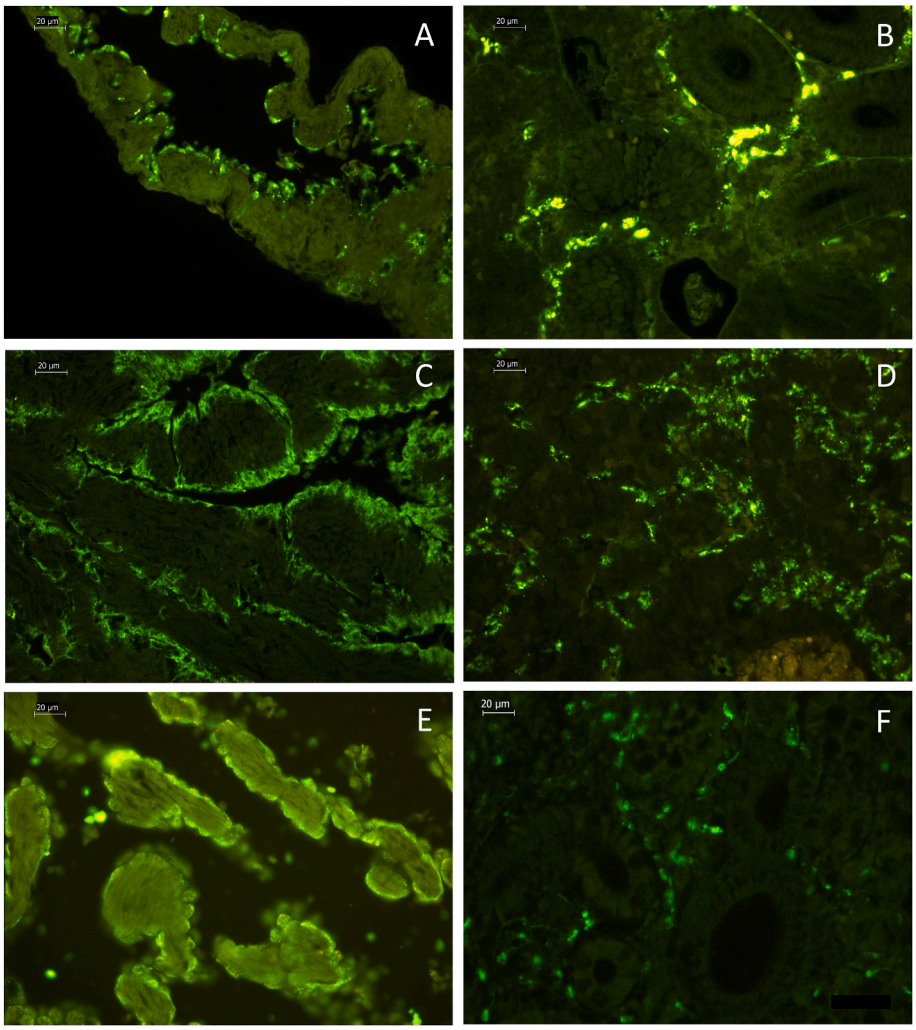

Fig. 3. Scavenger endothelial cells in the heart and kidney of a) *Scomber scombrus* heart, b) *Scomber scombrus* kidney, c) *Hippoglossus hippoglossus* heart, d) *Hippoglossus hippoglossus* kidney, e) *Lepomis gibbosus* heart and f) *Lepomis gibbosus* kidney 24 h after intravenous injection of FITC-formaldehyde treated albumin (n = 3).

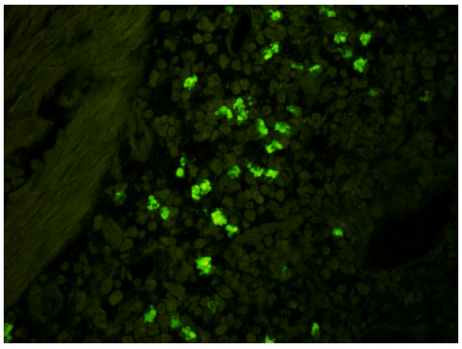

Fig. 4. Fluorescent cells present in the nodular epicardial tissue of the *Acipenser baerii* heart 24 h after intravenous injection of FITC-formaldehyde treated albumin (n = 3).

3.5. Kidney

In Salmoniformes, all species studied possessed stained cells appearing as endothelial cells lining blood sinusoids of the head and trunk kidney as shown in Fig. 5.

In Labriformes (*Ctenolabrus rupestris*) and Perciformes (*Gymnocephalus cernua*), fluorescence microscopic examination of tissue sections revealed intense fluorescence in the endothelial cells lining the blood sinusoids of the head and trunk kidney as shown in Fig. 5.

All species investigated in the Cypriniformes contained fluorescent stain in the endothelial cells lining the blood sinusoids of the head and trunk kidney as shown in Fig. 5.

In Anguilliformes (*Anguilla anguilla*), Siluriformes (*Ameiurus nebulosus*), and Esociformes (*Esox lucius*), the sinusoidal endothelial cells of the head and trunk kidney were clearly filled with the fluorescent label as shown in Fig. 5.

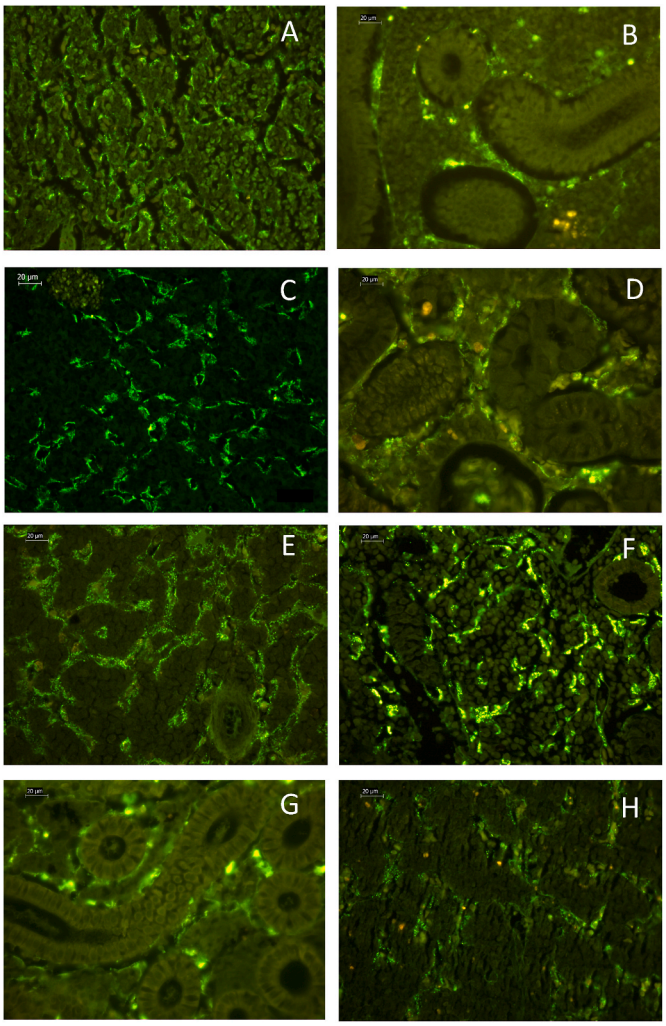

Fig. 5. Scavenger endothelial cell in the kidney of a) *Anguilla anguilla*, b) *Blicca bjoerkna*, c) *Leusicus idus*, d) *Ameiurus nebulosus*, e) *Thymallus thymallus*, f) *Salvelinus fontinalis*, g) *Salmo trutta*, and h) *Gymnocephalus cernua* 24 h after intravenous injection of FITC-formaldehyde treated albumin (n = 3).

4. Discussion

The scavenger endothelial cells of vertebrates are an important class of endocytic active cells responsible for clearance of foreign and physiological waste macromolecules by receptor mediated endocytosis. The SEC of all investigated vertebrates expresses a functional scavenger receptor [3]. The scavenger receptors belong to pattern recognition receptors (PRRs), which were originally defined for their ability to bind forms of low-density lipoprotein (LDL), and are subdivided into 8 classes (A–H) [15,16,30]. These receptors are extracellular glycoproteins, which mediate phagocytosis/endocytosis of negatively charged ligands [17]. The different classes are based on their architecture. Uptake of chemically modified serum albumin has been shown to be governed by scavenger receptors of different classes [18] – thus not solely restricted to one family only. As such, it is difficult to do targeted functional studies on each SR class by, for example, administration of chemically modified substances or other ligands. Overall, we speculate that several fish SRs in different classes were able to bind the same ligand. Furthermore, the scavenger receptors are not exclusive to SEC since a variety of different cells express SA. SRs play also a significant role in host defence by recognizing numerous microbial antigens and activating downstream immune responses to fight and eliminate the pathogens (Areschoug et al., 2008).

The liver of tetrapods (amphibia, reptiles, birds and mammals) is a perfect location for the clearance of macromolecules transported to the liver by the portal vein from the intestine in addition to the large amounts of waste macromolecules transported to the liver from the normal homeostasis process of the body. In humans, the cells lining the liver sinusoids covers an area of hundreds of square meters [10] where the blood is cleared continuously by e.g. receptor mediated endocytosis. The location of these cells in tetrapods is where they are expected to be, considering the function of clearing the blood from potentially harmful substances and to present foreign material to the immune system [3]. Studies on fish and lower vertebrates has complicated the assumed that there may be relationship between SEC localization and phylogeny. In fish, the cells express similar functional receptors for physiological waste as they possess functional scavenger receptors recognizing e.g., bacterial lipopolysaccharides, from G^- bacteria and are able to metabolize endocytosed proteins into smaller energy rich molecules like acetate and lactate [14,19]. However, the SECs are localized in different organs compared to higher vertebrate species. Even within bony fishes, there are different patterns with respect to SEC localization.

An obvious question to ask: is there a need of SECs in the liver of fish? The main function of fish liver is similar to the mammals, but the fish liver lacks any population of macrophages such as Kupffer cells in higher vertebrates [20,21]. In mammals, approximately 80 % of the macrophages are present in the liver as Kupffer cells [22]. The highest number and occurrence of mammalian Kupffer cells are found in the periportal area and, as such, constitute the main macrophage population that are exposed to bacteria and especially bacterial products such as endotoxins transported from the gut to the liver [23]. Contrary to mammalian species, there are no resident macrophages in the fish liver. Thus, the main function of the scavenger endothelial cells is also to clear the portal blood from the intestine for potentially harmful substances, such as LPS. In mammals LPS may indeed activate the immune system and in worst case, result in physiological dysfunction and even death if present in substantial concentrations. Berczi et al. [24] calculated the deadly dosage of LPS in different animals after intravenous injection. The LD_{50} in calf was calculated to 0.0005 μg per kg tissue. There are differences between animal species with respect to LPS tolerance as described in Berczi et al. [24], but none of the dosages given to these animals will cause any harm to fish. A dosage of several milligrams per kg fish can be injected intravenously into fish without any negative effect on survival [14]. This points to the anciently physiological adapted responses to potentially harmful and/or antigenic substances between fish and mammals [24]. It is suggested that LPS tolerance is attributed to the TLR4 receptor and that the fish TLR4 does not recognize LPS [25]. However, we do not speculate if the lack of LPS recognition by TLR4 receptor is the reason for the lack of scavenger endothelial cells in the fish liver.

In the Siberian sturgeon, large fluorescent cells, indicative of high level of FITC-FSA uptake, were observed in the nodular tissue covering the pericardium of the heart. This special nodular tissue of the heart in sturgeon has previously been defined as a lymphoid tissue ([26]. This tissue was first described by Hertwig [27] and is later reported as an epicardial lympho-haematopoietic tissue by Icardo et al. [28]. This nodular tissue has to be studied further to identify the cells responsible for the uptake and establish its role in the metabolism of soluble waste macromolecules must be established, and put in an immunological context.

In the current study we did not find scavenger endothelial cells in the liver of any of the bony fish species investigated. We found the scavenger endothelial cells present in the heart of marine species as described earlier [3,29] but in the heart of the *Percichthys trucha* and *Perca fluviatilis* which both are freshwater species. Moreover, in some fish species the cells were present in both the heart and kidney, and species where the SECs were located only in the kidney, both in marine and freshwater species. In this work we set out to find or to understand if there was a clear phylogenetical relationship between fish order and anatomical

localization of SEC, or whether the localization was dependent on whether the fish were salt- or freshwater adaption. Some of these species were selected by chance and others due to their anticipated relevant position.

We have earlier suggested that the scavenger endothelial cells of the cod heart are involved in energy metabolism by their very active endocytosis and effective degradation of circulating waste macromolecules down to lactate and acetate. The lactate and acetate are used to produce mitochondrial fuel in neighbouring high energy consuming parenchymal cells [null]; [4]. Note that *Gadus morhua* does not have a coronary circulation supplying oxygenated blood to the compact part of the ventricle with. The mackerel (*Scomber scombrus*) have a coronary circulation but still there are SECs in the heart. We do not know if the SECs we have found in other organs of other species are involved in energy metabolism or if the energy metabolism can explain the localization of these cells.

We conclude that there is neither a clear phylogenetic trend to explain anatomical localization of SECs, nor any pattern in terms of habitat (salinity preferences). In a cautious manner, the anatomical localization of SECs seemed to be relatively consistent with phylogeny at the level of order and family, apart from Perciformes. It must be noted that this order is highly diverse and species-rich with more than 4000 species (~11 % of all known fish species).

CRediT authorship contribution statement

Tore Seternes: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Trygve T. Poppe:** Conceptualization, Methodology, Formal analysis, Investigation. **Jarl Bøgwald:** Conceptualization, Writing – original draft, Writing – review & editing. **Arve Lynghammar:** Conceptualization, Writing – original draft, Writing – review & editing. **Roy A. Dalmo:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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