The Intestinal Epithelium of Salmonids

Transepithelial Transport, Barrier Function and Bacterial Interactions

Akademisk Avhandling
för filosofie doktorsexamen i zoofysiologi som enligt
naturvetenskapliga fakultetens beslut kommer att försvaras
offentligt fredagen den 2 juni 2006, kl. 10.00 i föreläsningssalen,
Zoologiska institutionen,
Medicinaregatan 18, Göteborg

av

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Published by the Department of Zoology, Zoophysiology
Göteborg University, Sweden

Printed by Vasastadens Bokbinderi AB, Göteborg 2006

ISBN 10: 91-628-6834-9

ISBN 13: 978-91-628-6834-5

Dissertation Abstract

Jutfelt, Fredrik (2006). The Intestinal Epithelium of Salmonids - Transepithelial Transport, Barrier Function and Bacterial Interactions

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The salmonid intestinal epithelium is important for growth and health of the fish. The epithelium is exposed to a multitude of internal and external factors that can influence its function. During the parr-smolt transformation and subsequent seawater transfer, the epithelium adapts for an osmoregulatory role and the fish starts drinking seawater (SW). Endocrine signals increases the intestinal water uptake partly through an up-regulation of Na^+,K^+-ATPase activity. It is shown that the epithelial paracellular permeability decrease concurrent with the increase in water transport, suggesting that water flow is directed from a paracellular to a more transcellular route. The rational for this could be the increase in epithelial exposure to the environment at SW entrance. Tightening the paracellular route could be a mechanism to reduce paracellular transfer of harmful substances and pathogens.

A major salmonid pathogen is the bacterium *Aeromonas salmonicida*, which cause losses in both aquaculture and in wild populations. It is not known, however, by which route the *A. salmonicida* enters the fish. *A. salmonicida* has been positively demonstrated in the intestinal lumen but it has been controversial whether or not the bacteria cross the epithelial barriers. It is demonstrated that *A. salmonicida* can translocate across the intestinal barrier, indicating the intestine as a functional route for bacterial infection in salmonids. It is concluded that *A. salmonicida* employs many virulence mechanisms, such as exotoxins, endotoxin and cell bound factors, to disrupts epithelial morphology and function and promote translocation. During the later phases of parr-smolt transformation the epithelial barrier integrity decreased and translocation of pathogens increased. The increased disease susceptibility during this life stage could thus partly be caused by a decreased barrier function.

Vegetable lipids are used as replacement for fish oil in salmonid aquaculture, but there are concerns about how the new diets affect the intestinal epithelium. The epithelial functions presently investigated indicate a slight increase in permeability, supporting earlier histological reports of epithelial disruptions but not to the same extent. Nutrient uptake and barrier function during the parr-smolt transformation was significantly improved by a vegetable lipid-containing diet, indicating that this inclusion may be beneficial in the freshwater (FW) stage. The fatty acid profile of the natural diet for salmonids in FW is more similar to a blend of vegetable oils than to the profile of marine feed ingredients, routinely used in salmonid aquaculture. This may be the rationale for the positive effects. Salmon fed sunflower oil, however, showed long term elevation of plasma cortisol levels indicating a chronic stress. As chronic stress is known to depress immune function, specific vegetable lipids potentially stressful to the fish may also affect their health and welfare. Thus, while vegetable lipids at certain life stages are feasible substitutes for fish oil, possible long term stress effects by vegetable oils should be considered.

In conclusion, the salmonid intestinal epithelium is a sensitive and dynamic tissue which is affected by external factors, such as pathogen bacteria, environment and diet, but which also can be endogenously regulated to compensate for this disturbance.

Keywords: Epithelial barrier function, smoltification, cortisol, osmoregulation, *Aeromonas salmonicida*, Atlantic salmon, rainbow trout, bacterial translocation, intestine, Ussing chamber, vegetable lipids.

ISBN 10: 91-628-6834-9; ISBN 13: 978-91-628-6834-5

List of papers

Paper I

Sundell K., Jutfelt F., Augustsson T., Olsen R-E., Sandblom E., Hansen T. and Björnsson B. Th. (2003) Intestinal transport mechanisms and plasma cortisol levels during normal and out of season parr-smolt transformation of Atlantic salmon, *Salmo salar*. Aquaculture 222, 265-285.

Paper II

Jutfelt F., Olsen R.E., Björnsson B. Th., Sundell K. (2006) Parr-smolt transformation and dietary vegetable lipids affect intestinal nutrient uptake, barrier function and plasma cortisol levels in Atlantic salmon. Aquaculture. Accepted.

Paper III

Ringø E., Jutfelt F., Kanapathippillai P., Bakken Y., Sundell K., Glette J., Mayhew T. M., MyklebustR., Olsen R.E. (2004) Damaging effect of the fish pathogen *Aeromonas salmonicida* ssp. *salmonicida* on intestinal epithelium enterocytes of Atlantic salmon (*Salmo salar* L.). Cell Tissue Res. 318:2, 305-311.

Paper IV

Jutfelt F., Olsen R.E., Glette J., Ringø E., Sundell K. (2006) Translocation of viable *Aeromonas salmonicida* across the intestine of rainbow trout. Journal of fish diseases. 29:5, 255-262.

Paper V

Jutfelt F., Sundh H., Burr S., Mellander L., Sundell K. (2006) Mechanisms of bacterial translocation across the intestine of rainbow trout and effects of *Aeromonas salmonicida* virulence factors. Manuscript.

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Introduction

The intestinal epithelium is a single cell layer protecting the organism against harmful agents in the lumen, and at the same time it is a site for nutrient, water and ion uptake. Integrity of the barrier and uptake mechanisms is crucial for the health and growth of the animal. However, many internal and external factors can influence the epithelium in both harmful and beneficial ways. The focus of the present thesis is to elucidate how factors such as pathogens, diet and developmental stage affect the physiological function of the intestinal epithelium in salmonids.

The intestinal epithelium

Intestinal morphology

The main functions for the gastrointestinal (GI) tract are: to store food and water, to process the ingested food and water; to absorb water, osmolytes and nutrients from the external medium, and to excrete waste. In vertebrates, the GI canal is constituted of several distinct regions that differ in morphology and histology as well as in physiological functions. Following prey capture and manipulation by teeth or other parts of the oral cavity, the esophagus transports the food to the stomach. The stomach is absent in some vertebrates, such as some species of fish, in which the intestine directly follows the esophagus. Stomach secretions typically contain proteolytic enzymes as well as hydrochloric acid. After mixing and processing in the stomach, the bolus is emptied into the intestine where absorption occurs. There are large differences in the morphology and physiology of the intestinal regions between the vertebrate groups and between different feeding strategies within the same vertebrate group. Mammals have several distinct regions of the intestine, whereas other vertebrate groups such as the cyclostomes only have one (Nilsson 1983). In fish, intestines vary in length from 0.4 to >38 times the body length. The percentage plant material in the diet is the major determining factor for intestinal length, where intestines of herbivorous fish generally are longer than those of carnivorous fish (Buddington *et al.* 1997; Clements and Raubenheimer 2006). Teleost intestines commonly have two regions, in this thesis referred to as the anterior and the posterior regions, separated by the ileocolonic junction (Clements and Raubenheimer 2006). At the anterior end of the anterior intestinal region, many fish species have numerous pyloric caeca which extend the surface area (Clements and Raubenheimer 2006; Veillette *et al.* 2005). Functionally, the anterior region and the pyloric caeca are the primary sites for nutrient uptake (Nordrum *et al.* 2000),

whereas the posterior region has less nutrient absorptive capacity and more phagocytotic activity (Buddington and Diamond 1987; Ezeasor and Stokoe 1981) The pinocytosis of proteins by the posterior region has been suggested to have nutritional importance (Clements and Raubenheimer 2006).

Layers of the intestinal wall

Despite the many specialized regions of the GI tract, cross-sectional tissue organization remains fairly similar throughout the intestines of vertebrates. The vertebrate intestine consists of several histologically distinct tissue layers with correspondingly distinct functions. Lining the lumen is the epithelium, the barrier between the exterior and interior medium (Figure 1), which is attached to the connective tissue layer of the basement membrane. The surface area of the mammalian intestinal epithelium is expanded through fingerlike villi. Fish intestinal epithelia are also expanded through folding, but lack the typical crypts of the mammalian villi. The term mucosal folds will hence be used

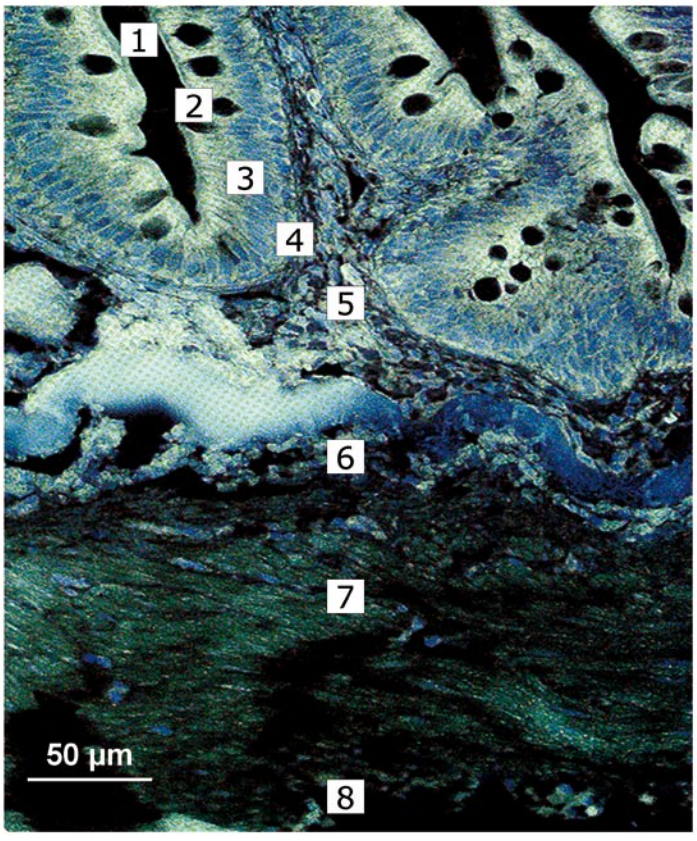

Figure 1.
Fluorescence microscopy image using triple filters (FITC, DAPI and Texas Red). Cross sectional view of the anterior intestine of rainbow trout. Layers of the intestinal wall:
1. Lumen
2. Goblet cells (black)
3. Enterocytes
4. Basement membrane
5. Lamina Propria
6. Submucosa
7. Circular and longitudinal muscle layers
8. Serosal layer removed

when referring to the fish epithelial folding. The epithelium, together with the underlying *lamina propria* constitutes the mucosa. Adjacent to the lamina propria is the connective and contractile muscularis mucosa which separates the lamina propria from the submucosa. Within the submucosal layer, the submucosal nerve plexus is found. Further away from the lumen, the circular muscle layer is followed by the myenteric nerve plexus and the longitudinal muscle layer. The perimeter of the intestine is lined by the serosa, a connective tissue layer attached to the mesenteric tissue.

The intestinal circulation

In addition to the regular gas exchange, nutrient and waste transport that occur in all tissues, the intestinal circulation also performs the task of removing absorbed substances from the epithelium of vertebrates. Arterioles and venules extend into the lamina propria of the mucosal fold tips in close proximity to the epithelium. The veins leaving the intestine collect in the portal vein leading to the liver for nutrient metabolism, detoxification and immune functions (Guyton and Hall 2000). The close contact between arterioles and venules in the villi in mammals has been shown to function as a counter-current gas exchanger that reduces oxygen content in the villi tips (Haglund 1994). If a similar counter-current mechanism is functional in fish is not known. Fish differ from other vertebrates in that they lack a true lymph system and instead have a secondary circulation. This circulatory system is connected to arterioles but have high resistance sphincters that reduce entry of red blood cells. The function of the secondary circulation is not well known, but it has been speculated that it may aid in osmoregulation and possibly be a precursor for the lymphatic system (Clements and Raubenheimer 2006).

Epithelial morphology

The epithelial layer consists mainly of absorptive columnar cells, referred to as enterocytes, with the inclusion of mucus-secreting goblet cells and endocrine cells (Figure 1). On the apical (luminal) surface of the enterocytes are numerous extensions called microvilli (Figure 2), and the whole apical surface of the epithelium is referred to as the brush border membrane (BBM). The microvilli greatly extend the surface area of the apical membrane which increases the area for absorption and membrane-bound digestive and absorptive enzymes (Clements and Raubenheimer 2006). Adjacent enterocytes are joined together at the apical end of the lateral surface by junctional complexes. The junctional complexes consist of anchoring adhesion belts and desmosomes, and located closest to the lumen are the occluding tight junctions (TJ).

The limit for the paracellular permeability is set by the occluding TJ, which are vital for the function of the epithelial monolayer. The tight junctional complexes are chains of transmembrane junctional proteins forming continuous seals between the adjoining epithelial cells (Anderson 2001). By the use of freeze-fracture electron microscopy, TJ complexes have been described as Ø10 nm particles spaced at 18 nm (center to center). Aqueous pores are thought to cross the TJ strands with different permeability in different tissues and regions of the intestine. The pores of the intestinal TJs display positive cation selectivity.

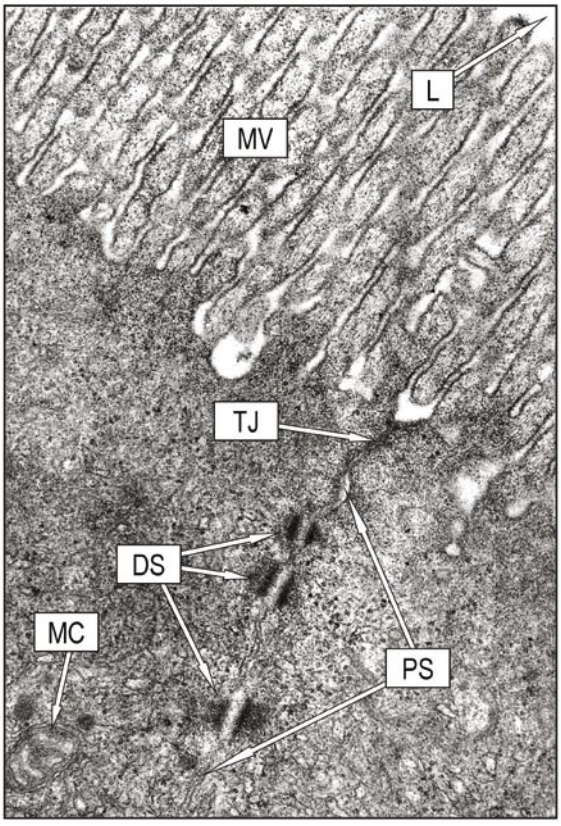

Figure 2.
Transmission electron micrograph of the anterior intestinal brush border membrane from rainbow trout. The intestinal segment was mounted into Ussing chambers for 150 minutes of regular control experiment, before fixation. Visible structures are: microvilli (MV), tight junctions (TJ), desmosomes (DS) and mitochondria (MC). Enlargement ×20.000. (TEM image: Sundell unpublished).

More than one TJ pore size may be found simultaneously in the same tissue. The proteins of the TJ complexes can be divided into three functional and morphological groups:

1. The extracellular proteins that span the paracellular gap and form the actual paracellular barrier. These consist of occludin and claudins. While occludin was originally thought to be the major component of the extracellular TJ complex, several claudins are now the prime candidates (Schneeberger and Lynch 2004).

Claudins are, like occludin, tetraspan membrane proteins with two loops, but unlike the similarly sized loops of occludin, one of the claudin loops is shorter than the other. Over- and under-expression of different claudins influences cation permeability through epithelia, indicating that claudins are responsible for the previously observed cation selectivity through the TJs (Johnson 2005). Long term regulation of TJ permeability is considered to include changes in the amount of TJ proteins (Johnson 2005).

2. TJ plaque proteins connect the transmembrane proteins to the actin cytoskeleton and are thought to be responsible for the rapid regulation of TJ permeability. The cytosolic plaque proteins consist of several families with the major family being the zona occludens proteins (ZO 1-3).

3. Cytosolic and nuclear proteins which interact with TJ plaque proteins to regulate among other things the paracellular permeability.

Together, these proteins constitute the regulated physical barrier connecting the epithelial cells. The regions of the intestinal tract differ in their luminal content and may thus need different TJ permeabilities in the different regions. Indeed, the paracellular permeability correspondingly differs between intestinal regions in mammals (Baumgart and Dignass 2002) as well as in fish (Schep *et al.* 1997).

Epithelial Barrier function

Extrinsic barrier

Commensal bacteria inhabit every ecological niche of the intestinal lumen and bacterial counts can reach 10^{12} cells g^{-1} mammalian luminal content. In humans, bacterial cells outnumber the human cells (Mueller and Macpherson 2006) whereas the bacterial concentration in fish intestinal luminal content is lower, ranging from 10^5 to 10^8 cells g^{-1} (Cahill 1990; Ringø *et al.* 1995). Under normal circumstances, the bacteria remain harmless in the lumen, and may even facilitate the digestion or production of nutrients otherwise inaccessible to the host (Ringø *et al.* 1992). Gut bacteria benefit from the symbiosis by the intestinal environment and available nutrients (Ismail and Hooper 2005). Commensal bacteria may also protect the host from pathogenic bacteria by means of ecological competition for resources or through direct inhibition by the secretion of peptide bacteriocins, H_2O_2 or organic acids such as lactic acid (Montagne *et al.* 2004; Ringø and Gatesoupe 1998). In fish, bacteria isolated from the gut inhibit the growth *in vitro* of bacterial fish pathogens including *Aeromonas salmonicida* (Irianto and Austin 2002; Jöborn *et al.* 1999; Robertson *et al.* 2000). *In vivo*, diets containing lactic acid bacteria may confer some resistance against

pathogenic bacteria. Juvenile Atlantic cod (*Gadus morhua*) fed a diet containing lactic acid bacteria were more resistant to infection, an effect speculated to be caused by ecological competition in the gut (Gildberg *et al.* 1997). Atlantic salmon and rainbow trout were more resistant during a disease challenge test with *A. salmonicida* (Robertson *et al.* 2000). Besides the ecological competition as an effect of probiotic bacteria, stimulation of the gut immune system by the endogenous bacterial flora has also been suggested as a protective mechanism (Irianto and Austin 2002). Some studies have shown a lack of protective effect of probiotic lactic acid bacteria (Gildberg *et al.* 1995).

In the epithelium, goblet cells secrete mucus (Figure 1), a viscous fluid containing mucin glycoproteins, to the apical surface of the epithelium. The mucus physically removes bacteria from the BBM by the constant flow away from the BBM and by receptor sites on the glycoproteins where bacterial adhesion factors attach (Maxson *et al.* 1994; Montagne *et al.* 2004). Deletion of mucus secretion reduces the barrier function of this layer (Maxson *et al.* 1994). Mucus may also be a homing cue for pathogenic bacteria in order to locate epithelia. Indeed, the fish pathogen *Vibrio anguillarium* has positive chemotaxis towards mucus from fish intestinal epithelia (Larsen *et al.* 2001).

Intrinsic barrier

The intestinal tract is an entry point for infection by pathogenic organisms in vertebrates. During the 1960's, Wolochow and coworkers investigated the intestinal epithelial barrier in rats (*Rattus norvegicus*) and concluded that bacteria instilled intra-intestinally later could be isolated from the lymph-nodes and blood. This process was termed translocation (Wolochow *et al.* 1966) and later defined as the transport of viable bacteria from the lumen to extra intestinal sites (Berg 1995), but is presently also used to describe transport of bacteria across the intestinal epithelium only. In the present thesis, the term bacterial translocation is used when referring to transport across the entire intestinal wall with all its layers of barriers.

The intrinsic barrier function consists of the physical epithelial wall working in concert with other mechanisms to prevent organisms to enter the host tissues. Starting from the luminal side, the epithelial monolayer with tight junctional complexes creates the primary physical barrier between the lumen and lamina propria (Clayburgh *et al.* 2004). As described above, tight junctional pores (~7- to 15-Å Ø) are too small for bacteria (~1 μm or 10.000 Å) to pass. However, some bacterial toxins may still be able to cross the physical barrier (Dalmo and Bogwald 1996; Popoff 2005).

Immunological barrier

The mucosal immune system (or gut-associated lymphoid tissue, GALT) of the jawed vertebrates comprises two functional groups, the innate and the adaptive immune system (Forchielli and Walker 2005). The innate immune system creates the first line of defense with rapid response and clearance of pathogens (Collier-Hyams and Neish 2005; Muller *et al.* 2005), whereas the adaptive immune system is slower but produces specific responses to the pathogen and maintains the specific response for a longer time period (Cheroutre and Madakamutil 2005; Nejdfors 2000). In fish, the innate immune system is suggested to have higher importance than in mammals, possibly because the specific defense is slower in ectothermic vertebrates (Ellis 2001; Magnadottir 2006). Antibody production in salmonids takes weeks while bacterial infections can kill in days, which demonstrates that the innate immune system is of high importance for salmonids (Ellis 2001).

Total exclusion of microorganisms by the epithelium is probably not possible. Some passage of bacteria across the epithelium is inevitable and may even, at least in mammals, be actively promoted by the host (Kucharzik *et al.* 2000). Specialized regions of the mammalian epithelium called Peyer's patches (also often referred to as the follicle associated epithelium; Peyer's patches will be used in the present thesis) continuously sample gut microbiota in order to prime the immune responses and antibody production towards the antigens present (Acheson and Luccioli 2004). Among the epithelial cells of the Peyer's patches are M-cells specialized for antigen sampling. These cells have high phagocytotic and transcytotic activity of luminal antigen from the apical to the basolateral side of the M-cells. There, intraepithelial and lamina propria lymphocytes and T-cells sample the antigens and induce the appropriate immune responses such as production of cytokines and specific antibodies (Kucharzik *et al.* 2000) and antigen recognition retention in memory cells (Cheroutre and Madakamutil 2005). Birds have a system for antigen sampling which is similar to the mammalian Peyer's patches (Pohlmeyer *et al.* 2005), whereas it is unclear if the structures and functions exists in reptiles or amphibians (Hart *et al.* 1988).

It has been suggested that Peyer's patches and M-cells are absent in the fishes (Buddington *et al.* 1997; Rombout 1998). However, the American paddlefish (*Polyodon spathula* Walbaum) has mucosal lymphoid follicles which form structures resembling the mammalian Peyer's patches (Fänge 1984). The function of the Peyer's patches-like structures of the paddlefish has not been investigated, but histological data suggests a function similar to the Peyer's patches of mammals (Petrie and Peterman 2005). The spiral valve of the elasmobranch intestine has mucosal lymphocyte accumulations which have a structure similar to Peyer's patches. The function of these structures is not determined (Hart *et*

al. 1988). The teleost mucosal immune system is more diffuse, consisting of antigen processing macrophages in the epithelium and lamina propria. The posterior intestine is considered to have more intraepithelial macrophages than the anterior region. These cells are suggested to function as antigen presenting cells (Buddington *et al.* 1997; Pettersen 2003). Intestinal B-cells and T-cells have been found in teleosts (Hart *et al.* 1988; Rombout 1998; Zapata *et al.* 2006), but surprisingly, are lacking sometimes (Wermenstam and Pilstrom 2001). Whether the antigen sampling function of the mammalian M-cells is performed by other epithelial cell types in fish, such as the enterocytes, is suggested but still poorly understood (Hart *et al.* 1988; Rombout 1998).

Transport across the epithelium has been demonstrated in salmonids for luminal macromolecules (Brudeseth and Evensen 1995; Georgopoulou *et al.* 1988; O'Donnell *et al.* 1994) as well as bacterial cells (Hart *et al.* 1988; Ringø 2006; Vigneulle and Laurencin 1991). The higher phagocytotic activity of the posterior region in comparison to the anterior region of fish intestines, as described below, has been suggested to include an antigen sampling function by transferring antigen from the lumen to macrophages and lymphoid cells in the epithelium and the lamina propria (Hart *et al.* 1988; Rombout 1998). Functionally, antigens presented orally can activate immune functions and induce production of specific antibodies and storage of antigen by memory cells (Georgopoulou and Vernier 1986; Nikoskelainen *et al.* 2003; Rombout 1998). Anal intubation of inactivated pathogenic bacteria in carp (*Cyprinus carpio*) has been shown to induce high levels of serum antibodies, as well as having a protective effect in subsequent challenge tests (Ellis 1995). The protection in those challenge tests were comparable to the protection after injection of the antigen, demonstrating that the carp posterior intestine has effective antigen sampling and presenting functions (Ellis 1995).

Lipopolysaccharide (LPS) is a constituent of the cell surface of gram-negative bacteria (Schletter *et al.* 1995). As many gram-negative bacteria are pathogenic to vertebrates, LPS is used by animals for early detection of infection by gram-negative bacteria. LPS detection systems thus have a protective function as a warning system, the importance of which has been demonstrated by animals with dysfunctional LPS detection systems; as they are highly sensitive to infections by gram-negative bacteria (Schletter *et al.* 1995). However, if the host immune response is too powerful, the animal can be harmed by the inflammation (Iliev *et al.* 2005; Munford 2005; Schletter *et al.* 1995). The innate immune system of teleosts, like that of other vertebrates, uses toll like receptors (TLR) to specifically detect pathogens (Plouffe *et al.* 2005). LPS is a potent inducer of the fish innate immune system through TLR activation (Iliev *et al.* 2005).

Mechanisms of bacterial translocation

In order to infect the host, bacteria first have to cross the multiple barriers of the intestinal wall. After growth and adhesion to the BBM, the epithelium has to be crossed. Transcytosis, where bacterial cells are phagocytosed at the apical surface and exocytosed at the basolateral side of the epithelium is suggested to be the dominant route for translocation in mammals (Cossart and Sansonetti 2004), although paracellular translocation has also been suggested especially in areas with a damaged epithelium (Baumgart and Dignass 2002; Berg 1995; Nadler and Ford 2000). Several mammalian bacterial pathogens utilize M-cells for epithelial passage (Kucharzik *et al.* 2000), since bacteria from many species are transcytosed without inactivation of the pathogen. An important virulence factor for these pathogens then becomes the ability to avoid or survive phagocytosis by the immune cells (Cossart and Sansonetti 2004). Transcytosis can also occur through cells that may not normally phagocytose bacteria. A bacterial virulence factor for injecting substances into eukaryotic cells is called the type III secretion system (TTSS). TTSSs are pore-like protein-structures across the bacterial envelope that include protruding needle-like structures which are able to penetrate host cell membranes (Buttner and Bonas 2003; Hueck 1998). When the bacteria come in close contact to the eukaryotic cell, the TTSS extend through the cell membrane of the host and substances can be injected directly into the cytoplasm (Hueck 1998). The injected substances are proteins that may be necessary for infection to occur. Functions of the injected substances include host cell membrane-bound receptors for the bacteria (Nougayrede *et al.* 2003), inhibition of phagocytosis (Anderson and Schneewind 1999; Burr *et al.* 2005; Hueck 1998), cytotoxic and apoptosis-inducing effects (Hueck 1998), killing of neutrophils by necrosis (Mecsas and Strauss 1996) and disruption of the actin cytoskeleton (Lesnick and Guiney 2001). In mammals, several species of gram-negative bacteria use contact dependent type III secretion of toxins for disruption of the intestinal epithelial barrier (McNamara *et al.* 2001; Parsot 2005), and activation of epithelial phagocytosis (Cossart and Sansonetti 2004; Guiney 2005; Mecsas and Strauss 1996; Parsot 2005; Popoff 2005).

Transepithelial transport mechanisms

Transfer of substances across the intestinal epithelium can occur through either the transcellular route or the paracellular route. Paracellular transport is thought to be due to diffusion and solvent drag. The solvent drag hypothesis has been proposed by Pappenheimer (1993), and predicts that sugars and amino acids may, in part, be transported paracellularly. The proposed mechanism consists

of transcellular sodium ion uptake across the epithelium which creates high osmolality in the lateral spaces. Water would thus be transferred through the TJs, which in turn could bring hydrophilic nutrients across the TJ through bulk flow (Pappenheimer 2001). The solvent drag hypothesis has been criticized by many as being of little if any physiological importance (Ferraris and Diamond 1997; Kellett 2001) and will thus not be discussed further in the present thesis. Paracellular diffusion of substances is driven by electrochemical gradients. The diffusional rate is limited by the TJ permeability, which can differ between tissues. The anterior region of the salmonid intestines has higher diffusion rate than the posterior region (Schep *et al.* 1998).

Water transport

Intestinal water transport in the vertebrates is thought to be driven by osmotic gradients only, as no active water transporters have been found in any organism. There is still some controversy, however, about which route water crosses the intestinal epithelium and how this is regulated (Loo *et al.* 2002; Reuss and Hirst 2002). The "standing-gradient" hypothesis (Figure 3C) predicts water transport to occur across the epithelium by the force of local osmotic gradients which are actively created in the lateral spaces of the epithelium. According to this view, solutes (mainly sodium), is actively secreted from the enterocytes into the lateral space between adjacent enterocytes to create a high local osmolality. This local hyperosmolality draws water from the lumen transcellularly and through the TJ to varying degree (Diamond 1979; Holtug *et al.* 1996).

Models of intestinal water transport conflicting with the standing-gradient hypothesis have emerged (Reuss and Hirst 2002). The major recent controversy is regarding the role of "water pumps" as some authors call the sodium-coupled glucose carrier SLGT1 (Loo *et al.* 2002). Intestinal SLGT1 transporters have been suggested to transport 200-260 water molecules together with the single glucose molecule and the two sodium ions, resulting in a stoichiometry of 70-90 molecules of water for each solute. This uptake can take place against an osmotic gradient because it is driven by the electrical and chemical gradient for sodium, thus earning the name "water pump" (Schultz 2001). This mechanism has been suggested to account for half of the intestinal water uptake in humans (Loo *et al.* 2002). The water pump hypothesis have been criticized, and the stoichiometric coupling is claimed to be explained entirely by osmotic flow (Duquette *et al.* 2001; Schultz 2001). Further research is needed to confirm or discard the water pump hypothesis.

Aquaporines (AQP) are water channels which greatly increase the cell membrane permeability for water in several tissues. Currently, at least six aquaporine isoforms have been found in the digestive system and four of these

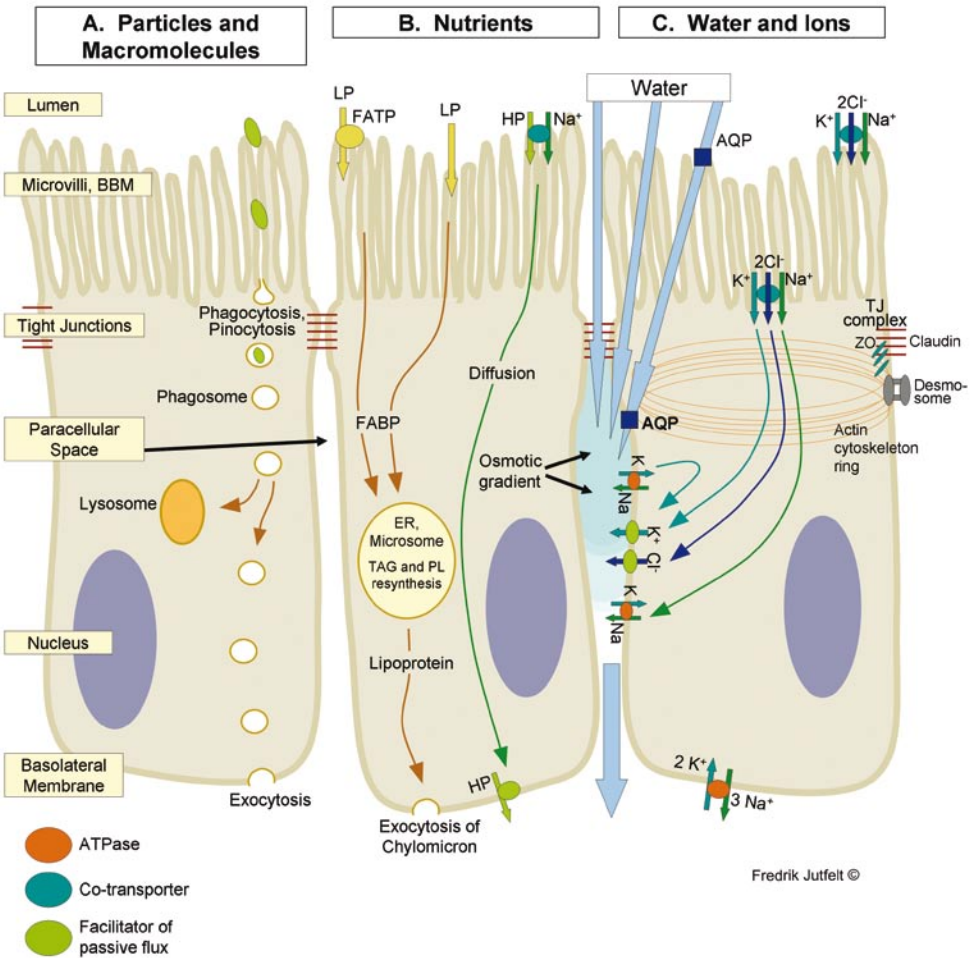

Figure 3.
Schematic view of intestinal transepithelial transport in fish. Three enterocytes are shown. Morphological structures in yellow boxes.
A. Transepithelial transport of particles and macromolecules starts with phagocytosis or pinocytosis at the brush border membrane (BBM). Phagosomes are transported either to digestive lysosomes or through the cell to the basolateral membrane (BLM) where exocytosis occurs.
B. Transepithelial transport of nutrients. Lipophilic molecules (LP) cross the BBM by diffusion over the lipid bilayer of the BBM and through fatty acid transport proteins (FATP). Triacylglycerol (TAG) and phospholipid (PL) resynthesis occur in the endoplasmatic reticulum (ER) before lipoprotein package and export of chylomicrons through exocytosis at the basolateral membrane. Hydrophilic nutrients (HP) such as amino acids and glucose cross the BBM by secondary active transport (sodium co-transport is shown) and cross the BLM by carrier proteins.
C. Na+,K+-ATPase activity at the BLM remove intracellular Na+. The NKCC co-transporter carries 2 Cl-, Na+ and K+ across the BBM. Cl- and K+ exit through ion channels across the BLM. The osmotic gradient created in the paracellular space, with the highest osmolality apically drives water diffusion transcellularly and through tight junctions (TJ). Aquaporins (AQP) may increase the BBM and/or BLM water permeability.

(AQP3, 4, 8 and 9) are expressed in the intestinal epithelium of mammals (Matsuzaki *et al.* 2004). The hypothesis has been proposed that aquaporins in the intestinal epithelium could be the primary route for water absorption within the standing-gradient hypothesis (Schultz 2001). There is, however, little experimental proof supporting the view that AQP are important components of epithelial water uptake in the intestine of mammals (Matsuzaki *et al.* 2004). In fish, reports are emerging on the presence of aquaporins in the intestine (Santos *et al.* 2004). The Japanese eel (*Anguilla japonica*) expresses at least two aquaporine isoforms in the intestinal epithelium which are similar to the mammalian AQP1 (Aoki *et al.* 2003) whereas the European eel (*Anguilla anguilla*) expresses AQP1 and AQP3 homologues in the GI tract (Lignot *et al.* 2002; Martinez *et al.* 2005b). The function of eel AQP have been suggested to include epithelial water uptake, and that the AQP is regulated for the purpose of euryhaline osmoregulation (Aoki *et al.* 2003).

Nutrient uptake

Nutrients can cross the membrane of epithelial cells by diffusion or by membrane transporters (Figure 3B). The diffusion rate increases linearly with concentration, whereas transport by membrane transporters becomes saturated at higher concentrations. Energy for nutrient uptake against an electrochemical gradient can be harnessed from ions (usually Na^+) moving with an electrochemical gradient. The actual source for the energy is the ATP hydrolysis by the Na^+,K^+-ATPase (Collie 1995) removing intracellular Na^+ and thus creating the electrochemical Na^+-gradient (Ferraris and Diamond 1997). In fish, a few membrane-bound nutrient transporters have been found. Glucose is transported by a transporter protein with similar functional and genetic characteristics as the mammalian glucose transport system (SGLT1 in the BBM and GLUT2 in the basolateral membrane); (Buddington *et al.* 1997; Collie 1995). In carnivorous fish, such as the salmonids, the rate of sugar absorption is low with little scope for up-regulation (Buddington *et al.* 1997; Clements and Raubenheimer 2006).

Amino acids (AA) can be transported as free AA's, as small peptides, or as larger proteins. Mammalian AA transporters are Na^+-dependent or Na^+-independent. The four categories of AA's, neutral, basic, acidic and imino, are transported by the corresponding Na^+-dependent BBM transporter (Silk *et al.* 1985; Thomson *et al.* 2001a). In addition, there are two Na^+-independent transporters, one for neutral and basic AA's and another one for acidic AAs (Ray *et al.* 2002). The presence of Na^+-dependent AA transporters in fish has been confirmed (Figure 3B), although the studies are few and scattered over several species of fish, making general conclusions difficult (Collie

1995). Di- and tripeptides have been suggested to constitute a large part in the absorption of digested protein, and Na^+-independent pathway has been suggested (Buddington *et al.* 1997; Collie 1995). Protein endocytosis occurs in the posterior intestine of several fish species (as described below) (Ezeasor and Stokoe 1981; Georgopoulou *et al.* 1988) which may serve nutritional purpose (Vernier 1990) as well as immunological (as discussed below).

Lipophilic substances can cross the epithelial cells by diffusion through the lipid bi-layers. Most of the lipid uptake in fish occurs in the pyloric caeca and anterior intestine, whereas the posterior intestine is suggested to have less lipid absorptive function (Vernier 1990). The major dietary lipids are triglycerides, whereas phospholipids and cholesterol amount to only a few percent (Olsen 1997). Digested triglycerides and phospholipids are absorbed into the enterocytes (Figure 3B) as free fatty acids (FFA), glycerol and 2-monoglycerides (2-MAG). In mammals, transport of FFAs across the BBM has been suggested to be partly mediated by transmembrane fatty acid transport proteins (FATP) (Stahl 2004; Thomson *et al.* 2001a). After entry, FFA and 2-MAG are converted into complex lipids, mainly triacylglycerol (TAG) and phospholipids (PL), before being packaged in lipoproteins and exported into the circulation of the lymphatic system. Long-chain fatty acids (LCFA), mainly polyunsaturated fatty acids (PUFA) can be transferred to the circulation as FFAs (Thomson *et al.* 1993). In fish, the lipid uptake is thought to occur in a similar manner (Olsen 1997; Oxley *et al.* 2006).

Macromolecule and particle transport across the mammalian intestinal epithelium can occur through pinocytosis and phagocytosis (Figure 3A), with subsequent exocytosis on the opposite side of the epithelium (Kucharzik *et al.* 2000). In fish, phagocytotic epithelial cells have been found (Hart *et al.* 1988; Ringø 2006; Ringø *et al.* 2003; Vernier 1990). Phagocytosis of bacteria, occurs in all regions of the salmonid intestinal tract including the pyloric caeca (Ringø 2006; Ringø *et al.* 2001; Ringø *et al.* 2003). However, the posterior intestine has been suggested to have higher phagocytotic activity than the other intestinal regions (Clements and Raubenheimer 2006; Hart *et al.* 1988; Olsen *et al.* 2001; Rombout 1998).

Salmonids

Parr-smolt transformation

The Atlantic salmon, like many salmonids, are anadromous fish *i.e.* they spawn in fresh water (FW) while they spend their adult stage in seawater (SW). After hatching in streams and rivers, the Atlantic salmon stay in FW during

the juvenile life stages lasting one to several years before undergoing a SW preparatory transformation called parr-smolt transformation or smoltification. During the parr-smolt transformation, a number of changes occur in the salmon physiology, morphology and behavior, regulated by endocrine signals, mainly cortisol, growth hormone, IGF-1 and thyroid hormones (McCormick 2001; McCormick and Saunders 1987). The parr, dark-colored, benthic and territorial fish changes to become smolts, silvery, pelagic and schooling fish with slender bodies. The alteration to the physiology is also substantial. In FW, fish are hyperosmotic to the environment. Water constantly enters the fish over the body surface by osmosis, and leaves the body mainly as dilute urine. Ions are mainly acquired from ingested food, but also actively absorbed across the gills, to counter diffusional and excretory losses. Life in SW poses opposite challenges, with water leaving the hypoosmotic fish while ions enter the body. Drinking rates and ion-coupled fluid uptake rates in the intestine are high. Excess monovalent ions are mainly secreted by the branchial SW chloride cells (McCormick and Saunders 1987) and divalent ions are mainly excreted through the urine and faeces (Mashall and Grosell 2006).

The Atlantic salmon drinking rates are low in FW, and increase dramatically when the fish enters hyperosmotic SW. The intestinal capacity for absorption of luminal water increases during parr-smolt transformation, in preparation for the increased drinking rates after SW migration. Water uptake over the intestine is dependent on ion gradients, and during this developmental period, the cellular ion transporting activity of the Na^+,K^+-ATPase activity increases in the mucosa, presumably a preparatory adaptation for the high ion transporting demands on the intestine once in seawater. What other changes the epithelium undergoes in order to increase the water transporting capacity is unclear, but increase in membrane PUFA fatty acids has been observed in salmonids after transfer to SW (Leray *et al.* 1984). Increased proportion of PUFA has been shown to increase the water permeability of membranes (Lande *et al.* 1995), indicating that the salmonid epithelial membrane permeability for water increases by SW acclimation. It can be speculated that the large changes in intestinal epithelial functions, and the major endocrine changes which occur during the parr-smolt transformation may compromise the barrier functions. After SW migration, increased ingestion of water increases the risk of pathogen entry into the GI tract. It has been documented in research and in practical aquaculture situations, that salmonids are more susceptible to infections during the parr-smolt transformation and after sea water transfer (Inglis *et al.* 1993; Smith 1993). It remains untested if a compromised intestinal barrier function can account for some of the observed increases in disease susceptibility. These issues, however, have not yet been properly addressed in fish research.

The pathogen *Aeromonas salmonicida*

Furunculosis

Furunculosis is a bacterial disease in salmonids. Fish can acquire either chronic or acute furunculosis. Acute furunculosis is characterized by septicaemia (blood poisoning) without external symptoms, usually followed by death; whereas the chronic form includes characteristic furuncle boils as well as darkening of the skin, lethargy, anemia and paling of the gills. Internally, there can be inflammation of the intestinal blood vessels, paling of the liver, swelling of the spleen, and the kidney can become liquefied. The fish loose apetite, leading to empty intestines (Inglis *et al.* 1993). *Aeromonas salmonicida* is the gram-negative bacteria causing both the acute and the chronic forms of furunculosis. Five *A. salmonicida* sub-species have been isolated; *salmonicida, achromogenes, smithia, masoucida* and *pectinolytica* (Cipriano and Bullock 2001; Pavan *et al.* 2000). The present thesis focuses on the subspecies *A. salmonicida* subsp. *salmonicida* which is also termed typical *A. salmonicida* and which cause typical furunculosis.

Virulence factors

A number of bacterial factors are essential for virulence by the *A. salmonicida*. These factors can be divided into exotoxins, endotoxins, adhesion proteins and type III secretion systems (Figure 4). The exotoxins and endotoxins are extracellular products (ECP) found in the bacterial medium. The cell envelope of *A. salmonicida* is covered by the S-layer, a outer surface layer of proteins (Garduno *et al.* 1995). The S-layer confers virulence (Beveridge *et al.* 1997; Noonan and Trust 1997) by the proposed mechanisms of improving adhesion to the host cell membranes (Garduno *et al.* 2000) and by providing resistance to protease digestion (Noonan and Trust 1997). Present on the surface are also long extruding type IV pili (Figure 4), known from studies on other *Aeromonas* species in mammals to be an adhesion factor to intestinal epithelial cells and possibly also to other bacterial cells (Kirov *et al.* 1999). Similar pili have been found in *A. salmonicida* and also demonstrated to be important virulence factors for infection in the rainbow trout (Masada *et al.* 2002). The possible importance of type IV pili in epithelial adhesion and epithelial translocation in fish has not yet been elucidated.

A large variety of exotoxins, *i.e.* toxins actively secreted from the bacteria into the surrounding medium, have been described for *A. salmonicida* (Ellis 1991; Gudmundsdottir *et al.* 2003). One group of toxins consists of membrane damaging toxins (lysins) including the glycerophospholipid: cholesterol acyltransferase (GCAT) (Bricknell *et al.* 1997; Gudmundsdottir *et al.* 2003; Lee and Ellis 1990). The GCAT forms large (2000 kDa) complexes with

lipopolysaccharide (LPS). These complexes have been considered to be among the most potent of the examined extracellular products. The GCAT:LPS complex lyses several cell types, but mortality is correlated with the hemolytic activity, indicating that anemia is one of the major causes of death (Lee and Ellis 1990). Another group of *A. salmonicida* toxins are the proteases, including a collagen digesting metalloprotease (Arnesen *et al.* 1995), and the serine protease

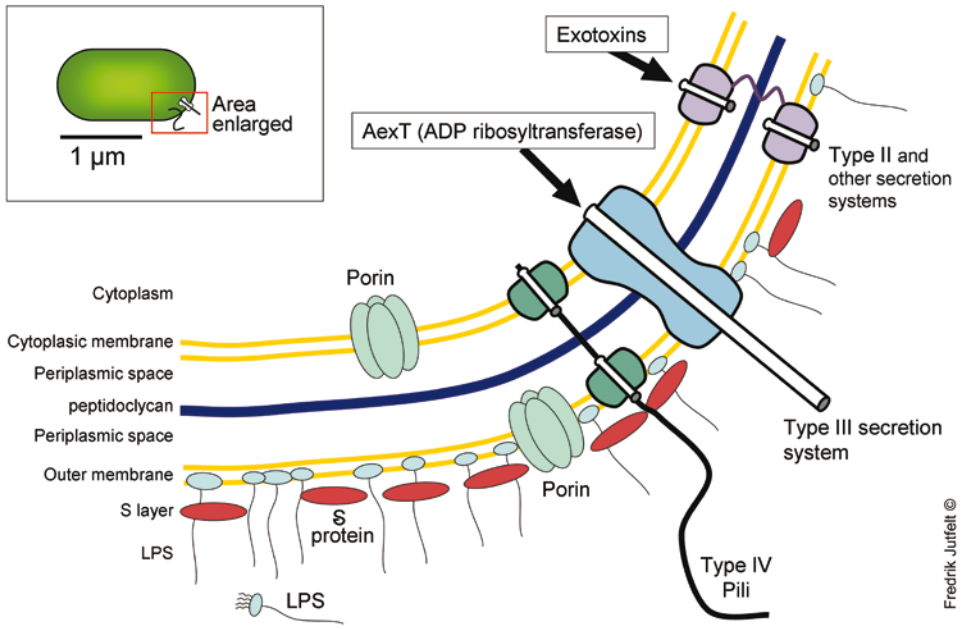

Figure 4.
Structures of the A. salmonicida envelope. The insert figure shows shape and size of *A. salmonicida*. A part of the bacterial wall is enlarged to present the surface and associated structures. Lipopolysaccharide (LPS) and S-layer proteins covers the external surface. Type II secretion systems, Type III secretion system, Type IV Pili and porins are also present on the surface.

P1 (also called caseinase) which is the major proteinaceous component of the ECP (Ellis 1991). Functionally, the serine protease may digests proteins for bacterial nutrient uptake, but it may also cause thrombosis (Arnesen *et al.* 1995; Ellis 1991) and immune system suppression (Hussain *et al.* 2000) in the host animal. However, genetic deletion of GCAT and serine protease did not reduce virulence after IP injection. Nor did the deletion reduce infectivity as assessed by cohabitant challenge (Vipond *et al.* 1998), which suggests that the major exotoxins are not involved in the primary entry of the host. Endotoxin or lipopolysaccharide (LPS) is another major component of the ECP (Ellis

1991). LPS may not have enzymatic functions or direct toxicity (Schletter *et al.* 1995), but is a highly potent inducer of the immune system. The resulting inflammation can be harmful and even fatal in mammals (Munford 2005).

Recently, a TTSS has been found in *A. salmonicida*. Functionally, the TTSS of *A. salmonicida* is relatively unknown. Burr (2005) has demonstrated that knocking out the TTSS gene expression of *A. salmonicida* completely abolished virulence after injection in rainbow trout, compared to injections of wild-type *A. salmonicida*. One virulence mechanism shown to be lost in the TTSS knock-out mutant *A. salmonicida* is the ability to avoid phagocytosis by leukocytes, but it is possible that other TTSS-dependent virulence mechanisms are also lost (Burr *et al.* 2005). It has not been tested if *A. salmonicida* use TTSSs for crossing the epithelial barrier in salmonids.

Aquaculture

Salmonid aquaculture is an important industry in several countries. Salmonids are carnivorous species and the production of fish is heavily reliant on wild-caught feed-fish. As the aquaculture industry continues to grow, even higher feed production will be needed in the future (FAO 2004). However, as the world-wide available feed stocks are already utilized to a maximal level, a shift in feed production to higher inclusion of alternative feed ingredients *i.g.* vegetable products, is required (Naylor *et al.* 2000). As the fat content of the feed is usually high, large quantities of fish oil are used for aquaculture, and a shortage of fish oil is predicted for the near future (FAO 2004). Aquaculture research has been successful in using vegetable ingredients such as oils in the diets, at least in regard to growth rates and survival (Bell *et al.* 2002; Bell *et al.* 2001; Bell *et al.* 2003; Izquierdo *et al.* 2003; Sargent *et al.* 1999). While a high percentage of vegetable lipid replacement can be used, the resulting flesh fatty acid profile changes and starts to reflect the vegetable lipid profiles (Björnsson *et al.* 2004; Caballero *et al.* 2002; Izquierdo *et al.* 2005; Tocher *et al.* 2000). This may be of concern for the consumer, as some of the health benefits for humans in consumption of fish are lost (Bell *et al.* 2001; Sargent 1997; Torstensen *et al.* 2004). Although growth rates of the fish in experimental studies have been high, some adverse effects on the fish health have occurred. Lipid droplets can be accumulated in the intestinal enterocytes, which has been suggested to disturb epithelial functions (Caballero *et al.* 2002; Olsen 1999). The fatty acid profiles of polar lipids in the intestinal mucosa have been shown to be altered by vegetable lipids in the diet, but the effects on epithelial functions have not been investigated (Björnsson *et al.* 2004).

The high fish density and stressful handling in aquaculture tanks and net pens make the fish more vulnerable for infections which can cause outbreaks of diseases (Barton and Iwama 1991; Vandenberg 2004). Acute stress causes decreased intestinal epithelial barrier function (Olsen *et al.* 2002; Olsen *et al.* 2005), which may lead to increased disease susceptibility. The aquaculture-related stress and high population densities in salmonid farming, in combination with the use of vegetable ingredients in the diet, may cause synergistic disturbances of the intestinal epithelium that could further increase disease susceptibility.

Vaccination is a common practice in salmonid aquaculture which is very successful in reducing outbreaks of infectious disease. However, the vaccination process with injection and handling stress can also cause adverse effects (Midtlyng 1997). Abdominal adhesions and reduced growth and flesh quality are common after IP vaccination (Cipriano and Bullock 2001; Ellis 1997). A preferable vaccination method would be oral vaccinations. Different oral vaccination techniques have been tested with varying degree of success (Ellis 1995; 1998; Irianto *et al.* 2003; Vandenberg 2004). The basic interactions between bacteria and the salmonid intestinal epithelium are not well understood. Research focused on the mechanisms of bacteria-epithelial interactions could lead to the development of functional oral vaccines for salmonid aquaculture.

Scientific Aims

The overall objective of the present thesis has been to increase the scientific understanding of the physiology of the intestinal epithelium in salmonids. Three important aspects were targeted.

Pathogenic bacteria are a problem for both wild salmonids and salmonids in aquaculture. The major route of infection for the pathogenic bacterium *A. salmonicida* is not conclusively settled. Several indications, however, point to the intestine as an important entry point. If and how the pathogenic bacteria cross the intestinal epithelium in salmonids has not been directly examined. The aims for the present thesis were thus to:

> Investigate if **translocation of bacteria** occurs in salmonids, to determine if the intestine is a possible route for bacterial infections.
>
> Elucidate by which mechanisms bacteria translocate across the intestinal barrier and how possible **bacterial virulence factors** affect the epithelium.

The salmonid intestinal epithelium undergoes dramatic change during the parr-smolt transformation. Both absorption and barrier function must be maintained despite the physiological alterations. If intestinal integrity is maintained throughout the smoltification process is unknown. The aim of the present thesis was thus also to:

> Gain insight into how the **parr-smolt transformation** affects the epithelial permeability, barrier function and nutrient uptake.

Fish stocks used for aquaculture feeds are declining and new feed sources are needed for a sustainable aquaculture. Feed manufacturers are thus including increasingly larger fractions of vegetable ingredients such as vegetable lipids. Vegetable lipid-containing diets can, however, disturb intestinal ultrastructure and change intestinal membrane lipids. How epithelial functions are affected has not been elucidated. The aim was thus to:

> Examine how dietary **vegetable lipids** affect the epithelial barrier function and nutrient transport.

Results and discussion

Methodological Considerations

Physiological Studies of the Intestinal Epithelium

The ultimate reason for studying physiological mechanisms is to understand the physiology of the organism *in vivo*, but the means to reach this goal may vary. When designing experiments to elucidate physiological mechanisms in an organism, the decision has to be made on the most appropriate method for the presented hypothesis. One of the first decisions to be made is whether to use an *in vivo* model or the alternatives such as *in vitro* or molecular models. In the present thesis, the focus on the mechanisms of the intestinal epithelium as a functional organ made the *in vitro* approach, often preceded by *in vivo* treatments, suitable for several parts of the present studies.

The Ussing chamber technique is used to study viable epithelia and measure different epithelial mechanisms. This methodology was developed by the Danish ion transport researcher Hans Ussing, and described in 1951. It has since been improved in many aspects, but the same basic principles are still used. The original paper describes a study on frog skin epithelia (Ussing 1951), but now the method is used on a number of tissues from many species. Presently, modified Ussing chambers (Grass and Sweetana 1988) are commonly used for intestinal epithelial studies in mammals.

One major issue with *in vitro* methods is how closely the physiology of the organ *in vitro* corresponds to the physiology of the organ *in vivo*. In the Ussing chambers, continuous measurements of the electrical properties: transepithelial resistance (TER), transepithelial potential (TEP) and short-circuit current (SCC) of the epithelium are made (Loretz 1995; Yang *et al.* 2000). While the original Ussing method short-circuited the epithelia (Ussing 1951), the present method applies short durations of alternating positive and negative direct current pulses and with concurrent recordings of the resulting voltage responses (Wikman and Artursson 1995). The current-voltage pairs are used to calculate the linear least-square fit, in which the slope represents the TER, the voltage axis intercept the TEP and the SCC is calculated by SCC= -TEP/TER (Paper I).

The TER is used for assessment of the paracellular permeability *i.e.* the tight junctional "tightness" (Papers I, II, IV and V). The TER is altered in longer time periods, as time for protein expression is required (Papers I, II). However, it can also be rapidly (minutes to hours) altered by factors introduced to the chambers (Jutfelt, unpublished results; Lindmark *et al.* 1998). The electrical parameters are excellent tools for constant integrity and viability monitoring

(Kurkchubasche *et al.* 1998; Oxley *et al.* 2006). TER as a measure of paracellular permeability is important for determining epithelial integrity *in vitro*. The TEP and SCC are measurements of potential difference and net ion flux, respectively (Loretz 1995). Other methods for studying the intestinal epithelium *in vitro* such as the gut sac and everted gut sac methods or enterocyte suspensions lack the possibility for concurrent viability measurements. Apart from electrical parameters, other tests of tissue integrity were used, such as measurements of paracellular permeability with ^{14}C-labeled mannitol which shows if the epithelium is damaged (Paper I) and active transport of nutrients, confirming a metabolically active epithelium (Paper II).

One major difference between the intestine *in vivo* and *in vitro* is the blood circulation. Instead of blood circulation for gas and nutrient exchange in the tissues, Ringer buffer is used to bathe the tissue surface. This can possibly lead to ischemia and damage in the deep tissues. In order to improve gas transport, the diffusion distance is minimized by removal of the serosal layer (Papers III, IV and V). *In vivo*, the transfer of molecules across the epithelium occurs only over the short distance from the lumen to the circulation. Removal of the serosal layer in jejunal segments from rat, increase the transport rate of several marker molecules, demonstrating that the serosal layer can constitute a transport barrier *in vitro* (Hägg 2000). *In vivo*, the serosal layer should not decrease the permeability for most substances, as they reach the venous circulation after epithelial passage. Removal of the serosal layer may thus better represent the *in vivo* situation, by making the diffusion distance shorter. In the Ussing chambers, the tissue is placed under pressure around the margins of the exposed epithelium, effectively closing the vessels outside the exposure window of the Ussing chambers. As the small vessels are aligned perpendicular to the intestine to collect at the mesenteric borders, the venules may or may not be closed depending on the location of the mesenteric border in the Ussing chamber window. It is possible that removing the serosal layer increase the permeability for some substances by disrupting blood vessels in the intestinal segments, allowing substances to escape the blood vessels into the serosal Ringer solution more rapidly, and reducing the risk of trapping substances in veins closed by the chamber edges. The serosal Ringer can thus be considered an extension of the intestinal circulation, as substances reach this half-chamber after crossing the epithelium.

For assessments of nutrient transport in the Ussing chambers, several developmental steps had to be made. Amino acids are hydrophilic molecules which make them convenient to work with in the aqueous solution of the Ringer buffer. Free fatty acids (FFA), on the other hand, are lipophilic substances with low solubility in Ringer. To dissolve FFA in Ringer, micelle formation with

the bile salt taurocholate (TC) was chosen as the most *in vivo*-like means of FFA administration (Olsen 1997). TC micelle formation with the fatty acid 16:0 required long sonication times, whereas the unsaturated fatty acids more easily formed micelles. This difference in physical properties can result in different concentrations of dissolved FFA reaching the epithelium, and may thus induce a factor which is difficult to control (Oxley *et al.* 2006). The same difference should apply also to the *in vivo* situation. During the establishment of the method, the effect of TC on epithelial integrity and viability was tested. It was noted that the tissue viability as measured by TEP and SCC markedly dropped at concentrations above 15 mM, whereas 10 mM was shown to produce stable viability during 90 minutes. As the epithelial polarity is preserved in the Ussing chamber, the epithelium was only exposed to TC from the mucosal side, compared with isolated enterocyte suspensions. This is a clear advantage compared with cell suspensions, as isolated enterocytes exposed to 10 mM TC rapidly die (Perez 1999). This indicates that there is a difference in tolerance to TC between the apical and basolateral membranes. This difference could be due to the separation of membrane lipids by TJ between the apical and basolateral membrane, where the apical membrane shows TC tolerance whereas the basolateral membrane is TC sensitive (Zegers and Hoekstra 1998). The use of Ussing chambers for studies of lipid absorption may thus preserve viability better than cell suspensions. Free fatty acids in Ringer solutions with Ca^{2+} and Mg^{2+}may cause precipitation, requiring the use of Ringer free of Ca^{2+} and Mg^{2+}. Ca^{2+} and Mg^{2+} are needed to maintain tissue viability in the Ussing chambers (Björnsson *et al.* 2004). However, when only the mucosal Ringer was Ca^{2+}- and Mg^{2+}-free, the tissue viability was unaffected during the 90 minute experiments (Paper II; Oxley *et al.* 2006). Ussing chambers can thus be a valuable approach for studying lipid epithelial transports in fish (Oxley *et al.* 2006), as also shown for mammals (Charney *et al.* 1998; Shiau 1990; Westergaard and Dietschy 1976).

The Ussing chamber technique was also considered for the study of bacterial translocation across the intestine, as Ussing chambers have been used for measuring translocation of pathogens in mammals (Dickinson *et al.* 1999; Kurkchubasche *et al.* 1998; Velin *et al.* 2004). A sensitive technique for detecting the bacteria after translocation was first developed. The most common technique in mammalian studies is collection of the serosal medium with subsequent nutrient agar plating and colony counting. Using *A. salmonicida* without antibiotic resistance made agar plating and colony counting difficult, as the methodology is not sterile and other species were more numerous in the serosal samples (Paper IV). The plated cultures were often overgrown with many species of bacteria making counts of *A. salmonicida* impossible.

Instead, the bacteria were labeled with fluorescein isothiocyanate (FITC), and the fluorescence of the translocated bacteria was measured (Paper IV). The fluorescence measurements, using a spectrofluorometer microplate reader made it possible to detect as few as 50 labeled bacterial cells when determined in dilution series. As the entire serosal chamber volume was collected and the cells concentrated, only 50 translocated bacterial cells during the 90 minute experiments were thus needed for positive detection. This makes the method very sensitive, as most (>90%) of the intestinal segments examined had translocation of FITC-labeled bacteria above the detection limit (Papers II, IV and V).

Several studies, including studies within the present thesis (Papers I, IV and V) have noted a discrepancy between hydrophilic marker molecule diffusion and electrical ion diffusion (electrical conductivity). This was in the present studies measured as TER *i.e.* the inverted conductance. Marker molecule diffusion could increase while ion permeability decreased, or vice versa. This could be explained by small ion-selective pores carrying ions during the short electric pulses of conductance measurements and larger, but much less abundant, pores for larger molecules (Van Itallie and Anderson 2004). The small pores would thus be the major determining factor for conductivity whereas the larger pores would be more important for marker molecule permeability. With separate regulation of the pore populations, the observed discrepancy would be explained. Another factor which may differentially affect resistance and permeability is mucus secretion. The electrical resistance increases with increasing mucus secretion in the chinook salmon (*Oncorhynchus tshawytscha*) (Schep *et al.* 1998) and rainbow trout (Paper V). It is possible that the paracellular permeability for mannitol is affected differently by the mucus than the electrical resistance; an effect that could explain the seemingly paradoxical discrepancy between some resistance and permeability measurements.

Bacterial Translocation - How and Why

The question of the major infection route for *A. salmonicida* in salmonids has been addressed by researchers using several different techniques, but the subject has not been satisfactory settled. Experimental infection have shown that disruption of the integument by artificial wounding can increase the risk for systemic infections and mortality in Atlantic salmon (Svendsen and Bøgwald 1997). Other studies suggest that the gills may be an infection route (Ellis 1997; Inglis *et al.* 1993). Several studies have isolated *A. salmonicida* from the intestinal lumen of salmonids (Cipriano *et al.* 1997; Hiney *et al.* 1994; Markwardt

and Klontz 1989), suggesting that the pathogen can reach and colonize the intestine. Infections through the oral route have been demonstrated (Cipriano and Bullock 2001; Rose *et al.* 1989), whereas other studies have reported resistance against orally induced infections (Perez *et al.* 1996; Tatner *et al.* 1984). With the bacteria capable of colonizing the intestinal tract, the question then arises of whether it can cross the intestinal barrier and infect the host. The *in vitro* studies performed with live *A. salmonicida* in the present thesis show that translocation across the barrier do occur in the isolated metabolically active and viable intestine (Papers II, IV and V).

Why does translocation occur?

Why do bacteria cross the multiple defense barriers in the salmonid intestinal wall? This issue can be discussed from two different perspectives: the bacterial and the host. For the bacteria, the evolutionary benefit for increased infectivity is the possibility to multiply to large numbers, in order to maximize spreading to new hosts, by using host nutrients. For the host, the need for specific immune defenses against pathogens may require selective sampling of gut microbes, as is known in mammals (Acheson and Luccioli 2004; Kucharzik *et al.* 2000). However, the antigen sampling function is at the same time used by some pathogenic bacteria to cross the epithelium. In order to reach the systemic circulation and cause infection, the bacteria must survive the host mucosal immune system which normally neutralizes translocated bacteria (Cossart and Sansonetti 2004). Avoiding macrophage phagocytosis is something that *A. salmonicida* has been shown capable of (Burr *et al.* 2005).

A proposed model of A. salmonicida translocation

A. salmonicida virulence factors (Figure 4) include several functions that may be required for passing the intestinal barriers. Virulence by *A. salmonicida* after IP injection at least partly require type IV pili (Masada *et al.* 2002). This is the same type of pili that promote adhesion to intestinal epithelial cells by other *Aeromonas* species, as shown in mammals (Kirov *et al.* 1999). It is thus possible that the type IV pili are used by *A. salmonicida* for attachment to the epithelial BBM (Figure 5.1), a process thought to be important for virulence among intestinal pathogenic bacteria in mammals (Berg 1995). There are high concentrations of commensal bacteria attached to the epithelial cells, both to the BBM microvilli tips and between microvilli, in salmonids (Ringø *et al.* 2001; Ringø *et al.* 2003), indicating that bacteria are able to attach to the BBM. If *A. salmonicida* are able to multiply and form BBM-attached colonies, secreted exotoxins could subsequently cause damage of the epithelium. Electron micrographs

of epithelium exposed to *A. salmonicida* for 90 minutes *in vitro* indicate that enterocytes of the anterior region of the Atlantic salmon intestine can become detached from the epithelial sheet (Paper III). Gaps in the epithelium caused by the shedding of enterocytes could thus be a route through the intestinal epithelium (Figure 5.3). Such damage to the epithelium should, if widespread, be possible to detect as increased permeability using TER and/or permeability markers. However, extensive epithelial damage caused by the mammalian pathogen *Salmonella typhimurium* has not been detected as decreased TER in rat intestinal segments mounted in Ussing chambers (Kurkchubasche *et al.* 1998), indicating that the resistance can be upheld during bacteria-induced epithelial damage. In the present studies exposing the epithelium to *A. salmonicida* (Papers III, IV and V), the permeability did not noticeably increase. This suggests that the damaging effect is local. Permeability-increasing damage may also be masked by a general decrease in permeability by secretion of mucus, a process used by the salmonid epithelium as a response to *A. salmonicida* (Lødemel *et al.* 2001), and shown to cause a decreased permeability as measured by electrical resistance in salmonids (Schep *et al.* 1997; Schep *et al.* 1998).

The damage to the intestinal epithelium (Paper III), has been suggested to be caused by extracellular products. The extracellular proteases may be able to affect the cell-to-cell adhesive junctions. Shedding of enterocytes may also be caused by apoptosis or necrosis after bacterial entry into the epithelial cells (Mayhew *et al.* 1999), as is seen in *Salmonella*-infected intestinal epithelia (Guiney 2005). The lack of effect on the paracellular permeability, TER and P_{app} after ECP exposure (Paper V) may indicate that the damage to the epithelium is caused by the bacterial cells (Paper III) instead of the suggested extracellular toxins. It is also possible that the histological damages observed are isolated events that are too rare to affect the total paracellular permeability.

Extracellular products from *A. salmonicida* was found to reduce the transepithelial potential and the short-circuit current of intestinal segments (Paper V), indicating that extracellular products affect the ion transporting function of the epithelium. The effect could be inhibition of ion channels or pumps, or a general decrease in the energy metabolism of the cells. It is concluded that none of the major enzymes known to be secreted from *A. salmonicida* (described above) caused the effect, as removal of molecules larger than 10 kDa by filtration, failed to remove the effect. Unknown smaller substances are thus suggested to cause the observed decrease in active transporting mechanisms of the enterocytes. Whether the effect is harmful to the epithelium, and constitutes a bacterial strategy for affecting the epithelial barrier, is unknown. It is possible that the ECP can the energy metabolism of the cell that may lead to necrosis and thus possibly the shedding of cells (Paper III; Mayhew *et al.* 1999).

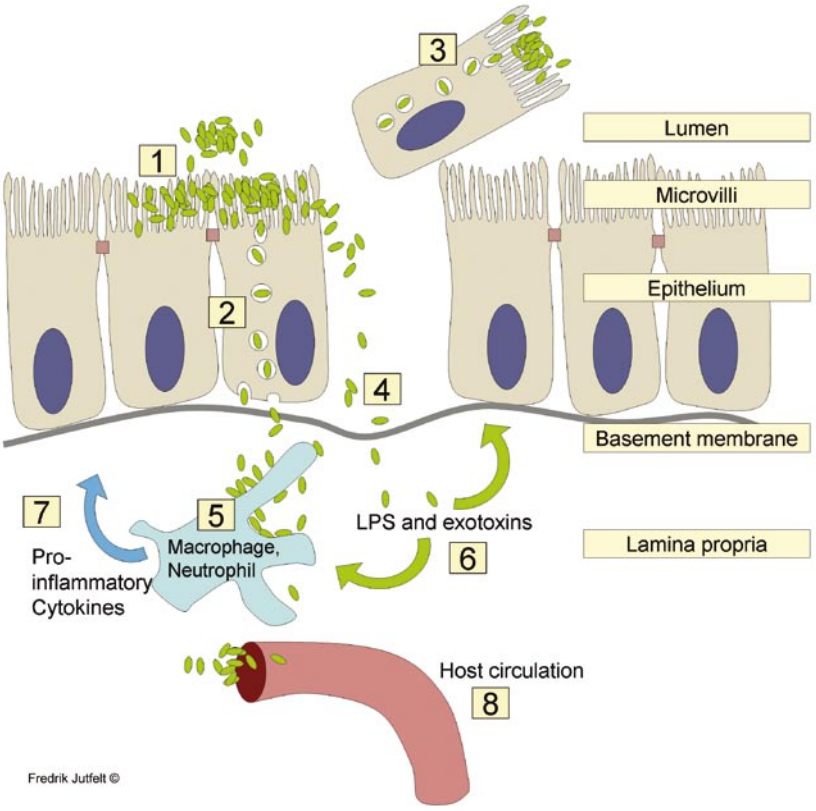

Figure 5.
Suggested model for translocation of the *A. salmonicida* across the intestinal epithelium. Demonstrated and possible mechanisms:
1. Attachment and overgrowth of A. salomonicida (Ringø 2003), possibly involving type IV pili (Masada 2002) and the S-layer (Garduno 2000). Exotoxins negatively affect the transepithelial ion fluxes (Paper V).
2. Transcellular transport indicated by different rates and influences on paracellular permeability and bacterial/microsphere translocation rates (Papers II, IV and V). Histological evidence of transcytosis of bacteria (Rombout 1998). TTSS may increase transcytosis (Cossart 2004) or cause apoptosis (Guiney 2005).
3. Shedding of enterocytes, possibly creating holes in the epithelium (Paper III). Bacterial extracellular proteases (Ellis 1991) may be involved in the detachment. Apoptotic or necrotic processes may also cause the shedding (Dickinson 1999; Mayhew 1999).
4. Entry through damaged epithelium occurs in mammals (Kurkchubache 1998), and possibly in salmonids (Paper II).
5. Survival of bacteria, despite macrophages in the local immune system by TTSS (Burr 2005) and the S-layer (Garduno 2000).
6. LPS and other bacterial substances may be detected by Toll-like receptors of the epithelial/ immune cells that release pro-inflammatory cytokines (Niklasson 2006) leading to decreased barrier function (Paper V). Tissue damage by exotoxins (Popoff 2005; Ellis 1991).
7. Locally released cytokines may cause inflammation and possibly increase bacterial translocation (paper V). Proinflammatory cytokines may also induce apoptosis (Guiney 2005).
8. Entry into the gut vascular system, logically inferred by the rapid appearance in the serosal fluid (papers II, IV and V). During systemic infection, survival and virulence of A. salmonicida require TTSS (Burr 2005). Host symptoms and death caused by extracellular toxins (Ellis 1991).

If disruption of the epithelium is a mechanism for bacterial translocation of *A. salmonicida*, it is probably not the only mechanism, as transcytosis of *A. salmonicida* across the intestinal epithelium is also a highly likely route (Figure 5.2). Phagocytotic entry into the epithelial cells by *S. typhimurium* and the subsequent disruption of the rat epithelium has been shown (Kurkchubasche *et al.* 1998), indicating that the two pathways are not mutually exclusive. Numerous data (Papers II, IV and V) show translocation rates independent of the P_{app} and TER *i.e.* the regional pattern is different and the responses to factors can differ between these parameters. For example, the posterior intestinal region has twice the TER and half the P_{app} of the anterior region, whereas the translocation rate for bacteria was similar or higher in the posterior region. If paracellular diffusion (through TJ or damaged epithelia) was the main pathway for translocation, the anterior intestine would be expected to have higher translocation rates than the posterior region. The posterior intestinal epithelium of salmonids has been suggested to have higher phagocytotic and transcytotic activity than the anterior region (Hart *et al.* 1988; Rombout 1998). This indicates that the relatively high bacterial translocation rate seen in the posterior region is governed through transcytosis.

Several bacterial pathogens in mammals use TTSS for the passage of the intestinal epithelium. After the discovery of a functional TTSS in *A. salmonicida*, it has been speculated that the TTSS is used during the epithelial translocation in salmonids (Paper V). Heat-inactivation (42ºC) of *A. salmonicida* caused a reduced ability to translocate. TTSS requires the bacteria to detect and respond to host cell contact, indicating that the secretion is actively controlled by the bacteria. As TTSS require active control over secretion (Hueck 1998), it may not function after heat-inactivation. One or several of the many proteins needed for TTSS function may thus become dysfunctional. It is also possible that some other virulence mechanisms used for translocation become damaged by the heat-inactivation, disabling the efficient translocation seen for the live bacteria. It is thus possible that TTSS is required for the initial passing of the host epithelium. The subsequent stage of surviving the host mucosal immune system is likely to be TTSS-dependent, as inhibition of macrophage phagocytosis of *A. salmonicida* requires TTSS (Burr *et al.* 2005).

In mammals, LPS can have adverse effects on the epithelium. The LPS detecting function in vertebrates has evolved to produce early and rapid immunological response to infection by gram-negative bacteria. One effect of serosal LPS exposure of the mammalian epithelium is increased paracellular permeability as well as increased translocation of macromolecules and bacteria (Dickinson *et al.* 1999; Osman *et al.* 1998). In rainbow trout, serosal LPS administration *in vitro* caused an increased transepithelial transport of bacterial-

sized plastic microspheres (Paper V). LPS lacks direct toxicity, indicating that the effect is mediated through the host immune system (Figure 5.5). Indeed, LPS from *A. salmonicida* has been shown to induce systemic cytokine release in mice (Gudmundsdottir and Gudmundsdottir 2001), and in rainbow trout, IP injection of *E. coli* LPS increased TNF-alpha mRNA expression in the intestinal mucosa after 12 hours (Niklasson 2006). TNF-alpha have been shown to increase TJ permeability in the intestinal epithelium in mammals (Schmitz *et al.* 1999). These observations together may suggest that the observed increase in particle transport could be mediated through a local immune system response such as proinflammatory cytokine release also in the trout intestine. The evolutionary rationale for increasing transport of bacteria and particles after LPS detection may be considered counterintuitive, but it is suggested to involve increased antigen sampling for induction of the adaptive immune system. It has also been suggested that the effect is a negative consequence of increased oxygen consumption and/or decreased blood flow in mucosal tissues, causing ischemia and acidosis in the epithelium (Haglund 1994; Salzman *et al.* 1994) or a consequence of loosening of TJ to promote transepithelial migration of neutrophils (Parkos *et al.* 1994; Reaves *et al.* 2001). Endothelial TJ dilate during inflammation to promote immune cell migration into damaged tissue (Tharp 1989). As the epithelial TJ respond to the same dilating signals, such as pro-inflammatory cytokines, the effect on epithelia may be a side-effect from the mechanism aimed at increasing the endothelial permeability. The mechanism for this epithelial increase in paracellular permeability of the mammalian intestine has been suggested to be internalization of TJ proteins into the epithelial cells as a response to proinflammatory cytokines, possibly trough pinocytosis (Chiba *et al.* 2006). These mechanisms have not been investigated in fish, but the increase in particle transport without effects on the paracellular transport (Paper V) indicates increased transcellular transport without changes of the TJ during the short *in vitro* experiments, although in a longer time perspective the permeability could be affected. If LPS is involved in the intestinal translocation of *A. salmonicida*, its role is likely limited to the LPS released after the initial penetration of the epithelium, as only serosal LPS-exposure causes increased transepithelial transport. LPS could thus increase the translocation of more bacteria after the initial bacteria reach the lamina propria (Thomson *et al.* 2001b). When reaching the lamina propria, exotoxin enzymes can digest connective tissue and exotoxin lysins lyses cells. Passage across endothelia into the host circulation may thus be aided by secreted exotoxins. Survival of *A. salmonicida* despite the immune system at all stages of infection is likely dependent on TTSS-mediated secretion of anti-phagocytotic proteins (Burr *et al.* 2005).

In conclusion, *A. salmonicida* expresses several of the known virulence

factors utilized for transepithelial translocation by mammalian gram-negative pathogenic bacteria. In addition, translocation of viable bacteria has been demonstrated in the present thesis. The intestinal tract is thus suggested to be a functional entry point for *A. salmonicida* in salmonids.

The Epithelium and the Parr-Smolt Transformation

Water transport and the parr-smolt transformation

The intestine of salmonids faces a shift in the ingested water salinity at seawater (SW) migration. As the intestine is the major water absorbing organ in SW, the intestine pre-adapts during the parr-smolt transformation (Papers I and II) (Veillette *et al.* 1995). During the parr-smolt transformation, the water transport across the intestine increases, partly due to increased mucosal Na^+,K^+-ATPase activity (Paper I). After SW transfer, the paracellular permeability decrease, as demonstrated by the increased transepithelial electrical resistance (TER) (Paper I) and decreased P_{app} for mannitol (Paper I; Sundell unpublished results). The mechanism is likely to involve tightening of the tight junctions (TJ) (Paper I), possibly by upregulation of TJ claudins (Schneeberger and Lynch 2004; Turner 2000). Due to the increased drinking in SW (Fuentes *et al.* 1996), the intestine may become exposed to more pathogens and harmful substances. The decreased paracellular permeability could thus be a mechanism for reducing the transepithelial transfer of unwanted substances (Papers I and II). The tightening of TJ could also have the osmoregulatory function of enhancing the osmotic gradient from the lumen to the basolateral space, as diffusive leakage of Na^+ from the basolateral spaces through TJ could be decreased. The opposite situation with increased water excretion as a result of increased paracellular permeability with concomitant Na^+ leakage has been shown in mammals (Holtug *et al.* 1996). However, the increased serosa-negative potential in salmonids acclimated to SW may indicate that the Na^+ leakage is higher in SW (Collie 1985; Loretz 1995).

The decrease in paracellular water transport with simultaneous increased water transport suggests that the route of water transport is shifted from a paracellular route in FW (Collie 1985; Loretz 1995) to a predominantly transcellular route in SW (Paper I). This view has support in the increased HUFA content of the salmonid BBM after SW transfer (Leray *et al.* 1984). Increased HUFA content of membranes leads to increased fluidity known to cause increased transmembrane diffusion rates of water (Lande *et al.* 1995). It is thus suggested that the route of water transport is shifted to a higher degree

of transcellular transport by the tightening of the TJ and actively increasing HUFA brush border membrane contents, and that the purpose is decreasing uptake of harmful agents.

Higher transcellular water permeability could also be caused by an upregulation of aquaporins (AQP) in the enterocyte membranes (Matsuzaki *et al.* 2004). AQPs incorporated into the apical and/or the basolateral membranes of the enterocytes could potentially increase the water transport (Aoki *et al.* 2003). The functions of eel intestinal AQPs have been suggested to include epithelial water uptake that may be regulated for the purpose of euryhaline osmoregulation (Aoki *et al.* 2003). When comparing seawater and freshwater acclimated eels, little change in AQP3 expression could be detected (Lignot *et al.* 2002). The AQP1 homologue in Japanese eel, however, was present in the gastrointestinal epithelium (Martinez *et al.* 2005a; b), and was suggested to be upregulated in the intestine in SW-adapted eels indicating a role for AQP1 in the SW-induced increase in water uptake. In esophageal epithelia of the European eel, AQP1 expression was induced by cortisol (Martinez *et al.* 2005a; b). As cortisol is a well known SW-adapting hormone, this cortisol-dependent upregulation of AQP can be suggested to be of importance for the hypoosmoregulatory ability (Martinez *et al.* 2005b). These results together, suggest that euryhaline fish may use AQPs during SW acclimation to improve the transcellular water permeability, and thus to increase water uptake capacity. Research on AQPs in the fish intestine, and its role in osmoregulation, is in the initial stages. Eels and other species of fish may also express other still unknown aquaporine isoforms that may be important for intestinal water uptake and osmoregulation. In salmonids, intestinal aquaporins could potentially be upregulated during the parr-smolt transformation to accommodate the shift from paracellular to transcellular water transport as suggested in Paper I. However, aquaporin expression in the salmonid intestinal epithelium has not yet been described.

Barrier function and the parr-smolt transformation

Cortisol has many functions, some of which influence intestinal physiology. The physiological changes occurring during parr-smolt transformation are in part orchestrated by cortisol (McCormick 2001; McCormick and Saunders 1987). During parr-smolt transformation, the Atlantic salmon plasma levels of cortisol greatly increase during several weeks (Papers I and II; McCormick and Saunders 1987). Besides being a developmental hormone, cortisol is also secreted during stress (Barton and Iwama 1991; Wendelaar Bonga 1997) and may stay slightly elevated during prolonged periods of stress (Wendelaar Bonga 1997). The developmental effects on the intestinal functions by cortisol may

differ from the stress-related effects. It is probable that the temporal pattern of cortisol secretion during stress differs from the developmental cortisol peak, and that this difference determines the effect on the target organs. It can be speculated that the low variance in cortisol plasma levels during the parr smolt transformation (Papers I and II) is an indication of relatively stable levels over the time period of hours and days. The plasma levels of cortisol after acute stress peak in the timeframe of minutes and return to low levels in hours, whereas during chronic stress, the plasma levels are low but constantly elevated (Barton and Iwama 1991; Wendelaar Bonga 1997). Experimentally increased plasma levels of cortisol after IP injection of slow-release cortisol implants in rainbow trout (Veillette *et al.* 1995) negatively affect the intestinal epithelial barrier function, as measured by TER and P_{app} for mannitol (Lund 1997; Sundell unpublished data). In addition, acute stress, with the associated short duration cortisol surge, also increases the paracellular permeability in Atlantic salmon and rainbow trout intestines (Olsen *et al.* 2002; 2005). Hence, both long and short duration of elevated cortisol plasma levels may disturb epithelial functions. It is possible that the cortisol peak during parr-smolt transformation (Papers I and II) and the additional peak after SW migration (Paper I; McCormick 2001; McCormick and Saunders 1987) may compromise the epithelial integrity. In fact, the bacterial translocation across the intestinal wall of Atlantic salmon was shown to increase during the parr-smolt transformation (Paper II), possibly due to disturbed epithelial function. During the parr-smolt transformation of the Atlantic salmon and the subsequent SW transfer, the disease resistance have been reported to decrease (Inglis *et al.* 1993; Smith 1993). Cortisol may thus affect the epithelial barrier function which, in turn, could lead to increased disease susceptibility (Paper II).

Increased disease susceptibility during the parr-smolt transformation could also be a result of immune suppression by cortisol (Schreck 1996). The immune system is modulated by cortisol. Acute stress redistributes lymphocytes to the periphery, whereas chronic stress can decrease the immune competence (Tort *et al.* 2004). It is thus possible that the long term increase in cortisol plasma levels during the parr-smolt transformation can decrease immune functions (Yada and Nakanishi 2002). During the parr-smolt transformation of coho salmon (*Oncorhynchus kisutch*), lymphocyte concentrations in blood and spleen decreased (Maule *et al.* 1987). Cortisol-releasing implants produced similar changes, indicating that the immune suppression during parr-smolt transformation was cortisol-incuced (Maule *et al.* 1987). In Atlantic salmon however, parr-smolt transformation, with the associated cortisol peak, did not reduce plasma lysozyme activity or leukocyte activity (Olsen *et al.* 1993). This may indicate species or methodological differences.

Dietary Vegetable Lipids and Epithelial Nutrient Uptake

Barrier function and vegetable lipids

Vegetable lipids in the diet of salmonids in aquaculture can function as partial replacement for fish oils (Bendiksen *et al.* 2003; Björnsson *et al.* 2004). However, the intestinal epithelium, as the first tissue to come in contact with the lipids, may be affected by them. Arctic charr (*Salvelinus alpinus*) fed linseed oil showed accumulation of lipid droplets in the enterocytes (Olsen 1999). Similarly, lipid droplets were found in rainbow trout (Caballero *et al.* 2002), as well as in gilthead seabream (*Sparus aurata*) (Caballero *et al.* 2006) after a vegetable lipid-containing diet, indicating that the effect is wide-spread across carnivorous fish fed vegetable lipids. A likely cause for the lipid droplets is higher rate of lipid uptake than export in the enterocytes. The mechanism behind the lipid droplet accumulation in the enterocytes could be dysfunctional or inadequate lipoprotein export, suggested to be caused by lack of dietary polar lipids (Olsen *et al.* 1999; Salhi *et al.* 1999). The lipid droplets can grow large enough to damage the enterocytes, and the epithelial ultrastructure can become disrupted (Olsen 1999). Physiologically, the epithelial barrier function could be predicted to become negatively affected by the disruption of enterocytes (Björnsson *et al.* 2004; Olsen *et al.* 2000). The total paracellular permeability was only slightly affected by three vegetable lipid diets compared to a fish oil based diet (Figure 6). The epithelial damage as assessed by electron microscopy (EM) (Björnsson *et al.* 2004; Olsen 1999) appeared greater than the minor effect detected on the paracellular permeability. One reason could be that during the long period of exposure to the experimental diets, other epithelial barrier functions

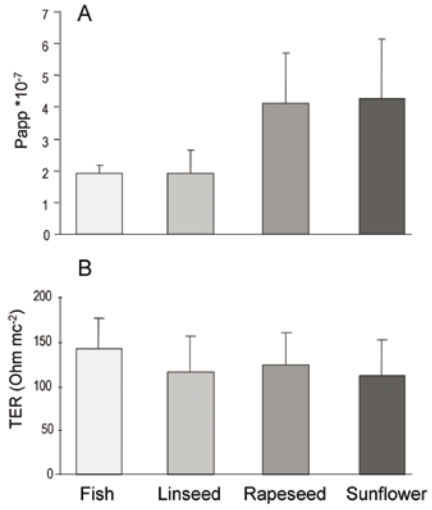

Figure 6.
A. Paracellular permeability for mannitol and B. transepithelial electrical resistance in the anterior region of the rainbow trout intestine fed diets containing fish oil, linseed oil, rapeseed oil or sunflower oil for 10 weeks. Mean ± SEM, (n=8).

successfully counteract the adverse effects. The mechanisms could be increased mucus secretion (Schep *et al.* 1997) or lowering of paracellular permeability by upregulation of TJ proteins (Van Itallie and Anderson 2004). The disruption caused by the lipid droplets may also be occurrences too localized to affect the total transepithelial permeability.

With the risk of a structurally damaged epithelium, as shown by EM in previous studies, and physiologically slightly higher paracellular permeability (Figure 6), the bacterial translocation could be expected to increase by dietary vegetable lipids. However, the bacterial translocation was instead significantly lower in the group fed dietary sunflower oil (SO) compared with the control diet (Paper II). Besides droplet formation, other possibly adverse effects of dietary vegetable lipids have been shown. The incorporation of vegetable fatty acids into polar lipids (Björnsson *et al.* 2004) could affect membrane fluidity (Lande *et al.* 1995) as well as function of the enterocyte membrane-bound proteins (Di Costanzo *et al.* 1983). Vegetable lipid fatty acids incorporated into fish macrophages can decrease the phagocytotic activity *in vitro* (Montero *et al.* 2003). It is thus possible that the observed decrease in bacterial translocation in the SO-fed fish is related to altered membrane function, which may result in reduced transcytosis. It seems likely then, that the disturbed epithelium does not increase the bacterial translocation through simple diffusion across the damaged areas. Instead, phagocytotic translocation is reduced trough alterations of the membrane fluidity or the function of membrane-bound proteins. It is not known whether this effect actually reduces disease sensitivity in fish fed vegetable lipids, but in bacterial disease challenge tests, fish fed dietary vegetable lipids had as low (Bransden *et al.* 2003) or even lower mortality compared with fish fed marine lipid control diets (Bransden *et al.* 2003; Lødemel *et al.* 2001).

In mammals, fatty acid deficiency can increase the intestinal translocation of bacteria, which may affect disease susceptibility (Barton *et al.* 1992). Different dietary fatty acid profiles can also directly suppress immune functions in humans (de Pablo *et al.* 2002; Lee *et al.* 2003), and may also thus decrease host resistance against pathogens (de Pablo *et al.* 2002). In seabream, vegetable lipids in the diet can reduce the phagocytotis of pathogenic bacteria by macrophages (Montero *et al.* 2003), suggesting that dietary lipids can modulate the immune defense also in fish.

Vegetable lipid diets as stressors

Sunflower oil as the major lipid source in the diet fed to Atlantic salmon caused long-lasting stress as measured by plasma cortisol levels (Paper II). The cortisol levels were significantly elevated already before the onset of smoltification and continued to stay significantly above the control group throughout the parr-

smolt transformation (Figure 7). Chronically elevated cortisol levels by dietary vegetable lipids have not been reported before. However, vegetable lipids increased the cortisol response to acute stress in seabream (Montero *et al.* 2003). It is unknown by which mechanism vegetable lipids cause stress. However, as dietary vegetable lipids may alter the membrane fluidity of many tissues in carnivorous fish, the cortisol response could be a result of altered membrane function leading to reduced functionality in the affected organ. This could

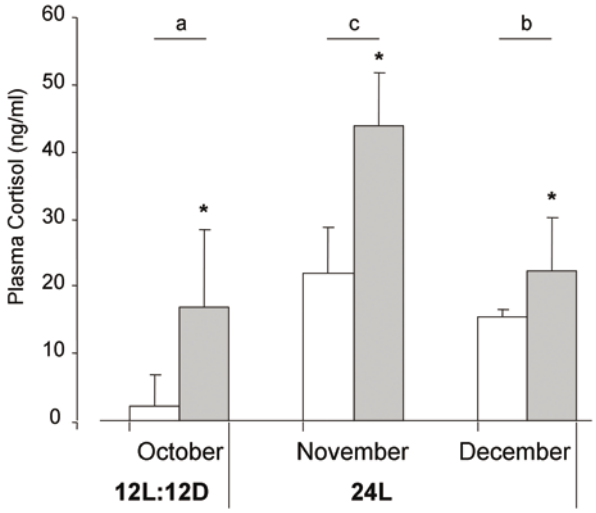

Figure 7.
Plasma levels of cortisol of 0+ age Atlantic salmon fed a fish oil based diet (open bars) or a sunflower oil based diet (filled bars). Sampling was performed during photoperiod-manipulated parr-smolt transformation under ambient temperature conditions. First sampling point was after five weeks on 12L: 12D light regime Oct. 20. After six weeks on 12L: 12D, the fish were subjected to an abrupt change to continuous light (24L). Second sampling was performed after three weeks on 24L Nov. 19 and third sampling was performed after 6 weeks on 24 L Dec. 10. Data are shown as means +-SEM (n=9). Different letters above data points indicate significant differences in time ($p<0.05$). Asterisks denotes significant differences ($p<0.05$) between FO and SO-fed fish.

induce cortisol release through one or several intermediate steps. Suppression of the immune system in carnivorous fish fed vegetable lipids has been shown (Montero *et al.* 2003) and suggested to be caused partly by altered membrane function, possibly by affecting phagocytotic processes, antibody binding or cytokine production (Balfry and Higgs 2001). Vegetable lipids can also reduce the availability for eicosanoid precursors, leading to dysfunctional eicosanoid

signaling (Balfry and Higgs 2001; Dosanjh *et al.* 1998; Tocher *et al.* 2000), which may stress the fish.

Elevated plasma cortisol levels during a prolonged time can be considered to be an indicator for chronic stress in mammals (Möstl and Palme 2002; Vanitallie 2002) as well as in fish (Barton and Iwama 1991; Flik *et al.* 2006; Wendelaar Bonga 1997). Long-lasting stress in fish have several adverse effects on the animal. The endocrine signaling during acute stress can induce energy mobilization as well as immune system modification and suppression (Barton and Iwama 1991; Tort *et al.* 2004; Wendelaar Bonga 1997). During influence of a long-term stressor, the fish immune system may become suppressed, increasing disease sensitivity and reducing fish growth. The reproductive capacity can also decrease (Mommsen *et al.* 1999; Wendelaar Bonga 1997). In mammals, the effects on the intestine includes decreased intestinal barrier function, shown as increased paracellular permeability and increased bacterial translocation (Groot *et al.* 2000; Santos *et al.* 2001; Söderholm and Perdue 2001; Velin *et al.* 2004). Both acute stress (Olsen *et al.* 2002; 2005) and chronically elevated cortisol levels (Lund 1997; Sundell, unpublished data) cause increased paracellular permeability in salmonids. In the present thesis, chronically elevated cortisol levels were not found to decrease barrier function (Paper II). The mechanism is suggested to be a reduction in phagocytotic activity caused by alterations in the membrane lipids, in combination with dietary lipids, as discussed above. As long-term elevation of cortisol levels is a sign of chronic stress, it is suggested that stress levels should be considered when investigating new diets in fish.

Nutrient uptake and vegetable lipids

The transepithelial uptake of two amino acids (AA), two fatty acids (FA) and glucose was assessed in fish fed either a sunflower oil diet or a control diet. The uptake rates of glucose and AAs were roughly one order of magnitude higher in the anterior than the posterior region of the intestine (Paper II). Fatty acid uptake rates also differed between regions with the same pattern, but to a lesser extent. These results support the view that the anterior region is the primary site for nutrient uptake in salmonids, possibly together with the pyloric caeca (Buddington and Diamond 1987), in salmonids. Within the anterior region, transport of the AA L-leucine was consistently twice the rate of L-proline uptake. This difference is probably due to higher transepithelial transporter capacity for L-leucine than for L-proline. One possible explanation to this observation is a difference in AA composition of the normal diet. Nutrient transporter capacity is suggested to be adapted to the normal diet as expressing redundant nutrient transporters carries an evolutionary cost (Diamond 1991). A study examining AA concentrations in the lumen of the anterior intestine

of salmonids fed a normal commercial diet showed the predicted difference, L-leucine concentrations were twice as high as L-proline concentrations (Berge *et al.* 2004).

With the change of habitat upon SW migration, a change in diet follows. Whereas the freshwater diet consist mainly of insects and crustaceans, the marine diet is based mainly on crustaceans and fish (Bell *et al.* 1994). The FA composition of the two diets greatly differs, with the limnic diet being rich in 18:2(n-6) and 18:3(n-3) and the marine diet richer in long chain highly unsaturated FAs (HUFA), typically including 20:5(n-3) and 22:6(n-3). This shift in diet is subsequently reflected by the muscle tissue composition (Bell *et al.* 2002; Bell *et al.* 2001; Tocher *et al.* 2000). Since the FA composition of the membrane polar lipids affect the membrane fluidity (Di Costanzo *et al.* 1983), the shift in diet may thus affect the membrane functions and possibly the function of the entire organ (Tocher 2003). The first organ to come into contact with these lipids is the GI tract. The phospholipid FA profile of the anterior and posterior intestine of salmonids has been shown to change four weeks after a shift in dietary FA profile (Björnsson *et al.* 2004).

Amino acid transport (L-leucine and L-proline) was positively affected by the sunflower oil (SO) based diet before and during the parr-smolt transformation (Paper II). By the end of the parr-smolt transformation, the effect was reversed with the fish oil fed fish showing the greater transport rates. This shift corresponds in time and developmental stage to the expected dietary change at SW migration (Bell *et al.* 1994). It seems, thus, as the SO diet increase the amino acid uptake during the normal fresh water stages whereas the marine fish oil increase the transport rate at, or slightly before, SW migration. It can thus be speculated that vegetable lipid diets with similar FA composition as the limnic diet, may improve nutrient uptake during the salmonid parr stage, and may ultimately improve the growth and health of the fish. In fact, positive effects on growth (Torstensen *et al.* 2005) and osmoregulation by vegetable lipids in salmonid parr have been shown (Bell *et al.* 1997; Tocher *et al.* 2000).

Relevance to Aquaculture and the Environment

Out-of-season parr-smolt transformation

Atlantic salmon grow rapidly after SW transfer (Handeland and Stefansson 2001; Hansen 1998). In the wild, the fish stay in the parr stage for one to several years, whereas in traditional salmon aquaculture the parr-smolt transformation occurs after one year (Hansen 1998). A relatively new practice is to induce parr-smolt transformation in underyearling fish by photoperiod manipulation which

mimics a short winter period followed by spring, during which the fish start the parr-smolt transformation (Papers I and II; Björnsson *et al.* 2000). The practice has been successful in preparing the fish for SW transfer. The out of season parr-smolt transformation induces typical changes in condition factor, salinity tolerance and endocrine profiles (Papers I and II; Berge *et al.* 1995; Björnsson *et al.* 2000). In the present thesis, the two aquaculture strategies have been compared. Previous studies on physiological alterations, such as SW tolerance and gill Na^+,K^+-ATPase activity, occurring in underyearling smolts were shown to be comparable to the natural parr-smolt transformation (Paper I; Handeland and Stefansson 2001). In line with this, all physiological parameters (growth hormone and cortisol profiles, gill and intestinal Na^+,K^+-ATPase activity, water transport and intestinal permeability) assessed in the present thesis suggests that the physiological processes during light-induced out-of-season parr-smolt transformation are comparable with the natural parr-smolt transformation (Papers I and II). This strengthens the view that out-of-season Atlantic salmon parr-smolt transformation induces the desired hypoosmoregulatory changes needed for early SW transfer in modern Atlantic salmon aquaculture.

Vegetable lipid diets

With the increasing use of vegetable lipids in feed production for aquaculture, there is a need for investigating how this affects the fish. The increase in plasma cortisol levels by a SO-containing diet could be an indication of chronic stress (Paper II). Chronic stress and increased cortisol levels reduce immune functions in salmonids (Maule *et al.* 1987; Wendelaar Bonga 1997). Together, these observations suggest that the effects of new dietary ingredients on salmonid stress levels should be carefully monitored, in order to maintain the health and welfare of farmed fish.

During the freshwater stage of the Atlantic salmon life, their limnic diet is rich in 18:2(n-6) and 18:3(n-3), *i.e.* similar to the FA profiles of many vegetable lipid diets (Bell *et al.* 1994). The rationale for feeding parr marine lipids can thus be questioned (Bendiksen *et al.* 2003; Tocher *et al.* 2000). Indeed, a sunflower oil-containing diet reduced bacterial translocation and increased AA uptake rates (Paper II), indicating possible mechanisms that may promote disease resistance and growth. While growth rates are not increased by vegetable lipid diets in relatively short term studies (12 weeks) (Paper II; Jutfelt, unpublished results), disease susceptibility can be reduced by vegetable lipids in the feed (Lødemel *et al.* 2001). Salmonids transferred to SW maintain health and growth rates also in SW, even when fed high inclusion levels of blended vegetable lipids (Dosanjh *et al.* 1998; Torstensen *et al.* 2005). However, there is concern for losing some of the human health benefits from marine lipids (Bell *et al.* 2002; Sargent 1997).

One suggested solution is a finishing "washing out" diet with high inclusion of marine lipids (Bell *et al.* 2003). This strategy combines the environmental benefits of reducing the use of marine lipids while still producing fish with the health benefits of marine lipids (Torstensen *et al.* 2004).

Oral vaccines

The finding that bacteria and particles are translocated across the entire intestinal wall (Papers II, IV and V) lends credibility to the prospect of developing oral vaccines for fish. Antigens, in particles or resistant to digestion by other means (Ellis 1998; Vandenberg 2004), could thus be introduced orally and taken up by the anterior and posterior intestine. The local intestinal immune system and other parts of the fish immune system would be exposed to the antigen, which may trigger the adaptive immune system (Dalmo and Bogwald 1996; Ellis 1997).

Environmental aspects

In this thesis, several results indicate that a shift towards greater inclusion of vegetable lipids is possible. As such a shift decreases the need for fishing of wild feed-fish species, a more sustainable aquaculture can be obtained (Naylor *et al.* 2000). Intestinal functions were shown to be maintained, although monitoring of fish stress and health should be made (Paper II).

Conclusions

The intestine is an important organ for growth and health of the fish. At the intestinal epithelium, nutrients, osmolytes and water are absorbed, while adverse agents are excluded. These multiple functions must occur throughout the salmonid life cycle, despite dramatic alterations in diet and the external milieu. However, several external and internal factors can affect both the barrier function and the absorptive function. The factors examined in the present thesis, and their effects on the barrier function, are summarized in Figure 8.

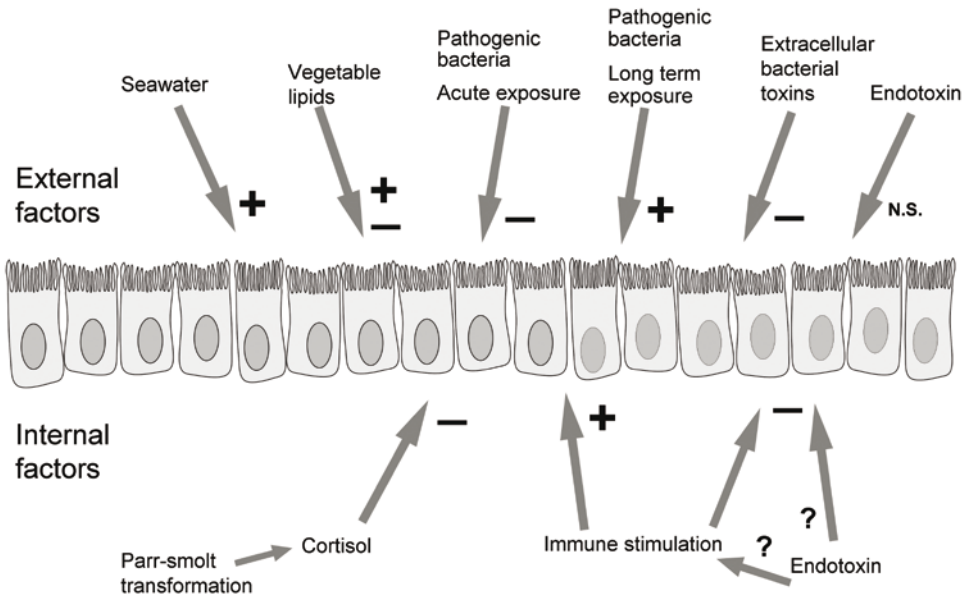

Figure 8.
Summary of the effects on the epithelial barrier function by the major external and internal factors tested in the thesis. The symbol + represents an increase in barrier function (either paracellular permeability or translocation of bacteria and particles) and the symbol - represents decreased barrier function. Question marks denote intermediate steps. N.S. = not significant. See text for discussion.

The pathogenic bacterium *Aeromonas salmonicida* ssp. *salmonicida* was shown to translocate across the intestinal epithelium. Earlier studies have reported that *A. salmonicida* can be isolated from the intestinal lumen of salmonids. The inference of these data is that the intestine comprises a functional bacterial port for host entry. The intestinal route of entry is thus suggested to be of importance when investigating lateral transmission of bacterial diseases in fish. The mechanisms of transepithelial transfer of *A. salmonicida* were investigated

and it is concluded that the pathogen utilizes several virulence factors for crossing the mucosal barrier. The main route for bacterial translocation is suggested to be transcellular transport, likely through transcytosis which is actively promoted by the bacteria. *A. salmonicida* in the intestinal lumen induce epithelial disruption that may allow access to the lamina propria, which could thus be a second mode of mucosal passage. Knowledge of the mechanisms used for epithelial passage could provide means for preventing the spreading of bacterial diseases in fish.

During the parr-smolt transformation, the Atlantic salmon intestine is modulated for life in seawater. Drinking rate and fluid uptake rate increases, and the route of transepithelial water transport is suggested to be shifted from a predominantly paracellular route to a more transcellular route. This is in agreement with the standing-gradient hypothesis for intestinal water uptake, and transcellular water transport can be increased through membrane fluidity changes or possible aquaporins.

During stages of parr-smolt transformation, bacterial translocation increased. This reduced barrier function coincides with increased disease succeptibility in Atlantic salmon, and may thus indicate a mechanims for the higher succeptibility during smoltification.

Sunflower oil in the feed induced chronically elevated cortisol levels. This is of concern for the fish health and well-being, as the fish immune system can be suppressed by both chronically elevated cortisol levels and by dietary vegetable lipids.

Vegetable lipids in the diets of salmonids can influence the epithelial morphology as well as function. Despite conspicuous morphological damage to the epithelial cells, epithelial integrity is maintained with only slight increase in paracellular permeability. During the freshwater stage, feeding dietary vegetable lipids may even be beneficial to the fish as the FA profiles of some vegetable oils better resemble that of the natural salmonid parr diet. Indeed, Atlantic salmon parr fed a sunflower oil-containing diet showed increased amino acid uptake rates which may positively affect growth. The mucosal barrier against pathogenic bacteria was also improved, suggesting a mechanism for an improved disease resistance.

In conclusion, the intestinal epithelium, with its important barrier- and transporting function, is actively regulated to maintain its functions. It can, however, be affected by external factors such as salinity, diet and pathogens.

Acknowledgements

I would like to thank the following:

First of all I would like to acknowledge my supervisor Kristina Snuttan Sundell. Thank you for all your support and for always taking your time to help. You have an amazingly positive approach to everything, even my mistakes. Nothing can go wrong now!

Susanne Holmgren, head of the department, for providing the excellent working conditions. And for the fun arguments over that last little literature point. We'll call it a draw.

Stefan Nilson, former head of the department, for being the best possible role model and the most entertaining lecturer.

My Assistant supervisor Thrandur Björnsson for the valuable support and collaboration, and all the fun pub visits abroad.

Erik, for being a true friend. Never forget our first scientific studies! Henke, for being several true friends. Jon, for twa trey url and the best party. Lena, for being one of the boys. Jenny, for allowing me to copy your thesis! Cheers.

Susana, for being an absolutely perfect room mate for me.

The rest of the FEL-group, Ingibjörg, Lisa, Barbro, for fruitful cooperation and for reading my manuscripts. Lars for the best reference of the thesis.

All PhD students at Zoologen for all the fun, you know who you are.

The technical and administrative staff for taking such good care of this place.

Bernth for always spreading happiness. Birgit, for always being kind.

The co-authors that I have worked with, especially Rolf Erik, Anthony, Einar and Sarah. Johan Glette, for his contributions to my work and his friendship. Thank you!

My friends in the real world for telling me to quit school already.

My mother Ninni, my father Dag and my sister Sanna for always believing in me, and telling me that I can do anything I set my mind to.

And most of all, my love Ida.

Fiancial support was given by the EU through project GUTINTEGRITY (Q5RS-2000-31656) under the Quality of Life and Management of Living Resources program. Further support was provided by Helge Ax:son Johnsons Foundation, NorFA, Lars Hiertas Minne Foundation abd the Agricultural Sciences and Spatial Planning to K. Sundell (22.3/2003-1241).

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49	Maja Pesonen	1992	Xenobiotic metabolizing enzymes in rainbow trout (*Oncorhynchus mykiss*) kidney and liver; characterization and regulation by xenobiotics
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79	Viktoria Johansson	2005	Behavioural effects and central nervous system actions of growth hormone in salmonid fish
80	Joachim Sturve	2005	Studies of DT diaphorase in fish. Characterisation and biomarker application

81	Sara Aspengren	2006	Melanophores -functional and morphological studies of intracellular transport and transfer of melanosomes
82	Jenny Turesson	2006	Oxygen chemoreflexes in fish – with emphasis on glutaminergic control mechanisms in the medulla
83	Anna Holmberg	2006	Ontogeny of gut motility and the enteric nercous system in *Danio rerio* and *Xenopus laevis*