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Effect of bioactive products on innate immunity and development of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae

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Submitted for partial fulfillment of degree of Master of Science in Natural Resource Science – Biotechnology and Aquaculture

Oktober 2008

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Abstract

Halibut larvae were treated with various bioactive products and the effects on selected components of the innate immune system investigated. Effects on growth, survival and normal development of larvae were also studied. The bioactive products which were tested were chitosan and protein hydrolysates from cod, blue whiting and pollock. The larvae where treated with bioactive products from the onset of feeding or from 4-5 weeks after the onset of feeding and throughout the first feeding period. The products were either added to the environment of the larvae, to the environment of the live feed or incorporated into the fatty acid mixture used for enrichment of the live feed. High mortality rates of larvae are commonly observed during the first weeks in feeding and previous research indicates that this may be partly caused by high bacterial numbers or the composition of the bacterial community. The specific immune response is not fully developed until after the first feeding period, thus, innate immune responses are of great importance. The overall results indicate that treating larvae with fish protein hydrolysates through the live feed resulted in stimulation of the innate immunity of first feeding halibut larvae. However, the treatment did not lead to improved growth or survival of larvae during the first feeding period.

Keywords: Halibut larvae, innate immune response, IgM, C3, lysozyme, fish protein hydrolysates, chitosan

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1. Introduction

The Atlantic halibut (*Hippoglossus hippoglossus* L.) is a promising species in aquaculture, because of its high filet output, high prices and low annual wild catches (Kvåle *et al*., 2007). The first stages of feeding have been one of the main obstacles in production of halibut juveniles and other marine fish species with high mortality rates commonly observed. Previous research indicates that stress caused by high numbers of bacteria and the bacterial community may cause problems during this delicate stage of the development (Olafsen, 2001). Research in the collaboration of Matis ohf., the University of Akureyri and Fiskey Ltd. have therefore focused on reducing the bacterial load in the larval environment during the first feeding period. High numbers of bacteria are commonly found in the environment of eggs and larvae that ingest bacteria by drinking long before exogenous feeding commences (Falk-Petersen, 2005). Furthermore, various groups of bacteria are introduced into the larval environment through the live feed used during the first weeks of feeding and bacterial numbers may adversely affect normal larval development (Olafsen, 2001). By stimulation of the immune system, the larvae will be better prepared for the overall bacterial load of the environment during first feeding. Stimulation of immune response may therefore lead to increased survival and/or growth of larvae. Specific immune response is, however, not fully developed in first feeding halibut larvae that therefore have to rely on the innate immune system during the first developmental stages (Magnadóttir *et al.,* 2005; Lange *et al.,* 2006). It is therefore important to find a way to enhance the innate immune responses of larvae during first feeding. Various research have been carried out on the immune system of fish (Magnadóttir *et al*., 2004; Elward & Gasque, 2003; Langston *et al*., 2002: Lange *et al*., 2001; Skjermo *et al*., 1995). Selected innate parameters of the immune system have been examined, such as the complement factor 3 (C3) (Lange *et al*., 2004a; Lange *et al*., 2004b; Fishelson *et al*., 2001) and lysozyme (Seppola *et al*., 2008; Bowden *et al*., 2004; Misra *et al*., 2004; Balfry & Iwama, 2004). The specific immune system and ontogeny of various fish species have also been extensively studied (Dautremepuits *et al*., 2006; Magnadottir *et al*., 2005; Zapata *et al*., 2005; Grove *et al*.,

2006; Schrøder *et al*., 1998; Bøgwald *et al*., 1996), IgM (Grove *et al*., 2006; Magnadóttir, 1998) as well as maternal transfer of immune parameters (Mulero *et al*., 2007). Further research will increase our understanding of the immune responses of fish larvae. When is the most appropriate time to stimulate and how do immunstimulants activate the unspecific immune system of marine larvae are questions that need to be answered.

The study describes the effects of bioactive products that were selected as possible immune-stimulants of first feeding halibut larvae. The bioactive products investigated were protein hydrolysates of cod, blue whiting and pollock in addition to a chitosan product, known to enhance immune stimulation in humans (www.primex.is). Various products of fish protein hydrolysates (FPH) (Kotzamanis *et al*., 2007; Murray *et al*., 2003; Bøgwald *et al*., 1996 Gildberg *et al*., 1996 Siwicki *et al*., 1994) as well as chitosan (Dautremepuits *et al*., 2004; Snaar-Jagalska *et al*., 2003) have been found to show bioactivity in fish. Previous research indicates that fish peptide hydrolysate show various bioactive effects. Peptides extracted from blue whiting (Jónsdóttir, 2005) as well as cod muscle, eyes and spleen (Hermannsdóttir, 2005) have been found to show bactericidal effects. Furthermore, earlier research show that a special group of protein, called lectins, recognize and bind certain types of carbohydrates on the surface of bacteria and thereby activate the innate immune response in many fish species (Nikolakopoulou & Zarkadis, 2006; Turner, 1996). Derivates of chitin have also been found to show bactericidal effects and contribute to faster renewal of damaged tissue and stimulate the innate immune response in many species, including fish (Primex, 2005).

The use of bioactive products is an environmentally friendly method for stimulation of the innate immune response of fish larvae. The activity of the selected products may, however, be expected to depend on various environmental parameters such as the pH of the tank water environment and the soluability of the product in sea water.

1.1. Development of larvae during first feeding

Marine fish larvae undergo major morphological and cellular changes during the first months of their life (Zambonino Infante & Cahu, 2001). The larval stage is related to both muscular and gill development and the activity generally increases with age, presumably associated with the search for food (Falk-Petersen, 2005). The liver is first a roundish mass that enlarges and becomes more wedge-shaped. The pancreas contains one islet of Langerhans but will gradually elongate and surround the intestine. At the onset of exogenous feeding the larvae begin to produce their own prolactin, thyroid hormones, growth hormones and cortisol which play a key role in further development and growth of the larvae (Falk-Petersen, 2005). In marine species, the lymphoid organs develop in the following order; head kidney, spleen and thymus (Chantanachookhin *et al*., 1991; Padrós & Crespo, 1996). Small lymphocytes are however first seen in thymus and later in the head kidney and spleen (Chantanachookhin *et al*., 1991).

From hatching to adulthood, the development of the fish alimentary canal changes from a straight, undifferentiated gut to a complex and segmented digestive tract (Govoni *et al.*, 1986). At the onset of exogenous feeding the stomach is lacking (Pedersen, 1993) although there is an area in the digestive tract that later differentiates to the stomach (Luizi *et al.*, 1999). This area appears to be heavily folded compared to the intestine, and is caudally oriented in a prolongation of the oesophagus where the pyloric caecae is still absent. After the first 30 days of exogenous feeding, the stomach is beginning to form in halibut larvae but various studies disagree with respect to the appearance and functionality of the gastric glands. According to Luizi *et al.* (1999) the gastric glands are formed between 35 and 50 days post onset of first feeding (dpff) of halibut larvae but are not functional at that stage. However, according to Murray *et al.* (1994) functional gastric glands are formed earlier or approximately 16 dpff. Before the stomach is fully functional, the larvae seem to compensate for the lack of gastric pre-digestion by active intracellular digestion in the rectal epithelial cells, following pinocytosis of macromolecules from the lumen (Dabrowski & Dabrowska, 1981; Govoni *et al.,* 1986; Loewe & Eckman, 1988; Bengstone *et al.*, 1993; Luizi *et al.*, 1999). The glands in the stomach are partly functional but the acidic protease activity is not established until weaning, approximately 50-70dpff (Zambonino Infante & Cahu, 2001).

During the larval stage, the muscularis externa is only a thin layer in the oesophagus and the stomach, but considerably thicker closer to the pylorus. At the junctions, the simple constriction observed in young larvae develops into a muscular sphincter marking the end of the stomach and the beginning of the intestine (Luizi *et al.*, 1999).

The intestines are identified as the section of the alimentary canal between the pyloric sphincter and the ileorectal valve (Figures 1 and 2). Up to 35 dpff a wide area of the intestines follows directly behind the pyloric constriction. At this stage in the development, some storage pockets are forming in the tube-like digestive tract. In between these two features, the pyloric sphincter and the ileorectal valve, the intestine forms a single loop that accounts for at least two thirds of the length of the alimentary canal (Pittman *et al.*, 1990; Luizi *et al.*, 1999). During the course of metamorphosis, the intestine slowly pushes forward caused by the differentiation of the stomach. Meanwhile, the pockets following the pyloric sphincter form the pyloric caecae by expansions of the intestine which grow in the opposite direction to the movement of the digestive chyme. All the caecae are located around the pyloric sphincter and develop at a steady pace during the entire metamorphosis. When the pyloric caecae develops, the folding of the mucosa increases and the wave-like structures observed in younger larvae turn into conical folds (Luizi *et al.*, 1999).

The rectum is separated from the intestine by the ileorectal valve. Also the ileorectal valve is composed of an expansion of the intestinal coating into its lumen. Soon after beginning of exogenous feeding, the valve partially obstructs the intestine and by the end of metamorphosis it is large enough to completely close the lumen. The anal region shows very little change during larval development except for thickening of the muscularis externa (Luizi *et al.*, 1999).

The thickness of the mucose in the digestive tract of halibut larvae changes during development, with increased folding, absorptive activity and the number of goblet cells (Luizi *et al.*, 1999). It has been shown in many other species that the enterocytes are fully functional at hatching (Segnar *et al.*, 1994). At all stages, the transition between the oesophageal epithelium and the rest of the digestive tract, whether it is already differentiated into a stomach or not, is well defined by the absence of goblet cells and a single layer of small prismatic epithelial cells of the digestive tract following the oesophagus (Luizi *et al.*, 1999).

Pre-metamorphic stage (up to day 35-40 PFF)

Metamorphic climax (between 35-40 and 50-55 days PFF)

Post-metamorphic stage (after day 50-55 PFF)

Luizi *et al.*, 1999
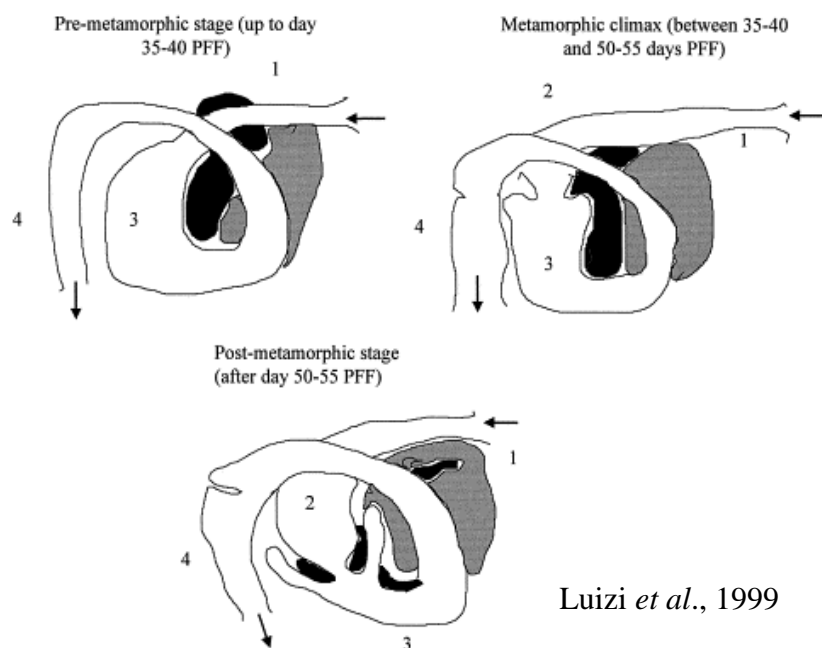
Figure 1. Schematic representation of the digestive tract of halibut larvae and its extramural glands during the larval development (PFF: days post onset of first feeding). The pancreas is represented in black and the liver in grey. 1 = the oesophagus; 2 = stomach; 3 = the intestine; 4 = the rectum. The direction of the digestive chyme movement is symbolized by the arrows.

The nutrient ingredients of the gastrointestinal tract are digested by the different types of enzymes produced in the stomach, exocrine pancreas and intestine. The intestinal cells also carry out the absorption and transport of nutrients from the lumen. The liver and pancreas have been shown to be functional at the onset of exogenous feeding in halibut larvae (Kjørsvik & Reiersen, 1992). The secretory function of the exocrine pancreas progressively develops and is functionally efficient at 21 days post hatch (dph), well before exogenous feeding starts (Zambonino Infante & Cahu, 2001).

The intestinal cells (enterocytes) produce two groups of enzymes: the cytosolic enzymes (mainly peptidases) found in the cell cytoplasm, and the brush border membrane enzymes, which are linked to the cell membrane (Zambonino Infante & Cahu, 2001). Different types of membranous enzymes can be detected, including peptidases, disaccharidases and esterases. The enzymes of the enterocyte cytosol have higher activity in younger larvae (up to 21 dpff) compared to older (21-28 dpff). The enzyme activity of the enterocytes characterizes the normal maturation of enterocytes in developing fish larvae as well as in other vertebrate species (Zambonino Infante & Cahu, 2001). The different enzyme types are found throughout the gastrointestinal tract

and are often complementary, where the enzymatic process leads to the total digestion of diet components allowing the absorption or transport of nutrients by enterocytes (Zambonino Infante & Cahu, 2001).

Metamorphosis is a step in the development of halibut larvae. After approximately 40-50 days of feeding on live feed, the larvae start to metamorphose and gradually turn into benthic juveniles. This metamorphic process includes the lateral flattening of the body, asymmetric colorating of the sides (the left side, the downside, being white and the right side, the top side, taking on a cryptic color) and the migration of the left eye up to the right side at the time as the fish becomes bottom dwelling (Figure 2). Then the juveniles are ready to eat and digest dry feed (50-70 dpff) (Hamre *et al.,* 2007). Many factors have been shown to affect the development of flatfish larvae and factors directly related to the diet are the thyroid hormones, vitamin A and fatty acid composition. These factors are furthermore ligands to nuclear receptors that participate in the control of development (Hamre *et al*., 2005a). Flatfish larvae that are fed rotifers and *Artemia* often develop malpigmentation, resulting in juveniles with their ocular side partially or totally white and sometimes with dark pigmentation on the blind side. This also applies to Atlantic halibut larvae, which in addition often show impaired eye migration, where the left eye fails partly or totally to migrate to the right side during metamorphosis (Hamre *et al.*, 2005b). At Fiskey Ltd., larvae developing incomplete eye migration are on average 0-10 % and 2-8 % of the larvae are white in color at the end of first feeding (Smáradóttir, *personal communication*).
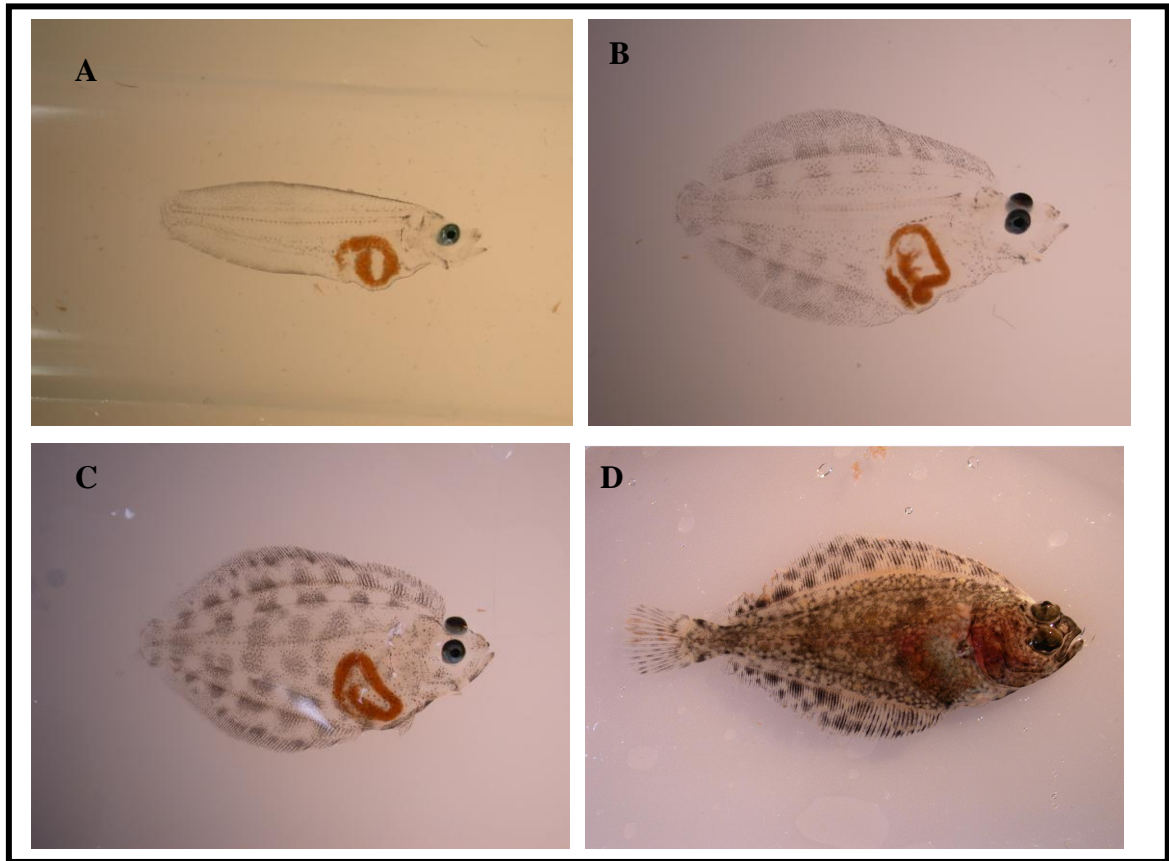
Figure 2. Halibut larvae at various developmental stages: ~15 to 20 days post onset of first feeding (dpff) (A), ~30 dpff (B), ~45 dpff (C) and metamorphosed juveniles ~70 dpff (D).

Understanding the feed and feeding requirements is the basis for successful production of larvae. The feed represents the main cost factor in the production of larvae and alternative feed and feed supplements are therefore important fields of study (Kvåle *et al.,* 2007; Hamre *et al.,* 2005a). In their natural environment, halibut larvae feed on copepods, however, in aquaculture, enriched *Artemia* is used during first feeding of larvae (Hamre *et al.,* 2005a) despite the fact that the digestive tract of larvae fed *Artemia* has been found to develop more slowly compared to larvae fed copepods (Luizi *et al.,* 1999).

Artemia is commonly used during first feeding of marine larvae. *Artemia* is a seawater shrimp found worldwide in saltwater and belonging to the phylum Arthropoda, class Crustacea. Approximately 90 percent of the world's commercial harvest of brine shrimp cysts (the dormant stage) comes from the Great Salt Lake in Utah (Treece, 2000). However, the lake's cyst production is heavily influenced by freshwater inflow, and the supply as well as quality varies dramatically (Treece, 2000). The *Artemia* are

extremely euryhaline, withstanding salinities from 3 ppt to 300 ppt and they can even survive short periods of time in fresh water, but without the ability to reproduce (http://www.artemia-international.com). Furthermore, *Artemia* can survive temperatures ranging from 15 to 55°C. The *Artemia* have two modes of reproduction. Sometimes nauplii (first *Artemia* swimming stage) hatch in the ovisac of the mother and are born alive. However, when the body of water where the adult *Artemia* are living begins to dry up with the following increase in salinities, the embryos are encysted in a hard capsule or cyst for protection and then hatch later when the conditions have improved (Treece, 2000). Dry conditions cause the encysted embryo to enter a dormant state, which allows it to withstand complete drying, temperatures over 100°C and near absolute zero, high energy radiation, and a variety of organic solvents (Treece, 2000). The dehydrated cysts can be stored for months and years without losing their ability to hatch (http://www.artemia-international.com).

1.2. The immune system

Normally the fish larvae are exposed to microorganisms immediately after hatching, and an effective immune system is therefore of great importance (Law & Dodds, 1997). In spite of limited pathogen recognition, the strength of the innate defense system against pathogens is impressive (Magnadóttir, 2006). The innate immune system's recognition of non-self and danger signals is served by a limited number of germ-line encoded pattern recognition receptors/proteins, which recognize pathogen associated molecular patterns such as bacterial and fungal glycoproteins and lipopolysaccharides as well as intracellular components released through injury or infection (Medzhitov & Janeway, 2002; Elward & Gasque, 2003). This is demonstrated by the very efficient immune defense of invertebrates, which exclusively rely on innate parameters for coping with a large variety of pathogens in diverse environmental conditions (Magnadóttir *et al.,* 2005). The innate immune system is also important in activating and adapting immune responses. In recent years this communication between the innate and the adaptive system has received increased attention in mammalian research (Magnadóttir, 2006; Fearon, 1997). The activation of innate recognition

components, through the stimulation of phagocytes, production of cytokines and chemokines, and activation of the complement system and various cell receptors, leads to the stimulation of T- and B-cells and antigen presenting cells (Lo *et al.,* 1999). Once activated the recognition molecules can induce opsonization and phagocytosis of the pathogen, stimulate natural cytotoxic cells or activate different signaling or executive processes like the complement system, the lytic pathway or an acute phase response. Recognition molecules, like lysozyme or α2 macroglobulin can also partake in direct elimination of the pathogen (Magnadóttir, 2006). Although less studied in fish, a similar communication probably takes place between the innate and the acquired immune system (Dixon & Stet, 2001). The components of the innate immune system are commonly divided into physical parameters in addition to cellular and humoral factors. The humoral parameters can be both cell associated receptors or soluble molecules of plasma and other body fluids (Magnadóttir, 2006). The key cells of the innate immune system are the phagocytic cells (granulocytes, neutrophils and monocytes/macrophages) and the non-specific cytotoxic cells (Evans *et al.,* 2001; Neumann *et al.,* 2001). Epithelial and dendritic cells also participate in the innate defense in fish (Press *et al*., 1994; Dalmo *et al.,* 1996). Innate immune parameters have been used as indicators of the effects of inherent or external factors on the immune system and the disease resistance of fish. Several external and internal factors can influence the activity of innate immune parameters. A change in temperature, crowding stress and handling can for example suppress the activity, but several food additives and immunostimulants can enhance various parameters of the innate immune response (Magnadóttir, 2006).

Relatively little is known about the ontogeny and the functioning of the immune system in fish larvae (Ellis, 1998; Dalmo *et al.,* 2000), however, our understanding of the components of the innate defense system of fish is growing (Magnadóttir, 2006; Magnadóttir *et al.,* 2005). The ontogenic development of the immune system of fish has primarily involved studies of the development of the different lymphoid organs of the immune system (thymus, kidney, spleen) and acquired immune parameters such as B- and T-lymphocytes and the expression or secretion of IgM (Zapata *et al.,* 1997; Ellis, 1998). Early life stages of fish do not appear to have developed immunocompetence, e.g. the synthesis of immunoglobulin, therefore, the organisms may rely on their ability to combat infectious diseases in a non-specific manner (Fletcher, 1982; Dogget & Harris, 1987). Furthermore, it has been shown that the ontogenetic development of the

immune system varies in different fish species, and it is questionable to apply the data for one species to another (Morgan & Gasque, 1996). The ontogenic studies of innate parameters of fish have so far been mainly limited to the first appearance of macrophages and phagocytic activity (Magnadóttir, 2006). The development of the specific immune system of fish in ontogeny is believed to be correlated with the weight of fish rather than time after hatching (Dalmo *et al*., 2000). In general the acquired immune system develops late in marine species compared to freshwater species which therefore depend on innate defense for the first 2-3 months after hatching (Schrøder *et al.,* 1998).

1.2.1. *Immunoglobulin M (IgM)*

Immunoglobulins (Ig) are the primary humoral components of the specific immune system. The first appearance of cytoplasmic and surface IgM in lymphocytes differs between species even though it can be complicated to compare data from various analyzing methods (Magnadottir *et al*., 2005). The ability to develop an antibody response does not necessarily correlate with the first detection of IgM positive cells. Although the time lapse between the detection of cellular IgM and functional immunocompetence has not been studied in many fish species, it has been observed in both fresh water and marine species (Dos Santos *et al*., 2000; Petrie-Hanson & Ainsworth, 1999). While most Ig^{+} cells are probably represented by B-cells or plasma cells, it should be noted that other cell populations in fish have been reported to show immunohistochemical reactivity for Ig. Macrophages (Imagawa *et al.,* 1991; Lamers *et al.,* 1986), neutrophils (Pettersen *et al*., 2000) and non-specific cytotoxic cells (NCC) have been reported to be Ig[+], probably through binding of Ig to Fc receptors, phagocytosis of immune complexes or the cells in question are plasma cells (O´Dowd *et al*., 1998; O´Dowd *et al.*, 1999). In general, the appearance of B-lymphocytes and immunoglobulins is late in marine species compared to fresh water species (Chantanachookhin *et al*., 1991). Commonly, fish larvae have reached about 20-30 mm in length when IgM is first expressed, its appearance being generally more dependent on size rather than age (Grönvendt & Espelid*,* 2003) although this is not always the case (Dos Santos *et al*., 2000).

Teleost IgM may exist as membrane bound or secreted forms and these forms of IgM seem to differ in molecular weight (MW) (Hordvik *et al*., 1992). Teleost IgM consists of two subunits, the heavy chain (μ) and the light chain (L). In several teleost species, both μ and L chains have been found as different isotypes. Two to five L chain isotypes or MW variants have been reported, which may differ in MW, antigenic epitopes and primary structure (Håvarstein *et al.,* 1988, Lobb *et al.,* 1984, Sanchez *et al*., 1989; Sanchez & DominQuez, 1991). An initial characterization of halibut Ig has indicated that it is similar to the Ig of other investigated fish species (Magnadóttir, 1998). However, detailed studies of teleost Ig have shown considerable variation between species. In teleost fish, the predominant Ig has traditionally been described as IgM-like (Pilstrøm & Bengten, 1996). However, a large chimeric Ig chain, which shows gene sequence similarity to human and murine IgD heavy chain, has been described in Atlantic salmon (*Salmo salar* L.) (Hordvik *et al*., 1999) and Atlantic cod (*Gadus morhua*) (Stenvik & Jørgensen, 2000).

The native form of teleost IgM seems predominantly to be a tetrameric structure (μ^2L^2) consisting of four Ig monomers (μ^2L^2)$_4$ (Pilstrøm & Bengten, 1996). The degree of disulfide bonding seems to be variable in a number of examined teleost species. The tetrameric teleost IgM has been shown to split into mono-, di-, and trimers, depending on the species (Bromage *et al*., 2004). The nature of IgM can vary between teleost species in many respects, including the number of redox forms and the existence and number of isoforms of heavy and light chains. Halibut possesses immunoglobulin of IgM-like nature, consisting of heavy and light chain subunits. The IgM is mainly tetrameric but is also found in mono-, di- and trimeric redox forms (Grove *et al*., 2006). However some inconsistencies are between findings on molecular weight on halibut IgM. According to Magnadottir (1998), the MW of halibut IgM is 832-870 kDa whereas Grove and co-workers (2006) found the MW to be 780 kDa.

Some speculations about the relatively late appearance of autologous humoral IgM may be compensated by maternally derived immunoglobulins that have been demonstrated in the eggs and embryos of several fish species like salmon (Olsen & Press, 1997), sea bass (Breuil *et al.,* 1997; Picchietti *et al*., 2004), tilapia (Mor & Avtalion, 1988; Takemura, 1993), carp (Suzuki *et al*., 1994) and plaice (Bly *et al*., 1986) but not in cod (Magnadottir *et al.,* 2004). Even though the transfer of maternal Ig has been identified in a number of fish species, the concentration of IgM in eggs was

only 0,3-6,3 µg/g wet weight which is approximately thousand times less than the concentration in maternal plasma (Olsen & Press, 1997). However, experimental immunization of maternal fish has not been found to provide the offspring protection from disease or bacterial infections (Lillehaug *et al*., 1996; Tanaka *et al*., 1999). This is in accordance with the fact that the concentration of maternal immunoglobulin during the larval stages is generally greatly reduced from that observed during the ovarian stages. Maternal IgM in sea bass for example is deposited in the ovary during vitellogenesis and reaches maximum concentrations during ovulation. At hatching this level had reduced about 100-fold and by 5 dph no maternal IgM was detected in the sea bass larvae (Picchietti *et al*., 2004; Pepin *et al*., 1997). Similar situation has been demonstrated in tilapia (Takemura, 1993) and Atlantic salmon (Olsen & Press, 1997). Most of the maternally derived IgM of eggs is structurally and antigenically identical to the maternal serum IgM, with respect to size and the tetrameric (Kanlis *et al.,* 1995; Hayman & Lobb, 1993) as well as dimeric (Suzuki *et al*., 1994) and monomeric (Yousif *et al*., 1995; Pepin *et al*., 1997; Avtalion & Mor, 1992) forms in several fish species.

1.2.2. Complement factor 3 (C3)

The complement system is one of the first lines of immune defense and a modifier of acquired immunity (Nakao & Yano, 1998). The complement system consists of a group of at least 20 - 30 serum proteins that co-operate with other defense mechanisms (Nakao & Yano, 1998). The complement system is activated through any of the three pathways demonstrated in Figure 3; the antibody-dependent classical pathway, the antibody-independent alternative pathway, and the lectin pathway triggered by the interaction of mannose-binding lectin (MBL) or ficolins with polysaccharides (Sunyer & Lambris, 1998; Magor & Magor, 2001). Ontogenic studies on halibut include immunohistochemical studies of the ontogeny of the complement component C3 (Lange *et al*, 2001; Langston *et al*, 2002; Lange & Magnadóttir, 2003) as well as haemolytic activity (Bowden *et al*, 2000) and characterization of halibut C3 (Colten, 1994). The ontogeny of the complement component C3 of halibut and cod has recently been described using immunohistochemical method (Lange *et al*, 2004a; Lange *et al* 2004b; Lange *et al* 2004c).

Boshra & Sunyer, 2007
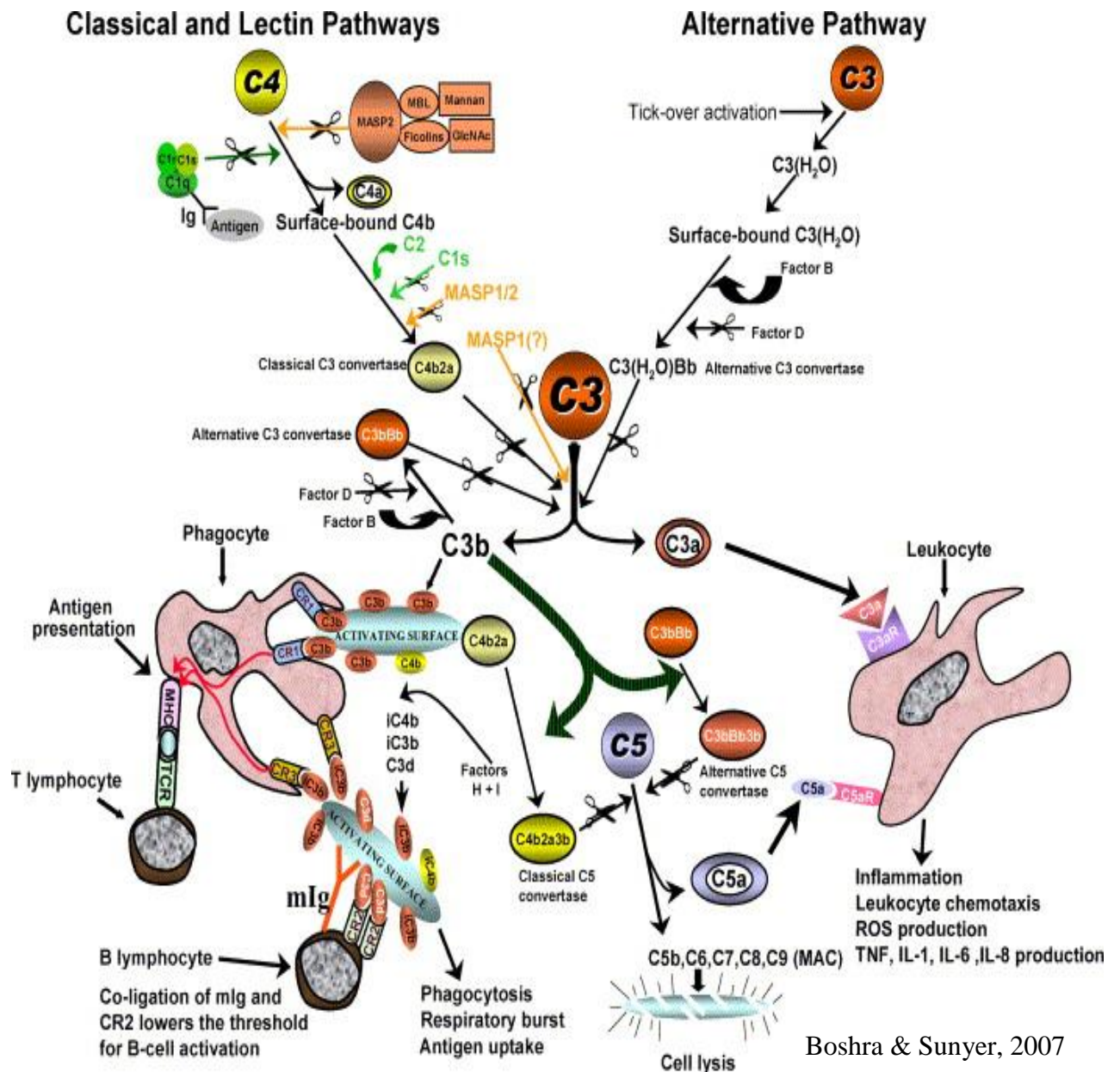
Figure 3. "Complement activation pathways and functions. Activation of the complement system through any of the three existing pathways (classical, alternative or lectin) leads to the activation of C3 forming C3b and C3a. C3b covalently binds to complement activating surfaces (i.e., bacteria, fungi, viruses). Bound C3b can be degraded into iC3b by factor I in the presence of factor H. C3b and iC3b bind to complement receptors (CR1, CR3) and promote phagocytosis, respiratory burst, and antigen-uptake processes. C4 activated through the classical or lectin pathways can also bind to an activating surface and promote its uptake, however the number of C4 molecules binding to a surface is always many folds less than that of C3 molecules. Antigen containing covalently bound C3b or C4b molecules (or their degradation fragments) can be further processed and presented to T-lymphocytes. Antigen containing bound Ig and C3d lead to the colligation of the B cells receptor (BCR or mIg) and complement receptor type 2 (CR2/CD21) on B cells, which in turn lowers the threshold for B cell activation. In addition, C3b/C4b bound to a microorganism can lead to the formation of the membrane attack complex (MAC) which results in cell lysis. C5a and C3a anaphylatoxins generated during complement activation play a key role in inflammatory processes." (Boshra & Sunyer, 2007)

The complement system has also been shown to play a role in the clearance of apoptotic material by enhancing the phagocytosis of apoptotic cells. These functions are critical in the maintenance of normal homeostasis and during developmental processes (Fishelson *et al*., 2001). C3 is the central complement component and interacts with many proteins, including some that participate in or control cell adhesion and cell-to-cell communication (Del Rio-Tsonis *et al*., 1998). Halibut C3 has been isolated and has been found to be a two-chain (α-chain, 115 kDa; β-chain, 68 kDa) glycoprotein with an intrachain thioester bond in the α-chain (Lange & Magnadóttir, 2003). These characteristics are similar to those of mammalian C3, which through its thioester bond can covalently bind to target cells (Law & Dodds, 1997). The local synthesis of C3, as well as of other complement components in tissues other than the liver, may play an important role in local inflammatory processes (Colten, 1994) and also be involved in the clearance of apoptotic cells during tissue construction (Fishelson *et al.*, 2001).

1.2.3. *Lysozyme*

Lysozyme plays an important role in the innate immune response and is an important factor in protecting fish against bacterial pathogens (Yousif *et al*., 1994; Lie *et al*., 1989). Its specific action is to attack the peptidoglycan layer of bacterial cell walls, by hydrolysis of N-acetylmuramic acid and N-acetylglucosamine, resulting in bacterial cell lysis. Leucocytes such as monocytes, macrophages and polymorphonuclear granulocytes are known to synthesize and secrete lysozyme in fish (Murray & Fletcher, 1976). Lysozyme is present in mucus, lymphoid tissue, serum and other body fluids of most fish species (Grinde *et al*., 1988; Lie *et al*., 1989; Lange *et al*., 2001). It has also been detected in oocytes, fertilized eggs and larval stages of several fish species (Yousif *et al*., 1991; Kudo, 1992; Takemura & Takano, 1995; Takemura, 1996; Brown *et al*., 1997; Cecchini *et al*., 2000 Takahashi *et al*., 1986; Lie *et al*., 1989; Murray & Fletcher, 1976). Lysozyme isolated from fish has been found to be effective as a bacteriolytic agent against both Gram-positive and Gram-negative fish pathogens (Grinde *et al*., 1988; Yousif *et al*., 1994). Thus, the importance of lysozyme is due to its antibacterial properties and because it is located in areas that are in frequent contact with pathogens. Bowden *et al*. (2004) compared the basal serum lysozyme levels in Atlantic halibut and observed highly different summer and winter levels of the enzyme

as has also been shown in the wild caught dab (*Limanda limanda*) (Hutchinson & Manning, 1996). Studies have revealed the presence of maternal lysozyme in many fish species (Balfry & Iwama, 2004; Kanis *et al*., 1976; Withler *et al*., 1987). Balfry and Iwama (2004) detected a positive correlation between maternal lysozyme activity (kidney and serum) and the lysozyme activity of unfertilized eggs of coho salmon. Furthermore, a significant lysozyme activity appeared at very early stages of the development.

1.2.4.　　　　*Intestinal immunity*

The intestinal immunity is important for the survival of larvae due to heavy bacterial load of the environment and the live feed. The gastrointestinal tract (GIT) is furthermore the largest immunologic organ in the vertebrate body (Takahasi & Kiyono, 1999). The fish GIT contains innate (Douglas *et al.,* 2001) and specific immune functions (Grove *et al.,* 2006; Matsunaga, 1998; Rombout *et al.,* 1993). Polymorphonuclear leukocytes, lymphocytes and plasma cells located in the epithelial layer and lamina propria of the mucosa function to provide innate as well as acquired immunity. The underlying submucosa is composed of vascular and connective tissue and sometimes contains mucous glands (Hébert *et al*., 2002). In halibut, the relatively limited numbers of IgM+ cells which are present in the posterior intestine are predominantly in the epithelium rather than in the lamina propria. Furthermore, granulocytes and macrophages are located under the intestinal epithelium (Grove *et al*., 2006). GIT immune function are regulated by a combination of local, intra-GIT and inter organ signaling networks (Klein, 1998; Shanahan, 2000) and by inputs from the central nervous system (Ottaway, 1991). For example, high densities culture and extreme environmental conditions impose stress and the surges in glucorticoids and other stress-associated hormones can compromise immune functions (Bly *et al*., 1997; Ruane *et al*., 1999; Yada & Nakanishi, 2002).

1.3. Immunostimulants

Previous studies have revealed that the use of non-specific stimulants which enhance elements of the innate immune system can lead to increased protection against different pathogens and result in improved larval survival (Ellis, 1988; Press & Lillehaug, 1995; Dalmo *et al.*, 2000). Stimulation of the innate immune defence is therefore attractive for improved microbial management in juvenile production (Vadstein, 1997).

An immunostimulant may be defined as an agent that stimulates the innate immune mechanism when given alone, or the specific mechanism when given with an antigen (Vadstein, 1997). Immunomodulation may therefore be directed at both the specific and the innate immunity. Vaccination is probably the best-known method of specific immunostimulation, entailing increased resistance against a specific antigen or pathogen. Due to a poorly developed immune system, fish larvae primarily rely on the innate immune parameters; however, for a short period the larvae can also depend on maternal immunity for specific defence. Experimental results have shown that maternal immunity may be manipulated by immunization of the broodstock and that increased resistance to infections may be obtained (Vadstein, 1997). Thus, direct immunostimulation of larvae must be aimed at the nonspecific part of the immune system, and several substances are known to have this ability (Vadstein, 1997). Phagocytosis is one of the main mediators of innate immunity to pathogens including bacteria, viruses and parasites in fish (Dügenci *et al.,* 2003). The most important cells involved in this defence are the phagocytes that are supported by several soluble factors, such as the complement system and lysozyme (Dalmo *et al.*, 1996; Verlhac *et al.*, 1998; Yano, 1996). It is also well known that the innate immune system in fish can be triggered by various immunostimulants such as levamisole (Jeney & Anderson, 1993), glucan (Jørgensen & Robertsen, 1995; Engstad *et al.*, 1992; Jeney *et al.*, 1997), glucan plus vitamin C (Verlhac *et al.*, 1996, 1998), yeast RNA (Sakai, 1999), lipopolysaccharide (Dalmo & Seljelid, 1995; Solem *et al.*, 1995), growth hormones (Sakai *et al.*, 1996), zeranol (Keles *et al.,* 2002), chitosan (Siwicki *et al.,* 1994) alginate (Skjemo & Berg, 2004) and medicinal plants (Dügenci *et al.*, 2003). Lipopolysaccharides (LPS) from the cell wall of Gram negative bacteria, may act as immunostimulants by triggering a number of mechanisms, while e.g. mannuronic acid polymers may have a more restricted stimulatory effect (Espevik *et al.*, 1993). Alginates

containing high percentages of mannuronic acid polymers are known to stimulate human monocytes to increased cytokine production (Otterlei *et al*., 1991). High-M alginate has been found to enhance the survival of Atlantic halibut yolk sac larvae and turbot juveniles (Vadstein *et al*., 1993; Skjemo *et al*., 1995).

Successful immunostimulation of various fish species has been obtained by injection, bath and oral administration (Vadstein, 1997). The practical use of nonspecific immunostimulation in fish larviculture depends on the ability to stimulate without injection, although it has been shown that injections with glucan and chitin gave an increased resistance to *Aeromonas salmonicida* infection compared to administration by immersion. However, too few conditions were tested to be able to draw any firm conclusions regarding the possible advantages of administration by injection (Anderson & Siwicki, 1994). It is not known if immersion and oral administration entail complete stimulation of the nonspecific defence or stimulation of only the mucosal immune system. Intestinal uptake and organ redistribution of high-molecular-weight polysaccharides (glucan and poly-glucose) after oral administration have been documented (Dalmo & Seljelid, 1995; Sveinbjörnsson *et al*., 1995). It is therefore possible that immersion and oral administration may result in complete stimulation, but with certain limitations depending on the immunostimulants used as well as the fish species. In addition to the positive effects as judged by immune parameters and challenge tests, immunostimulation has also been shown to abolish the negative effects of immune suppression (Vadstein, 1997).

1.4. Bioactive products

1.4.1. Protein hydrolysates

Hydrolysates can be defined as proteins that are chemically or enzymatically broken down into peptides of varying sizes (Skanderby, 1994). Although production is massive worldwide, the proper control of the process and the exact mechanism behind protein hydrolysis is in most cases not fully understood. However recent advances have given researchers insight into the connection between the process or extent of hydrolysis

and the physicochemical mechanisms responsible for specific functional properties of the hydrolyzed proteins (Kristinsson & Rasco, 2000).

Chemical hydrolysis of proteins is achieved by cleaving peptide bonds with either an acid or a base solution. Several processes have been proposed for the acid or alkaline hydrolysis of fish muscle (Hale, 1972; Kristinsson & Rasco, 2000). Protein hydrolysis using strong chemicals and solvents is performed at extreme temperatures and pH, generally yielding products with reduced nutritional qualities, poor functionality, and that are restricted to use as flavor enhancers (Webster *et al.*, 1982; Loffler, 1986). Biochemical hydrolysis of fish proteins is performed using enzymes for hydrolyzing the peptide bonds. This can be carried out using proteolytic enzymes already present in the fish viscera and muscle (endogenous proteases), or by adding enzymes from other sources (Kristinsson & Rasco, 2000).

Peptides are chains of 2-50 amino acids that are connected with peptide bond. Oligopeptides contain less than 10 amino acids but peptides containing 10-50 amino acids are often referred to as multi peptides whereas larger chains are considered proteins (Gunnlaugsdóttir *et al.*, 2005). Peptides are involved in various life cycles such as hormone release, control of blood sugar, metabolism in bones and in assorted nerve and protection processes (Pihlanto-Leppälä, 2001). Fish peptides can be produced by breaking down proteins with either fermentation or hydrolysate and there are some indications that those peptides have bioactivity (Pihlanto-Leppälä, 2001). As a nutritional factor, the proteins are broken down to smaller peptides by digestive enzymes and the peptides then disposed. Food enriched with bioactive products can physiologically affect the heart as well as the vascular system, the nerve system and the immune system in addition to the general absorption as nutrients (Pripp *et al.*, 2005).

Proteolytic modification of food proteins to improve palatability and storage stability of the available protein resources is an ancient technology (Adler-Nissen, 1986). Until few years ago, only peptides derived from milk and herbal proteins were used in the food industry, both as flavor enhancers and bactericidal supplements (Pripp *et al.*, 2005). Peptides derived from seafood, have recently gained increased interest, due to their bactericidal (Roller & Covill., 2000; Pranoto *et al.*, 2005; Kotzamanis *et al.*, 2007) and immunostimulating effects (Bøgwald *et al.*, 2002; Murray *et al.*, 2003; Dautremepuits *et al.*, 2004; Kim & Rajapakse, 2005; Snaar-Jagalska *et al.*, 2003) as well as significance as food suppliments (Cahu *et al.*, 1999; Murray *et al.*, 2003;

Kristinsson & Rasco, 2000). Furthermore, large amounts of protein-rich byproducts originate from the seafood industry and can no longer be discarded directly to the ocean, due to environmental legislations, resulting in high cost of refining the material before it is discarded (Aspmo *et al*., 2005). The value of the proteinaceous fish wastes can be increased by convertion to hydrolysates with different bioactivity (Kristinsson & Rasco, 2000; Aspmo *et al*., 2005). Pharmaceutical developments that are based on active peptides derived from marine organism are furthermore considered interesting for use as food supplements and functional food. The biggest problem with using fish protein and peptides to increase the nutrition value in the food industry is, however, the strong and bitter taste of the product (Kristinsson & Rasco, 2000).

One of the main focuses in aquaculture is to increase the value of the fish by using the whole product, including the viscera and other byproducts (Slizyte *et al*., 2004; Slizyte *et al*., 2005; Gbogouri *et al*., 2004). Several studies have been carried out involving peptides derived from cod, both with respect to nutritional value (Cahu *et al*., 1999; Murray *et al*., 2003), bactericidal effects (Kotzaminis *et al*., 2007) and as immunostimulants (Bøgwald *et al*., 2002; Murray *et al*., 2003). Bøgwald *et al*. (2002) reported that cod muscle hydrolysate had stimulating effect on Atlantic salmon head kidney leucocytes, *in vitro* as well as *in vivo*. Leucocytes cultured in the presence of peptides for a period of 2 to 9 days sustained the enhanced capacity of superoxide anion production compared to leucocytes grown in the absence of the peptides (Bøgwald *et al*., 2002).

Protein hydrolysates derived from blue whiting have been found to show antibacterial activity (Jónsdóttir, 2004; Hermannsdóttir, 2005) and induce an inhibition of cancer cell growth (Picot *et al*., 2006). Peptide hydrolysates of blue whiting are therefore interesting product with respect to bioactivity.

The nutritional composition of a protein hydrolysate derived from pollock has been investigated and the results revealed that it contained high levels of taurine, potassium and B-vitamin which gives high nutritional value (Liaset & Espe, 2008). Protein hydrolysates derived from pollock muscle have been reported to enhance lysozyme activity of sea bass after feeding for 30 or 60 days and treatment for 60 days was found to compensate haemolytic activity (Liang *et al*., 2006). Furthermore, only 15% of pollock hydrolysates in the diet provoked the immunostimulating effects, higher

or lower levels of pollock hydrolysates did not significantly affect the immune response (Liang *et al*., 2006).

1.4.2.   *Chitosan*

Chitin, the raw material in the production of chitosan, is a white, hard and inelastic structural polysaccharide found in cell walls of fungi and in exoskeletons of crustaceans. The molecular structure of chitin is identified as a high-molecular weight linear polymer of N-acetyl-ᴅ-glucosamine units (GlcNAc) linked by β-1,4 bonds. The hydrophobic nature of chitin makes it insoluble in water as well as in most organic solvents. In contrast, chitosan, the N-deacetylated form of chitin, is readily soluble in dilute organic acids at low pH (Primex, 2005). The most important parameter that determines the solubility of chitosan is the degree of deacetylation (DD). Conversion of chitin into chitosan increases DD, and thereby alters the charge distribution of the chitosan molecules. In general, the degree of acetylation (DA) of chitin is approximately 90% and following partial or fully deacetylation with alkaline treatment, it is converted into chitosan. In addition to the DD, the degree of polymerization (DP) also contributes to the alteration of physico-chemical properties of chitosan. Unlike most polysaccharides, chitosan is positively charged following removal of acetyl units from the ᴅ-glucosamine residues. This chemical feature allows chitosan to bind strongly to negatively charged surfaces and is responsible for many of the observed biological activities. In addition to that, the non-toxicity, biodegradability and biocompatibility of chitosan promote their biological applications compared to other synthetic polymers (Kurita, 1998).

Due to the rather unstable glycosidic bonds, chitosan and other polysaccharides can also be cleaved by hydrolyzing agents. Degradation of O-glycosidic linkages of chitosan by different methods leads to the production of chitosan oligosaccharides (COS) varying in the DP as well as number and sequences of glucosamine (GlcN) and GlcNAc units. Some of these methods include acid hydrolysis (Il'ina & Varlamov, 2004), enzymatic hydrolysis (Kuroiwa *et al*., 2002; Zhang *et al*., 1999), oxidative degradation (Shirui *et al*., 2004), ultra sonic degradation (Chen & Chen, 2000), chemoenzymatic (Akiyama *et al*., 1995) and recombinant approaches (Samain *et al*., 1997).

Chitosan and its derivatives have been found to show various functional properties and various products have been used in many fields, as bactericidal agents (Covil *et al.,* 2000; Pranoto, *et al,* 2005), immunostimulants (Dautremepuits *et al.*, 2004; Snaar-Jagalska *et al.*, 2003; Feng *et al.*, 2004), in cosmetics (Majeti & Kumar, 2000), biomedicine (Felt *et al.*, 1998) and in agriculture (Yamada *et al.*, 1993). Furthermore, the biodegradable, non-toxic and non-allergenic nature of chitosan products especially encourages its potential use as a bioactive material (Kurita, 1998). Even though various products of chitosan are known to have important functional activities, poor solubility makes them difficult to use in food and biomedical applications. Unlike chitosan, its hydrolyzed products and chitosan oligosaccharides (COS) are readily soluble in water due to their shorter chain lengths and free amino groups in D-glucosamine units (Jeon *et al.*, 2000). The low viscosity and greater solubility of COS at neutral pH have attracted the interest of many researchers to utilize chitosan in its oligosaccharide form. Especially, studies on the use of COS in food and nutrition arenas have emphasized their ability to improve food quality and human health progression. Recent advances have insighted into the health benefits of COS, including lowering of blood cholesterol (Macchi, 1996) and blood pressure (Giustina & Ventura, 1995), protective effects against infections (Tokoro, 1989), controlling arthritis (Lee *et al.*, 2003) and enhancing antitumor properties (Nishimura *et al.*, 1984). Chitosan coated diet has been shown to improve water quality in fish farm environment of olive flounder and stimulate immune responses (Cha *et al.,* 2008). Furthermore, it has been shown that chitosan can be used to deliver DNA vaccine by feeding the fish chitosan nanoparticles. The results indicated that sea bass orally vaccinated with chitosan-DNA (pVAOMP38) complex showed moderate protection against experimental *Vibrio anguillarum* infection (Kumar *et al.,* 2007).

2. Materials and methods

All experiments were carried out at Fiskey Ltd., the largest individual producer of halibut juveniles on a global scale since 1998. The experimental setup, surveillance and sample collection was carried out by the staff at Fiskey Ltd., while the student was responsible for the sample analysis and evaluation of the results, carried out at the laboratory of the University of Akureyri and Matís Ltd.

2.1.   Bioactive products used in the study

Three different products of fish protein hydrolysates (FPH), obtained by enzyme hydrolysis of fish muscle proteins into smaller peptides were used in the study. The effects of the products were studied in three separate experiments, using blue whiting peptides (BP), cod peptides (CP) and pollock peptides (PP) in addition to a chitosan product manufactured by Primex Ltd.

The cod peptide hydrolysates, produced by Jónína Þ. Jóhannsdóttir, scientist at Matís ohf. in Akureyri, and the pollock peptides manufactured by Iceprotein Ltd., were produced by an enzyme hydrolysis which was carried out under alkaline condition. The Alcalase 4.1 enzyme (Novozymes) was used for producing the cod hydrolysate, and Protomex (Novo Nordisk a/s) in addition to the Alcalase 4.1 enzyme (Novozymes) was used for producing the Pollock hydrolysate (95:5, respectively). The pH is kept stable by regularly adding alkali to the solution throughout the reaction process to avoid salt formation during the reaction and in order to keep the pH stable at the optimal level for the enzyme (pH 8.5). To stop the reaction, the pH is adjusted to 6.5 and the solution incubated at 80°C for 20 minutes for neutralization of the enzyme. The solution is then left to cool down at room temperature (RT) over night. During the hydrolysis, precipitation will occur due to proteins that are not hydrolysed or only partially

hydrolysed but the peptides with the requested degree of hydrolysis can be found dissolved in the liquid phase of the solution (Kristinsson *et al.*, 2000).

The blue whiting peptide hydrolysate used in the study was produced by Margrét Geirsdóttir, a scientist and PhD student at Matis ohf. The product was produced within the Seafood Plus project, funded by the European Commission (http://www.seafoodplus.org/Project_4_1_PROPEPHEAL.62.0.html). The peptide hydrolysate was produced using the Hultin-Process method which is based on changes in the pH of the solution in a chilled environment (Kristinsson & Rasco, 2000).

Chitin and chitosan were extracted from the shell of crustaceans using stepwise chemical methods. In the first step, shrimp or crab shells are treated with 3–5% aqueous NaOH solution to remove proteins attached to the shells and thereby prevent the contamination of the chitin products with proteins. Deproteinized shells are then neutralized and calcium is removed by treatment with 3–5% aqueous HCl solution to obtain a white or slightly pink chitin precipitate. The chitin is then N-deacetylated using 40–45% aqueous NaOH to form chitosan of a cationic nature. The resulting crude sample is then dissolved in 2% acetic acid and the supernatant neutralized with 3-5% aqueous NaOH solution to obtain purified chitosan as a white precipitate (Hirano, 1996). After alkaline deacetylation, some of the amino groups may remain acetylated and distributed randomly along the whole polymer chain. The solubility and bioactive properties of various chitosan products depend on the degree of deacetylation (DD), with higher DD providing increased solubility (Kim & Rajapakse, 2005). The chitin product used in this study was manufactured by Primex Ltd, product number G060703 (40% DD).

2.2.   Production of halibut larvae at Fiskey Ltd

The spawning of the parental fish is controlled by means of photoperiod manipulation which yields three different spawning groups and access of eggs more or less the whole year around. The fertilized eggs are placed in tanks in the dark (0.25 m^3)

with gentle aeration for 14 days at 5.0-5.3°C. The eggs are then transferred to silos (10 m^3) and approximately 24 hours later the larvae hatch. At that time the larvae are about seven mm in length and are delicate and poorly developed organisms (Fiskey Ltd). The yolk sac larvae remain in the silos at 5.0-5.3°C for approximately 50 days, nourishing on the content of the yolk sac, and are then transferred to first feeding tanks (3.5 and 7.0 m^3). At this stage the larvae are not able to digest formulated feed and are fed live *Artemia franciscana* nauplii (Great Salt Lake, Utah, USA). The *Artemia* is enriched using a mixture of fatty acids and vitamins prior to feeding to the larvae twice a day for approximately 65 days at 10.2-10.6°C. Towards the end of this period, the larvae go through metamorphosis and become benthic juveniles weighing approximately 0.3g. The juveniles are then transferred to weaning tanks where dry feed is gradually introduced. When the juveniles reach the size of 5g they can be transported to on-growing. The entire process from the fertilization of eggs until weaning unto dry feed lasts for approximately four months (Figure 4).

Fiskey Ltd.

Figure 4. The life cycle of farmed Atlantic halibut at Fiskey Ltd.

The mortality of first feeding larvae was recorded on a daily basis throughout the period. Larval growth was evaluated on approximately weekly basis using dry weight of approximately 150 (at the onset of first feeding) to 15 (towards the end of the first feeding period) larvae from each tank. The larvae were dried for 4-5 days at 70-75°C and then weighed (dry weight of larvae). The survival and success of metamorphosis was evaluated at the end of the first feeding period and the quality of the juveniles was evaluated with respect to satisfactory eye migration and pigmentation when all juveniles had been transferred to weaning tanks. The survival was calculated as the percentage of larvae originally transferred to the first feeding tanks. Larvae that have not developed into juveniles after 70 days + in first feeding tanks are not expected to be successful and the survival is usually poor (Smáradóttir, *personal communication*).

The first feeding larvae are offered *Artemia* nauplii during the first two months of feeding. The shell is removed using a chlorine solution (1:5 of 15% Natrium hypochloride, NaOCl, diluted in water) in a five step process including hydration, decapsulation, washing, deactivation and dehydration. Hatching of the *Artemia* eggs is a 24 hour process at 27-30°C and salinity of approximately 34.5 ‰. The *Artemia* larvae are then rinsed carefully using cold freshwater and transferred to new containers where the enrichment (24 hours) takes place. Prior to enrichment, the density of the *Artemia* culture is adjusted to approximately 300 animals in each mL. After enrichment for 24 hours (24 h *Artemia*) the *Artemia* is rinsed with cold water and approximately 1/3 of the culture is fed to the larvae (morning feeding) while 2/3 are transferred to a new container and an enrichment medium added before ongrowing for approximately 8 hours (32 h *Artemia*) at 15°C. A special fatty acid and vitamin emulsion is used for enrichment of the *Artemia* at Fiskey Ltd., developed in collaboration with scientists at the University of Iceland and Lysi hf.

2.3. Experimental design

The experiments were designed in collaboration between Fiskey Ltd., the student and scientists at Matis ohf. and the University of Akureyri. Experiments have to be organized with respect to the supply of eggs and larvae, and with a minimum risks taken with respect to the commercial production of larvae and juveniles. The staff at Fiskey Ltd. took care of the daily surveillance of the experiments and the treatments were terminated or changed if there were any signs of the treatment schedules negatively affecting the larvae. Control tank units with commonly practiced treatment of larvae were used for comparison in all experiments.

2.3.1. *Treatment of the live feed (Artemia)*

In the first experiment, two different methods were studied with respect to the delivery of the bioactive products to first feeding larvae. Adding the products to the tank water environment of larvae was compared to the method of using the live feed (*Artemia*) to deliver the products to larvae. In the two subsequent experiments, the method using the *Artemia* as vector was selected. The products were then added to the fatty acid enrichment medium of the live feed, in most cases through both daily feedings (24 h and 32 h *Artemia*, respectively).

In the first experiment, the *Artemia* eggs were hatched (24 hours cultures) and then divided into smaller experimental units of 5 L for enrichments using the fatty acid emulsion. Two litres of seawater were added to each experimental unit and 3 L of the newly hatched *Artemia* then added to each bag. Hydrolysat from blue whiting in concentration of 0.02 g/L or hydrolysates from cod in the concentration of 0.33 g/L were then added to the cultures. After culturing for 24 hours, samples were collected and the same concentration of the peptides added to the cultures again prior to further growout for approximately 8 hours (32 h *Artemia*). The experimental design is shown in Figure 5.
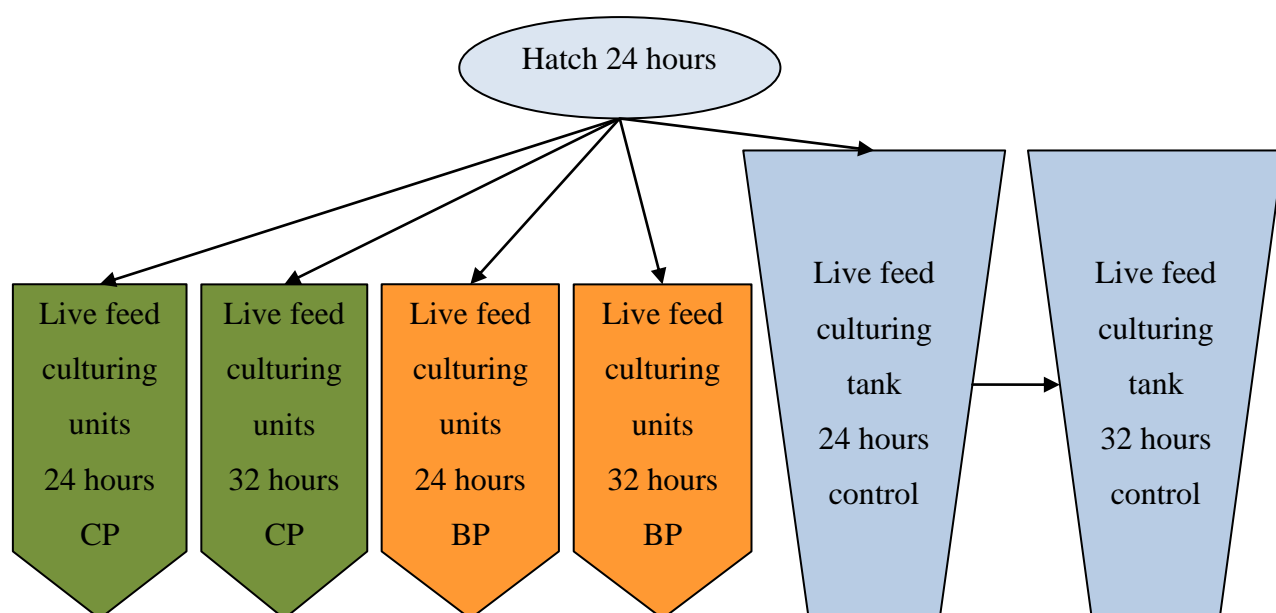
Figure 5. Culturing and enrichment of the live feed using blue whiting (BP) or cod peptides (CP). For the 24 hour cultures of Artemia*, the peptides were added to the culture only once and repeatedly for the 32 hour cultures, after culturing for 24 hours..*

In the experiments using chitosan and pollock peptides (PP), the *Artemia* eggs were hatched and then enriched with chitosan (0.2 g/L) or PP (0.02 g/L) by adding the peptides to the the fatty acid emulsion prior to offering to the *Artemia*. The PP enriched *Artemia* was offered to the larvae in the first of two daily feedings and the chitosan enriched *Artemia* in both daily feedings. The experimental design is shown in Figure 6.
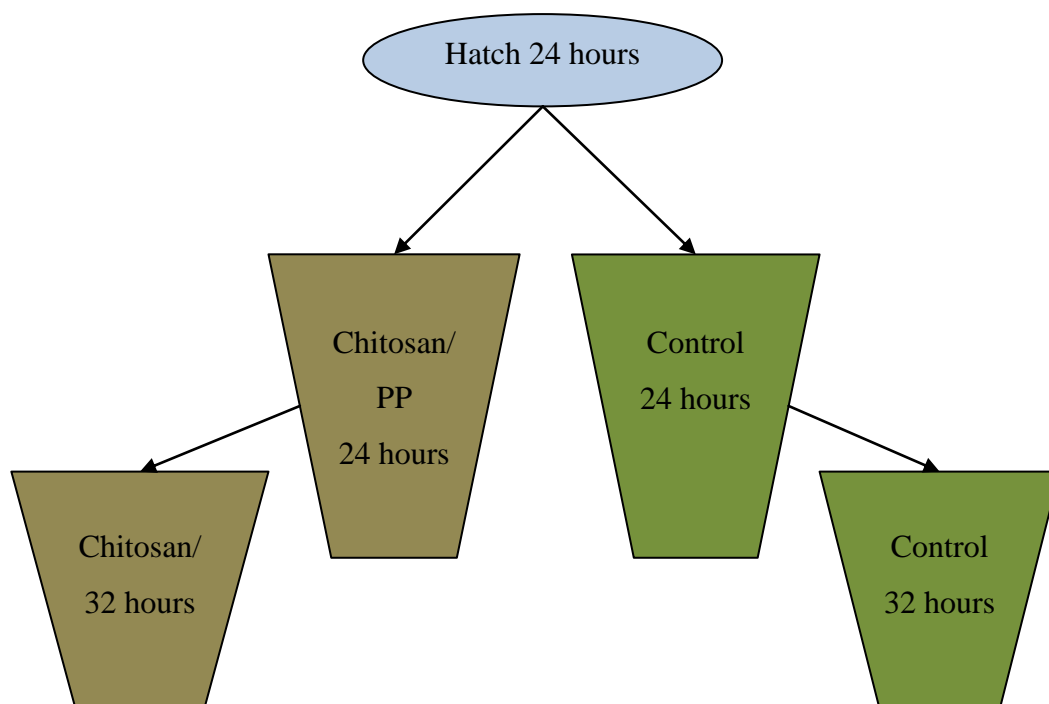
Figure 6. Culturing of the live feed and enrichment using chitosan or pollock peptides (PP). The newly hatched Artemia *were grown at 30.°C for 24 hours (24 h* Artemia*). Approximately 2/3 of the cultures were then transferred to new containers and grown further for 8 hours at 15°C (32 h* Artemia*). For the 24 hour cultures of* Artemia*, the peptides were added to the culture only once but repeatedly for the 32 hour cultures, after culturing for 24 hours.*

2.3.2.   Treatment of first feeding larvae

Three separate experiments were carried out with treatment of first feeding larvae using four different bioactive products. The effects of the treatments were evaluated with respect to stimulation of selected immunological parameters as well as with respect to the overall success of the treated larvae compared to larvae in the control tank units.

Experiment 1: The larvae in this experiment all originated from the same silo, collected to a 3.500 L tank at the onset of feeding at approximately 50 dph. The larvae were then transferred to the experimental tank units (100 L) at 34 dpff (96-184 larvae/tank) and were given four days to acclimatize before the treatment was started at 38 dpff. The experiment was carried out in duplicate, with untreated larvae in two tanks (control group), larvae in two tanks treated with blue whiting peptides through the live feed (0.02 g/L), larvae in two tanks treated with cod peptides through the live feed (0.33 g/L) and larvae in two tanks treated with cod peptides added to the environment (0.033

g/L) (Figure 7). After treatment for 6 days, the concentration of the cod peptides added to the tank water was reduced to 0.0165 g/L due to formation of excess organic wastes. The larvae were fed treated *Artemia* in both daily feedings until 66 dpff and samples collected at 34 dpff, 42 dpff, 48 dpff and 60 dpff.
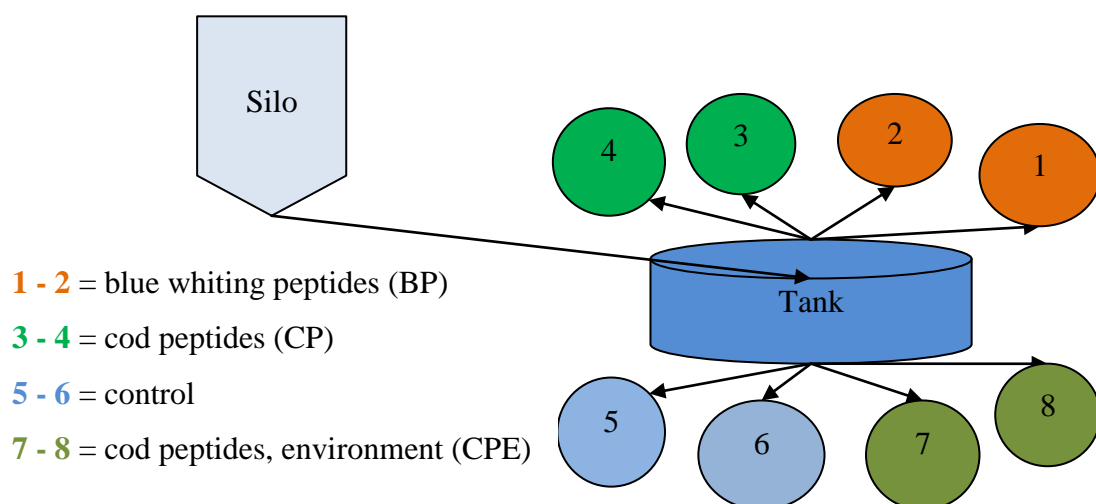
Figure 7. The experimental setup of larvae treated with peptides derived from blue whiting (BP), cod (CP) and cod peptides through the environment (CPE). All larvae used in the experiment originated from the same silo (sibling tank units).

Experiment 2: The experiment was carried out in 3.500 L production units. The treatment was started immediately after transferring of yolk sac larvae to first feeding tanks (0 dpff) and was carried out towards the end of the first feeding period (49 dpff). The larvae were treated with chitosan through the live feed (0.2 g/L) in both daily feedings during the first four weeks of feeding (0–28 dpff). Feeding chitosan enriched live feed in both daily feeding was obviously too much as the feeding activity of larvae was evidently reduced after four weeks of feeding. Control *Artemia* was therefore fed between 29–33 dpff while the larvae recovered and the chitosan treatment then continued from 34 dpff onwards in only one of the two daily feedings (the morning feeding). The treated larvae and the control larvae originated from the same silo (sibling tank units). Furthermore, an additional tank unit was used as a control (Figure 8). Samples were collected weekly throughout the period, starting at 0 dpff and the last sample collected at 49 dpff.
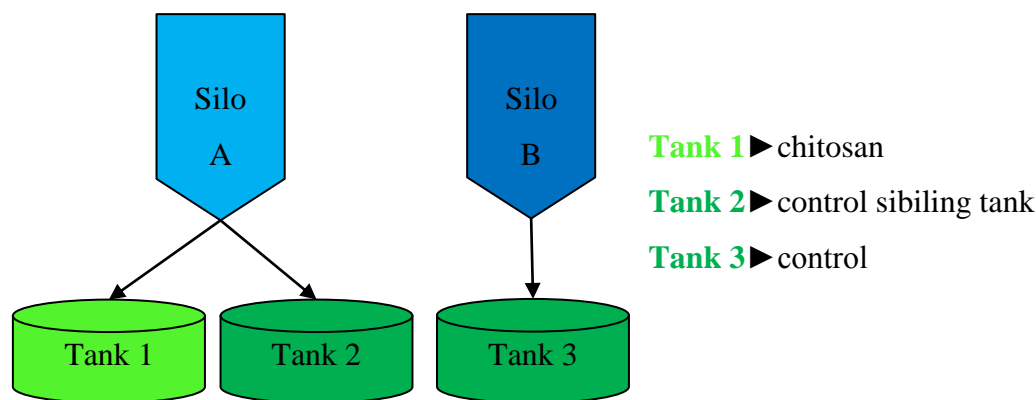
Figure 8. The experimental design for the chitosan treatment of first feeding larvae.

Experiment 3: The experiment was carried out in 3.500 L production units of first feeding larvae. The treatment with the bioactive products was started the day after the transfer of the yolk sac larvae to first feeding tanks and carried on throughout the first feeding period (1-57 dpff). The larvae in the experimental tank unit and the control tank unit originated from the same silo (sibling tank units). The larvae were treated with PP through the live feed in one of the two daily feedings (0.02 g/L) and samples were collected weekly throughout the period. The experimental design is shown in Figure 9.
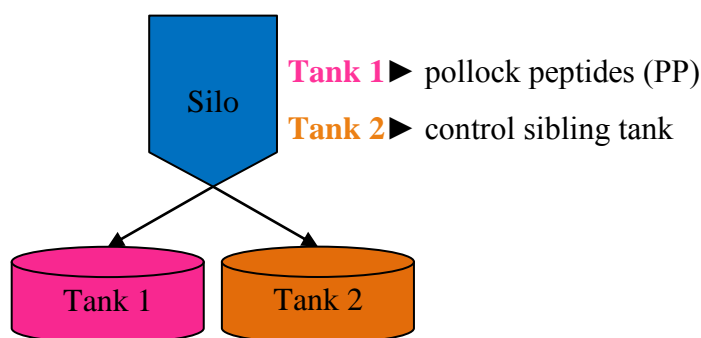
Figure 9. The experimental design for first feeding larvae treated with hydrolysate derived from pollock (PP)

2.4.   Sampling and preparation of samples

Larvae were collected using disinfected pocket net and then transferred to a sterile jar filled with water from the respective tank. A group of approximately 100 (0 dpff) to

15 (at the end of first feeding) larvae were sampled at weekly intervals throughout the first feeding period. Samples were transported on ice to the laboratory where they were processed within four hours of collection.

The larvae were killed with an overdose of the anaesthetic agent (Hypnodil; 51μg/ml final concentration) which was added to the jars, let stand for 2-5 minutes and the larvae then separated from the solution by pouring the content of the jar through a sterilized sieve.

For the immunohistochemistry analysis, 5 larvae from each sample in experiment 3 (PP treated and untreated larvae) were embedded in a plastic tube (15mm x 30mm) and then covered in TissueTek medium. The blocks were snap frozen in liquid nitrogen for approximately 15 sec and then stored at -80°C for later analysis.

For the enzyme linked immune sorbent assay (ELISA), western blot (WB) and protein analysis, the larvae were surface sterilized using 0.1 % v/v benzalkonium chloride and then rinsed three times in sterile solutions of 1% NaCl. The larvae were then enumerated and weighted into a sterile jar and a sterile solution of peptone seawater (0.1 % w/v) added up to a tenfold dilution. The larvae were then homogenized for 3x10 sec at 8000 rpm with 10 sec intervals (Ultra-Thurrax). Two 1 ml aliquots were then pipetted into eppendorf tubes and frozen at -80°C for later analysis.

To make sure that tissue and cell membranes were sufficiently homogenized, the thawed samples were sonicated prior to further analysis. The microtip of the sonicator was then placed in the middle of the eppendorf tube and without touching the tube walls. The microtip was set on pulse for 1 second and pulse off for 1 sec for at total of 59 sec. In order to avoid excess heating; the samples were kept on ice during sonication. The samples were then centrifuged at 1000 rpm for 10 min in order to precipitate larger proteins and tissue remains. The liquid phase was collected and the samples were then ready for further analysis using ELISA, WB and protein analysis.

2.5.   Sandwich ELISA

The 96 well microtiter plates (Nunc) were coated with unlabeled antibody, rabbit anti-halibut IgM, diluted in carbonate buffer (20 μg/mL). The antibody solution (100

μL) was then added to each well and the plate incubated overnight at room temperature (RT) in a humidified chamber to allow complete binding of the antibody and to avoid evaporation. The remaining sites for protein binding were then saturated by incubation with 2 % bovine serum albumin diluted in phosphate buffered saline (PBSA) with 0.05% Tween20, 200 μL/well for 1 hour at RT. The sample solution were further diluted using 0.2 % PBSA/0.05% Tween20, 100 μL then added to each well and the plates incubated for 2 h at RT in a humidified chamber. The capture antibody, mouse anti halibut IgM, was diluted 1:1000 in 0.2 % PBSA/0.05%Tween20. A 100 μl of the solution were added to each well before incubation for 2 h at RT in a humidified chamber. A 100 μl of the labeled secondary antibody (goat anti-mouse IgG; Sigma A-1902), diluted to the appropriate concentration (1:1000) using 0.2 % PBSA/0.05% Tween20, were added to each well and the plates incubated for 2 hours at RT in a humidified chamber. Finally, 100 μl of the substrate solution were added to each well (1mg/ml p-nitrophenyl phosphate diluted in substrate buffer) and the optical density at 405 nm read after incubation for 60 min at RT, using a Thermo multiscan Ex ELISA plate reader. Between every step, the wells were washed three times using PBS/0.05% Tween20 solution. All samples were run in quadruple and individual samples tested undiluted as well as in a tenfold dilution using 0.2 % PBSA/0.05% Tween20. For negative control, 100μl of 0.2% PBSA/0.05% Tween20 was added to the wells instead of the capture antibody and halibut IgM was used as a positive control on every plate (0.024 ng/mL – 1.56 ng/mL)
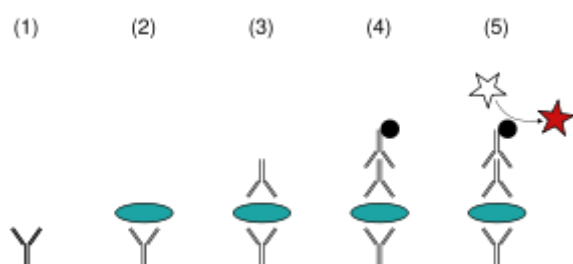
Figure 10. A schematic figure showing the different steps of a sandwich ELISA: antibody coating (1), saturation (2), capture antibody (3), labeled secondary antibody (4) and a substrate reaction (5).

To calculate and adjust the concentration of protein in the samples, a protein standard was prepared according to the Bradford method of protein quantification using Bradford reagent (Sigma B6916). The protein concentration of the antibodies was

adjusted to 20µg/ml. The positive control (halibut IgM) was tested in twofold dilutions of 1:1.000 – 1:1.024.000 and the protein content calculated based on the standard curve made of BSA.

2.6. Immunohistochemistry (IHC)

The samples were cryosectioned using Leica CM 1800, producing 8-10 µm thick sections. The sections were collected onto polysine microscope slides (Menzel gläser, Art.no.J2800AMNZ), the first one right after sectioning past the eyes. Three sections in a row were arranged one on each slide and the next 10 sections discarded before collecting the next three sections on the same slides. Sections were also collected in between for negative controls. The process was repeated until cutting to the distal end of the digestive system when only the muscle and no intestines appeared in the sections. The slides were dried for one hour and then stored at -80°C until stained.

The immunohistochemistry was carried out using horse radish peroxidase (HRP) and fluorescent conjugated secondary antibodies. The primary antibody was tested in various dilutions and the 1:100 dilutions then selected for analysis of all samples. The tissue sections mounted on the polysine coated microscope slides were thawed and warmed up to RT before fixing in ice cold acetone for 10 minutes and then stained.

Using HRP labeled secondary antibody, sections were washed in PBS after fixation and then incubated for 15 minutes at RT in a 3% solution of H_2O_2 diluted in PBS in order to block endogenous peroxidase activity. The sections were then washed in PBS and active sites for protein binding then blocked using 10% normal goat serum (Sigma G9023) diluted in PBS/0.05% Tween20 for 30 minutes at RT before washing in PBS/0.05% Tween20. The sections were then incubated with the primary antibody (rabbit anti-halibut IgM, mouse anti-halibut C3 or rabbit anti-cod lysozyme diluted in a solution of 0.2 % normal goat serum in PBS/0.05% Tween20) for 1 hour at RT in a humidified chamber. The sections were washed in PBS/0.05% Tween20 and then incubated with the HRP labeled secondary antibody solution (goat anti- rabbit Ig, Dako P0161 and rabbit anti-mouse Ig, Dako P0448) diluted in 0.2% normal goat serum in PBS/0.05% Tween20 for 1 hour at RT in a humidified chamber. The sections were

washed in PBS/0.05% Tween20 and then incubated in Na-acetaat buffer for 10 minutes at RT in a humidified chamber. Excess liquid was then poured off and the sections incubated in a solution of 3-amino-9-ethyl-cabazole (AEC) for 10 minutes. The sections were then rinsed with distilled water, counterstained with hematoxylin for 10 seconds and then rinsed again with running tap water for 10 minutes. Red color represents a positive reaction and the nuclei will stain blue.

The sections analyzed using fluorescent labeled secondary antibody were air dried for 30 minutes after the fixation. The sections were washed in PBS-Tween20 and then incubated in a solution of 10% normal goat serum diluted in PBS/0.05% Tween20 for 30 minutes to block non-specific binding of the immunoglobulins. The sections were then washed prior to incubation in a solution of the primary antibody (rabbit anti-halibut IgM, mouse anti-halibut C3 or rabbit anti-cod lysozyme) diluted in a 0.2 % solution of normal goat serum in PBS/0.05% Tween20 for 1 hour at RT in a humidified chamber. The sections were washed in PBS-Tween 20 and then incubated with a solution of fluorescent labeled secondary antibody, goat anti-rabbit Ig (Sigma F-0382) or rabbit anti-mouse Ig (Sigma F-0257) diluted in PBS/0.05% Tween20 for 30 minutes at RT in humidified chamber. From this step on, the sections had to be protected from light by covering the chamber with aluminum foil. The sections were then washed with PBS-Tween 20 for 3x2minutes.

Finally, all sections were mounted using Clarion Mounting Medium (Sigma, USA) and analyzed using Leica DMRA2 microscope with pictures taken using Leica DC300F digital camera. Both staining methods were used for the analysis of both small (20-29dpff) and larger (43-50 dpff) larvae. However, the response using the HRP method was found to be too weak for analyzes of the small larvae and the response using the fluorescent method too intensive for analyzes of the larger larvae. Consequently, the fluorescent labeling is presented for the smallest larvae only and the HRP staining for the larger larvae only.

2.7. Western blotting

All gels were run in duplicate, with proteins in one gel electrophoretically transferred to a nitrocellulose membrane and stained using specific antibody while the proteins on the other gel were stained using Coomassie brilliant blue (CBB).

The appropriate amounts of a sample buffer were added to each sample (1μl buffer: 4μl sample) and the samples then heated at 100°C for 5 minutes for denaturation of the proteins before loading onto the gels. The proteins in the samples were then separated by electrophoretic transfer in a 12 %SDS-PAGE gels (BioRad, 161-1102) using a constant current of 100mA for 4 hours. A size marker (BenchMark^{TM}Protein ladder, 10747-012), a possitive control (purified C3 or IgM) and a negative control (buffer) were run on each gel. The proteins in the gel were made visible by incubating the gels in a 0.1% aqueous solution of Comassie Brilliant blue. The proteins in the gel were then electrophoretically transferred onto a nitrocellulose membrane using BioRad-Trans-blot SD and using a constant current of 1.9-2.5mA per cm^2 of gel are for 60 minutes. Non-specific binding was then blocked by incubating the membrane in a 2% solution of BSA in 10% tris buffered saline (TBS) for 2 hours using mild rotation. The membranes were then incubated in a solution of primary antibody (rabbit anti-halibut IgM or mouse anti-halibut C3) diluted in 0.2% bovine serum albumin in TBS (TBSA) on rotation over night at RT. The membranes were then incubated in a solution of alcalic phosphate labeled secondary antibody (goat anti-rabbit Ig or rabbit anti-mouse Ig, Sigma) diluted in 0.2% TBSA, on rotation for 1 hour at RT. Then the membranes were incubated in alkalic phosphate (AP) substrate buffer before alkalic phosphatase activity was developed in a solution of AP-substratbuffer containing 7.5 mg/mL final concentration of nitroblue tetrazolium chloride (NBT) and 165 mg/mL final concentration of 5- bromo-4-chloro-3-indolyl phosphate p-Toluidine salt (BCIP). In between each step, the membranes were washed for 3x10 minutes in a solution of 0.05% Tween 20 in TBS under mild rotation.

2.8.   Protein analysis

The protein content of the samples was analyzed using the Experion ^{TM} Pro260 (BIORad 700-7000) which is an automatic electrophoresis station analysing both the total concentration as well as the size distribution of proteins (10-260 kD) of a sample. Each analysis kit contains a single use chip and the reagents required to perform the protein electrophoresis. All kit reagents were mixed according to the manufacturers´ recommendations (BioRad, 700-7000). The samples were prepared by adding 2 μl of a sample buffer to 4 μl of samples in a 0.5 ml microcentrifuge tube, vortex briefly and the solution then microcentrifuged at 10.000 x g for a few seconds. The blank was prepared the same way, using 4 μl of phosphate buffered saline (PBS) instead of a sample. The sample tubes and the Pro260 ladder were then heated at 95–100°C for 3–5 min, cooled down and microcentrifuged for 15 sec at 10.000 x g. Then 84 μl of distilled H_2O were added to each tube which then were vortexed briefly and the samples then ready to be loaded onto the chip. In each chip there are 10 wells for samples, four wells for the gel stain, one well for the gel and one well for the ladder. Priming and loading of the chip were performed as described in the manual from the manufacturer (BioRad, 700-700).

2.9.   Solutions

Carbonate buffer:

1.59g Na_2CO_3,

2.93g $NaHCO_3$

Fill up to 1000 ml with distilled H_2O (dH_2O) and pH then adjusted to 9.6 using 2 M HCl

Phosphate buffered saline (PBS) (stock solution):

53.72 g Na_2HPO_4,

7.72 g NaH_2PO_4

85 g NaCl

Fill up to 1000 ml with dH_2O, mix to dissolve completely and pH then adjusted to 7.4 using 2 M HCl. The stock solution is diluted tenfold before use.

Washing-buffer: PBS containing 0.05% Tween 20

1% PBSA: PBS containing 1% Bovine serum albumin (BSA)

0.2% PBSA: PBS containing 0.2% BSA

Substrat buffer:

97 ml (1M) dietanolamin added to 900 ml of dH_2O and pH adjusted to 9.8 using 6M HCl.

100 mg of $MgCl_2$ * 6 H_2O mg (0.005M) is added and the solution then stored in a dark bottle at $4°C$ *(in the dark)*

3% Hydrogen Peroxide: 10 ml of 30% H_2O_2 added to 90 ml PBS

Blocking Solution: 10% w/v normal goat serum diluted in PBS/0.05% Tween20

NA-acetaat buffer: 6.8 g Na-acetat added to 1 L d H_2O and bring pH to 5 using acetic acid (2 M)

AEC (stock solution):

    260 mg AEC (3-amino-9-ethyl-carbazole)

    15 ml DMF (N<N-dymethylformamide)

    Stored in portions of 750 µl at -20°C

AEC substrate solution for use:

    32.5 ml Na-acetaat buffer

    750 µl AEC stock solution

    16.25 µl 30% H_2O_2

Running buffer (10x stock solution):

    25 mM Tris

    192 mM glycine

Blotting buffer:

A fresh solution is made for each run, containing 100 ml of 10x running buffer, 200 ml 20% Methanol and fill up to 1000ml with dH_2O before adjusting pH to 8.3

Tris Buffered Saline (TBS) (stock solution):

    100mM Tris,

    0.9% NaCl

Fill up to 1000ml with dH_2O and pH adjusted to 8.3. The solution is stored in a dark bottle that is carefully covered from light by wrapping in an aluminium foil.

Alkaline Phosphatase (AP) Substratbuffer (stock solution):

    0.1 M Tris,

    0.1 M NaCl,

    50 mM $MgCl_2$

pH is adjusted to 9.5 and stored at 4°C (for maximum 30 days).

AP substrate solution (a fresh solution is made for every run):

    10 ml AP substrate buffer

44 µl NBT (Nitroblue Tetrazolium Chloride). A stock solution is made, containing 7.5 mg/ml of NBT dissolved in dH_2O and stored in 500µl aliquotes at -80°C.

33 µl BCIP (5-Bromo-4-chloro-3-indolyl-phosphate p-Toluidine salt). A stock solution is made, containing 165 µg/ml BCIP dissolved in 100% dymethylformamide (DMF) and stored in 500µl aliquots at -80°C.

3. Results

Selected bioactive products were used for treatment of halibut larvae during first feeding. The larvae were either treated through the tank water environment or through enrichment of the live feed before offering to larvae in one or both of the two daily feedings. The bioactive products selected were chitosan G060703 and peptide hydrolysates of cod, pollock and blue whiting muscle. The effects of the treatments were evaluated with respect to IgM, C3 and lysozyme in homogenates of larvae as well as in thin cross-sections of larvae, using monoclonal antibodies and the ELISA method or immunohistochemistry. The survival, growth and quality of larvae within each group were evaluated at the end of the first feeding period.

3.1.   Enrichment of *Artemia* using bioactive products

A number of pre-trials were carried out, studying the effects of various concentrations of blue whiting and cod peptide hydrolysates during culturing of *Artemia*. The results showed that the various concentrations of the bioactive products tested did not affect the quality of the *Artemia* measured as mobility, color and growth of the live feed. The method and timing of treatment of the *Artemia* was also examined and the results indicated that adding the products to the environment of the live feed, may lead to increased numbers of cultivable bacteria in the *Artemia* cultures. The analysis of the bacterial community was not a part of this work and the results therefore not shown or discussed here. Adding the bioactive products to the fatty acid and vitamin mixture used for enrichments of the live feed, proved to be easily implemented and in experiments 2 and 3, the bioactive peptides were therefore added to the fatty acid enrichment medium prior to emulsification of the mixture as this method of treatment may be expected to carry products effectively to the larvae. The treatment of the larvae using 0.33 g/L of the cod peptide hydrolysate resulted in a total collapse of larvae in

both tanks, but the concentrations used did not affect the quality of the live feed. There were, however, indications that the *Artemia* fed fatty acid enrichment medium containing pollock peptides seemed to grow larger compared to feeding the fatty acid enrichment medium alone (result not shown).

3.2. Experiment 1 – Treating larvae with peptide hydrolysates from blue whiting and cod

The larvae were treated from 34 dpff to 60 dpff, either through the tank water environment or through treatment of the live feed. The effects of treatment were evaluated by measuring the concentration of IgM in homogenates of ~30 (34 dpff) to 10 (65 dpff) larvae at individual samplings. The results are presented as mean values of two tanks ±S.D. The effects of treatment were also evaluated with respect to growth and normal development of the larvae. IgM was detected in low concentrations in larvae from 34 dpff, using the ELISA method (Table 1).

Table 1. IgM in larvae at various days post onset of first feeding (dpff) through first feeding (μg IgM/g larvae). A pool of larvae 34 dpff was divided into 8 tanks, with each treatment carried out in duplicate and sampling at various dpff.
BP = blue whiting peptides, CP = cod peptides, CPE = cod peptides through the tank water environment.

	34 dpff	42 dpff	48 dpff	70 dpff
BP		0.003 ± 0.001	0.014 ± 0.001	0.016 ± 0.0
CPE		0.004 ± 0.002	0.013 ± 0.001	0.015 ± 0.001
CP	0.001	0.005 ±0.000		
Control		0.003 ± 0.001	0.013 ± 0.003	0.014 ± 0.001

Similar concentrations of IgM were found in larvae from all groups at the various sampling dates. After treatment for four days, a total collapse of larvae occurred in tanks with the CP treatment and further sampling consequently terminated. The treatments with the BP and CPE were continued to the end of first feeding (60 dpff) and an

additional sampling carried out post the end of the first feeding period (70 dpff). Similar concentrations of IgM were observed in larvae from all groups at the last two samplings, somewhat higher than at 42 dpff.

Treating larvae with 0.1 g/L of BP through the live feed did not affect the growth (Table 2) or survival of the larvae (results not shown). Treating larvae with 0.5 g/L CP through the live feed resulted in a total collapse of larvae in both tanks only 4 days into the experiment (42 dpff). Treating larvae with CP through the tank water (CPE) resulted in elevated amounts of organic wastes in the tank water environment and that may have negatively affected the growth of larvae in this group.

Table 2. The weight of larvae in mg (dry weight) at the beginning and at the end of the experiment (±S.D.). A pool of larvae at 34 days post onset of first feeding (dpff) was divided into 8 experimental tank units, with each treatment carried out in duplicate.
BP = blue-whiting peptide CPE = cod peptides through the environment.

	34 dpff	**70 dpff**
BP		37.0 ± 1.0
CPE	12.8	32.0 ± 1.0
Control		39.5 ± 4.5

Treating with BP resulted in the highest ratio of larvae not metamorphosed at the end of first feeding (29%), considerably higher than in the control group (12.5%) but similar to the CPE group (25%) (Figure 11). The ratio of poorly metamorphosed juveniles was similar in all groups (~11.5 %).
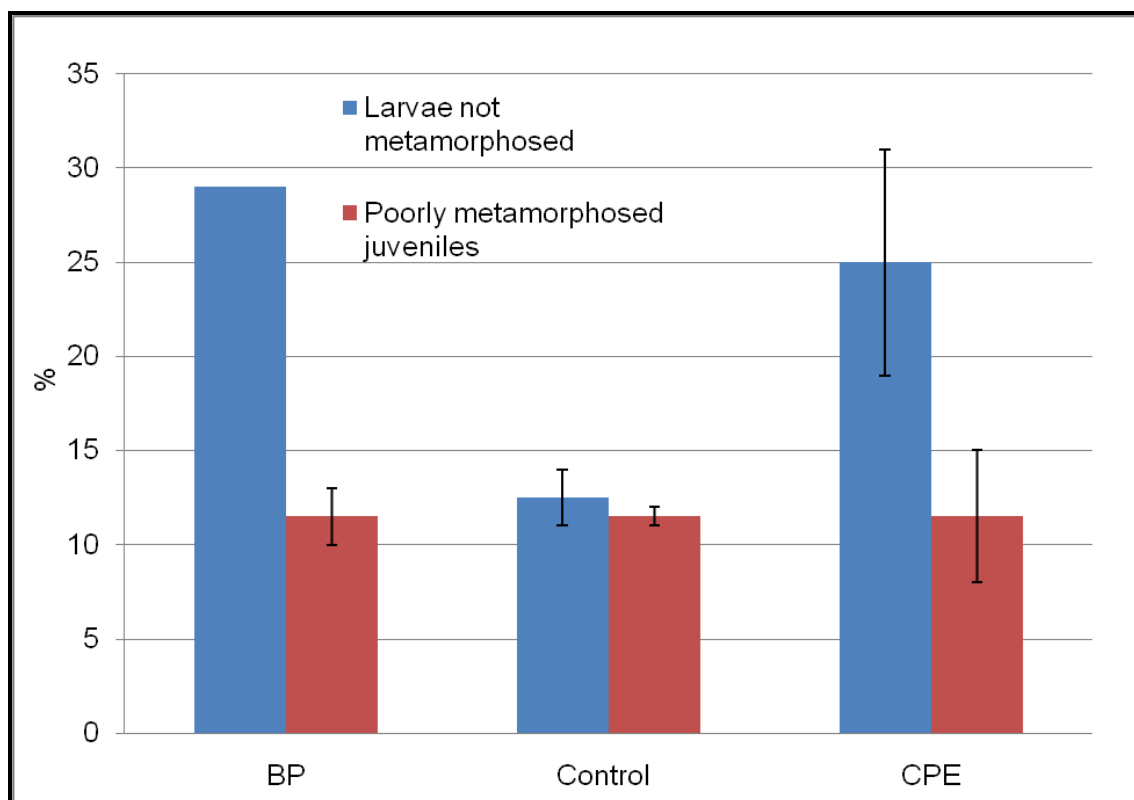
Figure 11. Success of metamorphosis of larvae at the end of the first feeding period. The columns show the mean % of larvae not metamorphosed (blue bars) and poorly metamorphosed juveniles (red bars) in each group (±S.D.). BP = blue whiting peptides, CPE = cod peptides through the environment.

3.3.   Experiment 2 – Treating larvae with chitosan G060703

Larvae were treated from 0 dpff to 49 dpff by adding the product to the fatty acid and vitamin mixture used for enrichment of the live feed prior to offering to larvae. The effects of treatment were evaluated by measuring the concentrations of IgM in homogenates of ~100 (0 dpff) to 10 (65 dpff) larvae at individual samplings. The effects of treatment were furthermore evaluated with respect to growth and normal development of the larvae at the end of first feeding.

Using the ELISA method, IgM was first detected in larvae at 35 dpff (Figure 12) which is approximately the time when the chitosan treated larvae stopped eating.
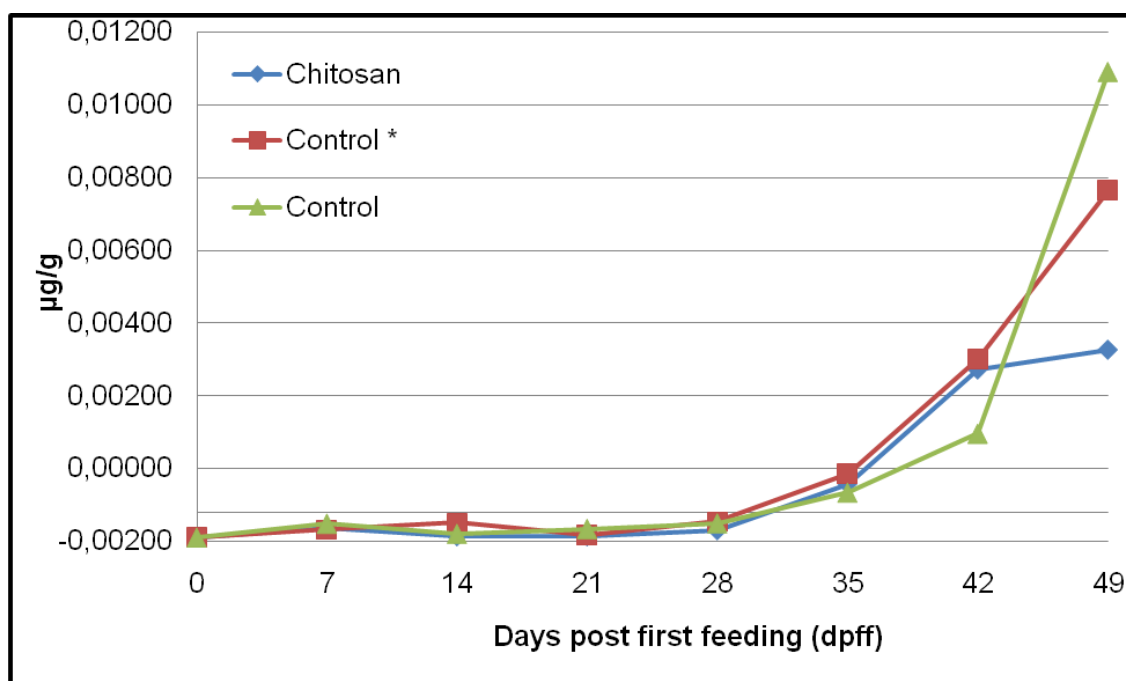
Figure 12. Concentration of IgM in larvae from the onset of feeding and throughout the first feeding period, measured by the ELISA method (µg IgM in each g of larvae). Included is the mean weight of larvae in all tanks during the period, measured at various dpff throughout first feeding.
* Larvae originating from the same silo as the chitosan treated larvae (sibling tank units)

Increasing concentrations of IgM were measured in larvae between 35 dpff and 49 dpff when the experiment was terminated. Similar concentrations were detected in larvae up to 42 dpff, but higher concentration of IgM was detected in the control larvae compared to chitosan treated larvae at 49 dpff. At the end of the experiment, the lowest concentrations of IgM were measured in the chitosan treated larvae. The results indicate that treatment with chitosan negatively affected the concentrations of IgM detected in larvae.

Feeding larvae *Artemia* treated with 0.2 g chitosan in each liter of the live feed cultures seemed to negatively affect the growth and normal development of larvae (Figures 13 and 14). However, larval survival was not affected (result nor shown). The chitosan treatment was discontinued at 29 dpff due to a recess in the feeding activity of the larvae. After a four days recovery period for active feeding to recommence while feeding untreated *Artemia*, the treatment was continued (35 dpff), however, only through one of the two daily feedings. A considerable reduction in weight gain of the chitosan treated larvae was observed during the last week compared to the control

larvae (Figure 13), resulting in the lowest weight of larvae in the chitosan treated group at the end of the experiment.
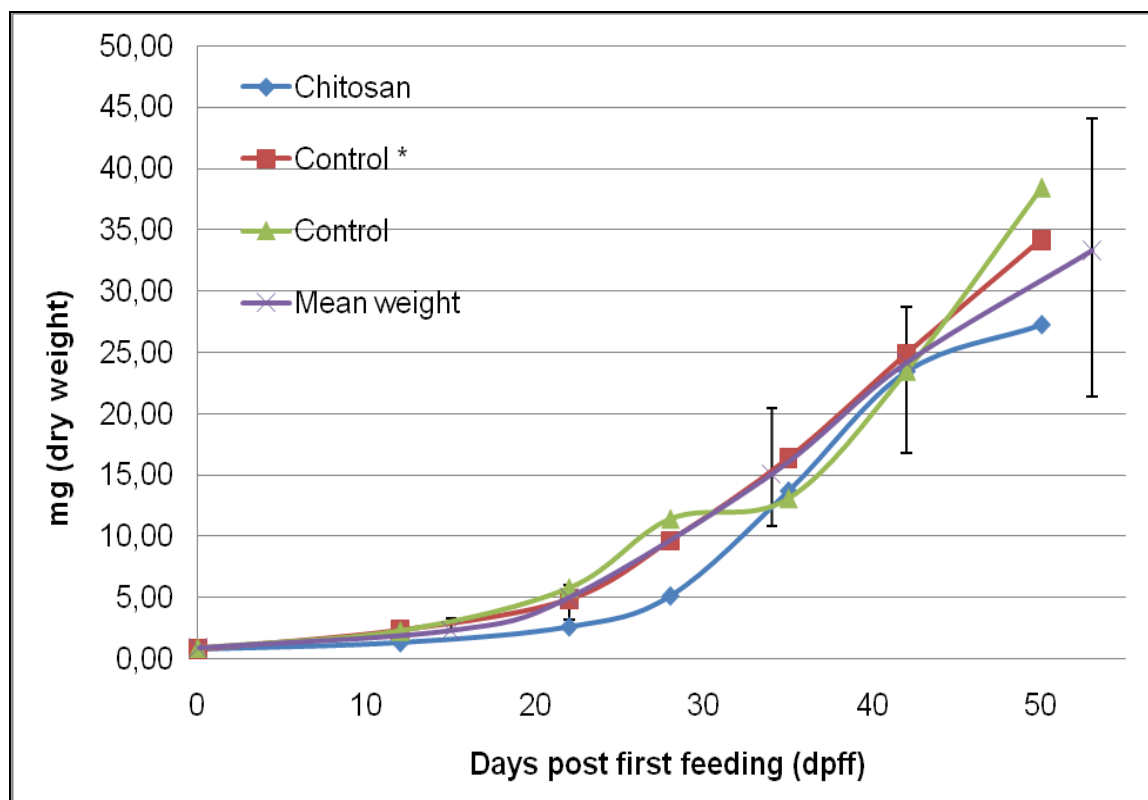
Figure 13. Weight of larvae during the experimental period (0-50 days post onset of first feeding, dpff). The weight was measured in mg dry weight of ~150 (0 dpff) to 15 (~50 dpff) larvae from each tank. Also shown is the mean weight of larvae in all tanks during the period.
* Larvae originating from the same silo as the chitosan treated larvae (sibling tank units).

The ratio of larvae not metamorphosed and poorly metamorphosed juveniles was higher in the chitosan treated group (60% and 30%, respectively) compared to both control groups (Figure 14). The ratio of not metamorphosed larvae varied in the control tanks (24% and 12%) but the percentage of poorly metamorphosed juveniles was similar.
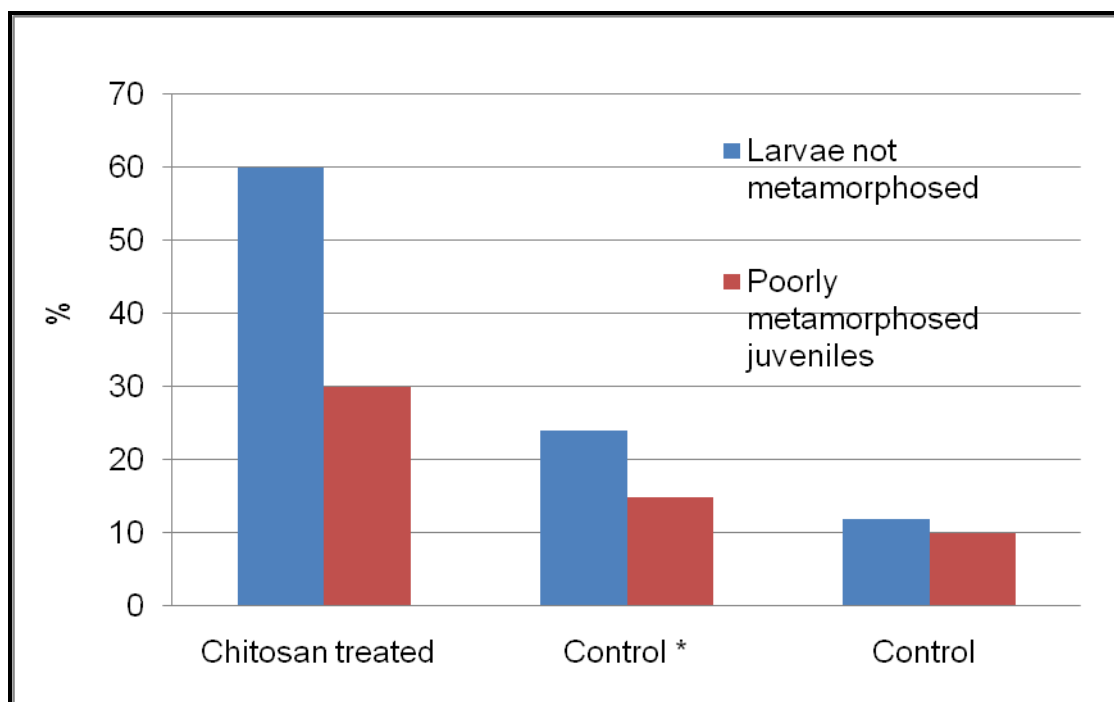
Figure 14. Normal development of larvae at the end of the first feeding period.
* Larvae originating from the same silo as the chitosan treated larvae (sibling tank units).

3.4. Experiment 3 – Treating larvae with pollock peptide hydrolysates

The larvae were treated from 0 dpff to 57 dpff, through treatment of the live feed. The effects of treatment were evaluated by measuring the concentration of IgM in homogenates of ~100 (0 dpff) to 10 (65 dpff) larvae. The presence of IgM, C3 and lysozyme was evaluated using immunohistochemistry studies of a minimum of five larvae from each tank at individual samplings. The results of the fluorescent staining are shown for the smallest larvae only (0-29 dpff), as the response using the HRP staining was very weak and hard to detect. However, the response in the larger larvae (43-50 dpff) was far too strong using the fluorescent staining and the results of the HRP staining therefore only presented. The main emphasis was on the presence of the selected immune parameters within the digestive system of larvae. The location within the digestive system shown in each figure was determined with respect to recognition of organs and cells. The negative controls were from similar locations in the digestive tract as shown in each group of figures with the location based on the number of collected

sections. The effects of treatment were also evaluated with respect to growth and normal development of the larvae at the end of the first feeding period.

3.4.1. Effects of treatment on the presence of IgM in larvae.

Using the ELISA method, low concentrations of IgM were detected in larvae from both groups at the onset of feeding (Figure 15). IgM was not detected in larvae between 8 dpff and 36 dpff when IgM was again detected in larvae from both groups.
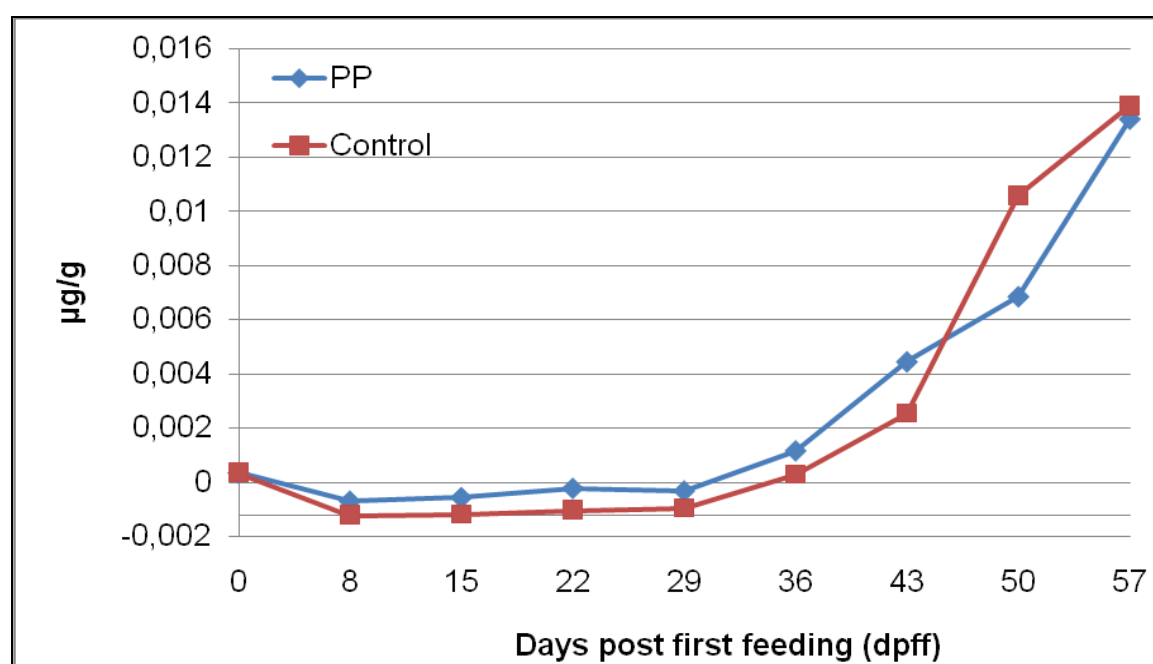
Figure 15. Concentration of IgM in larvae from the onset of feeding and throughout the first feeding period, measured by the ELISA method (µg IgM in each g of larvae). PP = pollock peptides and control larvae originating from the same silo as the treated larvae (sibling tank units).

Increasing concentrations of IgM were measured in larvae during the last three weeks of first feeding. Treatment with PP was not found to affect the measured concentrations of IgM.

IgM was detected in larvae already at the onset of feeding. (Figure 16). A positive response is observed in the kidney, spleen, the muscularis externa of the digestive tract, the connective tissue as well as other cellular layers of the digestive system of the larvae.
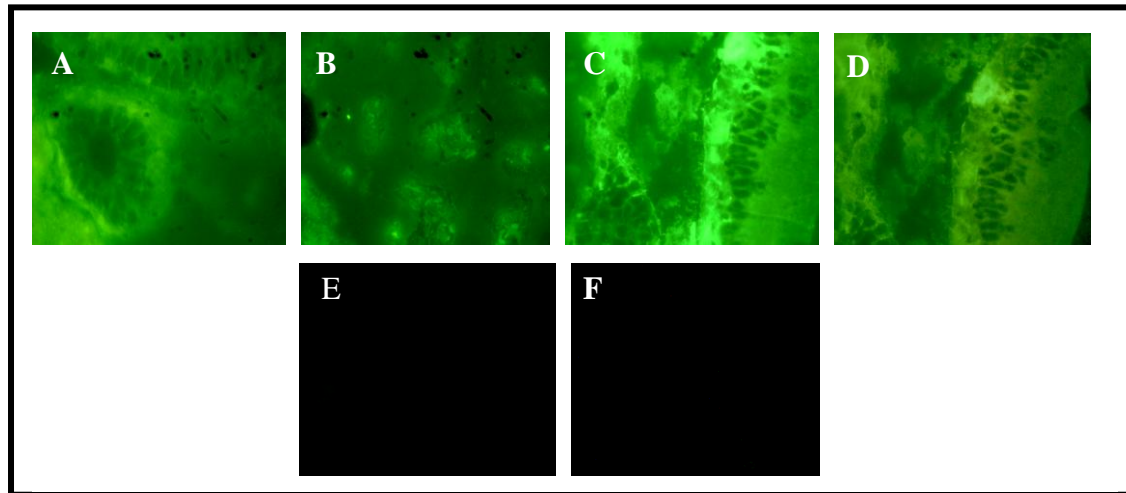
Figure 16. Fluorescent staining of IgM in sections of larvae at the onset of first feeding. The figure shows kidney (A), spleen (B), the digestive system (C and D) and negative controls of larvae at the onset of feeding (E) (1000x magnification).

After eight days of treatment with PP (8 dpff), IgM seems to be more prominent in treated larvae compared to the control larvae (Figure 17). However, no difference is observed with respect to the external layer of mucosa when treated and untreated larvae are compared.

Figure 17. Fluorescent staining of IgM in sections of larvae 8 days post onset of first feeding (dpff). The figure shows the digestive system of treated larvae (A-F), control larvae (G-I) and negative controls of treated and untreated larvae (M and N, respectively) (1000x magnification).

At 29 dpff, no obvious differences in the presence of IgM were detected in the connective tissue beneath the epithelium cells (Figure 18). A slight difference may be detected in the mucosa membrane of the digestive tract when the two groups are compared. There is an indication that IgM may be more prominent in between the epithelium cells in the treated larvae compared with the control larvae. No obvious

differences were detected in the five treated larvae examined, but the presence of IgM was highly variable in the five control larvae examined (Figure 18).

Figure 18. Fluorescent staining of IgM in sections of larvae 29 days post onset of first feeding (dpff). The figure shows the digestive system of treated larvae (A-F), control larvae (G-I) and negative controls of treated and untreated larvae (M and N, respectively) (1000x magnification).

Figure 19 shows the oesophagus, presumably at the junction to the stomach. No obvious differences were observed when the larvae from the two groups were compared. The positive reaction may be slightly stronger in the control larvae but further down the digestive tract the response is quite variable when individual larvae are compared (result not shown). Higher individual variations were observed in larvae from the untreated group compared to the treated group.
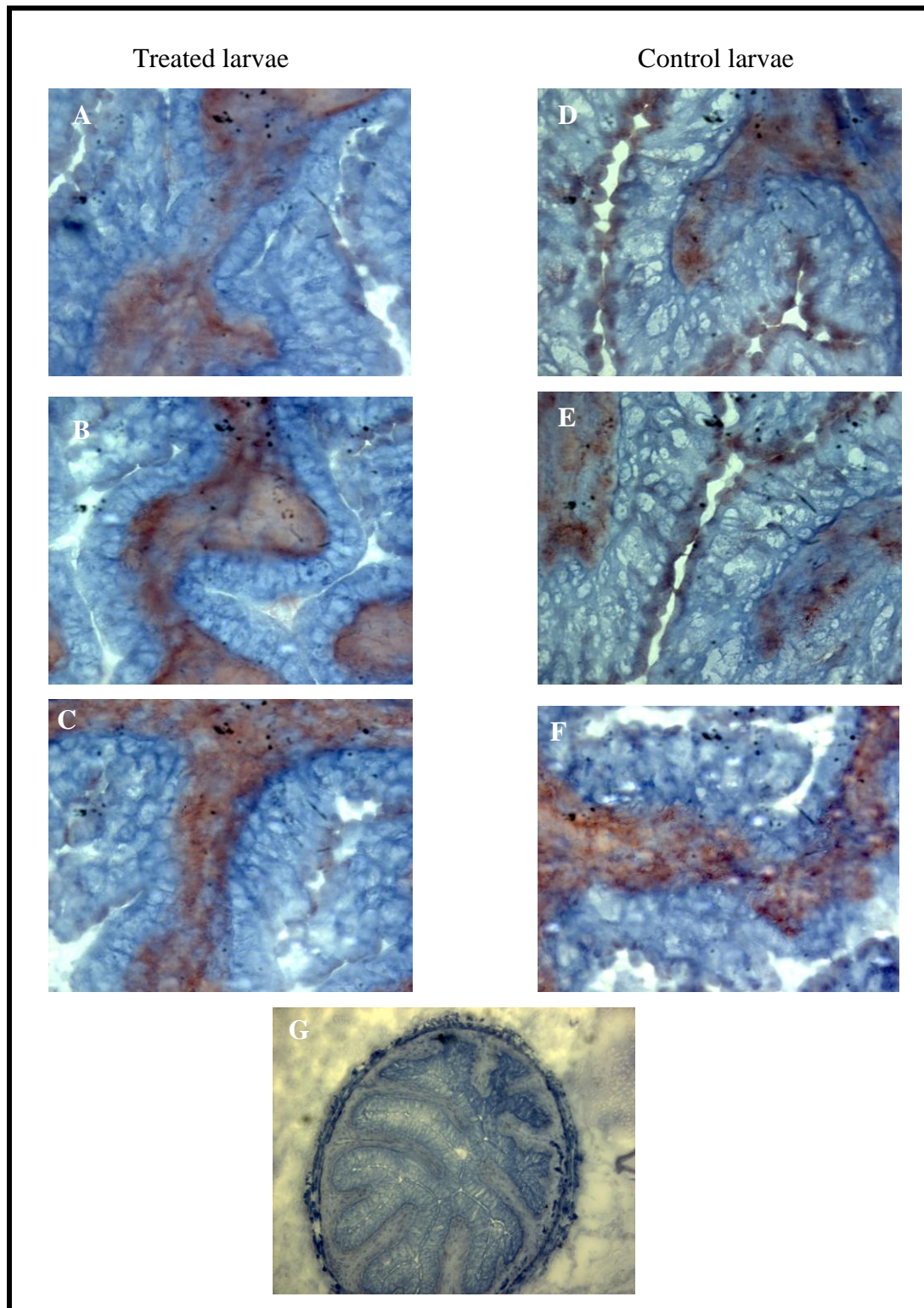
Figure 19. HRP staining of IgM in sections of larvae 43 days post onset of first feeding (dpff). The figure shows the oesophagus of treated larvae (A-C) and control larvae (D-F) (1000x magnification) and a negative control of treated larvea (G) (400x magnification).

A closer look at the intestines reveals similar reaction in the treated and untreated larvae at 50 dpff (Figure 20).

Treated larvae   Control larvae

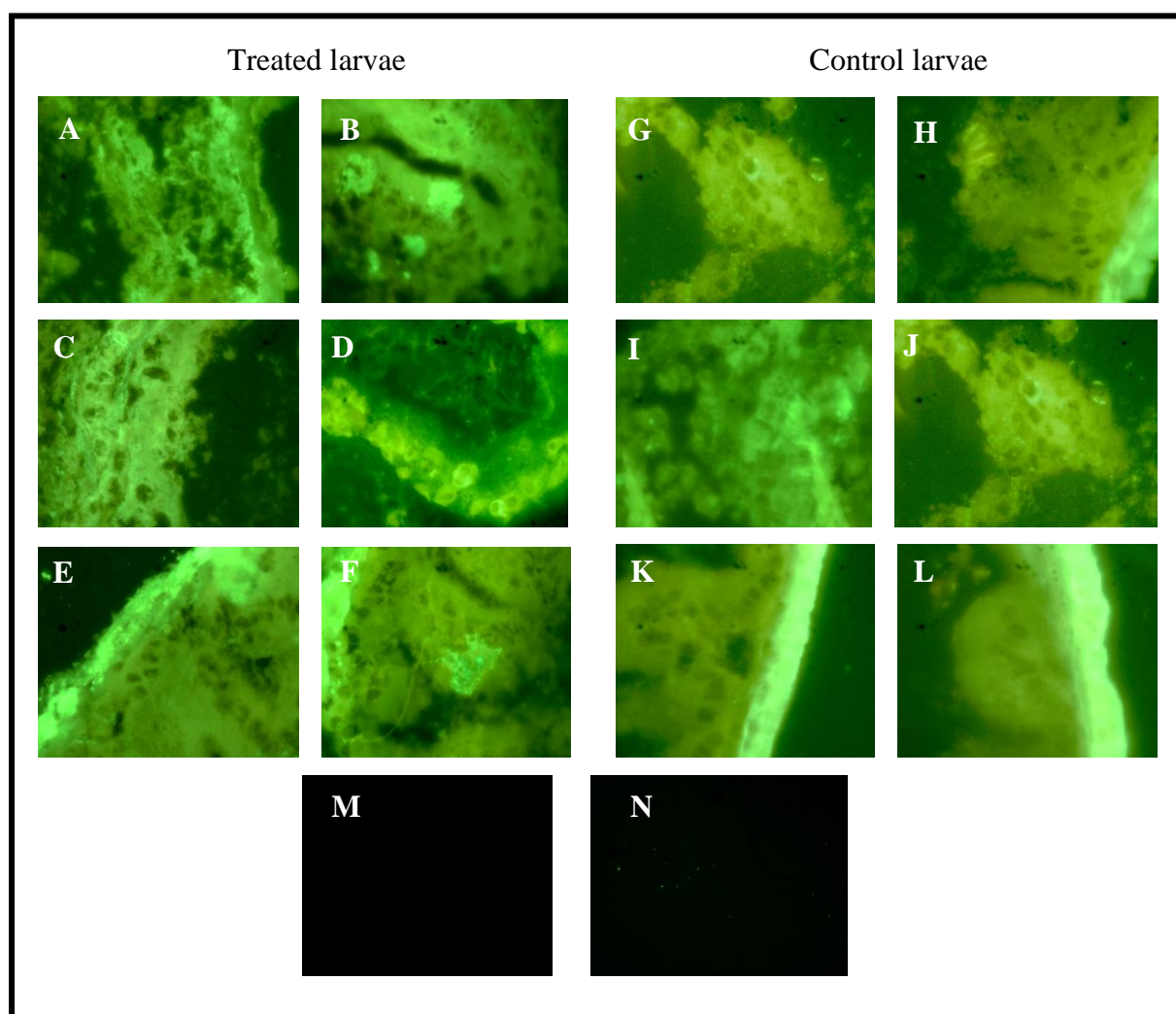*Figure 20. HRP staining of IgM in sections of larvae 50 days post onset of first feeding (dpff). The figure shows the intestines of treated larvae (A-B), 5 control larvae (C-D) and a negative control of treated larvae (E) (1000x magnification).*

3.4.2. *The effect of treatment on the presence of C3 in larvae.*

C3 was present in larvae already at the onset of feeding (Figure 21). A positive response was observed in the digestive system as well as in the kidneys.
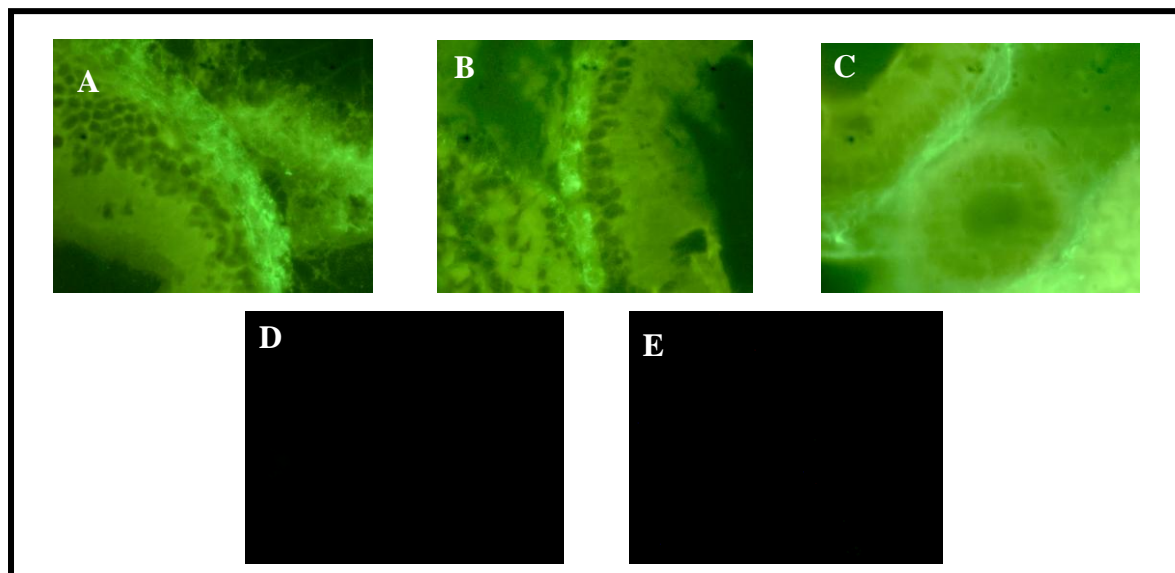
Figure 21. Fluorescent staining of C3 in sections of larvae at the onset of feeding. The figure shows the digestive system (A and B) and kidney (C) of larvae and negative controls of the digestive system and kidney of treated larvae (D and E, respectively). (1000x magnification).

After 8 days of treatment with PP (8 dpff), C3 is more prominent in the digestive system of treated compared to untreated larvae (Figure 22). In untreated larvae, C3 appears to be present only in the connective tissue but a positive reaction may be detected between the epithelium cells of the digestive system in the treated larvae.
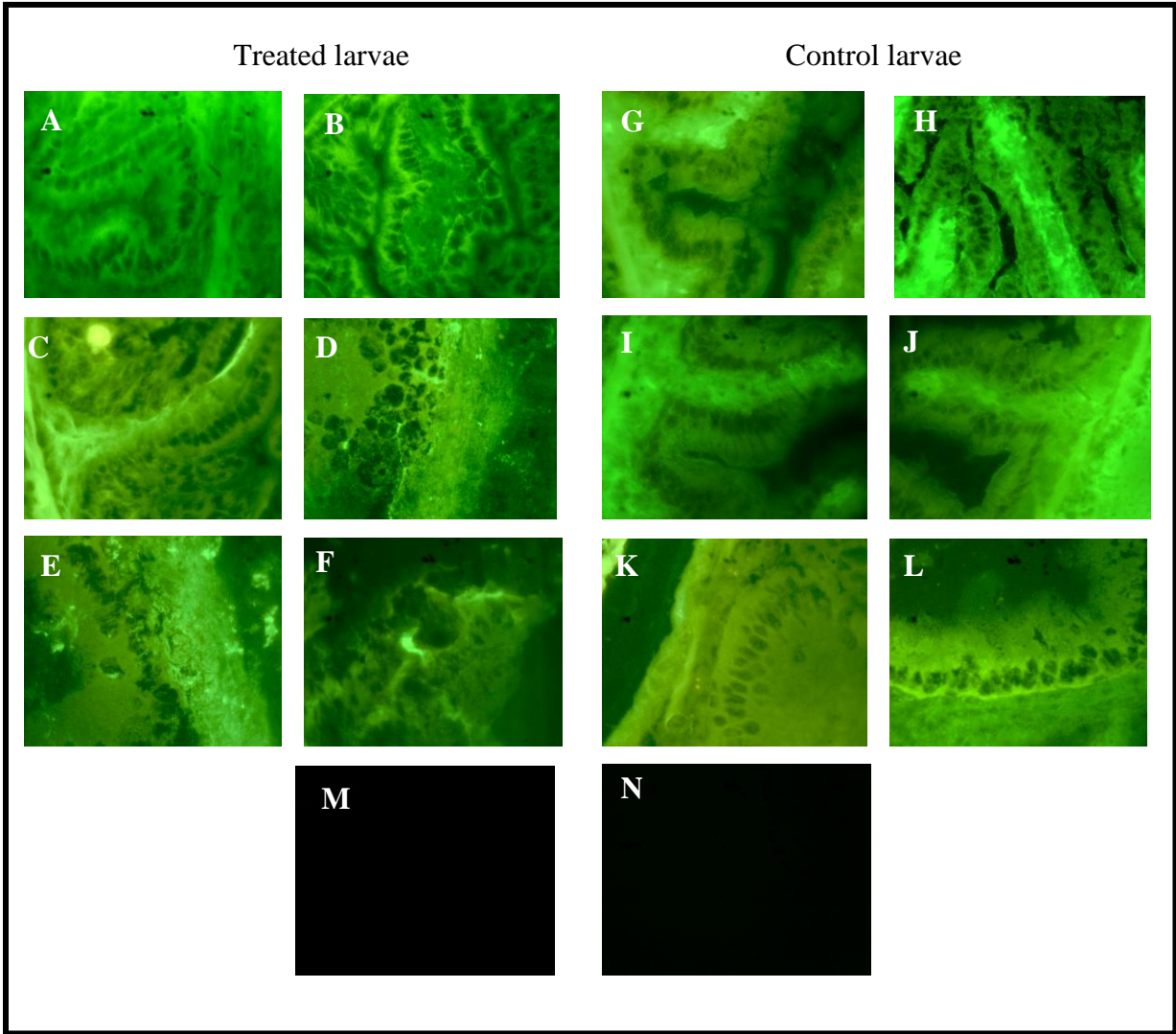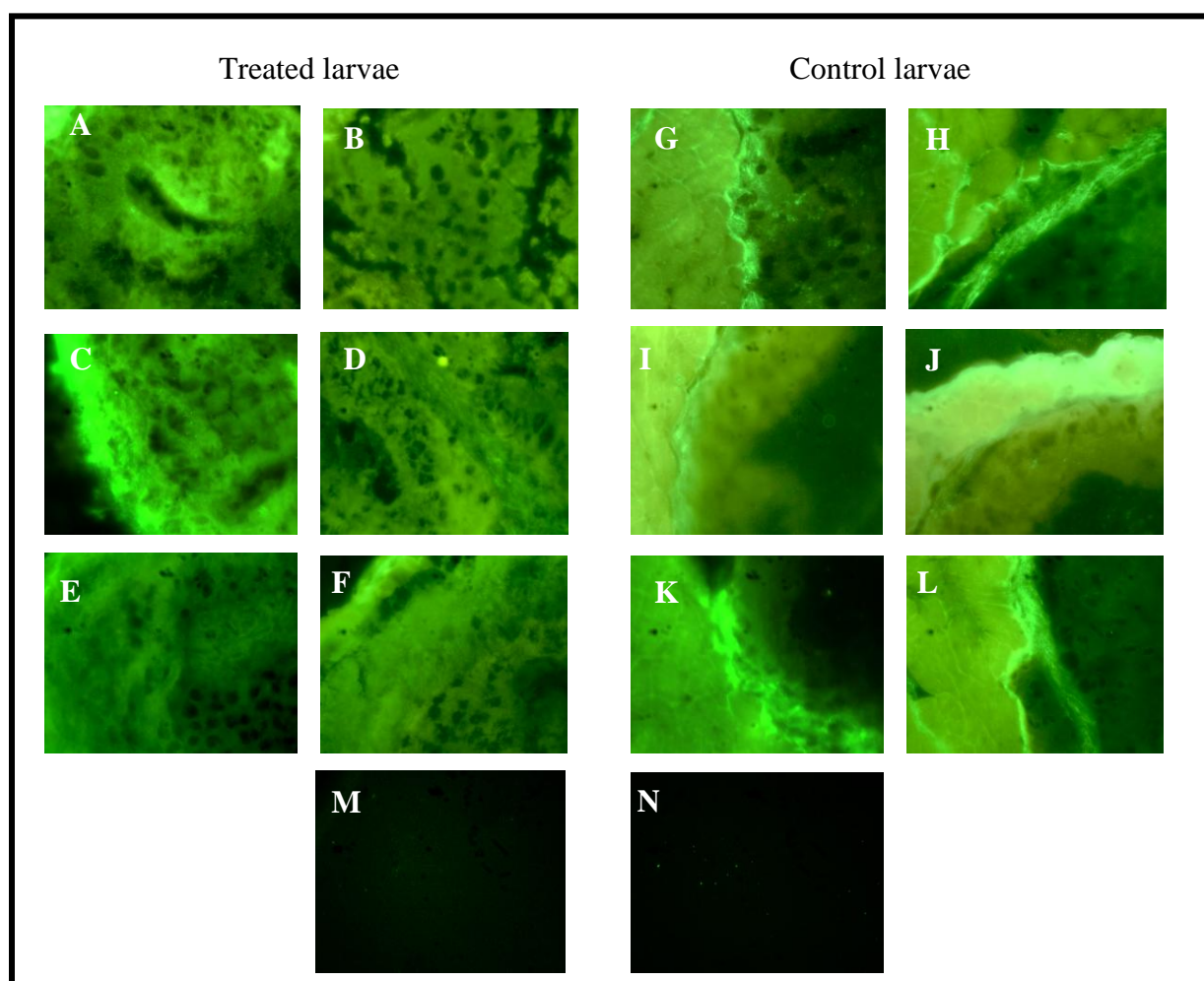

Figure 22. Fluorescent staining of C3 in sections of larvae 8 days post onset of first feeding (dpff). The figure shows the digestive system of treated larvae (A-F), control larvae (G-L) and negative controls of treated and untreated larvae (M and N, respectively). (1000x magnification).

After 29 days of treatment (29 dpff), there are indications that treatment with PP resulted in the stimulation of C3 production in the digestive system of larvae C3 (Figure 23). The positive reaction seems to be stronger in all the treated larvae that were examined compared to the same number of untreated larvae examined.
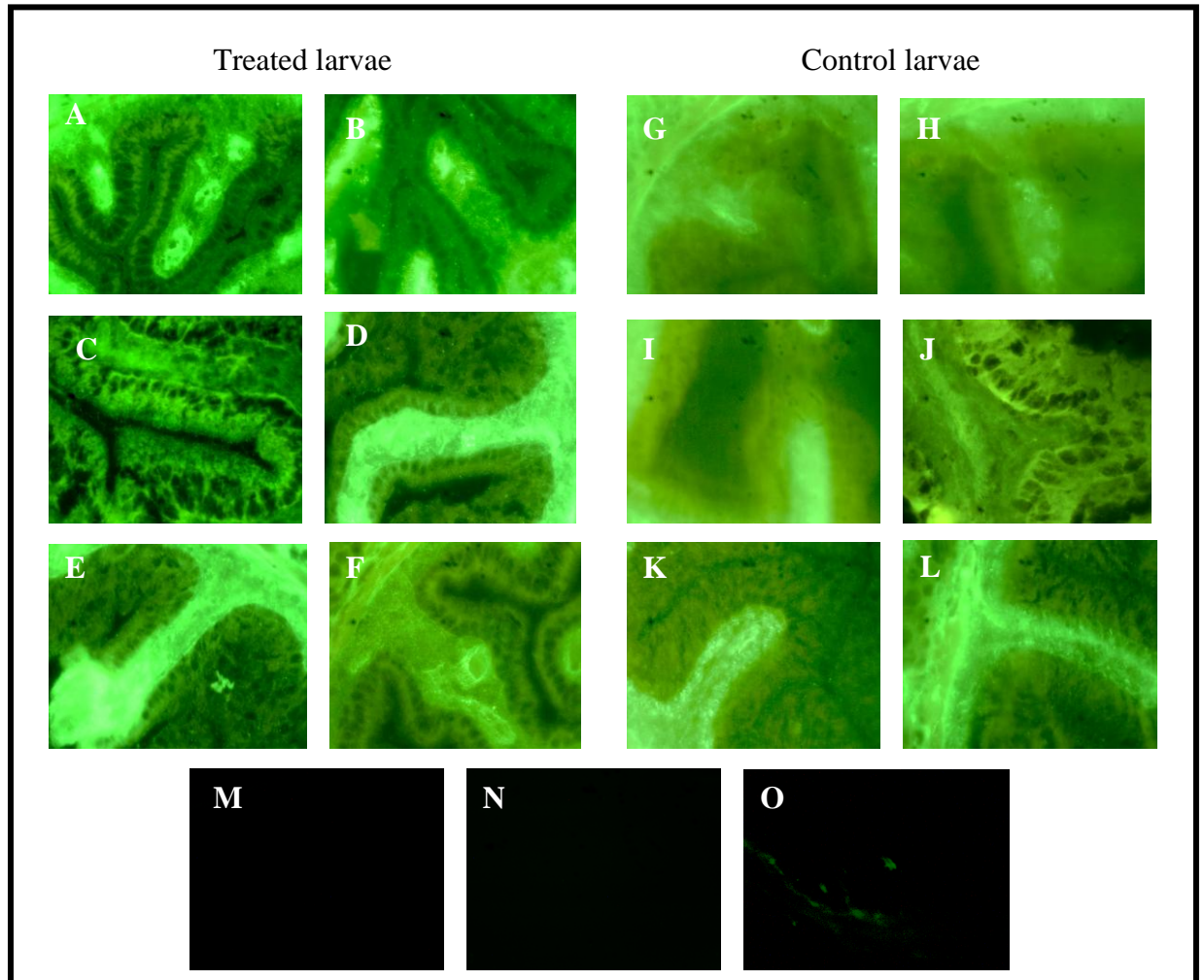
Figure 23. Fluorescent staining of C3 in oesophagus in larvae 29 days post onset of first feeding (dpff). The figure shows the digestive system of treated larvae (A-F), control larvae (G-L) and negative controls of treated (M, N) and untreated (O) larvae (1000x magnification).

The positive reaction in the intestines seems to be stronger and more widespread in the treated compared to untreated larvae (Figure 24). A positive reaction can be detected in the muscularis externa as well as in the connective tissue of treated larvae, but is mainly restricted to the muscularis externa in larvae from the control group.
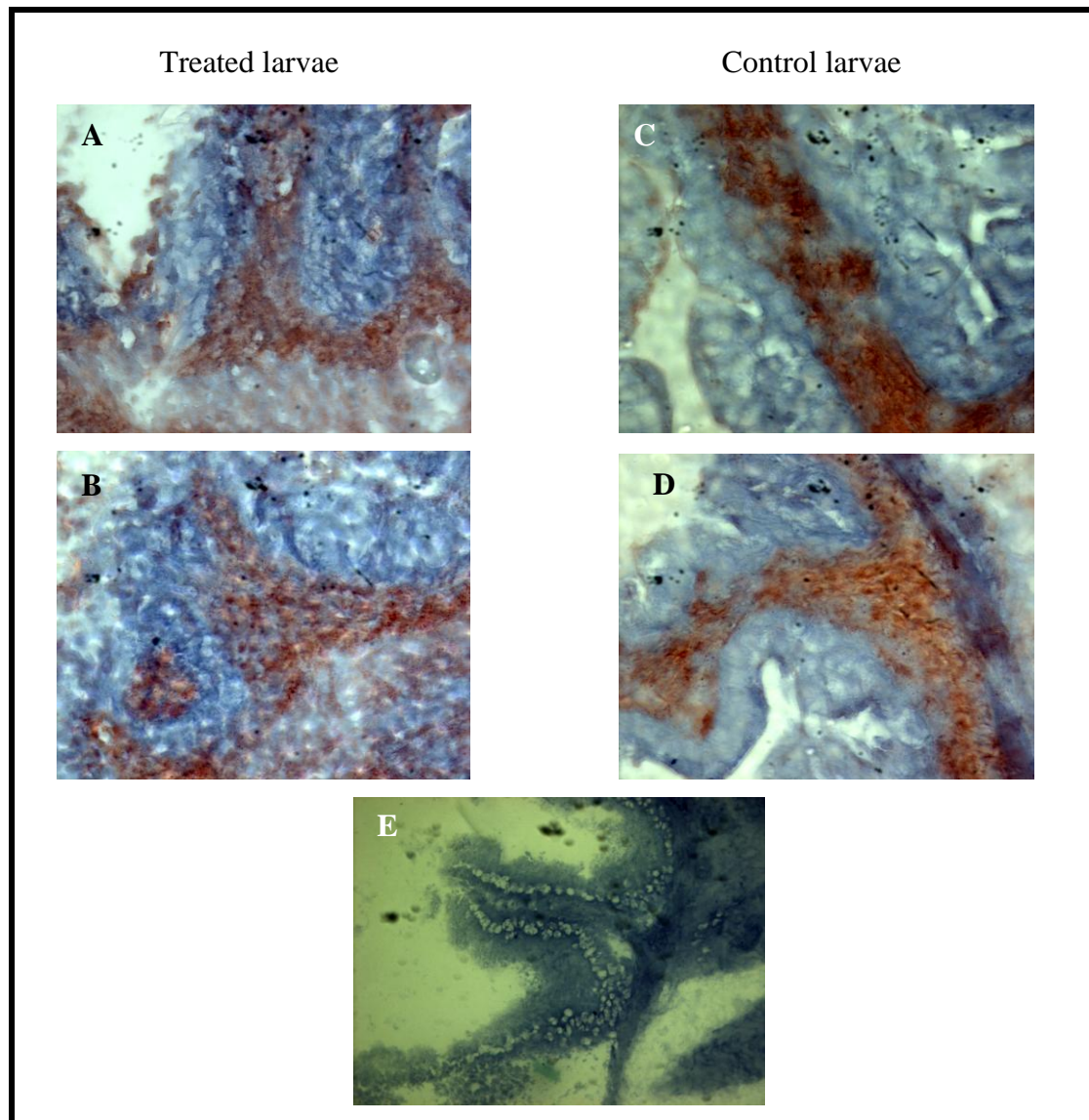

Figure 24. Fluorescent staining of C3 in sections of larvae 29 days post onset of first feeding (dpff). The figure shows the digestive system of treated larvae (A-F), control larvae (G-K) and negative controls of treated and untreated larvae (L and M, respectively) (1000x magnification).

The response in the oesophagus indicates stronger positive reaction in the treated larvae compared to the control (Figure 25). The positive reaction in the untreated larvae appears only as a thin line in the connective tissue beneath the epithelium and in the goblet cells but in the treated larvae the response is detected in the connective tissue as a whole.

Figure 25. HRP staining of C3 in sections of larvae 43 days post onset of first feeding (dpff). The figure shows the oesophagus of treated larvae (A-D), control larvae (E-H) (1000x magnification) and negative controls of treated and untreated larvae (I and J, respectively) (400x magnification).

Figure 26 shows a closer view of the regions shown in Figure 25, (C, D, G and H). The positive reaction in the treated larvae is stronger, indicating higher concentrations of C3.

Figure 26. HRP staining of C3 in sections of larvae 43 days post onset of first feeding (dpff). The figure shows a closer view of the regions shown in Figure 25, showing th oesophagus of treated (C, D) and untreated (G, H) larvae (1000x magnification).

At 43 dpff, the pyloric caecae are appearing and the foldings are turning conical (Figure 27). The positive reaction seems to be stronger in the treated compared to the untreated larvae. The difference in the brightness of the color may, however, be misleading.
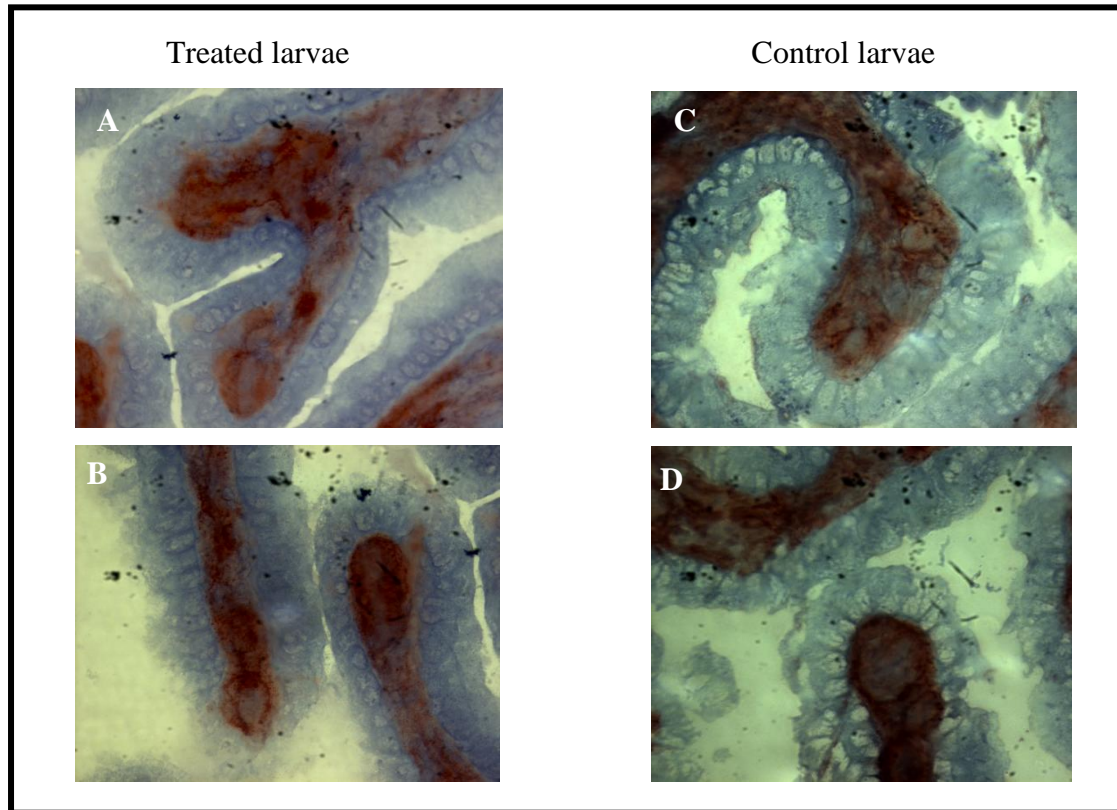
Figure 27. HRP staining of C3 in sections of larvae 43 days post onset of first feeding (dpff). The figure shows the pyloric caecae of treated larvae (A-B) and control larvae (C-D) (1000x magnification).

The intestines contain small folds in the uppermost part and at 43 dpff and C3 seems to be present only in a thin layer beneath the goblet cells and the epithelium layer (Figure 28). No obvious differences were detected in treated and untreated larvae with respect to the presence of C3.

Figure 28. HRP staining of C3 in sections of larvae 43 days post onset of first feeding (dpff). The figure shows the intestines of treated larvae (A-D), control larvae (E-H) and a negative control of treated larva (I) (1000x magnification).

A considerably weaker reaction with respect to the presence of C3 was observed in the oesophagus of larvae at the end of the first feeding period (Figure 29) compared to the sampling a week before (Figures 25 and 26). Hardly any reaction was observed in

the untreated larvae at this sampling, but a clear positive response was seen in larvae treated with PP.

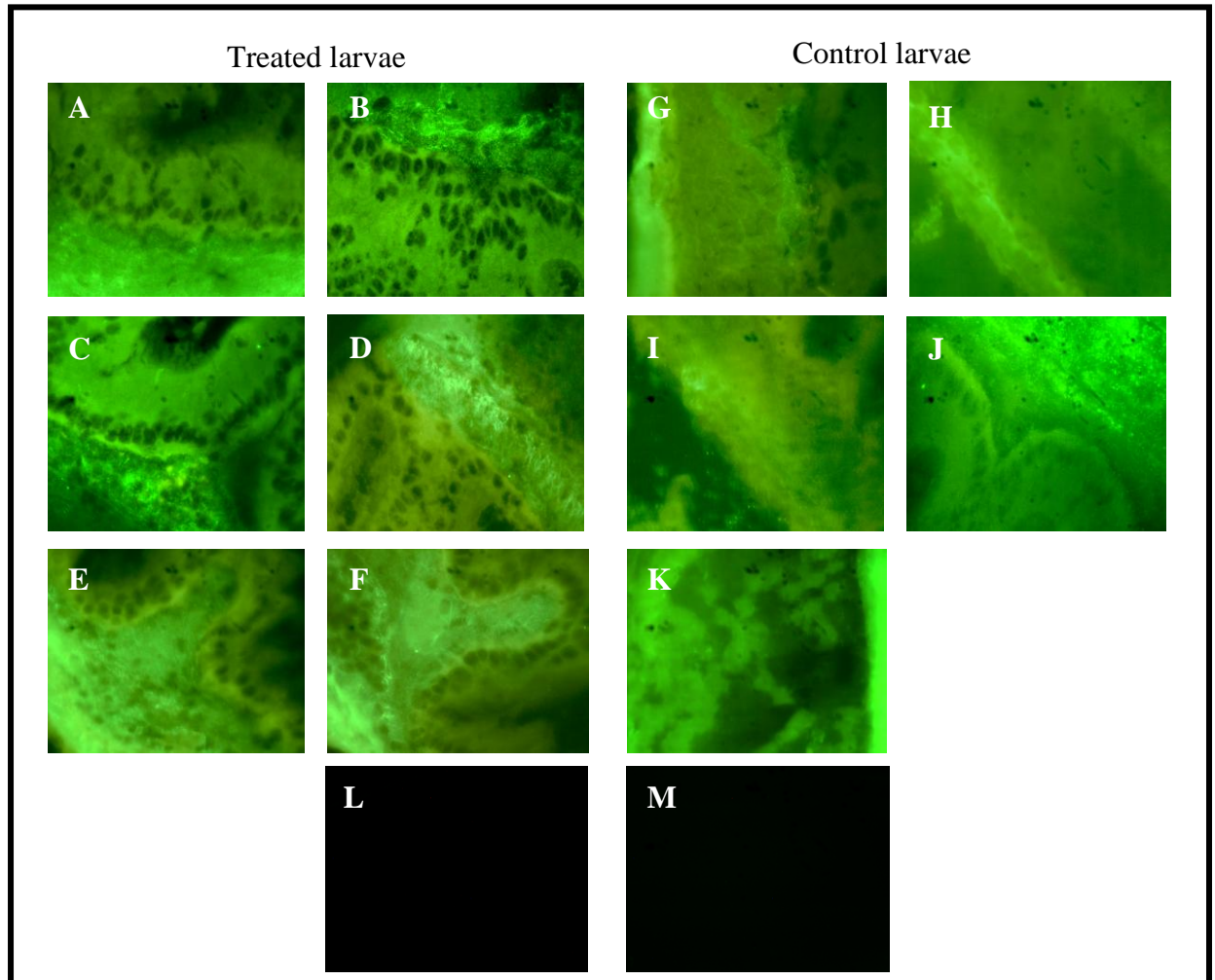*Figure 29. HRP staining of C3 in sections of larvae 50 days post onset of first feeding (dpff). The figure shows the oesophagus of treated larvae (A-C), control larvae (D-F) (1000x magnification) and a negative control of treated larvae (G) (400x magnification).*

In the intestines, the reaction at 50 dpff (Figure 30) is similar to the reaction observed at the last sampling a week before (Figure 28). No obvious differences can be detected when treated and untreated larvae are compared.
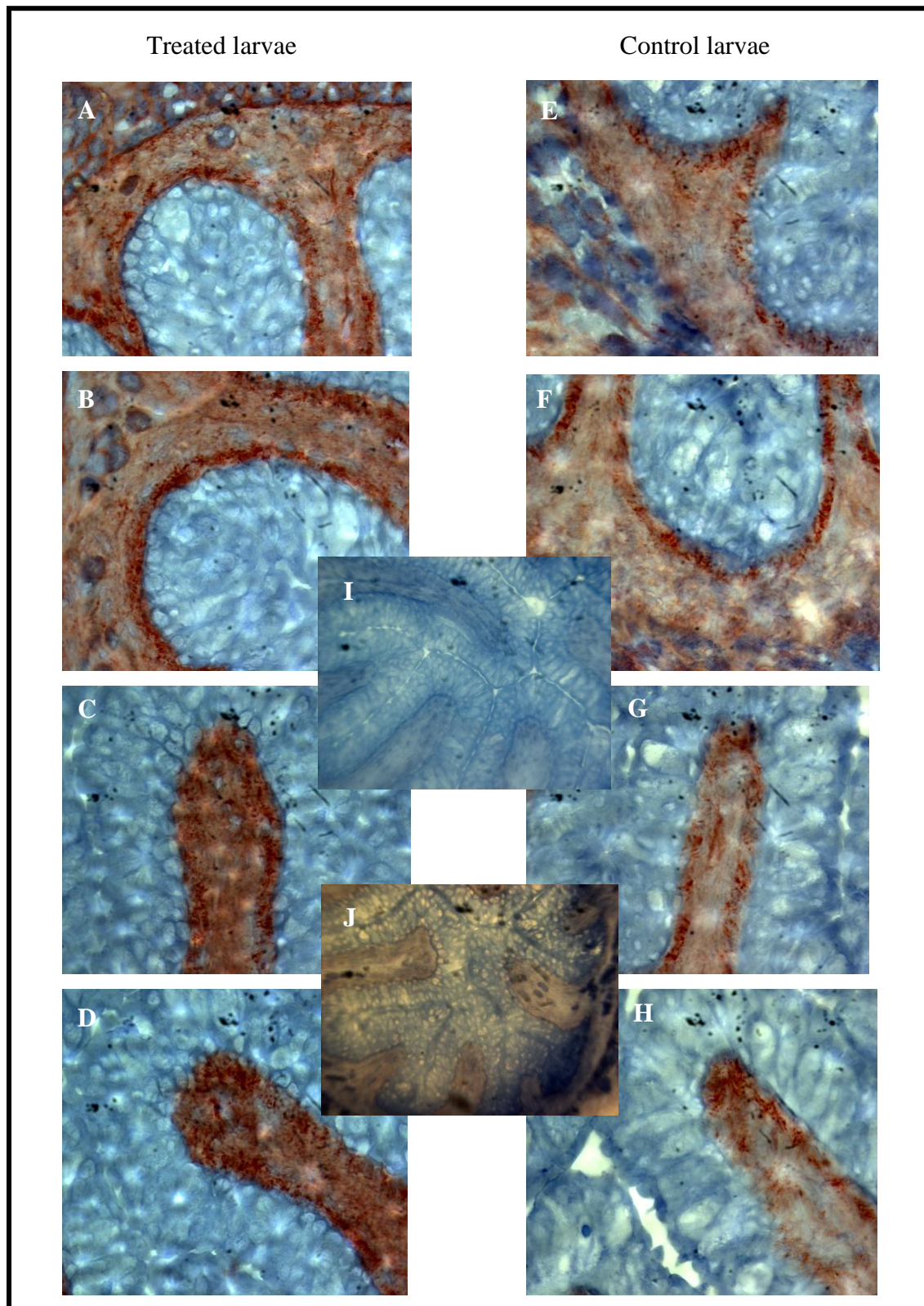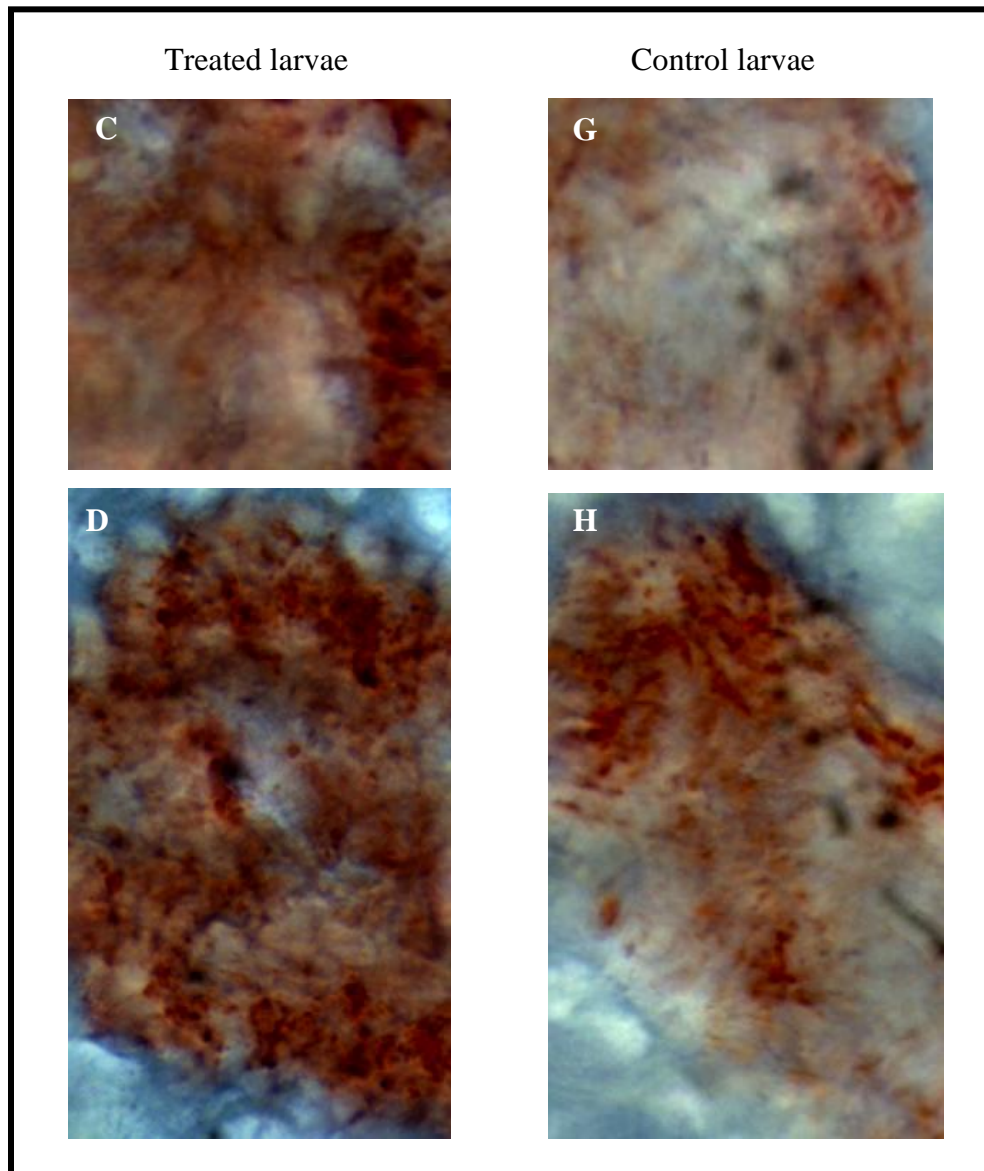

Figure 30. HRP staining of C3 in sections of larvae 50 days post onset of first feeding (dpff). The figure shows the intestines of treated larvae (A-B) and control larvae (C-D) (1000x magnification).

3.4.3. Effects of treatment on the presence of lysozyme in larvae.

At the onset of feeding, lysozyme seems to be present in the digestive system of larvae (Figure 31), but considerable differences in the magnitude of the reaction were observed when individual larvae were compared (result not shown). A strong positive reaction with respect to lysozyme was seen in the digestive system of some of the five larvae examined in each group, but hardly detectable in other larvae.
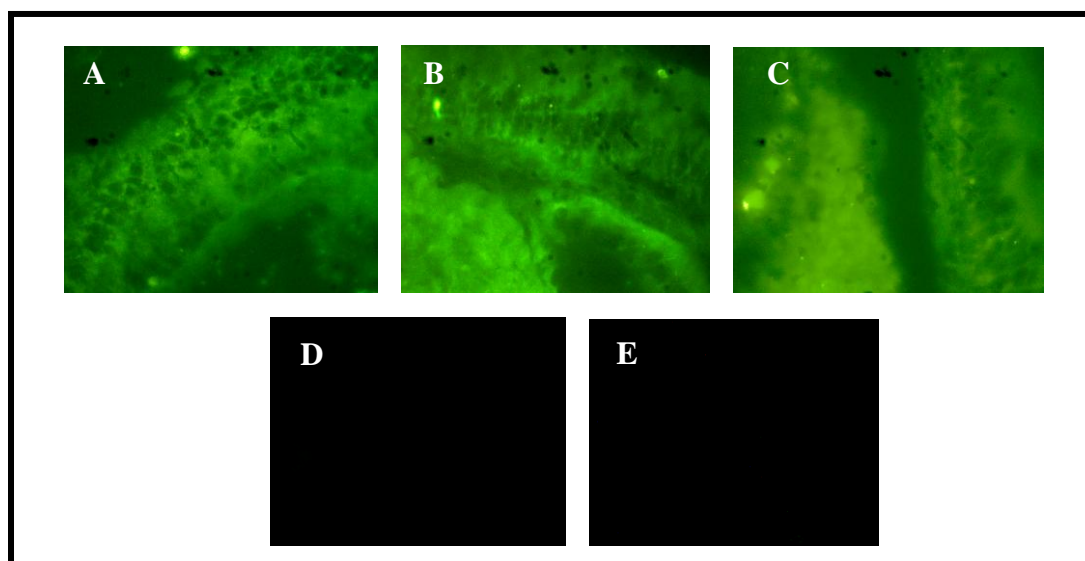
Figure 31. Fluorescent staining of lysozyme in sections of larvae at the onset of first feeding.The figure shows the digestive system (A-C) and negative controls from similar location in the digestive tract of treated (D) and untreated (E) larvae (1000x magnification).

After the first eight days in feeding (8 dpff), there are indications of a stronger positive reaction with respect to lysozyme in treated larvae compared to untreated larvae (Figure 32). The strongest response was observed in the contents of the digestive system, in the muscularis externa and surrounding the epithelium cells of the digestive system.
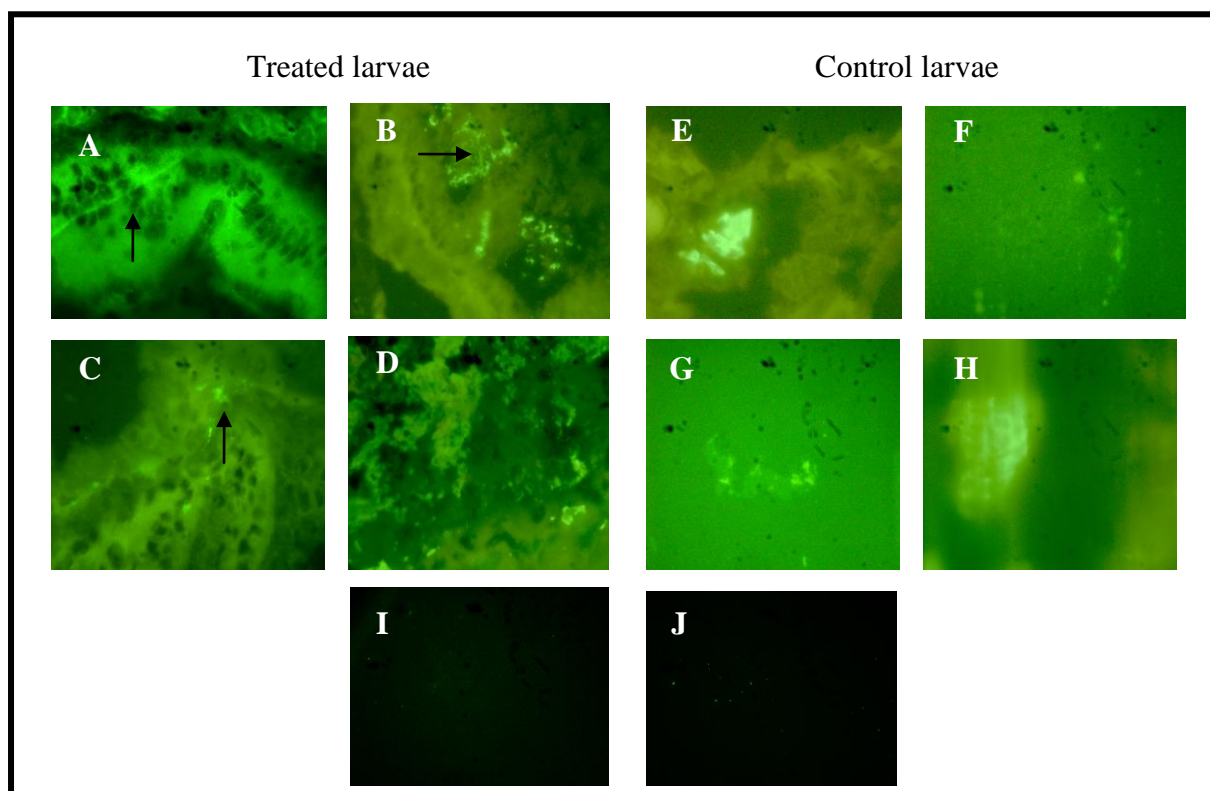
Figure 32. Fluorescent staining of lysozyme in sections of larvae 8 days post onset of first feeding (dpff). The figure shows the digestive system of treated larvae (A-D), control larvae (E-H) and negative controls from treated and untreated larvae (I and J, respectively) (1000x magnification). The arrow show Epithelium cells (A), content (B) and muscularis externa (C).

At 15 dpff, a similar reaction to lysozyme was observed in the digestive tract of treated and untreated larvae (Figure 33).
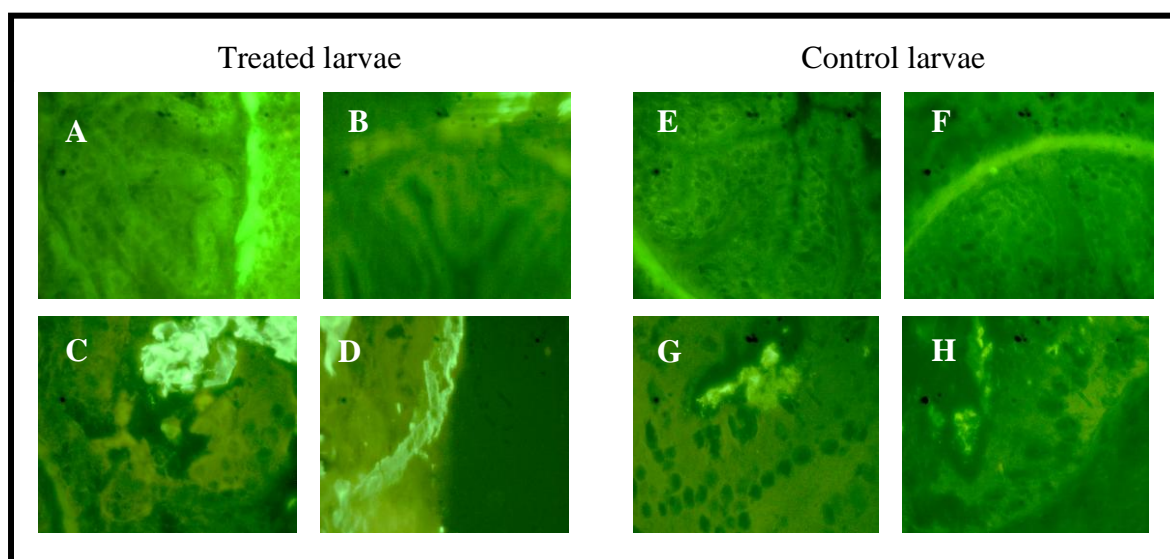
Figure 33. Fluorescent staining of lysozyme in sections of larvae 15 days post onset of first feeding (dpff). The figure shows the digestive system of treated larvae (A-D) and control larvae (E-H) (1000x magnification).

At 29 dpff, a similar reaction to lysozyme was observed in the digestive system of treated and untreated larvae (Figure 34). However, individual variations were observed in the untreated larvae.
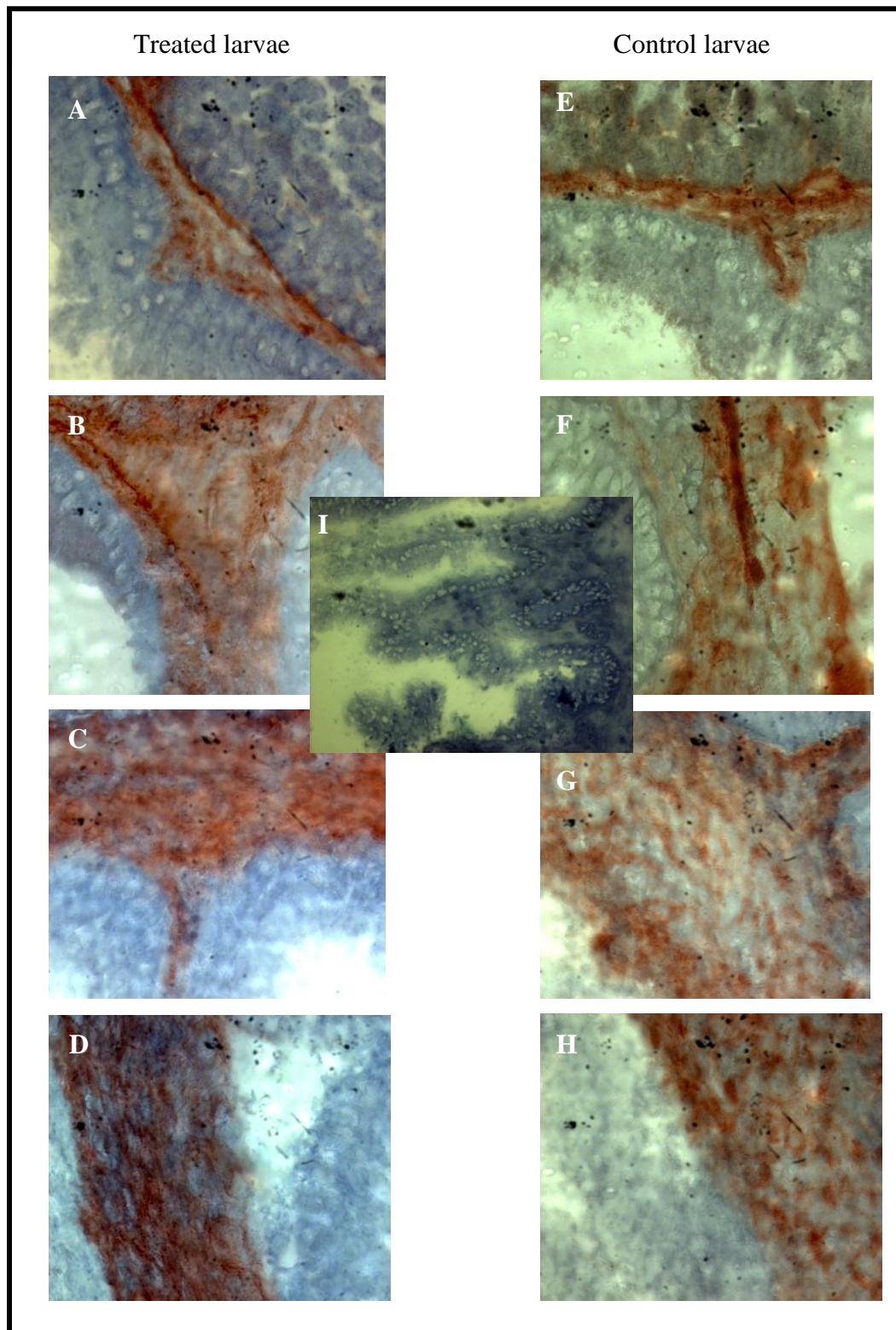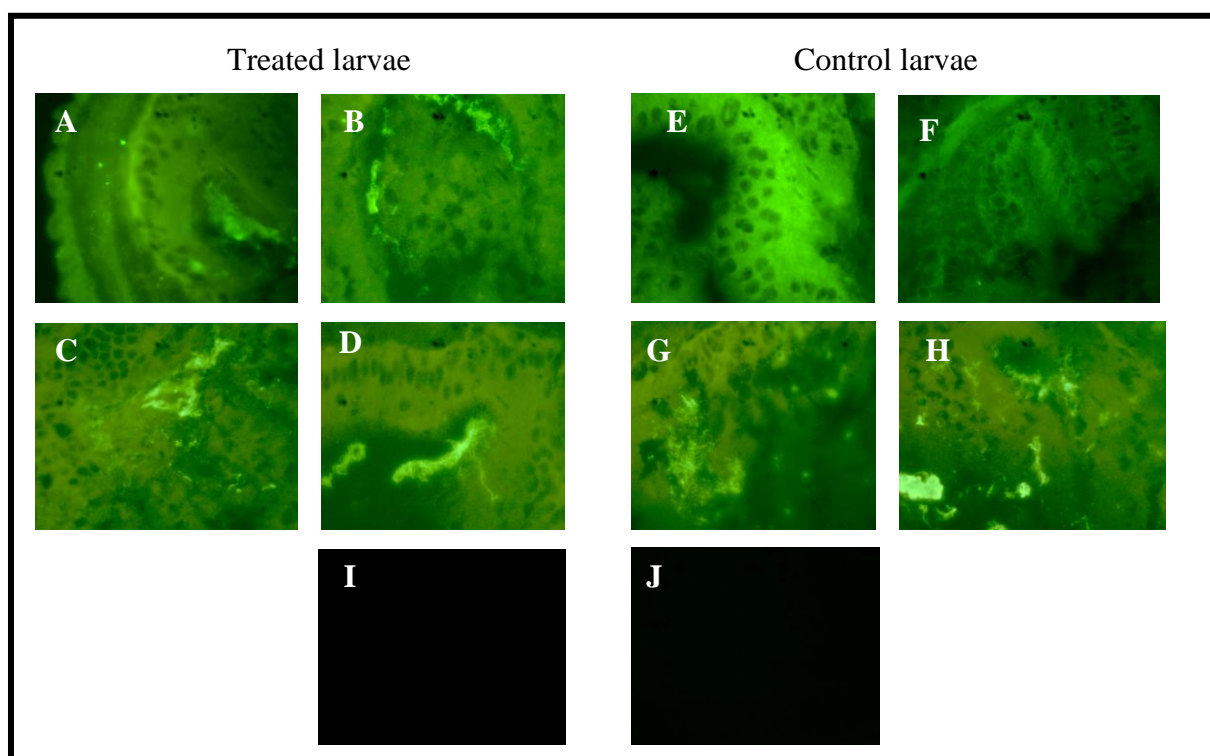

Figure 34. Fluorescent staining of lysozyme in sections of larvae 29 days post onset of first feeding (dpff). The figure shows the digestive system of treated larvae (A-D), control larvae (E-H) and a negative control from each group (I and J) (1000x magnification).

A weak positive response was detected in the stomach of larvae from both groups at 43 dpff (Figure 35). The response with respect to lysozyme was stronger in the connective tissue beneath the goblet cells and in the epithelium layer of the stomach in treated larvae compared to the control group.
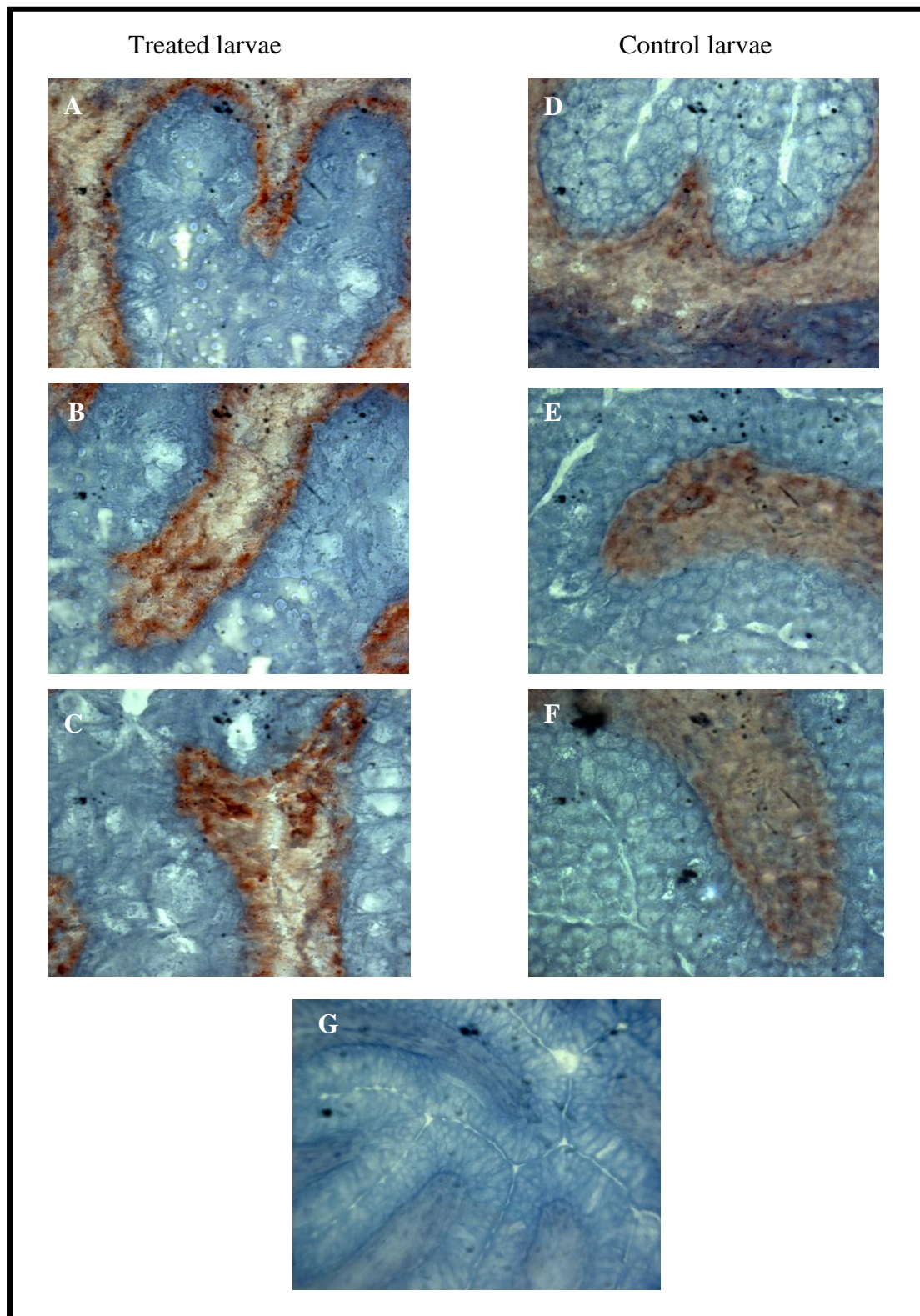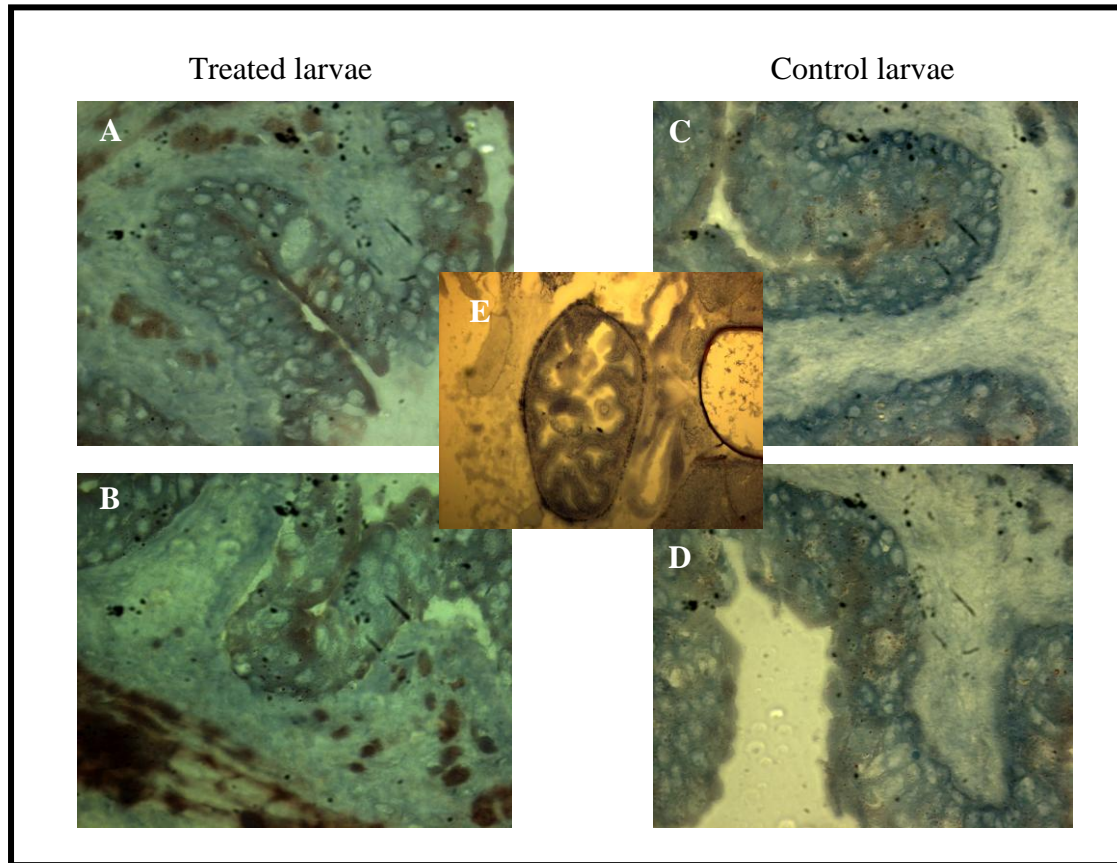

Figure 35. HRP staining of lysozyme in sections of larvae 43 days post onset of first feeding (dpff). The figure shows the stomach of treated larvae (A-B) and control larvae (C-D) (1000x magnification) and a negative control of treated larva (E) (100x magnification).

A strong positive reaction with respect to lysozyme was observed in the connective tissue beneath the goblet cells, in the epithelium layer as well as in the lumen of the intestines of treated larvae (Figure 36). A weak positive response was observed in the untreated larvae and only in the intestinal lumen.
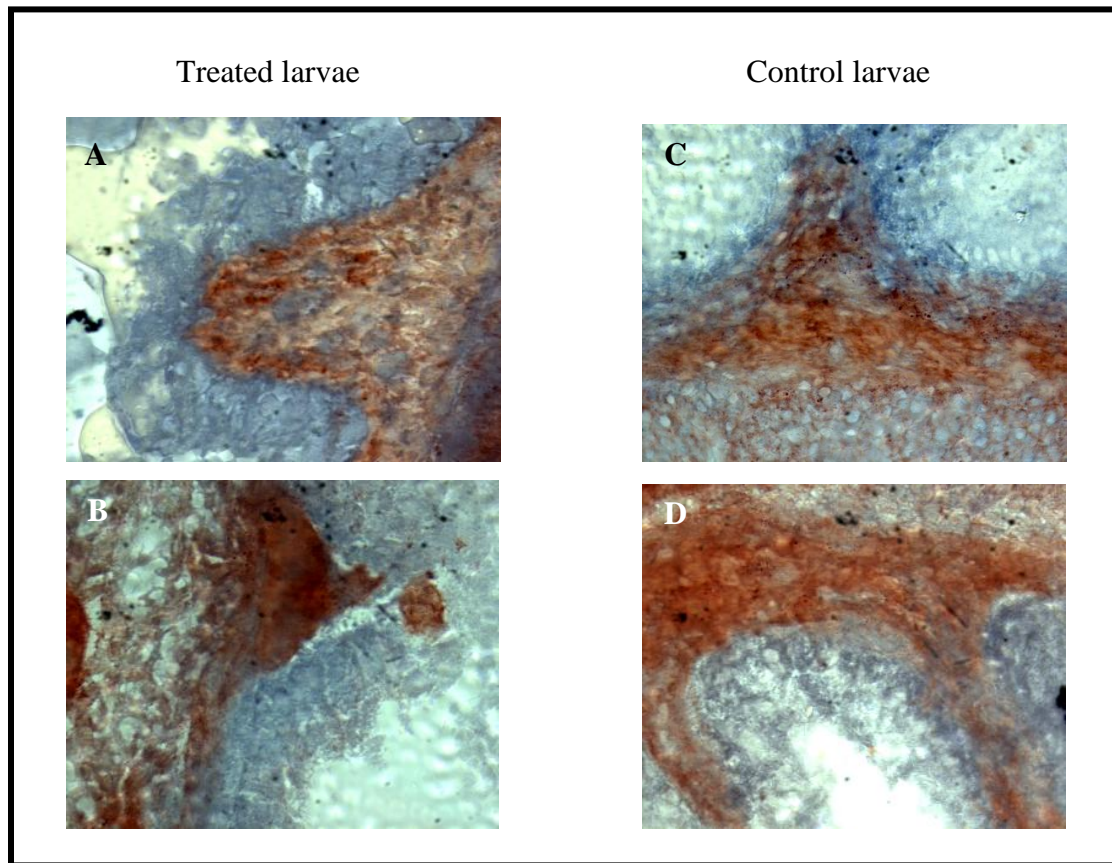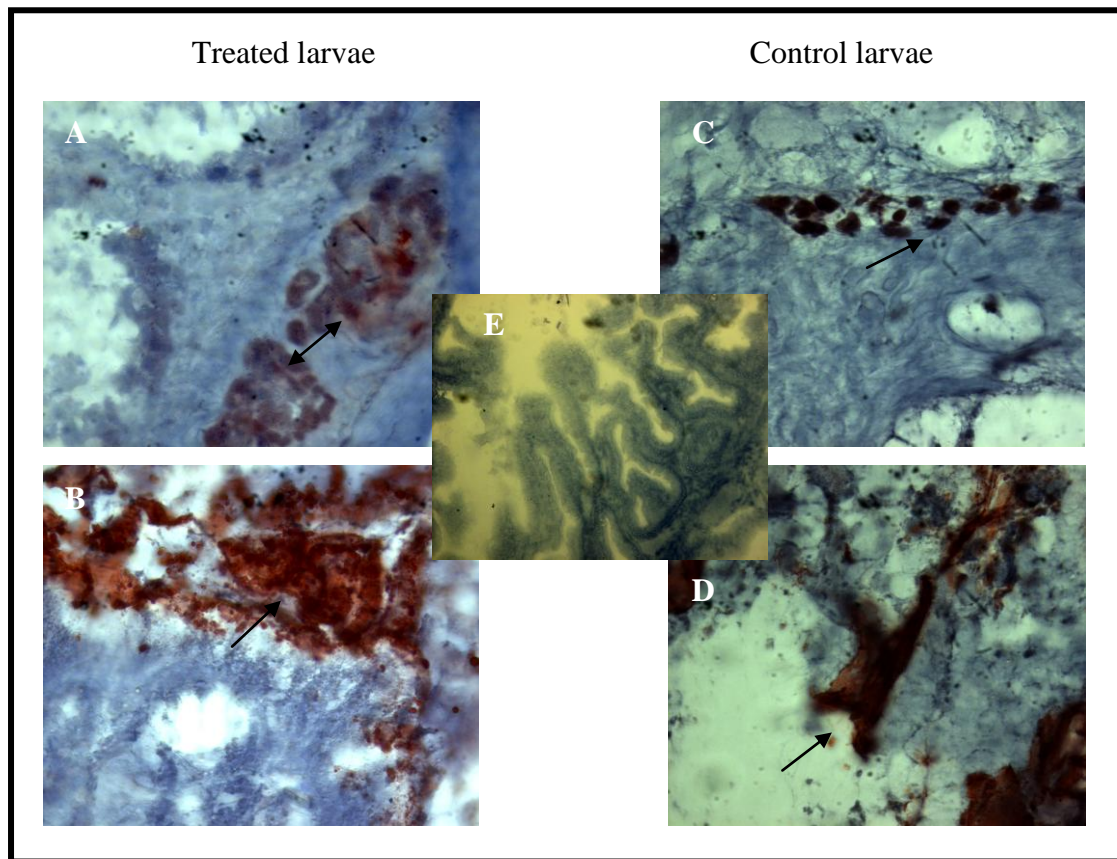
Figure 36. HRP staining of IgM in sections of larvae 43 days post onset of first feeding (dpff). The figure shows the intestines of treated larvae (A-B) and control larvae (C-D) (1000x magnification) and a negative control of treated larvae (E) (magnification 400x). The arrows shows connective tissue beneath the goblet cells (A,C) and the lumen (B,D).

A distinct positive response with respect to lysozyme was detected in treated larvae at 50 dpff (Figure 37). The response in untreated larvae was barely distinguishable and similar to the negative control. The response was mainly detected in the connective tissue as well as beneath and in-between the goblet cells.
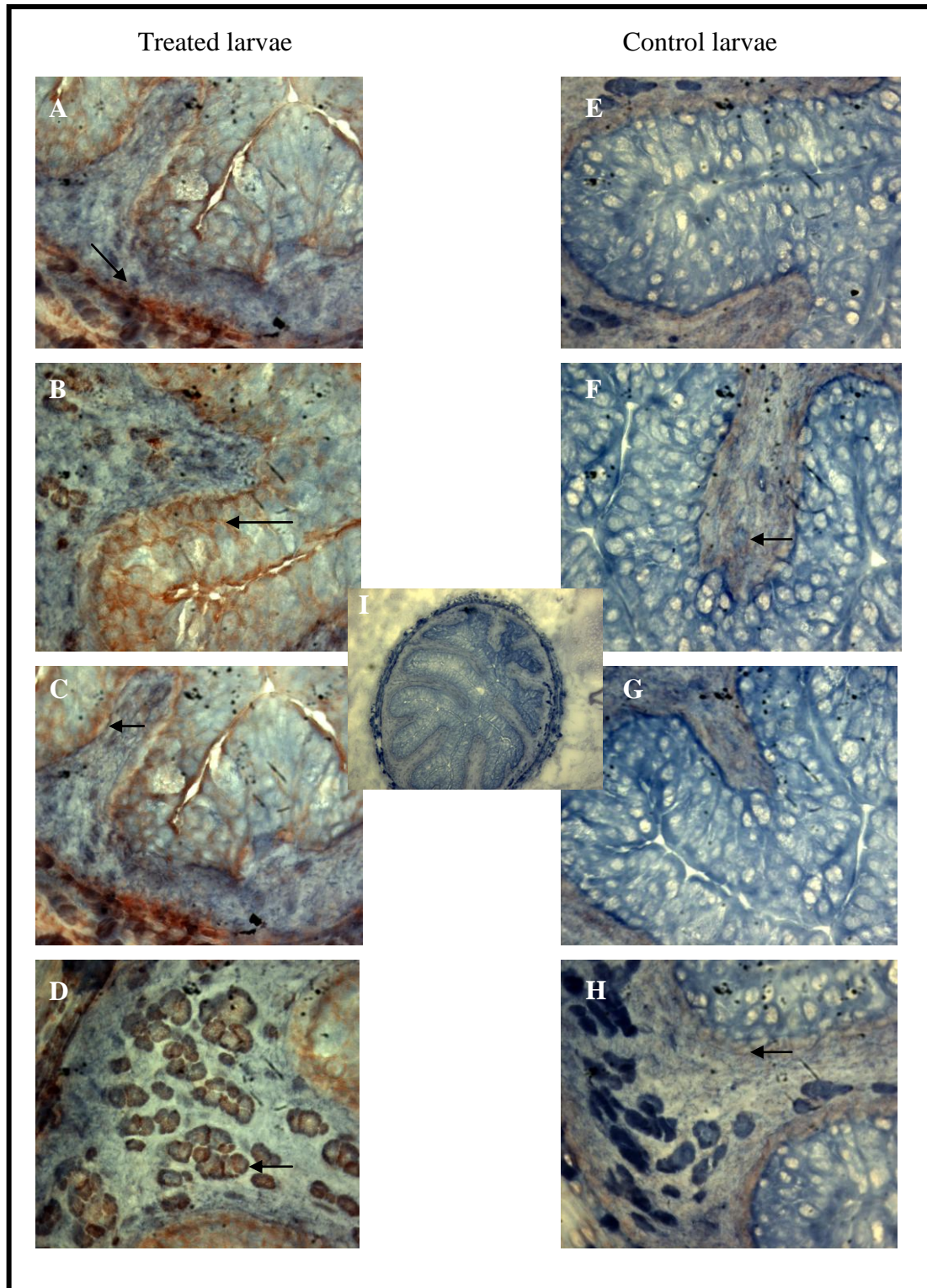
Figure 37. HRP staining of lysozyme in sections of larvae 50 days post onset of first feeding (dpff). The figure shows the oesophagus at the junctions to the stomach in treated larvae (A-D) control larvae (E-H) (1000x magnification) and a negative control of treated larvae (I) (magnification 200x). The arrows shows positive response in connective tissue (A,D,F), inbetween the goblet cells (B) and beneath the epitermal layer (C,H).

3.4.4.   *Evaluation of larval success*

Treating larvae using 0.02 g/L of PP through the live feed did neither affect the growth (Figure 38) nor the survival of the larvae (result not shown). However, the treatment resulted in higher ratio of larvae not metamorphosed (Figure 39). Larval growth was evaluated on approximately weekly intervals from the onset of (0 dpff) and until the end of the first feeding period (57 dpff). The success of metamorphosis was evaluated at the end of the first feeding period (57 dpff), with respect to deviation in eye migration and pigmentation and the ratio of larvae successfully developing into juveniles.

At the end of the first feeding period, the weight of larvae in the control group (39.5 mg) was higher than the weight of treated larvae (35.9 mg). The weight of larvae in both groups was similar to the mean weight of larvae in all tanks during the period (Figure 38).
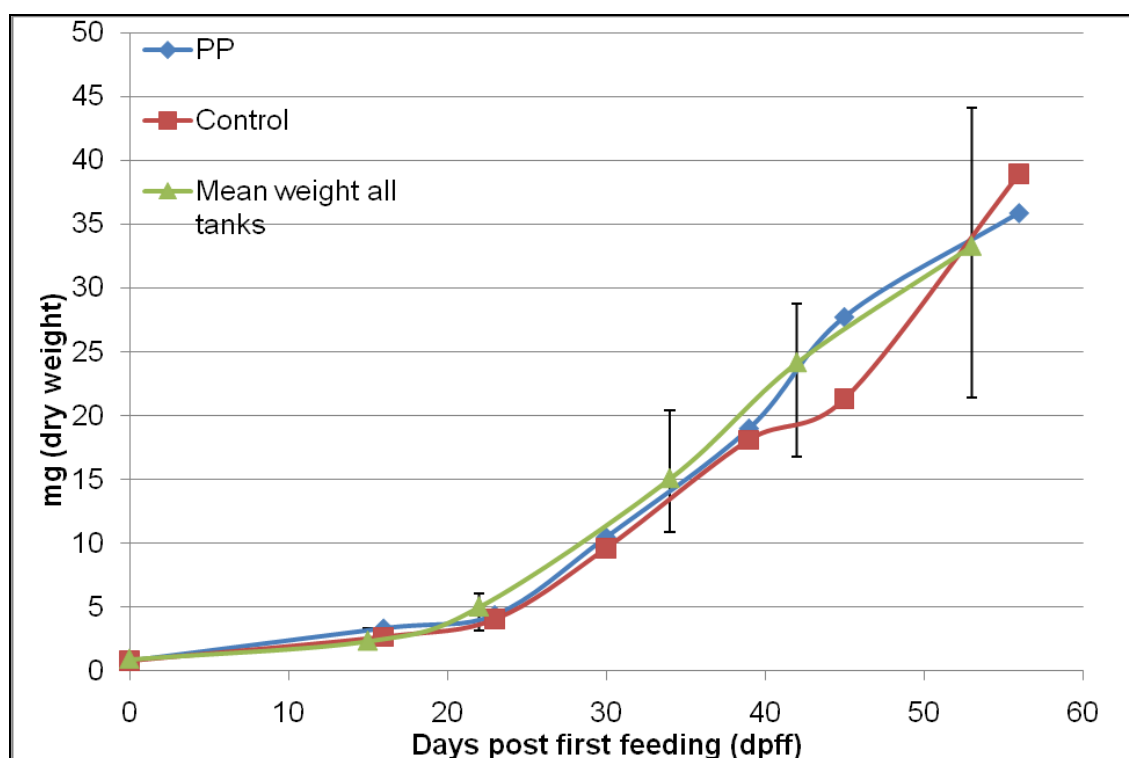
Figure 38. The weight of larvae in the experimental tank units at various days post onset of first feeding (dpff) throughout first feeding (mg dry weight). Included is the mean weight of larvae in all tanks during the period, measured at various dpff throughout first feeding. PP = Pollock peptides

The ratio of larvae not metamorphosed at the end of first feeding was over four times higher in the PP treated group compared to the control group (Figure 39). The ratio of poorly metamorphosed juveniles was similar in both groups.
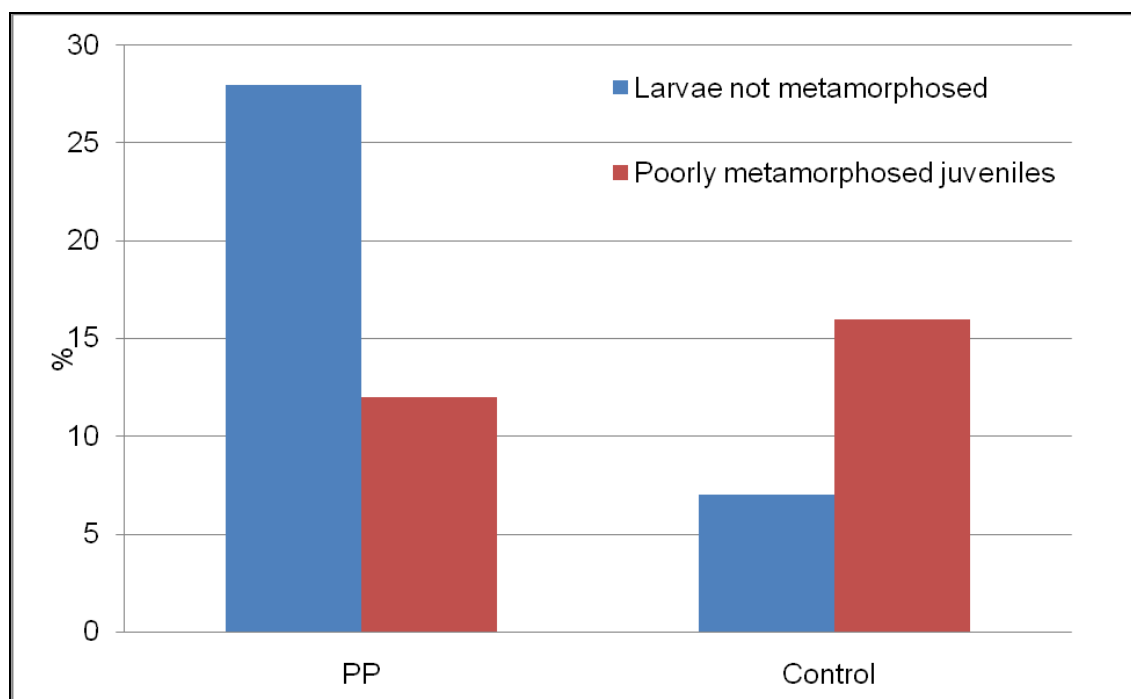
Figure 39. Metamorphosis evaluated towards the end of the first feeding period (57 days post onset of first feeding, dpff). Shown is the ratio of larvae not successfully metamorphosed and poorly metamorphosed juveniles. PP = Pollock peptides

3.5.   Western blotting and protein analysis

Proteins from homogenates of larvae, separated by electrophoresis, were found to be smeared over a large area of the gel, rather than appearing as distinct bands within the gel. The duration of the electrophoretic transfer was therefore carried out in various time lengths and the preparation of the samples was examined step by step. Different setups were tested as well as various methods for preparation of the samples, without managing to acquire successful separation of sample proteins. Electrophoretic transfer of the proteins onto a nitrocellulose membrane therefore resulted in smeared bands stained by the specific antibodies. As a consequence, the immunohistochemistry method was selected for analysis of samples in the last experiment.

Furthermore, a considerable effort was spent on analysis of the protein content of samples as well as in solutions of the bioactive products used in the study, using an automatic electrophoresis station, Experion^{TM} System. The ideology was to locate proteins of the same size as the immune parameters in question (IgM and C3) and with the possibility to quantify proteins of these size groups in samples of treated and untreated larvae. Various samples were tested, including dilutions of the bioactive products, samples of the live feed and larvae treated with the products, a BSA, IgM and C3 standards in addition to a negative control (dH$_2$O). Variable results were obtained when re-running individual samples and the equipment was not found to work adequately or give sufficiently accurate results. After numerous consultations with the manufacturer, a new protein marker was donated, and tested in all 10 wells obtaining identical and reproducible results. The samples were then rerun, but with unsuccessful and non reproducible results.

4. Discussion

The Atlantic halibut (*Hippoglossus hippoglossus* L.) is an eligible species in commercial aquaculture. However, the mortality rates of halibut larvae are high and more studies are needed in order to increase the survival rates (Schulze *et al.,* 2006; Olafsen, 2001). Among other aspects, the immune system of larvae has been studied in relation to their development (Biering, 1997; Bricknell *et al.,* 2000: Ingilae *et al.,* 2000; Lange *et al.,* 2001). The specific immune response is commonly slow and there are indications that specific parameters develop late in the developmental process in halibut among other fish species (Zapata *et al.,* 2005; Magnadottir *et al.,* 2005). It is therefore important to find ways to stimulate the innate immune response of fish larvae. A better understanding of the innate immune parameters of the larvae can provide indications of how and when these parameters should be stimulated (Bricknell & Dalmo, 2005; Sakai, 1999). The more advanced molecular tools available in today's research opens for the possibility of more specific studies of the interwoven and complex immunological responses that will hopefully result in increased quality of the larvae and contribute to higher success in marine aquaculture. Further understanding of the various factors that can influence the growth and metamorphosis of larvae is needed in order to contribute to a higher ratio of fast growing and successfully metamorphosed juveniles and thereby maximize the profit for the industry.

This project was a part of a bigger project where the common aim was to contribute to increased survival of halibut larvae during first feeding. Four bioactive products were studied with respect to their ability to stimulate selected components of the immune system of halibut. The study was carried out in three separate experiments and through a series of analyzes using the ELISA method and immunohistochemistry (IHC) where specific antibodies directed against selected components of the innate immune system of larvae were used. The bioactive products were fed through the live feed and the main emphasis therefore on the immune factors located in the digestive system of the larvae. It must, however, be noted that individual variations will always

exist, not least due to the age difference of individual larvae within each tank which normally can be up to eight days. As a consequence, the developmental stage of the digestive system in individual larvae can vary.

To summarize, increasing levels of IgM were detected in larvae from ~29 dpff and onwards. Furthermore, low levels of IgM were detected in larvae already at the onset of feeding but IgM was not detected in larvae sampled between 8 and 26 dpff, indicating that the IgM detected in larvae at the onset of feeding may be maternal IgM. Previous studies in salmon have shown that the levels of maternal IgM were quickly reduced after hatching, reaching non-detectable levels just prior to the onset of feeding when autologous IgM production started (Olsen & Press, 1997). Furthermore, maternal IgM have been seen dispersed throughout the yolk of the embryo as well as being localized within the external membrane of the egg in channel catfish, which may indicate separate functional roles (Hayman & Lobb, 1993). Maternal IgM may also be restricted to the egg membrane as observed in salmon (Olsen & Press, 1997). However, the lack of protection observed in the maternal antibodies of larvae and fry indicates a different or additional role attributed to the maternal IgM (Magnadottir *et al.*, 2005). A possible primary role of maternal antibodies could therefore be to protect the eggs against vertical transfer of certain pathogens. Maternal IgM may furthermore aid phagocytosis or the activation of complement pathways during the early developmental stages and IgM may even function simply as a nutritional yolk protein (Magnadottir *et al.*, 2005).

An increase in the levels of IgM has been reported during the early stage of fish larvae, where Ig+ cells are present and show immunohistochemical reactivity for Ig (Imagawa *et al.*, 1991; Lamers, 1986; Pettersen *et al.*, 2000; OʹDowd *et al.*, 1998; OʹDowd *et al.*, 1999). In salmon, the presence of IgM on the surface of lymphocytes appears at approximately 45 dph and coincides with the onset of feeding (Ellis, 1977). However, although surface Ig positive B cells were detectable in 14 dph old carp, they were not able to develop into plasma cells until 30 dph (Koumans-van Diepen *et al.*, 1994). In the present study, increasing concentrations of IgM were detected from 29 dpff and througout the first feeding period (29-70 dpff). Increasing concentrations of IgM were detected in all groups with the exception of the chitosan treated larvae where the detected levels of IgM were considerably lower compared to the other groups. The

highest levels of IgM were measured in the BP treated larvae and realtively higher than in the control group. A similar increase in the detected concentrations of IgM was observed in PP treated larvae and the control group. The negative effects of the chitosan treatment indicate some kind of detrimental effects and the treatment then reduced to only one of the two daily feedings of larvae. In this group, lower levels of IgM were detected in treated larvae compared to the control group. It can therefore be concluded that treatment with chitosan (0.2 g/L) did not stimulate the production or expression of IgM and even lead to reduced expression or production of IgM in the larvae. Furthermore, treating larvae with CP resulted in a total collapse of all larvae within both experimental tank units after only four days of treatment. The quality of the treated *Artemia*, measured as the color and mobility of the live feed as well as the numbers of cultivable bacteria in the cultures, was neither affected by treatment with CP nor chitosan. Previous findings have reported that elevated concentrations of bioactive products can adversely affect the immune system and harm or even kill the treated organism (Vadstein, 1997). The concentration of the bioactive products used is therefore a key issue to consider.

Increased levels of IgM were observed in the CPE treated larvae after only four days of treatment. However, considerable differences were observed in the concentration of IgM in larvae from the two separate tanks with the CPE treatment. The lower levels detected were similar to the levels detected in larvae from the control group. As a consequence it is difficult to state if treatment of larvae using the cod peptide hydrolysates through the environment stimulated the production of IgM.

C3 was detected throughout the larvae when treated with PP as well as in the untreated larvae. No differences were observed with respect to the presence or distribution of C3 in treated and untreated larvae with the exception of the digestive tract where stronger response may be detected in the treated larvae. The presence of C3 was detected already at the onset of feeding (50 dph) but only with a faint response, indicating low levels present at this stage. Previous studies show that at the onset of feeding, C3 was detected in columnar epithelial cells of the gut (oesophagus, stomach and intestine) as well as in epithelial and mucosal cells of the skin in halibut larvae (Magnadóttir *et al.,* 2005). The present results strongly indicate that the addition of the pollock peptides to the live feed resulted in increased levels of C3 in larvae. The main

difference observed in the presence of C3 in treated and untreated larvae was in the space between the epithelial cells lining the digestive tract of larvae. A similar response was observed in the muscularis externa of the digestive system in treated and untreated larvae, but the reaction was more prominent in the connective tissue beneath the epithelial cells in the treated larvae compared to the untreated larvae. It turned out to be difficult to get clear pictures of the corresponding sections of control larvae where the response was substantially weaker and in some larvae no response was detected. All the treated larvae, however, showed very clear and strong response. This indicates that treating larvae with PP through the live feed for one month (0-29 dpff) resulted in increased production of C3. Previous studies have shown that the use of non-specific stimulants may enhance various parameters of the innate immune system and lead to improved protection against different pathogens as well as increased larval survival (Austin & Austin, 1987; Ellis, 1988; Press & Lillehaug, 1995; Dalmo *et al.,* 2000). In the present study, the difference between treated and untreated larvae is still detectable further into first feeding (43-50 dpff). The difference in the presence of C3 in treated and untreated larvae, however, depends on the location in the digestive tract. In the uppermost part of the digestive tract, i.e. in the oesophagus and stomach, the presence of C3 was more prominent in treated larvae compared to untreated, but further down the digestive tract no differences were observed when treated and untreated larvae were compared. Increased understanding has been gained on the complement system in fish larvae and the expression of C3 in different organs at various stages of the development (Lange *et al.,* 2004a; Lange *et al.,* 2004b). Immunohistochemical studies directed at the presence of C3 in halibut and cod larvae indicate that C3 may be locally produced in various organs during development (Lange *et al.,* 2004a; Lange *et al.,* 2004b). Results furthermore indicate that C3 is expressed at an earlier stage in the development in certain organs and C3 has even been detected in the yolk sac (Magnadottir *et al.,* 2005). Furthermore, the complement factors play a role in the formation and generation of different organs in fish and the complement components are synthesized locally in other tissues besides the liver, as seen in other species (Lange & Magnadóttir, 2003). If the complement factor is locally synthesized, this may explain the variation in time when C3 is detected in various parts of the larvae in the present results as the organs of the immune system are not all fully developed all at the same time.

Already at the onset of feeding, lysozyme was detected in the digestive tract as well as in other parts of the larvae, such as in the skin mucus. Results from previous studies show that lysozyme has been detected in mucus and serum (Balfry & Imawa, 2004; Bowden *et al*., 2003; Lange *et al.,* 2001; Yousif *et al.,* 1991; Murray & Fletcher, 1976; Grinde *et al*., 1988; Lie *et al.,* 1989), ova (Yousif *et al.,* 1991; Murray & Fletcher, 1976) and lymphoid tissue (Grinde *et al*., 1988; Lie *et al.,* 1989) of a number of fish species. The present result indicate higher concentrations of lysozyme in treated compared to untreated larvae after eight days of treatment with PP through the live feed. Further into the first feeding, no differences were observed in the presence of lysozyme in treated compared to untreated larvae, but higher concentrations may be detected again in treated larvae compared to untreated at 43 dpff. The results may therefore indicate that the treatment of first feeding larvae with PP stimulates the production of lysozyme. Previous studies have shown that lysozyme activity may be enhanced in various fish species after immunostimulation with promising agents like e.g. β-glucan (Misra *et al.,* 2004; Engstad *et al.,* 1992, Jørgensen *et al*., 1993, Thompson *et al.,* 1995, Debaulney *et al.,* 1996), leading to increased resistance to various pathogens (Thompson *et al.,* 1995). Furthermore, increased lysozyme activity has been detected in the offspring of immunized parents compared to non-immunized (Hanif *et al.,* 2004).

The results of immunohistochemistry studies of larvae treated with PP through the live feed indicate higher concentrations of IgM, C3 as well as lysozyme in the digestive system compared to untreated larvae. Treating larvae through the live feed may be expected to evoke responses primarily in the digestive system. Hydrolysate from cod muscle as well as empty stomachs of cod have previously been found to stimulate the activity of Atlantic salmon head kidney leucocytes in addition to the benefits in growth performance (Bøgwald *et al.,* 1996; Gildberg *et al.,* 1996). Even though the bioactive compounds used in this study did not show any growth benefits, there are indications that the products stimulate parameters of the innate immune system of halibut larvae during first feeding. However, Murray *et al.* (2003) did not observe any positive effects of a dietary protein hydrolysate on the innate immune functions of juvenile coho salmon. There have, however, been reports of biologically active peptides with immunostimulating and antibacterial properties being produced during the hydrolyzing procedure (Coste *et al.,* 1992; Bøgwald *et al.,* 1996; Gildberg *et al.,* 1996; Daoud *et al.,*

2005). The present results indicate that the FPH used in this study may have immunostimulating affects in first feeding halibut larvae.

The treatment of larvae with the bioactive products used in the study was found to affect the growth, survival and normal development of the larvae to a various extent. Treating larvae with fish peptide hydrolysates did not lead to improved survival of larvae. The final weight of larvae treated with BP was lower compared with the control larvae and the weight of the larvae treated with CPE was considerably lower. Furthermore, the weight of larvae fed PP was slightly lower than the weight of larvae in the control tank but higher than the mean weight of larvae in all tanks during the period.

Fish protein hydrolysates (FPH) have been used as substitutes for fish meal in aquaculture feeds in order to enhance the growth and survival of marine fish (Liang *et al*., 2006; Kotzamanis *et al.,* 2007). The present results, however, do not indicate a growth enhancing effects of the FPH used in the study. The studies of FPH as a supplement in aquaculture feeds has mainly been carried out in juveniles and adult fish and have been reported to support higher growth in various species (Liang *et al*., 2006; Kotzamanis *et al.,* 2007) and induce increased survival (Day *et al.,* 1997). However, Rønnestad *et al.* (2000) suggested that the faster absorption of the free amino acids present in excess in some diets, might lead to amino acid imbalance but others have assumed that a fast flow of short peptides through the gut wall might saturate the larval digestive system (Kolkovski & Tandler, 2000). These assumptions are in agreement with the findings of Zambonino Infante *et al.* (1997), who reported that the dietary incorporation of a fishmeal hydrolysate at a 20% inclusion level, significantly improved the survival and growth of European sea bass larvae compared to a diet including 40% fishmeal hydrolysate. Several other studies have investigated the effects of dietary protein hydrolysate on the growth of Atlantic salmon (Lall, 1991; Parrish *et al.,* 1991; Heras *et al.,* 1994; Berge & Storebakken*,* 1996), rainbow trout (Aksnes *et al.,* 2006), goldfish (Szlaminska *et al.,* 1991), tilapia (Lapie & Biqueras-Benitez, 1992; Fagbenro *et al.,* 1994) and carp larvae (Carvalho *et al.,* 1997).

The final weight of the chitosan treated larvae was lower than in the control group and considerably lower than the mean weight of larvae from all tank units during the period. However, similar survival rates of larvae were observed in the chitosan treated and the control sibling tank (71% and 73%, respectively), but somewhat lower than in

the other control tank (84%). Dietary chitin as well as chitosan supplementation has been found to depress the growth of tilapia (Shiau & Yu, 1999). The properties of the chitosan depend on the degree of deacetylation (DD) and the appropriate concentrations of chitosan will therefore have to be selected with respect to the DD. Several studies have shown that fat absorption is one of the properties of chitosan (Gades & Stern, 2005) and the poor growth observed in the present study may thus be related to the fat absorption of chitosan added to the fatty acid enrichment medium of the live feed.

The success of metamorphosis was evaluated at the end of the first feeding period (~65dpff), when the larvae were transferred to weaning. The success was evaluated with respect to satisfactory eye migration and pigmentation. The overall success of larvae in the experiments was found to vary considerably between groups as well as between different tank units within the same group (Jóhannsdóttir et al., 2007). The quality of the fertilized eggs is important with respect to the survival and quality of the larvae and juveniles (Salze et al., 2005). High degree of variation was, however, observed when duplicate tank units receiving the same treatment were compared and may be caused by various environmental or feed related aspects. The three experiments were carried out at different time of the year and therefore using larvae from various spawning groups, which adds further to the variability observed when all three experiments are compared. The ratio of larvae not metamorphosed at the end of the first feeding period was higher in the FPH treated larvae compared to the controls in the various experiments. The highest ratio of larvae not metamorphosed at the end of first feeding was found in the chitosan treated group and considerably higher than in the control group. Hence, the treatments were not found to improve normal development of the larvae. Furthermore, the ratio of poorly metamorphosed juveniles in the BP, CPE and PP groups were similar to the control groups, but considerably higher in the chitosan treated larvae. Various factors can affect the success of metamorphosis and most studied are the fatty acid composition (Bell et al., 2003; Estevez et al., 1999; McEvoy et al., 1998; Copeman et al., 2002: Hamre et al., 2002), vitamin A (Miki et al., 1998; Estevez & Kanazawa, 1995) and thyroid hormones (Shi et al., 1996; Hamre et al., 2005a). In the present study, all larvae were fed live feed enriched with a special mixture of fatty acids and vitamins. The only difference between treated and untreated larvae was therefore the bioactive products added to the enrichment medium or to the tank water environment of larvae.

The results indicate that the bioactive products used, with the exception of the pollock peptide hydrolysate, may have influenced the absorption of fatty acids or vitamins and therefore negatively affecting the development and metamorphosis of larvae.

5. Conclusion

An increase in the concentration of IgM was observed in larvae from 29 dpff, with the highest levels detected at 70 dpff, indicating increased production of IgM following further development. Low levels of IgM were furthermore detected in larvae at the onset of first feeding and may be related to maternal IgM. Treating larvae with peptide hydrolysates of blue whiting resulted in higher levels of IgM detected in larvae compared to the other groups. Using specific antibodies against C3 and lysozyme, revealed an overall stronger response in various parts of the digestive tract of treated larvae compared to the control group.

The overall results indicate that treating larvae with fish protein hydrolysates through the live feed, resulted in stimulation of the innate immunity of first feeding halibut larvae. As a conclusion, larvae should only be fed live feed treated with the selected products in one of the two daily feedings, at least during the first weeks after the onset of feeding. The appropriate concentrations for treatment will have to be examined further and adjusted, not least with respect to elevated numbers of bacteria that may follow the increased overall biological load of the tank water environment. The selected chitosan product should be used in much lower concentrations than studied in the present work.

Feeding halibut larvae live feed treated with various fish protein hydrolysates or chitosan did not enhance the growth or survival of the larvae during the period studied. Treating larvae with BP and PP resulted in a final weight of larvae similar to the control groups. The treatments, however, resulted in a higher ratio of larvae not successfully metamorphosed at the end of first feeding. The treatments therefore did not improve the normal development of larvae. The selected treatment schedules of chitosan and CP seemed to negatively affect the larvae, indicating too high concentrations used for the treatments.

6. Reference

Adler-Nissen J., 1986. Enzymatic hydrolysis of food proteins. *New York: Elsevier Applied Science*.

Akiyama K., Kawazu K., Kobayashi A., 1995. A novel method for chemo-enzymatic synthesis of elicitor-active chitosan oligomers and partially N-acetylated chitin oligomers using N-acetylated chitotrioses as substrate in a lysozyme-catalyzed transglycosylation reaction system. *Carbohydrate Research, 279: 151–160.*

Aksnes A., Hope B., Jönsson E., Björnsson B.T., Albreksten S., 2006. Size-fractionated fish hydrolysate as feed ingredient for rainbow trout (*Oncorhynchus mykiss*) fed high plant protein diets. I: Growth, growth regulation and feed utilization. *Aquaculture, 261: 305-317.*

Anderson D.P., Siwicki A.K., 1994. Duration of protection against *Aeromonas salmonicida* in brook trout immunostimulated with glucan or chitosan by injection or immersion. *The Progressive Fish-Culturist 56: 258-261.*

Artemia International LLc: http://www.artemia-international.com

Aspmo S.I., Horn S.J., Eijsink V.G.H., 2005. Enzymatic hydrolysis of Atlantic cod (*Gadus morhua*) viscera. *Process Biochemistry 40: 1957-1966.*

Austin B., Austin D.A., 1987. *Bacterial Fish Pathogenes: Disease in Farmed and Wild Fish, pp. 111-195. Chichester: E. Horwood Ltd.*

Avtalion R.R., Mor A., 1992. Monomeric IgM is transferred from mother to egg in tilapias. Israel *Journol of Aquaculter (Bamidgeh) 44:93-98.*

Balfry S.K., Iwama G.K., 2004. Observations on the inherent variability of measuring lysoszyme activity in coho salmon (*Oncorhynchus kisitch*). *Comparative Biochemistry and Physiology, Part B 138: 207-211.*

Bell J.G., McEvoy L.A., Estevez A., Shields R.J., Sargent J., 2003. Optiminsing lipid nutrition in first-feeding flatfish larvae. *Aquaculture 227: 211-220.*

Bengston D.A., Borrus D.N., Leibovitz H.E., Simpson K.L., 1993. Studies on structure and function of the digest system of *Menidia beryllina* (Picses, Atherinidae). In:

Walther B.T., Fyhn H.J., (Eds.), *Physiological and Biochemical Aspects of Fish Development. University of Bergen, Norway, pp. 199-208.*

Berge G.M., Storebakken, T., 1996. Fish protein hydrolyzate in starter diets for Atlantic salmon (*Salmo salar*) fry. *Aquaculture 145: 205–212.*

Biering E., 1997. Immune response of the Atlantic halibut (*Hippoglossus hippoglossus* L) to infectious pancreatic necroris virus (IPNV). *Fish and Shellfish Immunology 7: 137-149.*

BioRad, The Experion™ Pro260, analysis kit, instruction manual:
http://www.genmall.com.tw/products/HB/Experion%20Pro260%20Analysis%20Kit.pdf

Bly J.E., Grimm A.S., Morris I.G., 1986. Transfer of passive immunity from mother to young in a teleost fish: haemagglutinating activity in the serum and eggs of plaice, *Pleuronectes platessa* L. *Comparative Biochemistry and Physiology A;84:309-313.*

Bly J.E., Quiniou S.M., Clem L.W., 1997. Environmental effects on fish immune mechanism. *Developments in Biological Standardization 90: 33-43.*

Boshra H, Li J, Sunyer JO, 2007. Recent advances on the complement system of teleost fish. *Fish and Shellfish Immunology 20(2): 239-262.*

Bowden T.J., Lochart K., Smail D.A., Ellis A.E., 2003. Experimental challange of post-smolts with IPNV: mortalities do not depend on population density. *Journal of Fish Diseases 26: 309-312.*

Bowden T.J., Butler R., Bricknell I.R., 2004. Seasonal variation of serum lysozyme levels in Atlantic halibut (*Hippoglossus hippoglossus* L.). *Fish and Shellfish Immunology 17: 129-135.*

Bowden T.J., Lester K., MacLachlan P., Bricknell I.R., 2000. Preliminary study into the short term effects of adjuvants on Atlantic halibut (*Hippoglossus hippoglossus* L.). *Bulletin of the European Association for Fish Pathologists 20(3): 148–51.*

Breuil G., Vassiloglou B., Pepin J.F., Romestand B., 1997. Ontogeny of IgM-bearing cells and changes in the immunoglobulin M-like protein level (IgM) during larval stages in sea bass (*Dicentrarchus labrax*). *Fish and Shellfish Immunology 7: 29-43.*

Bricknell I., Dalmo R.A., 2005. The use of immunostimulants in fish larval aquacultere. *Fish and Shellfish Immunology 19(5): 457-472.*

Bricknell I.R., Bowden T.J., Verner-Jeffreys D.W., Bruno D.W., Shields R.J., Ellis A.E., 2000. Susceptibility of juveniles and sub-adult Atlantic halibut (*Hippoglossus hippoglossus* L) to infection by *Vibreo anguillarum* and efficacy of protection induced by vaccination. *Fish and Shellfish Immunology 10: 319-327*.

Bromage E.S., Ye J.M., Owens L., Kaattari I.M., Kaattari S.L., 2004. Use of staphylococcal protein A in the analysis of teleost immunoglobulin structural diversity. *Developmental and Comparative Immunology Developmental and Comparative Immunology 28: 803-814*.

Brown L.L., Cox W.T., Levine R.P., 1997. Evidence that the causal agent of bacterial cold-water disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. *Disease of Aquatic Organisms 29: 213-218*.

Bøgwald J., Dalmot, R.A., McQueen Leifson, R., Stenberg, E., Gildberg, A., 1996. The stimulatory effect of a muscle protein hydrolysate from Atlantic cod, Gadus morhua L., on Atlantic salmon, Salmo salar L., head kidney leucocytes. *Fish and Shellfish Immunology 6: 3–16*.

Bøgwald J., Jaakola A., Rinne A., Kalkkinen N., Helin J., Ylönen A., 2002. Purification and characterization of novel kininogens from spotted wolffish and Atlantic cod. *European Journal of Biochemistry. 269(11): 2639-2646*.

Cahu C.L., Zambonino Infante, J.L., Quazuguel, P., Le Gall, M.M., 1999. Protein hydrolysate vs. fish meal in compound diets for 10-day old sea bass *Dicentrarchus labrax* larvae. *Aquaculture 171: 109–119*.

Carvalho A.P.C., Escaffre A.M., Olivia-Teles A., Bergot P., 1997. First feeding og common carp larvae on diets with high levels of protein hydrolysates. *Aquaculture International 5: 361-367*.

Cecchini S., Terova G., Caricato G., Saroglia M., 2000. Lysosome activity in embryos and larvae of sea bass (*Dicentrarchus labrax* L.), spawned by broodstocks fed with vitamin C enriched diets. *Bulletin of the European Association for Fish Pathologists 20: 120-124*.

Cha S.H., Lee J.S., Song C.B., Lee K.J., Jeon Y.J., 2008. Effects of chitosan-coated diet on improving water quality and innate immunity in the olive flounder, *Paralichthys olivaceus. Aquaculture 278 (1-4): 110-118*

Chantanachookhin C., Seikai T., Tanaka M., 1991. Comparative study of the ontogeny of the lymphoid organs in three species of marine fish. *Aquaculture 99: 143-155.*

Chen R.H., Chen J.S., 2000. Changes of polydispersity and limiting molecular weight of ultrasound-treated chitosan. *Adv Chitin Science 4: 361–366.*

Colten H.R., 1994. Immunology: Drawing a double-edged sword. *Nature 371: 474–5.*

Copeman L.A., Parrish C.C., Brown J.A., Harel M., 2002. Effects of docosahexanoic, eicosapentaenoic and arachidonic acids on the early growth, survival, lipid composition and pigmentation of yellowtail flounder (*Limanda ferruginea*): a live food enrichment experiment. *Aquaculture 210: 285-304.*

Coste M., Huneau, J.F., Mahe, S., Tomé, D., 1992. Interactions between milk protein peptides and intestinal mucosa. In: Paubert-Braquet, M., Dupont, C., Paoleti, R. (Eds.), Foods, Nutrition and Immunity. *Dynamic Nutrition Research, Basel, Krager 1: 96–103.*

Dabrowski K., Dabrowska H., 1981. Digestion of protein by rainbow trout (*Salmo gairdneri Rich*) and absorption af amino acids within the alimentary tract. *Comparative Biochemistry and Physiology 69A: 99-111.*

Dalmo R.A., Ingebrigsten K., Sveinbjornsson B., Seljelid R., 1996. Accumulation of immunomodulatory laminaran (beta (1,3)-D-glucan) in the heart, spleen and kidney of Atlantic cod, *Gadus morhua. Journal of Fish Diseases 19:129-136.*

Dalmo R.A., Kjerstad A.A., Arnesen S.M., Tobias P.S., Bøgwald J., 2000. Bath exposure of Atlantic halibut (*Hippoglossus hippoglossus L.*) yolk sac larvae to bacterial lipopolysaccharide (LPS): Absorption and distribution of the LPS and effect on fish survival. *Fish and Shellfish Immunology 10: 107-128.*

Dalmo R.A., Seljelid R., 1995. The immunomodulartory effect of LPS, laminaran and sulphated laminaran [β(1,3)-D-glukan] on Atlantic salmon, *S. salar,* macrophages in vitro. *Journal of Fish Diseases 18: 175-185.*

Daoud R., Dubois, V., Bors-Dodita, L., Nedjar-Arroume, N., Krier, F., Chihib, N.E., Mary, P., Kouach, M., Briand, G., Guillochon, D., 2005. New antibacterial peptide derived from bovine hemoglobin. *Peptides 26: 713–719.*

Dautremepuits C., Betoulle S., Paris-Palacios S., Vernet G., 2004. Humoral immune factors modulated by copper and chitosan in healthy or parasitized carp (*Cyprinus carpio L.*) by *Ptychobothrium* sp. (Cestoda). *Aquatic Toxicology* 68: *325-338.*

Dautremepuits C., Fortier M., Croietiere S., Belhumeur P., Fournier M., 2006. Modulation of juvenile brook trout (*Salvelinus fontinalis*) cellular immune system after *Aeromonas salmonicida* challenge. *Veterinary Immunology and Immunopathology 110(1-2): 27-36.*

Day O.J., Howell, B.R., Jones, D.A., 1997. The effect of dietary hydrolyzed fish protein concentrate on the survival and growth of juvenile Dover sole, *Solea solea* (L.), during and after weaning. *Aquaculture Research 28: 911–921.*

Debaulney M.O., Quental C., Fournier V., Lamour F., Gouvello R.L., 1996. Effect of long-term oral administration of β-glucan as an immunostimulant or an adjuvant on some non-specific parameters of the immune response of turbot, *Scophthalmus maximus. Disease of Aquatic Organisms 26: 139-147.*

Del Rio-Tsonis K., Tsonis P.A., Zarkadis I.K., Tsagas A.G., Lambris J.D., 1998. Expression of the third component of complement, C3, in regenerating limb blastema cells of urodeles. *The Journal of Immunology 161: 6819-6824.*

Dixon B., Stet R.J.M., 2001. The relationship between major histocompatibility receptors and innate immunity in teleost fish. *Developmental and Comparative Immunology 25: 683-700.*

Dogget T. A., Harris, J. E., 1987. The ontogeny of gut-associated lymphoid tissue in immunity and tolerance in fish. In *Microbial Diseases of Fish* (R. J. Roberts, ed.)

Dos Santos N.M.S., Romano N., De Sousa M., Ellis A.E., Rombout J.H.W.M., 2000. Ontogeny of B and T cells in sea bass (*Dicentrarchus labrax* L.). *Fish and Shellfish Immunology 10: 583-596.*

Douglas S.E., Gallant J.W., Gong Z., Hew C., 2001. Cloning and developmental expression of a family of pleurocidin-like antimicrobial peptides from winter flounder, *Pleuronectes americanus* (Walbaum). *Developmental and Comparative Immunology 25: 137– 147.*

Dügenci S.K., Arda N., Candan A., 2003. Some medicinal plants as immunostimulants for fish. *Journal of Ethnopharmacology 88(1): 99-106.*

Ellis A.E., 1977. The laucocytes of fish: a review. *Journal of Fish Biology 11: 453-491.*

Ellis A.E., 1988. Ontogeny of the immune system in teleost fish. In *Fish Vaccination (A.E. Ellis, ed) pp. 20-31. London: Academic Press.*

Ellis A.E., 1998. Ontogeny of the immune system. In: Handbook of vertebrate immunology, pp: 26-30.

Elward K, Gasque P., 2003. ''Eat me'' and ''don't eat me'' signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system. *Molecular Immunology 40: 85-94.*

Engstad R.E., Robertson B., Frivold E., 1992. Yeast glucan induces increase in lysozyme and complement-mediated haemolytic activity in Atlantic salmon blood. *Fish and Shellfish Immunology 2: 287-297.*

Espevik T., Otterlei M., Skjåk-Bræk G., Ryan L., Wright S.D., Sundan A., 1993. The involvement of CD 14 in stimulation of cytokine production by uronic acids polymers. *The European Journal of Immunology 23: 255-261.*

Estevez A., Kanazawa A., 1995. Effect of n-3 PUFA and vitamin A *Artemia* enrichment on pigmentation sucess of turbot, *Schopthalmus maximus* (L.). *Aquaculture Nutritionritition 1: 159-168.*

Estevez A., McEvoy L.A., Bell J.G., Sargent J.R., 1999. Growth, survival, lipid composition and pigmentation of turbot (*Schopthalmus maximus)* larvae fed live-prey enriched in arachidonic and eicosapentaenoic acids. *Aquaculture 180: 321-343.*

Evans D.L., Leary J.H., Jaso-Friedmann L., 2001. Nonspecific cytotoxic cells and innate immunity: regulation by programmed cell death. *Developmental and Comparative Immunology 25: 791-805.*

Fagbenro O., Jauncey K., Haylor G., 1994. Nutritive value of diets containing dried lactic acid fermented fish silage and soybean meal for juvenile *Oreochromis niloticus* and *Clarias gariepinus. Aquatic Living Resources 7: 79-85.*

Falk-Petersen I.B., 2005. Comparative organ differentiation during early life stage of marine fish. *Fish and Shellfish Immunology 19: 397-412.*

Fearon D.T., 1997. Seeking wisdom in innate immunity. *Nature 388: 323-324.*

Felt O., Buri P., Gurny R., 1998. Chitosan: A unique polysaccharide for drug delivery. *Drug Development and Industrial Pharmacy 24: 979–993.*

Feng J., Zhao L., Yu Q., 2004. Receptor-mediated stimulatory effect of oligochitosan in macrophages. *Biochemical and Biophysical Research Communications 317: 414–420.*

Fishelson Z., Attali G., Mevorach D., 2001. Complement and apoptosis. *Molecular Immunology 38: 207-219.*

Fletcher T.C., 1982. Non-specific defense mechanisms in fish. *Journal of Fish Diseases 8: 35–41.*

Gades M.D. & Stern J.S., 2005. Chitosan supplementation and fat absorption in men and women. *Journal of the American Dietetic Association 105(1): 72-77.*

Gbogouri G.A., Linder M., Fanni J., Parmentier M., 2004. Influence of hydrolysis degree on the functional properties of salmon byproducts hydrolysates. *Food Chemistry Toicol 69: 615-622.*

Gildberg A., Bøgwald, J., Johansen, A., Stenverg, E., 1996. Isolation of acid peptide fractions from a fish protein hydrolysate with strong stimulatory effect on Atlantic salmon (Salmo salar) head kidney leucocytes. *Comparative Biochemistry and Physiology 114B: 97–101*

Giustina A., Ventura P., 1995. Weight-reducing regimens in obese subjects: Effects of a new dietary fiber integrator. *Acta Toxicological Therapeutics 16: 199–214.*

Govoni J.J., Boehlert G.W., Watanabe Y., 1986. The physiology of digestion in fish larvae. *Environmental Biology* of *Fishes 16: 59-77.*

Grinde B., Lie Ø., Poppe T., Salte R., 1988. Species and individual variation in lysozyme activity in fish of interest in aquaculture. *Aquaculture 68: 299-304.*

Gröntvendt R.N., Espelid S., 2003. Immunoglobulin producing cells in the spotted wolfish (Anarhichas minor Olafsen). *Developmental and Comparative Immunology 27: 569-578.*

Grove S., Johansen R., Reitan L.J., Press C.McL., 2006. Immune- and enzyme histochemical characterization of leukocyte populations within lymphoid and mucosal tissues of Atlantic halibut (*Hippoglossus hippoglossus*). *Fish and Shellfish Immunology 20: 693-708.*

Gunnlaugsdóttir H. & Þorkelsson G., 2005. " Lífvirk efni í íslensku sjávarfangi." *Verkefnisskýrsla RF 6-05.*

Hale M.B., 1972. Making fish protein concentrate by enzymatic hydrolysis. In: Kristinsson H.G., Rasco B.A., 2000. Fish protein hydrolysates: production, biochemical and functional properties. *Critical reviews in food science and nutrition 40: 43-81.*

Hamre K., Holen E., Moren M., 2007. Pigmentation and eye migration in Atlantic halibut (*Hippoglossus hippoglossus L.*) larvae: new findings and hypotheses. *Aquaculture Nutrition 13: 65-80.*

Hamre K., Baeverfjord G., Harboe T., 2005(a). Macronutrient composition of formulated diets for Atlantic halibut (*Hippoglossus hippoglossus, L.*) juveniles, II: protein/lipid levels at low carbohydrate. *Aquaculture 244: 283-291.*

Hamre K., Moren M., Solbakken J., Opstad I., Pittman K., 2005(b). The impact of nutrition on metamorphosis in Atlantic halibut (*Hippoglossus hippoglossus* L.). *Aquaculture 250: 555-565.*

Hamre K., Opstad I., Espe M., Solbakken J., Hemre G.I., Pittman K., 2002. Nutrient composition and metamorphosis success of Atlantic halibut (*Hippoglossus hippoglossus,* L) larvae fed natural zooplankton or *Artemia. Aquaculture Nutritions 8: 139-148.*

Hanif A., Bakopoulos V., Dimitriadis G.J., 2004. Maternal transfer of humoral specific and non-specific immune parameters to sea bream (*Sparus aurata*) larvae. *Fish and Shellfish Immunology 17: 411-435.*

Håvarstein L.S., Aasjord P.M., Ness S., Endresen C., 1988. Purification and partial characterization of an IgM-like serum immunoglobulin from Atlantic salmon (*Salmo salar*). *Developmental and Comparative Immunology 12: 773-785.*

Hayman J.R., Lobb C.J., 1993. Immunoglobulin in the eggs of the channel catfish (Ictalurus punctatus). *Developmental and Comparative Immunology 17: 241-248.*

Hébert P., Ainsworth A.J., Boyd B., 2002. Histological enzyme and flow cytometric analysis of channel catfish intestinal tract immune cells. *Developmental and Comparative Immunology 26: 53– 62.*

Heras H., McLeod C.A., Ackman R.G., 1994. Atlantic dogfish silage vs. Herring silage in diets for Atlantic salmon (*Salmo salar*): growth and sensory evaluation of fillets. *Aquaculture 125: 93-106.*

Hermannsdóttir R., 2005. Áhrif lífvirkra efna á ríkjandi bakteríur í lúðueldi. BSc theses, *Resource Science, University of Akureyri.*

Hordvik I., Voie A.M., Glette J., Male R., Endresen C., 1992. Cloning and sequence analysis of two isotypic IgM heavy chain genes from Atlantic salmon, *Salmo salar L. The European Journal of Immunology 22: 2957-2962.*

Hordvik J., Thevarjan J., Samdal I., Bastani N., Krossøy B., 1999. Molecular cloning and phylogenetic analysis of the Atlantic salmon immunoglobulin D gene. *Scandinavian Journal of Immunology. 50: 202-210.*

Hutchinson T.M., Manning M.J., 1996. Seasonal trends in serum lysozyme activity and total protein concentration in dab (*Limanda limanda* L.) sampled from Lyme Bay, U.K. *Fish and Shellfish Immunology 6: 473-482.*

Il'ina A.V., Varlamov V.P., 2004. Hydrolysis of chitosan in lactic acid. *Applied Biochemistry and Microbiology 40: 300–303.*

Imagawa T., Hashimoto Y., Kon Y., Sugimura M., 1991. Immunoglobulin containing cells in the head kidney of carp (*Cyprinus carpio L.*) after bovine serum albunin injection. *Fish and Shellfish Immunology 1: 173-185.*

Ingilae M., Arnesen J.A., Lund V., Eggset G., 2000. Vaccination of Atlantic halibut *Hippoglossus hippoglossus* L., and spotted wolffish *Arnarhichas minor* L., against atypical *Aeromonas salmonicida. Aquaculture 183: 31-44.*

Jeney G., Anderson D.P., 1993. An in vitro technique for surveying immunostimulants in fish. *Aquaculture 112: 283-287.*

Jeney G., Galeotti D.P., Jeney Z., Anderson D.P., 1997. Prevention of stress in rainbow trout (*O. mykiss*) fed diets containing different doses of glucan. *Aquaculture 154: 1-15.*

Jeon Y.J., Shahidi F., Kim S.K., 2000. Preparation of chitin and chitosan oligomers and their applications in physiological functional foods. *Food Review International 61: 159–176.*

Jóhanssdóttir J.Þ., Smáradóttir H., Coe J., Hermannsdóttir R., Pétursdóttir M., Björnsdóttir R., 2007. Notkun lífvirkra efna í lúðueldi. *Matís report 51-07. ISSN 1670-7192*

Jónsdóttir A.M., 2005. Lífvirk efni í lúðueldi. BSc theses, *Resource Science*, *University of Akureyri*.

Jørgensen J., Robertsen B., 1995. Yeast β-glucan stimulates respiratory burst activity of Atlantic salmon (*Salmo salar L.*) macrophages. *Developmental and Comparative Immunology 19: 43-57.*

Jørgensen J.B., Sharp G.J.E., Secombes C.J., Robertsen B., 1993. Effect of a yeast-cell-wall glucan on the bactericidal activity of rainbow trout macrophages. *Fish and Shellfish Immunology 3: 267-277.*

Kanis E., Refstie T., Gjedrem T., 1976. A genetic analysis of egg, alevin and fry mortality in salmon (*Salmo salar*), sea trout (*Salmo trutta*), and rainbow trout (*Salmo gairdneri*). *Aquaculture 8: 259-268*

Kanlis G., Suzuki Y., Tauchi M., Numata T., Shirojo Y., Takashima F., 1995. Immunoglobulin in oocytes, fertilized eggs and yolk sac larvae of red sea bream. *Fish Science 61:787-790.*

Keles O., Candan A., Bakirel T., Karatas S., 2002. The investigation of the anabolic efficiency and effect on the non-specific immune system of zeranol in rainbow trout (*Oncorhnchus mykiss,* Walbaum). *Turkish Journal of Veterinary and Animal Science 26: 925-931.*

Kim Se-K., Rajapakse N., 2005. Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydrate Polymers 62: 357-368.*

Kjørsvik E., Reiersen A.L., 1992. Histomorphology of the early yolk-sac larvae of the Atlantic halibut (*Hippoglossus hippoglossus L.)* – an indication of the timing of functionality. *Journal of Fish Biology 41: 1-19.*

Klein J.R., 1998. Hormone regulation of immune homeostasis: local or long distance? *Biochemical Pharmocology 56: 1-5.*

Kolkovski S., Tandler A., 2000. The use of squid protein hydrolysate as a protein source in microdiets for gilthead seabream Sparus aurata larvae. *Aquaculture Nutrition. 6: 11–17.*

Koumans-van Diepen J.C.E., Taverne-Thiele J.J., van Rens B.T.T.M., Rombout J.H.W.M., 1994. Immunocytochemical and flow cytometric analysis of B cells and plasma cells in carp (*Cyprinus carpio* L.); an ontogenetic study. *Fish and Shellfish Immunology 4(1): 19-28*

Kotzamanis Y.P., Gisbert E., Gatesoupe F.J., Zambonino Infante J., Cahu C., 2007. Effects of different dietary levels of fish protein hydrolysates on growth, digestive enzymes, gut microbiota, and resistance to *Vibrio anguillarum* in European sea bass (*Dicentrarchus labrax*) larvae. *Comparative Biochemistry and Physiology 147: 205-214.*

Kristinsson H.G., Rasco B.A., 2000. Fish protein hydrolysates: production, biochemical, and functional properties. *Critical reviews in food science and nutrition 40: 43-81.*

Kudo S., 1992. Enzymatic basis for protection of fish embryos by the fertilization envelope. *Experientia 48: 277-281.*

Kumar S.R., Ahmed I.V.P, Parameswaran V., Sudhakaran R., Babu S.V., Hameed S.A.S., 2007. Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in Asian sea bass (*Lates calcarifer*) to protect from *Vibrio* (*Listonella*) *anguillarum. Fish and Shellfish Immunology*, **In Press, Corrected Proof**, *Available online 16 December 2007*

Kurita K., 1998. Chemistry and application of chitin and chitosan. *Polymer Degradation and Stability 59: 117–120.*

Kuroiwa T., Ichikawa S., Sato S., Hiruta O., Sato S., Mukataka S., 2002. Factors affecting the composition of oligosaccharides producedin chitosan hydrolysis using immobilized chitosanases. *Biotechnology Progress 18: 969–974.*

Kvåle A., Nordgreen A., Tonheim S.K., Hamre K., 2007. The problem of meeting dietary protein requirements in intensive aquaculture of marine fish larvae, with emphasis on Atlantic halibut (*Hippoglossus hippoglossus L.*). *Aquaculture Nutrition 13: 170-185.*

Lall S.P., 1991. Nutritional value of fish silage in salmonid diets. *Bulletin of the Aquaculture Association of Canada 91: 63-74.*

Lamers C.H.J., 1986. Histophysiology of primary immune response against *Aeromonas hydrophila* in carp (*Cyprinus carpio L.*). *Journal of Experimental Zoology 238: 71-80.*

Lange S., Bambir S., Dodds A.W., Magnadóttir B., 2004(b). An immunohistochemical study on complement component C3 in juvenile Atlantic halibut (*Hippoglossus hippoglossus* L.). *Developmental and Comparative Immunology 28(6): 593-601.*

Lange S., Bambir S., Dodds A.W., Magnadóttir B., 2004(a). The ontogeny of complement component C3 in Atlantic halibut (*Hippoglossus hippoglossus* L.) an immunohistochemical study. *Developmental and Comparative Immunology 28: 593-601.*

Lange S., Bambir S.H., Dodds A.W., Bowden T., Bricknell I., Espelid S., Magnadóttir B., 2006. Complement component C3 transcription in Atlantic halibut (*Hippoglossus hippoglossus L.*) larvae. *Fish and Shellfish Immunology 20: 285-294.*

Lange S., Dodds A.W., Magnadóttir B., 2004(c). Isolation and characterisation of complement component C3 from Atlantic cod (*Gadus morhua* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.). *Fish and Shellfish Immunology 16: 227-239.*

Lange S., Gudmundsdottir B.K., Magnadottir B., 2001. Humoral immune parameters of cultured Atlantic halibut (*Hippoglossus hippoglossus* L.). *Fish and Shellfish Immunology 11: 523-535*

Lange S. & Magnadóttir B., 2003. The spontaneous haemolytic activity of Atlantic halibut (*Hippoglossus hippoglossus* L.) and Sea bass (*Dicentrarchus labrax*) serum. *Comparative Biochemistry and Physiology Part B Biochemestry and Molecular Biology 136(1):99–106.*

Langston A.L., Hoare R., Stefansson M., Fitzgerald R., Wergeland H., Mulcahy M., 2002. The effect of temperature on non-specific defence parameters of three strains of juvenile Atlantic halibut (*Hippoglossus hippoglossus* L.). *Fish and Shellfish Immunology 12: 61–76.*

Lapie L.P., Biqueras-Benitez C.M., 1992. Feeding studies on tilapia (*Oreochromis sp.*) using fish silage. *FAO-Fisheries. Reports 470: 165-177.*

Law S.K.A., Dodds A.W., 1997. The internal thioester and the covalent binding properties of the complement proteins C3 and C4. *Protein Science 6: 263-274.*

Lee S.H., Suh J.S., Kim H.S., Lee J.D., Song J.S., Lee S.K., 2003. Evaluation of radiation synovectomy of the knee by means of intraarticular injection of holmium-166-chitosan complex in patients with rheumatoid arthritis: Results at 4-month follow-up. *Korean Journal of Radiology 4: 170–178.*

Liang M., Wang J., Chang Q., Mai K., 2006. Effects of different levels of fish protein hydrolysate in the diet on the nonspecific immunity of Japanese sea bass, *Lateolabrax japonicas (Cuvieret Valenciennes, 1828). Aquaculture Research 37: 102-106.*

Liaset B., Espe M., 2008. Nutritional composition of soluble and insoluble fractions obtained by enzymatic hydrolysis of fish-raw materials. *Process Biochemistry 43: 42-48.*

Lie Ø., Evensen Ø., Sørensen A., Frøysadal E., 1989. Study on lysozyme activity in some fish species. *Disease of Aquatic Organisms 6: 1-5.*

Lillehaug A., Sevatdal S., Endal T., 1996. Passive transfer of specific maternal immunity does not protect Atlantic salmon (*Salmo salar* L.) fry against yersiniosis. *Fish and Shellfish Immunology 6: 521-535.*

Lo D., Feng L., Carson M.J., Crowley M., Pauza M., Nguyen A., 1999. Integrating innate and adaptive immunity in the whole animal. *Immunology Review 169: 225-239.*

Lobb C.J., Olson M.O.J., Clem L.W., 1984. Immunoglobulin light chain classes in a teleost fish. *The Journal of Immunology 132: 1917-1923.*

Loewe H., Eckmann R., 1988. The ontogeny of the alimentary tract of coregonid larvae normal development. *Journal of Fish Biology 33: 841-850.*

Loffler A., 1986. Proteolytic enzymes: sources and applications. *Food Technoology 40: 63.*

Luizi F.S., Gara B., Shields R.J. and Bromage N.R. 1999. Further description of the development of the digestive organs in Atlantic halibut (*Hippoglossus hippoglossus*) larvae, with notes on differential absorption of copepod and *Artemia* prey. *Aquaculture. 176: 101–116.*

Macchi G., 1996. A new approach to the treatment of obesity: Chitosan's effects on body weight reduction and plasma cholesterol levels. *Acta Toxicol Therapeutics 27: 303–320.*

Magnadóttir B., 1998. Comparison of immunoglobulin (IgM) from four fish species. *Búvísindi, Icelandic Agriculter Science 12: 47-59.*

Magnadóttir B., 2006. Innate immunity of fish (overview). *Fish and Shellfish Immunology 20(2): 137-151.*

Magnadóttir B., Lange S., Steinarsson A., Gudmundsdóttir S., 2004. The ontogenic development of innate immune parameters of cod (*Gadus morhua* L.). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 139(2): 217-224.*

Magnadottir B., Lange S., Gudmundsdottir S., Bøgwald J., Dalmo R.A., 2005. Ontogeny of humoral immune parameters in fish. *Fish and Shellfish Immunology 19: 429-439.*

Magor B.G., Magor K.E., 2001. Evolution of effectors and receptors of innate immunity. *Developmental and Comparative Immunology 25: 651–682.*

Majeti N.V., Kumar R., 2000. A review of chitin and chitosan. *Reactive and Functionial Polymers 46: 1–27.*

Matsunaga T., 1998. Did the first adaptive immunity evolve in the gut of ancient jawed fish? *Cytogenet Cell Genet 80: 138– 141.*

McEvoy L.A., Estevez A., Bell J.G., Shields R.J., Gara B., Sargent J.R., 1998. Influence of dietary levels of eicosapentaenoic and arachidonic on the pigmentation success of turbot (*Schopthalmus maximus*) and halibut (*Hippoglossus hippoglosuss*). *Bulletin of the Aquaculture Association of Canada 4: 17-20.*

Medzhitov R. & Janeway Jr. C.A., 2002. Decoding the patterns of self and nonself by the innate immune system. *Science 296: 298-300.*

Miki N., Taniguchi T., Hamakawa H., 1998. Effect of rotifer enriched with fat soluble vitamins on occurrence of albinism in hatchery reared ´Hirame´ *Paralichthys olivaceus* (preliminary report). *Suisan Zoshoku 36: 91-96.*

Misra C.K., Das B.K., Pradhan J., Pattnaik P., Sethi S., Mukherjee S.C., 2004. Changes in lysosomal enzymes activity and protection against *Vibrio* infection in *Macrobrachium rosenbergii* (De Man) post larvae after bath immunostimulation with β-glucan. *Fish and Shellfish Immunology 17: 389-395.*

Mor A., Avtalion R.R., 1988. Evidence of transfer of immunity from mother to eggs in tilapias. *Israel Journol of Aquaculter (Bamidgeh) 40:22-28.*

Morgan B.P., Gasque P., 1996. Expression of complement in the brain: role in health and disease. *Immunology Today 17: 461-466.*

Mulero I., García-Ayala A., Meseguer J., Mulero V., 2007. Maternal transfer of immunity and ontogeny of autologous immunocompetence of fish: A minireview. *Aquaculture 268: 244-250.*

Murray C.K., Fletcher T.C., 1976. The immunohistochemical localization of lysozyme in plaice (*Pleuronectes platessa* L.) tissues. *Journal of Fish Biology 9:329-334.*

Murray H.M., Wright G.M., Goff G.P., 1994. A comparative histological and histochemical study of the stomach from three species of pleuronectid, The Atlantic halibut, *Hippoglossus hippoglossus*, the yellowtail flounder, *Pleuronectes ferruginea*, and the winter flounder, *Pleuronectes americanus*. *Canadian Journol of Zoology 72: 1199-1210.*

Murray A.L., Pascho R.J., Alcorn S.W., Fairgrieve W.T., Shearer K.D., Roley D., 2003. Effects of various feed supplements containing fish protein hydrolysate or fish

processing by-products on the innate immune functions of juvenile coho salmon (*Oncorhynchus kisutch*). *Aquaculture 220: 643–653.*

Nakao M., Yano T., 1998. Structural and functional identification of complement components of the bony fish, carp (*Cyprinus carpio*). *Immunology Review 166: 27–38.*

Neumann N.F., Stafford J.L., Barreda D., Ainsworth A.J., Belosevic M., 2001. Antimicrobial mechanisms of fish phagocytes and their role in host defense. *Developmental and Comparative Immunology 25: 807-825.*

Nikolakopoulou K., Zarkadis I.K., 2006. Molecular cloning and characterisation of two homologues of Mannose-Binding Lectin in rainbow trout. *Fish and Shellfish Immunology 21: 305-314.*

Nishimura K., Nishimura S., Nishi N., Saiki I., Tokura S., Azuma I., 1984. Immunological activity of chitin and its derivatives. *Vaccine 2: 93–99.*

O´Dowd A.M., Ellis A.E., Secombes C.J., 1998. Binding of immune complexes to Atlantic salmon peripheral blood leucocytes. *Developmental and Comparative Immunology 22: 439-448.*

O´Dowd A.M., Ellis A.E., Secombes C.J., 1999. Binding of soluble immune complexes to fractionated Atlantic salmon (*Salmo salar L.*) leucocytes. *Veterinary Immunology and Immunopathology 68: 149-157.*

Olafsen J.A., 2001. Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture 200(1-2): 223-247.*

Olsen Y.A., Press C.McL., 1997. Degradation kinetics of immunoglobulin in the egg, alevin and fry of Atlantic salmon, *Salmo salar* L., and the localisation of immunoglobulin in the egg. *Fish and Shellfish Immunology 7: 81-91.*

Ottaway C.A., 1991. Neuroimmunomodulation in the intestinal mucosa. *Gastroenterology Clinics of North America 20: 511-529.*

Otterlei M., Østgård K., Skjåk-Bræk G., Smidsrød O., Soon-Shion P., Espevik T., 1991. Induction of cytokine production from human monocytes stimulated with alginate. *Journal of Immunotherapy10: 286-291.*

Padrós F., Crespo S., 1996. Ontogeny of the lymphoid organs in the turbot *Scophthalmus maximus;* a light and electron microscope study. *Aquaculture 144: 1-16.*

Parrish C.C., Li H., Indrasena W.M., Ackman R.G., 1991. Silage feeds in Atlantic salmon farming: flavour volatiles and lipid composition, feeding trials, and taste panels. *Bulletin of the Aquaculture Association of Canada 91(1): 75-84.*

Pedersen B.H., 1993. Protein digestion in herring (*Clupea harengus*) larvae: trypsinogen secretion and effects of a transitory food restriction on mortality, growth and digestive enzyme content. In: Walther B.T., Fyhn H.J. (Eds.), *Physiological and Biochemical Aspects of Fish Development. University of Bergen, Norway, pp. 220-225.*

Pepin J.F., Brueil G., Rigobert P., Romestand B., 1997. Detection and characterization of immunoglobulins in the eggs of the sea bass (*Dicentrarchus labrax*). *Ichtyophysiol Acta 20: 77e85.*

Petrie-Hanson L., Ainsworth A.J., 1999. Humoral immune responses of channel catfish (*Ictalurus punctatus*) fry and fingerlings exposed to *Edwardsiella ictaluri. Fish and Shellfish Immunology 9: 579-589.*

Pettersen E.F., Bjerknes R., Wergeland H.I., 2000. Studies of Atlantic salmon (*Salmo salar L.*) blood, spleen and head kidney leucocytes using specific monoclonal antibodies, immunohistochemistry and flow cytometry. *Fish and Shellfish Immunology 10: 695-710.*

Picchietti S., Taddei A.R., Scapigliati G., Buonocore F., Fausto A.M., Romano N., 2004. Immunoglobulin protein and gene transcripts in ovarian follicles throughout oogenesis in the teleost *Dicentrachus labrax. Cell and Tissue Research 315: 259-270.*

Picot L., Bordenave S., Didelot S., Fruitier-Arnaudin I., Sannier F., Thokelsson G., Bergé J.P., Guérard F., Chabeaud A., Piot J.M., 2006. Antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines. *Process Biochemistry 41: 1217-1222.*

Pihlanto-Leppälä A., 2001. Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. *Trends in Food Science and Technology 11: 347-356.*

Pilstrøm L., Bengten E., 1996. Immunoglobulin in fish – genes, expression and structure. *Fish and Shellfish Immunology 6: 243-262.*

Pittman K., Skiftesvik A.B., Berg L., 1990. Morphological and behavioural development of halibut, *Hippoglossus hippoglossus* (L) larvae. *Journal of Fish Biology 37: 455-472.*

Pranoto Y., Rakshit S.K., Salokhe V.M., 2005. Enhancing antimicrobial activity of chitosan films by incorporating garlic oil, potassium sorbate and nisin. *Food Science and Technology 38(8): 859-865*

Press C.McL., Lillehaug A., 1995. Vaccination in European salmonid aquaculture: a review of practices and prospect. *The British veterinary journal151: 45-69.*

Press C.McL., Dannevig B.H., Landsverk T., 1994. Immune and enzyme histochemical phenotypes of lymphoid and nonlymphoid cells within the spleen and head kidney of Atlantic salmon (*Salmo salar* L.). *Fish and Shellfish Immunology 4: 79-93.*

Primex, retrieved 03.09.2005, http://www.primex.is/chitininni.htm

Pripp A.H., Isaksson T., Stepaiak L., Sorhaug T., Ardö Y., 2005. Quantitative structure activity relationship modelling of peptides and proteins as a tool in food science. *Trends in Food Science and Technology 16: 484-494.*

Roller S. & Covill N., 2000. The antimicrobial properties of chitosan in mayonnaise og mayonnaise-based shrimp salads. *Journal of Fodd Protection 63(2): 202-209.*

Rombout J.H., Taverne-Thiele A.J., Villena M.I., 1993. The gutassociated lymphoid tissue (GALT) of carp (*Cyprinus carpio* L.): an immunocytochemical analysis. *Developmental and Comparative Immunology 17: 55– 66.*

Rønnestad I., Conceição L.E.C., Aragão C., Dinis M.T., 2000. Free amino acids are absorbed faster and assimilated more efficiently than protein in postlarval Senegal sole (*Solea senegalensis*). *Journal of Nutrition 130: 2809–2812.*

Ruane N.M., Wendelaar Bonga S.E., Balm P.H., 1999. Differences between rainbow trout and brown trout in the regulation of the pituitary-interrenal axis and physiological performance during confinement. *General and Comparative Endocrinology 115: 210-219.*

Sakai M., 1999. Current research status of fish immunostimulants. *Aquaculture 172: 63-92.*

Sakai M., Kobayashi M., Kawauchi H., 1996. In vitro activation of fish phagocytic cells by GH, prolactin and somatolactin. *Journal of Endocrinology 151: 113-118.*

Salze G., Tocher D.R., Roy W.J., Robertson D.A., 2005. Egg quality determinants in cod (*Gadus morhua L.*): egg performance and lipids in egg from farmed and wild broodstock. *Aquaculture Research 36: 1488-1499.*

Samain E., Drouillard S., Heyraud A., Driguez H., Geremia R.A., 1997. Grame-scale synthesis of recombinant chitooligosaccharides in *Escherichia coli*. *Carbohydrate Research 302: 35–42.*

Sanchez C. & Dominquez J., 1991. Trout immunoglobulin populations differing in light chains revealed by monoclonal-antibodies. *Molecular Immunology 28: 1271-1277.*

Sanchez C., Dominquez J., Coll J., 1989. Immunoglobulin heterogeneity in the rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Diseases 12: 459-465*.

Schrøder M.B., Villena A.J., Jørgensen T.Ø., 1998. Ontogeny of lymphoid organs and immunoglobulin producing cells in Atlantic cod (*Gadus morhua* L.). *Developmental and Comparative Immunology 22: 507-517.*

Schulze A.D., Alabi A.O., Tattersall-Sheldrake A.R., Miller K.M., 2006. Bacterial diversity in a marine hatchery: Balance between pathogenic and potentially probiotic bacterial strains. *Aquaculture 256(1-4): 50-73*

Seafood plus, retrieved 15.10.2006 :
http://www.seafoodplus.org/Project_4_1_PROPEHEAL.62.0

Segnar H., Storch V., Reinecke M., Kloas W., Hanke W., 1994. The development of functional digestive and metabolic organs in turbot, *Scophthalmus maximus. Marine Biology 119: 471-486.*

Seppola M., Larsen A.N., Steiro K., Robertsen B., Jensen I., 2008. Characterisation and expression analysis of the interleukin genes, IL-1β, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.) *Molecular Immunology 45(4): 887-897*

Shanahan F., 2000. Nutrient tasting and signaling mechanism in the gut: V. Mechanism of immunologic sensation of intestinal contents. *American Journal of Physiology 278: 191-196.*

Shi Y.B., Wong J., Puzianowska-Kuznicka M., Stolow M.A., 1996. Tadpole competence and tissue-specific temporal regulation of amphibian metemorphosis: roles of thyroid hormone and its receptors. *BioEssays 18: 391-399.*

Shiau S.Y. & Yu Y.P., 1999. Dietary supplementation of chitin and chitosandepresses growth in tilapia, Oreochromisniloticus x O-aureus. *Aquaculture 179(1-4): 439-446.*

Shirui M., Xintao S., Florian U., Michael S., Dianzhou B., Thomas K., 2004. The depolymerization of chitosan: Effects on physicochemical and biological properties. *The International Journal of Pharmaceutics 281: 45–54.*

Siwicki A.K., Anderson D.P., Rumsey G.L., 1994. Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. *Veterinary Immunology and Immunopathology 41: 139-159.*

Skanderby M., 1994. Protein hydrolysates: their functionality and applications. *Food Science and Technology International 10: 141.*

Skjermo J., Bergh Ø., 2004. High-M alginate immunostimulation of Atlantic halibut (*Hippoglossus hippoglossun* L.) larvae using *Artemia* for delivery, increases restistance against vibriosis. *Aquaculture 238: 107-113.*

Skjermo J., Defoort T., Dehasque M., Espevik T., Olsen Y., Skjåk-Bræk G., Sorgeloos P., Vadstein O., 1995. Immunostimulation of juvenile turbot (*Scophtalmus maximus L.*) using an alginate with high mannuronic acid content administered via the live food organism *Artemia. Fish and Shellfish Immunology 5: 531-534.*

Slitzyte R., Nguyen J.V., Rustad T., Storrø I., 2004. Hydrolysis of cod (*Gadus morhua*) by-products: influence of initial heat inactivation, concentration and separation conditions. *Journal of Aquatic Food Product Technology 13: 31-48.*

Slizyte R., Dauksas E., Falch E., Storrø I., Rustad T., 2005. Characteristics of protein fractions generated from hydrolysed cod (*Gadus morhua*) by-products. *Process Biochemistry40: 2021-2033.*

Slizyte R., Rustad T., Storrø I., 2005. Enzymatic hydrolysis of cod (*Gadus morhua*) by-products Optimization of yield and properties of lipid and protein fractions. *Process Biochemistry 40(12): 3680-3692.*

Smáradóttir H., 2008. *Personal communications*

Snaar-Jagalska B.E., Krens S.F.G., Robina I., Wang L-X., Spaink H.P., 2003. Specific activation of ERK pathways by chitin oligosaccharides in embryonic zebrafish cell lines. *Glycobiology 13: 725-732.*

Solem S.T., Jørgensen J.B., Robertson B., 1995. Stimulation of respiratory burst and phagocytic activity in Atlantic salmon (*S. salar* L.) macrophages by lipopolysaccharide. *Fish and Shellfish Immunology 5: 475-491.*

Stenvik J., Jørgensen T.Ø., 2000. Immunoglobulin D (IgD) of Atlantic cod has a unique structure. *Immunogenetics 51: 452-461.*

Sunyer J.O., Lambris J.D., 1998. Evolution and diversity of the complement system of poikilothermic vertebrates. *Immunology Review 166: 39–57.*

Suzuki Y., Orito M., Furukawa K., Aida K., 1994. Existence of low molecular weight immunoglobulin M in carp eggs. *Fish Science 60:159-162.*

Sveinbjörnsson B., Smedsrød B., Berg T., Seljelid R., 1995. Intestinal uptake and organ distribution of immunomodulatory aminated β-1,3-$_D$polyglucose in Atlantic salmon (*Salmo salar* L.). *Fish and Shellfish Immunology 5: 39-50.*

Szlaminska M., Escaffe A.M., Charlon N., Bergot P., 1991. Preliminary data on semisynthetic diets for goldfish (*Carassius auratus* L.) larvae. In: Kaushik S.J., Luquet P. (Eds.), *Fish Nutrition in Practice*. Biarrotz, France, 24-27 June 1991. INRA, Paris, Les Colloques, 61, pp. 607-612.

Takahashi Y., Itami T., Konegawa K., 1986. Enzymatic properties of partially purified lysozyme from the skin carp. *Bullentin of the Japanese Society of Scientific Fisheries 52: 1209-1214.*

Takahashi I., Kiyono H., 1999. Gut as the largest immunologic tissue. *JPEN. Journal of Parenteral and Enteral Nutrition 23: S7– S12.*

Takemura A., 1993. Changes in an immunoglobulin M (IgM)-like protein during larval stages in tilapia, Oreochromis mossambicus. *Aquaculture 115: 233-241.*

Takemura A., 1996. Immunohistochemical localization of lysozyme in the prelarvae of tilapia, Oreochromis mossambicus. *Fish and Shellfish Immunology 6: 75-77.*

Takemura A., Takano K., 1995. Lysozyme in the ovary of tilapia (*Oreochromis mossambicus*): its purification and some biological properties. *Fish Physiology and Biochemistry 14: 415-421.*

Tanaka T., Furukawa K., Suzuki Y., Aida K., 1999. Transfer of maternal antibody from mother to egg may have no protective meaning for larvae of red sea bream Pagrus major, a marine teleost. *Fish Science 65: 240-243.*

Thompson K.D., Cachos A., Inglis V., 1995. Immunomodulating effects of glucans and oxytetracycline in rainbow trout, *Oncorhynchus mykiss,* on serum lysozyme and protection. *Diseases in Asian Aquaculture 11: 433-439.*

Tokoro A., Kobayashi M., Tatewaki N., Suzuki K., Okawa Y., Mikami T., 1989. Protective effect of N-acetyl chitohexaose on Listeriamonocytogenes infection in mice. *Microbiology and Immunology 33: 357–367.*

Treece G.D., 2000. *Artemia* Production for Marine Larval Fish Culture, *SRAC Publication No. 702.*

Turner M.W., 1996. Mannose-binding lectin: the pluripotent molecule of the innate immune system. *Review Immunology Today 17(11): 532-540.*

Vadstein O., 1997. The use of immunostimulation in marine larviculture: possibilities and challenges. *Aquaculture 155: 401-417.*

Vadstein O., Øie G., Olsen Y., Salvesen I., Skjermo J., Skjåk-Bræk G., 1993. A strategy to obtain microbial control during larval development of marine fish. In: Vadstein O., 1997. The use of immunostimulation in marine larviculture: possibilities and challenges. *Aquaculture 155: 401-417.*

Verlhac V., Gabaudan J., Obach A., Schuep W., Hole R., 1996. Influence of dietary glucan and vitamin C on non-specific and specific immune responses of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture 143: 123-133.*

Verlhac V., Obach A., Gabaudan J., Schuep W., Hole R., 1998. Immunomidulation by dietary vitamin C and glucan in rainbow trout (*O. mykiss*). *Fish and Shellfish Immunology 8: 409-424.*

Webster J.D., Ledwaed D.A., Lawrie R.A., 1982. Protein hydrolysates from meat industry by-products. *Meat Science 7: 147.*

Whitler R.E., Clarke W.C., Riddell B.E., Kreiberg H., 1987. Genetic variation in freshwater survival and growth of Chinook salmon (*Onco-orhynchus tshawytscha*). *Aquaculture 64: 85-96.*

Yada T., Nakanishi T., 2002. Interaction between endocrine and immune system in fish. *International Review of Cytology 220: 35-92.*

Yamada A., Shibbuya N., Komada O., Akatsuka T., 1993. Induction of phytoalexin formation in suspension-cultured rice cells by N-acetylchitooligosaccharides. *Bioscience, Biotechnology, and Biochemistry 57: 405–409.*

Yano T., 1996. The non-specific immune system: humoral defense. In: *The Fish Immune System: Organism, Pathogen, and Environment. San Diego, pp. 105-156.*

Yousif A.N., Albright L.J., Evelyn T.P.T., 1991. Occurrence of lysozyme in the eggs of coho salmon Oncorhynchus kisutch. *Disease of Aquatic Organisms 10: 45-49.*

Yousif A.N., Albright L.J., Evelyn T.P.T., 1994. In vitro evidence for the antibacterial role of lysozyme in salmonid eggs. *Disease of Aquatic Organisms 19: 15-19.*

Yousif A.N., Albright L.J., Evelyn T.P.T., 1995. Immunological evidence for the presence of an IgM-like immunoglobulin in the eggs of coho salmon *Oncorhynchus kisutch. Disease of Aquatic Organisms 23: 109-114.*

Zambonino Infante J.L., Cahu C.L., 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. *Comparative Biochemistry and Physiology Part C: Toxicoly & Pharmacology 130: 477-487.*

Zambonino Infante J.L., Cahu C.L., Péres A., 1997. Partial substitution of diand tripeptides for native proteins in sea bass diet improves Dicentrarchus labrax larval development. *Journal of Nutrition 127: 608–614.*

Zapata A., Diez B., Cejalvo T., Frías C.G., Cortés A., 2005. Ontogeny of the immune system in fish. *Fish and Shellfish Immunology 20: 126-136.*

Zapata A.G., Torroba M., Varas A., Jime´nez E., 1997. Immnity in fish larvae. *Developments in Biological Standardization 90: 23-32.*

Zhang H., Du Y., Yu X., Mitsutomi M., Aiba S., 1999. Preparation of chitooligosaccharides from chitosan by a complex enzyme. *Carbohydrate Research 320: 257–260.*