The Effects of Nucleotide-Containing Product Maxi-Gen^{TM} Plus on Stress Level, Immune-Related Gene Expression, Growth Performance and Intestinal Histology of In-Season and Off-Season Atlantic Salmon (*Salmo salar*) Smolts During Smoltification and Desmoltification Periods

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Abstract

Trials were conducted to determine the effects of dietary nucleotides fed as Maxi-Gen™ Plus on stress level, immune-related gene expression, growth performance and intestinal histology of in-season and off-season Atlantic salmon smolts. Twenty-four hour, 40ppt salinity tests were conducted to measure the hypo-osmoregulatory ability. Expression of immune-related genes response to polyIC injection was investigated. Growth performance was evaluated as weight gain, food consumption and FCR. Intestinal structure parameters were determined. Diets contained 0.40 to 0.60% Maxi-Gen™ Plus resulted in enhanced growth performance. Hypo-osmoregulatory ability during smoltification gradually enhanced with increased inclusion of Maxi-Gen™ Plus from 0.05 to 0.60%, which indicated an extended "smolt window". Diets contained 0.20 to 0.25% Maxi-Gen™ Plus resulted in enhanced anti-viral immune response of fish after polyIC injection. Diet contained 0.60% Maxi-Gen™ Plus had lower plasma cortisol level of fish. The intestinal structure was not enhanced by feeding Maxi-Gen™ Plus.

List of Abbreviations used

	Abbreviation
Adenosine monophosphate	AMP
Analysis of variance	ANOVA
Ante-dependence	Ante (1)
Bacterial kidney disease	BKD
Bayesian information criterion	BIC
Body weight	BW
Celsius	°C
Completely randomized design	CRD
Compound symmetry	Cs
Corrected akaike information criterion	AICC
Cytidine monophosphate	CMP
Deoxyribonucleic acid	DNA
Enzyme-linked immunosorbent assay	ELISA
Feed conversion ratio	FCR
Fork length	FL
Guanosine monophosphate	GMP
Heterogeneous compound symmetry	Csh
Heterogeneous toeplitz	Toeph
Immunoglobulin M	IgM
Infectious hematopoietic necrosis virus	IHNV
Infectious pancreatic necrosis virus	IPNV
Infectious salmon anaemia virus	ISAV

Inosine monophosphate	IMP
Interferon stimulated gene 15	ISG15
Major histocompatibility complex	MHC
Melanoma-differentiation-associated gene 5	MDA5
Tricaine methane sulfonate	MS222
National center for biotechnology information	NCBI
No reverse transcription control	NRT
No template control	NTC
Phosphate-buffered saline	PBS
Polyriboinosinic polyribocytidylic acid	PolyIC
Radioimmunoassay	RIA
Randomized complete block design	RCBD
Retinoic-acid-inducible protein I	RIG-I
Ribonucleic acid	RNA
Salmonid alphavirus	SAV
Toeplitz	Toep
Transport-associated proteins	ABCB2
Type 1 interferon	IFN-α
Type II interferon	IFN-γ
Undisturbed controls	UC
Uridine monophosphate	UMP

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Chapter 1: Introduction

Canadian finfish aquaculture has been continuously growing with a significant increase in total value from 500 million dollars in 2003 to 740 million dollars in 2012 (Aquaculture Statistics, 2013). Atlantic salmon has become the dominant species in the Canadian aquaculture industry with an annual production of over 108,000 tonnes with a value of 599 million dollars in 2012 (Aquaculture Statistics, 2013). Coho (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawytscha*) were the main farmed species when the salmon aquaculture first started in the west coast in the early 1970s. They were soon replaced by Atlantic salmon due to its faster growth in seawater and higher density tolerance (Olin et al., 2010). Atlantic salmon production had dramatically increased on both the Pacific and Atlantic coasts during the 1990s, which greatly contributed to the overall growth of the finfish aquaculture (Olin et al., 2010). The annual Canadian exports of Atlantic salmon have remained stable at around 500 million dollars since 2003 (Aquaculture Statistics, 2013).

Smoltification, also referred to as parr-smolt transformation, is a critical phase in the salmon production cycle associated with a complex series of physiological, morphological and behavioral changes of fish preceding the transfer from freshwater to seawater (Stefansson et al., 2008). An effective smoltification process is an important factor that affects the survival and growth of Atlantic salmon smolts in the marine environment (Folmar and Dickhoff, 1980; Stefansson et al., 2008). Smolting is completed in spring stimulated by the increase in water temperature and photoperiod (Folmar and Dickhoff, 1980; McCormick, et al., 2009). Soon the fish reach the “smolt window”, a limited period of about 4 to 6 weeks when the fish can be transferred to seawater and

quickly adapt to the marine environment (McCormick et al., 1998a). If the fish are not transferred within the window, they will start desmolting to become postsmolts with a rapid loss of seawater tolerance (Stefansson et al., 2008).

Atlantic salmon are faced with a series of stressors in aquatic environments. The stressors can negatively impact the overall health of fish and culminate in failure to acclimate to seawater and increased mortality (McCormick, et al., 2009; Madaro et al., 2015). The completion of smoltification is a stressful period for Atlantic salmon as evidenced by an elevated plasma cortisol level (Sundell et al., 2003). Salmon can be compromised by numerous abiotic and biotic stressors such as poor water quality, high temperature, high rearing density, inappropriate photoperiod, rough handling and diseases as they complete smoltification (Mesa et al., 1999; Price and Schreck, 2003a). Compromised smolts cost the aquaculture industry up to 12 million dollars annually, either by exhibiting high mortality (up to 17%) after seawater transfer, or depressed growth generally associated with osmoregulatory imbalance (Zydlewski et al., 2010; Dietrich et al., 2013; Hatchery International, 2013).

Nucleotides are a series of organic molecules that play various roles in cellular metabolism. They are the building blocks of nucleic acids: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), which are the storage molecules of genetic information (Nelson and Cox, 2012). All endogenous cellular components and biomolecules are products that are determined by the nucleotide sequence of the nucleic acids within the cells (Nelson and Cox, 2012). Increased attention has been focused on adding dietary nucleotides into fish feed to reduce the susceptibility of fish to various stressors and diseases by enhancing their overall immunity (Burrells et al., 2001a; Ringo et al., 2012).

Use of dietary nucleotides enhanced the intestinal morphology of Atlantic salmon, which was correlated with the enhanced growth (Burrells et al., 2001b). Nucleotide-containing diets effectively enhanced the growth performance of juvenile rainbow trout (*Oncorhynchus mykiss*) (Tahmasebi-Kohyani et al., 2012). In addition to enhancing the immunity of the fish, dietary nucleotides reduced the plasma cortisol level of juvenile rainbow trout infected with infectious pancreatic necrosis virus (IPNV) (Leonardi et al., 2003). When fish are stressed, additional demands are put on nucleotide requirements (Burrells et al., 2001b). To produce smolts that reliably perform well following seawater transfer, the effects of dietary nucleotides fed as Maxi-Gen^{TM} Plus on enhancing the growth and stress tolerance of Atlantic salmon smolts during smoltification were evaluated in the current study.

No published literature was found describing the effects of dietary nucleotides during smoltification of salmonids. If dietary nucleotides can enhance stress tolerance and reduce disease-risk of fish during smoltification, their inclusion into transfer diets may promote the growth and survival of fish after seawater transfer. Dietary nucleotides, hence, have the potential as effective feed ingredients to greatly improve efficiency of Atlantic salmon production.

Chapter 2: Literature review

2.1 Atlantic salmon during smoltification

2.1.1 Smoltification and desmoltification

During the life cycle of anadromous salmonids, smoltification is associated with a series of changes in morphology, physiology and behavior (Stefansson et al., 2008). Smoltification transforms the darkly pigmented, bottom-dwelling parr into the pelagic, silvery smolts that are ready for the seawater transfer (Folmar and Dickhoff, 1980).

Changes in colouration and body shape are the two main visible characteristics that occur during smoltification. Salmon parr are identified by the darkly pigmented bars on the lateral surface. During the transformation these bars gradually become visually obstructed by the accumulation of purines. The purine layers show the silver appearance on the surface of the transformed smolts (Johnston and Eales, 1967). Accompanied with colour changes during smoltification is the change of body from round-shaped parr to slender streamlined smolt (Vanstone and Markert, 1968).

Increased salinity tolerance, swimming and feeding patterns change, and the tendency to migrate to seawater are the major behavioral changes associated with smoltification (Folmar and Dickhoff, 1980). Differences in salinity preference are mainly due to the state of physiological development of the fish. The increased salinity tolerance of the fish that developed during smoltification is strongly associated with the increase in gill Na^+, K^+-ATPase activity and increase in the size and number of gill chloride cells (McCormick et al., 1998a). The osmoregulatory ability of fish at different salinities is size and age dependent (Parry, 1960; Folmar and Dickhoff, 1980). Atlantic salmon over two years of age (12 to 15 cm) showed higher salinity tolerance and survival than one-

year old fish (1.5 to 10 cm) after transfer to seawater (Parry, 1960). Salmon parr swim against the water current in order to maintain their positions in the stream where they establish and maintain their bottom feeding territories while smolts tend to form aggregates and schools and migrate downstream (Folmar and Dickhoff, 1980).

In salmonid aquaculture, fish spend around one and a half years in freshwater before smoltification occurs (Folmar and Dickhoff, 1980). A "smolt window" exists when the fish have developed seawater tolerance and are ready for transfer to seawater (McCormick et al., 1998a). Urke et al., (2014b) suggested that the "smolt window" for Atlantic salmon lasted for a minimum of 4 to 6 weeks. If the fish are not transferred into seawater within the "smolt window", a process called desmoltification will occur. Normally, desmoltification is considered as a sign of salmonids losing their ability to adapt to seawater. Changes associated with desmoltification include decreased hypo-osmoregulatory ability, re-establishment of positive rheotaxis and endocrine changes (Stefansson et al., 2008). The response of fish prevented from exposure to seawater indicates the closing the "smolt window". Fish during desmoltification are referred to as postsmolts. Postsmolts gradually lose their smolt characters morphologically even though they do not revert to the morphology of parr (Stefansson et al., 2008). The growth of postsmolts in freshwater is independent of the decreased hypo-osmoregulatory ability associated with desmoltification (Duston, 1994). Transferring postsmolts into seawater resulted in reduced feed consumption, growth and growth hormone levels, all of which indicated the fish losing their ability to survive in the marine environments (Arnesen et al., 2003).

2.1.2 Off-season smolts

The process of smoltification can be accelerated by advanced photoperiod and increased water temperature (Muir et al., 1994; McCormick et al., 2009). Induced early smoltification of Atlantic salmon using artificial photoperiod has been applied by salmon farms to produce off-season smolts (Berrill et al., 2003). Off-season Atlantic salmon smolts subjected to constant light (24h light) exhibited better growth than those subjected to simulated natural photoperiod and short photoperiod (9h light:15h dark) with similar mortality among all the treatment groups after they were transferred into seawater (Duncan et al., 1999). Atlantic salmon juveniles subjected to constant long photoperiod (16h light:8h dark) and high water temperature (16°C) showed higher growth than those subjected to simulated natural conditions before smoltification (Gaignon and Quemener, 1992). Off-season Atlantic salmon smolts triggered by two-month constant long photoperiod (17h light:7h dark) showed satisfactory survival and growth after they were transferred to sea-cages in November (Duston and Saunders, 1995). Low temperature had negative effects on the physiological response of fish to increased day length as the increase in gill Na^+, K^+-ATPase activity of off-season smolts was delayed at 2°C compared with those reared at 10°C (McCormick et al., 2000). In conclusion, application of modified photoperiod is effective to induce out of season smoltification of Atlantic salmon.

2.1.3 Stressors on salmon during smoltification

Salmonids are faced with a series of environmental stressors and they are particularly sensitive to these stressors during smoltification as well as during subsequent seawater transfer (Price and Schreck, 2003b; Sundh et al., 2009; Nilsen et al., 2013). The

environmental stressors can be high water temperature, low oxygen level, water pH, physical damage to the fish and the presence of diseases and toxic substances in the water. All these stressors can negatively affect both the physiological and behavioral characteristics of the fish during the process of smoltification (Price and Schreck, 2003a). Sometimes the stressors are compounded with the fish experiencing a combination of two or more stressors. Nilsen et al. (2013) indicated that the exposure to acidic water (pH =5.7) and aluminum by Atlantic salmon smolts in fresh water resulted in a significant decrease in the ability of fish to recover from physiological disruption and more than two weeks were required for the fish to fully recover from their injuries. The exposure of Chinook salmon undergoing parr-smolt transformation to toxic substances, such as fire retardant chemicals, damaged gill tissues and caused significant mortalities (Dietrich et al., 2013). Saltwater preference of stressed juvenile Chinook salmon was significantly decreased compared to the unstressed fish (Price and Schreck, 2003a). Physical damage, such as a descaling injury, caused significant osmotic perturbations of Atlantic salmon smolts during the peak of the smolting period (Zydlewski et al., 2010). Anderson (1996) demonstrated that exposing rainbow trout and brown trout (*Salmo trutta*) to environmental stressors, such as metals and chemicals, resulted in reduced feed intake and growth, increased cortisol-induced immunosuppression and increased susceptibility to diseases.

Some production-related stressors such as reduced water flow, hyperoxygenation and high stocking density that may occur in the intensive salmon farming could affect the fish during smoltification. Stress caused by vaccinating Atlantic salmon parr significantly reduced the weight of the fish by 23% compared to the unvaccinated fish one year after

seawater transfer, indicating a growth depression of fish subjected to vaccination (Midtlyng and Lillehaug, 1998). Hyperoxygenation (up to 180% saturation) and reduced water flow (1.5L/kg/min to 0.25L/kg/min) were stressful to Atlantic salmon prior to seawater transfer and resulted in increased mortality when the fish were challenged with IPNV (Sundh et al., 2009). Coho salmon (*Oncorhynchus kisutch*) smolts reared at high stocking density (41kg/m^3) had lower plasma thyroxine levels, gill Na^+, K^+-ATPase activity and osmoregulatory ability than those reared at low density, indicating signs of an impaired smoltification process (Schreck et al., 1985). However, Hosfeld et al. (2009) found no negative impacts on the growth of Atlantic salmon reared at densities up to 86 kg/m^3 during smoltification with water flow rate and water qualities maintained at optimal levels. The salmon farming industry can suffer significant losses if these production-related stressors are not minimized when the fish are transferred from freshwater hatcheries to seawater production sites.

The stress caused by pathogens is one of the biggest issues in both wild salmon populations and the salmon aquaculture industry, as disease agents can greatly affect the survival and growth of fish (Mesa et al., 1999). Bacterial kidney disease (BKD) is a serious disease of salmonids that has received considerable attention due to its contribution to significant mortality events of salmonids in the Pacific Northwest (Price and Schreck, 2003b). The hypo-osmoregulatory ability of coho salmon smolts infected with BKD was seriously compromised, resulting in failure of infected fish to survive after transported into seawater (Moles, 1997). Price and Schreck (2003b) demonstrated a decreased saltwater preference of juvenile chinook salmon that were infected with BKD

during the parr-smolt transformation, showing a significant negative relationship between BKD infection level and saltwater preference of the fish.

2.1.4 Plasma cortisol level

Cortisol is a type of corticosteroid that can serve as a stress indicator of fish. Its plasma concentration significantly increased when fish were stressed (Mommsen et al., 1999). The rise of plasma cortisol in response to the acute stress was considered as a short-term adaptive mechanism to generate alertness and energy for the fish to get over the stress and maintain their homeostasis (Schreck, 1981; Mommsen et al., 1999). Fast et al. (2008) demonstrated that the plasma cortisol level of Atlantic salmon significantly increased from 5 to 70 ng/ml one hour after being subjected to short-term handling stress. The basal (unstressed) plasma cortisol level of juvenile Atlantic salmon ranged from 3 to 6 ng/ml (Fast et al., 2008). Similar basal cortisol level (4 ng/ml) was found in Atlantic salmon parr (Carey and McCormick, 1998). McCormick et al. (1998b) stated a higher basal plasma cortisol level (5-9 ng/ml) of Atlantic salmon parr.

Chronically elevated plasma cortisol suppresses the immune system of fish. The susceptibility of brown trout to furunculosis infection was increased without significantly reducing the numbers of lymphocytes after the fish were injected with cortisol (Pickering and Pottinger, 1985). Similarly, the lymphocytes of Atlantic salmon was qualitatively affected by the cortisol as the number of immunoglobulin-positive cells were reduced after cortisol injection (Espelid et al., 1996). Petochi et al. (2008) reported a decrease in the percentage of B and T lymphocytes in the peripheral blood of sea bass (*Dicentrarchus labrax*) 5 weeks after intraperitoneal implantation of cortisol, indicating a negative effect of cortisol on the acquired immunity of fish.

During the normal smolting season, the plasma cortisol level of Atlantic salmon (initial level of 7 ng/ml in mid-April) gradually increased during smolting with a transient peak (40 ng/ml) in early May (Sundell et al., 2003). A similar pattern was found in off-season smolts as an elevation of cortisol (from 3 to 22 ng/ml) occurred after the application of artificial long photoperiod to induce smolting (Sundell et al., 2003; Jutfelt et al., 2007). A second elevation in plasma cortisol level (from 18 to 55 ng/ml) was found when the fish were transferred into seawater (Figure 2.1; Sundell et al., 2003).

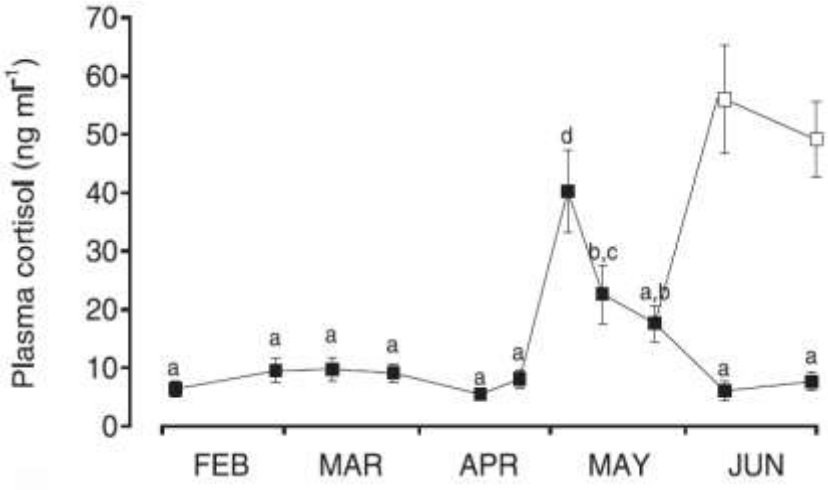

Figure 2.1 Plasma cortisol level of 1^{+} age Atlantic salmon during natural smoltification before (filled symbols) and after transfer to seawater (open symbols) (Sundell et al., 2003).

The role of cortisol during smoltification is somewhat ambiguous. Bisbal and Specker (1991) indicated that elevated cortisol level (160 to 167 ng/ml) stimulated the hypo-osmoregulatory ability of juvenile Atlantic salmon as the cortisol-implanted fish exhibited enhanced gill Na^{+}, K^{+}-ATPase activity (22 μmol Pi/ mg protein/h, tripled over that of the control fish) as well as reduced mortality rate during smoltification. Gill Na^{+}, K^{+}-ATPase activity of Atlantic salmon postsmolts was elevated by around 40% following cortisol implantation (Cornell et al., 1994). Elevated cortisol stimulated and mediated the

increase in the fluid uptake rate (50μl/cm^2/hr, compared to 20 μl/cm^2/hr of control fish) of the posterior intestine of Atlantic salmon during smoltification (Cornell et al., 1994; Veillette et al., 1995).

Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are two common ways of measuring plasma cortisol. RIA is an in vitro assay used to measure concentrations of organic substances, such as hormones, enzymes and antigens by the use of their corresponding antibodies (Yalow, 1980). To perform the RIA, plasma samples, cortisol-specific antibodies and radioactive antigen standards were incubated together for the antigen-antibody binding reaction. The cortisol from the samples compete with the antigen standards for the antibody-binding sites and reduce the ratio of antibody-bound antigen standards to free antigen standards. The plasma cortisol concentration is determined by measuring the radioactivity of remaining free antigen standards after the reaction (Yalow, 1980).

ELISA is an in vitro assay used to measure concentrations of substances by the use of their corresponding antibodies and specific enzymes (Ma et al., 2006). According to the instructions of cortisol ELISA kit (Neogen Corp., US), plasma samples and enzyme conjugates are added into the microplate wells that contain cortisol-specific antibodies and incubated for the antigen-antibody binding reaction. The cortisol from the samples compete with the enzyme conjugates for the antibody-binding sites. After the incubation the unbound materials (cortisol and conjugates) are washed off and the colorless substrate is added into the wells. The bound enzyme conjugates convert the substrate to a blue-colored product. The plasma cortisol concentration is determined by measuring the absorbance reading of the wells. The extent of color development is inversely

proportional to the amount of cortisol presented in the samples.

2.1.5 Intestine of Atlantic salmon

The gastrointestinal tract of Atlantic salmon is the major site for food digestion and nutrient absorption. It is composed of five regions: pyloric caeca, first segment of the mid intestine surrounded by pyloric caeca, first segment of the mid intestine posterior to pyloric caeca (mid gut), second segment of the mid intestine posterior to pyloric caeca (hind gut or distal intestine) and posterior segment (Figure 2.2; Lokka et al., 2013). The intestinal wall consists of four layers with the innermost layer called mucosal fold or villi, which greatly contributes to the enlarged surface area of the intestine (Wilson and Castro, 2010). For vertebrates, increased surface area and the consequential enhanced absorption of nutrients are due to the folding of intestine as well as the presence of villi and microvilli on the inner surface (Kisia, 2011).

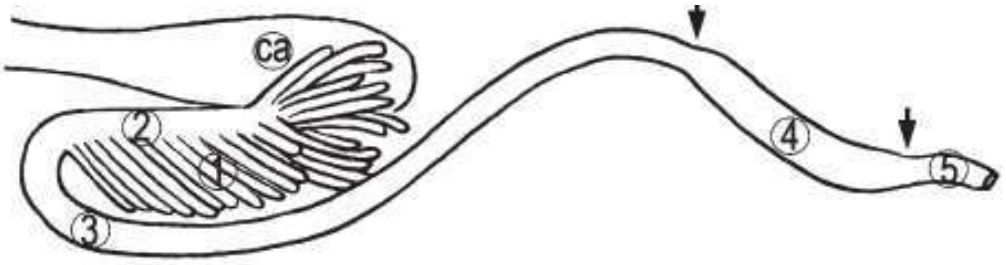

Figure 2.2 Gastrointestinal tract of Atlantic salmon. Ca: cardiac stomach. 1: pyloric caeca. 2: first segment of mid intestine. 3: first segment of mid intestine posterior to pyloric caeca. 4: second segment of mid intestine posterior to pyloric caeca. 5: posterior segment (Lokka et al., 2013).

In addition to being a digestive organ, the intestine of salmonids is also a major osmoregulatory organ that maintains the balance between water and ions. During smoltification, the intestine needs to be developed for the switch of its role from preventing water inflow in freshwater to actively absorbing water in seawater (Sundell et

al., 2003; Jutfelt et al., 2007). During the parr-smolt transformation, the fluid transport of middle intestine of Atlantic salmon exhibited a significant decrease while the posterior intestinal fluid transport dramatically increased, indicating a superior osmoregulatory role for the posterior intestine (Veillette et al., 1993). According to Sundell et al. (2003), the increased intestinal fluid transport of Atlantic salmon during smoltification was related to increased gill Na^+, K^+-ATPase activity and decreased paracellular permeability of the posterior intestine. Veillette et al. (2005) indicated a significantly increased intestinal fluid uptake via the pyloric caeca after chinook salmon were transferred to seawater, suggesting its important role in maintaining water and ionic balance.

2.2 Nucleotides

2.2.1 General knowledge about nucleotides

Nucleotides are a group of small intracellular molecules that have multiple functions in cellular metabolism as well as storing genetic information. They are composed of three basic components: a nitrogenous base, a pentose sugar and at least one phosphate group. There are 5 types of nucleotides classified by the different nitrogenous bases they contain: adenosine monophosphate (AMP), cytidine monophosphate (CMP), uridine monophosphate (UMP), inosine monophosphate (IMP) and guanosine monophosphate (GMP). The basic function of nucleotides is to serve as the building blocks of both DNA and RNA. The nucleotide sequence in DNA fragments determines the expression of its associated RNA through the transcription process, which can further affect the structures of every single biomolecule and cellular component within cells. In addition to their roles as the subunits of nucleic acids, nucleotides serve as energy carriers in metabolic transactions, important chemical bridges in cell-hormone responses and structural

components of a variety of metabolic intermediates and coenzymes (Nelson and Cox, 2012).

2.2.2 Use of dietary nucleotides in fish

2.2.2.1 Digestion and absorption of nucleotides

For single-stomached animals, nucleotides are formed and degraded at a constant rate in all types of organ tissues, especially in those with high turnover rates (Cosgrove, 1998; Sauer et al., 2011). The digestion and absorption of dietary nucleotides include several complex enzymatic processes such as hydrolyzation as only nucleosides and bases can be absorbed through the small intestine (Sauer et al., 2011). Nucleoproteins in the food are degraded to nucleic acids with the presence of proteases. The nucleic acids are then further degraded by nucleases into single nucleotides. Intestinal alkaline phosphatase converts nucleotides to nucleosides by removing their phosphate groups (Quan and Uauy, 1991). The nucleosides are taken up by the small intestine, released into tissues and ready to be utilized for nucleotide production (Sauer et al., 2011). Despite the fact that the presence of three most important nucleotide-digestion-related enzymes (protease, alkaline phosphatase and nuclease) in fish intestine have been identified, limited information can currently be found about the digestion and absorption of nucleotides by fish (reviewed by Li and Gatlin, 2006).

2.2.2.2 Dietary requirements for nucleotides

Generally, animals have access to nucleotides via three major pathways: de novo synthesis, salvage pathway and exogenous supply (feed) (Cosgrove, 1998; Sauer et al., 2011). De novo synthesis is a metabolically costly process that consumes energy while salvage pathway uses less energy to generate nucleotides through linking ribose

phosphate regions to free bases (Burrells et al., 2001b; Sauer et al., 2011). Even though most tissue cells have access to nucleotides through de novo synthesis, some cells such as immune cells have limited synthesis capacity and thus have to rely on pre-formed or exogenously supplied nucleotides (Quan, 1992; Sauer et al., 2011). When fish are in stressful conditions either due to going through significant developmental changes or being affected by stressors such as pathogens, exogenous nucleotides are required since self-synthesized nucleotides via the de novo process may not be timely and quantitatively adequate to meet the increased requirements (Burrells et al., 2001b; Borda et al., 2003; Reddy, 2012). These exogenous nucleotides can either be directly used by the cells as a backup of self-synthesized nucleotides or provide free bases for the salvage pathway (Quan, 1992; Sauer et al., 2011). It was suggested that animals require diets that contain certain amount of nucleotides as diets lacking dietary nucleotides resulted in a perturbation of cell proliferation due to an increase in intracellular deoxyuridine monophosphate/deoxythymidine triphosphate ratios (Jackson et al., 1997).

Requirements of dietary nucleotides have been demonstrated for salmonids and some other species such as channel catfish since the addition of nucleotides in fish feed resulted in an increased stress tolerance as well as enhanced immunity when the fish were challenged with various stressors and viral infection (Burrells et al., 2001a; Burrells et al., 2001b; Low et al., 2003; Welker et al., 2011). However, it is hard to quantify the exact amount of dietary nucleotides that are required by fish as it greatly depends on the developmental stages as well as the anti-stress status of each individual (reviewed by Li and Gatlin, 2006). An inclusion level of 2.7% nucleotides enhanced the stress resistance of channel catfish while an inclusion level of as low as 0.03% nucleotides increased the

disease resistance of rainbow trout and Atlantic salmon (Burrells et al., 2001a; Welker et al., 2011).

2.2.2.3 Immunity and stress tolerance

During the last two decades increased attention has been focused on adding dietary nucleotides into fish feeds to enhance the overall immunity of fish against various stressors and diseases, and to strengthen the survival and growth of fish (reviewed by Li and Gatlin, 2006; Ringo et al., 2012). Diets supplemented with dietary nucleotides enhanced the resistance of several species to both stressors and diseases, which resulted in an increase in the overall health of the fish (reviewed by Li and Gatlin, 2006; Ringo et al., 2012).

Burrells et al. (2001a) demonstrated the positive effect of dietary nucleotides on increasing the resistance of salmonids to both bacterial and viral diseases as well as ectoparasitic infestations. Rainbow trout fed dietary nucleotides for 3 weeks had lower mortality (31%) than those fed the control diet (49%) after being challenged with *V. anguillarum* infection (Burrells et al., 2001a). Cumulative mortality of nucleotide fed Atlantic salmon was reduced after the infectious salmon anaemia virus (ISAV) challenge (Burrells et al., 2001a). Feeding dietary nucleotides reduced the mortality of coho salmon infected with *Piscirickettsia salmonis* (Burrells et al., 2001a). Atlantic salmon fed dietary nucleotides had fewer sea lice (*Lepeophtheirus salmonis*) attached on them (Burrells et al., 2001a). The use of dietary nucleotides enhanced the immune system of turbot (*Scopthalamus maximus L.*) by positively up-regulating a series of immune related genes, such as immunoglobulin M (IgM), in the fish (Low et al., 2003). Nile tilapia (*Oreochromis niloticus*) fed diets supplemented with dietary nucleotides showed higher

bacterial disease resistance as higher nitric oxide production by peripheral blood monocytes was found in nucleotide fed fish subjected to *Aeromonas hydrophila* challenge (Barros et al., 2015).

An increased stress tolerance in channel catfish (*Ictalurus punctatus*) associated with improvement of the immunosuppressive effects of the stress response on non-specific immunity was reported when nucleotides were added into diets (Welker et al., 2011). Juvenile Atlantic salmon fed a nucleotide-containing diet had lower plasma chloride levels than the control group three weeks after the exposure to seawater, indicating a better osmoregulatory capacity of the fish after seawater transfer (Burrells et al., 2001b). Feeding dietary nucleotides reduced the stress level of juvenile rainbow trout by lowering the plasma cortisol level (9.2ng/ml and 28.0ng/ml at day 7 post first and second infection respectively, compared to 18.2ng/ml and 56.2ng/ml of fish fed the control diet) of the fish challenged with IPNV (Leonardi et al., 2003). Fingerling rainbow trout fed dietary nucleotides had lower plasma cortisol level (73.0ng/ml at 1 h and 29.3ng/ml at 3 h) than the fish fed the control diet (124.6ng/ml at 1 h and 45.1ng/ml at 3 h) after being subjected to handling stress (Tahmasebi-Kohyani et al., 2012). Feeding dietary nucleotides enhanced the resistance of sole (*Solea solea*) subjected to the handling stress as the nucleotide fed fish had lower plasma cortisol (24ng/ml) and glucose (4.68mM) levels than those fed the control diet (50ng/ml and 4.95mM, respectively) one hour post-stress (Palermo et al., 2013).

2.2.2.4 Growth performance and intestinal development

The addition of dietary nucleotides in fish diets was associated with improved growth and enhanced intestinal structures of several species (reviewed by Li and Gatlin, 2006).

Feeding dietary nucleotides resulted in a higher weight gain of both small (43g initially) and large (205g initially) Atlantic salmon after seawater transfer (Burrells et al., 2001b). Fingerling rainbow trout fed nucleotide-supplemented diets had a higher weight gain percentage (151.7-192.1%) and feed efficiency (0.73-0.95) than those fed the control diet (98.6% and 0.62, respectively) (Tahmasebi-Kohyani et al., 2012). An enhanced feed intake (3.6g/100g of body weight for the nucleotide fed group, compared to 2.47g/100g of body weight for the control group) was reported by Kubitza et al. (1997) when feeding juvenile largemouth bass (*Micropterus salmoides*) with the diet supplemented with nucleotides, which suggested that dietary nucleotides could serve as an effective feed intake enhancer.

Morphological changes in the fish intestine were observed by gut histology examination (Øverland et al., 2009). Studies have evaluated the effects of dietary nucleotides on the intestinal morphology of fish. Burrells et al. (2001b) reported an increase in the height of intestinal fold (18.7, 18.0 and 21.4% increases in the proximal, mid and distal areas of the gut, respectively) and total gut surface area of Atlantic salmon fed a nucleotide-containing diet for 3 weeks. Juvenile sea bream (*Pagrus major*) fed nucleotide-supplemented diet had better growth (final weight of 40mg) along with the increased numbers of intestinal villi compared to those fed the control diet (final weight of 27mg) (Borda et al., 2003). Juvenile turbot fed diets supplemented with 0.1% nucleotides showed a higher fold height (650μm, compared to 559μm in distal intestine for the fish fed non-nucleotide-containing diet) (Peng et al., 2013). Cheng et al. (2011) reported an increase in the fold height (620.1 μm) of proximal intestine of red drum (*Sciaenops ocellatus*) fed 0.5% dietary nucleotides than that (551.3 μm) of the fish fed the control

diet. Improved growth performance of fish caused by feeding dietary nucleotides might be due in part to the enhanced intestinal fold morphology (Burrells et al., 2001b).

2.2.2.5 Effects of single type of nucleotides on fish

A balanced intracellular nucleotide pool is essential to maintain the stability and accuracy of DNA replication and repair within the cells (Das et al., 1985; Fasullo and Endres, 2015). Research on the effects of dietary nucleotides on fish is still in its infancy and almost all the growth and immune studies focused on evaluating the effects of a balanced mixture of nucleotides, which led to a lack of knowledge on the contributions of single type of nucleotide. Limited information was found about the comparison between different types of nucleotides, which made it difficult to determine their individual nutritional values and requirements by fish.

Studies were conducted to evaluate the effects of single nucleotide type as feed attractant to fish. Mackie and Adron (1978) first demonstrated the potency of IMP as a gustatory feeding stimulant. The gustatory sensitivity of aigo rabbitfish (*Siganus fuscescens*), isaki grunt (*Parapristipoma trilineatum*), kampachi amberjack (*Seriola dumerili*), jack mackerel (*Trachurus japonicus*) and masaba chub mackerel (*Scomber japonicus*) to nucleotides was tested. IMP, UMP and AMP had stimulatory effects at a range of 10^{-3}-10^{-1} mmol/kg (Ishida and Hidaka, 1987). IMP, GMP and UMP were all effective as feed attractants to jack mackerel at a dose of 0.1mmol/kg feed (Ikeda et al., 1991). IMP at a dose of 10.4mmol/kg feed enhanced the feed intake of largemouth bass (Kubitza et al., 1997). AMP effectively stimulated the feeding response of giant tiger prawn (*Penaeus monodon*) at doses above 10^{-3} mmol/kg (Coman et al., 1996).

2.2.3 Commercial nucleotide-enriched products

The use of dietary nucleotides and their potential effects on aquaculture species have been studied for more than two decades (reviewed by Ringo et al., 2012). Products enriched with nucleotides have been concurrently developed by different companies. They were added into fish feed with the purpose of enhancing the growth performance and stress resistance of fish.

Optimun® is a yeast-derived nucleotide-containing product manufactured by Chemoforma Ltd., Augst, Switzerland. The product contains a mixture of nucleotides (AMP, CMP, UMP, IMP and GMP) at a combined inclusion level of 0.03%. It has been evaluated by examining the growth performance, stress response and intestinal developments of rainbow trout, coho salmon, Atlantic salmon and turbot at a recommended level of 0.2% (Burrells et al., 2001a; Burrells et al., 2001b; Low et al., 2003; Tahmasebi-Kohyani et al., 2012).

Ascogen P® is a yeast based commercial product made by Canadian Bio-Systems Inc., Calgary, Alberta, Canada. Although no information can be found about the nucleotide inclusion level within the product, a level of 0.5% was recommended by the company to be added into fish diet. The product was evaluated by examining the growth and immune response of tilapia (*Sarotherodon niloticus*), red drum and hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) (Ramadan et al., 1994; Cheng et al., 2011; Ringo et al., 2012).

Maxi-Gen™ Plus is another commercial feed supplement manufactured by Canadian Bio-Systems Inc. It is a yeast-derived product that contains an inclusion of 1.5% total nucleotides along with 28% crude protein and 22% carbohydrates. The product serves as

a source of exogenous free nucleotides that can be rapidly absorbed by the animals (Quan, 1992; Sauer et al., 2011). Approaches such as spray drying and targeting dry matter greater than 88% were conducted to ensure the stability of nucleotides in the product. Maxi-Gen^{TM} Plus has been commercially used as an ingredient in the diets of swine, poultry, cattle and fish mainly as a "growth booster" or an "immune enhancer" at a recommended level of 0.05 to 0.1%. It was used in the current study as the test ingredient to evaluate its effects on the overall growth, immunity and the stress tolerance of Atlantic salmon smolts during smoltification.

2.3 Methods to evaluate the response of fish to dietary nucleotides

2.3.1 Salinity challenge test

Developed salinity tolerance, also known as the hypo-osmoregulatory ability in seawater, is a major change in salmonids during the smoltification period that ensures the survival of fish in the marine environment (Parry, 1960). The mechanisms of the hypo-osmoregulation of fish in seawater is to maintain their osmosis at a steady state through excretion, swallowing and active absorption of water or ions selectively (Parry, 1966). The acclimation of euryhaline species such as Atlantic salmon to the marine environment is achieved by increasing water intake and subsequent water absorption through the intestine to compensate for the osmotic water loss to the environment (Perrott et al., 1992; Marshall and Grosell, 2005). The hypo-osmoregulatory ability of salmon first appears at the parr stage and become fully developed as they transform to smolts (Parry, 1960). Urke et al. (2014a) demonstrated that the development of salinity tolerance occurred from early May to June for both wild and farmed Atlantic salmon. For Atlantic salmon, the blood osmotic concentration ranges from 328 mOsm/kg in freshwater to 344

mOsm/kg in seawater with a gradual increase following the seawater transfer (Parry, 1961; Parry, 1966).

Enhanced stress resistance of salmonids can be recognized by the development of seawater tolerance when they undergo smoltification. The completion of smolting, the development of hypo-osmoregulation and the subsequent marine survival can all be negatively impaired by some environmental stressors such as increased water temperature, presence of contaminants and exposure to acid and metals (Price and Schreck, 2003a; McCormick et al., 2009). The osmoregulatory ability of juvenile sockeye salmon (*Oncorhynchus nerka*) was disturbed due to stress from rough handling as the plasma sodium concentration was higher in stressed groups (215mEq/L, compared to 165Mq/L in control fish) (Ban, 2001). The plasma chloride levels, tissue moisture and gill Na^+, K^+-ATPase activity of Atlantic salmon were significantly impaired when the fish were reared at lower temperature (4°C), indicating a compromised smoltification process (Handeland et al., 1998). The parameters above were not affected by different salinities (28 or 34ppt) during smoltification (Handeland et al., 1998). Handeland and Stefansson (2002) found that acclimating off-season Atlantic salmon smolts to different salinities (6.0, 13.1 and 20.0ppt) before seawater transfer did not positively affect the hypo-osmoregulatory ability or gill Na^+, K^+-ATPase activity, suggesting that salinity is not a key factor affecting the smolting process. Descaling injury significantly impaired the hypo-osmoregulation of Atlantic salmon as the osmolality of descaled fish (above 400 mOsm/kg) was 70 mOsm/kg higher than the control fish at the peak of smolting (Zydlewski et al., 2010).

Salinity challenge tests can be conducted to determine the hypo-osmoregulatory ability

of fish by challenging them with high salinities and measuring the plasma osmolality after the challenge (Duston et al., 2011). These tests can also be used to evaluate the timing of the development of seawater tolerance as well as the duration of the "smolt window" through repeatedly measuring the plasma osmolality on a regular basis (Urke et al., 2014a and 2014b). High gill Na^+, K^+-ATPase activity and normal plasma ion levels of hybrid Atlantic salmon smolts were observed during a 96h, 32ppt salinity challenge (Urke et al., 2013). The genotype and salinity challenge timing have been shown to significantly affect the plasma osmolality and muscle water content of different Atlantic salmon stocks during the 96h, 35ppt salinity test (Duston et al., 2011). Juvenile Atlantic salmon implanted with cortisol showed an elevated osmoregulatory ability as well as a reduced mortality rate after exposed to a 96h, 37ppt test (Bisbal and Specker, 1991).

2.3.2 Viral mimic test and gene expression

Polyriboinosinic polyribocytidylic acid (polyIC) is a synthetic immune-stimulant that induces the maturation of dendritic cells and activating their immune functions (Verdijk et al., 1999). The mechanism of polyIC stimulating the antimicrobial immune responses of fish is through mimicking the double-stranded RNA structure of viruses (viral mimic), which will be recognized by the immune detectors such as toll-like receptors in the specialized immune cells or by retinoic-acid-inducible protein I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5) that are present in the somatic cells (Alexopoulou et al., 2001; Kato et al., 2006). Viral mimic test can be conducted to characterize the immune system and its related gene expression in fish. The test includes two steps: stimulating fish with polyIC as an intraperitoneal injection, followed by the analysis of immune-related gene expression (Rise et al., 2008). The changes in

expression of immune-related genes stimulated by polyIC injection are analyzed using RT-qPCR (Rise et al., 2008).

During the last decade, studies have been done to evaluate the polyIC-induced immune responses and their related gene expression by Atlantic salmon and other species. Mx, an indicator of interferon responses in fish, is an antiviral protein that protects cells from being infected with viruses such as IPNV and rhabdoviruses through inhibiting the replication of these viruses (Caipang et al., 2003; Larsen et al., 2004). The expression of Mx and other antiviral protein encoding genes is stimulated by type 1 interferon (IFN-α), a secreted protein that participates in the defense against viruses (Robertsen, 2006). Both Atlantic salmon parr and post-smolts had an increased expression of Mx protein encoding gene in liver tissues, 24 hours after being injected with polyIC, and with the expression rate peaking at 3 days post-injection (Lockhart et al., 2004). Similar results were reported by Das et al. (2009) where the Mx transcript levels of the kidney, liver, gill and blood of Atlantic salmon parr were elevated up to 7 weeks after polyIC injection. Jensen et al. (2002) demonstrated that polyIC injection resulted in increased relative amount of Mx protein in the major organs of Atlantic salmon, which corresponded with reduced mortality rate when the fish were challenged with ISAV. Injecting a mixture of cpG oligodeoxynucleotides and polyIC had positive effects on Atlantic salmon against the viraemia caused by salmonid alphavirus (SAV) up to 7 weeks post-injection (Strandskog et al., 2011).

In addition to the Mx protein encoding gene, other immune-related genes and their encoding products are involved in the immune responses of Atlantic salmon against various viral infections. IFN-α is a protein that plays a major role in antiviral immune

system of vertebrates through interfering with viral replication within the infected cells as well as triggering the expression of other antiviral molecules (e.g. Mx) (Robertsen, 2006). IFN-α encoding gene within Atlantic salmon kidney cells (in vitro) was up-regulated by 7 fold, 3 days after polyIC stimulation (Jorgensen et al., 2006). The expression of IFN-α gene in the liver and head kidney of Atlantic salmon was significantly up-regulated (170 fold and 90 fold increase, respectively), 24 hours after polyIC injection (Kileng et al., 2008). Type II interferon (IFN-γ) is another type of interferon produced by T lymphocytes that plays a major role in adaptive immune responses (Robertsen, 2006). The expression of IFN-γ in the head kidney of Atlantic salmon (in vivo) exhibited a 193 fold increase 24 hours after the fish were subjected to polyIC injection (Kileng et al., 2008). Interferon stimulated gene 15 (ISG15) is one of the most predominant ubiquitin-like proteins that is induced following IFN-α stimulation. It functions in the antiviral interferon system by conjugating to different target proteins (Rokenes et al., 2007). The transcription of ISG15 was significantly induced in Atlantic salmon head kidney cells (in vitro) 12-36 hours after polyIC application. Atlantic salmon subjected to polyIC injection showed induced expression of ISG15 in their head kidneys 24-48 hours post-injection as detected by Northern blot analysis (Rokenes et al., 2007). MHC class I and ABCB2 are the two major genes activated by interferons and participate in the major histocompatibility complex (MHC) class I pathway, through which intracellular antigenic peptides within the cytoplasm are transported into the endoplasmic reticulum by the transport-associated proteins (ABCB2) and subsequently presented to $CD8^+$ T lymphocytes by MHC class I molecules (Jorgensen et al., 2006; Jorgensen et al., 2007). Jorgensen et al. (2006) demonstrated that the transcription of both MHC class I and

ABCB2 within Atlantic salmon kidney cells (in vitro) were increased (2.5 and 4.8 fold, respectively) after exposure to polyIC for 5 days. MHC class II molecules were induced by interferons and participate in the MHC class II pathway through presenting extracellular antigenic peptides to $CD4^+$ T lymphocytes (Jorgensen et al., 2007). The expression of MHC class II gene within Atlantic salmon leukocyte cells (in vitro) was increased by 7 fold, 48 hours after polyIC treatment (Larsen et al., 2013). However, Jorgensen et al. (2007) indicated that MHC class II gene was not significantly induced in the liver, spleen and head kidney tissues of Atlantic salmon infected with ISAV.

For other species, such as Atlantic cod (*Gadus morhua*), higher Mx protein level was found in the organs of fish injected with polyIC compared with those of the control fish (Das et al., 2008). Immune genes of Atlantic cod such as ISG15 and MHC class I were up-regulated by the polyIC injection (Rise et al., 2008). Genes associated with the brain transcript expression of Atlantic cod such as ISG15 and IL8 were significantly up-regulated following the polyIC injection (Rise et al., 2010). An increase in ambient temperature has been reported to have stimulative effects on the spleen transcriptome expression by Atlantic cod responding to the polyIC injection (Hori et al., 2012). Rainbow trout pre-injected with polyIC had high survival rate (95.2%) after being challenged with infectious hematopoietic necrosis virus (IHNV) and the IHNV-specific antibodies were found among the survivors (Kim et al., 2009).

2.4 Focus of the literature

Before transfer into seawater, Atlantic salmon parr undergo smoltification, which is induced by increased water temperature and photoperiod. Salmon parr are transformed into smolts as a pre-adaption to the marine environment (Folmar and Dickhoff, 1980). If

the fish are not successfully transferred within the "smolt window", desmoltification would occur as a commitment by fish to abandon moving to the marine environment (McCormick et al., 1998a; Stefansson et al., 2008).

Evidenced by the significantly elevated plasma cortisol levels, smoltification has been shown to be a stressful period for the fish as they undergo a series of morphological, physical and behavioral changes including increased gill Na^+, K^+-ATPase activity and subsequently developed salinity tolerance (McCormick et al., 1998a; Sundell et al., 2003; Stefansson et al., 2008). The combined effects of smoltification, environmental challenges as well as production-related stressors significantly impaired the survival and growth of Atlantic salmon after the transfer to seawater, which could cause a significant production loss (Anderson, 1996; Price and Schreck, 2003b; Sundh et al., 2009; Dietrich et al., 2013).

Dietary nucleotides have multiple beneficial effects on fish in terms of growth, organ development and overall health (Low et al., 2003; Li and Gatlin, 2006; Tahmasebi-Kohyani et al., 2012). However, most of the information arises from their effects on the overall immunity and stress resistance of fish against various stressors and diseases (Burrells et al., 2001a; Leonardi et al., 2003; Ringo et al., 2012). Additional requirements for nucleotides from feed have been demonstrated when fish were affected by stressors (Burrells et al., 2001b; Borda et al., 2003).

Very limited information was found relating the effects of dietary nucleotides to the stress response and antiviral status of Atlantic salmon during smoltification. The focus of the current study is to evaluate the effects of dietary nucleotides on Atlantic salmon during smoltification.

Chapter 3: Objectives and Hypotheses

3.1 Trial 1

3.1.1 Objective

To determine the effects of different levels of dietary nucleotides fed as Maxi-Gen^{TM} Plus (0, 0.05, 0.10, 0.15, 0.20 or 0.25%) of the diet on the plasma osmolality, immune-related gene expression, growth performance and gut histology of in-season Atlantic salmon smolts during smoltification.

3.1.2 Hypotheses

1. The plasma osmolality of nucleotide fed fish following the salinity challenge test will be lower than that of fish fed the control diet.

2. The expression of immune-related genes in nucleotide fed groups will be up-regulated at a higher level than that of fish in the control group.

3. Fish fed nucleotides will exhibit better growth performance than those fed the control diet.

4. Intestinal morphology of the fish will be enhanced by feeding nucleotides.

3.2 Trial 2

3.2.1 Objective

To determine the effects of an extended range of Maxi-Gen^{TM} Plus (0, 0.15, 0.30 or 0.45%) of the diet on the plasma osmolality, growth performance and gut histology of off-season Atlantic salmon smolts during smoltification.

3.2.2 Hypotheses

1. The plasma osmolality of fish fed higher levels of nucleotides following the salinity challenge test will be lower than that of fish fed the control diet.

2. Feeding higher levels of nucleotides will improve the growth performance and intestinal morphology of fish.

3.3 Trial 3

3.3.1 Objective

To determine the effects of a further extended range of Maxi-Gen^{TM} Plus (0, 0.2, 0.4 or 0.6%) of the diet on the plasma osmolality, plasma cortisol level and growth performance of in-season Atlantic salmon smolts during smoltification.

3.3.2 Hypotheses

1. The plasma osmolality of fish fed higher levels of nucleotides will be lower than that of fish fed the control diet.

2. The plasma cortisol level of fish will be reduced by feeding nucleotides.

3. Fish fed Maxi-Gen^{TM} Plus at an inclusion level of 0.45% or higher will show better growth performance than those fed the control diet.

Chapter 4: Materials and Methods

The study consisted of three trials. Trial 1 was a natural smolting trial conducted from April to July, 2014. Trial 2 was an off-season trial conducted from August, 2014 to February, 2015. Trial 3 was another normal-season trial conducted from April to August, 2015.

4.1 Experimental fish

All fish (Saint John River stock) were sourced from Big Falls Fish Growers Ltd., Wolfville, NS. Fish used in trial 1 arrived on April 24th, 2014. They were reared in tanks until May 11th, 2014 for the purpose of acclimating to the tank conditions. Fish used in trial 2 arrived on August 12th, 2014. They were reared in tanks until October 25th, 2014 before the start of trial. Fish used in trial 3 arrived on March 26th, 2015. They were reared in tanks until April 23rd, 2015. Fish were not fed during the first 3 days after transportation then were fed commercial pellets (Corey Nutrition Company Inc., NB, Canada) and control diet before distribution. The fish were anesthetized using MS222 (tricaine methane sulfonate), then batch-weighed (10 fish per batch) before distribution into the tanks. The fish were not fed on the day they were distributed. Three meals (0900h, 1200h and 1600h) were manually provided daily to the fish. The fish were fed to satiation at each feeding time. All feed provided to the fish was pre-weighed.

In trial 1, 1200 Atlantic salmon parr (initial weight 60.0 ± 1.3g, mixed sexes) were randomly and equally distributed into 24, 500L recirculation tanks (50 fish per tank). In trial 2, 1504 Atlantic salmon parr (initial weight 46.2 ± 1.1g, mixed sexes) were randomly and equally distributed into the same 16 recirculation tanks with 94 fish per tank. In trial 3, 1472 Atlantic salmon parr (initial weight 47.1 ± 1.3g, mixed sexes) were

randomly and equally distributed into the same 16 recirculation tanks with 92 fish per tank.

The experiments were conducted according to animal care protocol (file: 2014-019). The fish were cared for following standard operating procedures approved by the local Animal Care and Use Committee using the Canadian Council on Animal Care guidelines on the care and use of fish in research, teaching and testing (Canadian Council on Animal Care, 2005).

4.2 Diet formulation and preparation

4.2.1 Trial 1

The test ingredient Maxi-Gen^{TM} Plus was kindly provided by Canadian Bio-Systems Inc. Six dietary treatments were used in trial 1: the control diet and 5 other diets that contained 0.05, 0.10, 0.15, 0.20 or 0.25% inclusion of Maxi-Gen^{TM} Plus (Table 4.1). Except for Maxi-Gen^{TM} Plus, all the ingredients are typically used in commercial Atlantic salmon feed. The only difference between the control diet and the other diets was the replacement of part of the 2.5% celite (a non-nutritional, space filler) by different levels of Maxi-Gen^{TM} Plus. The approach was taken to facilitate easy incorporation of the Maxi-Gen^{TM} Plus. The nucleotide levels in the test diets were calculated to be 0, 7.5, 15, 22.5, 30 and 37.5 ppm respectively from the Maxi-Gen^{TM} Plus. All 6 diets were formulated to meet or exceed the nutritional requirements for Atlantic salmon according to National Research Council (NRC, 2011). Calculated nutrient contents in each diet and the nutritional requirements for Atlantic salmon were shown in Table 4.2.

Table 4.1 The formulations of control and test diets containing different levels of Maxi-Gen™ Plus used in trial 1 (% as fed basis).

Ingredients	Levels of Maxi-Gen™ Plus (%)					
	0	0.05	0.10	0.15	0.20	0.25
Fish oil	20.00	20.00	20.00	20.00	20.00	20.00
Fish meal	17.50	17.50	17.50	17.50	17.50	17.50
Poultry byproduct meal	13.20	13.20	13.20	13.20	13.20	13.20
Empyreal 75®[1]	12.00	12.00	12.00	12.00	12.00	12.00
Blood meal	10.00	10.00	10.00	10.00	10.00	10.00
Wheat gluten meal	10.00	10.00	10.00	10.00	10.00	10.00
Whey	9.00	9.00	9.00	9.00	9.00	9.00
ARBO® TemStik[2]	5.00	5.00	5.00	5.00	5.00	5.00
Betafin® S1[3]	1.00	1.00	1.00	1.00	1.00	1.00
Dicalcium Phosphate	0.50	0.50	0.50	0.50	0.50	0.50
Bio-Phytase 5000[4]	0.50	0.50	0.50	0.50	0.50	0.50
Lysine HCl	0.30	0.30	0.30	0.30	0.30	0.30
Choline chloride	0.30	0.30	0.30	0.30	0.30	0.30
Special premix[5]	0.25	0.25	0.25	0.25	0.25	0.25
Celite®[6]	0.25	0.20	0.15	0.10	0.05	0.00
Maxi-Gen™ Plus[7]	0.00	0.05	0.10	0.15	0.20	0.25
Vitamin/mineral premix[8]	0.20	0.20	0.20	0.20	0.20	0.20

1. Empyreal 75® is a corn protein concentrate manufactured by Cargill Corn Milling Inc., Nebraska, USA.

2. ARBO® TemStik is a product manufactured by Tembec Inc., QC, Canada. The product contains lignosulphonate that serves as a natural binding agents.

3. Betafin® S1 is a highly purified source of feed grade anhydrous betaine manufactured by Danisco Animal Nutrition Corp., Wiltshire, UK.

4. Bio-Phytase 5000 is a phytase-containing product manufactured by Canadian Bio-Systems Inc., Calgary, Alberta, Canada.

5. Special premix contains: vitamin E 250 mg, vitamin C 200 mg, astaxanthin 60 mg, selenium 0.22 mg and wheat shorts 1988mg (per kg).

6. Celite® is a diatomaceous earth-derived product that contains crystalline silica. It is manufactured by Celite Corp., California, US.

7. Maxi-Gen™ Plus is a nucleotide-containing product manufactured by Canadian Bio-Systems Inc., Calgary, Alberta, Canada.

8. Vitamin/mineral premix contains: manganese 125 mg, iron 84 mg, zinc 77.5 mg, copper 2.5 mg, iodine 7.5 mg, vitamin A 5000 IU, vitamin D 4000 IU, vitamin K 2 mg, vitamin B12 4 μg, thiamin 8 mg, riboflavin 18 mg, pantothenic acid 40mg, niacin 100mg, folic acid 4 mg, biotin 0.6 mg, pyridoxine 15 mg, inositol 100 mg, ethoxyquin 42 mg and wheat shorts 1372 mg (per kg).

Table 4.2 The calculated nutrient contents in diets containing graded levels of Maxi-Gen™ Plus used in trial 1 (% as fed basis).

	Levels of Maxi-Gen™ Plus (%)						Requirements[a]
	0	0.05	0.10	0.15	0.20	0.25	
Digestible energy (Kcal/kg)	4572	4572	4572	4572	4572	4572	4400
Crude protein (%)	46.4	46.4	46.4	46.4	46.4	46.4	44
Lipid (%)	23.8	23.8	23.8	23.8	23.8	23.8	/
Nucleotides[b] (ppm)	26.6	34.1	41.6	49.1	56.6	64.1	/
Arginine (%)	2.2	2.2	2.2	2.2	2.2	2.2	1.8
Histidine (%)	1.1	1.1	1.1	1.1	1.1	1.1	0.8
Isoleucine (%)	1.7	1.7	1.7	1.7	1.7	1.7	1.1
Leucine (%)	3.2	3.2	3.2	3.2	3.2	3.2	1.5
Lysine (%)	3.5	3.5	3.5	3.5	3.5	3.5	2.4
Methionine (%)	1.2	1.2	1.2	1.2	1.2	1.2	0.7
Methionine + Cysteine (%)	1.6	1.6	1.6	1.6	1.6	1.6	1.1
Phenylalanine (%)	2.1	2.1	2.1	2.1	2.1	2.1	0.9
Phenylalanine + Tyrosine (%)	2.2	2.2	2.2	2.2	2.2	2.2	1.8
Threonine (%)	1.5	1.5	1.5	1.5	1.5	1.5	1.1
Tryptophan (%)	0.4	0.4	0.4	0.4	0.4	0.4	0.3
Valine (%)	2.2	2.2	2.2	2.2	2.2	2.2	1.2
Calcium (%)	0.2	0.2	0.2	0.2	0.2	0.2	0.5
Phosphorus (%)	0.7	0.7	0.7	0.7	0.7	0.7	0.5
Ash (%)	4.1	4.1	4.1	4.1	4.1	4.1	/

a: Nutritional requirements for Atlantic salmon, National Research Council (NRC, 2011).
b: The total nucleotide contents in the diets were calculated as the sum of the nucleotide contents of feed ingredients (fish meal, poultry byproduct meal, blood meal, whey and Maxi-Gen™ Plus) available to be found (Li et al., 2015).

4.2.2 Trial 2

Based on the results from trial 1, an extended range of Maxi-Gen™ Plus was examined in trial 2. Four dietary treatments were used in the current trial (Table 4.3): the control diet and 3 test diets that contained 0.15%, 0.30% or 0.45% inclusion of Maxi-Gen™ Plus. The nucleotide levels in the diets from Maxi-Gen™ Plus were calculated to be 0, 22.5, 45 and 67.5 ppm respectively. Same ingredients were used in this trial as in trial 1.

Table 4.3 The formulations of control and test diets containing different levels of Maxi-Gen™ Plus used in trial 2 (% as fed basis).

Ingredients	Levels of Maxi-Gen™ Plus (%)			
	0	0.15	0.30	0.45
Fish oil	20.00	20.00	20.00	20.00
Fish meal	17.50	17.50	17.50	17.50
Poultry byproduct meal	13.20	13.20	13.20	13.20
Empyreal 75®[1]	12.00	12.00	12.00	12.00
Blood meal	10.00	10.00	10.00	10.00
Wheat gluten meal	10.00	10.00	10.00	10.00
Whey	8.80	8.80	8.80	8.80
ARBO® TemStik[2]	5.00	5.00	5.00	5.00
Betafin® S1[3]	1.00	1.00	1.00	1.00
Dicalcium Phosphate	0.50	0.50	0.50	0.50
Bio-Phytase 5000[4]	0.50	0.50	0.50	0.50
Celite®[5]	0.45	0.30	0.15	0.00
Maxi-Gen™ Plus[6]	0.00	0.15	0.30	0.45
Lysine HCl	0.30	0.30	0.30	0.30
Choline chloride	0.30	0.30	0.30	0.30
Special premix[7]	0.25	0.25	0.25	0.25
Vitamin/mineral premix[8]	0.20	0.20	0.20	0.20

1. Empyreal 75® is a corn protein concentrate manufactured by Cargill Corn Milling Inc., Nebraska, USA.

2. ARBO® TemStik is a product manufactured by Tembec Inc., QC, Canada. The product contains lignosulphonate that serves as a natural binding agents.

3. Betafin® S1 is a highly purified source of feed grade anhydrous betaine manufactured by Danisco Animal Nutrition Corp., Wiltshire, UK.

4. Bio-Phytase 5000 is a phytase-containing product manufactured by Canadian Bio-Systems Inc., Calgary, Alberta, Canada.

5. Celite® is a diatomaceous earth-derived product that contains crystalline silica. It is manufactured by Celite Corp., California, US.

6. Maxi-Gen™ Plus is a nucleotide-containing product manufactured by Canadian Bio-Systems Inc., Calgary, Alberta, Canada.

7. Special premix contains: vitamin E 250 mg, vitamin C 200 mg, astaxanthin 60 mg, selenium 0.22 mg and wheat shorts 1988mg (per kg).

8. Vitamin/mineral premix contains: manganese 125 mg, iron 84 mg, zinc 77.5 mg, copper 2.5 mg, iodine 7.5 mg, vitamin A 5000 IU, vitamin D 4000 IU, vitamin K 2 mg, vitamin B12 4 μg, thiamin 8 mg, riboflavin 18 mg, pantothenic acid 40mg, niacin 100mg, folic acid 4 mg, biotin 0.6 mg, pyridoxine 15 mg, inositol 100 mg, ethoxyquin 42 mg and wheat shorts 1372 mg (per kg).

All 4 diets were formulated to meet or exceed the nutritional requirements for Atlantic salmon (Table 4.4) (NRC, 2011). As Maxi-Gen™ Plus level was increased in the diet the Celite® level was reduced to ensure a 0.45% inclusion level of the combination of these two ingredients in the diets.

Table 4.4 The calculated nutrient contents in diets containing graded levels of Maxi-Gen™ Plus used in trial 2 (% as fed basis).

	Levels of Maxi-Gen™ Plus (%)				Requirements[a]
	0	0.15	0.30	0.45	
Digestible energy (Kcal/kg)	4566	4566	4566	4566	4400
Crude protein (%)	46.3	46.3	46.3	46.3	44
Lipid (%)	23.8	23.8	23.8	23.8	/
Nucleotides[b] (ppm)	26.0	49.1	71.6	94.1	/
Arginine (%)	2.2	2.2	2.2	2.2	1.8
Histidine (%)	1.1	1.1	1.1	1.1	0.8
Isoleucine (%)	1.7	1.7	1.7	1.7	1.1
Leucine (%)	4.2	4.2	4.2	4.2	1.5
Lysine (%)	3.5	3.5	3.5	3.5	2.4
Methionine (%)	1.2	1.2	1.2	1.2	0.7
Methionine + Cysteine (%)	1.6	1.6	1.6	1.6	1.1
Phenylalanine (%)	2.1	2.1	2.1	2.1	0.9
Phenylalanine + Tyrosine (%)	2.2	2.2	2.2	2.2	1.8
Threonine (%)	1.5	1.5	1.5	1.5	1.1
Tryptophan (%)	0.4	0.4	0.4	0.4	0.3
Valine (%)	2.2	2.2	2.2	2.2	1.2
Calcium (%)	0.2	0.2	0.2	0.2	0.5
Phosphorus (%)	0.7	0.7	0.7	0.7	0.5
Ash (%)	4.1	4.1	4.1	4.1	/

a: Nutritional requirements for Atlantic salmon, National Research Council (NRC, 2011).
b: The total nucleotide contents in the diets were calculated as the sum of the nucleotide contents of feed ingredients (fish meal, poultry byproduct meal, blood meal, whey and Maxi-Gen™ Plus) available to be found (Li et al., 2015).

4.2.3 Trial 3

Based on the results from trials 1 and 2, a further extended range of Maxi-Gen™ Plus was examined in trial 3. Four dietary treatments were used in trial 3 (Table 4.5): the

control diet and 3 test diets that contained 0.20%, 0.40% or 0.60% inclusion of Maxi-Gen™Plus.

Table 4.5 The formulations of control and test diets containing different levels of Maxi-Gen™ Plus used in trial 3 (% as fed basis).

Ingredients	Levels of Maxi-Gen™ Plus (%)			
	0	0.20	0.40	0.60
Fish oil	20.00	20.00	20.00	20.00
Fish meal	17.50	17.50	17.50	17.50
Poultry byproduct meal	13.20	13.20	13.20	13.20
Empyreal 75®[1]	12.00	12.00	12.00	12.00
Wheat gluten meal	10.00	10.00	10.00	10.00
Blood meal	9.85	9.85	9.85	9.85
Whey	8.80	8.80	8.80	8.80
ARBO® TemStik[2]	5.00	5.00	5.00	5.00
Betafin® S1[3]	1.00	1.00	1.00	1.00
Celite®[4]	0.60	0.40	0.20	0.00
Maxi-Gen™ Plus[5]	0.00	0.20	0.40	0.60
Dicalcium Phosphate	0.50	0.50	0.50	0.50
Bio-Phytase 5000[6]	0.50	0.50	0.50	0.50
Lysine HCl	0.30	0.30	0.30	0.30
Choline chloride	0.30	0.30	0.30	0.30
Special premix[7]	0.25	0.25	0.25	0.25
Vitamin/mineral premix[8]	0.20	0.20	0.20	0.20

1. Empyreal 75® is a corn protein concentrate manufactured by Cargill Corn Milling Inc., Nebraska, USA.

2. ARBO® TemStik is a product manufactured by Tembec Inc., QC, Canada. The product contains lignosulphonate that serves as a natural binding agents.

3. Betafin® S1 is a highly purified source of feed grade anhydrous betaine manufactured by Danisco Animal Nutrition Corp., Wiltshire, UK.

4. Celite® is a diatomaceous earth-derived product that contains crystalline silica. It is manufactured by Celite Corp., California, US.

5. Maxi-Gen™ Plus is a nucleotide-containing product manufactured by Canadian Bio-Systems Inc., Calgary, Alberta, Canada.

6. Bio-Phytase 5000 is a phytase-containing product manufactured by Canadian Bio-Systems Inc., Calgary, Alberta, Canada.

7. Special premix contains: vitamin E 250 mg, vitamin C 200 mg, astaxanthin 60 mg, selenium 0.22 mg and wheat shorts 1988mg (per kg).

8. Vitamin/mineral premix contains: manganese 125 mg, iron 84 mg, zinc 77.5 mg, copper 2.5 mg, iodine 7.5 mg, vitamin A 5000 IU, vitamin D 4000 IU, vitamin K 2 mg, vitamin B12 4 μg, thiamin 8 mg, riboflavin 18 mg, pantothenic acid 40mg, niacin 100mg, folic acid 4 mg, biotin 0.6 mg, pyridoxine 15 mg, inositol 100 mg, ethoxyquin 42 mg and wheat shorts 1372 mg (per kg).

The nucleotide levels in the diets from Maxi-Gen™ Plus were calculated to be 0, 30, 60 and 90 ppm respectively. Same ingredients were used in this trial as those used in trial 1 and trial 2. All 4 diets were formulated to meet or exceed the nutritional requirements for Atlantic salmon (Table 4.6) (NRC, 2011). As Maxi-Gen™ Plus level was increased in the diet the Celite® level was reduced to ensure a 0.6% inclusion level of the combination of these two ingredients in the diets.

Table 4.6 The calculated nutrient contents in diets containing graded levels of Maxi-Gen™ Plus used in trial 3 (% as fed basis).

	Levels of Maxi-Gen™ Plus (%)				Requirements[a]
	0	0.20	0.40	0.60	
Digestible energy (Kcal/kg)	4559	4559	4559	4559	4400
Crude protein (%)	46.2	46.2	46.2	46.2	44
Lipid (%)	23.8	23.8	23.8	23.8	/
Nucleotides[b] (ppm)	26.0	56.6	86.6	116.6	/
Arginine (%)	2.2	2.2	2.2	2.2	1.8
Histidine (%)	1.1	1.1	1.1	1.1	0.8
Isoleucine (%)	1.7	1.7	1.7	1.7	1.1
Leucine (%)	4.1	4.1	4.1	4.1	1.5
Lysine (%)	3.5	3.5	3.5	3.5	2.4
Methionine (%)	1.2	1.2	1.2	1.2	0.7
Methionine + Cysteine (%)	1.6	1.6	1.6	1.6	1.1
Phenylalanine (%)	2.1	2.1	2.1	2.1	0.9
Phenylalanine + Tyrosine (%)	2.2	2.2	2.2	2.2	1.8
Threonine (%)	1.5	1.5	1.5	1.5	1.1
Tryptophan (%)	0.4	0.4	0.4	0.4	0.3
Valine (%)	2.2	2.2	2.2	2.2	1.2
Calcium (%)	0.2	0.2	0.2	0.2	0.5
Phosphorus (%)	0.7	0.7	0.7	0.7	0.5
Ash (%)	4.1	4.1	4.1	4.1	/

a: Nutritional requirements for Atlantic salmon, National Research Council (NRC, 2011).
b: The total nucleotide contents in the diets were calculated as the sum of the nucleotide contents of feed ingredients (fish meal, poultry byproduct meal, blood meal, whey and Maxi-Gen™ Plus) available to be found (Li et al., 2015).

All the diets were made at the Chute Animal Nutrition Centre, Faculty of Agriculture, Dalhousie University. The ingredients used for each diet were mixed according to the formulations (Table 4.1, 4.3 and 4.5). After mixing the diets were steam pelleted using a 3 mm die in a California pellet mill (San Francisco, US). The pellets were dried in the oven at 60^{o}C for 4 hours then cooled down to the room temperature and stored at -20^{o}C until fed. Pellets with 3 mm diameter were used in all trials. Samples of all diets were taken for subsequent analysis.

The dry matter, crude protein, crude fat and ash content within each diet were analyzed in the nutrition lab, Faculty of Agriculture, Dalhousie University. All the diet samples were put into oven for drying at 65^{o}C for 24 hours. After that the dry mater content was calculated (weight after drying/weight before drying*100) (AOAC, 2011; method no. 934.01). Crude protein content (N x 6.25) was analyzed in duplicate using the FP-528 nitrogen/protein determinator (LECO Corp., Canada) (AOAC, 2011; method no. 968.06). Crude fat content was analyzed in duplicate using the ANKOM^{XT15} extraction system and ANKOMRD Dryer (ANKOM Technology Inc., NY, US) (AOAC, 2011; method no. 920.39). The samples were put in duplicate into the Isotemp® Muffle Furnace (Fisher Scientific Inc., US) for ashing at 450^{o}C overnight. After that the ash content was calculated (weight after ashing/weight before ashing) (AOAC, 2011; method no. 942.05).

4.3 Rearing conditions

Three identical 8-tank recirculation systems were used for rearing fish in trial 1. Two recirculation systems were used in each of trials 2 and 3. Each system consisted of 5 main components: rearing tanks (500L), mechanical filter (sand filter), biological filter (bio-filter), oxygen saturator and temperature controller. The water that exited from the tanks

(containing solids, low O_2 and high CO_2) was pumped into the mechanical filter to remove the undissolved solids, then by gravity into the biological filter within which the dissolved solids were removed and the CO_2 was vented off. After that the water was supersaturated with O_2 while passing through the oxygen saturator. The O_2-saturated water was pumped back into the tanks. The total volume of each system was 4575 L. each tank was covered with a hood to eliminate the risk of fish stressed by people passing by.

Ambient fresh water was recirculated through the tanks with the average water temperature of 10 ± 0.6ºC and dissolved oxygen level ranged from 94% to 125% saturation throughout all three trials. Water temperature was maintained using a UTCHW-8.5 temperature controller (Universal Marine Industries Inc., BC, Canada). Dissolved oxygen level and water temperature were monitored daily using an YSI Pro20 dissolved oxygen meter (YSI Inc., Ohio, US). The make-up water was added into each system at a rate of 180 ± 20 ml/minute (5.7% water replaced per day). The average water flow rate within each tank was 14 ± 1.5L per minute. The average light intensity (water surface) within each tank was 40 ± 7lux. Water salinity was increased and maintained at around 1.9ppt in trial 1 starting on May 26th, 2014 to control fungus growth within the systems. Salinity treatment (adding seawater into the systems to temporarily increase the salinity to around 2.5ppt) was applied in trial 2 whenever mortality occurred with fungus infection. Repeated formalin treatment (3 days in a row, adding diluted formalin into the systems at 130µl/l for 1h) was applied on May 31st and June 29th, 2015 in trial 3 after mortality outbreaks occurred.

In trials 1 and 3, simulated natural day length for latitude 45°N was applied throughout the trial. In trial 2, one month of short photoperiod (8h light:16h dark, from September 24th to October 21st, 2014) was followed by two months of long photoperiod (16h light:8h dark, from October 22nd to December 20th, 2014) applied to produce off-season smolts that underwent smoltification from September, 2014 to February, 2015 (McCormick et al., 2000). The long photoperiod was maintained after the stimulation period until the end of the trial (February 14th, 2015) to simulate the natural photoperiod during the natural smolting season (April to June).

4.4 Treatment and tank distribution

In trial 1, 24 rearing tanks were randomly allocated to one of six treatments (4 tanks/ treatment) with each treatment represented at least once in each system. All 24 tanks were used to observe the growth performance of the fish. Within each treatment, fish in two of the 4 tanks were randomly selected to be used to conduct the salinity tests (salinity-rearing tanks). The other two tanks were used to conduct the viral mimic test (viral mimic-rearing tanks).

In trials 2 and 3, one row of four tanks was considered as a block (4 blocks in total). Within each block, the 4 treatments were randomly allocated to one of four tanks. All 16 tanks were used to observe the growth performance and to conduct the salinity tests.

Tank is the experimental unit in all 3 trials.

4.5 Growth Performance

All the fish were batch-weighed (20 fish/batch) to calculate the body weight gain. In trial 1, the fish from all the tanks were batch-weighed on May 11th (day 0), June 8th (day 28), July 6th (day 56) and July 28th (day 78), 2014. In trial 2, the fish were batch-weighed on

October 25th (day 0), November 22nd (day 28), December 20th (day 56), 2014, January 17th (day 84) and February 14th (day 112), 2015. In trial 3, the fish were batch-weighed on April 24th (day 0), May 22nd (day 28), June 19th (day 56) and August 24th (day 122), 2015. Feed consumption was recorded biweekly by substituting the feed remaining (end of each two-week interval) from the feed weighed at the beginning of each interval. Feed conversion ratio (FCR) was calculated as total feed consumed/ total weight gain of fish within the tank. Mortality was recorded every day and the weight of mortalities was measured. The growth performance of the fish were determined by the daily body weight gain/fish, daily feed consumption as % of body weight and FCR (calculated as followed):

Daily weight gain/fish = (total weight (current) of fish/numbers of fish – total weight (previous) of fish/number of fish)/days within the period

Daily feed consumption as % of body weight = (total feed consumption within the period/average body weight of fish within the period)/days within the period *100

FCR = total feed consumption/total weight gain of fish within the period

4.6 Salinity Challenge Test

Twenty-four hour, 40ppt salinity challenge tests were repeated biweekly in all the trials to assess the hypo-osmoregulatory ability of Atlantic salmon during smolting and post-smolting periods (Duston et al., 2011). Higher salinity used in the current study can fully challenge the hypo-osmoregulatory ability of fish while not likely to cause any mortality during this short period. Four salinity tests were conducted starting on May 27th, June 10th, June 24th and July 8th, 2014 in trial 1. Five tests were conducted on December 12th, December 26th, 2014, January 9th, January 23rd and February 6th, 2015 in trial 2. Four tests were conducted on May 22nd, June 9th, June 19th and July 3rd, 2015 in trial 3.

In each test, 6 fish were randomly selected from each salinity-rearing tank per treatment and transferred to the corresponding salinity challenge test tanks (clear acrylic, 75cm x 30cm x 36cm deep, vol. 70L) for 24 hours in 40ppt salinity. All the salinity test tanks shared the same recirculated seawater made by mixing filtered seawater (from Sandy Cove, NS, 31ppt) with artificial sea-salt (Spectrum Brands Inc., Virginia, US). The water temperature in each test tank was maintained between 10 to 11°C by two chillers and the dissolved oxygen level was maintained between 100 to 115% saturation by supplying air into the water. At the end of each test, the mortalities were recorded while the survivors were netted from the tank and euthanized by MS-222. The fork length (FL) and body weight (BW) of each fish were recorded. Blood samples were taken from the severed caudal vessels of the fish with heparinized glass tubes into 1.5 ml tubes. The samples were immediately put on ice until the centrifugation. The samples were centrifuged at 3000 rpm for 5 min. After centrifugation, the plasma was separated from the blood samples and stored in the freezer at -20°C until analysis. The samples were analyzed in duplicate using a 5004 Automatic Freezing Point Osmometer (Precision Systems Inc., NJ, US) to determine the plasma osmolality (Duston et al., 2011).

4.7 Viral Mimic Test

Viral mimic test was conducted in trial 1 to determine the effects of dietary nucleotides on the immune-related gene expression of Atlantic salmon responding to the polyIC injection. Sampling was conducted in July 28th, 2014 after which RT-qPCR was used to evaluate expression levels in targeted immune genes.

4.7.1 PolyIC stimulation and sampling

Spleen tissues of 6 randomly selected euthanized fish were collected from each viral

mimic-rearing tank as undisturbed controls (UC) before the polyIC stimulation. After collecting UC samples, the remaining fish were lightly anesthetized by MS-222. Within each tank, 16 fish (about 60g by the time of injection) were injected with 240 μl of 0.5 mg/ml pIC in phosphate-buffered saline (PBS) (2 mg polyIC/g body weight) while another 16 fish in the same tank were injected with 240 μl of PBS (saline control) (Booman et al., 2014). Fin clip was conducted to differentiate the polyIC-injected fish from the saline-injected ones (adipose and anal fins clipped for polyIC and saline groups, respectively). Spleen samples of 6 fish from each group within each tank were sampled at 24 hours post-stimulation. Tools (forceps, scissors and scalpels) were sprayed with RNase AWAY® (Molecular BioProducts Inc., US) and wiped by Kimwipes delicate task wipers (Kimberly-Clark Worldwide Inc., US) before sampling each fish to avoid the presence of RNase. The spleen tissues were collected in 1.5ml RNase-free tubes, flash frozen in liquid nitrogen and stored at -80^{0}C until RNA extraction were conducted (Rise et al., 2010).

4.7.2 RNA extraction and cDNA synthesis

All samples were homogenized using MS-100 Micro Smash™ (TOMY Digital Biology LTD., Tokyo, Japan) before extraction. Total RNA was extracted using an RNEasy Mini kit (Qiagen, Ontario, Canada) following the manufacturer's instructions. Extracted RNA was eluted in a final volume of 50 μl RNase-free water and stored in 1.5 ml tubes at -80 ^{o}C. RNA quality and integrity were checked using Nano 6000 chip on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., US). Samples with degraded RNA (RNA integrity number less than 8) were rejected for the current study. RNA concentrations were determined using a Nanodrop ND-1000 spectrophotometer.

The cDNA synthesis was performed using 1 µg of total RNA mixed with 4 µl of iScript™ RT Supermix (Bio-Rad Ltd., Canada) in 1.5ml tube. Nuclease-free water was added to make the total volume of the mixtures to 20 µl. No template control (NTC) samples were made by mixing 4 µl of iScript™ RT Supermix with 16 µl nuclease-free water. No reverse transcription control (NRT) samples were made by mixing 1µl of total RNA of a randomly selected sample with 4 µl of iScript™ RT Supermix, No-RT Control (Bio-Rad Ltd., Canada) and nuclease-free water (added to make the total volume of 20 µl). Contents of each 1.5 ml tube were then mixed using a vortexer, spun by a centrifuge at 8000 x g for 10 seconds and incubated for 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C and 3 minutes at 12°C to synthesize cDNA. After the incubation 20 µl of nuclease-free water was added into the tubes and mixed. The synthesized cDNA was divided into 2µl aliquots and stored at -80°C.

4.7.3 Primers for selected genes

Immune-related genes of Atlantic salmon (IFN-α, IFN-γ, Mx, MHC class I, ABCB2, ISG15 and MHC class II) were selected as target genes based on previous studies (Hynes et al., 2011; Jorgenson et al., 2007). β-actin, EF1-α, EIF4H, LSM8, MRTO4, DYNLL1 were selected as potential housekeeping genes for normalization (Olsvik et al., 2005; Sutherland et al., 2014). The sequences of all the primers (Table 4.7) used in the study were checked and verified for specificity using the BLASTn algorithm and the GenBank database of the National Center for Biotechnology Information (NCBI).

Table 4.7 Real-time PCR primers used in the current study.

Genes	Direction	Sequence 5'-3'	Amplicon	Acc. No.
IFN-α	F	TGGGAGGAGATATCACAAAGC	163	NM00112-
	R	TCCCAGGTGACAGATTTCAT		3570
IFN-γ	F	TTCAGGAGACCCAGAAACACTAC	125	AJ841811
	R	TAATGAACTCGGACAGAGCCTTC		
Mx	F	TGATCGATAAAGTGACTGCATTCA	80	SSU66477
	R	TGAGACGAACTCCGCTTTTTCA		
MHC	F	CTGCATTGAGTGGCTGAAGA	175	AF504022
class I	R	GGTGATCTTGTCCGTCTTTC		
ABCB2	F	CCAATAGTATGTCAAGCCTGT	151	SSZ83327
	R	ATACGACATCACGGCCTCCA		
ISG15	F	TGTTAGGTGTCAATGGGAGCAA	151	AY926456
	R	TGTGTCTGGCCCTTTTCGTT		
MHC	F	ATGGTGGAGCACATCAGCC	68	X70166
class II	R	CTCAGCCTCAGGCAGGGAC		
β-actin	F	CCAAAGCCAACAGGGAGAAG	91	BG933897
	R	AGGGACAACACTGCCTGGAT		
EF1-α	F	TGCCCCTCCAGGATGTCTAC	59	BG933853
	R	CACGGCCCACAGGTACTG		
EIF4H	F	AGAACCCTCTGACGAGGAGAG	104	BT058726
	R	ATATGGCAGAGTTGGGGTTG		
LSM8	F	TTGACCAGACCATCAACCTG	117	BT050261
	R	CAGCAACGTTGTCTCCTCTG		
MRTO4	F	GGGAGACACACTAACCCCTG	109	BT046843
	R	GTCGCTCGTTTCAGAGTTCC		
DYNLL1	F	ACATCGAGAAAGACATCGCC	111	BT149975
	R	TCTCATGGGTCACGTAGCTG		

The primers were manufactured by Integrated DNA Technologies Inc., US. The efficiency (E) of each primer set was determined by performing 4 serial 5-fold dilutions of standard cDNA (made by mixing 25 random samples from all treatment groups) in triplicates in a qPCR reaction using a C1000 Touch^{TM} thermal cycler (Bio-Rad Inc., Canada). The inter-assay reproducibility of each primer set was maintained under 10%. Melt curves for each primer set were made to determine the optimal annealing temperature with no presence of primer-dimer products (single peak on the curve). The

thermocycling protocol was 95ºC for 3 minutes followed by 40 cycles of 95ºC for 15 seconds and 60ºC (for annealing) for 1 minute. If the melt curve of the primer set showed two peaks (indication of primer-dimer), higher annealing temperatures (62ºC, 65ºC or 68ºC) were tested until only the higher temperature single peak was present. The primer sets for IFN-γ and EIF4H were rejected from the current study as the melt curves always showed two peaks at different annealing temperatures.

4.7.4 Quantitative real-time PCR

Samples from 0, 0.20 and 0.25% Maxi-Gen™ Plus groups in trial 1 were selected for the RT-qPCR analysis as the fish fed 0.20 and 0.25% Maxi-Gen™ Plus showed better osmotic stress tolerance during the salinity tests than that of the fish fed the other Maxi-Gen™ Plus inclusion levels.

Transcript expression levels of selected genes were quantified by RT-qPCR using the Ssofast™ EvaGreen® Supermix (Bio-Rad Ltd., Canada) and the Bio-Rad CFX96™ Real-Time PCR Detection systems (Bio-Rad Ltd., Canada). To quantify gene expression in different samples, the cDNA of each sample were run in triplicates in a semi-skirted 96 well PCR plates (Neptune Inc., New Mexico, US). Each plate well contained 2 µl of 10-fold diluted cDNA, 5 µl Ssofast™ EvaGreen® Supermix, 0.1 µl of 10 µM forward primer, 0.1 µl of 10 µM reverse primer and 2.8 µl nuclease-free water. Two plates were used for each gene with 32 samples on the first plate and the rest (including NTC and NRT) on the second plate. Three samples were randomly selected to be put on both plates for interrun calibration. The RT-qPCR assay was run on the Bio-Rad CFX96™ Real-Time PCR Detection systems with the thermal profile of 95ºC for 3 minutes followed by 40 cycles of 95ºC for 15 seconds and 60ºC (62ºC for LSM8 and DYNLL1) for 1 minute.

4.7.5 RT-qPCR data analysis

The RT-qPCR data was organized and sorted using the Bio-Rad CFX Manager™ software according to the instruction manual. Baseline and cycle threshold (crossing point) were set manually to get the C_T values for each sample. No C_T value greater than 40 (rejected if any) appeared for all the samples (Jorgensen et al., 2007).

The data was transferred to qbase$^+$ v2.3 real-time PCR analysis software to calculate the relative mRNA transcriptions of target genes. GeNorm was used to determine the stability of reference genes and β-actin, EF1-α, LSM8, DYNLL1 were selected for normalization as they had the lowest geNorm M values. The replicate variability within the three replicates for each sample was set at 0.75. If the replicate variability exceeded 0.75, one value that was most dissimilar to the other two was rejected. The specific amplification efficiency determined for each primer sets was used to calculate the relative expression. The relative expression was calculated relative to the levels in the control group. Values greater than 1 indicated an up-regulation of gene expression while values less than 1 indicated a down-regulation of gene expression,

4.8 Intestinal histology

The intestinal histology of fish was evaluated to determine the effects of dietary nucleotides on the alteration of intestinal structures as well as the nutrient metabolism of the fish (Øverland et al., 2009). Intestinal samples were taken on July, 2014 and February, 2015 for trials 1 and 2, respectively.

For the sampling, 3 fish from each tank were euthanized by MS-222 and dissected to expose the intestinal tract. Mid and distal intestine samples (2-3 cm, on the middle of both regions) were immediately collected and fixed in 10% neutral buffered formalin.

Histology slides were prepared in the pathology laboratory, Hancock Veterinary Building, Department of Agriculture, Government of Nova Scotia. A piece of each sample was placed in a cassette and soaked in 10% buffered formalin. The cassettes were rinsed and fixed with paraffin in the Tissue-Tek TEC^{TM} (Sakura Finetek USA Inc., Torrance, US). After fixing, the longitudinal section (5 μm) from each sample was cut using a microtome (Leica RM2255, Nussloch, Germany). The longitudinal sections were placed on glass slides (one section/slide) and stained with haematoxylin and eosin using the Tissue-Tek TEC^{TM}.

Histology slides were scanned using the Nikon Super Coolscan 4000ED (Nikon Inc., Japan) in the image lab, Haley Institute, Faculty of Agriculture, Dalhousie University. Ten intact shaped simple villus were selected from each slide to be measured. The length, width and apparent surface area of each villus were measured using the SigmaScan Pro 5 software. The average values of the 10 villi selected from each slide were used for the statistical analysis. Villus height (mm) was measured from the base (the edge between villi and submucosa) to the apex of the villi (A). Villus width (mm) was measured at the midpoint of each villi (B). Villus apparent surface area (mm^2) was measured as the closed area circled (covering the whole villus above submucosa) using the SigmaScan Pro 5 software (C) (Figure 4.1).

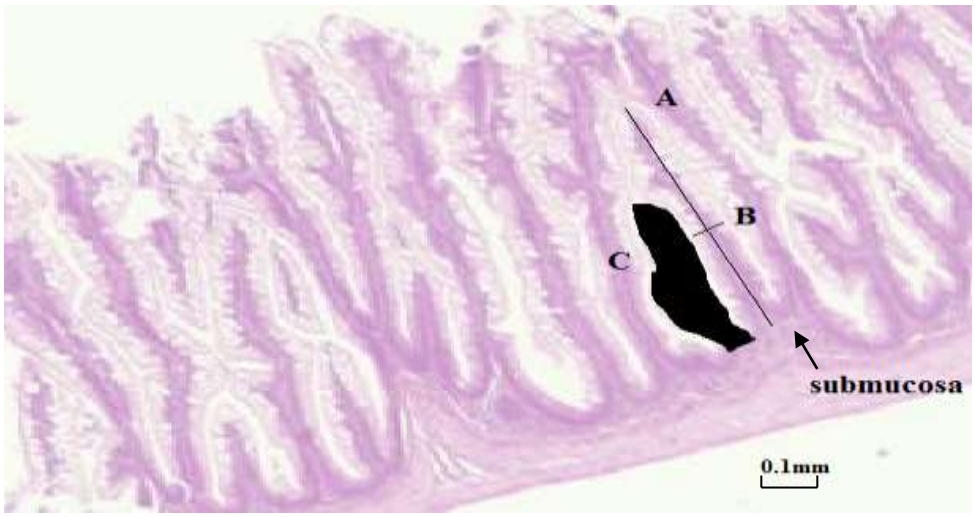

Figure 4.1 Longitudinal section of Atlantic salmon distal intestine and intestinal parameter measurements: A: villi length; B: villi width and C: villi apparent area (the photo was taken from the current study).

4.9 Plasma cortisol level

The basal plasma cortisol levels of fish were measured in trial 3 to evaluate the effects of dietary nucleotides on reducing the stress level of Atlantic salmon smolts during smoltification. Samples were collected biweekly starting from May, 2015 (day 29).

During each sampling time, 2 fish were randomly netted from each tank and immediately euthanized by MS-222. The FL and BW of each fish were recorded. Blood samples were taken from the severed caudal vessels of the fish using heparinized glass tubes (Fisher Scientific Inc., US) into 1.5 ml tubes. The samples were put on ice until the centrifugation. The samples were centrifuged at 3000 rpm for 5 min immediately after the blood collection. After centrifugation, the plasma was separated from the blood samples and stored in 1.5 ml tubes at -20°C in the freezer until analysis.

The total cortisol was extracted from the plasma samples by adding 100 µl of plasma into 1 ml of petroleum ether to allow the phases to separate. The organic phase (upper layer) was transferred into clean glass tubes and evaporated with a stream of nitrogen gas. The

residue was dissolved in 100 µl, 5-fold diluted extraction buffer (provided in the kit). The extract was diluted 100 fold by adding 10 µl of extract into 990 µl diluted extraction buffer.

The plasma cortisol concentrations were measured using the 96-well cortisol enzyme-linked immunosorbent assay kit (#402710, Neogen Corp., Lexington, USA) following the manufacturer's instructions (Fast et al., 2008). The assay used in the current study was validated for Atlantic salmon by Fast et al. (2008). Fifty µl of extracted samples were added into cortisol antibody-coated microplate wells (provided in the kit) in duplicate along with 50 µl diluted enzyme conjugate (provided in the kit). The plate was gently shaken, covered with plastic film and incubated at room temperature for 1 hour. After incubation the contents of the plate were poured off and the plate was washed three times with 300µl diluted wash buffer (provided in the kit). Each plate well was then incubated with 150µl substrate (provided in the kit) at room temperature for 30 minutes. After incubation the plate was read in a microplate reader at an absorbance of 650 nm to get the absorbance value for each sample. Two wells with only substrate added were used as blanks to account for substrate background. Standard absorbance-concentration curve (plasma concentration on y axis, absorbance value on x axis) and its corresponding equation (R^2 greater than 95%) were made using Excel 2013 to correlate 8 serially diluted standard stock solution (0, 0.04, 0.1, 0.2, 0.4, 1, 2 and 10 ng/ml) with their respective absorbance values. One standard curve was made for each of the microplate used. The absorbance value of each sample was converted to the plasma cortisol concentration using the standard curve. The concentrations were multiplied by 100 (dilution factor) to get the final concentrations.

4.10 Statistical analysis

Trial 1 was designed as a completely randomized design (CRD) with Maxi-Gen^{TM} Plus inclusion level as the main factor. Repeated measures were used to evaluate the effects of time when it is considered as a factor. Factorial arrangement (3 x 3) was applied for the viral mimic test to evaluate the interaction between the treatment (0, 0.20 and 0.25% Maxi-Gen^{TM} Plus inclusion levels) and injection (undisturbed control, saline control and polyIC). The statistical models were as follows:

Without repeated measures:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij} \qquad (i = 1, 2, 3, 4, 5, 6)$$

Where: Y_{ij} = response variable
μ = the overall mean
α_i = the effect of treatment
ε_{ij} = the random error

With repeated measures:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk} \qquad (i = 1, 2, 3, 4, 5, 6; j = 1, 2, 3, 4)$$

Where: Y_{ijk} = response variable
μ = the overall mean
α_i = the effect of treatment
β_j = the effect of time
$(\alpha\beta)_{ij}$ = the interaction effect of treatment and time
ε_{ijk} = the random error

With factorial arrangement:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk} \qquad (i = 1, 2, 3; j = 1, 2, 3)$$

Where: Y_{ijk} = response variable
μ = the overall mean
α_i = the effect of dietary treatment
β_j = the effect of injection
$(\alpha\beta)_{ij}$ = the interaction effect between treatment and injection
ε_{ijk} = the random error

The growth performance (daily weight gain, daily feed consumption as % of body weight and FCR) and plasma osmolality from the salinity tests were statistically analyzed by analysis of variance (ANOVA) using the Mixed procedure of SAS 9.3 with the significant level of 0.05 (Littell et al., 1996). Repeated measures were used on the growth performance and plasma osmolality. Intestinal parameters (villus length, width and apparent surface area) were analyzed by one-way ANOVA using Minitab 17 statistical software. Immune-related relative gene expression from viral mimic test was analyzed by two-way ANOVA using the Mixed procedure of SAS 9.3 with the significant level of 0.05 (Littell et al., 1996).

Trials 2 and 3 were designed as randomized complete block design (RCBD) with a row of 4 tanks considered as a block. Maxi-Gen^{TM} Plus inclusion level was the main factor for both trials. The block effect was considered as a random effect. Repeated measures were used to evaluate the effects of time when it is considered as a factor. The statistical models were as follows:

Without repeated measures:

$$Y_{ijk} = \mu + \alpha_i + \gamma_j + \varepsilon_{ijk} \qquad (i = 1, 2, 3, 4; j = 1, 2, 3, 4)$$

Where: Y_{ijk} = response variable
μ = the overall mean
α_i = the effect of treatment
γ_j = the random effect of block
ε_{ijk} = the random error

With repeated measures:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \varepsilon_{ijkl} \qquad (i = 1, 2, 3, 4, 5, 6; j = 1, 2, 3, 4; k = 1, 2, 3, 4)$$

Where: Y_{ijkl} = response variable
μ = the overall mean
α_i = the effect of treatment
β_j = the effect of time

$(\alpha\beta)_{ij}$ = the interaction effect of treatment and time
γ_k = the random effect of block
ε_{ijkl} = the random error

The growth performance (daily weight gain, daily feed consumption as % of body weight and FCR), plasma osmolality from the salinity tests and plasma cortisol level of the fish were statistically analyzed by ANOVA using the Mixed procedure of SAS 9.3 with the significant level of 0.05 (Littell et al., 1996). Repeated measures were used on the growth performance, plasma osmolality and plasma cortisol. Intestinal parameters (villus length, width and apparent surface area) were analyzed by one-way ANOVA using Minitab 17 statistical software.

For all 3 trials, the three assumptions (normality, constant variance and independence) were validated for each response variable. The main effect of treatment or time was ignored when the interaction effect of these two factors was significant. If ANOVA showed significant difference ($P < 0.05$), Tukey-Kramer test was used to conservatively evaluate the differences among the treatment means ($\alpha = 0.05$) (Gbur et al., 2012).

When repeated measures were applied, five covariance structures: compound symmetry (cs), Toeplitz (toep), heterogeneous compound symmetry (csh), heterogeneous toeplitz (toeph) and ante-dependence (ante (1)) were compared to determine the best-fitting model. The one with the smallest Corrected Akaike Information Criterion (AICC) and Bayesian Information Criterion (BIC) numbers (best-fitting) was selected for ANOVA.

Chapter 5: Results

5.1 Analyzed nutrient compositions of diets

All the diets used in the 3 trials shared similar levels of dry matter, crude protein, crude fat and ash contents. The dry matter contents ranged from 93.8 to 96.8% probably due to daily differences in humidity on the day of sampling (Table 5.1). The crude protein contents ranged from 48.6 to 51.0% across all 3 trials (Table 5.1), which were about 4% higher than calculated values shown in Table 4.2, 4.4 and 4.6 for trial 1, 2 and 3 respectively. The crude fat ranged from 20.7 to 24.6% across the 3 trials mainly due to different sources of some ingredients used but were in a much narrower range within each trial (Table 5.1). The analyzed crude fat levels were similar to calculated values (23.8%, Table 4.2, 4.4 and 4.6). The analyzed ash contents ranged from 5.8 to 7.2% (Table 5.1), which were higher than calculated values (4.1%, Table 4.2, 4.4 and 4.6).

Table 5.1 Nutrient analysis of the diets used in trials with Atlantic salmon smolts (% as fed basis±SE).

Diets	Dry matter (%)	Crude protein (%)	Crude fat (%)	Ash (%)
Trial 1				
Control diet	95.5±0.35	50.8±0.24	21.2±0.21	6.3±0.06
0.05% Maxi-Gen^{TM} Plus	94.0±0.35	50.5±0.24	21.7±0.21	6.1±0.06
0.10% Maxi-Gen^{TM} Plus	94.3±0.35	49.6±0.24	22.1±0.21	5.9±0.06
0.15% Maxi-Gen^{TM} Plus	95.9±0.35	50.9±0.24	22.2±0.21	6.0±0.06
0.20% Maxi-Gen^{TM} Plus	93.8±0.35	50.4±0.24	20.7±0.21	6.1±0.06
0.25% Maxi-Gen^{TM} Plus	95.2±0.35	50.5±0.24	21.5±0.21	5.8±0.06
Trial 2				
Control diet	96.8±0.28	49.9±0.19	23.7±0.32	6.9±0.09
0.15% Maxi-Gen^{TM} Plus	95.8±0.28	50.1±0.19	24.6±0.32	6.5±0.09
0.30% Maxi-Gen^{TM} Plus	96.7±0.28	51.0±0.19	23.5±0.32	6.8±0.09
0.45% Maxi-Gen^{TM} Plus	95.8±0.28	49.9±0.19	22.4±0.32	6.1±0.09
Trial 3				
Control diet	94.0±0.31	49.6±0.23	23.9±0.33	7.2±0.05
0.20% Maxi-Gen^{TM} Plus	93.9±0.31	48.6±0.23	23.3±0.33	6.9±0.05
0.40% Maxi-Gen^{TM} Plus	95.0±0.31	50.0±0.23	22.5±0.33	7.2±0.05
0.60% Maxi-Gen^{TM} Plus	95.0±0.31	49.1±0.23	23.3±0.33	7.0±0.05

5.2 Trial 1

5.2.1 Growth performance

No interaction effect between treatment and period was found throughout the trial for daily body weight gain, daily feed consumption and FCR ($P \geq 0.05$). No difference among treatments were found ($P \geq 0.05$) for daily body weight gain, daily feed consumption and FCR. The effect of period was significant on the daily body weight gain, daily feed consumption and FCR (Table 5.2).

Table 5.2 P-values for treatment, period and treatment × period on daily body weight gain, daily feed consumption and FCR of Atlantic salmon smolts in trial 1 fed 0, 0.05, 0.10, 0.15, 0.20 or 0.25% Maxi-Gen^{TM} Plus at 10^{o}C for period 0-28, 29-56 and 57-78 days on test (May 11th to July 28th, 2014).

Parameters	Treatment	Period	Treatment x Period
Daily weight gain	0.87	<.0001	0.97
Daily feed consumption	0.99	<.0001	0.99
FCR	0.25	0.0019	0.34

Significantly different when $P < 0.05$.

The initial mean body weight ranged from 59.3 to 61.1g on day 0. Body weight gain averaged 0.04g per day during the first 4 weeks, which was less than the weight they gained per day during the next 4 weeks (0.10g from day 29 to day 56). The fish gained the most weight (0.18g per day) from day 57 to day 78 (Table 5.3). Mean body weight at the end of each period was shown in Appendix A.

No treatment differences occurred for daily feed consumption. The fish consumed 0.11% of body weight per day during the first 4 weeks, 0.18% during the next 4 weeks and 0.25% from day 57 to day 78. Daily feed consumption gradually increased as the experiment progressed (Table 5.4).

Table 5.3 Initial weight (g, mean±SE), daily body weight gain (g/fish, mean±SE) and final weight (g, mean±SE) of Atlantic salmon smolts fed 0, 0.05, 0.10, 0.15, 0.20 and 0.25% Maxi-Gen^{TM} Plus at 10^{o}C for period 0-28, 29-56 and 57-78 days on test (May 11th to July 28th, 2014).

Period (days)	Level of Maxi-Gen^{TM} Plus (%)						Average
	0	0.05	0.1	0.15	0.2	0.25	
Initial weight	59.7±0.64	60.3±0.64	61.1±0.64	59.3±0.64	59.7±0.64	59.8±0.64	60.0±0.64
Final weight	67.1±0.55	68.7±0.55	67.7±0.55	67.2±0.55	67.8±0.55	66.8±0.55	67.9±0.55
0-28	0.04±0.03	0.03±0.03	0.03±0.03	0.03±0.03	0.06±0.03	0.03±0.03	0.04±0.01^{c}
29-56	0.10±0.03	0.12±0.03	0.08±0.03	0.12±0.03	0.08±0.03	0.08±0.03	0.10±0.01^{b}
57-78	0.16±0.03	0.19±0.03	0.16±0.03	0.17±0.03	0.19±0.03	0.18±0.03	0.18±0.01^{a}
Average	0.10±0.02	0.12±0.02	0.09±0.02	0.11±0.02	0.11±0.02	0.09±0.02	

a-b: Means within a column with different superscript letters are significantly different ($P < 0.05$).

Table 5.4 Daily feed consumption (as % of body weight, mean±SE) of Atlantic salmon smolts fed 0, 0.05, 0.10, 0.15, 0.20 and 0.25% Maxi-Gen^{TM} Plus at 10^{o}C for period 0-28, 29-56 and 57-78 days on test (May 11th to July 28th, 2014).

Period (days)	Level of Maxi-Gen^{TM} Plus (%)						Average
	0	0.05	0.1	0.15	0.2	0.25	
0-28	0.10±0.04	0.11±0.04	0.09±0.04	0.12±0.04	0.11±0.04	0.11±0.04	0.11±0.02^{c}
29-56	0.18±0.04	0.18±0.04	0.17±0.04	0.17±0.04	0.17±0.04	0.19±0.04	0.18±0.02^{b}
57-78	0.27±0.04	0.23±0.04	0.22±0.04	0.25±0.04	0.26±0.04	0.23±0.04	0.24±0.02^{a}
Average	0.18±0.03	0.18±0.03	0.16±0.03	0.18±0.03	0.18±0.03	0.18±0.03	

a-c: Means within a column with different superscript letters are significantly different ($P < 0.05$).

For FCR, 2.16g of feed was consumed to produce 1g of weight gain during the first period. FCR decreased to 1.32 and 1.44 respectively during the next two periods (Table 5.5), indicating a better FCR as the experiment progressed.

The mortality rate in trial 1 was 3.8% and the mortalities occurred in all the tanks (no system effect). The mortalities were evenly distributed among the treatment groups (no treatment effect). The mortalities were evenly spread over the trial (no period effect).

5.2.2 Intestinal histology

The body weight of fish sampled for intestinal histology ranged from 70.0 to 97.4g on day 78. No differences ($P \geq 0.05$) were found for the villi length, width and surface area in either mid or distal intestine of the fish fed different inclusion levels of Maxi-Gen^{TM} Plus. The average villi length, width and area of the mid intestine were 0.62 mm, 0.09 mm and 0.06 mm^2, respectively. The average villi length, width and area of the distal intestine were 0.66 mm, 0.10 mm and 0.06 mm^2, respectively (Table 5.6).

Table 5.5 Feed conversion ratio (mean±SE) of Atlantic salmon smolts fed 0, 0.05, 0.10, 0.15, 0.20 and 0.25% Maxi-Gen™ Plus at 10°C for period 0-28, 29-56 and 57-78 days on test (May 11th to July 28th, 2014).

Period (days)	Level of Maxi-Gen™ Plus (%)						Average
	0	0.05	0.1	0.15	0.2	0.25	
0-28	1.70±0.36	2.11±0.42	2.46±0.36	2.57±0.36	0.73±0.27	2.77±0.36	2.16±0.15[a]
29-56	1.67±0.36	1.00±0.36	1.12±0.42	1.28±0.42	0.90±0.52	1.98±0.42	1.32±0.17[b]
57-78	0.94±0.42	2.33±0.52	1.33±0.52	1.31±0.52	1.33±0.52	1.38±0.42	1.44±0.20[b]
Average	1.44±0.22	1.81±0.26	1.64±0.25	1.72±0.25	1.20±0.26	2.04±0.23	

a-b: Means within a column with different superscript letters are significantly different ($P < 0.05$).

Table 5.6 Body weight (g, mean±SE) and villi length (mm, mean±SE), width (mm, mean±SE) and surface area (mm^2, mean±SE) on the mid and distal intestine of Atlantic salmon smolts fed 0, 0.05, 0.10, 0.15, 0.20 and 0.25% Maxi-Gen™ Plus on day 78 (July 28th, 2014).

	Parameters	Level of Maxi-Gen™ Plus (%)						Average	P-values
		0	0.05	0.1	0.15	0.2	0.25		
Mid Intestine	Length	0.60±0.03	0.57±0.03	0.61±0.03	0.53±0.03	0.65±0.03	0.74±0.03	0.62±0.02	0.29
	Width	0.09±0.01	0.10±0.01	0.09±0.01	0.10±0.01	0.09±0.01	0.10±0.01	0.09±0.01	0.99
	Area	0.06±0.01	0.06±0.01	0.06±0.01	0.05±0.01	0.06±0.01	0.08±0.01	0.06±0.01	0.74
Distal Intestine	Length	0.58±0.03	0.68±0.03	0.75±0.03	0.62±0.03	0.75±0.03	0.61±0.03	0.66±0.01	0.13
	Width	0.10±0.01	0.10±0.01	0.09±0.01	0.10±0.01	0.10±0.01	0.08±0.01	0.10±0.01	0.44
	Area	0.06±0.01	0.07±0.01	0.07±0.01	0.06±0.01	0.07±0.01	0.05±0.01	0.06±0.01	0.22
Body Weight (g)		71.0±6.3	75.6±6.3	97.4±6.3	72.9±6.3	70.0±6.3	88.7±6.3	79.3±2.6	

5.2.3 Plasma osmolality following 24h, 40ppt salinity challenge

Mean plasma osmolality following the 24h, 40ppt salinity tests was significantly affected by the interaction effect between treatment and period ($P < 0.05$). No significant differences were found in plasma osmolality among the treatments on day 16. However on day 30, all the nucleotide-containing diets resulted in a significantly lower plasma osmolality (ranged from 343 to 358 mOsm/kg) than the control diet (373 mOsm/kg) while 0.20% inclusion level resulted in a significantly lower plasma osmolality (343 mOsm/kg) than that of 0.05, 0.1 and 0.15% inclusion levels (355, 358 and 357 mOsm/kg, respectively). On day 44, all the nucleotide-containing diets again had a lower plasma osmolality (range from 355 to 372 mOsm/kg) than the control diet (386 mOsm/kg) ($P < 0.05$). Inclusion level of 0.25% was significantly lower in plasma osmolality (355 mOsm/kg) than 0.1 and 0.15% inclusion levels (372 and 366 mOsm/kg, respectively). On day 58, 0.25% inclusion level (332 mOsm/kg) resulted in a significantly lower plasma osmolality than that of the rest of diets (range from 344 to 354 mOsm/kg) (Table 5.7).

With respect to period, plasma osmolality for all 6 treatment groups increased from day 16 to 44 and followed by a decrease on day 58 (Table 5.7).

Table 5.7 Plasma osmolality (mOsm/kg, mean±SE) of Atlantic salmon smolts fed 0, 0.05, 0.10, 0.15, 0.20 and 0.25% Maxi-Gen™ Plus measured following 24h, 40ppt salinity tests on day 16, 30, 44 and 58 starting on May 27th, 2014.

Day	Level of Maxi-Gen™ Plus (%)						Average
	0	0.05	0.1	0.15	0.2	0.25	
16	338±3.7^{i-l}	340±3.7^{h-l}	334±3.7^{kl}	338±3.7^{jkl}	338±3.7^{i-l}	343±3.7^{h-l}	338±1.5
30	373±3.7^{b}	355±3.7^{c-f}	358±3.7^{cde}	357±3.7^{cde}	343±3.7^{h-k}	349±3.7^{e-i}	356±1.5
44	386±3.7^{a}	364±3.7^{bcd}	372±3.7^{b}	366±3.7^{bc}	356±3.7^{c-f}	355±3.7^{d-g}	366±1.5
58	354±3.7^{d-g}	350±3.7^{e-h}	344±3.7^{g-k}	348±3.7^{e-j}	346±3.7^{f-j}	332±3.7^{l}	346±1.5
Average	363±1.8	352±1.8	352±1.8	352±1.8	346±1.8	345±1.8	
	Treatment		Period		Treatment x Period		
P-values	0.001		<.0001		0.017		

a-l: Treatment by period means with different superscript letters are significantly different (P < 0.05).

5.2.4 Expression of immune-related genes response to polyIC injection

The optimal annealing temperatures and primer set efficiency of both immune-related genes and housekeeping genes used in the current study were determined (Table 5.8). Single peak was shown on the melt curve for most of the genes except for LSM8 and DYNLL1 (62^{o}C) when the annealing temperature was set as 60^{o}C. The primer efficiency of the genes ranged from 82.3 to 100% (Table 5.8).

Table 5.8 Optimal annealing temperatures (^{o}C) and primer set efficiency (%) of immune-related genes (Mx, IFN-α, ISG15, ABCB2, MHC class I and MHC class II) and housekeeping genes (β-actin, EF1-α, LSM8, MRTO4, DYNLL1) of Atlantic salmon used in the current study.

Genes	Annealing temperature (^{o}C)	Primer efficiency (%)	Melt curve
Mx	60	92.9	Single peak
IFN-α	60	94.8	Single peak
ISG15	60	93.8	Single peak
ABCB2	60	93.6	Single peak
MHC class I	60	92.2	Single peak
MHC class II	60	82.3	Single peak
β-actin	60	88.5	Single peak
EF1-α	60	98.5	Single peak
MRTO4	60	100	Single peak
LSM8	62	87.2	Single peak
DYNLL1	62	93.9	Single peak

Interaction effect between treatment and injection was significant ($P < 0.05$) for Mx, IFN-α, ISG15. The effect of treatment was significant for ABCB2 and MHC class I ($P < 0.05$). The effect of injection was significant for ABCB2 ($P < 0.05$). No effect ($P \geq 0.05$) of either treatment or injection occurred for MHC class II (Table 5.9).

Table 5.9 P-values for treatment, injection and treatment × injection on relative expression of immune-related genes of Atlantic salmon smolts fed 0, 0.20 and 0.25% Maxi-Gen^{TM} Plus during viral mimic test on July 28th, 2014.

Genes	Treatment	Injection	Treatment x Injection
Mx	0.0389	<.0001	0.0158
IFN-α	0.5080	0.0253	0.0034
ISG15	0.0272	<.0001	0.0054
ABCB2	0.0386	<.0001	0.7712
MHC class I	0.0089	0.1724	0.4288
MHC class II	0.4748	0.8133	0.1974

Significantly different when $P < 0.05$.

5.2.4.1 Mx

Mx protein encoded by Mx gene fought against viral infection by inhibiting the replication of viruses (Caipang et al., 2003; Larsen et al., 2004). No differences ($P \geq 0.05$) occurred in the relative expression of Mx between undisturbed and saline group while polyIC injection resulted in higher relative expression (5.19 fold increase) than the other two groups (0.87 and 0.68 fold, respectively). Within polyIC group, fish fed 0.20 and 0.25% Maxi-Gen^{TM} Plus had higher relative expression (6.10 and 5.77 fold, respectively) than that of the fish fed the control diet (3.69 fold). The diets did not result in differences of relative expression in either undisturbed or saline group (Table 5.10).

Table 5.10 Relative expression (mean±SE) of Mx of Atlantic salmon smolts fed 0, 0.20 and 0.25% Maxi-Gen^{TM} Plus and subjected to different injection (undisturbed, saline and polyIC) during viral mimic test on July 28th, 2014 (the values were relative to that of non-injected fish fed the control diet).

Injection	Level of Maxi-Gen^{TM} Plus (%)			Average
	0	0.20	0.25	
Undisturbed	1.00±0.47^{c}	0.74±0.38^{c}	0.87±0.42^{c}	0.87±0.24
Saline	0.50±0.38^{c}	0.60±0.38^{c}	0.94±0.38^{c}	0.68±0.22
PolyIC	3.69±0.42^{b}	6.10±0.38^{a}	5.77±0.42^{a}	5.19±0.23
Average	1.73±0.24	2.48±0.22	2.53±0.23	

a-c: Treatment by injection means with different superscript letters are significantly different ($P < 0.05$).

5.2.4.2 IFN-α

IFN-α encoded by IFN-α gene participated in antiviral response of fish through interfering with viral replication within infected cells and stimulating the expression of other antiviral molecules (Robertsen, 2006). No difference ($P \geq 0.05$) occurred for the relative expression of IFN-α between undisturbed and saline group. In 0.25% Maxi-Gen^{TM} Plus feeding group, fish injected with polyIC had higher relative expression (1.73 fold) than that of non-injected fish and the fish injected with saline (0.13 and 0.30 fold, respectively). Within polyIC group, fish fed 0.25% Maxi-Gen^{TM} Plus showed higher relative expression (1.73 fold) than that of the fish fed the control diet (0.37 fold) (Table 5.11).

Table 5.11 Relative expression (mean±SE) of IFN-α of Atlantic salmon smolts fed 0, 0.20 and 0.25% Maxi-Gen^{TM} Plus and subjected to different injection (undisturbed, saline and polyIC) during viral mimic test on July 28th, 2014 (the values were relative to that of non-injected fish fed the control diet).

Injection	Level of Maxi-Gen^{TM} Plus (%)			Average
	0	0.20	0.25	
Undisturbed	1.00±0.31^{ab}	1.11±0.25^{ab}	0.13±0.28^{b}	0.75±0.16
Saline	0.44±0.25^{b}	0.51±0.25^{ab}	0.30±0.25^{b}	0.42±0.15
PolyIC	0.37±0.28^{b}	0.95±0.25^{ab}	1.73±0.28^{a}	1.02±0.15
Average	0.60±0.16	0.86±0.15	0.72±0.15	

a-b: Treatment by injection means with different superscript letters are significantly different ($P < 0.05$).

5.2.4.3 ISG15

ISG15 protein encoded by ISG15 gene contributed to the anti-viral response of fish by conjugating to target antigens (Rokenes et al., 2007). No difference ($P \geq 0.05$) occurred for the relative expression of ISG15 between undisturbed and saline group while polyIC group resulted in higher relative expression (13.51 fold) than the other two groups (0.68 and 0.40 fold, respectively). Within polyIC group, fish fed 0.20% Maxi-Gen^{TM} Plus had

higher relative expression (18.62 fold) than that of fish fed the control diet and 0.25% Maxi-Gen^{TM} Plus (9.98 and 11.94 fold respectively) (Table 5.12).

Table 5.12 Relative expression (mean±SE) of ISG15 of Atlantic salmon smolts fed 0, 0.20 and 0.25% Maxi-Gen^{TM} Plus and subjected to different injection (undisturbed, saline and polyIC) during viral mimic test on July 28th, 2014 (the values were relative to that of non-injected fish fed the control diet).

Injection	Level of Maxi-Gen^{TM} Plus (%)			Average
	0	0.20	0.25	
Undisturbed	1.00±1.59^{c}	0.61±1.37^{c}	0.44±1.23^{c}	0.68±0.34
Saline	0.26±1.12^{c}	0.42±1.23^{c}	0.52±1.12^{c}	0.40±0.67
PolyIC	9.98±1.23^{b}	18.62±1.23^{a}	11.94±1.23^{b}	13.51±0.71
Average	3.75±0.77	6.55±0.74	4.30±0.69	

a-c: Treatment by injection means with different superscript letters are significantly different ($P < 0.05$).

5.2.4.4 ABCB2

ABCB2 protein encoded by ABCB2 gene participated in MHC class I pathway against viral infection by transporting antigenic peptides into the endoplasmic reticulum (Jorgensen et al., 2006). No difference ($P \geq 0.05$) appeared for the relative expression of ABCB2 between undisturbed and saline group while polyIC group had higher relative expression (2.4 fold) than the other two groups (1.28 and 1.09 fold, respectively). No difference was found on the relative expression of fish fed 0.20% Maxi-Gen^{TM} Plus and 0.25% Maxi-Gen^{TM} Plus. Fish fed 0.25% Maxi-Gen^{TM} Plus had higher relative expression (1.82 fold) than that of the fish fed the control diet (1.35 fold) (Table 5.13).

Table 5.13 Relative expression (mean±SE) of ABCB2 of Atlantic salmon smolts fed 0, 0.20 and 0.25% Maxi-Gen^{TM} Plus and subjected to different injection (undisturbed, saline and polyIC) during viral mimic test on July 28th, 2014 (the values were relative to that of non-injected fish fed the control diet).

Injection	Level of Maxi-Gen^{TM} Plus (%)			Average
	0	0.20	0.25	
Undisturbed	1.00±0.24	1.36±0.19	1.49±0.24	1.28±0.13^{b}
Saline	1.00±0.19	0.96±0.19	1.32±0.21	1.09±0.12^{b}
PolyIC	2.05±0.21	2.51±0.19	2.66±0.21	2.40±0.12^{a}
Average	1.35±0.12^{b}	1.61±0.11^{ab}	1.82±0.13^{a}	

a-b: Means within a column or a row with different superscript letters are significantly different ($P < 0.05$).

5.2.4.5 MHC class I

MHC class I molecule encoded by MHC class I gene participated in MHC class I pathway against viral infection by presenting the antigenic peptides $CD8^+$ T lymphocytes (Jorgensen et al., 2007). No difference ($P \geq 0.05$) occurred for the relative expression of MHC class I among the three injection groups. Difference was found among the treatment groups as the fish fed 0.25% Maxi-Gen^{TM} Plus had higher relative expression (1.31 fold) than that of the fish fed 0.20% Maxi-Gen^{TM} Plus (1.14 fold) (Table 5.14).

Table 5.14 Relative expression (mean±SE) of MHC class I of Atlantic salmon smolts fed 0, 0.20 and 0.25% Maxi-Gen^{TM} Plus and subjected to different injection (undisturbed, saline and polyIC) during viral mimic test on July 28th, 2014 (the values were relative to that of non-injected fish fed the control diet).

Injection	Level of Maxi-Gen^{TM} Plus (%)			Average
	0	0.20	0.25	
Undisturbed	1.00±0.20	0.76±0.16	1.54±0.18	1.10±0.10
Saline	0.84±0.16	0.74±0.16	1.15±0.16	0.91±0.09
PolyIC	1.09±0.18	1.14±0.16	1.25±0.18	1.16±0.10
Average	0.97±0.10^{ab}	0.88±0.09^{b}	1.31±0.10^{a}	

a-b: Means within a row with different superscript letters are significantly different ($P < 0.05$).

5.2.4.6 MHC class II

MHC class II molecules encoded by MHC class II gene participated in the MHC class II pathway through presenting antigenic peptides to $CD4^+$ T lymphocytes (Jorgensen et al.,

2007). No difference appeared for the relative expression of MHC class II among the different injection groups. No difference ($P \geq 0.05$) was found on the relative expression among the different treatment groups (Table 5.15).

Table 5.15 Relative expression (mean±SE) of MHC class II of Atlantic salmon smolts fed 0, 0.20 and 0.25% Maxi-Gen^{TM} Plus and subjected to different injection (undisturbed, saline and polyIC) during viral mimic test on July 28th, 2014 (the values were relative to that of non-injected fish fed the control diet).

Injection	Level of Maxi-Gen^{TM} Plus (%)			Average
	0	0.20	0.25	
Undisturbed	1.00±0.21	0.72±0.18	1.21±0.19	0.98±0.11
Saline	1.26±0.18	1.11±0.18	0.81±0.18	1.06±0.10
PolyIC	1.09±0.19	1.04±0.18	0.81±0.19	0.98±0.11
Average	1.12±0.11	0.96±0.10	0.95±0.11	

5.3 Trial 2

5.3.1 Growth performance

No interaction effects between treatment and period were found ($P \geq 0.05$) throughout the trial for daily body weight gain, daily feed consumption and FCR. Treatments were different ($P < 0.05$) for daily body weight gain and daily feed consumption. The effect of period was significant on the daily body weight gain, daily feed consumption and FCR (Table 5.16).

Table 5.16 P-values for treatment, period and treatment × period on daily body weight gain, daily feed consumption and FCR of off-season Atlantic salmon smolts fed 0, 0.15, 0.30 and 0.45% Maxi-Gen^{TM} Plus at 10^{o}C for period 0-28, 29-56, 57-84 and 85-112 days on test (October 25th, 2014 to February 14th, 2015).

Parameters	Treatment	Period	Treatment x Period
Daily weight gain	0.008	<.0001	0.53
Daily feed consumption	0.003	<.0001	0.09
FCR	0.96	0.001	0.92

Significantly different when $P < 0.05$.

Body weight gain was similar for the control diet and diets containing 0.15 and 0.30% Maxi-Gen^{TM} Plus. Diet containing 0.45% Maxi-Gen^{TM} Plus had a higher weight gain

(0.61g per day) than that of the control diet (0.54g per day). The fish gained on average 0.47 and 0.48g per day during 0-28 and 57-84 days respectively, which were less than the weight gained (0.55g per day) during day 29 to 56. Fish gained the most weight (0.79g per day) during day 85 to 112 (Table 5.17). Mean body weight at the end of each period was shown in Appendix B.

Table 5.17 Initial weight (g, mean±SE), daily body weight gain (g/fish, mean±SE) and final weight (g, mean±SE) of off-season Atlantic salmon smolts fed 0, 0.15, 0.30 and 0.45% Maxi-Gen^{TM} Plus at 10^{o}C for period 0-28, 29-56, 57-84 and 85-112 days on test (October 25th, 2014 to February 14th, 2015).

Period (days)	Level of Maxi-Gen^{TM} Plus (%)				Average
	0	0.15	0.30	0.45	
Initial weight	47.0±0.49	46.7±0.49	46.1±0.49	45.2±0.49	46.2±0.49
Final weight	107.8±0.79	110.5±0.79	110.2±0.79	113.2±0.79	110.3±0.79
0-28	0.47±0.02	0.47±0.02	0.46±0.02	0.48±0.02	0.47±0.01^{c}
29-56	0.51±0.02	0.55±0.02	0.56±0.02	0.60±0.02	0.55±0.01^{b}
57-84	0.45±0.02	0.50±0.02	0.48±0.02	0.49±0.02	0.48±0.01^{c}
85-112	0.74±0.02	0.76±0.02	0.79±0.02	0.86±0.02	0.79±0.01^{a}
Average	0.54±0.01^{b}	0.57±0.01^{ab}	0.57±0.01^{ab}	0.61±0.01^{a}	

a-c: Means within a column or a row with different superscript letters are significantly different ($P < 0.05$).

Daily feed consumption was not different ($P \geq 0.05$) between the control diet and the diets containing 0.15 and 0.30% Maxi-Gen^{TM} Plus. However, the diet with 0.45% Maxi-Gen^{TM} Plus inclusion had a higher feed consumption (0.62% body weight per day) than the control diet (0.56% body weight per day). The lowest feed consumption was found during 57-84 days (0.47% body weight per day) while the highest feed consumption (0.68% body weight per day) occurred during the first period (Table 5.18).

Table 5.18 Daily feed consumption (as % of body weight, mean±SE) of off-season Atlantic salmon smolts fed 0, 0.15, 0.30 and 0.45% Maxi-Gen^{TM} Plus at 10^{o}C for period 0-28, 29-56, 57-84 and 85-112 days on test (October 25th, 2014 to February 14th, 2015).

Period	Level of Maxi-Gen^{TM} Plus (%)				Average
(days)	0	0.15	0.30	0.45	
0-28	0.67±0.01	0.69±0.01	0.65±0.01	0.71±0.01	0.68±0.01^{a}
29-56	0.59±0.01	0.65±0.01	0.64±0.01	0.69±0.01	0.64±0.01^{b}
57-84	0.43±0.01	0.48±0.01	0.49±0.01	0.50±0.01	0.47±0.01^{d}
85-112	0.55±0.01	0.54±0.01	0.58±0.01	0.59±0.01	0.57±0.01^{c}
Average	0.56±0.01^{b}	0.59±0.01^{ab}	0.59±0.01^{ab}	0.62±0.01^{a}	

a-d: Means within a column or a row with different superscript letters are significantly different ($P < 0.05$).

For feed conversion ratio, no differences occurred among the diets throughout the trial. The FCR during 85-112 days (0.72) was lower than those during 29-56 and 57-84 days (0.78 and 0.81, respectively), indicating a better FCR as the experiment progressed (Table 5.19) similar to trial 1.

Table 5.19 Feed conversion ratio (mean±SE) of off-season Atlantic salmon smolts fed 0, 0.15, 0.30 and 0.45% Maxi-Gen^{TM} Plus at 10^{o}C for period 0-28, 29-56, 57-84 and 85-112 days on test (October 25th, 2014 to February 14th, 2015).

Period	Level of Maxi-Gen^{TM} Plus (%)				Average
(days)	0	0.15	0.30	0.45	
0-28	0.77±0.03	0.78±0.03	0.75±0.03	0.77±0.03	0.77±0.01^{ab}
29-56	0.79±0.03	0.80±0.03	0.76±0.03	0.77±0.03	0.78±0.01^{a}
57-84	0.79±0.03	0.80±0.03	0.83±0.03	0.84±0.03	0.81±0.01^{a}
85-112	0.73±0.03	0.72±0.03	0.72±0.03	0.70±0.03	0.72±0.01^{b}
Average	0.77±0.01	0.77±0.01	0.77±0.01	0.77±0.01	

a-b: Means within a column with different superscript letters are significantly different ($P < 0.05$).

The mortality rate in trial 2 was 1.6% and was evenly distributed among the two systems (no system effect). No differences occurred for mortality rate among the treatment groups (no treatment effect). The mortalities were evenly spread over the trial (no period effect).

5.3.2 Intestinal histology

No differences ($P \geq 0.05$) were found on the villi length, width and area in either mid or distal intestine of the fish fed different inclusion levels of Maxi-Gen^{TM} Plus ($P \geq 0.05$). The average villi length, width and area of the mid intestine were 0.56 mm, 0.12 mm and 0.06 mm^2, respectively. The average villi length, width and area of the distal intestine were 0.90 mm, 0.14 mm and 0.12 mm^2, respectively (Table 5.20).

Table 5.20 Body weight (g, mean±SE) and villi length (mm, mean±SE), width (mm, mean±SE) and surface area (mm^2, mean±SE) on the mid and distal intestine of off-season Atlantic salmon smolts fed 0, 0.15, 0.30 and 0.45% Maxi-Gen™ Plus on day 112 (February 14th, 2015).

	Parameters	Level of Maxi-Gen™ Plus (%)				Average	P-values
		0	0.15	0.30	0.45		
Mid Intestine	Length	0.54±0.01	0.56±0.01	0.54±0.01	0.58±0.01	0.56±0.01	0.60
	Width	0.11±0.01	0.12±0.01	0.12±0.01	0.11±0.01	0.12±0.01	0.89
	Area	0.06±0.01	0.06±0.01	0.06±0.01	0.06±0.01	0.06±0.01	0.98
Distal Intestine	Length	0.90±0.04	0.69±0.04	0.99±0.04	1.02±0.04	0.90±0.05	0.06
	Width	0.14±0.01	0.13±0.01	0.13±0.01	0.15±0.01	0.14±0.01	0.30
	Area	0.12±0.01	0.11±0.01	0.12±0.01	0.13±0.01	0.12±0.01	0.38
Body weight (g)		116.4±6.3	121.7±6.3	122.5±6.3	112.1±6.3	118.2±3.0	

5.3.3 Plasma osmolality following 24h, 40ppt salinity challenge

No interaction effects between treatment and period ($P \geq 0.05$) occurred for the plasma osmolality. The effects of both treatment and period were significant ($P < 0.05$). No differences were found among the three nucleotide-containing diets throughout the trial but were all significantly lower (346, 345 and 347 mOsm/kg, respectively) than the control diet (355 mOsm/kg) (Table 5.21).

No differences appeared for the average plasma osmolality of fish on day 50 and 64. The plasma osmolality then kept increasing from 330 to 374 mOsm/kg from day 64 to 106 (Table 5.21).

Table 5.21 Plasma osmolality (mOsm/kg, mean±SE) of off-season Atlantic salmon smolts fed 0, 0.15, 0.30 and 0.45% Maxi-Gen^{TM} Plus measured following 24h, 40ppt salinity tests on day 50, 64, 78, 92 and 106 starting on December 12th, 2014.

Day	Level of Maxi-gen Plus (%)				Average
	0	0.15	0.30	0.45	
50	336±3.4	326±3.4	332±3.4	333±3.4	331±2.2^{d}
64	334±2.3	328±2.3	328±2.3	330±2.3	330±1.8^{d}
78	354±4.6	345±4.6	340±4.6	350±4.6	347±2.7^{c}
92	373±5.7	357±5.7	356±5.7	351±5.7	359±3.2^{b}
106	378±4.9	375±4.9	370±4.9	371±4.9	374±2.8^{a}
Average	355±2.4^{a}	346±2.4^{b}	345±2.4^{b}	347±2.4^{b}	
	Treatment x Period		Treatment		Period
P-values	0.70		0.01		<.0001

a-d: Means within a column or a row with different superscript letters are significantly different ($P < 0.05$).

5.4 Trial 3

5.4.1 Growth performance

No interaction effects between treatment and period ($P \geq 0.05$) occurred throughout the trial for daily body weight gain, daily feed consumption and FCR. Treatment differences appeared ($P < 0.05$) for daily body weight gain, daily feed consumption and FCR. The effects of period were significant ($P < 0.05$) for daily body weight gain, daily feed consumption and FCR (Table 5.22).

Table 5.22 P-values for treatment, period and treatment × period on daily body weight gain, daily feed consumption and FCR of Atlantic salmon smolts fed 0, 0.20, 0.40 and 0.60% Maxi-Gen^{TM} Plus at 10^{o}C for period 0-28, 29-56 and 57-122 days on test (April 24th to August 24th, 2015).

Parameters	Treatment	Period	Treatment x Period
Daily weight gain	0.01	<.0001	0.72
Daily feed consumption	0.0003	<.0001	0.78
FCR	0.01	<.0001	0.08

Significantly different when $P < 0.05$.

For body weight gain, no differences occurred ($P \geq 0.05$) between the control diet and the diet containing 0.20% Maxi-Gen^{TM} Plus. Diets containing 0.40 and 0.60% Maxi-Gen^{TM} Plus resulted in higher weight gain (0.30g per day) than the control diet (0.24g per day). The fish gained 0.07g per day on average during the first period, which was less than the weight gained (0.11g per day) during the second period. The fish gained the most weight (0.66g per day) during 57-122 days (Table 5.23). Mean body weight at the end of each period was shown in Appendix C.

Table 5.23 Initial body weight (g, mean±SE), daily body weight gain (g/fish, mean±SE) and final weight (g, mean±SE) of Atlantic salmon smolts fed 0, 0.20, 0.40 and 0.60% Maxi-Gen^{TM} Plus at 10^{o}C for period 0-28, 29-56 and 57-122 days on test (April 24th to August 24th, 2015).

Period (days)	Level of Maxi-Gen^{TM} Plus (%)				Average
	0	0.20	0.40	0.60	
Initial weight	47.8±0.56	46.0±0.56	47.7±0.56	47.1±0.56	47.1±0.56
Final weight	91.7±1.37	92.3±1.37	99.1±1.37	98.9±1.37	95.7±1.37
0-28	0.05±0.02	0.06±0.02	0.08±0.02	0.08±0.02	0.07±0.01^{c}
29-56	0.08±0.02	0.11±0.02	0.13±0.02	0.12±0.02	0.11±0.01^{b}
57-122	0.61±0.02	0.63±0.02	0.69±0.02	0.70±0.02	0.66±0.01^{a}
Average	0.24±0.01^{b}	0.27±0.01^{ab}	0.30±0.01^{a}	0.30±0.01^{a}	

a-c: Means within a column or a row with different superscript letters are significantly different ($P < 0.05$).

Daily feed consumption were similar between the control diet and the diet containing 0.20% Maxi-Gen^{TM} Plus. However diets containing 0.40 and 0.60% Maxi-Gen^{TM} Plus resulted in higher feed consumption (0.41% body weight per day for both groups) than the control diet (0.35% body weight per day). No difference appeared for feed consumption by fish during the first two periods while a higher feed consumption (0.70% body weight per day) occurred during the last period (Table 5.24).

Table 5.24 Daily feed consumption (as % of body weight, mean±SE) of Atlantic salmon smolts fed 0, 0.20, 0.40 and 0.60% Maxi-Gen^{TM} Plus at 10^{o}C for period 0-28, 29-56 and 57-122 days on test (April 24th to August 24th, 2015).

Period	Level of Maxi-Gen^{TM} Plus (%)				Average
(days)	0	0.20	0.40	0.60	
0-28	0.20±0.02	0.20±0.02	0.23±0.02	0.24±0.02	0.22±0.01^{b}
29-56	0.21±0.02	0.25±0.02	0.26±0.02	0.27±0.02	0.25±0.01^{b}
57-122	0.64±0.02	0.69±0.02	0.73±0.02	0.72±0.02	0.70±0.01^{a}
Average	0.35±0.01^{b}	0.38±0.01^{ab}	0.41±0.01^{a}	0.41±0.01^{a}	

a-d: Means within a column or a row with different superscript letters are significantly different ($P < 0.05$).

For the feed conversion ratio, no significant difference appeared between the control diet and the diet containing 0.20% Maxi-Gen^{TM} Plus. Diets containing 0.40 and 0.60% Maxi-

Gen^{TM} Plus had higher FCR (1.13 and 1.14, respectively) than the control diet (1.33). The FCR during the first period (1.62) was higher than that during the second period (1.18). The lowest FCR (0.79) was found on the last period, indicating a better FCR as the experiment progressed (Table 5.25).

Table 5.25 Feed conversion ratio (mean±SE) of Atlantic salmon smolts fed 0, 0.20, 0.40 and 0.60% Maxi-Gen^{TM} Plus at 10^{o}C for period 0-28, 29-56 and 57-122 days on test (April 24th to August 24th, 2015).

Period	Level of Maxi-Gen^{TM} Plus (%)				Average
(days)	0	0.20	0.40	0.60	
0-28	1.88±0.08	1.54±0.08	1.54±0.08	1.52±0.08	1.62±0.04^{a}
29-56	1.36±0.08	1.22±0.08	1.03±0.08	1.10±0.08	1.18±0.04^{b}
57-122	0.77±0.08	0.79±0.08	0.82±0.08	0.80±0.08	0.79±0.04^{c}
Average	1.33±0.05^{a}	1.18±0.05^{ab}	1.13±0.05^{b}	1.14±0.05^{b}	

a-b: Means within a column or a row with different superscript letters are significantly different ($P < 0.05$).

5.4.2 Plasma osmolality following 24h, 40ppt salinity challenge

No interaction effect between treatment and period existed ($P \geq 0.05$) for plasma osmolality of fish. The effects of both treatment and period were significant ($P < 0.05$). All three nucleotide-containing diets resulted in significantly lower plasma osmolality at 359, 355 and 351 mOsm/kg respectively, compared to the control diet at 369 mOsm/kg. No differences appeared between the diets with 0.20 and 0.40% Maxi-Gen^{TM} Plus inclusions. Diet with 0.60% Maxi-Gen^{TM} Plus resulted in lower plasma osmolality (351 mOsm/kg) than that of the diet with 0.20% Maxi-Gen^{TM} Plus (359 mOsm/kg) (Table 5.26).

The plasma osmolality of the fish significantly increased from 332 to 370 mOsm/kg between day 29 and 57. The plasma osmolality then stabilized at around 370 mOsm/kg on day 57 and 71 (Table 5.26).

Table 5.26 Plasma osmolality (mOsm/kg, mean±SE) of Atlantic salmon smolts fed 0, 0.20, 0.40 and 0.60% Maxi-Gen^{TM} Plus measured following 24h, 40ppt salinity tests on day 29, 47, 57 and 71 starting on May 22nd, 2015.

Day	Level of Maxi-Gen^{TM} Plus (%)				Average
	0	0.20	0.40	0.60	
29	339±4.2	336±4.2	327±4.2	326±4.2	332±2.2^{c}
47	371±4.2	352±4.2	360±4.2	357±4.2	360±2.2^{b}
57	382±4.2	372±4.2	364±4.2	361±4.2	370±2.2^{a}
71	386±4.2	376±4.2	370±4.2	359±4.2	373±2.2^{a}
Average	369±2.2^{a}	359±2.2^{b}	355±2.2^{bc}	351±2.2^{c}	

	Treatment x Period	Treatment	Period
P-values	0.3097	<.0001	<.0001

a-d: Means within a column or a row with different superscript letters are significantly different ($P < 0.05$).

5.4.3 Plasma cortisol levels

No treatment by period interaction effects occurred ($P \geq 0.05$) for plasma cortisol levels of fish. The main effects of both treatment and period were significant ($P < 0.05$). No difference was found among the nucleotide-containing diets while 0.60% Maxi-Gen^{TM} Plus in diet had a significantly lower plasma cortisol (15.1 ng/ml) than the control diet (17.0 ng/ml). The average plasma cortisol of the fish increased from 13.1 ng/ml (day 29) to 22.0 ng/ml (day 71) with a transient increase on day 47 (Table 5.27).

Table 5.27 Plasma cortisol (ng/ml, mean±SE) of Atlantic salmon smolts fed 0, 0.20, 0.40 and 0.60% Maxi-Gen^{TM} Plus measured on day 29, 47, 57 and 71 starting on May 22nd, 2015.

Day	Level of Maxi-Gen^{TM} Plus (%)				Average
	0	0.20	0.40	0.60	
29	13.5±0.68	12.8±0.68	13.1±0.68	13.1±0.68	13.1±0.37^{c}
47	16.8±0.68	15.7±0.68	15.4±0.68	14.1±0.68	15.5±0.37^{b}
57	13.8±0.68	13.5±0.68	14.0±0.68	12.5±0.68	13.4±0.37^{c}
71	24.1±0.68	22.2±0.68	21.2±0.68	20.7±0.68	22.0±0.37^{a}
Average	17.0±0.33^{a}	16.0±0.33^{ab}	15.9±0.33^{ab}	15.1±0.33^{b}	

	Treatment x Period	Treatment	Period
P-values	0.53	0.008	<.0001

a-c: Means within a column or a row with different superscript letters are significantly different ($P < 0.05$).

5.4.4 Mortality

In trial 3, unexpected mortality outbreaks (not seen in trials 1 and 2) occurred between May 30th and June 5th (day 36 to 42), and between June 26th and July 10th, 2015 (day 63 to 77) (Fig 5.1). A total of 128 and 64 mortalities were found within all rearing tanks during the two periods, respectively. The water qualities were measured and all the parameters were within the normal levels (Table 5.28).

The cumulative mortality within each treatment group showed similar trend throughout the trial. However fish fed 0.60% Maxi-Gen^{TM} Plus had lower mortality (30.7%) than the other three groups (37.5-38.5%) since June 5th, 2015, which indicated a positive effect of feeding high level of Maxi-Gen^{TM} Plus on enhancing the overall health of fish (Figure 5.1).

Fish reared in system 3 had higher mortality (41.0%) than those reared in system 1 (31.4%) throughout the trial (Figure 5.1). The water qualities were similar in two systems

except that higher total ammonia nitrogen and water pH were found in system 3 (Table 5.28).

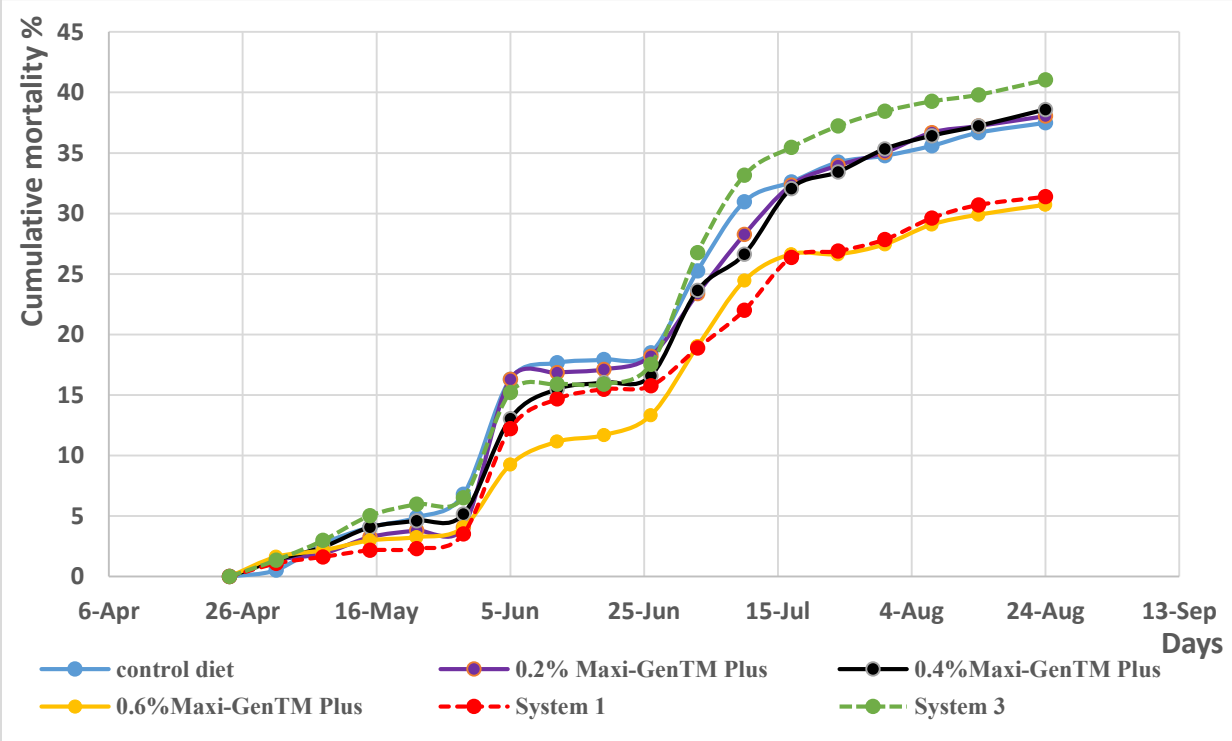

Figure 5.1 Cumulative mortality (%) of Atlantic salmon smolts fed 0, 0.20, 0.40 and 0.60% Maxi-Gen^{TM} Plus for a duration of 122 days in trial 3 starting on April 24th, 2015.

Table 5.28 Qualities of water collected from rearing system 1 and 3 measured on June 11th, 2015 (day 48).

Water qualities	System 1	System 3
Temperature (^{o}C)	10.1	10.5
Dissolved oxygen (%)	127	130
Total ammonia nitrogen (ppm)	1.0	1.5
Nitrite (ppm)	0.17	0.17
Alkalinity (ppm)	116	116
Water hardness (ppm)	64	64
pH	7.6	8.0

Chapter 6: Discussions

6.1 Growth performance

Based on the results from all three trials, Maxi-gen Plus inclusion ranging from 0 to 0.3% did not significantly enhance the daily weight gain of either in-season or off-season Atlantic salmon smolts (Table 5.3 and 5.17). However, 0.4 to 0.6% Maxi-gen Plus resulted in higher weight gain of both in-season and off-season smolts compared to the control diet (Table 5.17, 5.23). Burrells et al. (2001b) indicated that diet supplemented with 0.2% Optimun® (0.6 ppm total nucleotide content in the diet) resulted in an increased body weight of both small (43g initially) and large (205g initially) Atlantic salmon smolts (by 22.4 and 15.7%, respectively) than that of the control diet. However, the results from the present study indicated that a higher level of total nucleotide content (60-90 ppm from 0.4-0.6% Maxi-gen Plus) was required to enhance the body weight gain in fish with the initial weight of 46 to 47g (Table 5.17, 5.23).

A similar pattern occurred in all trials. Weight gain of the fish gradually increased as the experiment progressed (Table 5.3, 5.17 and 5.23). Handeland et al. (1998) showed that the growth rate (% day^{-1}) of Atlantic salmon smolts reared in seawater (8^{o}C, 34ppt) continuously increased from 0.42% to 1.15% in a 90-day duration. In trial 2, a transient increase on the weight gain was found during day 29 to 56 (Table 5.17), which might be due to the elevation in plasma growth hormone level concomitant with the occurrence of subsequent smolting process during this period. Growth hormone level of Atlantic salmon elevated in response to increased day length, which had a stimulatory effect on increase in growth and hypo-osmoregulatory ability of fish (Bjornsson et al., 1989). Long photoperiod manipulation applied in trial 2 (day 1 to 56) might stimulate the increase in

plasma growth hormone level of fish, which subsequently promoted the growth. Plasma osmolality measured on day 50 and 64 (331 and 330 mOsm/kg, respectively, Table 5.21) indicated the smolting process of fish within the period. The transient increase in growth associated with smolting in trial 2 was supported by Boeuf et al. (1989) demonstrating that an increase in plasma growth hormone levels (from 2.5 to 20 ng/ml) of Atlantic salmon occurred two weeks before the peak in Na^+, K^+-ATPase activity during smoltification.

The daily feed consumption of fish appeared to be positively related with the daily weight gain in all three trials as increased body weight gain was always accompanied with increased feed consumption of the corresponding Maxi-gen Plus inclusions. No difference occurred among the diets containing 0-0.3% Maxi-gen Plus inclusion while Maxi-gen Plus between 0.4 to 0.6% resulted in higher daily feed consumption than the control diet (Table 5.4, 5.18 and 5.24). Daily feed consumption of fish as % of body weight in trials 1 and 3 gradually increased as the trials progressed (Table 5.4 and 5.24), which indicated an adaptation of fish to the diets. Handeland et al. (2008) indicated that the feed intake of Atlantic salmon smolts increased from 0.01g/fish to 2.2g/fish along with the increase in body size (78g to 140g) 6 weeks after transfer to seawater. Similar results were found by Thodesen et al. (2001) indicating that the daily feed intake (as % body weight) of Atlantic salmon increased from 0.24 to 0.42% as the weight of fish increased from 148 to 191g in a duration of 70 days. In trial 2, however, the numbers gradually decreased as the trial progressed, which might be due to higher weight gain and better feed utilization efficiency of fish observed from the trial (Table 5.17, 5.18 and 5.19). Even though the fish consumed more feed as they grew, the proportion of it to their

body weight gradually decreased as the increase in body weight was higher than the increase in feed consumption of fish.

No information was found describing the effect of dietary nucleotides on the feed consumption of Atlantic salmon. Kubitza et al. (1997) reported an enhanced daily feed consumption (1.48 and 1.50% body weight, respectively) of juvenile largemouth bass fed diets supplemented with 2800 or 5600 ppm dietary nucleotides (IMP) while diet supplemented with 1400 ppm nucleotides did not differ from that of the control diet (1.27 and 1.2% body weight, respectively). The dosage of nucleotide used by Kubitza et al. (1997) to enhance the feed consumption of fish was much higher compared to the current study (60-90 ppm from 0.4-0.6% Maxi-gen Plus), which might indicate a lower dietary nucleotide requirement for Atlantic salmon to enhance feed consumption. Dietary nucleotides had stimulatory effects on the feed intake of some other species such as largemouth bass and jack mackerel (Ikeda et al., 1991; Kubitza et al., 1997). The positive relationship between the body weight gain and feed consumption found in the current study might indicate the role of dietary nucleotides as the feed attractant to stimulate the feed intake and subsequently increase the weight gain of Atlantic salmon smolts.

Both the weight gain and feed consumption of fish used in the current study were not as good as those reared in commercial production. Daily feed consumption of fish in all 3 trials (ranged from 0.11 to 0.70% of body weight) were much lower than the daily feed intake of Atlantic salmon with body weight of 50 to 110 g (1.97 to 2.18% of body weight) recommended by EWOS Canada Ltd., BC, Canada. Depressed feed consumption and weight gain occurred in the current study might result from elevated stress level of fish due to frequent handling. Plasma cortisol levels measured in trial 3 indicated an

elevated stress level of fish as the cortisol levels (13.1 to 22.0 ng/ml) were higher than that of the unstressed fish (9 ng/ml) demonstrated by Iversen et al. (1998). Stressed Atlantic salmon exhibited reduced growth performance such as lower body weight (156.7g, compared to 170.2g in non-stressed fish) at a duration of 64 days (Basrur et al., 2010), or reduced feed intake (24% of feed eaten, compared to 46% in non-stressed fish) (Pankhurst et al., 2008). McCormick et al. (1998) indicated the daily feed intake of stressed Atlantic salmon parr was reduced by 37 to 62%. Since Atlantic salmon smolts are more sensitive to the stress than parr, the impact of elevated stress level on reduced growth performance of fish used in the current study can be more severe (Carey and McCormick, 1998). Thodesen et al. (2001) reported an average daily feed consumption of 0.33% of body weight for Atlantic salmon (initially 148 g) at 6^{o}C, which was also lower than the recommended value (0.8 to 1.09% of body weight) by EWOS Canada Ltd. Based on the results from the current study and Thodesen et al. (2001), the growth of fish reared in the experimental conditions might not be comparable to that of fish reared in commercial production due to differences in rearing conditions as well as fish handling approaches.

The effect of dietary nucleotides on feed conversion ratio of Atlantic salmon had not been evaluated before the current study. An enhanced feed conversion ratio occurred when 0.4 and 0.6% Maxi-gen Plus were used in trial 3 (Table 5.25). However, 0.45% Maxi-gen Plus inclusion did not result in better FCR than the control diet in trial 2 (Table 5.19). Fish from trial 2 had better FCR (0.72 to 0.81) than those from trial 3 (0.79 to 1.62) (Table 5.19 and 5.25). The ineffectiveness of 0.45% Maxi-gen Plus on FCR in trial 2 might indicate that feeding dietary nucleotides could improve the FCR of fish to a certain

level (around 0.8 according to the current study) and the promoting effect might gradually disappear along with the improvement on FCR. Similar results was found on the effect of period on the FCR of fish in all three trials. The FCR gradually improved from the first period to the last period in all trials (Table 5.5, 5.19 and 5.25), indicating an enhanced ability of fish to convert the feed consumed to the weight gain with time on feed. Handeland et al. (2008) found feed efficiency (weight gain/feed consumption), the inverse of FCR, of Atlantic salmon smolts increased from 0.4 to 1.2 (while FCR decreased from 2.5 to 0.8) during 90 days after transfer to seawater (10^{o}C).

The overall growth performance of fish in trial 2 (off-season trial) was better than those in trial 1 and trial 3 (in-season trials) as it exhibited higher body weight gain, higher feed consumption and lower FCR. Goncalves et al. (2013) reported that off-season Atlantic salmon smolts induced by photoperiod manipulation (2 month short photoperiod followed by 2 month long photoperiod) had higher weight (162.8g) than that of the smolts reared under natural photoperiod (151.3g) by the end of a 120-day freshwater trial. One possible reason for the difference between trial 2 and the other two trials would be the different daily photoperiod duration during the trials. In trials 1 and 3, simulated natural day length was applied throughout the trials. From April to August, the day length at the latitude 45^{o}N ranged between 12.9 and 15.5 hours with the average day length of 14.6 hours. In trial 2, long photoperiod (16h light: 8g dark) was applied throughout the trial (October, 2014 to February, 2015). McCormick et al. (1987) indicated that a decline in photoperiod (from continuous light to simulated natural day length) resulted in a reduced growth of Atlantic salmon smolts (50g, compared to 60g of fish subjected to continuous light) during smoltification. Similar results were found by Sigholt et al.

(1998) demonstrating that Atlantic salmon pre-smolts subjected to continuous light had higher final weight and specific growth rate (67.5g and 0.78, respectively) than those of the fish subjected to a 7-week short photoperiod (59.5g and 0.59, respectively). Enhanced growth of fish subjected to long photoperiod manipulation was associated with elevated plasma growth hormone levels (Bjornsson et al., 1989). Longer daily photoperiod manipulation used in trial 2 might promote the growth of fish. The other reason might be the difference in the duration of fish recovering from the stress caused by transport and acclimating the new environment before the trials were conducted. In trials 1 and 3, the fish had 17 (April 24th to May 11th, 2014) and 28 days (March 26th to April 23rd, 2015) to recover from transport stress and acclimate the tank environment while the fish in trial 2 had 75 days (August 12th to October 25th, 2014) for recovery and acclimation. Iversen et al. (1998) stated that loading and transport of Atlantic salmon smolts resulted in a significant increase in the concentrations of plasma cortisol (9 to 158 ng/ml), glucose (4 to 9 mmol/l) and lactate (1 to 3.5 mmol/l) and the levels remained high 48 hours after transport. Plasma cortisol level of fish measured on day 29 in trial 3 (13.1 ng/ml) was higher than that of unstressed fish (9 ng/ml) found in Iversen et al. (1998), which might indicate that the fish were still not fully recovered 57 days after being transported (Table 5.27). Similar results were found by Gatica et al. (2010) indicating that processes such as loading, transport and unloading of adult Atlantic salmon increased the plasma concentrations of cortisol (103.2 to 226.2 ng/ml), glucose (2.3 to 3.4 mmol/l) and lactate (1.0 to 1.4 mmol/l). Generally, the stress level of fish is inversely related to the duration of recovery and acclimation period. Since the fish from trial 2 had longer time to recover

from the transport stress and acclimate to the environment, they were more likely to exhibit better growth performance than those used in the other trials.

The growth performance of fish were similar in trials 1 and 3. However, the last period (day 57-122) in trial 3 exhibited dramatically higher body weight gain, feed consumption and improved FCR compared to any other period within the two trials. The enhanced growth performance within this period might be due to the reduced handling (sampling) frequency of fish. The fish were handled three times within each period (28 days) due to biweekly blood sampling and monthly batch-weighing in trial 1 and the first two periods in trial 3. However, only one blood sampling was conducted (day 71) within the last period (66 days) in trial 3. The significant reduction in the frequency of fish handling might explain the enhanced growth performance during the period as it resulted in less chance of fish to get injured during handling and more time to recover from the stress. McCormick et al. (1998b) indicated that repeated acute stress caused by handling (twice per day for 30 days) resulted in reduced weight (8g) and growth rate (2.2%/day) of Atlantic salmon parr compared to those of the non-stressed fish (13g and 3.1%/day, respectively). The handling frequency in the current study was lower than that in McCormick et al. (1998b). However, Carey and McCormick (1998) demonstrated that Atlantic salmon smolts were more sensitive to handling stress than parr as longer time was required for smolts to return to pre-stressed state after being stressed. The handling frequency in the current study might have been high enough to disturb the growth of fish.

6.2 Plasma osmolality following 24h, 40ppt salinity challenge

The effect of dietary nucleotides on the hypo-osmoregulatory ability of Atlantic salmon smolts during smoltification and desmoltification was first evaluated by their effect on

reducing the plasma osmolality of fish in the current study. Based on the results from all three trials, Maxi-Gen™ Plus at inclusion levels from 0.05% to 0.60% all resulted in lower plasma osmolality than the control diet (Table 5.7, 5.21 and 5.26). However, 0.05% to 0.10% Maxi-Gen™ Plus did not affect the plasma osmolality of fish consistently as they only resulted in lower plasma osmolality compared to the control diet on day 30 and 44 in trial 1 (Table 5.7). A range of 0.15 to 0.60% Maxi-Gen™ Plus consistently lowered the plasma osmolality throughout trials 2 and 3 (Table 5.21 and 5.26). Similar results were reported by Burrells et al. (2001b) indicating that diet supplemented with dietary nucleotides resulted in an enhanced osmoregulatory ability of Atlantic salmon smolts by lowering the plasma chloride concentrations. The fish fed nucleotide-containing diet had significantly lower plasma chloride (119.2 mmol/l) than that of the fish fed the control diet (139.1 mmol/l) 5 weeks after transfer to saltwater (Burrells et al., 2001b). The nucleotide content in the diet used by Burrells et al. (2001b) was 0.6 ppm (0.2% Optimun®), which was lower than the nucleotide levels used in the current study (7.5 to 90 ppm from 0.05 to 0.60% Maxi-Gen™ Plus).

No significant difference in plasma osmolality was found among the nucleotide fed groups in trial 2 (Table 5.21) while a significant difference was shown among the nucleotide fed groups in trials 1 and 3 (Table 5.7 and 5.26). Inclusion levels of 0.20 and 0.25% Maxi-Gen™ Plus resulted in lower plasma osmolality of fish than that of the fish fed 0.05, 0.10 and 0.15% Maxi-Gen™ Plus in trial 1 (Table 5.7). An inclusion level of 0.60% resulted in lower plasma osmolality than that of the fish fed 0.20% Maxi-Gen™ Plus in trial 3 (Table 5.26). Based on the results from the current study, the plasma osmolality of Atlantic salmon smolts during smoltification was inversely related to the

increase in the Maxi-Gen^{TM} Plus inclusion in the diets as the significant reduction in the plasma osmolality appeared when the inclusion level increased from 0.05 to 0.20% and from 0.20 to 0.60%.

A similar pattern was found in all trials on the effect of period on the plasma osmolality of fish (except for the last salinity test in trial 1) as the plasma osmolality gradually increased during each salinity test (Table 5.7, 5.21 and 5.26), which indicated a decreased hypo-osmoregulatory ability of Atlantic salmon smolts. Similar results were reported by Young et al. (1995) stating that the plasma osmolality of coho salmon during 24h, 32ppt salinity tests stabilized at 310 mOsm/kg from April to June, followed by an increase (310 mOsm/kg to 320 mOsm/kg) from June to September. McCormick et al. (2009) reported that the plasma osmolality of Atlantic salmon smolts during 24 h, 30ppt salinity tests ranged from 315 to 325 mOsm/kg from March to May, followed by an increase to 330 mOsm/kg in June. The unexpected decrease in plasma osmolality of fish during the last salinity test in trial 1 was not typically seen in the previous studies as well as the current study (trials 2 and 3) and reasons for this are unknown. The potential reason for the decrease might be the fish during the last period of trial 1 somehow regained their hypo-osmoregulatory ability and maintained their plasma osmolality at the normal level during the salinity test.

In the current study, the plasma osmolality of fish following the salinity tests were higher (ranged from 338 to 366 mOsm/kg in trial 1, 330 to 374 mOsm/kg in trial 2 and 332 to 373 mOsm/kg in trial 3) (Table 5.7, 5.21 and 5.26) than that of the fish reported by Young et al. (1995) and McCormick et al. (2009). The higher plasma osmolality might be due to the higher salinity (40ppt) used in the current study (compared to 32 and 30ppt

used by Young et al. (1995) and McCormick et al. (2009), respectively). Byrne et al. (1972) demonstrated that the exposure of Atlantic salmon to high salinity (30ppt) resulted in higher plasma osmolality (355 mOsm/kg) than that of the fish exposed to 15ppt salinity (325 mOsm/kg).

Decreased hypo-osmoregulatory ability (salinity tolerance) of fish is a sign of desmoltification process (Stefansson et al., 2008). The fish used in the current study were retained in freshwater throughout the experiment. Smolts would gradually lose their salinity tolerance if they were retained in freshwater (Hoar, 1988; Stefansson et al., 2008). Parry (1961) indicated that the plasma osmolality of Atlantic salmon ranges from 328 mOsm/kg in freshwater to 344 mOsm/kg in seawater. Plasma osmolality higher than 350 mOsm/kg, accordingly, might indicate a declined hypo-osmoregulatory ability of fish in seawater resulted from desmoltification.

In trial 1, the plasma osmolality of control group during the first salinity test on day 16 (338 mOsm/kg) was lower than 350 mOsm/kg while the value (373 mOsm/kg) was higher than 350 mOsm/kg during the second salinity test on day 30 (Table 5.7). However, the plasma osmolality of 0.20 and 0.25% Maxi-Gen^{TM} Plus groups did not exceed 350 mOsm/kg until the third test on day 44 (356 and 355 mOsm/kg, respectively) (Table 5.7). The difference between the control group and 0.20 and 0.25% Maxi-Gen^{TM} Plus groups indicated a delay of the occurrence of desmoltification, or more importantly, a 2-week extended "smolt window" for the fish to be transferred to seawater. In trial 2, similar results were shown as the time (day 92) for the plasma osmolality of fish fed 0.15, 0.30 and 0.45% Maxi-Gen^{TM} Plus to exceed 350 mOsm/kg was two weeks later than that of the fish fed the control diet (day 78) (Table 5.21). In trial 3, the plasma osmolality of fish

from all groups exceeded 350 mOsm/kg during the second test on day 47. However, the values for 0.20, 0.40 and 0.60% Maxi-Gen^{TM} Plus groups (352, 360 and 357 mOsm/kg, respectively) were much closer to 350 mOsm/kg than that of the control group (371 mOsm/kg) (Table 5.26), which still indicated a positive effect of feeding Maxi-Gen^{TM} Plus on extending the "smolt window". Plasma cortisol level of fish measured on day 47 was higher (15.5 ng/ml) than those measured on day 29 and 57 (13.1 and 13.4 ng/ml, respectively) (Table 5.27), which indicated an elevated stress level of fish during the period. Gatica et al. (2010) indicated that the plasma osmolality of stressed Atlantic salmon (275 to 297 mOsm/kg) was higher than that of the unstressed fish (267 mOsm/kg). Elevated cortisol level of fish during that period might explain the disappearance of extended "smolt window" caused by feeding Maxi-Gen^{TM} Plus in trial 3 as increased stress level might accelerate the increase in plasma osmolality of fish during smoltification.

6.3 Immune-related gene expression (viral mimic test)

The effect of dietary nucleotides on the expression of immune-related genes of Atlantic salmon smolts subjected to polyIC injection was first evaluated in the current study. Based on the results of viral mimic test in trial 1, 0.25% Maxi-Gen^{TM} Plus in the diet resulted in a higher expression of Mx, IFN-α and ABCB2 while 0.20% Maxi-Gen^{TM} Plus in the diet resulted in a higher expression of Mx and ISG15 compared to that of the fish fed the control diet 24 hours after polyIC injection (Table 5.10, 5.11, 5.12 and 5.13). Higher expression of immune-related genes of Atlantic salmon after polyIC injection caused by feeding Maxi-Gen^{TM} Plus indicated an enhanced immune response of fish against the virus infection.

For undisturbed and saline groups, no difference existed among the diets on the expression Mx, IFN-α and ISG15 while increased expression of ABCB2 appeared on 0.25% Maxi-Gen^{TM} Plus (Table 5.10, 5.11, 5.12 and 5.13). Diet with 0.25% Maxi-Gen^{TM} Plus resulted in higher expression of MHC class I than that of the control diet (not significant) and the diet with 0.20% Maxi-Gen^{TM} Plus among all injection groups (Table 5.14). The results indicated that feeding Maxi-Gen^{TM} Plus at a inclusion level of 0.25% did not increase the basal expression levels (before virus infection) of Mx, IFN-α and ISG15 while it resulted in an elevated basal expression levels of ABCB2 and MHC class I.

No difference was found among the diets on the expression of MHC class II (Table 5.15), which might suggest that feeding Maxi-Gen^{TM} Plus did not increase the expression of genes involved in MHC class II pathway of Atlantic salmon smolts.

Rokenes et al. (2007) indicated that the expression of ISG15 in the head kidney of Atlantic salmon was stimulated by both polyIC injection and IPNV infection. Similar results were found by Larsen et al. (2013) indicating that the expression of IFN-α in Atlantic salmon leukocyte cells (in vitro) was increased by both polyIC stimulation and ISAV infection. PolyIC injection had similar stimulatory effect on the expression of Atlantic salmon immune-related genes to the infection of viruses. The effect of feeding Maxi-Gen^{TM} Plus on enhancing the immune response of Atlantic salmon smolts after polyIC injection could simulate what takes place when the fish were infected with viruses after transfer to seawater.

The positive effect of dietary nucleotides on the immunity of Atlantic salmon smolts against ISAV infection was demonstrated by Burrells et al. (2001a) as it indicated a

reduced mortality of fish fed the nucleotide-containing diet (33%) than that of the fish fed the control diet (47%) 53 days after the ISAV challenge. The results found in the current study supported the findings from Burrells et al. (2001a) at the genetic level and demonstrated the underlying mechanisms of the beneficial effect of dietary nucleotides on the immunity of Atlantic salmon smolts.

Among the injection groups, the fish injected with polyIC had higher expression of Mx, IFN-α, ISG15 and ABCB2 compared to that of non-injected fish and the fish injected with saline (Table 5.10, 5.11, 5.12 and 5.13). The results from the current study were supported by previous studies demonstrating the effect of polyIC injection on increasing the expression of the genes above (Jorgensen et al., 2006; Kileng et al., 2008; Lockhart et al., 2004; Rokenes et al., 2007). No difference appeared between the polyIC group and the other two injection groups on the expression of MHC class I and MHC class II (Table 5.14 and 5.15). For MHC class I, different results were found by Jorgensen et al. (2006) indicating that the polyIC stimulation resulted in an increased expression of MHC class I in Atlantic salmon kidney cells (in vitro). Ingerslev et al. (2009) stated that Atlantic salmon smolts infected with IPNV had increased expression of MHC class I (4-16 fold) in spleen and liver than that of the control fish. An induced expression of MHC class I in the liver and spleen (3.23 and 2.78 fold, respectively) of Atlantic salmon challenged with ISAV was detected by microarray analyses (Jorgensen et al., 2008). In the current study, spleen samples were taken 24 hours after the polyIC stimulation. In Ingerslev et al. (2009) and Jorgensen et al. (2008), however, the samples were taken respectively at day 15, 24 and 37, and day 21, 28 and 37 after the virus infection. The difference in the timing of sampling might explain the difference in the expression of MHC class I as a

duration of 24 hours might not be long enough to result in a significant increase in the expression of MHC class I. Also, a dosage of 2 mg polyIC/g body weight of fish used in the current study might not be high enough to significantly stimulate the expression of MHC class I of the fish in 24 hours. For the expression of MHC class II, an in vitro study indicated that an increased expression of MHC class II appeared in the leukocyte cells of Atlantic salmon after polyIC stimulation (Larsen et al., 2013). However, the results found in the current study was supported by Jorgensen et al. (2007) indicating that no significantly induced expression of MHC class II was found in the liver and spleen of Atlantic salmon after the ISAV infection. Similar results were found by Jorgensen et al. (2008) indicating that no increase existed on the expression of MHC class II of Atlantic salmon infected with ISAV. The expression of MHC class II is not likely to be induced by polyIC injection as MHC class II molecules mainly participate in the immune response of fish through presenting extracellular antigenic peptides (bacteria) to $CD4^+$ T lymphocytes (Jorgensen et al., 2007). The different results found between the current study and Larsen et al. (2013) might due to the varieties between in vivo and in vitro studies as well as the difference in expression of the gene in different organs.

6.4 Intestinal histology

Based on the results of intestinal histology in trials 1 and 2, Maxi-Gen™ Plus levels from 0.05 to 0.45% did not significantly enhance the length, width and surface area on the mid and distal intestine of either in-season or off-season Atlantic salmon smolts compared with those of the fish fed the control diet (Table 5.6 and 5.20). Different results were found by Burrells et al. (2001b) indicating that diet supplemented with 0.2% Optimun® (0.6 ppm total nucleotide content in the diet) resulted in increased villi length in both mid

and distal intestine (by 18.0 and 21.4%, respectively) of Atlantic salmon than that of the fish fed the control diet. In the current study, however, the dietary nucleotides did not have a positive effect on the intestinal structure of the fish even with an inclusion level of 67.5 ppm (from 0.45% Maxi-Gen^{TM} Plus). The difference in the size of fish used in the studies might explain the different results found from the current study and Burrells et al. (2001b) as larger fish (205g initially) were used by Burrells et al. (2001b) than those used in the current study (60g initially in trial 1 and 46.2g initially in trial 2). Feed consumption of Atlantic salmon was positively related to the body size (Handeland et al., 2008; Thodesen et al., 2001). Even though higher nucleotide level in the diet was used in the current study, the total amount of nucleotides consumed by the fish might be less than that of the fish used by Burrells et al. (2001b) due to the difference in feed consumption relative to the body size. The amount of dietary nucleotides consumed by the fish in the current study might not be sufficient to affect the intestinal structure of the fish.

6.5 Plasma cortisol levels

Based on the results from trial 3, feeding Maxi-Gen^{TM} Plus had a positive effect on reducing the plasma cortisol level of Atlantic salmon smolts during smoltification. The plasma cortisol level of the fish gradually decreased along with the increase of Maxi-Gen^{TM} Plus inclusion levels from 0 to 0.60% with significant difference shown when 0.60% Maxi-Gen^{TM} Plus was added into the diet (Table 5.27). Higher plasma cortisol levels (13.1 to 22.0 ng/ml) were found in the current study compared to that of unstressed Atlantic salmon smolts (9 ng/ml) stated by Iversen et al. (1998), which indicated an elevated stress level of fish used in trial 3. The elevated plasma cortisol levels might be due to the fish going through smoltification or not fully recovered from the handling

stress. The effect of feeding dietary nucleotide on lowering the plasma cortisol levels of fish has been reported for species such as rainbow trout and sole (Leonardi et al., 2003; Palermo et al., 2013; Tahmasebi-Kohyani et al., 2012). The results from the current study confirmed the beneficial effect of dietary nucleotides on the plasma cortisol of Atlantic salmon. The total nucleotide content in the diets used by Leonardi et al. (2003), Palermo et al. (2013) and Tahmasebi-Kohyani et al. (2012) were 1.5, 8 and 0.6 ppm, respectively. However, 90 ppm nucleotides (from 0.60% Maxi-Gen^{TM} Plus) was required in the current study to significantly lower the plasma cortisol of fish, which indicated that higher level of dietary nucleotide might be required for Atlantic salmon to reduce the stress level compared to other species.

A gradual increase of plasma cortisol level of Atlantic salmon smolts during smoltification was demonstrated by Sundell et al. (2003). The results from the study indicated that the plasma cortisol level of Atlantic salmon smolts continuously increased during the smoltification and desmoltification periods. The transient increase in plasma cortisol on day 47 indicated an elevated stress level of fish during this period, might be associated with the mortality outbreak occurred between day 36 and 42 in trial 3. Jarvi (1989) stated that Atlantic salmon smolts stressed by the presence of predators had significantly higher mortality rate (88%) than that of unstressed fish (0%). Elevated stress level of Atlantic salmon caused by various stressors, in general, contributed to an increased mortality (Moles, 1997; Sundh et al., 2009).

Elevated plasma cortisol level of Atlantic salmon subjected to cortisol implantation has been reported to have stimulatory effect on the hypo-osmoregulatory ability of fish by increasing the gill Na^+, K^+-ATPase activity (Bisbal and Specker, 1991; Cornell et al.,

1994). In the current study, both the plasma osmolality and plasma cortisol of fish gradually increased over time. Feeding Maxi-Gen^{TM} Plus reduced the plasma osmolality and plasma cortisol simultaneously (Table 5.26, 5.27). Fish with higher plasma cortisol level (control group) also had higher plasma osmolality, which indicated a reduced hypo-osmoregulatory ability. Higher plasma cortisol level (not elevated by cortisol implantation), according to the results from the current study, did not result in enhanced hypo-osmoregulatory ability of fish.

6.6 Mortality

Twelve fish with extreme symptoms (body wall lesions, dark skin, fin erosions and abnormal movements) from rearing system 1 and 3 were selected, euthanized and sent to Atlantic Veterinary College, University of Prince Edward Island for examination on June 11th, 2015. Most fish showed systemic fat necrosis and associated histiocytic infiltrates, and increased red cell turnover, which indicated a nutritional problem due to the lack of feed intake or intake of detrimental ingredients.

The potential reason for the nutritional problem and subsequent mortality outbreaks might be due to the elevation of stress level of fish caused by handling. Both mortality outbreaks (day 36 to 42 and day 63 to 77) occurred about one week after batch-weighing days (day 28 and 56). The stress and potential injuries during batch-weighing might greatly contribute to the occurrence of mortality outbreaks.

Cumulative mortality in system 3 was higher mortality (41.0%) than that in system 1 (31.4%) throughout the trial (Figure 5.1), which might be due to the difference in water qualities within the systems. The total ammonia nitrogen and water pH were higher in system 3 (Table 5.28). All the fish used in trial 3 were held in system 3 during

acclimation period (28 days) after transport, which might result in an accumulation of organic waste in the system and subsequent changes in water qualities.

Fish fed 0.60% Maxi-Gen^{TM} Plus had lower mortality than the other three groups since the occurrence of first mortality outbreak, which suggested an enhanced overall health of fish during stressful period by feeding high level of Maxi-Gen^{TM} Plus (Figure 5.1).

Chapter 7: Conclusion

7.1 Conclusions

Enhanced growth performance, hypo-osmoregulatory ability, expression of immune-related genes and reduced plasma cortisol level occurred by feeding different inclusion levels of Maxi-Gen^{TM} Plus to Atlantic salmon smolts during smoltification and desmoltification periods.

Maxi-Gen^{TM} Plus at inclusion levels of 0.40 to 0.60% resulted in higher body weight gain, feed consumption and better feed conversion ratio of fish than those of the fish fed the control diet.

Maxi-Gen^{TM} Plus at inclusion levels of 0.05 to 0.60% resulted in an enhanced hypo-osmoregulatory ability of fish after transfer to seawater. The hypo-osmoregulatory ability was gradually enhanced when the inclusion level of Maxi-Gen^{TM} Plus was increased from 0.05 to 0.20%, and from 0.20 to 0.60%.

Feeding Maxi-Gen^{TM} Plus resulted in an extended "smolt window" of at least two weeks.

Maxi-Gen^{TM} Plus with inclusion levels of 0.20 to 0.25% resulted in an enhanced anti-viral immune response of Atlantic salmon smolts by increasing the expression of immune-related genes after polyIC injection.

An inclusion of 0.60% Maxi-Gen^{TM} Plus in diet resulted in lower plasma cortisol level of Atlantic salmon compared to the control diet, which indicated a reduced stress level of fish during smoltification and desmoltification.

Inclusion levels of 0.05 to 0.45% Maxi-Gen^{TM} Plus did not enhance the intestinal structures of Atlantic salmon during smoltification.

7.2 Future studies

Since the fish used in the current study did not exhibit good growth performance, which might result from frequent handling. Future studies are worth conducting to evaluate the effects of Maxi-Gen^{TM} Plus on the growth of fish that are not handled as much as in the current study.

The fish used in the current study were reared in freshwater during the whole duration of the experiment (except for the exposure to seawater during the salinity tests). The effect of Maxi-Gen^{TM} Plus on the performances of Atlantic salmon smolts reared in seawater is worth investigating in future studies.

A range of 0.05 to 0.60% Maxi-Gen^{TM} Plus inclusion levels were investigated in the current study. Since only 0.60% Maxi-Gen^{TM} Plus in the diet resulted in a significantly lower plasma cortisol level of fish compared to the control diet, evaluation of a further extended range of Maxi-Gen^{TM} Plus inclusion is useful to investigate the effect of dietary nucleotides on the plasma cortisol levels of Atlantic salmon smolts during smoltification.

Although the current study evaluated the effects of Maxi-Gen^{TM} Plus on the performances of Atlantic salmon smolts during smoltification and desmoltification. Future studies should evaluate the effects of Maxi-Gen^{TM} Plus on the performances of Atlantic salmon parr.

The control diets used in the current study were formulated to meet the nutritional requirements of Atlantic salmon. Future studies can be conducted using a more challenging control diet to address the effects of Maxi-Gen^{TM} Plus.

The digestibility of Maxi-Gen^{TM} Plus in the diet is worth evaluating in the future studies since no information can be currently found.

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Appendix A. Mean body weight (g, mean±SE) of Atlantic salmon smolts fed 0, 0.05, 0.10, 0.15, 0.20 and 0.25% Maxi-Gen^{TM} Plus at 10^{o}C in trial 1 on days 0, 28, 56 and 78 on test (April 24th to August 24th, 2015).

Day	Level of Maxi-Gen^{TM} Plus (%)						Average
	0	0.05	0.10	0.15	0.20	0.25	
0	59.7±0.64	60.3±0.64	61.1±0.64	59.3±0.64	59.7±0.64	59.8±0.64	60.0±0.64
28	60.8±0.28	61.1±0.28	61.9±0.28	60.1±0.28	61.4±0.28	60.6±0.28	61.1±0.28
56	63.6±0.49	64.5±0.49	64.2±0.49	63.5±0.49	63.6±0.49	62.9±0.49	63.9±0.49
78	67.1±0.55	68.7±0.55	67.7±0.55	67.2±0.55	67.8±0.55	66.8±0.55	67.9±0.55
Average	62.8±0.40	63.7±0.40	63.7±0.40	62.5±0.40	63.1±0.40	62.5±0.40	

Appendix B. Mean body weight (g, mean±SE) of off-season Atlantic salmon smolts fed 0, 0.15, 0.30 and 0.45% Maxi-Gen^{TM} Plus at 10^{o}C in trial 2 on days 0, 28, 56, 84 and 112 on test (October 25th, 2014 to February 14th, 2015).

Day	Level of Maxi-Gen^{TM} Plus (%)				Average
	0	0.15	0.30	0.45	
0	47.0±0.49	46.7±0.49	46.1±0.49	45.2±0.49	46.2±0.49
28	60.2±0.29	59.9±0.29	59.0±0.29	58.6±0.29	59.4±0.29
56	74.4±0.37	75.3±0.37	74.7±0.37	75.4±0.37	74.8±0.37
84	87.0±0.47	89.3±0.47	88.1±0.47	89.2±0.47	88.2±0.47
112	107.8±0.79	110.5±0.79	110.2±0.79	113.2±0.79	110.3±0.79
Average	75.3±2.36	76.3±2.36	75.6±2.36	76.3±2.36	

Appendix C. Mean body weight (g, mean±SE) of Atlantic salmon smolts fed 0, 0.20, 0.40 and 0.60% Maxi-Gen^{TM} Plus at 10^{o}C in trial 3 on days 0, 28, 56 and 122 on test (April 24th to August 24th, 2015).

Day	Level of Maxi-Gen^{TM} Plus (%)				Average
	0	0.20	0.40	0.60	
0	47.8±0.56	46.0±0.56	47.7±0.56	47.1±0.56	47.1±0.56
28	49.2±0.35	47.7±0.35	49.9±0.35	49.3±0.35	49.1±0.35
56	51.4±0.45	50.8±0.45	53.6±0.45	52.7±0.45	52.1±0.45
122	91.7±1.37	92.3±1.37	99.1±1.37	98.9±1.37	95.7±1.37
Average	60.0±2.70	59.2±2.70	62.6±2.70	62.0±2.70	