Master thesis in marine nutrition

Evaluating primary (cry1Ab) and secondary effects (deoxynivalenol) of GM maize when fed to zebrafish (*Danio rerio*)

Investigating growth, intestinal mRNA and white blood cell differentiation

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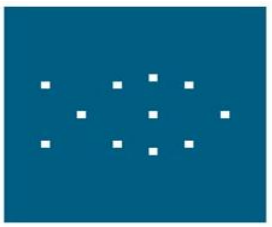

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Susanne Jørgensen

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Abstract

Several of the plants which are currently being used in fish feed in Norway today, are being genetically modified (GM) around the world. The use of GM plants is increasing worldwide, and whether these are safe to use in fish feed has been questioned. Deoxynivalenol (DON) is a highly prevalent mycotoxin contaminant found in crops. Previous findings suggest that GM maize has a higher level of this contaminant. This study aims to investigate whether GM maize (event MON 810) and low DON contamination affects performance, intestinal mRNA and white blood differentiation when fed to Zebrafish (*Danio rerio*).

Two separate trials were run simultaneously; (1) zebrafish were fed either GM maize (event MON 810) or the conventional near-isogenic parental line for 45 days, (2) zebrafish were fed diets with increasing concentration of synthetic DON (0.0, 0.1, 0.5, 1.5, 2, 3 ppm DON) for 45 days. All fish were weight and measured when terminated. The intestine was analyzed for difference in gene expressions using Quantitative Real Time RT PCR (qPCR), and white blood cell differentiation was performed on blood samples.

Feed acceptance was good for both trials. The fish fed GM maize had a higher growth than the non-GM group, although this increase was not significant. There fish feed 0.1 ppm DON had the highest growth for the DON trial, however there were no significant differences between the diets. No significant differences was observed for the mean normalized gene expressions (MNE) for the maize diets, although there was a trend towards increase of mitogen activated protein kinase (Mapk14) which could indicate ribotoxic stress. The MNE for the DON trial showed no significant difference or dose response to the increased DON concentrations in the diets. No effects were observed for the white blood cell differentiation for either of the trials.

In conclusion, an inclusion level of 19 % GM maize does not significantly affect fish performance, intestinal mRNA or white blood cell differentiation in zebrafish. Low concentrations of naturally contaminated or synthetically DON do not seem to affect zebrafish growth, intestinal mRNA transcript levels or white blood cell differentiation. The lack of effects indicates, compared to other investigated animals, that zebrafish is not very sensitive to DON contamination if feed.

1. Introduction

1.1 Genetically modified (GM) plants in aquaculture

During the last two decades the world of agriculture has encountered huge changes including introduction of genetically altered plants. The commercial use of genetically modified (GM) plants was initiated in 1996, and has increased each year since (James, 2011).

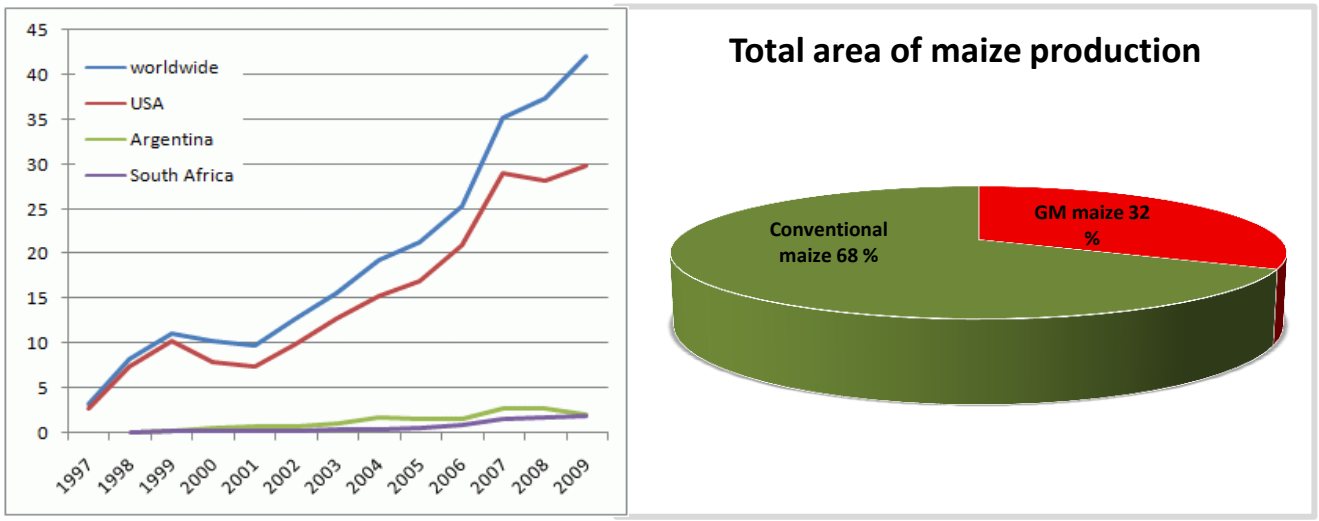

Figure 1.1: The graph show the cultivation increase (millions hectares) of GM maize in different areas (GMO-Compass, 2010), whilst the pie chart show how much of the total maize production is GM plants (James, 2012).

Organisms that have been genetically altered are collectively known as Genetically Modified Organisms (GMO). The Norwegian Gene Technology act from 1993 states that GMOs are "microorganisms, plants and animals whose genetic material has been altered using gene technology providing a gene combination that could not occur naturally or by mutations in nature" (lovdata, 2001). Today, the main GM plants are soybeans, maize, canola and cotton grown in 29 countries with USA, Brazil, Argentina and India being the four largest producers (James, 2012). The rapid increase in aquaculture production, along with intensification of the production cycle has created a great need for ingredients for fish feed (Watanabe, 2002, FAO, 2010). Carnivore fish such as salmon (*Salmo salar)* has traditionally been feed fish meal and fish oils from wild caught fish. However, the increased requirement of an intensified industry puts a strain on the reservoirs of the wild fish used for fish meal and fish oil production.

Alternatives to traditional feed ingredients have been investigated for several years, and the three main fish feed producers in Norway incorporates both marine and plant ingredients into their products. In 2010, Skretting reported a fish meal inclusion of only 15 %, and the marine ingredients were only 50 % in the equivalent feed from EWOS. Biomar has even established its own sustainability program to ensure that only sustainable ingredients are being used (Ewos, 2011, Skretting, 2010, Biomar).

Experiments on replacing fish meal with plant protein have shown that it is possible to almost entirely replace fish meal with plant protein as long as amino acids are balanced to meet the nutritional requirements of the species in question (Espe et al., 2006, Torstensen et al., 2008). The experiment by Torstensen et al. (2008) showed that the aquaculture industry could be a net producer of protein. Even though a high amount of plant protein gave a reduction in growth, by replacing 80 % of the fish meal with plant protein the salmon produced 2 kg protein per kg fish meal fed which is four times more efficient that diets where the protein source is 100 % based on fish material.

If today's development continues, will the future of aquaculture probably rely equally as much on agriculture, including plants obtained from genetic engineering, as wild fish populations.

1.2 What is genetic engineering

The amount of GM plants in production is increasing rapidly. Even though new and improved techniques are emerging continuously there are still some basic principles that apply for general gene modification. Initially the gene coding for the wanted trait has to be located and isolated. The gene code is cultured and a marker gene is attached. Thereafter the target and marker gene is injected into the DNA of the objects that is to be modified. The new DNA is placed in the cell, and by utilization of the marker gene the cell are controlled to ensure positive transformation (Peel, 2001). Unsuccessfully transformed cells are eliminated and new plants are regenerated from the single transformed cell. When the transformation is successfully completed the new transgenic plant is tested for its new trait and can from then on be bred conventionally (Stella G, 2000, Christou, 1996).

1.2.1 Gene transformation methods

The plant pathogen *Agrobacterium tumefaciens* causes crow-gall tumors upon infection of dicotyledonous plants (Hoekema et al., 1983). The large tumor inducing plasmid (Ti plasmid) is the causative agent creating the crow-gall tumors which contains Transfer-DNA (T-DNA) (Hoekema et al., 1983). Gene transfer using *Agrobacterium tumefaciens* quickly became popular after it was proven a successful method of transferring full length genes expressing antibiotic resistance into tobacco plants by Barton et al. (1983), the method however had its limitations (Christou, 1996). *Agrobacterium tumefaciens* worked well when introducing new genes to the dicotyledonous plants, nonetheless, because of its host specificity the results on monocotyledonous plant (maize, rice wheat, etc.) remained absent (Corbin and Klee, 1991). Years later, in 1988, a new method was introduced using high velocity micro projectiles to transport substances into cells. The biolistic method is based on a mechanism that can accelerate small tungsten particles to high velocity, making them able to cross cell walls (Klein et al., 1987, Klein et al., 1988). An important step in this procedure is that the bombarded cell survives and the micro particle can be transported by the cytoplasmic stream. Klein et al. (1987) showed that large RNA and DNA could be bound to the small tungsten particles (4µm), and therefore being able to successfully delivering biologically active RNA and DNA into the cells. The figure below shows a simplified illustration of the two methods for gene transformation.

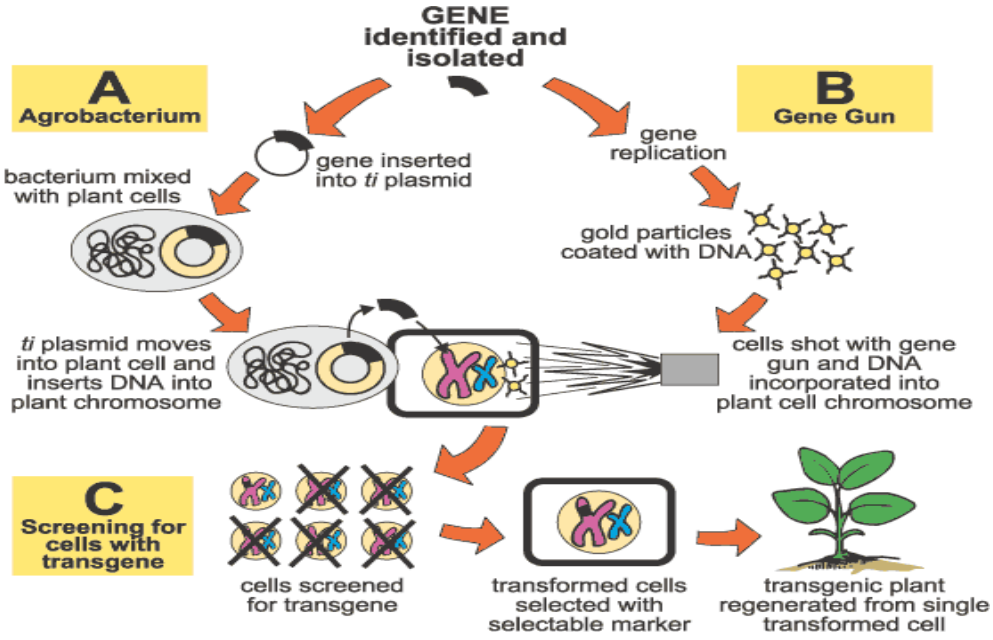

Figure 1.2: The picture illustrates the basic principles of the two main ways of gene modification which are described above (Peel, 2001).

1.3 Genetically modified maize (event MON 810) and Cry1Ab

The main purposes for genetically modifying maize have been focused around agronomic interest with insect resistance as the most common (Christou, 1996). *Bacillus thuringensis*-maize (*Bt*-maize) is a commercially available GM maize (Betz et al., 2000) created by micro projectile bombardment. The abbreviation *Bt* refers to the soil bacterium *Bacillus thuringensis.* This particular bacterium produces cry-proteins during its sporulation phase that is toxic to insects of the Lepidoptera, Diptera and Coleoptera species. The European corn borer, *Ostrinia nubilalis,* (EBC) belongs to the Lepidoptera species and is a commercially significant pest in maize agriculture (Koziel et al., 1993). Koziel et al (1993) showed that by incorporating the maize DNA with a synthetic gene that coded for the cry1Ab protein derived from the *B. thuringiensis* the maize could produce the toxic cry-protein. During field trials the hybrid line (elite inbred plants crossed with traditional lines) showed successful insect protection (Armstrong et al., 1995). Cry1Ab proteins bind to specific receptors in the corn borer's intestine, invading the cell membrane which then disintegrates (Shimada et al., 2006). *Bt* -maize is approved for use in several countries today, with the assumptions of it being target specific (Mcclintock et al., 1995) and only able to bind to the EBC intestine. The event MON810 produced by Monsanto was approved within the EU both as food and food additives and feed and feed additives in 1998, whilst it was approved as early as 1996 in the US (CERA, 2009). However, a study by Chowdhury et al. (2003) showed trace amounts of cry1Ab protein in the gastrointestinal contents of pigs feed GM maize.

Based on these findings, Shimada et al. (2006) investigated the effects of cry1Ab protein on bovine, porcine and human intestinal cells. The study showed that even though the cry1Ab protein could slightly bind to the brush boarder membrane the toxin had no adverse effects on the cells, supporting the initial theory that the cry 1Ab toxin is not harmful to other species, although indicating that cry1Ab can bind to other intestines than the ECB. Great concern has also been raised around the larvae of the monarch butterfly, *Danaus plexippus*, since they are closely related to the ECB. In enclosed laboratory experiments the monarch butterfly larvae showed a slight effect to the cry1Ab toxin from the event mon810 (Hellmich et al., 2001), but the levels in the laboratory where much higher than what would occur in nature (Hellmich et al., 2001, Anderson et al., 2004).

The knowledge gaps connected to the cry proteins and their potential risks of inducing adjuvant effects and possibly increase the intestinal permeability has resulted in a new health risk evaluation of cry proteins (VKM, 2012).

1.4 Genetically modified maize (event MON 810) in fish feed

Although several studies have aimed to investigate potentially adverse effects of GM maize as animal feed, few studies have focused on GM maize as an ingredient in fish feed (Sissener et al., 2011b). Sissener et al. (2011b) summarizes the fish feeding trials with GM products including GM maize (MON 810) that has been published in scientific literature. The first trial was performed using Atlantic salmon (*Salmo salar L.)* in an 8 month study. The study concluded that, at an inclusion level of 6 % GM (MON 810) , there was no apparent adverse effect of feeding GM-maize compared to traditional maize ingredients regarding growth, intestinal health and nutritional factors (Sanden et al., 2005, Sanden et al., 2006, Bakke-McKellep et al., 2008). A shorter study of 82 days, also performed with salmon, with increased inclusion levels from 15 to 30 % GM maize resulted in reduced feed intake and growth whilst there was an increase in liver size and distal intestinal mass. There were also observed increased activity of CuZnSOD in both liver and distal intestine, whilst there was a reduction in catalase (CAT) activity in the liver (Sagstad et al., 2007). There was an increase of maltase activity in the mid- and distal intestinal segment and increased glucose uptake in the pyloric caeca (Hemre et al., 2007). An interesting increase in granulocyte level was also observed in the fish fed GM maize (Sagstad et al., 2007). Increased CuZnSOD gene expression was also observed in zebrafish (*Danio rerio)* liver by Sissener et al. (2010). However, growth was increased for the GM feed group.

The research on GM maize on fish is inconclusive both regarding cause and effect, and safety of one GM event does not necessarily extrapolate to others. Further investigating the deviating results, Sissener et al., (2011a) analyzed the GM maize form the Sagstad et al., (2007) trial and found that the levels of the mycotoxin deoxynivalenol (DON) was much higher in the GM maize ingredient creating a possible explanation for the observed effects of the fish fed the GM maize feed suggesting that more knowledge is required on the effect of low mycotoxin contamination in feed in order to distinguish these from potential GM effects.

1.5 Mycotoxin contamination in plants

Mycotoxins are secondary metabolites of fungi as they appear to have no effect on the growth or development of the fungi as described by Pitt (1996). Their presence is estimated to have occurred for as long as crops have been grown, while occurrence was first determined in the early 1960s (Richard, 2007). Initially, the toxins were considered only a storage phenomenon where molding caused by incorrect storage was thought to be the reason.

Updated knowledge shows that several of the mycotoxins can be formed during the growing phase of the crops in the field (Richard, 2007). Large variability in mycotoxins is observed between locations and growing seasons both in GM and non-GM maize. The general trend is however, reduced levels of mycotoxins in *Bt* -maize compared with conventional maize varieties, due to better resistance against *Fusarium* spp. resulting in limited insect damage to the plant (Bakan et al., 2002, Papst et al., 2005, Dowd, 2000, Munkvold et al., 1997). The effect of the toxins on humans and animals can be both acute and chronic with toxicities ranging from death to deleterious effects upon the central nervous, cardiovascular and pulmonary systems, and upon the alimentary tract (Bennett and Klich, 2003). Mycotoxins may also be carcinogenic, mutagenic, teratogenic and immunosuppressive (Smith et al., 1995). Amongst the mycotoxins, aflatoxins have received the greatest attention. Aflatoxin B_1 is the most potent hepato carcinogen known and is also known to have caused acute poisoning with fatal outcomes (Pitt et al., 2000). Although several of the other mycotoxins are not as toxic, they are much more prevalent that aflatoxins. Deoxynivalenol (DON) is a trichothecene mycotoxins produced by species in the *fusarium* spp (Vesonder et al., 1973) and is one of the most common contaminants of wheat, maize and barley worldwide (Rotter, 1996, Bretz et al., 2006). Out of 200 randomly collected field samples in southern Europe were 139 positive for DON contamination with levels ranging from 0.253 to 3.14 ppm (mg/kg) (Griessler et al., 2010).

1.6 Deoxynivalenol

The growth of the *fusarium* species is associated with weather conditions, and the growth of DON favors cold and wet surroundings both in field and storage (Vesonder et al., 1973). Generally, lower levels of mycotoxins are found in *Bt* -maize, although regarding DON specifically, the relationship is not so clear (Ostry et al., 2010).

Analysis of several *Bt* -maize batches here at NIFES show increased DON in *Bt* -maize, but the variability is large (unpublished data).

When DON was discovered it was initially named vomitoxin based on the effect it had on swine fed contaminated feed. Pigs would refuse to eat the contaminated crops, and vomit when ingesting small amounts (Vesonder et al., 1973). Research shows that swine is highly sensitive to DON exposure, where consumption of 1-10 ppm causes decreased feed intake, reduced growth and impartment of the nutritional efficiency (Rotter, 1996, Young et al., 1983).

There have also been reports of changes is blood parameters in pig feed contaminated feed, although these changes are difficult to separate from the decreased feed intake and nutritional status (Young et al., 1983). Rotter et al. (1994) examined leucocyte level after feeding low doses of DON to swine and found that leucocyte level increased after one week, and decreased after 28 days for the diet containing 3 ppm DON.

In general, there are great differences between sensitivity both among species and specific life stages, commonly the animals are arranged by decreasing sensitivity as pigs > mice > rats >> poultry ≈ ruminants (Rotter, 1996, Richard, 2007).

There is a lack of knowledge regarding the effects of DON on aquatic animals as plant ingredients have traditionally not been used in feed for aquacultured animals. Recently an experiment by Hooft et al. (2011) showed that aquatic animals can be extremely sensitive. Rainbow trout (*Oncorhynchus mykiss*) fed concentrations of 0.3 – 2.6 ppm naturally contaminated DON showed decreased feed intake, reduced growth and lower feed efficiency with increasing DON concentration. There has also been an experiment with rainbow trout earlier which also states that this specie is highly sensitive to DON contamination (Woodward et al., 1983).

In addition to feed refusal, DON has been reported to cause reduction in tissue protein synthesis in kidneys, spleen and ileum of pigs, while no effects were observed in the liver, pancreas, duodenum or jejunum (Danicke et al., 2006). Reduced protein synthesis and inhibition of RNA and DNA at the ribosomal level are established effects of DON (Rotter, 1996, Robbana-Barnat et al., 1987, Kouadio et al., 2005). Kouadio et al. (2005) reported of alterations in the cell membrane permeability and structure in *in vivo* experiments with human intestinal cells.

The immune system is sensitive to DON-induced immunosuppression which arises from the vulnerability of the continually proliferating cells that participate in immune–mediated activities (Oswald et al., 2005).

Vaccine immunity is also lowered by DON which could have huge implications for vaccination effects in combination with feed contamination (Pinton et al., 2008). DON can also affect the mitochondrial metabolism through succinate dehydrogenase activity, which is a key enzyme in aerobic energy production in both eukaryotic and prokaryotic cells (Oyedotun and Lemire, 2004).

Cytotoxic effects were also found when investigating the effects on DON on different fish cell cultures (Pietsch et al., 2011). Reactive oxygen species (ROS) was reduced in all cell lines, even though there were distinct differences among species which illustrates that even amongst fish there is great difference in susceptibility.

1.7 Risk evaluations of GM feed ingredients and zebrafish as a model

Risk evaluations of GM feed ingredients must consider several elements, one being the question of substantial equivalence. Substantial equivalence relates to any unintended effect(s) introduced by the process of genetic engineering (Aumaitre, 2002). When performing risk evaluations of GM feed ingredients, near-isogenic lines are generally recommended as the non-GM control. However, there is no perfect control as shown by previous studies with differences in e.g mycotoxin levels between GM and the near-isogenic control line. When unintentional differences are present, where some may be due to the modification and others may be due to storage conditions or production it is challenging to interpret the cause of the observed differences in animals fed these plants. It is therefore always the investigators responsibility and challenge to try to distinguish between effects that are related to the modification and effects that are related to *e.g* storage conditions or production.

The zebrafish (*Danio rerio)* is a teleost which belongs to the *Cyprinidae* family which has been a successful model for studying vertebrate development for a long time (van der Sar et al., 2004). Zebrafish was initially used by developmental biologist as a model organism, however the advantages of the zebrafish has become apparent to other scientific areas including toxicology in more recent time (Hill et al., 2005).

The advantages of this fish, compared to other popular research animals like rodents and bigger fish, is that the small zebrafish reduces cost both regarding husbandry and feed amount. Having a sequenced genome has contributed to establishing the zebrafish as an adequate genetic model and has shown several examples of the structural and functional conservation of genes across all vertebrates. Despite its small size, analysis of the whole organ, tissue, or the intact organism is possible (Hernández and Allende, 2008). Regardless of its popularity, there are few studies investigating the nutritional requirements of zebrafish (Drew et al., 2008, Sissener et al., 2010, Gomez-Requeni et al., 2010). In the present study, the zebrafish were selected to evaluate dietary risks of GM maize (cry1Ab) and DON on zebrafish performance, mid intestine gene expressions and the white blood cell population. Our model may pinpoint if DON could be a confounding factor when performing GM feeding trials.

1.8 Mid-intestinal gene expression

Table 1.1 shows the ten genes that were selected to evaluate the differences in mid-intestine gene expressions for the fish. It also gives a brief explanation to the choice of each specific gene, which are based either on general knowledge concerning the regulatory effects of the gene or direct findings in previous trials with either GM maize or DON contamination.

Table 1.1: The different genes selected to evaluate intestinal health of the zebrafish.

Gene	**Reaction mechanisms and previous finding**
Cyclin G1	Controls cell progression in the cell cycle. Can be changed by alterations in growth parameters. Inhibition negatively regulates cell progression.
Proliferating cell nuclear antigen	The protein encoded by this gene is found in the nucleus and is a cofactor of DNA polymerase delta. The encoded protein acts as a homotrimer and helps increase the processivity of leading strand synthesis during DNA replication.
Apoptosis-related cysteine peptidase Caspase 6	Involved in the activation cascade of caspases responsible for apoptosis execution. Cleaves poly (ADP-ribose) polymerase in vitro, as well as lamins.
Interleukin 6 receptor	Deoxynivalenol contamination is linked to an up regulation of pro-inflammatory cytokines which could inhibit cytokine signaling and effect growth.
Cytochrome P450, family 1, subfamily A	A detoxification marker connected to phase 1 response. Induction of cyp1A can provide an early warning marker of exposure toxic pollutants.
Solute carrier family 5 (Na/glucose cotransporter), member 1	There was an increased glucose uptake in salmon feed GM maize (Hemre et al., 2007).
maltase-glucoamylase	Higher maltase enzyme activity was observed in (Hemre et al., 2007) where they used GM maize.
Danio rerio ghrelin/obestatin preprohormone	Ghrelin regulated appetite which is one of the major effects of deoxynivalenol contamination in feed.
Mitogen-activated protein kinase 1	Many natural toxins that inhibit translation such as DON are also effective activators of MAPKs via a mechanism known as ribotoxic stress.
Copper zinc superoxide dismutase	Belongs to enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide, making them important antioxidant defense in nearly all cells exposed to oxygen.

1.9 Aims of study

This study aims to distinguish between primary (cry1Ab) and secondary factors (DON) found in the GM maize feed ingredient by investigating:

1) If GM maize (event MON810) containing both factors (cry1Ab and naturally contaminated DON) is as safe as conventional maize when fed to zebrafish for 45 days by evaluating fish performance, intestinal mRNA levels and differential counts of the white blood cell population

2) If there is any dose-response effects of synthetic DON when fed to zebrafish for 45 days by evaluating fish performance, intestinal mRNA levels and differential counts of the white blood cell population

2. Materials and method

2.1 Animal experiment

The animal experiment was carried out at the Zebrafish laboratory facilities at the National Institute of Nutrition and Seafood research (NIFES). Zebrafish (*Danio rerio)* (figure 2.1) was of the inbreed strain AB wild type reared in the experiment facilities upon starting trial. The trial was approved by FDU (approval number ID 2426).

Figure 2.1 Zebrafish (*Danio rerio*) used for the present trial (fichchannel.com, 2010)

2.1.1 Experimental design

The experiment was carried out in AHAB multiple rack zebrafish system (Aquatic habitats, Aauatic Eco-Systems, Apoka, USA). Reverse osmosis and automatic salt adding was used to treat the intake water. Filtration was done by UV, mechanical and carbon-filter. Temperature, salinity and oxygen was registered every day and measured 28.5 ±0.5 °C, 500 ± 30µS/cm and above 90 % respectively. Photoperiod was 14 light:10 dark.

Bt -maize feeding trial

A total of 60 larvae were included in the *Bt*-maize feed trial. Prior to the trial initiation the fish were weighted and divided into two groups where one was fed GM maize and the other fed the equivalent near-isogenic line. Each diet consisted of tree individual 3 liter tanks (n = 3).

DON feeding trial

351 Zebrafish larvae were divided into six different groups. Each group was feed one of six diets only differing in the amount of added synthetic deoxynivalenol (DON).

Control (n=4); <20 µg/kg DON, low (n=4); 108 µg/kg DON, medium (n=5); 534 µg/kg DON, medium high (n=5); 1543 µg/kg DON, high (n=4); 2002 µg/kg don and high high (n=5); 3022 µg/kg don. The water supply for the control group was separated from the other in case of any leakage of DON from the feed into the water. All groups were kept in 1,5 liter tanks.

2.2 Feed ingredients and diets

Prior to feed preparation, the *Bt*-maize and the conventional hybrid line were analyzed for mycotoxins at the premier analytical services in London by liquid-mass spectrometry (LC-MS). The results are presented in Table 2.1.

Table 2.1 The amount of mycotoxins (µg/kg) in the two different maize ingredients (GM maize and non GM maize).

	Amount (µg/kg)	
Toxin	GM maize	Non GM maize
Deoxynivalenol (DON)	769	39
Aflatoxin B1	<0.1	1.8
Aflatoxin B2	<0.1	0.1
Nivalenol (NIV)	<10	10
Fumonisin B1	22	114
Fumonisin B2	<10	22
Fumonisin B3	<10	15
Zearalenone (F2, ZON, ZEA)	8.5	<3.0

The diets were produced in laboratories at NIFES. The diet production is based on the mixing of three different fractions. The gelatin/carophyll pink was dissolved in hot water (≈80°C) at a 9:1 ratio (water: gelatin, w/w) before all the dry ingredients and the oil mixture were carefully added. The feed blend was mixed in a conventional kitchen machine until homogenous. For the DON diets was Deoxynivalenol (Sigma Life Science, St Louis, MO, USA) dissolved in water and added to the gelatin/carophyll pink solution. The feed paste was poured onto a labeled baking paper covered tray and then dried at low temperatures (40°C) in an oven before it was grinded sieved and stored at -18 ºC. Maize was used as the main source of starch in the *Bt*-maize trial with an inclusion level of 19%. Dextrin was used as the main source of starch in the DON trial with an inclusion level of 16%. Table 2.2 and 2.3 shows the ingredients for the *Bt*-maize feed trial and DON trial, respectively.

Table 2.2: The approximate composition of the experimental diets containing GM maize and non-GM maize. The specification of each ingredient is listed in appendix I.

Ingredients	Composition (%)
Casein sodium salt	40
Gelatin	12
Cod liver oil	3
Rapeseed oil	9
Maize	19
Dextrin	4
Lecithin	2
Mineral mix	5
Vitamin mix	1
Aminoacid mix	1
Astaxanthin	1
Betain	0
cellulose	3
Analyzed	
Crude Protein	53
Crude Fat	13
Starch	16
Total carbohydrates	23

Table 2.3 The approximate composition of the experimental diets containing increasing amounts of deoxynivalenol. The specification for each ingredient is listed in appendix I.

Ingredients	Composition (%)
Casein sodium salt	43
Gelatin	12
Cod liver oil	3
Rapeseed oil	9
Dextrin	16
Cellulose	5
Lecithin	1
Mineral mix	5
Vitamin mix	1
Amino acid mix	1,4
Astaxanthin	1
Deoxynivalenol mg kg^{-1} (ppm)	0, 0.1, 0.5, 1.5, 2.0, 3.0
Betain	1
Sucrose	1
Analyzed	
Crude Protein	56
Crude Fat	13
Starch	17
Fiber	5
Analyzed	93
Not analyzed rest	7

2.2.1 Trichothecen in the feed

The feed samples were analyzed at the veterinary institute using gas Chromatography-mass spectrometry (GC-MS) according to the method of Langseth et al. (1998) with only minor changes. The whole sample was ground and homogenized. 25 g was extracted with 125 ml acetonitrile-water (84+16, v/v) for one hour, followed by purification on a Mycosep 225 column (Romer Labs, Whashington, USA). A 3 ml aliquot, corresponding to 0.6 g of the sample, was evaporated to dryness, and derivatised with pentafluoropropionic anhydride (PFPA). The MS was operated in electron impact (EI) mode, measuring 1-3 ions per compound in selected ion monitoring (SIM) mode. The trichothecenes nivalenol (m/z 896), deoxynivalnol (DON)(m/z 734), HT-2 toxin (m/z 555and 572) and T-2 toxin (m/z 407, 452, 468). The values given in the brackets are the ions of measurement of each toxin. Two internal standards were added to the extracts after purification to compensate for variation in the instrument response during the run. The internal standards that were used were fusarenon-X (for DON and NIV) and neosolaniol (for HT-2 and T2).

An external standard calibration curve was used for quantification. The detection limit was 20-30 µg/kg for the toxins. Table 2.4 and 2.5 shows the amount of DON, HT-2, NIV and T2 for the experimental diets for the *Bt* –maize feeding trial and DON fedding trial, respectively.

Table 2.4 The amount, µg/kg, of the mycotoxins Deoxynivalenol (DON), HT-2 toxin, Nivalenol (NIV) and T2 toxin in the two experimental diets.

	Mycotoxin (µg/kg)			
Feed	DON	HT-2	NIV	T2
GM maize	80	<20	<30	<30
Non GM maize	<20	<20	<30	<30

Table 2.5 The amount, µg/kg, of the mycotoxins Deoxynivalenol (DON), HT-2 toxin, Nivalenol (NIV) and T2 toxin in the diets containing increasing amounts of DON.

	Mycotoxin (µg/kg)			
Diet	DON	HT-2	NIV	T2
1	<20	<20	<30	<30
2	118	<20	<30	<30
3	534	<20	<30	<30
4	1543	<20	<30	<30
5	2002	<20	<30	<30
6	3022	<20	<30	<30

2.2.2 Feeding

The fish were fed twice every day, where first feeding was within the first hour of light and the second around eight hours later. The quantity of feed given to each tank was weighted each day to carefully control that all the groups had been given the same amount of feed. The fish were monitored during feeding to detect any difference in appetite between the groups. During the first weeks of the trial the larvae were fed approximately 10 % of their body mass based on the mean starting weight and estimated growing rate for zebrafish. The feed quantity was gradually reduced to 2-3 % of body mass at the end of the trial. Feed particle size was gradually increased from 315-400 µm to 560-700 µm at the end of the feeding trial.

2.3 Sampling

Bt -maize feeding trial

After 45 days of feeding (75 days post hatch), ten fish were sampled from each tank and weight and length determined. The intestine was carefully dissected out from six fish per tank and preserved in liquefied nitrogen and stored until analyzed (-80 ºC). Blood was collected from four fish.

DON feeding trial

After 45 days of feeding (75 days post hatch), seven fish were sampled from each tank and weight and length determined. The intestine was carefully dissected out from four fish per tank and preserved in liquefied nitrogen and stored until analyzed (-80 ºC). Blood was collected from three fish.

For both trials, the sampling was performed one tank at the time; therefore, to obtain the same feed status (14 hours post feeding), fish were fed at different hours according to a sampling schedule the last day of the trial. Prior to handling the fish were euthanized by immersion in a mixture of ice and water as described by Wilson et al. (2009). Each fish were weighed to the nearest 0.01 g, measured to the closest mm and sex was determined. The collected fish were randomly distributed to either dissection (intestine) or blood sampling. During dissection the intestine was separated from the connective tissue and the mid intestine was frozen in liquid nitrogen. The blood was collected by tail cutting. The tail was cut posterior to the anal fin and blood was collected into a heparinized capillary tube held towards the exposed caudal vessels. An approximately equal amount of the collected blood was carefully placed on the end of a glass slide to produce blood smears.

Another glass slide was placed in front of the drop and pulled towards the drop at a 45 ° angle to smear the blood droplet. When the second glass slide hits the drop it spreads and the glass slide is pushed gently forward to create a thin layer of cells. An illustration of the procedure is showed in the figure 2.3. The glass slides were left to air dry before they were fixed in methanol and stained.

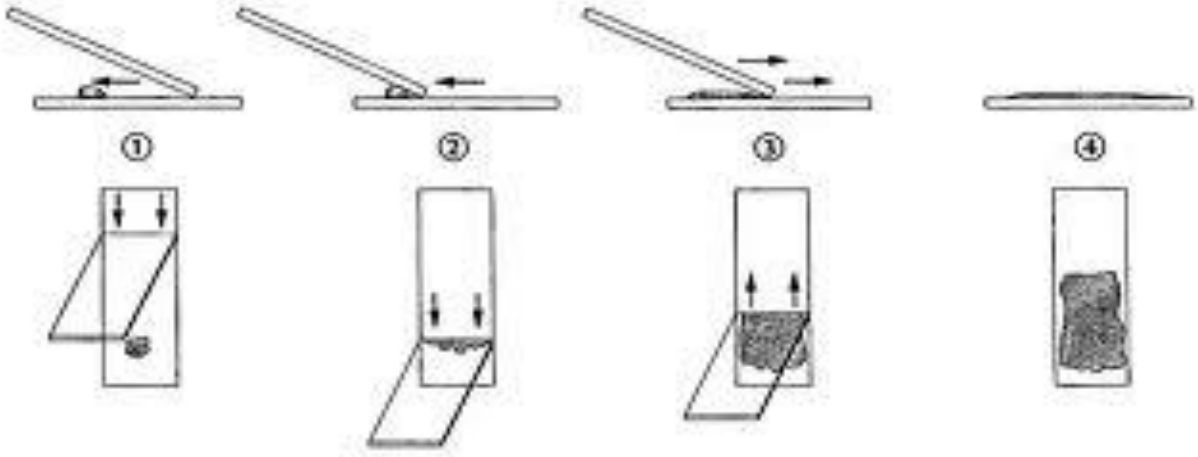

Figure 2.3: How to create a blood smear. (Cytopath, 2009)

2.3.1 Staining procedure

May-grünwald (Merck KGaA, Darmstadt, Germany) and Giemsa (Merck KGaA, Darmstadt, Germany) staining solutions were used to stain the blood smears. After being fixed in methanol, the glass slides were immersed into equal parts of May-grünwal color solution and Sørensens phosphate buffer solution (pH 6.8) for 5 minutes. The slides were directly transferred to a 1:10 mixture of Giemsa color solution and Sørensens phosphate buffer solution for 15 minutes. To rinse of the excess color the slides were washed several times by immersion in Sørensesns phosphate buffer solution before air dried and stored.

2.3.2 White blood cell count

The lymphocytes were characterized by the nucleus that fills most part of the cell, whilst the monocytes have a kidney shaped nucleus and therefore more visual cytoplasm. The granulocytes are characterized by the split or granulated nucleus (Lieschke et al., 2001). 100 white blood cells were counted for each blood smear. The cells were differentiated into lymphocytes, monocytes and granulocytes. The cells were differentiated at 40 X (Olympus BX51), and pictures were taken from each slide (Nikon DS Fi1). Figure 2.3 gives an illustration of the different leucocytes.

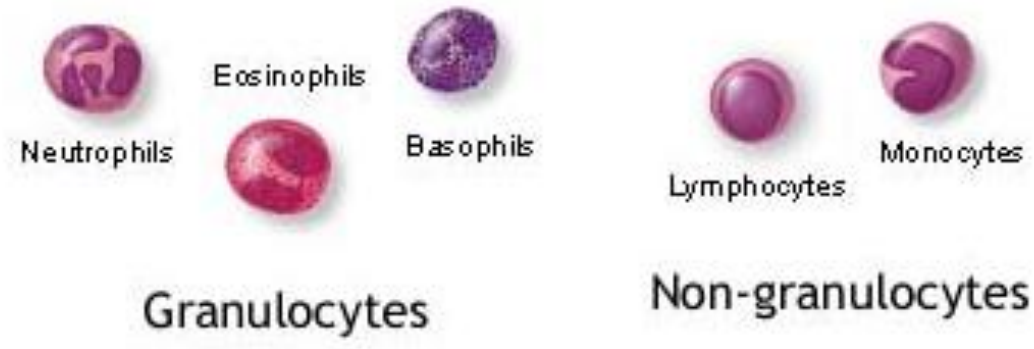

Figure 2.3: The picture shows the three different types of leucocytes that were differentiated between (Walgreens, 2011).

2.4 Cry 1Ab examination and Quantitative Real-Time reverse transcriptase PCR

2.4.1 Cry 1AB protein analysis

Principle

The test for cry1Ab protein is a direct Double Antibody Sandwich (DAS) ELISA. Antibodies specific to cry1Ab have been coated to the test wells of a micro plate. If cry1Ab protein or cry1Ac protein is present in the sample, it will bind to the antibodies and be captured on the micro plate. An enzyme conjugate is added to detect any captured protein. After a short incubation the micro plate is washed to remove any unbound enzyme conjugate and sample. TMB substrate is added to the micro plate. If the conjugate is present a color will be produced signifying the presence of cry1Ab or cry1Ac.

Procedure

The test samples consisted of the GMO-maize and the non GMO maize, the GMO feed and the non GMO feed.

In addition was there a fifth sample of extruded salmon feed that had been used for a previous feeding trial with GM maize, to investigate if the extrusion of feed affects the presence of cry-protein. The salmon feed sample was included to investigate breakdown of cry-protein during normal feed processes. All other components of the test were found in the *Bt* -cry1Ab/1Ac ELISA Kit (agdia©). An equal amount of the samples was weighted and mixed with 1 X PBST buffer at a ratio of 1:10 (tissue weight in g : buffer volume in ml). The enzyme conjugate was diluted with the RUB6 enzyme before 100 µl was dispensed into each test well. A color grid was created based on the provided control sample that was double diluted for each grid decrease. The test samples were added to the wells with four parallels for each sample. After incubation for 2 hours at room temperature the plate was emptied and thoroughly rinsed with the 1 X PBST buffer. When all excess liquid had been removed the TMB substrate was added to the test wells and the plate was incubated for 20 minutes. The results were evaluated both visually and measured with a plate reader at 650 nm.

2.4.2 RNA extraction by Qiazol

Principle

RNA is extracted using the principles first described by Chomczynski and Sacchi (1987). Their method is based on the chemical Trizol, whilst the extraction process for this study uses Qiazol which is a chemical equivalent to the original procedure except lower in cost.

Qiazol denatures the proteins and ruptures the cell walls allowing the nucleus protein to separate from the nucleic acid (Chomczynski and Sacchi, 2006). The tissue sample is homogenized in Qiazol. Chloroform is added to separate RNA from DNA which splits into a pink organic phase and a clear aqueous phase where the clear supernatant phase holds the RNA. The addition of isopropanol precipitates the total RNA.

Procedure

All areas and equipment used during RNA purification were treated with RNAse Zap to remove any RNAse that can contribute to degradation of the RNA. The whole tissue sample was transferred quickly to precellys tubes containing 1 ml of qiazol lysis reagent (QIAGEN, Norway) with 3 zirconium beads and homogenized at 600 rpm in 3 x 15 seconds with 10 seconds interval (Precellys 24 lysis & homogenization instrument, Bertin Technologies).

After homogenization, 200µl of chloroform was added, the tubes firmly shaken and incubated for 3 minutes at room temperature before centrifuged at 12000 g for 15 minutes at 4°C (Eppendorf Centrifuge 5415 R). The supernatant was transferred to 1. 5 ml RNase free tubes containing 500 µl of isopropyl alcohol and stored at 4°C for 1 hour.

Centrifuging the tubes at 12 00 g for 30 minutes at 4 °C gave small RNA pellets at the bottom of the tubes. The pellet was washed by removing the supernatant using a suction pipette (IBS Integra Biosciences, Vacuboy, Switzerland) and shaken with 1ml of ice cold 75 % EtOH with diethyl pyrohydrocarbonate, DEPC, treated water. The tubes were vortexed before centrifuged at 10 000 g for 5 minutes at 4°C. Again the supernatant was discarded. The RNA pellet was left to dry completely before it was dissolved in 30µl double distillated water (MilliQ biocel) and stored at -80 ° C.

2.4.3 RNA quantification and purity

Principle

The amount of RNA is measured using a spectrophotometer (Nanodrop ND 1000, Thermo Scientific). The nanodrop also gives an indication of purity of the samples. At 260 nm, RNA has it highest absorption and it is the most correct wavelength to indicate RNA concentration in the sample (Imbeaud et al., 2005). The ratio of the absorbance at 260 and 280 (A_{260}/A_{280}) indicates purity of the sample. However, the A260 measurement might be compromised by the presence of genomic DNA leading to over-estimation of the actual RNA concentration.

The A280 measurement will give an estimate of the presence of protein but provides no information on possible residual organic contaminants which is considered at 230 nm. Pure RNA will have A_{260}/A_{230} equal to A_{260}/A_{280} and greater than 1.8 (Imbeaud et al., 2005).

Procedure

1.5 µm of a sample was loaded onto the measurement pedestal of the Nanodrop spectrophotometer (ND 1000, Thermo Fish Scientific). Surface tension between the lower and upper pedestal held the sample in place during the measurement. The RNA concentration and ratios were directly presented on the connected computer screen.

2.4.4 Second precipitation

Principle

The samples that had low quality when measured on the nanodrop were precipitated a second time to improve purity. For the final results to be reliable the quality of the samples should be approximately equal.

Procedure

The samples were added 70 µl of double distillated water (ddH_2O), 250 µl of absolute ethanol and 10 µl of a 3M sodium acetate solution and stored in - 80°C over night. The next day the samples were centrifuged at 12 000 g for 30 minutes at 4°C resulting in formation of a new RNA pellet at the bottom of the tube. The supernantant was removed and the pellet was washed with 75 % EtOH with DEPC treated water, vortexed thoroughly and centrifuged at 10 000 g for 5 minutes at 4°C. After centrifugation the supernatant was removed, and after the pellet was dried completely was it dissolved in 30 µl ddH_2O.

2.4.5 DNAse treatment

To ensure that there were no DNA left in the samples they were treated with a DNA-free kit (Ambion® DNA free^{TM,} Invitrogen). Reagents for the entire procedure were found in the kit. Each sample was added 3µl DNase I buffer and 2µl DNase I enzyme and incubated at 37°C for 30 minutes. After incubation the samples were added 5 µl of the "slurry" part of the DNase inactivation reagent vortexed and incubated for 1 minute at room temperature before centrifuged at 10 000 g for 1 minute. The grey matter of the inactivation reagent created a mass at the bottom of the tube, leaving a clear supernatant to be transferred to a new 1.5 ml RNase free tube.

2.4.6 RNA quality on bioanalyzer

Principle

The bioanalyzer provides electrophoresis separation of small amounts of RNA when high voltage is lead to samples in solution. The separation is based on molecular weight and is detected via fluorescence. The amount of detected fluorescence correlates with RNA of a certain size and is visualized as an electropherogram (AgilenTechnologies, 2008).

An RNA Integrity Number (RIN) is given to each sample, which is a measure of degradation of the RNA based on the graphs and thus ruling out manual interpretation. The values range from 1 to 10, where RIN 1 indicates totally degraded RNA and RIN 10 signifies intact RNA.

Procedure

The quality of the RNA was measured using BioaAnalyser (RNA 600 Nano, Agilent Technologies, Germany) and the samples were prepared with a chip priming station (Agilent Technologies) and the 600 nano labchip kitt (Agilent Technologies).

0.5 µl of dye concentrate was added to a small tube containing 32.5 µl filtered gel.

The mix was thoroughly vortexed and centrifuged at 13 000 g for 10 minutes (Eppendorf centrifuge 5415 R) at room temperature. Samples with RNA concentration above 500 ng/µl was diluted with ddH_2O and all samples were denatured at 70 °C for 2 min before analyzing. The 600 Nano Chip was added the gel-dye mix, a nano marker and 5µl of each sample. Before it was run in the bioanalyzer was the chip votexed for at 2400 rpm for 1 minute in the IKA vortexer (IKA®Werke GmbH & Co. KG). An example of the result presented by the bioanalyzer is found in appendix III figure A.

2.4.7 Preparation of cDNA

Principle

In advance of quantitative real time polymerase chain reaction (qPCR) RNA is transcribed into complementary DNA (cDNA) by the enzyme reverse transcriptase.

Procedure

The cDNA tray (96 AB gene PCR plate, Thermo Scientific) consisted of standard solutions and the RNA samples, all in triplets. In order to prepare the standard solution, a "pool" of all the samples was made. The "pool" consists of 500 ng RNAfrom all the samples. The standards, 100 ng/µl, 50 ng/µl, 25 ng/µl, 12.5 ng/µl, 6.25 ng/µl and 3.125 ng/µl (± 5%) were prepared using the sample "pool" and ddH_2O water. All the RNA samples were diluted to a concentration of 25 ng/µl ± 5%. A real time reactions mix was added to the wells prior to the standards and the samples. The specific amount of the reagents for the reaction mix is listed in appendix IV B. 20µl of the reaction mix and 10 µl of a sample were added to each of the 96 wells in addition to two negative controls; non application control (nac) consisting of the reaction mix without the multiscribe enzyme and a non-template control (ntc) without RNA.

The Real Time plate was covered by an RNase free rubber mat before centrifuged at 50 x g for 1 minute (Eppendofr centrifuge 5810 R). The transcription took place in a PCR machine (Gene Amp PCR System 9700 PCR machine, Applied Biosystems) during a specific thermal cycling program. The specifications are showed in appendix IV C.

When the program was finished the RT plate was diluted with 30µl of ddH_2O, covered with a plastic film and stored at -20°C.

2.4.8 Preparation and testing of primers

Principles

The primes from Invitogen in table 2.1 were tested before they could be used for qPCR. The test is performed with reverse transcriptase (RT) and polymerase chain reaction (PCR) prepared in one tube. Thereafter the products are run in an agar gel to verify that the primer pair creates strong bonds.

Procedure

The primes were diluted with TE buffer based on the concentration of each primer. A master mix containing a RNA template, dNTP, RT-PCR enzyme mix and buffer (QIAGEN) was prepared. The amounts are presented in appendix V A. The reaction mix was added a forward and reveres primer. The tubes were subjected to a thermal cycle in the PCR machine (Gene Amp PCR System 9700 PCR machine, Applied Biosystems). The specific times and temperatures are showed in appendix V B.

The products were stored in 4°C over night. An agar gel was prepared with agar power and 1 x TAE heated in a conventional microwave owen. The products from the RT-PCR reaction were mixed with a loading buffer in a 1 to 6 ratio. Running buffer (1 x TEA) covered the whole gel. A DNA ladder/marker was loaded in the well next to the sample. After connecting the wires the voltage was set and the gel was run before it was photographed in gel doc.

2.4.9 Quantitative real time polymerase chain reaction

Principle

The quantitative Real Time polymerase chain reaction is based on the principles behind the PCR method which was developed by Kary Mullis during the 1980s. Basically, PCR can amplify small specific cDNA sequences and produce high numbers of identical sequences in a short time to be used for further analyses (Kubista et al., 2006).

The invention of Real Time PCR made it possible to monitor the amplification process (Nolan et al., 2006). There are basically three steps for gene quantification.

The first is the previous described reverse transcriptase reaction which turns DNA into cDNA, which is amplified through PCR and then detected using quantitative Real Time PCR. For each cycle during the PCR reaction the number of cDNA is doubled.

There is a need for two oligonucleotide primers which have the complimentary sequence to the DNA template to amplify the specific DNA strains. The reaction also requires the heat-stable DNA polymerase enzyme, the four nucleotide triphosphates (dNTPs) and magnesium ions in the buffer (Kubista et al., 2006). During each cycle the temperature is increased to separate the double stranded DNA (dsDNA), lowered to anneal the primers to the template, and increased again to optimize the temperature for the process where the dNTPs extends the primers as illustrated in figure 2. 4.

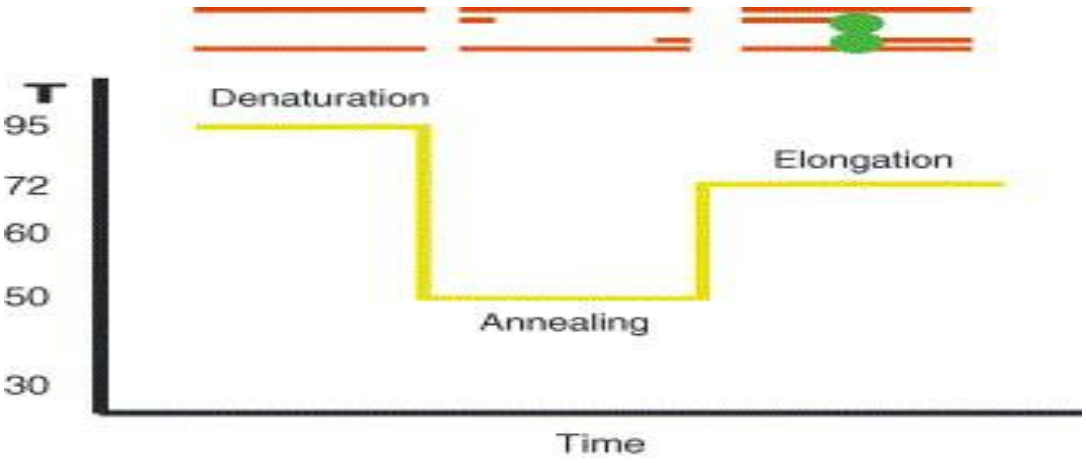

Figure 2.4: The PCR temperature cycle. Initially the temperature is raised to melt the dsDNA, and then lowered to let primers anneal and conclusively raised to let the polymerase extend the primers (Kubista et al., 2006).

To be able to detect the amount of DNA produced a fluorescent probe is added to the primer mix. The probe binds to the DNA products and gives for a fluorescent signal that correlates to the amount of DNA produced.

Figure 2.5 shows the different phases of the PCR reaction. During the first cycles the signal is low, then when the amount increases the signal increases exponentially until it reaches a plateau where is saturates due to limitations of the reagents (Kubista et al., 2006). It is not the saturation level that provides information on the amount of DNA present; it's the response curve that shows the difference in the initial amount of DNA.

The quantification is achieved by comparing the number of heat cycles needed to reach a set signal level referred to as the CT value as illustrated in figure 2.5.

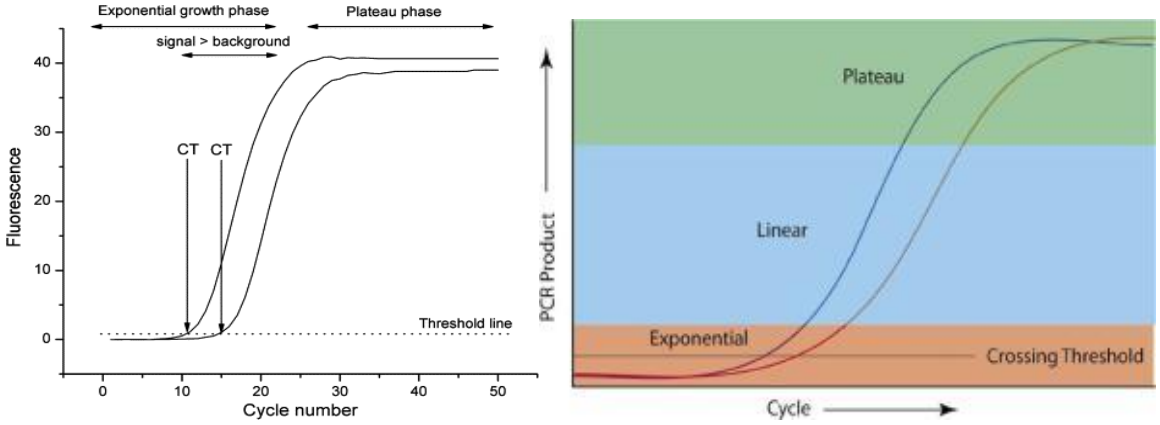

Figure 2.5: The phases of PCR on the left side (VanGuilder et al., 2008) and Cycle curves and CT values on the right side (Kubista et al., 2006).

The CT value is inversely proportional to the amount of a specific DNA sequence in the original sample, and can therefore be used to determine the relative quantity of the expressed gene. Housekeeping genes are used as an internal reference to the gene in question (Jain et al., 2006). The CT values of the housekeeping gene are used to normalize the differences among the different DNA sequences by compensating for biological differences. There are no universal housekeeping genes, usually genes which are expressed constant in the tissue for the sample in question is chosen (Kubista et al., 2006).

Procedure

The RT-plate with the previously prepared cDNA was centrifuged at 100 x g for 1 minute and vortexed at 1100 x g for 3 minutes before use.

The reaction mix consisted of forward and reverse primer pairs, SYBR GREEN master reagent and ddH_2O. The list of primers is presented below in table 2.1, and the reaction reagents are listed in appendix VI B.

8µl of the reaction mix and 2 µl of the template cDNA were transferred to a 384-well real-time PCR plate by a robot (Biomeck 3000 Laboratory Automation Workstation, Beckman Coulter).The qPCR reaction consisted of 45 cycles and was carried out by a LightCycler 480 (Roche). The belonging software presents amplification curves and CT values.

The calculated efficiency and error for the standard curves was acceptable at respectively 1.8-2.2 and below 0.04.

2.5 Equations and statistical analysis

The condition factor and specific growth rate for the fish were calculated to measure fish performance using the equations presented below.

$$Conditon\ factor = \frac{Weight(g) * 100}{Length^3(cm)}$$

$$Specific\ growth\ rate = \frac{(\ln initial\ weight(g) - \ln final\ weight(g)) * 100}{Days}$$

The real time quantitative PCR gene expression data from the mid intestine were normalized using the normalization factor acquired from geNorm algorithm.

The statistical analysis was performed using the graph pad prism (Version 5.04, Graph Pad software ®). One of the main criteria for analysis was that the data were obtained from a population that follows Gaussian distribution. The normalized data were therefore tested for normality using the d'agostino and pearson test from the graph pad prism 5 software.

Student's t-test was used on the data from the *Bt*-maize trial to determine if there were any significant differences between the parameters related to growth performance (weight, length, condition factor or specific growth rate), mean normalized expressions (MNE) of the mid-intestine and differential count of white blood cells.

One way analysis of variance (ANOVA) was used on the data obtained from the DON dose respons trial to identify if there were any difference between the parameters related to growth performance (weight, length, condition factor and specific growth rate) , MNE of the mid-intestine and differential count of the with blood cells. The same parameters were tested for linear regression to analyze for dose responses.

A significant cut of value was set at $p \leq 0.05$ for all statistical analysis. Significant differences for the ANOVA omnibus were further explored using Tukey's Honestly Significant Difference (HSD) test.

Table 2.1: The genes selected to evaluate mid-intestinal gene expression presented with forward and reverse primers and the RefSeq accession numbers for the sequences from which the primers were designed.

Gene	**Forward**	**Reverse**	**Accession nr**
Cyclin G1	GACTCCGCGTCATCGAGTCCG	AACGTCTCCGCACAGAAGCCAA	NM_199481
Proliferating cell nuclear antigen	TCGGGTGAGTTTGCCCGCATC	GCCCAGCTCTCCGCTAGCAGA	NM_131404
Caspase 6	AGGACAGCGCTTCAGCAGGACA	TGAGAGCCATTCCCCGTCTCTTGT	NM_001020497
Interleukin 6 receptor	TCAGCCAGAGGAGCAGGATGCC	TGTGTGACCCACTGCGGGGTT	NM_001114318
Sodium glucose co-transporter	GGACGCACTTGCCCTCCTCA	TCCCACCGCCAGAACCACCA	NM_200681
Maltase-glucoamylase	TGAGGGGAGAGGGCATGCGT	GCTGAGCGCAGGAGGCCATTT	XM_001919100
Cytochrome P450, family 1, subfamily A , Cyp1A	TCCACTCGATCGCTCCGGGTT	GCGGTTTAGGCGCATGAGCAGAT	NM_131879
Ghrelin/obestatin preprohormone	GTGCCGTGCCAGCAGCATGT	TGGCCTTCGACCCTGCGGTT	NM_001083872
Mitogen-activated protein kinase 14a, Mapk14	AGCTACTGCGGGGGACTCGT	CTTTCCCTGCTCGTCCGCCC	NM_131722
CuZn SOD	CGCATGTTCCCAGACATCTA	GAGCGGAAGATTGAGGATTG	Y12236
Eukaryotic translation elongation factor 1 alpha 1	AGACAACCCCAAGGCTCTCA	CTCATGTCACGCACAGCAAA	NM_001017795
B-actin	CGAGCAGGAGATGGGAAACC	CAACGGAAACGCTCATTGC	

3 Results

3.1 Feed ingredients and diets

The genetically modified (GM) maize had a DON concentration of 769 µg/kg, which is nearly twenty times higher than the non genetically modified (non GM) maize. However, the non-GM maize had minimal levels of the fumonisins and aflatoxins. Only trichothecene mycotoxins were analyzed in the feed, and the only toxin above the detection limit for the two diets was DON in the GM maize feed, with a value of 80 µg /kg feed.

Only the added synthetic DON was detected during analysis of the feed for the DON feeding trial, with values increasing from less than 20 up to 3000 µg DON $^{-1}$ kg feed.

3.2 ELISA Cry protein test

In the first row of the ELISA plate, row 1, well 3-13 shows the color changes caused by the standard sample with an established content of cry protein (cry1Ab). The first four wells in the second row, row 2, is test material from the non-GM maize whereas well 4-8 in row 2 consist of samples from the GM maize. Wells 9-12 in row 2 consist of test material from the non-GM feed, whilst the four first wells of row 3 contain test material from the GM feed. The final 8 wells in row 3 is the additional test material, the GM and non-GM salmon feed which has been heated during normal production processes like feed extrusion.

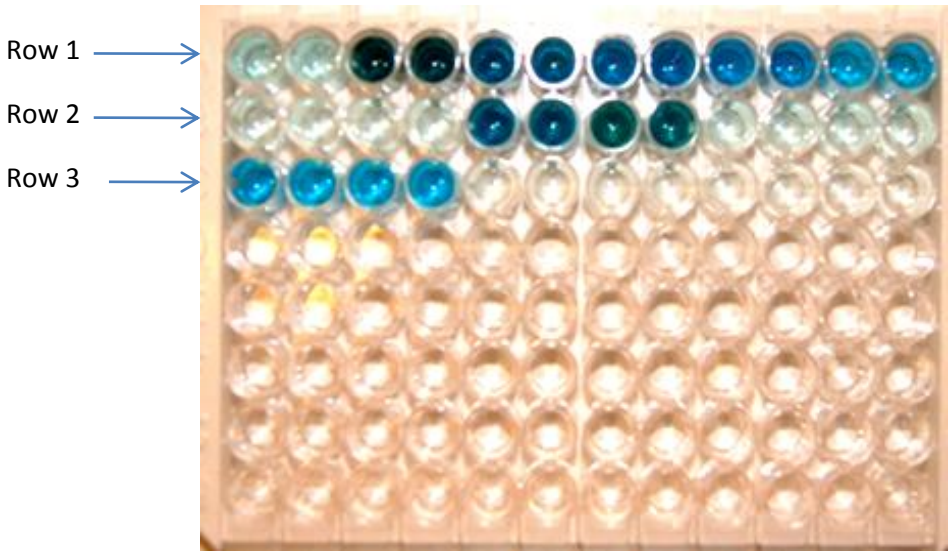

Figure 3.1: An ELISA test plate with standard samples compared with the feed maize material and from the non-GM and GM feed. The blue color in the test wells with GM maize and GM feed shows the presence of cry protein.

The color changes clearly illustrate the difference between the GM and non-GM ingredients and feed. There is a presence of the cry1Ab protein in the GM feed when comparing the change in color with the certified test material. The results for the heath treated salmon feed with GM maize confirms that the cry1Ab protein is not heat stable and destroyed during normal salmon feed processing.

3.3 Growth performance

Bt -maize feeding trial

Feed acceptance was high over all and no differences were seen in feeding behavior amongst the different diets. The final weights of the two different feeding groups were 200 and 187 mg for the GM group and non-GM respectively. The results are presented by the graphs in figure 3.2. There was no significant difference (p= 0.30) between the group fed GM maize and non-GM maize.

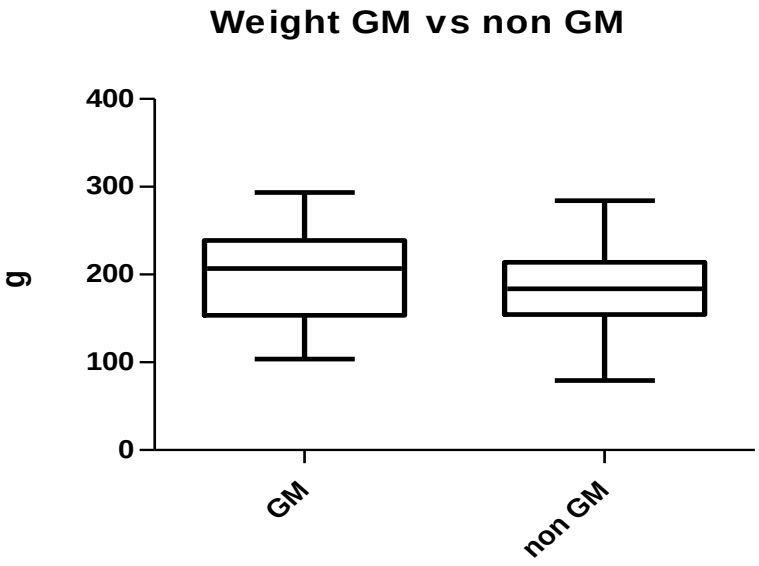

Figure 3.2: The graph shows the final average weight (75 days post hatching) for the fish within the two different feeding groups. Each Colum represents the average value for each group shown with maximum and minimum values ± SEM.

Table 3.4 shows the average final weight, length, condition factor and specific growth rate after 45 days. There were no significant differences in any parameters between the fish fed GM feed and non-GM feed.

Table 3.1: Growth performance for the two different diets given as mean (n=3) ±SD

Feed	Weigth (mg)	Length (mm)	Condition factor	Spesific growth rate
GM	200 ± 54	26 ± 2	1.0 ± 0.1	4.5 ± 0.4
non-GM	186 ± 55	26 ± 3	0.8 ± 0.1	3.7 ± 0.5
T-test	ns	ns	ns	ns

DON feeding trial

The feed acceptance was good for all the groups and all of the feed was consumed rapidly. We observed that the group fed the 3.0 ppm concentration consumed the feed somewhat faster and seemed hungrier compared to the other tanks. However this did not results in any significant difference in the final weight of the group (p=0.6). Mortality during the trial was negligible. Figure 3.3 shows the average final weight of the fish in each group at the end of the trial.

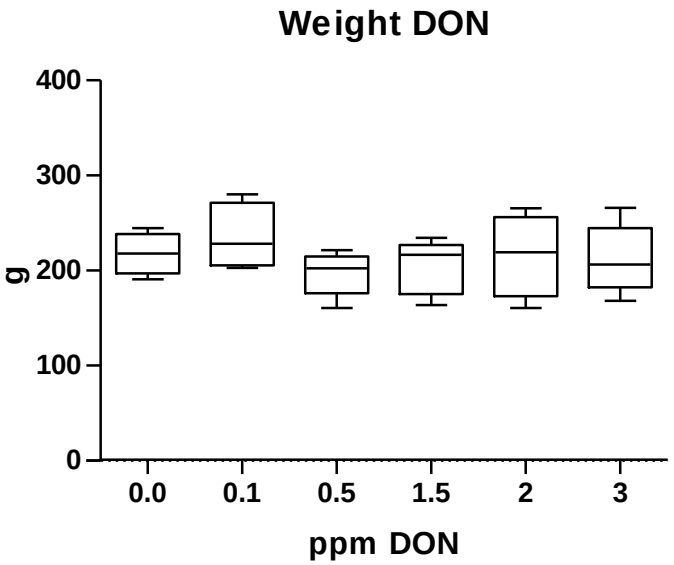

Figure 3.3: The graph shows the final average weight (75 days post hatching) for the fish in each group. Each bar represents the minimum and maximum values ± SEM for the groups.

Condition factor and specific growth rate was also calculated to evaluate performance for each of the groups. There were no significant differences between the evaluated performance parameters. The values for each diet are presented in table 3.5.

Table 3.2: Performance parameters for the different diets. Results are presented as mean ± SD

DON concentration (ppm)	Weight (g)	Length (mm)	Condition factor	Specific growth rate	ANOVA
0	218 ± 84	26.9 ± 4	1.1 ± 0.2	4.8 ± 0.2	ns
0.1	235 ± 86	27.2 ± 3	1.2 ± 0.2	5.0 ± 0.3	ns
0.5	197 ± 59	26.6 ± 2	1.0 ± 0.1	4.6 ± 0.3	ns
1.5	204 ± 70	26.5 ± 3	1.1 ± 0.2	4.7 ± 0.3	ns
2	216 ± 68	26.9 ± 3	1.1 ± 0.1	4.8 ± 0.4	ns
3	177 ± 76	26.7 ± 3	0.9 ± 0.1	4.8 ± 0.4	ns

3.4 Mid intestine gene expression

Bt -maize feeding trial

A set of transcripts encoding proteins involved in oxidative stress (CuZn sod, mapk 14, cyp1A), the cell cycle (caspase 6, cyclin G1, PCNA), the immune system (interleukin 6), appetite regulation (ghrelin) and intestinal function (maltase glucoamlyse, sodium glucose co-transporter) were quantified in the mid intestinal tissue. Mean normalized expression (MNE) for the investigated gene transcripts were between 0.5-1.3. Figure 3.4 show all of these transcripts presented as mean normalized gene expressions (MNE) ± standard error of the mean (SEM). Dietary cry1Ab and natural DON exposure did not affect any of these. The transcript encoding protein for mapk 14 and ghrelin were the only one showing a trend towards significant difference, with $p=0.06$ and $p= 0.16$ respectively, where the group feed GM maize were highest for both. The transcripts for interleukin 6, ghrelin and cyp1A are also slightly higher for the GM maize group. For the remaining genes, caspase 6, maltase-glucoamylase, sodium glucose co-transporter, cyclin and PCNA, the transcript results are nearly equal.

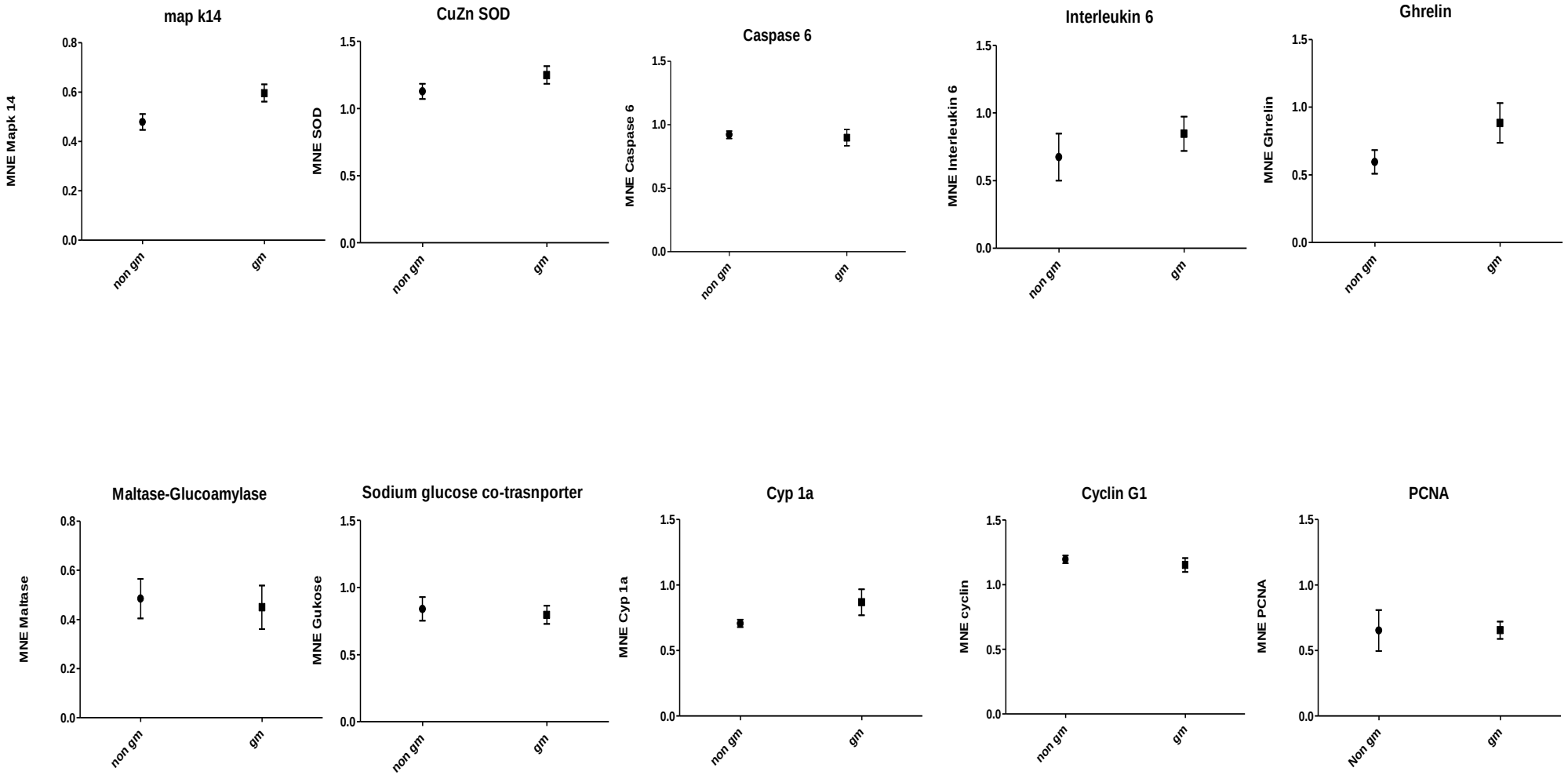

Figure 3.4: The MNE data for each gene transcript for the *Bt* -maize trial. The graphs are given as mean (n=3) ± SEM

DON feeding trial

A set of transcripts encoding proteins involved in oxidative stress (CuZnSOD, mapk 14, cyp1A), the cell cycle (caspase 6), the immune system (interleukin 6), appetite regulation (ghrelin) and intestinal function (maltase glucoamlyse, sodium glucose co-transporter) were quantified in the mid intestinal tissue. Mean normalized expression (MNE) for the investigated gene transcripts were between 0.5-1.5 for all genes. Figure 3.5 shows all of these transcripts presented as mean normalized gene expressions (MNE) ± standard error around the mean (SEM). There were no significant differences between the dietary groups, and there were no dose response to the increase in DON concentration. The dietary groups with low DON concentrations (0.0, 0.1, 0.5) have a trend towards slightly higher transcripts for both caspase 6, sodium glucose co-transporter, Mapk 14, CuZnSOD and cyp1A. Interleukin 6, ghrelin and maltase-glucoamylase all have scattered variations for all the groups.

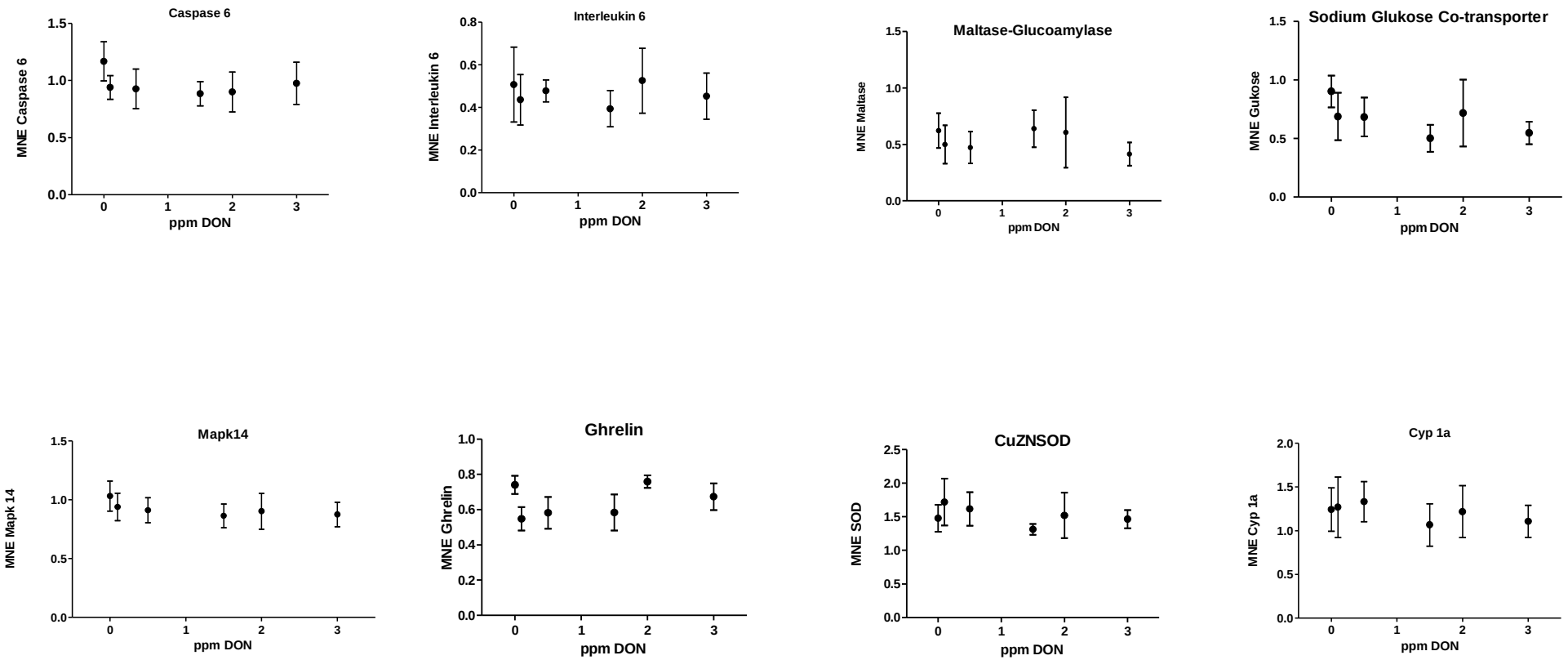

Figure 3.5: Average MNE for the gene transcript evaluating mid-intestinal health for increasing DON contamination (n=5) ± SEM

3.5 Differential counts of white blood cells

There were big differences within each group for the differential count of the white blood cells and there was no significant difference between the dietary groups for either of the trials.

The picture below illustrates the different types of cells that were categorized. The lymphocytes dominated with an occurrence of around 90 % on average.

Figure 3.2: Pictures of the different blood cells (X40) that were differentiated during blood cell count.

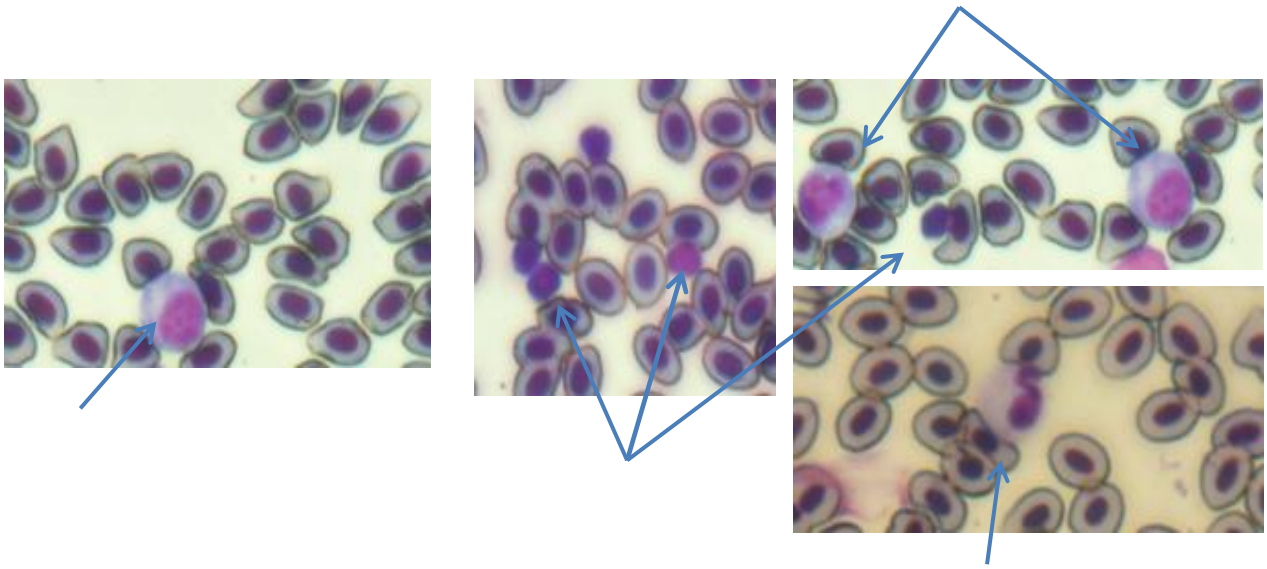

Table 3.6: The average values of the different white blood cells for both trials. The average values are based on individual fish for each of the dietary groups.

	DON cocentration (ppm)						Maize diets	
Leucocytes	0.0	0.1	0.5	1.5	2	3	Non-GM	GM
Lymphocytes	90 ± 5	87 ± 7	93 ± 3	91 ± 5	91 ± 6	88 ± 5	81 ± 14	89 ± 4
Monocytes	7 ± 4	11 ± 8	7 ± 3	8 ± 5	9 ± 6	8 ± 4	13 ± 10	9 ± 3
Granulocytes	3 ± 3	3 ± 8	0 ± 0	2 ± 5	1 ± 1	4 ± 3	5 ± 4	3 ± 1

4 Discussion

4.1 Methodological considerations

4.1.1 Feeding and design

Although each tank was given an equal amount of feed, there is no guaranty that the feed was distributed evenly amongst the fish. The fish were always hungry after feeding and perhaps less restricted feeding could have given alterations in our results. Hierarchy behavior in the tank, due to a restricted diet, could explain some of the difference within the groups

The experimental design for the DON feeding trial is strong as it has 4 and 5 replicates for each of the DON concentrations, however, there were only 3 replicates for the *Bt* –maize feeding trial. Additional tanks could have enhanced the trend towards difference observed in growth and mapk14.

Also, to improve the relevance of the *Bt* -maize feeding trial, additional diets should have been included so that the data could be compared to standard feed also.

4.1.2 Gene expression analysis

The isolation of RNA by the principles of acid guanidinium thiocyanate-phenol-chloroform extraction and gene expression quantification by quantitative Real-Time reverse transcription polymerase chain reaction are well established methods both in the scientific community (Chomczynski and Sacchi, 2006, Bustin, 2000), and at the molecular lab at NIFES where the samples were prepared. In addition are there several measurements during the sample preparation stages to ensure that the quality of the samples is satisfactory to ensure reliable results. The intestinal samples that were below 1.8 for the 260/230 ratio measured by the nanodropp, or below 7 for the RIN number after isolation of the RNA were eliminated from the trial. There are several elements to be aware of in the process leading up to QRT PCR, where a number of the problems that can occur are most likely to originate from the RNA isolation step (Bustin and Nolan, 2004). It is generally recommended in scientific literature to extensively test the RNA integrity for each sample, however, the routine at the lab only test a random selection of the isolated samples anticipating that if the quality of those are good, so are the rest of the samples that have been treated in the same way.

If some of the samples were degraded it could cause false differences when measured by the QRT PCR. The RNA integrity, defined by the RIN number, was excellent (above eight) for all the randomly collected samples that were tested for our trial indicating that there is a homogenous quality amongst the samples.

There were no known obstacles during the laboratory procedures that should reflect on the outcome of the results.

4.1.3 White blood cell differentiation

The blood smears were of very different quality as collecting blood from the zebrafish was not an easy task. The blood collected from the tail will most likely contain other body fluids as well, and possibly water from the outside of the fish. Because of this, the blood was not as viscous as it should be and it was difficult to perform the smears. The smears that were of low quality were not counted to obtain a reliable result. Other ways of collecting blood like heart puncture could have been explored before deciding on method.

4.2 Feed ingredients and diets

The level of deoxynivalenol (DON) in cereals found in the survey by the Norwegian food safety authority had an average DON contamination around 0.2-0.3 ppm (Clasen and Børsum, 2012). This level is lower than the naturally contaminated GM maize ingredient used for our study which was 0.7 ppm. An inclusion level of 19 % was used for our maize diets resulting in a DON concentration in the GM maize diet of around 0.1 ppm. This is much lower than the guidance value given for DON in complementary and complete feeding stuffs which is 5 ppm (EU, 2006). DON contamination of wheat, barley and maize has had an increasing focus in the later years, and there has been reports of growing problems which are suggested to be due to climate changes (McMullen et al., 1997). However, there is a lack of knowledge regarding the effects of DON and mycotoxins in general in aquaculture species. Adverse effects on performance and health has been found in Atlantic salmon fed DON levels below this guidance value (Döll et al., 2010). In addition to the presence of DON, the GM maize diet also contained the transgenic protein, cry1Ab. Exactly how much cry1Ab is expressed by the GM maize is dependent of the event, growth stage and tissue.

Few scientific investigations have been performed with focus on the stability of the amount of cry1Ab expressed from event MON810 which was used for our trial (Nguyen and Jehle, 2007). The producer of the event, Monsanto, has reported of stable values of cry1Ab from this event, however the average numbers reported from the producer deviates from the investigations made by Nguyen and Jehle (2007). Our maize was only qualitative tested to verify the presence of the toxin and did not determine the amount. For further discussion, I will assume that the average amount of cry protein produced in the whole plant from event MON 810 be 4 ppm wet weight, based on the results from Nguyen and Jehle (2007). This gives, with an inclusion level of 19 %, approximately 0.8 ppm feed. In a salmon study by Sanden et al. (2005) was the amount of cry1Ab in the maize ingredient measured to 0.11 to 0.13 ppm which is much lower than reported by both Monsanto and Nguyen and Jehle (2007). Sanden et al. (2005) concluded that the cry1Ab level in the feed, with an induction level of 12 %, did not affect the fish considerably. One of the reasons why few studies provide information on the amount of cry1Ab in the maize used for the trial might be because there are usually no distinctive observed effects. However, a recent study with human cells showed that a concentration of 100 ppm cry1Ab caused cell death, although much higher than what generally occurs, disagreeing that cry1Ab is totally harmless for other species that the European corn borer (Mesnage et al., 2012).

The increased level of DON that was found in our GM maize ingredient has also been reported in other studies (Sissener et al., 2011a). It was discussed by Sissener et al. (2011a) that the DON contamination could be a confounding factor in trials investigating effects of *Bt* -maize. However, higher levels of DON in *Bt* -maize is contradictory to several studies where *Bt* -maize expressing cry1Ab protein has resulted in lower mycotoxin concentration compared to the near-isogenic traditional maize (Bakan et al., 2002, Ostry et al., 2010). Zeralenone and fumonisin are clearly reduced in *Bt* -crops, while the relationship towards DON is not so distinctive (Ostry et al., 2010). Magg et al. (2002) investigated several locations over two years and found that even though *Bt* -maize significantly reduced the amount of damage on the maize crops overall, it did not consistently reduce the concentration of DON over all locations and years. The conflicting results indicate that the effects of the *Bt* -maize show a discrepancy between locations. This might be correlated to the proven variation in amount of cry1Ab expressed within the same event. Also, since the effect of the GM maize is usually compared to its near-isogenic parental line, the qualities of this might also influence the effect *Bt* -maize displays.

To be able to differentiate between primary effects (cry1Ab) and secondary effects (DON) in fish fed the GM maize diet, a regression design with increasing levels of DON was run in parallel with the maize trial. The diets in this study were spiked with increasing level of DON to specifically cover the levels of DON that was found in the contaminated GM maize and also what would naturally occur in contaminated maize (Griessler et al., 2010). The diets for the two trials were the same except for the starch source which was dextrin for the DON diets to eliminate further mycotoxin contaminations. The level of DON was also analyzed in the non-GM maize ingredient and was only 0.04 mg/kg. It was also found low levels of aflatoxin (0.02 mg/kg) and fumonisin (B1, B2, B3 0.15 mg/kg), these levels were however considered too low to have any effects in the fish at an inclusion level of 19 % maize.

4.3 Growth performance

All tanks in this study were given an equal amount of feed twice every day and all feed was rapidly ingested, indicating very good feed acceptance. The zebrafish given GM maize did not show any difference in feed acceptance when visually compared to the fish feed traditional maize. The same was observed in the DON trial except for the 3 ppm group which seemed hungrier. However, at the end of the trial there were no significant differences in growth, length, SGR or CF between any of the groups for either of the trials. Even though not significant, the average growth of the fish feed GM maize was slightly higher compared to the non GM group. This is consistent with findings by Sissener et al. (2010) where zebrafish fed GM maize had a higher weight. Increased growth was also found in a feeding trial with pigs using the same batch of maize as used in our study (Walsh et al., 2012). Results from the *Bt* -maize feeding trial indicate that the cry1Ab expressed by *Bt* -maize have little or no effect on feed intake and growth of the fish. And if cry1Ab has an effect, it mighth be speculated that it actually enhances growth. Otherwise else, perhaps the low DON levels could be enhancing the growth. In the DON trial, fish fed 0.1 ppm DON, the same concentration of DON as in the GM maize diet, had the highest average growth, although not significant. Even though DON has been shown to be imunostimulatory, it has not been found to increase growth (Pestka et al., 2004). When Atlantic salmon (*Salmo salar)* was given GM maize contaminated with DON the opposite was observed (Hemre et al., 2007).

The conflicting results indicate that different aquatic animals might have different responses to the *Bt* -maize regardless if the effects are caused by the cry1Ab protein or confounding factors. When comparing our growth data with the reference growth curve for zebrafish established by Gomez-Requeni et al. (2010), our fish had a lower weight that expected form their results. However, there are several components that differ between the conditions for our zebrafish. Their fish were fed ad libitum on formulated feed and *Artemia naulii* and were kept in bigger tanks with lower fish density that for our trial. These differences provide one explanation why the growth for our trial deviate from the growth in the Gomez-Requeni et al. (2010) trial.

4.4 Mid intestine – gene expression analysis

A total of eight genes were investigated for both feeding trials, which were selected based on reported effects of DON in other animals and results from previous studies with GM maize. Our results indicate that the zebrafish mid intestine is not very sensitive to low levels of cry1Ab in combination with natural DON or synthetic DON as none of the investigated gene transcripts were significantly different between the feeding groups.

Mitogen-activated protein kinases (mapks) are reported to be markers of ribotoxic stress and important transducers of downstream signaling events related to immune response and apoptosis (Pestka et al., 2004). Ribotoxic stress is a mechanism which has been linked to the presence of DON where DON can, by binding to the ribosomes, cause activation of the mitogen-activated protein kinases (Mapks). The activation of the Mapks control intracellular events and are related to immune responses and cell death (Pearson et al., 2001). The presence of DON might act differently on the same mechanism as it has been proven that low doses of DON can be imunostimulatory, whilst high doses cause immunosuppression (Pestka et al., 2004). The results from both trials indicate that this might be the case of our results. The fish feed GM maize with low DON concentration (0.1 ppm) showed a trend towards increase gene transcript levels of mapk14 compared to the non-GM group. The same was observed in the DON trial where the low concentrations (0.0, 0.1 and 0.5) had a higher MNE of mapk14 compared to fish feed higher DON concentrations (1.5, 2 and 3).

CuZnSOD, which is a cytoplasmic antioxidant involved in combating cellular oxidative stress (Krishnaswamy et al., 2010), was higher for the low DON doses and the *Bt*-maize.

This could indicate a protection of the cells against the toxic mechanisms of DON when only low doses are present.

The hormone ghrelin, which is involved in the seronergic system, is associated with hunger and appetite, increases before feeding and increase during meals (Cummings, 2006). An increased level of ghrelin stimulates appetite and could increase both meal size and frequency. There was a trend towards higher ghrelin expression in fish feed GM maize compared to the fish fed non-GM maize, which correlates well with the trend towards increased growth for this group. This may indicate that the amount of DON present in the feed is not enough to reduce the appetite of the fish. Keeping in mind that pigs are considered highly sensitive to DON contamination; reduced appetite was neither observed for pigs at this level (Prelusky et al., 1994). There are variations in MNE for ghrelin between all the groups in the DON trial, none of which do correlate to the growth of the dietary groups.

The level of starch is approximately the same in the GM and DON diets and similar to the starch level in the diets used in the salmon trial with GM maize by Hemre et al. (2007). In the study by Hemre et al (2007) increased maltase enzyme activity and glucose uptake was observed for the Atlantic salmon fed GM maize. These results were not reproduced in our trial as there were no significant differences between any of the diets. The differences might be explained by the investigated parameter, being gene transcription in our study and protein levels in the study by Hemre et al. (2007). Investigations on plasma glucose could be an additional way of investigating if there were changes in starch digestibility for the high DON concentrations. Furthermore, the findings by Hemre et al. (2007) are from salmon, and it is known that there are large differences in how a fish species digest starch in the feed (Krogdahl et al., 2005). Zebrafish might have a higher ability to digest starch than salmon, which might explain why there was now apparent difference in MNE of these two genes selected as markers of intestinal transport. Caspase 6 was investigated because it may be a sensitive biomarker both related to the primary factor (cry1Ab) and secondary factor (DON). DON is a known inducer of programmed cell death in relations to downstream signaling events of mapks (Pestka, 2008). Caspase 6 is part of a group consisting of apoptosis-related cysteine peptidases which are responsible for the apoptosis execution. To obtain homeostasis in tissues, both cell proliferation and cell death need to be controlled by regulatory genes, but in addition programmed cell death can be affected by outside stimuli (Soldani and Scovassi, 2002). Caspase 6 cleaves poly (ADP-ribose) polymerase, which plays a key role in cell

reparation, and destroys lamins which are support structures in the cell nucleus (Soldani and Scovassi, 2002). There is no indication of an overexpression of caspase 6 based on our data suggesting that there low doses do not increase apoptosis.

Dietary exposure to DON is known to induce rapid and temporary up regulation of pro-inflammatory cytokine expression in mice. The latter are known to induce several suppressors of cytokine signaling (SOCS), some of which impair the growth hormone (GH) signaling resulting in reduced growth (Amuzie et al., 2009). Interleukin 6 belongs to a family involved in phase-response during infection and is an essential mediator for immunotoxicity (Moon and Pestka, 2003). Increased levels of interleukin 6 could indicate that the fish experiences disturbed physiological homeostasis due to toxic substances (Heinrich et al., 1998). There were no significant differences on gene transcript levels of interleukin 6 between fish fed any of the diets, although the levels for the fish fed GM feed were slightly elevated. Activation of interleukin 6 is connected to the Mapk mechanism, and viewing the results alongside the trend towards increase of mapk14 for the same group indicates that there could be a minor response in the fish which could be increased with higher concentrations of DON.

The trend towards an increase of Cyp1A gene transcripts in fish fed the GM maize diet was not observed for the equivalent DON concentration in the DON trial. The Cyp1A function as a detox mechanism for DON and the trend towards an increased MNE for the fish fed the GM maize diet could be related to the low doses of DON, or the presence of cry1Ab protein since the difference was only observed in the *B*t-maize trial.

Proliferating Cell Nuclear Antigen, PCNA, is a protein used as a marker for cell proliferation potential. PCNA is found in eukaryotic cells and is essential for DNA replication by binding to polymerase-δ and D-cyclins to initiate cell cycle progression (Tiemann et al., 2003). A change in cell proliferation could signal further damage to the intestinal track (Sanden et al., 2005). However no such changes were seen in the present study, which correlate well with the lack of difference for cyclin G1 as well, which can function as an inhibitor of cellular proliferation (Kimura et al., 2001).

Since there are no significant results for the genes tested for the present study, it could be thought that the low doses of DON are not high enough to alter gene transcripts. However a study performed on broilers chicken showed that concentrations as low as 2.5 ppm could

significantly alter gene expression in the liver and jejunum (Dietrich et al., 2012), In addition it might be supposed that the zebrafish mid-intestine is not a target organ for DON damage.

Unpublished results on gene transcription level of liver taken from the same zebrafish as this study indicate that zebrafish liver is the place where the detoxifying of DON takes place (Sanden et al., 2012). The study by Hooft et al. (2011) supports this theory as they found damages to the liver, and not the intestine in rainbow trout (*Oncorhynchus mykiss)* fed 2.6 ppm DON. The fact that not all organs are effects by DON in the same way was also illustrated by varied effects on protein synthesis in different organs of pigs (Danicke et al., 2006).

4.5 Blood – white blood cell differentiation

Blood has been shown to be a target for DON (Borutova et al., 2008). And in a study by Sagasad et al. (2007) they found increased percentage of granulocytes in salmon feed *Bt* - maize. Observed effects on blood could therefore be caused by either DON or cry1Ab. An alteration in the granulocyte level could indicate an immune response in the blood. However, there were no significant differences between the amount of lymphocytes, granulocytes or monocytes amongst the different diets.

Considering the results for the *Bt*- maize feeding trial collectively, with regards to the use of GM maize in aquaculture, low doses of naturally contaminated DON in combination with cry1Ab do not seem too affect fish performance or intestinal gene transcripts. As illustrated by the ELISA test, the cry1Ab will be destroyed during feed extrusion, and the natural DON contamination of 0.1 ppm seems too low to cause effects.

A fact to be aware of when evaluating the results from the DON feeding trial is that there has been showed differences between naturally and synthetic DON in the feed (Trenholm et al., 1994), where naturally contamination has shown to cause greater effects than synthetic DON. When the cereals are naturally contaminated, which would be the case in normal settings, there will also most likely be other mycotoxins present. Therefore, even though the DON levels for this trial are representative for natural circumstances, the same levels should also be investigated using ingredients which are naturally contaminated with *fusarium* spp.

5 CONCLUSION

Based on the results from the two trials, have we come to the conclusion that;

1) Cry1Ab in the *Bt*- maize feed do not significantly affect growth, intestinal mRNA or white blood cell composition when feed to zebrafish,. The DON contamination of 0.1 ppm in the *Bt*-maize feed does not affect the measured parameters either.
2) The increasing concentrations of synthetic DON appear to be too low to significantly affect growth, intestinal mRNA or white blood cell differentiation in zebrafish.

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Appendix

Appendix I: Feed ingredients

The specifications of the ingredients for the two different feeding trials. All ingredients are the same except that for the *Bt* -maize trial was not potato starch (5) or DON (10) added.

1Casein from bovine milk (Sigma Life Science, St Louis, MO, USA)

2 Gelatin from porcine skin (Fluka BioChemica, Buchs, Switzerland)

3 Möller's Tran, Axellus AS, Oslo, Norway

4 Eldorado, Oslo, Norway

5 Potato starch, Hoff Norske, Gjøvik, Norway

6 Refined, Alfa Aesar, Karlsruhe, Germany

7 Mineral mix; 66% dicalcium phosphate dehydrate, 0.014% cobalt chloride hexahydrate, 0.04% copper sulphate, 30% potassium sulphate, 0.1% potassium iodide, 2% magnesium sulphate heptahydrate, 0.1% manganese sulphate, 5.7% sodium chloride, 0.01% sodium selenite, 1% zinc sulphate heptahydrate, 1% iron sulphate heptahydrate

8 Vitamin mix; 0.1% vitamin A (500.000 IU), 0.04% vitamin D3 (500.000 IU), 2% vitamin E, 0.1% vitamin K, 4.3% vitamin C, 40% choline, 0.15% thiamine, 0.19% riboflavin, 0.2% pyridoxine, 2% niacin, 4% inositol, 0.05% folic acid, 0.6% calcium panthotenat, 0.75% biotin, 0.3% cobalamin, 45.2% casein salt

9 AA mix; 4.3% taurine, 21.4% aspartic acid, 6.4% threonine, 8.5% glycine, 15.2% alanine, 4.3% valine, 5.8% methionine, 4.3% isoleucine, 8.5% leucine, 6.4% lysine, 8.5% arginine, 6.4% tryphtophan

10 Synonyms; 3α,7α,15-Trihydroxy-12,13-epoxytrichothec-9-en-8-one, Vomitoxin. Empirical formula, C15H20O6 (Sigma Life Science, St Louis, MO, USA)

11 Maize event mon810 produced in spain 2010/2011

Appendix II: RNA extraction

Table II A Chemicals and reagents used for RNA extraction.

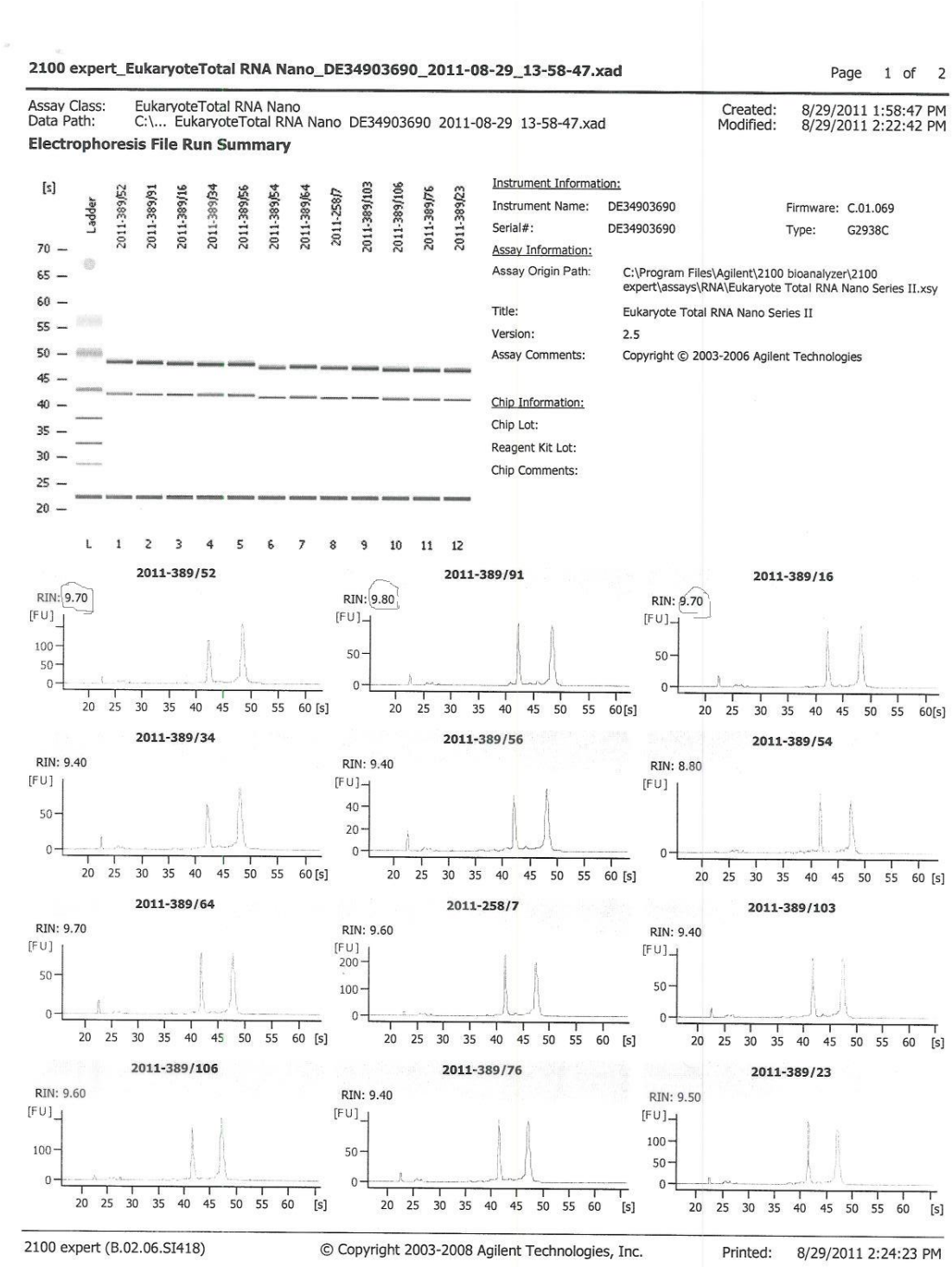
Product name	**Vendor**
Trizol	Invitrogen art.nr. 15596-026, USA
Chloroform	Merck, Germany
Isopropanol	Arcus, Norway
Ethanol	Arcus, Norway
DEPC (Diethyl pyrocarbonate)	Sigma art.nr. F32490
RNase free ddH2O	MilliQ Gradient, Lab-tec, Norway
RNase Zap	Sigma art.nr. R2020, USA

Appendix III: Agilent 2100 Bioanalyzer

Table III A Chemicals and reagents used for RNA quality measurements by the Agilent 2100 Bioanalyzer.

Product name	**Vendor**
RNA 6000 Nano Labchip kit	Agilent Technologies art.nt.5065-4476
RNA 6000 Ladder	Ambion art.nr. 7152
RNase free ddH2O	MilliQ Gradient, Lab-tec, Norway

Appendix III Figure A: An example of how the results from the agilent bioanalyzer are presented. The RIN are circled for the three firth graphs.



Appendix IV: RT-reaction

Table IV A Chemicals and reagents used in RT-reaction .

Product name	**Vendor**
TaqMan RT buffer 10X	Applied Biosystems art.nr. N808 0234
25 mM Magnesium chloride	Applied Biosystems art.nr. N808 0234
10 mM deoxyNTPs	Applied Biosystems art.nr. N808 0234
50 µM Oligo d(T)16 primer	Applied Biosystems art.nr. N808 0234
RNase inhibitor (20 U/µl)	Applied Biosystems art.nr. N808 0234
Multiscribe reverse transcriptase (50 U/ µl)	Applied Biosystems art.nr. N808 0234
RNase free ddH2O	MilliQ Gradient, Lab-tec, Norway

Table IV B RT- reaction mix for a 30 µl reaction mix .

Properties	**Reagent**	**Amount (µl) for 96 wells**
Reagents without enzymatic properties	RNase free water	267
TaqMan RT buffer 10X		150
25 mM Magnesium chloride		330
10 mM deoxyNTPs		300
50 µM Oligo d(T)16 primer		75
Enzymes	RNase inhibitor (20 U/µl)	30
Multiscribe reverse transcriptase (50 U/ µl)		50,1

Table IV C Reverse Transcriptase reaction conditions .

Step	**Temperature (°C)**	**Time (minutes)**
Incubation	25	10
Reverse Transcriptase	48	60
Reverse Transcriptase inactivation	95	5
End	4	∞

Appendix V: One step test of primers

Table V A: Amount of reagents for testing of primers.

Reagent	**Volume**	**Final concentration**
5 x QIAGEN One step RT-PCR buffer	10 µl	1 X
dNTP mix	10 µl	5 X
RNase fritt vann	19 µl	(final volume 50 µl)
Primer forward	3 µl	0.6 µM
Primer reverse	3 µl	0.6 µM
QIAGEN One step RT-PCR MIX	2 µl	
RNA template	1 µl	

Table V B: RT-PCR cycle for One step gene test.

Proses	Time	Temperature
Reverse transcriptase	30 min	50C
PCR activating	15 min	95C
3 step cycle		
Denaturizing	45 sek (30-60)	94C
Annealing	45 sek (30-60)	55C (50-68)
Extension	1 min	72 C
Number of cycles		33 (25-40)
Final extension	10 min	72C

Appendix VI : Quantitative Real-Time PCR

Table VI A Chemicals and reagents used for real-time PCR.

Product name	**Vendor**
SYBR GREEN Master	Roche, Norway
Primer	Invitrogen Ltd, UK
RNase free ddH2O	MilliQ Gradient, Lab-tec, Norway

Table VI B: SYBR GREEN reaction mix for Light Cycler 480 (10 µl reaction).

Reagent	Volume (µl)
RNase free water	331
Forward primer	11.4
Reverse primer	11.4
SYBR GREEN Master	570