**Master's thesis**

# Effects of fumonisin $B_1$ on performance of juvenile Baltic salmon (*Salmo salar*)

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**21.02.2013**

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Carrera García Erika: Effects of fumonisin $B_1$ on performance of juvenile Baltic salmon (*Salmo salar*)
Master's thesis: 22 p.
Supervisors: Dr. Juhani Pirhonen
Reviewers: Dr. Juhani Pirhonen, Dr. Mikko Kiljunen
February 2013

Key Words: Feed intake, fumonisin $B_1$, growth, liver, mycotoxins, salmon.

## ABSTRACT

Fumonisin $B_1$ is a mycotoxin produced by fungi of the genus *Fusarium* that frequently occurs on maize (*Zea mays*) and feeds containing it. The use of plant-based protein sources in feeds designed for aquaculture has increased due to their low costs compared with fishmeal. This trend has resulted in a global increase in feed formulations contaminated with mycotoxins, producing economical losses in aquaculture industry. The topic also causes concerns because of the potential health consequences that aquaculture products could have on human consumers. $FB_1$ mainly disrupts sphingolipid metabolism and also has immune suppressive effects. Fish mycotoxicosis produces a wide range of symptoms from poor growth rate and weight gain to reproductive, immune, liver and kidney disorders that can lead to mortality. Despite its importance, very little is known about the effects of $FB_1$ in Baltic salmon, *Salmo salar*. In this study growth performance, feed intake, mortality and liver histopathology of juvenile salmon exposed to $FB_1$ doses 0, 1, 5, 10 or 20 mg/kg feed was evaluated. The hypothesis was that $FB_1$ ingestion would reduce salmon growth, feed intake and would produce liver damage. At the end of the 10-week experiment no differences in the evaluated parameters were found. Species-specific differences in vulnerability because of variations in toxin metabolism could explain the results. However, due to the slow growth of fish during the trial additional research to confirm the results are suggested.

JYVÄSKYLÄN YLIOPISTO, Matemaattis-luonnontieteellinen tiedekunta

Bio- ja ympäristötieteiden laitos
Kalabiologia ja kalatalous

Carrera García Erika: Effects of Fumonisin $B_1$ on performance of juvenile Baltic salmon (*Salmo salar*)
Pro gradu: 22 s.
Työn ohjaajat: FT Juhani Pirhonen
Tarkastajat: FT Juhani Pirhonen, FT Mikko Kiljunen
Helmikuu 2013

Hakusanat: fumonisin $B_1$, kasvu, lohi, maksa, mykotoksiini, ravinnonotto

## TIIVISTELMÄ

*Fusarium* -suvun homeet tuottavat mykotoksiineja, joista yksi on fumonisin $B_1$ ($FB_1$). Näitä myrkkyjä on erityisesti maississa ja siitä tehtävissä ruuissa. Kalanviljelyssä on suuntaus käyttää yhä enemmän kasviperäisiä raaka-aineita kalajauhon sijasta, mikä on johtanut mykotoksiinivaaran kasvuun kalanrehuissa. Mykotoksiinit voivat olla haitta paitsi suoraan kaloille niin myös ihmisille, jotka syövät mykotoksiinirehua syöneitä kaloja. $FB_1$ häiritsee sfingolipidien metaboliaa mutta se voi myös vaikuttaa immunosupressiivisesti. Kaloissa mykotoksiinit voivat aiheuttaa monenlaisia oireita kuten heikentynyttä kasvua, ongelmia lisääntymisessä ja immuniteetissä sekä häiriöitä maksassa ja munuaisissa. Nämä ongelmat voivat johtaa lisääntyneeseen kuolleisuuteen. Mykotoksiinien merkityksestä huolimatta niiden vaikutuksista loheen (*Salmo salar*) ei juurikaan tiedetä. Tässä 10 viikon kasvatuskokeessa tutkittiin viiden $FB_1$ -tason (0, 1, 5, 10 or 20 mg/kg rehua) vaikutusta lohen kasvuun, ravinnonottoon, kuolleisuuteen ja maksan histopatologiaan. Hypoteesina oli, että $FB_1$ vähentää ravinnonottoa, hidastaa kasvua ja aiheuttaa maksavaurioita. Kokeen lopussa ei havaittu tilastollisesti merkittäviä eroja missään mitatussa tai lasketussa muuttujassa. Selityksenä saattaa olla lajienväliset erot herkkyydessä mykotoksiinille, mutta kalojen heikosta kasvunopeudesta johtuen tarvitaan uusi kasvatuskoe tulosten varmistamiseksi.

# Contents

## 1. INTRODUCTION

The use of plant-based protein sources in feeds designed for aquaculture has increased in the last decade. This is due to their lower costs compared with fishmeal (Spring & Fegan 2005, Santos et al. 2010, Encarnação 2011), and its production is believed to be declining (Josupeit 2010). Aditionally, during El Niño years it can fluctuate (Jackson 2012). Therefore, plant-based protein sources are considered a sustainable alternative to fishmeal based feeds (Spring & Fegan 2005, Encarnação 2011). Production of carnivorous species still requires considerable input of fishmeal (Naylor et al. 2000) but it has been replaced successfully in diets of some omnivorous and herbivorous species (Spring & Fegan 2005). However, this trend is not a panacea because plant-based aquafeed formulations are commonly contaminated with mycotoxins (Manning 2001, Spring & Fegan 2005, Santacroce et al. 2008, Santos et al. 2010, Encarnação 2011).

Mycotoxin contamination of feedstuffs is a serious threat to animal welfare and production, and can result in dramatic economic losses (Hooft et al. 2010, Santos et al. 2010, Iheshiulor et al. 2011). It has been estimated that 25% of the world's feed crops are affected by mycotoxins (Hooft et al. 2010, Iheshiulor et al. 2011), and that feed is contaminated with one or more mycotoxins (Santos et al. 2010). In the USA alone, it is believed that 90 million dollars in grain production are lost every year to mycotoxin contamination (Griessler & Encarnação 2009). Mycotoxins can be mutagenic (Anonymous 2002), hepatotoxic (Manning 2001, Santos et al. 2010), carcinogenic (Manning 2001, (Anonymous 2002, Spring & Fegan 2005), teratogenic (D´Mello et al. 1999) and often impair immune and reproductive function leading to poor growth rates and increases mortality in animals (D´Mello et al. 1999, Manning 2001, Spring & Fegan 2005, Santacroce et al. 2008, Santos et al. 2010). These effects depend on the species, age, sex, and nutritional and health conditions of the animal, as well as on the types of mycotoxins ingested, their concentration and time of exposure (Manning 2001, Spring & Fegan 2005, Santacroce et al. 2008, Griessler & Encarnação 2009, Santos et al. 2010).

Further, mycotoxins have been responsible for outbreaks of illnesses in humans (Pitt 1989). Historically, ergotism and toxic aleukia have been noteworthy as each resulted in thousands of deaths in Europe and the Soviet Union, respectively (Pitt 1989). Other outbreaks have killed tens of thousands of livestock in the Soviet Union and over one hundred thousand turkeys in England (Pitt 1989).

Mycotoxins are of increasing concern in aquaculture because mycotoxin contamination is persistent in fish flesh (Spring & Fegan 2005, Santacroce et al. 2008, Santos et al. 2010) and residues have been found in marketed fish products beyond acceptable levels (Santos et al. 2010).

What are mycotoxins, and where do they come from? Mycotoxins are produced by molds of the genera *Aspergillus*, *Penicillium* and *Fusarium* in substrate of plant origin (e.g. maize (*Zea mays*), rice (*Oryza* spp.), peanuts (*Arachis hypogaea*), cotton seeds (*Gossypium* spp.)) (Santacroce et al. 2008). Mycotoxins are secondary metabolites that accumulate in fungal spores, vegetative mycelium, and in the substratum. These metabolites are not essential for fungal metabolic functions, but act as competitive compounds that protect the mold against other organisms that could compromise its survival (Santacroce et al. 2008). These molds are common phytopathogens, responsible of crop diseases that have proven to be difficult to control. Indeed, crop contamination by mycotoxins is considered unavoidable (D'Mello et al. 1999).

Fungal growth occurs when the specific environmental conditions required by a given fungal species (humidity, temperature, oxygen) are met during crop growth, harvest, processing and storage (Manning 2001, Spring & Fegan 2005, Santacroce et al. 2008, Encarnação 2011). Conditions for fungal growth varies greatly along the cycle from crop growth to crop storage, leading to the possibility that different fungi with divergent environmental requirements will produce a diverse mix of mycotoxins accumulating on the plant substrate over the various stages of crop production (Spring & Fegan 2005).

## 1.1. *Fusarium* mycotoxins

*Fusarium* fungi are among the three most widespread genera (*Aspergillus*, *Penicillium* and *Fusarium*) in crops. These three genera of fungi produce more than 400 different mycotoxins that are known as the aflatoxins, ochratoxins, fumonisins, zearalenones and trichothecenes (Santos et al. 2010). This research will focus on one of the fumonisins, fumonisin $B_1$ ($FB_1$). $FB_1$ has been recognized as a problem in aquaculture because it represents 70% or more of the total fumonisin content in naturally contaminated feed (Griessler & Encarnação 2009).

### 1.1.1. Fumonisin $B_1$

Fumonisin $B_1$ which, is produced by the genus *Fusarium,* is the best known and most toxic of the *Fusarium* toxins (Manning 2001). $FB_1$ frequently occurs with $FB_2$ and they are mainly produced by *F. verticillioides* and *F. proliferatum*, which occur predominately in maize. These species produce kernel rot in maize that is associated with warm and dry environments (Anonymous 2002). Usually, broken kernels have higher fumonisin concentration than whole kernels, and increased fumonisin concentrations are found in maize gluten and fiber produced through wet milling. $FB_1$ has been detected also in asparagus (*Asparagus officinalis*), black tea (*Camellia sinensis*), barley (*Hordeum vulgare*), rice, sorghum (*Sorghum* spp.), soybean (*Glycine max*), wheat (*Triticum* spp.) and in cereal-based products (Anonymous 2002).

#### 1.1.1.1. Fumonisin $B_1$ mechanism of action

$FB_1$ disrupts sphingolipid metabolism. Sphingolipids are essential components of cellular lipoprotein membranes and they are involved in specific functions such as cell regulation, recognition and signaling. Fumonisins inhibit the enzyme sphinganine N-acyltransferase (ceramide synthase) activity, which catalyzes sphingolipid production (Anonymous 2002, Spring & Fegan 2005, Griessler & Encarnação 2009). The inhibition is particularly effective because there are two loci on the $FB_1$ molecule that can interact with the enzyme (Anonymous 2002). Reduction of sphingolipids by fumonisins alters cellular growth control, cell-cell interactions and also results in elevated levels of free sphingoid bases (Anonymous 2002, Griessler & Encarnação 2009).

Free sphingoid bases (sphinganine and sphingosine) are toxic and persist in kidney and liver because the bases persist longer than the toxin itself, inducing cell death and leading to the accumulation of sphinganine. Sphinganine concentration is positively correlated with kidney and liver damage (Anonymous 2002, Spring & Fegan 2005, Griessler & Encarnação 2009). $FB_1$ also increases complex lipids containing sphinganine. The ceramide produced from these lipids is dihydroceramide, which is incapable of signaling and therefore failing in inducing apoptosis of damaged liver cells. In other words, cells that are responsive to the decrease in levels of ceramide will survive and multiply, while those susceptible to increases in free sphingoid bases will die. Survival and proliferation of liver cells with damaged DNA increases the risk of liver cancer

(Anonymous 2002). $FB_1$ carcinogenicity has been tested in several animals including fish (Gbore et al. 2010).

$FB_1$ also disturbs phospholipid and fatty acid metabolism. Phospholipids are part of cell membranes and fatty acids are essential precursors of other lipids that regulate cell growth, differentiation, and death. $FB_1$ increases free sphingoid bases and their metabolites alter phosphatidic acid phosphatase and monoacylglycerol acyltransferase activity (Anonymous 2002). Therefore phospholipid and fatty acid biosyntheses are interrupted. Membranes with modified phospholipids also interact with cellular processes, shifting proteins that control cell cycle progression, resulting in higher cell death and distorted propagation of liver cells. In humans an indicator of $FB_1$ exposure is the elevation of free sphinganine in urine and blood and (Anonymous 2002).

The capacity of $FB_1$ to impair sphingolipid metabolism has been studied in plants, mammals including humans (Anonymous 2002), poultry (Anonymous 2002, Tardieu et al. 2006, Tardieu et al. 2007) and fish (Pepeljnjak et al. 2002, Spring & Fegan 2005). In all animal species analyzed, $FB_1$ has been proven to be both hepatotoxic and nephrotoxic (Anonymous 2002, Pepeljnjak et al. 2002, Tardieu et al. 2007). $FB_1$ has been also related to morphological anomalies due its capacity to interfere embryos sphingolipid metabolism (Anonymous 2002, D´Mello et al. 2009).

$FB_1$ can also have adverse affects on the central nervous system in carp (*Cyprinus carpio*) (Kovačić et al. 2009). $FB_1$ is a hydrophilic molecule with low molecular weight (500 Da), which can pass through the blood brain barrier (BBB) of young individuals. The BBB is a structure that allows selective entry of oxygen and glucose into the brain and spinal cord while preventing the entry of a spectrum of large, potentially toxic molecules. Once $FB_1$ reaches the brain, it produces edemas and cell degeneration (Kovačić et al. 2009).

Further, fumonisins also have immunosuppressive effects in many species (Kovačić et al. 2009, Iheshiulor et al. 2011) as in calves (*Bos* spp.) (Pepeljnjak et al. 2002), broilers (*Gallus gallus*) (Ledoux et al. 1992, Lumlertdacha et al. 1995), fish (Gbore et al. 2010, Encarnação 2011) and pigs (*Sus* spp.) (Anonymous 2002, Iheshiulor et al. 2011). $FB_1$ reduces the amount of macrophages, inhibiting immunological function activity against pathogens and diminishing levels of antibodies IgA and IgM. $FB_1$ can therefore lead to increased vulnerability to infectious diseases (D'Mello et al. 1999, Manning 2001).

#### 1.1.1.2. Fumonisin $B_1$ effects in animals

$FB_1$ toxicity varies significantly between animals. Equines (*Equus* spp.) are extremely vulnerable and ingestion of feed containing low dosages of $FB_1$ (5–10 mg/kg), and short exposures of only a few days can produce equine leukoencephalomalacia (ELEM). ELEM is a fatal condition in horses, mules and donkeys that affect the central nervous system that results in liquefaction of the brain (Manning, 2001, Anonymous 2011). Symptoms include pharyngeal paralysis and blindness that can appear as shortly as in a few hours or a couple of days (Anonymous 2011). In pigs, $FB_1$ causes liver, lung, cardiovascular (Anonymous 2002) and immune systems damage (Iheshiulor et al. 2011), and porcine pulmonary edema (PPE) (Manning, 2001, Anonymous 2011). PPE is a lethal disease caused by abnormal filling of liquid of the pleural cavity (Manning 2001, Anonymous 2011), which appears after 3-6 days of $FB_1$ intake at levels from 15–100 mg/kg (Anonymous 2011). PPE can be produced to embryos when the sows are fed $FB_1$ in advanced stages of pregnancy, suggesting also placental transfer (Anonymous 2011).

On the other hand, cattle and poultry are more resistant; broilers fed dosages over 200–400 mg/kg present feed intake reduction, weight loss and skeletal abnormalities but not tissue lesions (Anonymous 2011). In fish $FB_1$ exposure usually results in poor growth rate, lowered feed intake, liver and kidney damage, tumors, and impairment of immune system (Griessler & Encarnação 2009, Santos et al. 2010). In lab rats (*Rattus norvegicus*), fed 1 g/kg for 33 days had severe liver damage including bile duct proliferation, fibrosis and nodules. In addition, weight loss was observed at doses ≥ 20 $FB_1$ mg/kg body weight per day and kidney damage at dosages of 15 $FB_1$ mg/kg and 50 $FB_1$ mg/kg in males and females, respectively (Anonymous 2002).

#### 1.1.1.3. Fumonisin $B_1$ in humans

In humans $FB_1$ has been linked to esophageal cancer in China and South Africa (Manning 2001, Anonymous 2002). There has also been shown a positive correlation between maize consumption, and therefore $FB_1$ intake, and human immunodeficiency virus (HIV) pathology in sub-Saharan Africa (Williams et al. 2010). When $FB_1$ disrupt sphingolipids and ceramide metabolism, cellular membrane properties are altered, suggesting an increment in permeability and therefore virus attachment to human cells, which promote the virus transmission (Williams et al. 2010).

Fumonisins are found worldwide and the Joint FAO/WHO Expert Committee on Feed Additives has set 2 μg/kg body weight of daily intake as the maximum acceptable concentration of total fumonisins ($FB_1$ + $FB_2$ + $FB_3$) in human feeds (Anonymous 2002), while the US Food and Drug Administration has set it c. 4 mg/kg (Anonymous 2000). Worldwide, the incidence of fumonisins in maize have been found to be 324/729 (samples with $FB_1$ detected/ total samples) in North America, 126/138 in Latin America, 248/714 in Europe, 199/260 in Africa, 380/633 in Asia and 67/70 in Oceania (Anonymous 2002).

### 1.1.2. Other *Fusarium* mycotoxins

Moniliformin (MON) and Trichothecenes are other mycotoxins of *Fusarium* origin (Santos et al. 2010). MON is considered less toxic than $FB_1$. Weight gain of channel catfish (*Ictalurus punctatus*) fed feed containing 20 $FB_1$ mg/kg was significantly lower than those fed the same concentration of MON (Yildirim et al. 2000). Diets containing 70–150 mg/kg MON reduced growth in tilapia (*Oreochromis niloticus*) fingerlings, but no mortality or histopathological lesions were found (Tuan et al. 2003). However, MON has been implicated on poultry mortality. Within poultry, ducklings (*Anas platyrhynchos*) are more susceptible than turkeys (*Meleagris gallopavo*), which is also more sensitive than chicks (Anonymous 2002). MON is known to have cardiotoxic effects, which produce myocardial degeneration and necrosis in all species tested (D'Mello et al. 1999).

Deoxynivalenol, also called DON or vomitoxin, is the best known of the trichothecenes group. DON has the capacity to reduce feeding responses and cause vomiting (Smith 2008, Hooft et al. 2010). Additionally, DON reduces growth and feed intake, and leads to diarrhea, gastrointestinal hemorrhages, inflammations, and immune, and renal disorders (Döll et al. 2010; Hooft et al. 2010). In fish, at cellular level, DON prevents protein synthesis by binding to the 60S ribosomal unit (Döll et al. 2010, Hooft et al. 2010). In broilers, DON increases the relative weights of the gizzard, bursa of Fabricius, and heart (D'Mello et al. 1999).

From the trichothecenes group, T-2 toxin reduces feed intake, growth, hematocrit and hemoglobin levels in fish. Moreover, mortality is increased via increased susceptibility to bacterial disease (Santos et al. 2010). In swine, T-2 toxin may cause infertility and

during last months of gestation it can induce abortions within 2 days. In turkeys and broilers T-2 toxin reduces weight gain and causes oral damage (D'Mello et al. 1999). T-2 toxin has been reported to produce a decrease in egg yield and hatchability, mortality, inhibition of ovary maturation, and adrenal and thyroid gland damage in geese (*Anser* spp.) (D'Mello et al. 1999).

Fusaric acid is a mycotoxin that is poorly known. Fusaric acid has low to moderate toxicity, but may have a role in plant pathogenesis. In animals, fusaric acid toxicity occurs mainly in combination with other mycotoxins as a result of synergistic interactions. Fusaric acid was found to affect brain and pineal neurotransmitters, elevating levels of serotonin and tryptophan (Bacon et al. 1996, Manning et al. 2001).

Zearalenone (ZEN) is a mycotoxin that acts like oestrogen and disrupts the normal physiological role of endogenous estrogenic compounds (D'Mello et al. 1999, Manning 2001, Iheshiulor et al. 2011). ZEN is considered of low toxicity because $LD_{50}$ value is reached at levels of 2–10 mg/kg body weight in mice (*Mus* sp.). In mammals ZEN disrupts endocrine function (D'Mello et al. 1999). ZEN induces vulvovaginitis, enlargement of the uterus and mammary glands, reduces embryo survival, and produces testicular atrophy (D'Mello et al. 1999). In cattle ZEN decreased milk production and produced infertility (D'Mello et al. 1999). Nevertheless, the actions of fumonisins in fish are not well understood because few studies have been done in these animals (Griessler & Encarnação 2009).

### 1.2. Synergistic effects

Incidence of multiple mycotoxins in the same feed supply is likely, which can lead to synergistic effects. In other words, the effects of an individual toxin can be magnified or altered in the presence of another toxin (Spring & Fegan 2005, Griessler & Encarnação 2009, Hooft et al. 2010). Trials suggest that for a given DON dosage, negative consequences are increased when another pollutant is added (Hooft et al. 2010). Carlson et al. (2001) reported that rainbow trout exposed to diets with concentrations of $FB_1$ between 0 and 104 mg/kg for 34 weeks did not produce tumors in the liver or kidney. However, in fish exposed previously to Aflatoxin $B_1$ ($AFB_1$, produced by *Aspergillus* fungi), $FB_1$ dosages of just 23 mg/kg in feed, led to increase liver tumors in 42 weeks. Channel catfish, when fed diets of MON and $FB_1$ at 40 mg/kg each simultaneously, exhibited significantly lower weight gain (42% of the controls) than when fed the same concentrations of mycotoxins separately (16% and 23%, respectively) (Yildirim et al. 2000).

Fumonisins alone are not a significant problem in poultry. However, combinations of toxins can have serious effects (D'Mello et al. 1999). In chicken eggs, combination of fusaric acid and $FB_1$ was fatal while individually these mycotoxins had no effect on mortality. In broilers, the combination of $FB_1$ and DON increased serum protein and urea nitrogen while combination $FB_1$ and T-2 toxin increased serum calcium levels (D'Mello et al. 1999). Mixtures of T-2 toxin and $FB_1$ produced changes in haematological values in turkeys, while treatment with T-2 or $FB_1$ alone did not. MON caused mortality in broilers, but its effects were enhanced by $FB_1$. Indeed, acute mortality syndrome in broilers is produced by the combination of MON and $FB_1$ (D'Mello et al. 1999).

Synergism among co-occurring mycotoxins is more frequent than previously thought, and these effects remain largely unknown. This area requires additional research to facilitate proper assessment of risks to animal production.

### 1.3. Residual mycotoxins in products for human consumption

Mycotoxins are gaining importance in research due to the fact that they are persistent in derivative products from agriculture and aquaculture, and represent a potential health risk to humans. Recently, studies have shown that residues in commercially available fish products can exceed acceptable levels (Santos et al. 2010). El-Sayed & Khalil (2009) reported that sea bass (*Dicentrarchus labrax*) exposed orally to $AFB_1$ by gavage (0.018 mg/kg body weight) had $AFB_1$ accumulation in their edible muscles (flesh); these residuals increased gradually with time of exposure from 0.29 ± 0.10 $AFB_1$ mg/kg on day 0 to 4.25 ± 0.85 $AFB_1$ mg/kg on day 42. Han et al. (2010) reported that residual concentrations of 0.003 mg/kg occurred in gibel carp (*Carassius gibelio*) that were fed diets of 0.01 $AFB_1$ mg/kg for 3 months.

However, in both tilapia and channel catfish, no residual toxins have been found after exposure to higher levels, presumably due to differences in mycotoxin metabolism between species (El-Sayed & Khalil 2009, Encarnação 2011). Residues can be found also in eggs, meat and offal (D'Mello et al. 1999). Hens fed contaminated feed will lay eggs with detectable levels of DON, but the fate of DON metabolites remains unknown (D'Mello et al. 1999). Swine that were fed ZEN had noticeable residues in kidney and liver. Likewise, fumonisins can also be transferred to milk but this is believed to be at minimal levels (D'Mello et al. 1999).

### 1.4. Mycotoxins amelioration

Careful selection and adequate processing of raw materials are the best way to prevent mycotoxin contamination, yet it is difficult to guarantee the complete absence of these toxins. Mycotoxin remediation and detoxification procedures are under research and development. Adsorbents can be used to bind mycotoxins in the digestive tract, thereby reducing toxin absorption. The most common substances used for adsorption are clays, bentonites, zeolites, silicas and aluminum silicates, which are effective against aflatoxins only (Griessler & Encarnação 2009, Santos et al. 2010, Encarnação 2011). Enzymatic deactivation has been used to reduce negative influence of trichothecenes, ochratoxin A and zearalenone (Santos et al. 2010). For $FB_1$, charcoal has been tested, but it also binds valuable nutrients. New methodologies, such as biotransformation, involve the degradation of mycotoxins through natural metabolic pathways. These pathways lead to detoxification via enzymatic action, some of which have been isolated and then characterized from some microbial strains (Griessler & Encarnação 2009). Chemical substances such as calcium hydroxide monomethylamine, sodium bisulphate and ammonia are also under study D'Mello et al. 1999). However, the commercial capability of the new methods has yet to be determined (Griessler & Encarnação 2009).

### 1.5. Objectives

Few studies have investigated the effects of mycotoxins on fish despite their potential impacts on aquaculture productivity and human health. Salmon (*Salmo salar*) is one of the most farmed species worldwide and surprisingly, little is known about the possible responses of salmon to mycotoxins, especially to fumonisins. The aim of this study was to evaluate the effects of fumonisin $B_1$ ($FB_1$) on the growth, feed intake, and potential effects on the livers of Baltic salmon. My hypothesis was that exposure to different concentrations of $FB_1$ would reduce salmon growth and feed intake, and also would result in liver damage.

## 2. MATERIALS AND METHODS

### 2.1. Experimental setup

The research was conducted between 17 December 2011 and 5 May 2012 at the Department of Biological and Environmental Science of the University of Jyväskylä, Finland. The fish (River Neva strain) were acquired from Hanka-Taimen Ltd. The 389 fish initially weighed 31.8 ± 6.4 g (mean ± S.D.) with lengths of 14.6 ± 0.9 cm. Fish were randomly divided among 15 circular (74 cm diameter) green plastic tanks of 200 liters that were arranged within the laboratory space to avoid systematic differences between tanks. Tank conditions were maintained at 15 ºC, oxygen > 7 mg/l, $NH_3/NH_4 \leq 0.5$ mg/l, pH 7.2 ± 0.1, 800 ml/min water flow, and 12L:12D photoperiod. Heated well water was vigorously aerated with compressed air in an overhead tank and additionally in each tank using air pumps (Mouse M-103; Mareena Aquarium, India) and air diffusers. Temperature was recorded once a day, and ammonium concentration (Sera Kit GmbH, Germany), oxygen (YSI 550A DO, YSI, USA), and pH (ProODO, YSI Inc.) were tested weekly. Room light was provided by fluorescent bulbs over the tanks and controlled with a timer.

Fish were acclimatized for 9 weeks. During this period, fish were hand-fed commercial salmon feed, *ad libitum*, twice a day. Fish were treated for a *Flavobacterium* infection using Orimycin Vet antibiotic over a period of 10 days.

During the 10 week trial period, we tested 5 levels of fumonisin $B_1$ ($FB_1$): 0 (control), 1, 5, 10 or 20 mg/kg of feed. Each level of $FB_1$ was tested in 3 tanks. Sparos Lda. (Portugal) supplied the extruded experimental feed, which was of 2mm diameter.

Fish were individually weighed (to 0.01 g) and measured for length (to 1 mm) at the beginning and at the end of the 10 week trial period. For these measurements, fish were anesthetized using clove oil: ethanol mixture (clove oil concentration in the anesthetic solution 40 mg/l) and returned to their original tanks. Fish recovered from the anesthesia within approximately 5 minutes. During the experiment, fish were also weighed in batches (to 0.1 g) every 2 weeks (4 measurements).

Fish condition factor (K) was calculated as:

$$K = W / L^3 * 100$$

where $W$ was fish weight (g) and $L$ was fish length (cm). Growth was determined as specific growth rate (SGR):

$$SGR\ (\%/d) = 100 * (\ln W_2 - \ln W_1) / t,$$

where $W_1$ and $W_2$ were weights (g) at the first and last experimental day, respectively, and $t$ was time (days). Feed conversion ratio (FCR) was calculated as:

FCR = Amount of feed eaten (dry weight)(g) / weight gain (g).

Feed consumption was calculated as the difference between offered and uneaten pellets. Uneaten pellets were collected every day by siphoning the tank bottom, dried at 120 °C and weighed. Relative feed intake (RFI, percentage in body weight per day) and daily feed intake (FI) were determined respectively as:

RFI (% BW / d)= [100 * Amount of feed eaten (g) / biomass (g)] / $t$

where, for this case, biomass was average fish weight of a given period.

FI = Amount of feed eaten (g) / fish biomass (kg),

At the end of experiment all the fish were killed by overdose of clove oil:ethanol mixture (1:10). Fifteen individuals of each treatment (5 fish per tank) were weighed and the livers removed and weighed for hepatosomatic index (HSI) which was calculated as:

HSI = Liver wet weight (g) * 100 / $W$

Then, for water content analysis, livers were dried to constant weight at 70ºC and weighed (to 1 mg) and determined as:

Water content (%) = $W_w - W_d / W_w * 100$

where $W_w$ and $W_d$ were wet and dry weights (mg) respectively.

Statistical analyses were performed using PASW statistical software (version 18.0). To examine possible effects of the different feeding regimes in the salmon's growth performance, one-way ANOVA was used. Homogeneity of variances was examined using Levene's test and Kolmogorov-Smirnov test to check normality. Tukey's test was used for *post-hoc* comparisons between sample means. Additionally, linear regression analysis was performed for exploring possible relationships in the variables in response to $FB_1$ concentrations and Wilcoxon test (parametric test assumptions were not met) to test for possible differences between initial and final condition factor. $p$ values < 0.05 were considered significant.

### 2.2. Liver damage

At the end of the experiment, three individuals from each treatment were used for histological liver examination. Fish were killed by overdose of clove oil and the livers were removed. Livers were inspected for histopathological damage and then processed for lipid accumulation check up. For the last matter, a small piece (c. 5 x 5 x 5 mm) from the liver was cut and washed with phosphate buffered saline solution (PBS). Then it was mounted in a metal frame and frozen in isopentane, which previously was cooled in liquid nitrogen. Samples were sectioned at a thickness of 7 μm in Ames® Microtome cryostat maintained at -20ºC. Sections were mounted on microscope slides, and fixed in 4% paraformaldehyde (PFA) for 15 minutes, washed in PBS, and stained with a lipid specific dye, LD540 (provided by the Department of Biology of Physical Activity of Jyväskylä University) for one hour in complete darkness. Incidence of lipids was measured via fluorescence using a microscope. Two histological samples per liver were assessed for percentage of fluorescence associated with lipids and classified into 4 levels: level 1 (0–25%), level 2 (25–50%), level 3 (50–75%), and level 4 (75–100%).

## 3. RESULTS

### 3.1. Feed intake and growth

No fish in any of the control or experimental groups exhibited signs of bacterial infection or died due to the dietary treatments used during the trial.

There were no significant differences in fish size at the beginning of the experiment (ANOVA, weight, $p = 0.592$; length, $p = 0.390$; Table 1). The different $FB_1$ treatments had no effect on relative feed intake (ANOVA, $p = 0.451$; Fig. 1), daily feed intake (ANOVA, $p = 0.447$; Table 1) and in fish final weight or length (ANOVA, weight, $p = 0.460$; length, $p = 0.14$; Table 1). Furthermore, no significant differences were found in weight gain

(ANOVA, $p$ = 0.489; Table 1), feed conversion ratio (ANOVA, $p$ = 0.423; Fig. 2a) or specific growth rate over the experiment (ANOVA, $p$ = 0.641; Fig. 2b). Fish exhibited approximately linear growth with a mean specific growth rate of 0.5% per day during the study period (Fig. 3).

There was a significant difference in the final condition factor as the controls had significantly higher condition factor than fish fed the highest $FB_1$ concentration (Tukey, $p$ = 0.035; Fig. 4). However, no differences between initial and final condition factor for each treatment were found (Wilcoxon, $p > 0.05$). Finally, regression analyses showed that there were no significant relationships between the variables and the $FB_1$ concentrations ($p > 0.05$, $R^2 \leq 0.23$).

Table 1. Measured parameters of 0+ Baltic salmon, *S. salar,* fed diets with different concentrations (mg/kg of feed) of fumonisin $B_1$ ($FB_1$). Given values are group averages ± S.D., n=3.

| | **$FB_1$ 0** | **$FB_1$ 1** | **$FB_1$ 5** | **$FB_1$ 10** | **$FB_1$ 20** |
|---|---|---|---|---|---|
| Initial weight (g) | 32.60 ± 3.11 | 31.24 ± 1.39 | 31.15 ± 1.41 | 33.01 ± 0.93 | 31.47 ± 0.66 |
| Initial length (cm) | 14.74 ± 0.34 | 14.59 ± 0.11 | 14.54 ± 0.20 | 14.85 ± 0.15 | 14.65 ± 0.06 |
| Final weight (g) | 46.78 ± 4.80 | 43.79 ± 4.60 | 42.26 ± 1.88 | 46.17 ± 2.35 | 43.08 ± 2.39 |
| Final length (cm) | 16.37 ± 0.36 | 16.73 ± 0.57 | 15.86 ± 0.80 | 16.56 ± 0.37 | 16.33 ± 0.05 |
| Feed intake (g/kg/d) | 8.50 ± 0.52 | 8.53 ± 0.47 | 8.73 ± 0.30 | 8.23 ± 0.26 | 8.20 ± 0.30 |
| Total $FB_1$ intake (mg/kg) | 0 | 0.44 ± 0.01 | 2.22 ± 0.13 | 4.26 ± 0.33 | 8.47 ± 0.79 |
| Weight gain (g) | 14.18 ± 2.09 | 12.56 ± 3.25 | 11.11 ± 1.37 | 13.16 ± 1.58 | 11.61 ± 2.25 |

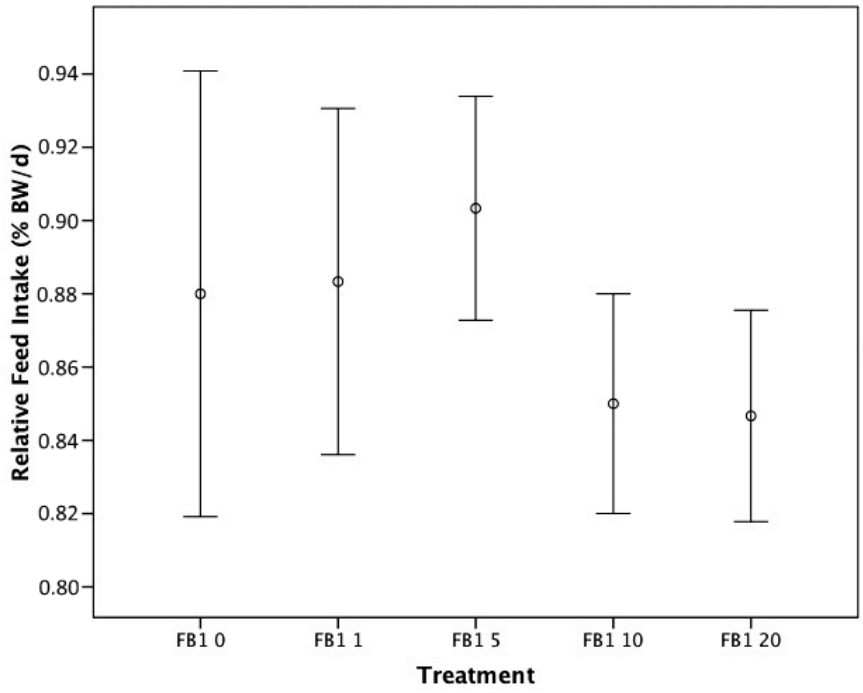


Figure 1. Average relative feed intake of 0+ Baltic salmon, *S. salar*, fed diets with different concentrations (mg/kg) of fumonisin $B_1$ during the 10-week experiment. There were no statistical differences between the treatments. Circles indicate mean and error bars indicate S.D., n=3. FB1: fumonisin $B_1$, BW: body weight.

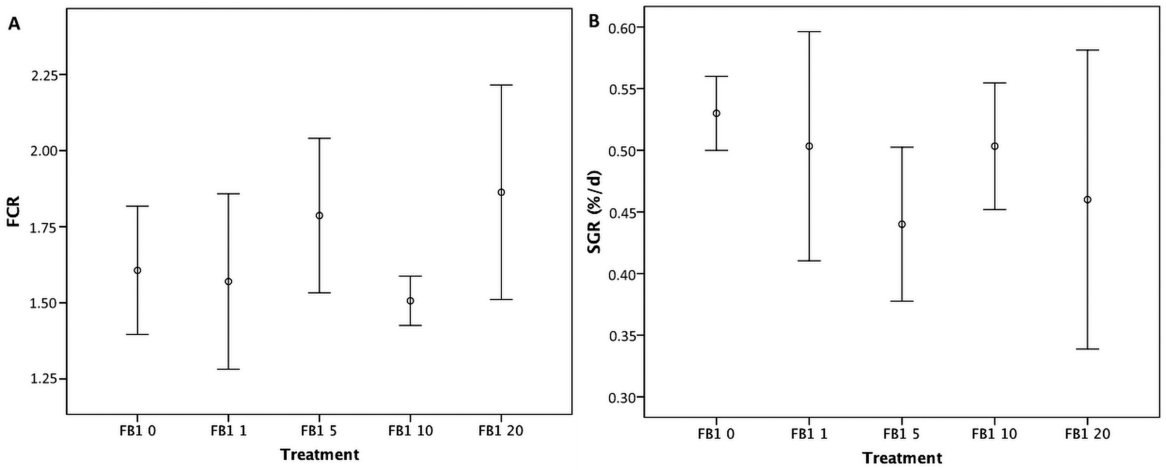


Figure 2. Average feed conversion ratio (a) and average specific growth rate (b) of 0+ Baltic salmon, *S. salar*, fed diets with different concentrations (mg/kg) of fumonisin $B_1$ during the 10 week experiment. There were no statistical differences between the treatments. Circles indicate mean and error bars indicate S.D., n=3. FCR: feed conversion ratio, SGR: specific growth rate, FB1: fumonisin $B_1$.

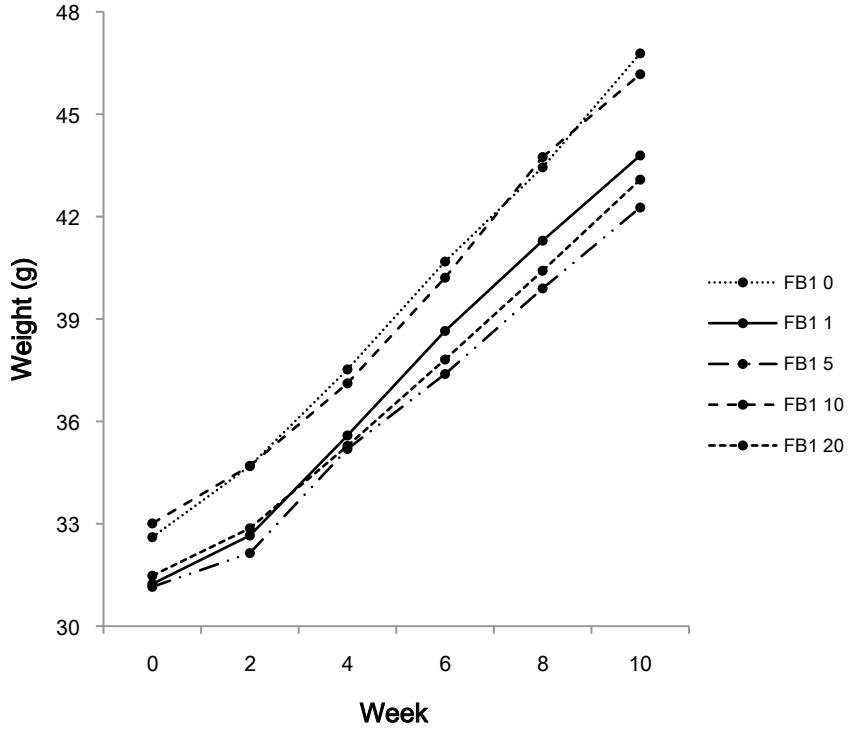


Figure 3. Average weight of 0+ Baltic salmon, *S. salar*, measured every two weeks during the 10 week experiment and fed diets with different concentrations (mg/kg) of fumonisin $B_1$ ($FB_1$). There were no statistical differences between treatments (n=3). Error bars omitted for clarity.

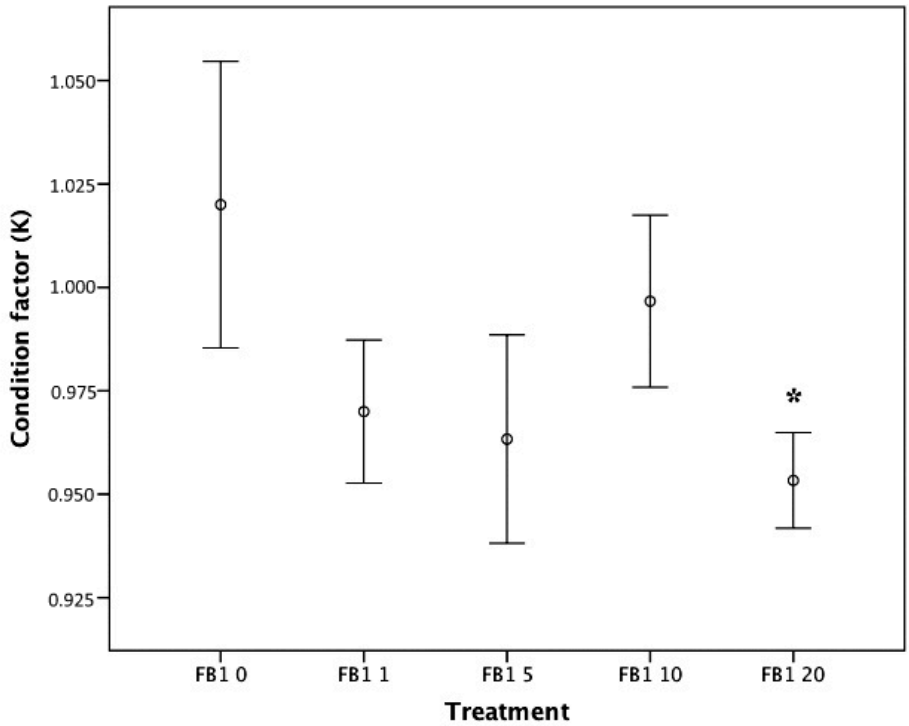


Figure 4. Final condition factor of 0+ Baltic salmon, subjected to diets with different concentrations (mg/kg) of fumonisin $B_1$ ($FB_1$) during 10 week trial. Circles indicate mean and error bars indicate S.D., n=3. * Significantly different (one-way ANOVA, $p = 0.032$; Tukey, $p = 0.035$) from the $FB_1$ 0 group.

### 3.2. Liver analysis and liver damage

The ratio of liver weight to fish body weight (HSI) between the different $FB_1$ treatments did not differ significantly during the experiment (ANOVA, $p = 0.309$, Table 2) and no relationship between the variables was found either ($p = 0.58$, $R^2 = 0.02$). Likewise, no differences in liver water content between treatments were found (ANOVA, $p = 0.354$, Table 2) and no liver lesions and fat accumulation was found in any sample regardless of the dietary treatment.

Table 2. Hepatosomatic index and liver water content of 0+ Baltic salmon, *S. salar,* fed diets with different concentrations (mg/kg of feed) of fumonisin $B_1$ ($FB_1$). Given values are group averages ± S.D., n=3.

| | **$FB_1$ 0** | **$FB_1$ 1** | **$FB_1$ 5** | **$FB_1$ 10** | **$FB_1$ 20** |
|---|---|---|---|---|---|
| Hepatosomatic index | 0.88 ± 0.04 | 0.84 ± 0.09 | 0.79 ± 0.03 | 0.89 ± 0.10 | 0.90 ± 0.04 |
| Liver water content (%) | 77.50 ± 0.36 | 76.39 ± 1.57 | 76.84 ± 0.29 | 76.34 ± 0.68 | 76.09 ± 0.77 |

## 4. DISCUSSION

This experiment examined the effects of a mycotoxin, fumonisin $B_1$, which affects the health and survival of many fish species in aquaculture (Manning 2001). $FB_1$ is a toxin produced by mold of the genus *Fusarium* that is found primarily in maize based feeds. Fumonisins occur worldwide, and in Europe levels between up to 250 $FB_1$ mg/kg of feed has been detected (Anonymous 2002). The current research examined the effects of a range of dosages of $FB_1$ in hatchery-reared salmon. No differences were found in feed

intake, final weight or length, feed conversion ratio, specific growth rate and hepatosomatic index in response to any of the dosages of $FB_1$, 1 to 20 mg/kg. The only statistically significant effect was on condition factor at the highest $FB_1$ dose, 20 mg/kg, which K was significantly lower than in the controls.

### 4.1. Growth

In this work the fish mean specific growth rate was about 0.5% per day. Austreng et al. (1987) model predicts growth rates of 3.62% per day for salmon of 31.8 g at 15 ºC. Moreover, McCormick et al. (1998) reported growth rates of 2.39–2.72% on salmon of similar size than in this study and held at 16 ºC but also lower values, about 1% per day, have been reported (Farmer et al. 1983).

Although the fish exhibited normal swimming behavior and appeared to be in good health, they did not responded well to feeding which was reflected as low growth rates. Poor palatability of the experimental feed is an unlikely explanation. During acclimatization, the fish were fed commercial pellets, which were also used in the hatchery. The fish ate and had the same behavior towards the commercial and experimental feed.

The fish farm provided a batch of salmon that had been growing well at their facility. After this experiment, another batch of Baltic salmon from different fish farm was brought into the same laboratory conditions and a similar result with poor feed intake and growth was obtained. In fact, this second batch of fish lost weight after a period of about 3 weeks in the laboratory. At present, we do not have an explanation for the low growth rates observed in these two batches of salmon. The tanks and the water system have been used successfully in many previous experiments with rainbow trout (*Oncorhynchus mykiss*).

Sørum & Damsgård (2004) obtained feed intake rates, on 0+ salmon of similar weight than in this study, of 6–11 g/kg per day, while in this study intake was less than 1g/kg. This supports the idea that the fish of this study were not eating eagerly. In addition, when compared total toxin intake between the current trial and Li et al. (2007), it is shown that the salmon of this trial ate 5 times less toxin despite of being 5 times heavier than Li's catfish. These data suggest that due to poor feed intake the fish were not exposed to the toxic effect of $FB_1$. Low exposure to $FB_1$ also explains the lack of differences in feed conversion ratio between treatments. Tuan et al. (2003) showed that exposure to $FB_1$ lead to increase in feed conversion ratio in Nile tilapia fed with dosages of 40 mg/kg or above compared to controls.

### 4.2. Effects of $FB_1$ in relation to fish size and time of exposure

Previous studies of $FB_1$ toxicity in other fish species have shown that age, weight, and time of exposure affect vulnerability to $FB_1$. Young channel catfish, *Ictalurus punctatus* (initial weight c. 1.2 g), were exposed to different levels of $FB_1$ (0.3, 20, 80, 320, 720 mg/kg in feed) for 10 weeks (Lumlertdacha et al. 1995). Dosages of 20 $FB_1$ mg/kg reduced weight gain compared to controls (Lumlertdacha et al. 1995). In larger catfish (initial weight c. 6.1 g), the threshold for $FB_1$ toxicity was higher: dosages of 40 $FB_1$ mg/kg and above resulted in statistically significant reduction in growth, feed consumption, and feeding efficiency over a period of 12 weeks (Li et al. 1994). Furthermore, 2-year-old channel catfish (initial weight c. 31 g) exposed for 14 weeks to the same levels as in the first example, gained less weight at concentrations of 80 $FB_1$ mg/kg and above. 50% mortality was observed at dosages of 320 $FB_1$ mg/kg (Lumlertdacha et al. 1995). In Nile

tilapia fingerlings, *Oreochromis niloticus*, (initial weight c. 2.7 g), dosages of 40 $FB_1$ mg/kg or higher reduced weight gain in an 8 week trial (Tuan et al. 2003).

The salmon used in our experiment were underyearlings with a mean weight of 31g. Salmon of this size may have been more resistant to the used concentrations of $FB_1$ toxin than the channel catfish in the experiments of Li et al. (1994) and Lumlertdacha et al. (1995), and higher concentrations will need to be tested in the future. Further, future work should look at the effects of longer duration exposures, greater than 10 weeks, to $FB_1$. Critically, the highest $FB_1$ level used in this trial, 20 $FB_1$ mg/kg, was the minimum level that caused effects in channel catfish (Li et al. 1994).

### 4.3. Effects of other mycotoxins in fish

There are differences in vulnerability to other mycotoxins among salmonid species. For example, rainbow trout is considered very susceptible to aflatoxin $B_1$ ($AFB_1$) (Manning 2001, Santacroce et al. 2008). Santacroce et al. (2008) explained that dosages of 0.5–1 $AFB_1$ mg/kg feed resulted in 50% mortality. Other study showed that levels of 0.02 $AFB_1$ mg/kg feed produced 96% incidence of liver tumors in rainbow trout. However, the same treatment had no effect on coho salmon (*Oncorhynchus kisutch*) (Manning 2001). These species differences are likely due, at least in part, because $AFB_1$ is processed differently in the two species; trout's liver is more efficient turning the mycotoxin into a more carcinogenic metabolite resulting in higher liver cancer rates (Santacroce et al. 2008).

Deoxynivalenol (DON), also known as vomitoxin, is another type of fumonisin, which affect feeding response and causes vomiting (Smith 2008, Hooft et al. 2010). Studies done in rainbow trout, (initial weight c. 24 g), concluded that this species is highly vulnerable to DON. Fish showed reduction in weight gain, feed intake and feed efficiency when treated with 0.3 DON mg/kg feed and higher dosages over a period of 8 weeks (Hooft et al. 2010). On the other hand, in Atlantic salmon (initial weight c. 405 g), concentrations of 3.7 DON mg/kg for 15 weeks resulted in 20% decrease in feed intake, 31% decrease in specific growth rate, and 18% higher FCR when compared to the controls (Döll et al. 2010).

### 4.4. Effect of $FB_1$ on condition factor

Condition factor is a measure used to describe fish health, particularly in relation to the degree of nourishment (Williams 2000, Nash et al. 2006). It is influenced by factors as age, sex and stage of maturation (Barnham & Baxter 1998, Jobling 2002). For salmonids, condition factor values range between 0.8 to 2.0 (Barnham & Baxter 1998) and tend to increase as fish grow and decrease with smoltification (McCormick et al. 1998b). Fish of this study were in between of normal ranges (c. 1) but McCormick et al. (1998) reported increased on condition factor from 1.06 to 1.25 on 0+ salmon parr during his research. On the other hand, condition factor did not change substantially between the beginning and the end of this trial regardless of the treatment, fact that is related with the poor feed intake and growth obtained. Nevertheless, significant differences in final condition factor were observed between salmon treated with the highest level of $FB_1$ (20 mg/kg) and the controls. Besides that some fish were possible becoming smolts by the end of the trial, reduction in condition factor could not be explained by smoltification process because it did not occur in all tanks. It is possible that the 10-week exposure to $FB_1$ was in the beginning stages of its toxic effects; as stated above, the time of exposure and dosages levels were short and low. But because there was no significant differences in other growth parameters measured, the meaning of this significant difference should be considered inconclusive.

### 4.5. Liver damage

Lipid content is inversely correlated with water content, meaning that an increment in the former will result in reduction of the later. In this study, no difference in liver water content was found regardless of the treatment. These findings were confirmed with the absence of liver lipid accumulation observed in the histological samples and that no significant differences in hepatosomatic index were found either. This is consistent with Carlson et al. (2001) study which showed that rainbow trout fry fed on any of 3.2, 23 or 104 $FB_1$ mg/kg diets for 32 weeks had not significant liver lesions compared with controls. Likewise, Tuan et al. (2003) in his examination on Nile tilapia, (initial weight c. 2.7 g), reported that exposure for 8 weeks to different concentrations of 10, 40, 70 or 150 $FB_1$ mg/kg feed, only the highest dosage increased substantially liver free sphinganine levels in comparison with controls but no histopathological lesions were found. On the other hand, Lumlertdacha et al. (1995) found that livers of 1 and 2 year old channel catfish fed 20 $FB_1$ mg/kg or more for 10 weeks had injuries as swollen liver cells, lipid vacuoles and necrosis. As mentioned before, the lack of liver damage could be explained by species differences to $FB_1$ vulnerability, short time of exposure to the toxin and/or to the low feed intake observed in this research.

## 5. CONCLUSIONS

This study did not support my hypotheses because the feeds containing $FB_1$ did not affect the growth, feed intake or produced liver damage on Baltic salmon. Nevertheless, it is known that different species show distinctive susceptibility to mycotoxins due to variation in toxin metabolism (Santacroce et al. 2008, Encarnação 2011). Unfortunately, the fish in all tanks (including the controls) had very poor appetite and grew very slowly when compared to SGR values 2–6 times higher reported in other studies (Farmer et al. 1983, McCormick 1998). The present results should be confirmed by additional experiments. Indeed, only a few studies have been done on effects by fumonisins on fish despite their potential deleterious effects on fish production and human health. Currently, salmon production worldwide exceeds 1000000 tons placing it as one of the most important species farmed. More than the 50% of the global salmon market comes from aquaculture (Jones 2004). Industry's objective is to minimize costs in feedstuffs using vegetable ingredients but to face the incoming mycotoxin risks more research on them and how they affect farming of salmonids is needed. Likewise, due to the difficulty to prevent mycotoxins occurrence in raw materials, extensive research on the means to neutralize them is desirable.

## ACKNOWLEDGMENTS

My deepest gratitude to Juhani Pirhonen for his patient guidance and supervision. Especially for trusting me and giving me the space to cope by my own while keeping me on track. I could not have had a better advisor and mentor. Also I would like to thank to Biomin® for the financial support and to those people that had collaborate me with the installation and lab work, and make this project possible: Ahti Karusalmi, Anette Lehtola, Juha Ahonen, Nina Honkanen and Paavo Rahkila.

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Appendix. Experimental setup for the 10 week feeding experiment on Baltic Salmon (*Salmo salar*).