β_2-microglobulin distribution in trout body fluids and release from intestinal epithelial cells in response to plant meal components

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

β_2-microglobulin (β2m) exists free of the major histocompatibility complex class I (MHC I) receptor in many bodily fluids. The amount of protein present in these fluids has been found to be a useful prognostic marker for various diseases but outside of its practical value not much is known about this form of β2m. In fish, soluble β2m has not been studied at all. Another unknown in fish is the effects that plants lectins might have on naturally carnivorous species in aquaculture. These plant proteins which bind to specific sugar groups found on cells have been shown to have a multitude of gastrointestinal and immune effects in mammals and can be found in the plant products being fed to carnivorous, cultured fish making them possible toxicants. The two studies of this thesis set out to pioneer knowledge on these subjects using rainbow trout as a model. The first investigation inspected the various body fluids of these fish for their free β2m content. Soluble β2m was found to be present in the plasma, the seminal fluid, ovarian fluid, and the mucus of the skin and intestines. This distribution shows that β2m could indeed make a good biomarker, not only for disease but also for pheromone release and alludes to some possible functions of soluble β2m while opening the way for future research on this form of the protein. The second study looked at the effects of lectins on the gut of rainbow trout by treating RTgutGC, an intestinal epithelial cell line derived from trout, with plant lectins from wheat (WGA) and soybean (SBA), among others. This study found WGA to be a potent inducer of morphological and cytotoxic effects in these cells while other lectins and plant factors were not. WGA was also observed to effect the expression of β2m and the α-chain of the MHC I receptor. This work suggests WGA ingested by trout through the wheat in their diet might be causing them harm and should be studied further. It is also interesting that both studies related β2m to the intestines of trout. This could allow soluble β2m to serves as a marker of WGA's effect or for WGA to aid in the study of free β2m.

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Dedication

This thesis is dedicated to my grandmother Marcella Hollohan (1921-2009); the most loving and understanding person I have ever known.

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List of Abbreviations

1°	Primary
16 S rRNA	16 Svedberg ribosomal RNA
2°	Secondary
AB	Alamar blue
ANF	Antinutritional factor
BCA	Bicinchoninic acid assay
bp	Basepairs
BSA	Bovine serum albumen
CD	Cluster of differentiation
cDNA	Complementary DNA
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl ester
Con A	Concanavalin A (jack bean lectin)
CRT	Calreticulin
CTL	$CD8^+$ cytotoxic T lymphocyte
DI	Distal intestine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
DPBS	Dulbecco's modified PBS
DSA	Jimson weed lectin
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FcRn	Fraction crystallizable neonatal receptor
FM	Fish meal
GalNAc	N-acetylgalactosamine
Glc	Glucose
GlcNAc	N-acetylglucosamine
HMGB1	High mobility group box 1
IHNV	infectious haematopoietic necrosis virus
IL	Interleukin
kDa	Kilodaltons
L-15	Leibovitz's media
L-15 - FBS	L-15 without FBS supplementation
LPS	Lipopolysaccharide
Man	Mannose
MH	major histocompatibility
MH I	class I major histocompatibility receptor
MH Iα	class I major histocompatibility receptor α-chain
MHC	major histocompatibility complex
mRNA	Messenger RNA
NBT/BCIP	Nitro-blue tetrazolium/ 5-bromo-4-chloro-3-indolyl

OF	Ovarian fluid
P	Passage
P/S	Penicillin/streptomycin
PBLs	Peripheral blood leukocytes
PBS	Phosphate-buffered saline
PBS-T	PBS with Tween 20
PCR	Polymerase chain reaction
PHA	Red kidney bean lectin
PI	Proximal intestine
p-NPP	p-Nitrophenyl phosphate
Poly(I:C)	Polyinosinic:polycytidylic acid
RBCs	Red blood cells
RNA	Ribonucleic acid
RTG-2	A rainbow trout gonadal fibroblast-like cell line
RTgill-W1	A rainbow trout gill epithelial cell line
RTgutGC	A rainbow trout intestinal epithelial cell line
RT-PCR	Reverse transcriptase PCR
RTS11	A rainbow trout spleen macrophage-like cell line
rβ2m	Recombinant β2m
SBA	Soybean lectin
SBM	Soybean meal
SBTI	Soybean trypsin inhibitors
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	Seminal fluid
TBS-T	Tris-buffered saline with Tween-20
U	Units
UBA	Classical MH I locus of salmonids
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
WGA	Wheat germ lectin
β2m	β_2-microglobulin

Chapter 1
General introduction

1.1 β_2-microglobulin

As a surface protein, β_2-microglobulin (β2m) plays an essential role in the immune system. It is best known as the light chain portion of the major histocompatibility complex class I (MHC I) receptor (Peterson et al. 1974, Nakamuro et al. 1973, Cresswell et al. 1974). Its noncovalent bond with the transmembrane MHC Iα chain allows for effective loading and surface expression of peptide antigens from within cells as well as their presentation to $CD8^+$ cytotoxic T lymphocytes (CTLs, Vitiello et al. 1990, Rock et al. 1991) and is therefore a vital contributor to antiviral immunity (Figure 1.1). Though less well known, β2m is also a critical partner for two other immune receptors: CD1 (cluster of differentiation 1) and FcRn (Fraction crystallizable neonatal receptor, Praetor and Hunziker 2002, Bauer et al. 1997). CD1 is another antigen presenting molecule that specializes in displaying lipids and FcRn is an IgG Fc receptor used to transfer maternal antibodies to the fetus.

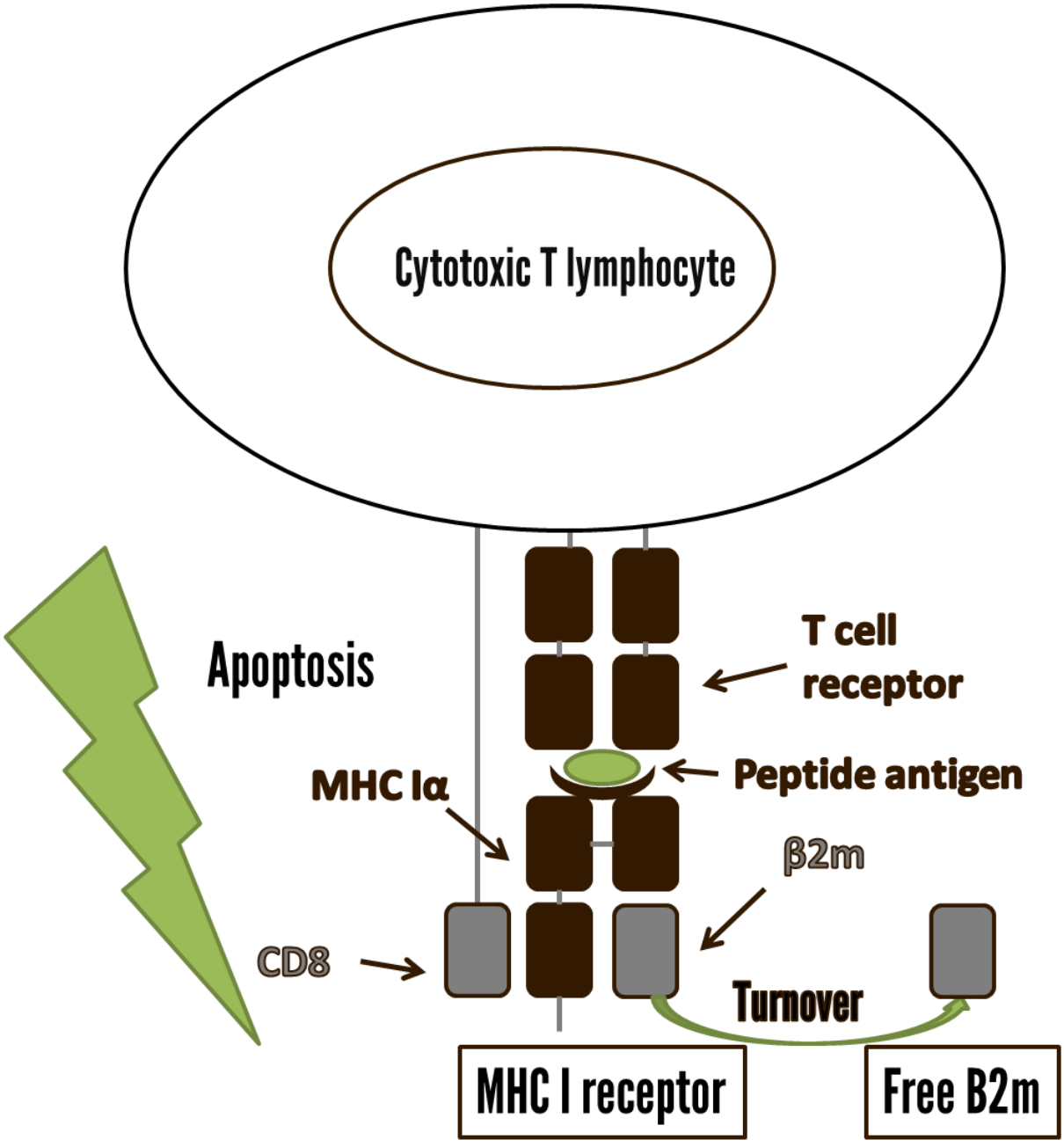

Figure 1.1 – Antigen presentation and turnover of major histocompatibility class I (MHC I) receptor As stated in the body text, the MHC I receptor, made up of both an α-chain (MHC Iα) and β2m, is found on the surface of all somatic cells where it presents peptide antigens to CTLs. This presentation relies on the peptide binding cleft of MHC Iα and recognition by the T cell receptor; CD8 is a necessary accessory molecule which binds specifically to the MHC Iα receptor. CTLs initiate apoptosis in the cell if the antigen is recognized. If the antigen is not recognized then the receptor is turned over and the noncovalently bound β2m subunit is released as a free protein.

β2m also exists in a soluble form that resides in bodily fluids. It was actually first isolated as an 11.7 kDa protein in the urine of proteinuria patients (Berggard and Bearn 1968) and later was discovered to be present in the plasma, cerebrospinal fluid, saliva, colostrum, amniotic fluid, synovial fluid, seminal fluid and urine (but at lower levels) of healthy people (Berggard and Bearn 1968, Evrin et al. 1971, Jonasson et al. 1974, Talal et al. 1975, Plesner and Bjerrum 1980). β2m is shed from the surface of cells with the turnover of MHC I receptors (Cresswell et al. 1974) and this is thought to be the source of free β2m under normal physiological conditions. When circulating plasma β2m reaches the kidneys close to 100% is taken up and catabolized by the proximal tubules leaving a small concentration to be excreted in the urine (Peterson et al. 1969, Bernier and Conrad 1969, Karlsson et al. 1980).

The potential use of soluble β2m as a biomarker for disease was appreciated soon after its discovery (Evrin and Wibell 1973) and it is now known to be a useful diagnostic and prognostic variable for many cancers and inflammatory diseases. Higher levels of β2m in plasma and other body fluids have been linked to multiple myeloma, heart disease, rheumatoid arthritis, Crohn's disease, Sjogern's syndrome and cancer/inflammation of the pancreas and urinary system and increased quantities usually correlate with greater severity of disease or poorer patient outcomes (Talal et al. 1975, Cuzick et al. 1985, Brenning et al. 1985, Zissis et al. 2001, Stefanovic et al. 1999, Kawai et al. 2010, Pezzilli et al. 1995). Given the nature of these diseases it has been hypothesized that greater release of β2m comes from activated immune cells or rapidly dividing tumour cells but the mechanisms are still debated (Stefanovic et al. 1999, Pezzilli et al. 1995).

The physiological significance of free β2m is not known and it might simply be a byproduct of MH I surface expression. Interestingly, though studies have hinted that this form of the protein may be a growth/apoptosis factor and may have alternative roles in immunity. Firstly, treatment with purified and recombinant β2m stimulates the growth of prostate and renal cancer cells, osteoclasts, osteoblasts and mesenchymal stem cells *in vitro* and in some cases *in vivo* (Zhu et al. 2009). Conversely, β2m exposure induces apoptosis in leukemic and breast cancer cells *in vitro*. It does so by a Fas and TNF-α independent mechanism which involves binding to an unknown receptor and potential cleavage of some currently unknown caspases (Mori et al. 2001, Gordon et al. 2003). It has also been shown to kill leukemic cells *in vivo* (Mori et al. 1999). Aside from its possible role as a cell growth/apoptosis mediator soluble β2m appears to evoke some immunologically-relevant effects *in vitro*. For instance, when dendritic cells (DCs) were treated with β2m it prevented their maturation; cells had fewer MHC receptors and costimulatory molecues and were poorer at activating T cells (Xie et al. 2003). *In vitro* work has also highlighted a potential role of β2m as a chemotaxin for cells making their way to the thymus to become T lymphocytes (Dargemont et al. 1989). Finally, β2m from saliva can bind to and agglutinate certain strains of *Streptococcus mutans* implicating it as an antibacterial peptide (Ericson 1984).

As with other vertebrates, β2m exists in fish. It was first cloned in the early 90's in tilapia, carp, and zebrafish (Dixon et al. 1993, Ono et al. 1993). In trout, there appear to be three *β2m* genes with four copies each. However, only one gene is primarily expressed and the

functional significance of small differences in the coding sequence between genes is unknown (Magor et al. 2004). In Atlantic salmon, mRNA expression of *β2m* seems to be higher in the intestine, gill, spleen and head kidney and lower in the liver and muscle which may be indicative of the distribution found in trout (Kales et al. 2006). It should be noted that translation of the *β2m* gene has been shown in all rainbow trout cell lines tested to date and is particularly high in the rainbow trout intestinal epithelial cell line, RTgutGC (Kawano et al. 2010).

1.2 Major histocompatibility complex genes

Major histocompatibility complex genes (*MHC*) were first recognized for their connection with transplant rejection (Strominger 2002). The genes were found to be very polymorphic, possessing many different alleles allowing them to be useful markers of individuality. Much later, it was recognized that several polymorphic genes within the complex coded for two receptor molecules, named MHC class I and class II, that play pivotal roles in providing adaptive immunity (Strominger 2002). The class II receptors are made up of two transmembrane proteins of similar size, are present only on specialized antigen presenting cells, and are responsible for presenting exogenous peptide antigens to $CD4^+$ helper T lymphocytes which they then activate for a specific immune response (Grommé and Neefjes 2002). This protects the body from extracellular (e.g. bacterial) invasion while class I receptors present endogenous peptides to $CD8^+$ cytotoxic T cells (CTLs) and thereby provide antiviral immunity (Grommé and Neefjes 2002). Since CTLs kill the cells they recognize, MHC class I receptors are present on nearly every cell type to provide an effective immune response (Grommé and Neefjes 2002). Also unlike class II and as was mentioned before,

class I receptors are composed of a larger transmembrane domain containing chain (known as the α-chain) responsible for holding peptides and the smaller noncovalently bound β2m. Both class I and class II molecules show a great degree of diversity in their peptide binding grooves which allows different alleles to hold different peptides and provide distinctive immunity (Rammensee et al. 1995).

Fish, like mammals, possess major histocompatibility genes although they are not organized in quite the same way. For a long time, transplant rejection and mixed-leukocyte reactions were known to occur in fish, trout included, and this ability to tell self from nonself hinted that *MHC* genes might be present (Hildemann 1957, Caspi and Avtalion 1984, Kaastrup et al. 1988). The genes were eventually uncovered in the common carp (Hashimoto et al. 1990) and later found in most other teleost species under study. Unlike in mammals, class I and class II genes are not linked in teleosts (Bingulac-Popovic et al. 1997) and so conventionally the "complex" is dropped from the name and they are simply referred to as *MH* genes (Shand and Dixon 2001). In trout, the classical gene for the MH Iα chain is encoded by a single polymorphic locus named *UBA* (Aoyagi et al. 2002).

It appears that the fish *MH I* genes function like their mammalian counterparts. Firstly, it seems that fish cells display complete MH I receptors, as β2m from the surface of catfish cells was shown to co-immunoprecipitate with the MH Iα chain (Antao et al. 1999). Also, transcription of both *β2m* and *MH Iα* was demonstrated to be upregulated in trout intestine and spleen when fish were infected with infectious hematopoietic necrosis virus (IHNV, Hansen and La Patra 2002) and the tissue distribution of MH Iα protein in trout resembles that of mammals (Dijkstra et al. 2003). Probably the most convincing evidence however has

come from a cell-mediated cytotoxicity study done on rainbow trout. Here it was shown that peripheral blood leukocytes (PBLs) from fish immunized against IHNV could kill IHNV-infected, MH I-matched RTG-2 cells while PBLs from a MH I-mismatched fish could not (Nakanishi et al. 2002). This study proves that at least in trout MH I plays an essential role in viral antigen presentation.

Since different *MHC* alleles have different peptide-binding motifs (Rammensee et al. 1995), possessing a wide variety of *MHC* alleles, or being heterozygous in the case of organisms like trout that have only one locus, should impart better parasite resistance on offspring than a lower MHC diversity. Therefore mate choices should theoretically take allelic differences at the *MHC* loci into account. This was first shown empirically to be the case in laboratory mice where males showed a preference for females that had a different *MHC* allele than their own (Yamazaki et al. 1976). Since then, evidence of mate choice involving MHC in other animals such as nonhuman primates (Setchell and Huchard 2010), birds (Bonneaud et al. 2006), and humans (Wedekind et al. 1995) has accumulated. Olfaction is thought to play an important role in detecting allelic differences. MHC olfactory signals could be the molecules themselves (Singh et al. 1987), their peptides (Leinders-Zufall et al. 2004), or products from MHC-specific microflora (Milinski et al. 2005).

MHC-based mate choice is also known to occur in fish. One particularly well studied model is the three-spined stickleback, a fish with multiple classical *MH* loci. Females of this species use olfactory cues to preferentially pick males that provide their offspring with a target number of different *MH* alleles thought to be optimal for pathogen immunity (Reusch et al. 2001, Aeschlimann et al. 2003). In this system it has been shown, as it has been with

mouse (Leinders-Zufall et al. 2004), that the peptides presented by MHC molecules act as important signals since the spiking of male odours with synthetic peptides was shown to change female MHC preferences (Milinski et al. 2005). MH disassortative mating was also demonstrated, under natural and semi-natural conditions respectively, in Atlantic salmon and brown trout which seem to have single *MH* loci like trout (Landry et al. 2001, Forsberg et al. 2007).

1.3 Plant use in salmonid aquaculture feed

In 2008, aquaculture accounted for 46% of global food fish supply and this industry's rapid growth in recent years has allowed the amount of fish consumed per capita to remain relatively static in the face of a growing human population (FAO 2010). In Canada, rainbow trout is the most cultured finfish after salmon with 7000 tonnes being produced in 2009, mostly in Ontario (Statistics-Canada 2009). Although globally aquaculture produces more pounds of fish than it uses up as inputs, for salmonid culture (which includes trout) the reverse is true (Tacon and Metian 2008); this group consumes large amounts of fish meal (FM) compared to other cultured fish and other farmed animals (IFFO 2011). This is a problem because the small pelagic fish from the ocean that are ground up to make fish meal are relatively scarce and therefore expensive and unsustainable.

To try to circumvent these issues, products from soybean, wheat, corn and other plants are included in salmonid feed to act as cheaper replacement sources of protein and carbohydrate (FAO 2009b, Figure 2.1). However, fish meal has not yet been completely replaced in trout or salmon feed as this causes a reduction in growth and nutrition as well as intestinal inflammation. The poorer growth seen with high levels of plant products is generally

ascribed to antinutritional factors (ANFs) present in plants, amino acid imbalances in plant protein and/or deficiencies in certain nutrients present only in fish meal (Rumsey et al. 1994).

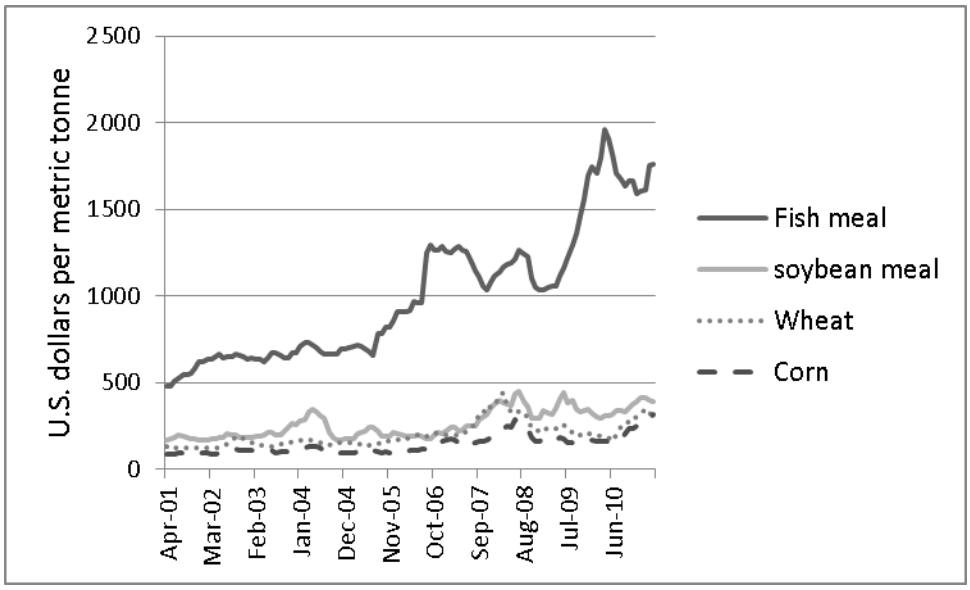

Figure 1.2 - Price of commodities used as sources of nutrition in aquaculture over last 10 years. Adapted from Index Mundi(2011).

The negative effects of feeding trout soybean meal (SBM), a common FM replacement, are well documented. Generally, growth of fish is impaired when SBM contributes 40% or more of the total protein in feed (Rumsey et al. 1994, Dabrowski et al. 1989, Romarheim et al. 2006, Ostaszewska et al. 2005). However, normal growth can be obtained at lower SBM replacement levels (Refstie et al. 2000, Morris et al. 2005, Oliva-teles et al. 1994) and has even occasionally been observed to be acceptable at concentrations above 40% (Cheng et al. 2003, Heikkinen et al. 2006). Nevertheless, when trout are fed higher levels of SBM they develop an acute intestinal gastroenteritis. This is blamed on the ANFs found in soybean. This inflammation is characterized by blunted and fused villi, cell infiltration of the lamina propria and submucosa, increased vacuolation, necrosis and sloughing of epithelial cells and is accompanied by systemic changes such as higher TNF-α mRNA levels in liver, higher

levels of plasma proteins and immunoglobulins, more circulating leukocytes and increased macrophage/neutrophil activity (Rumsey et al. 1994, Sealey et al. 2009).

Wheat is also commonly found in trout feed usually as wheat flour or middlings which act as a source of starch. Also, gluten meal from wheat, although not in widespread use, has been proposed as a FM/SBM replacement given its extremely high protein content (Hardy 2010). Wheat products seem to be able to sustain good growth in trout. When SBM is replaced by wheat middlings and wheat gluten meal, growth of trout is improved as long as the feed is supplemented with lysine (Davies et al. 1997). Also protein digestibility and various mineral uptake of wheat middlings, flour and gluten meal were all excellent when compared to a number of different FMs (Sugiura et al. 1998). It has also been shown that wheat gluten meal has no effect on the taste of rainbow trout fillets although it did slightly affect their colour (Skonberg et al. 1998). However, less conspicuous effects of wheat products on trout and other fish such as intestinal pathology have never been studied and therefore it is not known whether this plant can cause an inflammation similar to that produced by soybean.

1.4 Plant lectins

Lectins are proteins (or glycoproteins) that bind to carbohydrate groups specifically. Lectins from plants are generally regarded as antinutritional factors since ingested lectins can bind to the intestinal epithelium of rats and other mammals and cause toxicity, decreased growth, poorer digestion, reduced brush-border enzyme activity and degraded intestinal integrity among other effects (reviewed in Vasconcelos and Oliveira 2004). Their ability to mediate such reactions is greatly aided by their high resistance to proteolysis in the gut (Bardocz et al. 1995, Pusztai et al. 1993). Lectins are also known to be very immunostimulatory. In fact the

first lymphocyte mitogen ever discovered, PHA, is a plant lectin derived from red kidney beans (Nowell 1960).

Wheat germ lectin (WGA), as the name suggests, is a lectin isolated mostly from the germ portion of wheat (Peumans and Van Damme 1996). It is a homodimeric protein with two carbohydrate binding sites that are specific for the N-acetlyglucosamine sugar but bind to dimers and trimers of this sugar with even more affinity (Goldstein and Poretz 1986).

WGA has been shown have a slew of gastrointestinal effects. When fed to rats, it binds to the intestinal epithelium and reduces their growth and causes hyperplastic and hypertrophic growth of the small intestine (Pusztai et al. 1993). It can also disrupt the normal gut microflora by causing an overgrowth of *Escherichia coli* (*E. coli*), deepen the crypts between villi and cause sloughing of cells from the tips of villi (Pusztai et al. 1995, Lorenz-meyer et al. 1985). Furthermore, WGA can disrupt monolayers of the enterocyte-like human adenocarcinoma cell line Caco-2 suggesting it might make the gut more permeable *in vivo* (Ohno et al. 2006). More than this WGA can pass through the intestinal barrier and enter the circulatory and lymphatic systems; this is probably what allows it to enlarge the pancreas and have other systemic effects (Pusztai et al. 1993).

WGA's ability to transcend the gut's blockade allows it to interact with the immune system of animals and it has been shown to have immune effects both *in vivo* and *in vitro*. It is clear that the immune system reacts to WGA as antibodies against dietary WGA have been shown in the serum of mice as well as humans (Tchernychev and Wilchek 1996, Lavelle et al. 2000). *In vivo* it has also been shown to cause a striking atrophy of the thymus as well as a slight modulation of the immune response of rats intestinally challenged with ovalbumin

(Pusztai et al. 1993, Watzl et al. 2001). It also has an impressive list of *in vitro* immune effects: it causes the secretion of IL-4, IL-13 and histamine from basophils/mast cells (Shibasaki et al. 1992, Haas et al. 1999, Lopes et al. 2005), the release of reactive oxygen species from neutrophils (Karlsson 1999), the activation or inhibition of T cells under different conditions (Kilpatrick 1995) and the enhancement of phagocytosis by macrophages (Stolka et al. 2001).

Despite all of its known activity in mammals, the effects of WGA on trout, and fish in general, remain a mystery. This is disconcerting because trout and other aquaculture species are very likely exposed to this lectin in their feed as it can be found in flour at approximately 30-50 μg/g (Matucci et al. 2004, Radulescu et al. 2009). Moreover, WGA is also highly heat-resistant which would likely allow it to pass through feed processing intact (Peumans and Van Damme 1996, Matucci et al. 2004). It is therefore imperative that investigations such as this one be undertaken to ascertain the effects of WGA and other wheat components on fish.

Lectin from soybeans (SBA) is a tetrameric glycoprotein that contains two different subunits in equal proportions (Goldstein and Poretz 1986). Its four carbohydrate binding sites uniquely recognize N-acetylgalactosamine, its glycosides and oligosaccharides at the terminal end of carbohydrate groups (Goldstein and Poretz 1986).

From feeding trials in rats, it also appears that SBA can provoke gastrointestinal changes in these animals. Like WGA, SBA appears to reduce their total growth, increase the weight of their small intestines, and alter their intestinal microbiota (Pusztai et al. 1995, Armour et al. 1998). It can also bypass the intestinal barrier as it too can increase the size of the pancreas

and induce circulating antibodies against itself (Tchernychev and Wilchek 1996, Armour et al. 1998).

Aside from being able to evoke a systemic antibody response, few immune effects have been attributed to SBA. But, unlike WGA, some work on the interactions of SBA with the guts of rainbow trout and Atlantic salmon has been carried out due to its potential role in soybean-induced gastroenteritis. The first study was done by Hendriks et al. (1990) and established that SBA binds the intestine of Atlantic salmon *in vitro* with a particular affinity for the distal intestine, the known site of SBM-induced inflammation. SBA binding was then shown to solely occur in the distal intestine of both salmon and trout *in vivo* and these lectins alone (albeit at a high concentration of 35 g/kg) were shown to cause a distal intestinal pathology in Atlantic salmon with some of the traits of soybean-induced gastroenteritis (Buttle et al. 2001).

It does not seem likely however that SBA is the ultimate cause of the intestinal inflammation endured by trout. This conclusion comes from work done on Atlantic salmon. First, Van den Ingh et al. (1996) showed that alcohol extract from SBM which contained certain ANFs but no lectins recapitulated the acute gastroenteritis induced by SBM in salmon. Later work by Knudsen et al. (2007, 2008) pinned down another ANF, saponins, as the main causal factor. It is possible however that SBA may play a subsidiary role as two recent papers have reported that only in combination with lectins do soyasaponins and mixtures of soybean ANFs cause a thickening of the lamina propria in trout (Iwashita et al. 2008, Iwashita et al. 2009). Moreover, the etiology of the disease might differ between

rainbow trout and Atlantic salmon. Therefore SBA cannot be completely ruled out as a causal factor in the SBM-induced gastroenteritis of trout.

1.5 Purpose

This thesis had two main goals. The first aim of this work concentrated on elucidating the distribution of the β_2-microglobulin (β2m) in the bodily fluids of trout. The second goal was to further investigate the immunological and metabolic effects that plant lectins, particularly those from wheat and soybean, may have on the intestines of fish by testing the effects of purified lectins on the rainbow trout intestinal epithelial cell line RTgutGC.

Chapter 2

The distribution of β_2-microglobulin in the bodily fluids of rainbow trout

2.1 Introduction

β2m has long been known to be the light chain of the major histocompatibitiy complex class I (MHC I) immune receptor found on all nucleated cells of the body (Peterson et al. 1974, Nakamuro et al. 1973, Cresswell et al. 1974). In this role, it noncovalently attaches to the larger, transmembrane α-chain allowing it to bind endogenous peptide antigens and display them on the surface of cells. These antigen-MHC I complexes, if foreign, are recognized by $CD8^+$ cytotoxic T lymphocytes (CTLs) which will proliferate and activate to kill all cells presenting that specific antigen (Grommé and Neefjes 2002). This imparts the organism with a specific immunity against intracellular threats such as viruses. Therefore the MHC I receptor is a critical component of the immune system of higher organisms.

The MHC I receptor as well as the class II receptor, which has a similar function but for extracellular threats, are also well-known for their role in mate choice. Both receptors are highly polymorphic in their binding grooves giving different alleles the ability to bind different arrays of antigens and therefore provide disparate immunity to various pathogens (Rammensee et al. 1995). Organisms should then theoretically try to expand the number of unique *MHC* alleles their offspring receive by choosing mates that are genetically dissimilar at the *MHC* loci. This theory has proven true in mice, birds, humans and other primates which seem to be able to use olfactory cues to decipher the set of *MHC* alleles a potential partner has (Yamazaki et al. 1976, Setchell and Huchard 2010, Bonneaud et al. 2006,

Wedekind et al. 1995). MHC-based mate choice is also seen in fish. Both sticklebacks and Atlantic salmon, a fish closely related to trout, appear to look for mates with a number of dissimilar *MHC* alleles to their own (Reusch et al. 2001, Aeschlimann et al. 2003). In order to convey enough information the olfactory signals used are likely the MHC molecules themselves (Singh et al. 1987), the peptides they bind (Leinders-Zufall et al. 2004), or products from the bacterial microflora that are in some way MHC-dependent (Milinski et al. 2005).

β2m also exists in bodily fluids free from the class I receptor; in this form it is a useful biomarker and may have important physiological functions. β2m is shed from the surface of cells with the turnover of the class I receptor because it is noncovalently bound to the α-chain (Cresswell et al. 1974). Soluble β2m can be found in the plasma, urine, saliva, and cerebrospinal fluid of all human beings as well as the seminal fluid of males and the amniotic fluid and colostrum of pregnant and post-labour females (Jonasson et al. 1974, Talal et al. 1975, Plesner and Bjerrum 1980). With many malignant or inflammatory pathologies such as multiple myeloma, heart disease, rheumatoid arthritis, Crohn's disease, Sjogern's syndrome and cancer/inflammation of the pancreas and urinary system, increased quantities of soluble β2m usually correlate with greater disease severity or poorer outcomes making it a marker of great prognostic value (Talal et al. 1975, Cuzick et al. 1985, Brenning et al. 1985, Zissis et al. 2001, Stefanovic et al. 1999, Kawai et al. 2010, Pezzilli et al. 1995). The physiological significance of free β2m remains unknown and could be nil but interestingly *in vitro* studies have alluded to some potential immune functions. For instance, purified or recombinant β2m can prevent the maturation of dendritic cells (Xie et al. 2003), act as a chemoattractant for

immature T cells (Dargemont et al. 1989) and can even agglutinate bacteria, fix complement and act as an opsonin (Ericson 1984, Painter et al. 2009). Soluble β2m is also likely a factor involved in cell growth and death as it can cause the proliferation or apoptosis of a range of cancer cells both *in vitro* and *in vivo* (reviewed in Zhu et al. 2009).

In rainbow trout, the genes for both β2m and the MH class I α-chain (MH Iα) have been cloned (Shum et al. 1996, Shum et al. 2001); in teleosts, the "complex" is dropped from the name because unlike in mammals the class I and class II genes are not linked (Shand and Dixon 2001). Additionally, these fish exhibit MH I-based viral immunity since rainbow trout cells infected with infectious hematopoietic necrosis virus (IHNV) can be killed by PBLs from a trout immunized against the virus and possessing a matching *MH Iα* allele but not from an immunized trout that is an MH Iα-mismatch (Nakanishi et al. 2002).

Despite knowledge of the MH I receptor in trout and other fish, little is understood about their soluble β2m. Here western blots employing a polyclonal antiserum developed against a truncated recombinant rainbow trout β2m protein (Kales et al. 2006) will be used to discern the distribution of free β2m in the body fluids of trout and in one case, for comparison purposes, Arctic charr. This knowledge will serve as a basis for understanding the physiological purpose of soluble β2m in trout and its potential usefulness as a biomarker for inflammation or disease and might help illuminate pathways of environmental release of MH I-related molecules.

2.2 Materials and Methods

2.2.1 Fish

Rainbow trout were obtained from Silver Creek Aquaculture (Erin, ON) and kept in 450 and 50 gallon fresh-water flow-through tanks at the University of Waterloo with the well water temperature kept at a constant 13°C. During the week, they were fed a daily dry pellet ration (5 point floating trout chow from Martin Mills, Elmira, ON). Fish were sacrificed by an overdose with 2-phenoxyethanol.

2.2.2 Gastrointestinal content collection and protein extraction

The contents of the stomach, proximal intestine and distal intestine were taken from 3 fish in different stages of digestion: a female fish roughly 34 cm long that was fed approximately 1 h before euthanization, a male fish approximately 35 cm long that was fed approximately 2 h before euthanization to allow food digestion, and a male fish roughly 40 cm long that had been starved for 4 days prior to euthanization. To get intestinal contents the intestine was removed at the rectum and stomach and divided in two. The contents were squeezed out of the distal end of each piece into separate petri dishes and subsequently moved to 1.5 mL Eppendorf tubes. For the stomach, if there were solid contents inside than the contents were removed with forceps and placed in an Eppendorf tube, otherwise the liquid contents were squeezed out the esophageal opening onto a petri plate and then transferred to a tube. All contents were frozen at −20°C.

For protein extraction, contents were thawed, aliquoted and weighed. An equal volume of PBS (phosphate buffered saline; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), or PBS-T (PBS containing 0.05% (v/v) Tween-20) if the contents were mostly

mucus, was then added to each and samples were blended with a hand homogenizer for 30 s, after which tubes were centrifuged at 21,000 x g for 15 min and supernatants collected. Tubes were spun down at the same speed for an addition 10 min if particulates were still found in the supernatant. Extracts were stored at −20°C. To control for β2m in feed, protein was also extracted from trout pellets that were crushed with a mortar and pestle in either PBS or concentrated HCl (15 pellets/mL).

2.2.3 Bile Collection

Bile was taken from the fish fed 2 h prior to sacrifice: the gall bladder was located and punctured with a needle (21 gauge) in place and bile was suctioned into a syringe. The bile was then transferred to a 1.5 mL Eppendorf tubes and stored at −20°C.

2.2.4 Blood collection, separation and protein extraction

Blood was drawn from the caudal vein using a 21 gauge needle doused in heparin. The blood was then transferred to a 15 mL conical tube, 3 mL of histopaque 1077 (Sigma-Aldrich, St. Louis, MO) was added underneath the blood and the tube was centrifuged for 15 min at 800 x g. Following this, some of the plasma supernatant was collected and stored at −20°C and the tube containing the remaining blood components was spun down again. The red blood cells (RBCs) were then collected from the bottom pellet and the peripheral blood leukocytes (PBLs) were collected from the plasma-histopaque interface and both were spun down at 440 x g for 5 min. Following this supernatant removal, pellets were washed with 1 mL of Hyclone Dulbecco's modified phosphate-buffered saline (DPBS; Thermo Fisher Scientific, Waltham, MA). Both cell types were inspected for purity using a Nikon Eclipse TS100

inverted phase contrast microscope. Protein was extracted from the cells by adding an approximately 2x volume of cell lysis buffer (1% (v/v) NP-40, 150 mM NaCl, 50 mM Tris (pH 8.0)) supplemented with 0.2% (v/v) protease inhibitor cocktail (Sigma-Aldrich) to the cell pellets, pipetting/vortexing the cells and leaving the tubes on ice for 20-30 min. Lysed cells were then spun down at ~ 7500 x g for 15 min and the soluble protein in the supernatant was collected and stored at −20°C.

2.2.5 Epidermal mucus collection and protein extraction

Mucus was heaped together by scraping the skin of a fish (fork-length of ~ 22 cm) with metal scupulas and the shoveled into a 1.5 mL Eppendorf tube. The mucus was spun down at 400 x g for 5 min to collect it at the bottom of the tube and was stored at −20°C. Protein was extracted by adding a roughly equal volume of PBS-T to the freshly thawed mucus, vortexing vigorously and spinning down for 10 min at 950 x g. The supernatant was collected and stored at −20°C.

2.2.6 Milt collection, separation and protein extraction

Milt from rainbow trout and Arctic charr was kindly provided by the Alma Aquaculture Research Station at the University of Guelph courtesy of Michael Burke. The milt used was collected in November 2010 from anesthetized fish in their first or second spawning season by the application of a light pressure to belly and towards the vent. Milt was centrifuged at 700-1000 x g for 5 min to separate sperm from seminal fluid. Protein was collected from sperm as it was for blood cells (Section 2.2.4) but with a roughly equal volume of cell lysis buffer. Extracts and seminal fluid were stored at −20°C.

Sperm pellets from Arctic charr were washed in a separate experiment to rule in/out the possibility of sperm contamination by seminal fluid. To do this, milt from a single fish was separated into equal volumes among three tubes. From one tube, sperm lysate was collected as normal while sperm pellets from the remaining tubes underwent either one or two washes with 1 mL of DPBS (spun down at 1000 x g for 5 min) prior to protein extraction.

2.2.7 Protein Quantification

Bradford Assay

The protein content of some of the extracts and body fluids were assessed using a Bradford assay (Bradford 1976) according to Bio-Rad's (Hercules, CA) microtiter plate protocol. The standard dilution series was made up of bovine serum albumen (BSA) diluted from 0.5 mg/mL to 0.0658 in 2/3 dilution intervals and 10 μL of each standard and sample were added to the wells of a Nunc 96-well plate (Thermo Fisher Sceintific) in triplicate. Following this, 200 μL of prepared dye (5:1 mixture of the provided Bio-Rad dye and MilliQ water) was added to each well. Plates were then incubated for 10 min and read at 595 nm by a VersaMax™ spectrophotometer (Molecular Devices, Sunnyvale, CA).

BCA Assay

The protein levels of other extracts and body fluids were analyzed by a microplate BCA assay. The advantage of this assay is that it is unaffected by many detergents including NP-40 which can sometimes skew the results of the Bradford assay. Like the Bradford Assay, this protocol also used a BSA standard dilution series and 10 μL of each sample and standard was added to wells of a 96-well Nunc plate (Thermo Fisher Scientific) in triplicate. Similarly,

200 μL of dye was added to each well. The dye was made by mixing 50 parts of BCA reagent A with one part BCA reagent B (Thermo Fisher Scientific). Plates were incubated for 15 min at 37°C and read by a VersaMax™ microplate reader (Molecular Devices) at 562 nm.

2.2.8 Polyclonal antiserum

The polyclonal antiserum used was made against a truncated recombinant trout β2m protein (rβ2m), as described elsewhere (Kales et al. 2006). The rβ2m was produced in the BL21(DE3)pLysS *E. coli* expression system using part of a type 1 (functional and expressed) trout β2m coding sequence (accession # L47354) and injected into rabbits conjugated to KLH along with Freund's complete adjuvant. After exsanguination, the antiserum was affinity purified using a full-length rβ2m that had been produced in a similar manner.

2.2.9 Western Blot

SDS-PAGE was performed with 15% gels that had equal amounts of total protein in loading buffer (50 nM Tris (pH 6.8), 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% bromophenol blue and 10% glycerol) added to each well. RTgutGC culture supernatant was diluted 1:2 before loading. Proteins were transferred to nitrocellulose membranes (Bio-Rad) by semi-dry means using a transfer solution consisting of 25 mM Tris, 192 mM glycine and 20% (v/v) methanol. To ensure protein transfer and assess total protein distributions, membranes were stained for 10 min with Ponceau S stain (0.2% (w/v) Ponceau S powder and 5 % glacial acetic acid). The membranes were then washed with MilliQ water and scanned on a flatbed scanner. Next, membranes were blocked with 5% skim milk contained in "western TBS-T" (10 mM Tris, 100 mM NaCl, and 0.1% (v/v) Tween-20) for 1 h at room temperature with a possible

overnight storage at 4°C and then exposed to 1° and 2° antibodies diluted in this same blocking buffer for 1 h each. In terms of antibodies, a 1:200 dilution of anti-trout β2m was used as the 1° and a 1:3000 or 1:30,000 dilution of anti-rabbit IgG polyclonal goat antibody conjugated to alkaline phosphatase (Sigma-Aldrich) was used as the 2°. Bands were detected by exposure to NBT/BCIP and blots archived with a flatbed scanner.

2.2.10 Cell Culture

Conditions

This study employed the recently developed rainbow trout intestinal epithelial cell line RTgutGC (Kawano et al., 2011). These cells were grown in Leibovitz's L-15 basal medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S; 10,000 U/mL penicillin G and 100 mg/mL streptomycin sulfate; Sigma-Aldrich). Cells were kept at room temperature (20-22°C) in sealed 25 cm^2 or 75 cm^2 flasks (BD Falcon, Franklin Lakes, NJ).

Subcultivation

Confluent flasks were washed with 5 mL of PBS and then detached from the flasks by treatment with TrypLE (1.5 mL for 25 cm^2 flasks and 3 mL for 75 cm^2 flasks; Invitrogen, Carlsbad, CA) along with vigorous shaking. L-15 containing FBS and P/S was added to the cells which were collected and spun down for 5 min at 440 x g. After aspirating the supernatants, cell pellets were resuspended in an appropriate amount of supplemented L-15 and transferred to new flasks. No flask housed cells more than 3 times. Cells were usually split 1:2 although occasionally they were transferred undiluted or diluted 3:4. The media was

also changed in between passages on occasion. After a 1:2 splitting it typically took 7 days for the cells to become confluent again.

Collection of supernatant

The complete media of confluent 25 cm2 RTgutGC cultures was aspirated and cells were washed with 5 mL of DPBS. L-15 – FBS was then added to replace the media and cells were incubated for a given time, after which, cells were collected by treating cells with TrypLE (Invitrogen) and spinning them down as usual. The supernatants were collected and stored at −20°C.

Microscopy

Cells were visualized with a Nikon Eclipse TS100 inverted phase contrast microscope.

2.2.11 Cytotoxicity Assay

RTgutGC cells were taken from a confluent 75 cm^2 flasks and counted with a hemocytometer. The experiment was done with passage 88 (P88) cells. Cells were collected as in subcultivation (Section 2.2.10) but the L-15 medium used had no FBS supplementation (L-15 – FBS). Wells of a 96-well plate (BD Falcon) were seeded with 40,000 cells in 200 μL and allowed 24 h to attach to the plate. Following this, the plate was inverted to remove the media and each lane (8 wells) was given different treatments in L-15 – FBS. Treatments consisted of a recombinant truncated form of β2m (rβ2m) produced in the BL21(DE3)pLysS *E. coli* expression system (Kales et al. 2006) and lipopolysaccharide (LPS; Sigma; Aldrich) in PBS. Plates were incubated with treatments for 24 h. After this period, media was again

removed, wells were washed with DPBS and 100 μL of dye mixture (5% (v/v) Alamar blue (AB) and 4 μM 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) in DPBS) was added to the wells and left for 1 h in the absence of light. Following this, fluorescence was immediately detected with a Cytofluor® multi-well plate reader (Perseptive Biosystems, Framingham, MA) using an excitation and emission wavelengths of 530 nm and 595 nm for AB and 495 nm and 530 nm for CFDA-AM. AB and CFDA-AM were used to measure cellular metabolism and plasma membrane integrity respectively (Dayeh et al. 2003).

2.3 Results

2.3.1 β2m is found in the intestines but not in the stomach and may be present in bile

The stomach and intestines of the fish fed 2 hours prior to sacrifice (fish 1) were full of solid waste, that appeared light yellow and dark yellow respectively while the contents of the intestines in the fish fed only an hour prior to euthanization (fish 3) were made up entirely of a dark yellow mucus; the stomach of this fish was full of feed though, appearing as a light yellow solid. Bile taken from the first trout appeared for the most part bright yellow but did have some blood contamination. The stomach of the starved fish (fish 2) only contained mucus (translucent and colourless with white specks) and its intestines were full of a dark yellow mucus with a small amount of a brown solid. All gastrointestinal contents from all three fish were visually uncontaminated with blood.

In all three fish, western blots found no β2m in the stomach contents (Figure 2.1 A). In the fish that were not starved, obvious bands of the size of β2m were observable in the proximal intestinal contents as well as the distal intestine but at a lower intensity. The starved fish was

different in that it had a high degree of β2m in its proximal intestine but no detectable amount in its distal intestine. Bile from fish 1 also showed a faint band in the western blot but was contaminated with blood. A duplicate blot performed with the gastrointestinal contents and bile of fish 1 but using pre-immune serum showed no detection of any kind (blot not shown). Finally, whether it was treated with HCl or not, the feed given to the trout was devoid of any β2m.

2.3.2 β2m is released from RTgutGC

After 24 hours, β2m was detectable in the supernatant of a confluent 25 cm^2 flask of RTgutGC (Figure 2.1 B). No band of this size was seen when an anti-β2m western blot was performed on L-15 medium containing SBA or WGA strongly implying that this was a cell-dependent phenomenon.

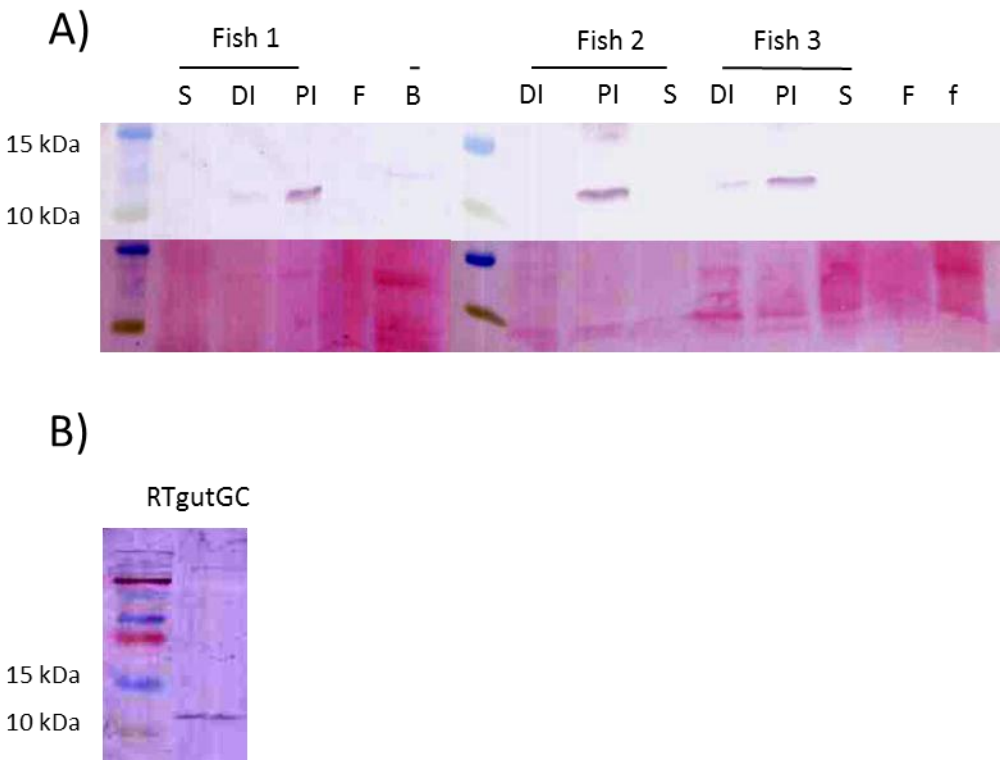

Figure 2.1 – Soluble β2m in rainbow trout intestinal lumen and RTgutGC culture medium.
All pictures are of anti-β2m western blots. The Ponceau stains performed on the membranes are shown below some blots. β2m is expected to be ~ 11.7 kDA in size. **A)** β2m distribution from a trout fed ~ 2 h before euthanization (Fish 1), starved 4 days prior to euthanization (Fish 2), and fed ~ 1 h before euthanization (Fish 3). Examined contents from stomach (S), proximal intestine (PI), and distal intestine (DI) as well as bile (B) and ground up and HCl-treated (F) or nontreated (f) feed . **B)** Supernatant from a 25 cm^2 flask of RTgutGC 24 h after media change to L-15 – FBS.

2.3.3 β2m is in the plasma, PBLs and epidermal mucus of trout

The blood components and epidermal mucus of a fourth rainbow trout were analyzed for the presence of β2m. Figure 2.2 shows that a relatively high concentration of β2m is found in the plasma, with a lower concentration associated with PBLs and no detectable presence in RBCs. A faint band was seen for RBCs however in a replicate blot using a higher concentration (1:3000) of 2° antibody (not shown). Under the microscope, the PBLs appeared to be contaminated with RBCs in about a 1:1 ratio while the RBCs did not show

any visible contamination with PBLs. β2m also appeared to be a constituent of rainbow trout epidermal mucus.

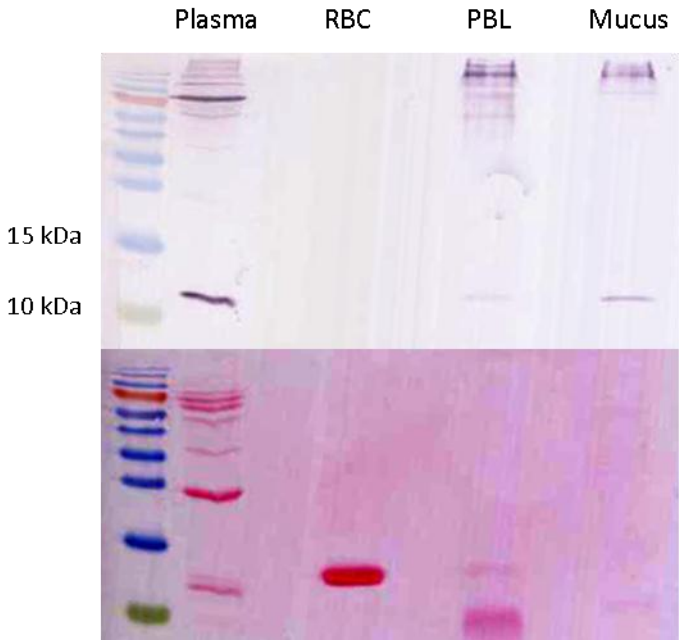

Figure 2.2 – Soluble β2m in blood and epidermal mucus
Anti-β2m western blot (above) with Ponceau (below) stain analyzing plasma, red blood cells (RBC), and peripheral blood leukocytes (PBL) separated from the blood of a single rainbow trout and the epidermal mucus gathered from this same fish.

2.3.4 β2m is in the seminal fluid, ovarian fluid and ovaries but not in sperm

Seminal fluid from the milt of rainbow trout and Arctic charr developed ~ 11.7 kDa bands in western blots (Figure 2.3 A and B). The sperm pellet of rainbow trout was free of β2m while a faint band was seen with the pellet from Arctic charr. However, protein extracted from charr sperm that had been washed did not show any bands in another anti-β2m western blot (Figure 2.3 C). β2m was also found in the female trout reproductive tissues and fluids; it was detected in ovary extract as well as in the ovarian fluid (OF) but at a higher concentration (Figure 2.3 A).

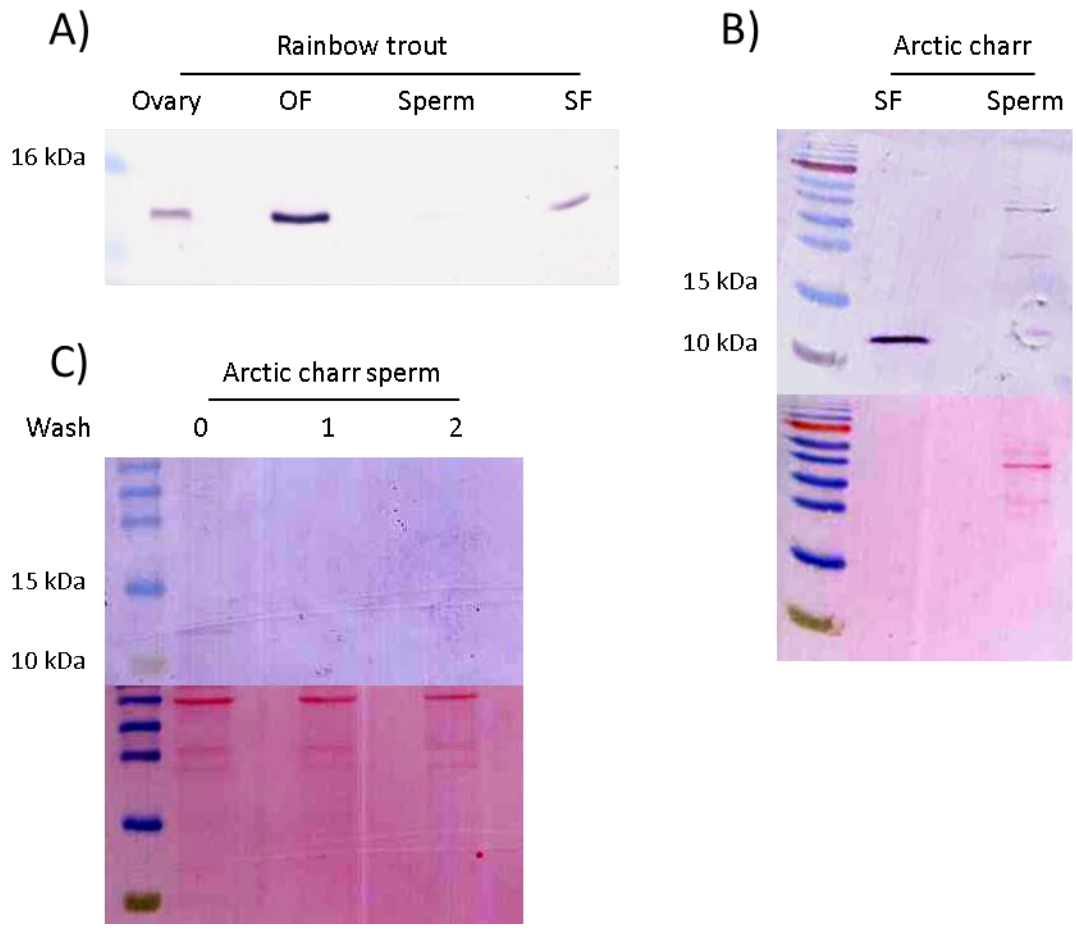

Figure 2.3 - β2m distribution in trout and Arctic charr reproductive tissues and fluids
All pictures are of anti-β2m western blots with Ponceau stains shown below some blots. **A)** Analysis of ovary and sperm extract from rainbow trout as well as ovarian fluid (OF) and seminal fluid (SF) (Kales 2006). **B)** Milt from Arctic charr split into seminal fluid (SF) and sperm. **C)** Sperm extract from charr. Pellets were given 0, 1, or 2 washes with DPBS.

2.3.5 rβ2m is not cytotoxic to RTgutGC cells and does not protect them from LPS-induced cytotoxicity

When RTgutGC cells were treated with soluble rβ2m at 80 µg/mL no difference in AB or CFDA-AM fluorescence was detected when compared to cells given only PBS indicating no cytotoxic effects (Figure 2.4). 50 µg/mL of LPS on the other hand, significantly reduced RTgutGC viability as assessed by both lowered metabolism (AB) and decreased membrane

activity (CFDA-AM), as previously reported (Kawano et al. 2011), and the monolayers of these cells appeared ravaged under the microscope. The addition of rβ2m (20 μg/mL or 80 μg/mL) did not reverse this LPS-induced cytotoxicity in any meaningful way (Figure 2.4).

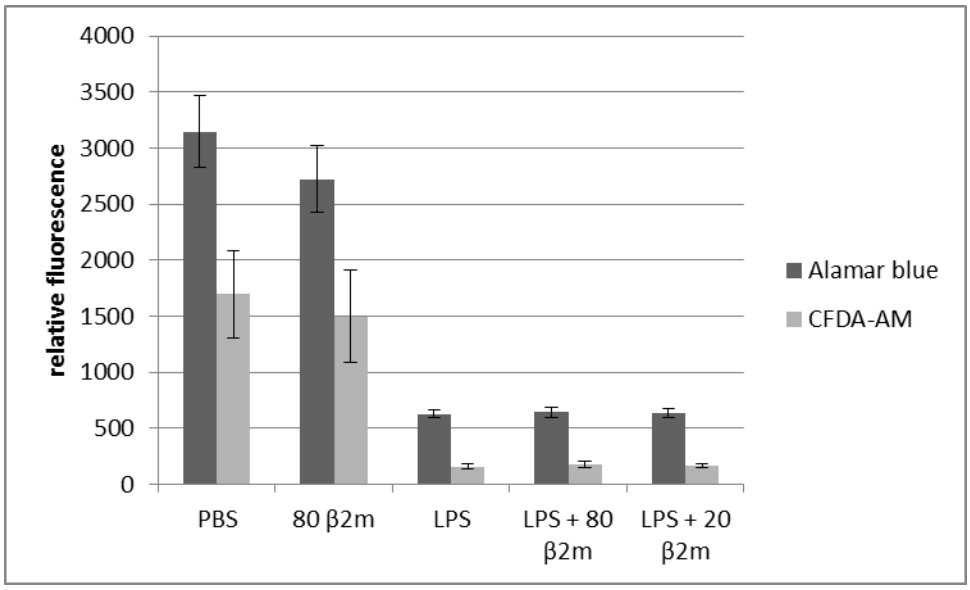

Figure 2.4 - Analysis of cytotoxic/protective effects of β2m on RTgutGC.
RTgutGC cells (P89) were treated with PBS, β2m, LPS or β2m in combination with LPS. The units for β2m are μg/mL and LPS was used at a concentration of 50 μg/mL. Each treatment contained an equal proportion of PBS and cells were exposed to treatments for 24 h. Viability was assessed with the fluorescent dyes Alamar blue and CFDA-AM. Error bars represent ± 1 standard deviation (SD).

2.4 Discussion

The main goal of this investigation was to elucidate some of the distribution of β2m in rainbow trout and this was accomplished in a number of areas of the body. To start, the gastrointestinal contents of trout demonstrated an interesting pattern: a high concentration of β2m in the contents of the proximal intestine (PI), a lower concentration in the contents of the distal intestine (DI), and none detectable at all in the stomach contents (Figure 2.1 A). This discovery is particularly intriguing as it is the first study to show free β2m in the lumen

of the intestines. The luminal β2m was derived from the trout itself since the western blots did not detect any of the protein in the feed that was given to the trout (Figure 2.1 A). This was true even when feed was mixed with HCl to simulate travel through the stomach. Furthermore, similar levels of β2m were detected in the intestine independent of the presence or absence of solid waste. Also, the estimated 11.7 kDa band from the western blot that is assumed to be the β2m peptide was the specific result of the anti-β2m antiserum as a duplicate blot using pre-immune serum from the same rabbit did not detect anything whatsoever (data not shown).

The pattern itself (absent in stomach, high in PI and low in DI) seems to suggest that a large amount of β2m gets released into the PI of the rainbow trout and that this then gets degraded or reabsorbed as it travels down the rest of the intestine. The liver and pancreas both empty their contents into the PI making them potential sources for this organ's abundance of β2m. To test the liver hypothesis, bile was investigated for its β2m content. A faint band was observed but this bile had been contaminated with blood which contains β2m (Figure 2.2 A) and so no conclusion on its β2m content can be drawn at this point. MH I α protein expression has been reported in the epithelium of the bile duct (Dijkstra et al. 2003) leaving open the possibility that β2m may be shed into the bile. However, this may not be strong proof since in humans β2m is expressed in hepatocytes and in bile duct epithelial cells as well but has not been found in bile (Nagafuchi and Scheuer 1986, Banner et al. 1996, Farina et al. 2011). Also, in comparison to other cell types, trout liver cell lines produce relatively less β2m protein (Kawano et al. 2010). β2m protein expression in the pancreas was

not examined and is unknown in fish but other proteomic analysis from humans suggests it is not present in the pancreatic juice either (Gronborg et al. 2007).

An alternative hypothesis is that β2m is shed from or secreted by the intestinal epithelial cells. This conjecture is buttressed by the observation that the rainbow trout intestinal epithelial cell line RTgutGC was shown here to be capable of releasing β2m extracellularly (Figure 2.1 B and later by an ELISA: Section 3.3.7). Moreover, β2m protein can be found in trout intestinal extract (Kales et al. 2006) and immunohistochemistry has shown that in the pyloric caeca of rainbow trout the epithelial cells display vivid staining for MH Iα while cells of the lamina propria do not (Dijkstra et al. 2003). Because the PI has a higher concentration of the protein, epithelial cells there would have to produce more of or perhaps all of the β2m for this proposal to hold. The fact that starved fish which presumably would have had less intestinal movement showed β2m only in its PI suggests all the β2m originates from this area. To clarify this situation, immunohistochemistry should be done to see if a protein expression gradient for β2m exists in the intestinal epithelium.

β2m levels were also analyzed in the blood (Figure 2.2). It was found in the plasma of trout showing that this feature of humans is evolutionarily conserved. Unsurprisingly, the western blot also showed that rainbow trout PBLs express β2m; this result was expected because surface expression of both β2m and MH Iα has been shown in trout leukocytes before (Kales et al. 2006, Dijkstra et al. 2003). This expression can be attributed solely to the PBLs even though they were contaminated with RBCs because pure RBCs did not show the same level of β2m expression.

Whether or not β2m is found in trout RBCs is less straightforward. Though RBCs did not show a band in the western blot when 1:30,000 dilution of 2° antibody was used (Figure 2.2), a faint band was detected with a 1:3000 dilution (not shown). Dijkstra et al. (2003) have witnessed a small amount of MH Iα surface expression in trout RBCs and in a separate experiment using the same polyclonal antisera used here, a 35 kDa band was observed by an anti-MH Iα western blot (Kales 2006). However, the latter study could not detect any β2m on the surface of trout RBCs (Kales et al. 2006). It might be that the assay used to determine surface expression in that study was less sensitive than that used by Dijkstra et al., or alternatively that the β2m in RBCs may remain inside the cells rather than coupling with MH Iα on the surface of the cells. Although the α-chain can reach the cell surface in small quantities without the accompaniment of β2m (Vitiello et al. 1990), fish RBCs are nucleated and therefore could have an immunological use for full MH I receptor surface expression. Furthermore, surface expression of the entire receptor has actually been documented in chicken RBCs which also have nuclei (Ziegler and Pink 1978). It is therefore plausible that surface MH I surface expression does occur in trout RBCs and that the previous assay was not sensitive enough to detect the β2m surface expression that would accompany this.

As with the intestinal mucus, the origin of β2m in the plasma of rainbow trout remains unclear. Given the much lower concentration of β2m found in PBLs it does not seem reasonable that shedding of the protein from their surface is the sole source of plasma β2m, especially if it is catabolized by the kidney as quickly as it is in humans (Karlsson et al. 1980). The answer may be that β2m is also shed from the endothelium. This is certainly a possibility since endothelial cells were shown by immunohistochemistry to stain brightly for

MH Iα in trout (Dijkstra et al. 2003).Conversely, in humans the liver has been proposed as the major producer of plasma β2m as hepatocytes appear to be able to secrete the protein (Ramadori et al. 1988). This could also potentially be the case in trout. In the liver of these fish MH Iα protein expression has been shown to occur in the endothelium of the central vein and the sinusoidal capillaries (Dijkstra et al. 2003).

Along with blood, mucus was taken from the skin of the same rainbow trout and interestingly this too was found to contain β2m (Figure 2.2). The epidermis of fish actually lies above the scales in direct contact with the mucus and in trout the epidermis has been found to contain leukocytes (Iger et al. 1995). MH Iα protein expression has also been shown in the apical epidermal cells (Dijkstra et al. 2003). Therefore, the epidermis and particularly epidermal leukocytes are prime candidates as sources of soluble β2m in the epidermal mucus of rainbow trout. β2m found in this mucus might also have practical value if free β2m proves to be a useful biomarker of disease and inflammation in fish as it is in humans since mucus from skin could be sampled quickly and noninvasively.

Finally, some interesting results came from examining the reproductive tissues and fluids of trout for β2m (Figure 2.3 A). This protein was detected in SF, in ovary extract and was particularly concentrated in OF (the ova remain to be tested however). As sperm do not have detectable β2m they are likely not the source of this protein in SF. It might be that the ultimate source of β2m in reproductive fluids is plasma since in humans levels of soluble MHC proteins found in the SF correlated with levels found in the plasma (Koelman et al. 2000). Given that all somatic cells should show some degree of MH I surface expression the β2m found in reproductive fluids might be shed from cells of the testis and sperm duct in

males and ovary and oviduct in females. In support of this, the rainbow trout gonadal fibroblast cell line RTG-2 was shown to express relatively high levels of β2m (Kawano et al. 2010). Similar to trout, Arctic charr also had β2m in its SF while having none in the sperm (Figure 2.3 B and C). This demonstrates that this phenomenon is conserved across the two salmonid species and adds some legitimacy to this allocation of β2m.

The distribution of β2m in trout might indicate the pathways of release of olfactory cues for MH I recognition. In fish, the olfactory signals could possibly be peptides released from the MH receptors, for which there is evidence in sticklebacks (Milinski et al. 2005), or the MH molecules themselves. Since β2m shedding corresponds with surface expression of the MH I receptor (Cresswell et al. 1974) and this would likely coincide with the release of bound peptide or the α-chain itself, it seems reasonable that routes of β2m excretion may be the same as those used by MH I olfactory cues. By this logic, the results of this study indicate that MH I olfactory molecules might be released from the skin, the gonads and the intestines of rainbow trout. The urinary system is another possible route but trout urine remains to be tested for β2m or MH Iα.

Indeed this study actually demonstrates that β2m molecules in fluids do tend to mark the presence of class I α-chains in trout and these molecules when found in the reproductive fluids might be more than simply olfactory cues. β2m was discovered in both trout plasma and SF and previously these fluids were found to contain shorter than normal (34 kDa) proteins that reacted with the anti-trout MH Iα polyclonal antisera showing a correlation does exist between the release of these two proteins in trout. Soluble α-chains of a similar molecular weight are common in human plasma (Adamashvili et al. 2003). They lack

transmembrane domains and are thought to be the result of proteolytic degradation (Dobbe et al. 1988, Haga et al. 1991). Soluble α-chains are also found in human SF (Koelman et al. 2000) and its presence here as with trout (and presumably Arctic charr) might serve as a distinguishing factor in female cryptic choice at fertilization. This appears to occur in humans as women with partners who are more closely related in terms of *MHC* alleles have higher spontaneous abortion rates (Ober et al. 1997). There is some evidence of MH-based cryptic female choice in fish as well. For instance, Arctic charr sperm from individuals heterozygous at the class IIb locus were shown to be more successful at fertilizing eggs than sperm from homozygous individuals (Skarstein et al. 2005).

With knowledge of the distribution of free β2m in trout, it is also possible to speculate on its physiological purpose rather than just its use as a marker. Firstly, β2m in the mucus (both intestinal and epidermal) may be antibacterial. This seems reasonable because fish mucus is known to contain many antibacterial peptides (Subramanian et al. 2008) and *in vitro* human β2m has been shown to bind to bacteria and agglutinate them (Ericson 1984). The β2m circulating in the plasma of trout on the other hand could act as a chemoattractant or a regulator of cell growth and death since recombinant or purified human β2m is known to cause chemotaxis in immature T cells and apoptosis or proliferation in a variety of human cancer cells (Zhu et al. 2009, Dargemont et al. 1989). Excitingly, β2m does seem to cause some chemotaxis in RTS11, a rainbow trout macrophage/monocyte cell line, adding credence to this potential function (Cynthia Tang, unpublished data). It is also possible in trout that different alleles of *β2m* might have different functions. To test this hypothesis, transcripts from different tissues could be sequenced and analyzed for any allelic bias.

The cytotoxicity assay performed in this study was designed to test the idea that β2m, which was shown to be released by RTgutGC, might be a mediator of RTgutGC cell growth/death. The experiment looked at rβ2m's direct cytotoxic impact on these cells as well as its ability to prevent the documented cytotoxic effect that LPS has on RtgutGC (Kawano et al. 2011) and it was found to neither kill nor protect the cells (Figure 2.4). This result does not exclude the possibility that β2m may have these effects in other cells and so other rainbow trout cell lines should be assayed. Also, the concentrations of β2m used (20 and 80 µg/mL) were quite a bit higher than those seen in human plasma under normal conditions (~1-2 µgEvrin 1972) and so even though similarly high concentrations of β2m have been shown to cause *in vitro* effects before (Zhu et al. 2009, Mori et al. 1999), trying some lower concentrations may be worthwhile and more appropriate.

In conclusion, this study has revealed for the first time the distribution of free β2m protein throughout the rainbow trout body and is the first ever to show that soluble β2m is present in the lumen of the intestines. The allocation hints at how β2m might be released and transported in various organs and organs systems (skin, gastrointestinal system, circulatory system) of trout and also what physiological roles soluble β2m might play. It also shows that in the future β2m could if desired could be used as a biomarker of disease and inflammation in fish since it can be detected noninvasively from the plasma or better yet the mucus of the skin. Lastly, free β2m might serve as an indicator of MH olfactory signals and therefore the distribution seen in trout has highlighted some potential pathways for the environmental release of these molecules. Much of this however remains speculation and so more research

is needed to fully appreciate the physiological significance of soluble β2m in trout and its potential use as a biological marker.

Chapter 3
The immune and other effects of plant lectins on RTgutGC

3.1 Introduction

The high cost and unsustainability of fish meal (FM) has led to its replacement by cheaper and more renewable products from plants such as soybean, wheat and corn in the feed of cultured fish, including the naturally carnivorous rainbow trout (FAO 2009b). However, FM still makes up 20-40% of the diet of trout as extensive substitution by plant products reduces fish yields or causes other problems in these fish (FAO 2009a). For instance, when soybean meal (SBM), a typical plant inclusion in trout feed, replaces more than 40% of FM, growth and nutrient utilization is reduced in trout (Rumsey et al. 1994, Dabrowski et al. 1989, Romarheim et al. 2006, Ostaszewska et al. 2005) and, additionally, these fish develop an acute intestinal inflammation. This gastroenteritis is characterized by changes in their gut histology such as blunted and fused villi, cell infiltration of lamina propria, increased vacuolation, necrosis and sloughing of intestinal epithelial cells and stimulation of the systemic immune system (Rumsey et al. 1994, Sealey et al. 2009).

Wheat is also found in trout feed usually in the form of flour or middlings (FAO 2009a). *In vitro* analysis demonstrated that higher levels of wheat flour in fish feed led to poorer digestibility (Bhattacharya et al. 1988) however good protein digestibility and growth have been shown with wheat products tested on trout *in vivo* at slightly lower concentrations (Davies et al. 1997, Sugiura et al. 1998). Despite this, there is the possibility that wheat products might have immunological effects on trout similar to SBM and higher levels of wheat, which could be included in trout feed in the future, might also have harmful effects.

One reason trout and other fish react negatively to high levels of plant products in their diet is the presence of bioactive components in these plants that are broadly labeled as antinutritional factors (ANFs, Rumsey et al. 1994). Lectins are one group of antinutritional factor found in plants. These nonenzymatic proteins are quite resistant to proteolysis by the gut and mediate their effects by binding to specific carbohydrate groups on the surface of cells. In mammals, lectins from wheat germ (WGA) when orally administered cause poor growth, alterations in the intestinal microflora, deepening of intestinal crypts, sloughing of intestinal cells into the lumen and hyperplastic and hypertrophic growth of the intestine itself (Pusztai et al. 1993, Pusztai et al. 1995, Lorenz-meyer et al. 1985). Furthermore, WGA can bypass the gut barrier and enter circulation and *in vitro* studies have shown it to have the capacity to activate various immune cells such as basophils, neutrophils, T cells and macrophages (Pusztai et al. 1993, Shibasaki et al. 1992, Haas et al. 1999, Lopes et al. 2005, Karlsson 1999, Kilpatrick 1995, Stolka et al. 2001). Lectin from soybean (SBA) when ingested has similar effects on growth, intestinal weight and microflora although few immunostimulatory effects have been reported (Pusztai et al. 1995, Armour et al. 1998).

Little work has looked at the effects of lectins in the diet of fish but studies looking for plant factors involved in salmonid SBM-induced gastroenteritis suggest SBA only plays a minor role. Although SBA can specifically bind to the distal portion of the intestines of rainbow trout and Atlantic salmon, the usual site of inflammation, and by itself can induce some histological changes that are reminiscent of the pathology induced with SBM (Buttle et al. 2001), it has also been shown that SBM fractions devoid of lectins and purified ANFs other than SBA can largely recapitulate the intestinal inflammation seen in salmon (van den

lngh et al. 1996, Knudsen et al. 2007, Knudsen et al. 2008). However, SBA may be a factor of secondary importance as two studies have shown that without it a full inflammatory reaction is not observed (Iwashita et al. 2008, Iwashita et al. 2009). Moreover, etiologies may differ between trout and salmon leaving open the possibility of SBA as a main causal factor in trout SBM-induced intestinal inflammation.

WGA, on the other hand, has not been examined at all in the context of fish gut interactions. This is concerning since trout and other fish are likely exposed to active WGA through their diets as flour is known to contain between 30 to 50 μg/mL of the lectin (Matucci et al. 2004, Radulescu et al. 2009). WGA also likely survives feed processing given its high heat-resistance (Peumans and Van Damme 1996, Matucci et al. 2004).

The major histocompatibility complex (*MHC*) genes which code for the class I and class II receptors exist in fish but are referred to as simply *MH* genes as they are not linked in a complex (Shand and Dixon 2001). The MHC class I receptor, made up of a transmembrane α-chain and a noncovalently bound β2m (Cresswell et al. 1974), is found on all nucleated cells and presents endogenous peptide antigens via the α-chain to $CD8^+$ cytotoxic T lymphocytes (CTLs) which when activated kill cells displaying these specific antigens; thus, this system provides the organism with specific viral and tumour immunity (Grommé and Neefjes 2002). The MH class I (MH I) receptor has the same structure as its mammalian counterpart (Antao et al. 1999). It also likely serves the same function since in trout its distribution mirrors that of mammals (Dijkstra et al. 2003) and it can be upregulated *in vivo* by infection with infectious hematopoietic necrosis virus (IHNV, Hansen and La Patra 2002). Furthermore, peripheral blood leukocytes from trout immunized with IHNV with matched

MH I to IHNV-infected cells could lyse these cells while cells from a MH I mismatched donor could not, showing that in trout something akin to CTLs is specifically recognizing MH I:antigen complexes and inducing apoptosis (Nakanishi et al. 2002).

The purpose of this study was to begin to understand what gastrointestinal consequences, if any, plant lectins in the diets of rainbow trout have on these fish by investigating the effects of purified WGA and SBA on the rainbow trout intestinal epithelial cell line RTgutGC. Other lectins (jack bean lectin = Con A, jimson weed lectin = DSA) and ANFs (Kunitz and Bowmann-Birk soybean trypsin inhibitors; SBTIs) were also used for comparison purposes. The effects of these lectins on morphology and cytoxicity were examined but also their impact on the expression of MH I-related and other immune genes because of the immunological properties that WGA and other lectins are known to possess.

3.2 Materials and methods

3.2.1 Lectins and other plant factors

Lectins were obtained from Sigma-Aldrich as lyophilized powders (Table 3.1). Stock solutions of 5 mg/mL (or 1 mg/mL in the case of DSA) were made by dissolving the powders in Dulbecco's modified phosphate-buffered saline (DPBS; Thermo Fisher Scientific). Soybean trypsin inhibitors (both Kunitz and Bowman-Birk types) were also purchased from Sigma-Aldrich in powder form and resuspended in DPBS at 5 mg/mL. Because DPBS was used as the solvent, all control treatments consisted of an equal volume of DPBS unless otherwise stated. All were stored as aliquots at −20°C.

Table 3.1 - Basic information on lectins used in this study

Plant source	Acronym	Sugar residue preference
Canavalia ensiformis (jack bean)	Con A	Man > Glc
Datura stramonium (jimson weed)	DSA	GlcNAc(β1,4GlcNAc)$_{1-2}$ > GlcNAc
Glycine max (soybean)	SBA	Terminal GalNAc oligomers/glycosides > Terminal GalNAc
Triticum vulgare (wheat, specifically the germ)	WGA	GlcNAc(β1,4GlcNAc)$_{1-2}$ > GlcNAc

Man = Mannose; Glc = Glucose; GlcNAc = N-acetylglucosamine; GalNac = N-acetylgalactosamine

(Goldstein and Poretz 1986)

3.2.2 Cell culture

Conditions

These experiments were mostly conducted with the rainbow trout intestinal epithelial cell line RTgutGC (Kawano et al. 2011) but some observations were made with RTgill-W1, a gill epithelial rainbow trout cell line (Bols et al. 1994).Cells were grown in either 25 or 75 cm^2 sealed tissue culture flasks (BD Falcon) in L-15 medium supplemented with 10% (v/v) FBS and 1% P/S (Sigma-Aldrich) and kept at room temperature (20-22°C).

Subcultivation

Confluent flasks of RTgutGC and RTgill-W1 were washed with 5 mL of PBS, treated with (1.5 mL for 25 cm^2 flasks and 3 mL for 75 cm^2 flasks; Invitrogen) and shaken vigorously until cells detached. Fully supplemented L-15 was then added and cells were collected by centrifugation for 5 min at 440 x g. After aspirating the supernatants, cell pellets were resuspended in an appropriate amount of supplemented medium and dispensed into their new flasks. Old flasks were reused up to a maximum of 3 times. Generally, cells were diluted 1:2 but were sometimes transferred undiluted or diluted 3:4 and confluent flasks occasionally had

their media changed. All cell lines were ready to be subcultivated approximately one week after they were split 1:2.

Microscopy

A Nikon Eclipse TS100 inverted phase contrast microscope was employed to examine cells. Images of cells were taken with a mounted Nikon Coolpix E8400 camera.

3.2.3 Western Blot

Western blots were done to detect changes in relative levels of β2m and MH Iα proteins in cell extracts of RTgutGC treated with various lectins and SBTIs.

Cell extract preparation

Protein was extracted from RTgutGC cells in L-15 – FBS treated with lectins or trypsin inhibitors for 24 h. Cells were collected from 25 cm^2 or 75 cm^2 flasks by scraping and centrifugation at 440 x g for 5 min. After the supernatants were aspirated, pellets were resuspended in 50 or 100 uL (for 25 cm^2 or 75 cm^2 flasks respectively) of cell lysis buffer (1% (v/v) NP-40, 150 mM NaCl, 50 mM Tris (pH 8.0) supplemented with 0.2% (v/v) protease inhibitor cocktail (Sigma-Aldrich). Resuspended pellets were mixed by vortexing/pipetting, placed on ice for 20-30 min and spun down at ~7500 x g for 15 min to remove insoluble debris.

Protein in cell extracts was quantified with the BCA assay mentioned elsewhere (Section 2.2.7). Briefly, 10 uL of a bovine serum albumen (BSA) standard and extract dilutions were added to the wells of a 96-well Nunc plate (Thermo Fisher Scientific) in triplicate and with equal proportions of MilliQ water and cell lysis buffer. The BCA reagent, composed of 50

parts reagent A and one part reagent B (Thermo Fisher Scientific), was dispensed (200 μL) into each well. Plates were then moved to 37°C for 15 min and the absorbance at 562 nm was read by a VersaMax™ microplate reader (Molecular Devices).

Polyclonal antisera

One of polyclonal antiserum used was made against a truncated recombinant trout β2m protein (rβ2m, Kales et al. 2006). The rβ2m was injected into rabbits conjugated to KLH along with Freund's complete adjuvant after being produced in the BL21(DE3)pLysS *E. coli* expression system using part of a type 1 (functional and expressed) trout β2m coding sequence (accession # L47354). After exsanguination, the antiserum was affinity purified using a full-length rβ2m that had been produced in a similar manner. The other rabbit polyclonal antiserum was raised against trout MH Iα in a similar fashion (Kawano et al. 2010, Kales 2006). The recombinant protein used in this case was produced from part of the coding sequence of a trout UCA 0401 allele (accession # AY523662) that corresponded to the extracellular portion of the protein. This antiserum was also affinity purified.

Blotting

Equal amounts of protein were added to loading buffer (50 nM Tris (pH 6.8), 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% bromophenol blue and 10% glycerol), boiled for 5 min and loaded into the wells of polyacrylamide gels (12 or 15%) in 15 μL volumes. After electrophoresis, proteins were semi-dry transferred to nitrocellulose membranes (Bio-Rad), Ponceau stained and scanned on a flatbed scanner (Section 2.2.8). Following this, 20 mL of blocking solution (5% skim milk in "western TBS-T": 10 mM Tris, 100 mM NaCl, and 0.1%

(v/v) Tween-20) was added to membranes. Membranes were incubated with blocking solution for 1 h at room temperature with shaking and could have also been stored overnight at 4°C in blocking solution before 1° primary antibody was added. Both 1° and 2° antibodies were diluted in blocking solution and added to membranes for 1 h with shaking also at room temperature. Membranes were washed extensively with western TBS-T after both of these incubation periods. The anti-β2m and anti-MH Iα antisera mentioned above diluted at 1:200 and 1:100 respectively served as 1° antibodies while goat anti-rabbit IgG (Sigma-Aldrich) diluted 1:30,000 was used as 2°. Bands were detected by exposure to NBT/BCIP and blots were scanned with a flatbed scanner.

Densitometry

ImageJ software (NIH) was used to assess band intensities from both western blots and Ponceaus. Pictures were manipulated in Adobe Photoshop in an unbiased fashion in order to increase contrast. Occasionally, instead of this, densitometry was performed on pictures of blots taken with a Nikon D700 camera and manipulated with RawTherapee image-editing software.

3.2.4 Cytotoxicty Assay

Cytotoxicity was measured in RTgutGC using the fluorescent dyes Alamar blue (AB) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), which measure cellular metabolism and membrane permeability respectively (Dayeh et al. 2003). First, cells were collected in L-15 – FBS and added to the wells of 96-well plates (BD Falcon) in 200 μL volumes containing 40,000 cells each. These plates were incubated for 24 h to allow cells to

attach to the bottom. Media was then removed from the wells through inversion of the plates, 200 μL of varying concentrations of lectins in L-15 – FBS were added to each column (8 wells), and plates were incubated for an additional 24 h. DSA was added at concentrations necessary to give them the same molarity as WGA concentrations. This ensured that these lectin treatments contained an equal number of total carbohydrate binding sites as both WGA and DSA contain two sites per molecule (Goldstein and Poretz 1986). After this, the treatments were displaced and the wells of plates washed with 200 μL of DPBS. After washing, 100 μL of an AB-CFDA-AM dye mixture (5% (v/v) of the former and 4 μM of the latter in DPBS) was added to every well. Subsequently, plates were kept in the dark for 1 h. The plates were then read for fluorescence with a Cytofluor® multi-well plate reader (Perseptive Biosystems), first with excitation and emission wavelengths respectively set to 530 nm and 595 nm for AB and then set to 495 nm and 530 nm respectively for CFDA-AM.

3.2.5 Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed on the total RNA from RTgutGC cells in order to look at changes in transcript levels of *β2m*, *MH Iα* and two cytokine genes (*IL-8* and *HMGB1*) with exposure to WGA over time.

RNA extraction

RNA was extracted from RTgutGC cells exposed to either WGA or DPBS in L-15 – FBS. Cells were exposed for 6, 12 or 24 h or not at all (0 h) and collected using the TrypLE method (Section 3.2.2). Extractions were done in a flow hood using an RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Homogenates were made by

adding 600 μL of RLT buffer to pellets and pipetting cells up and down through a sterile 21 gauge needle at least 5 times as suggested as one option in the manual. Also, the discretionary 1 min drying centrifugation step was performed while the final optional step was skipped. From the ~ 30 μL of RNA elution a 2 μL aliquot was taken aseptically for RNA quantification; this was performed in the University of Waterloo's core facility with a NanoDrop 100 spectrophotometer and its complementary software. The remaining RNA was stored at −80°C.

Complementat DNA (cDNA) synthesis

Single stranded cDNA was synthesized from RNA extracts using a Fermentas RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Each tube contained 1 μg of total RNA, 250 ng/uL of Oligo$(dT)_{18}$ primer, 20 U of RiboLock™ RNase inhibitor, 1 x (v/v) of reaction buffer, 1 mM of dNTP, and 200 U of RevertAid™ M-MuLV reverse transcriptase (RT) and were filled to 20 μL with RNase-free water. "No RT" controls contained everything but the RT and "no template" controls lacked only the 1 μg of RNA. Tubes were incubated for 1 h at 42°C for elongation and then heated to 70°C for 10 min to terminate the reaction using an Eppendorf mastercycler ep gradient machine (Hamburg, Germany). cDNA was stored in aliquots at −80°C.

Polymerase chain reaction (PCR)

PCR was performed on each cDNA sample in duplicate. Every 25 μL PCR reaction contained 1 μL of template (cDNA), 1 U of Taq polymerase, 600 nM of forward and reverse primers, 200 μM of each dNTP, reaction buffer at 1 x concentration, and 1.5 mM $MgCl_2$

(MP Biomedicals, Solon, OH). Using a DNA Engine thermocycler (Bio-Rad) tubes were initially heated to 95°C for 5 min and then subjected to a number of cycles of the following: 1) a 1 min 95°C denaturing, 2) a 1 min primer annealing stage of variable temperature, and 3) a 72°C DNA extension of variable duration. The annealing temperature used was primer-dependent and extension time depended on the length of the PCR product: 30 s for those under 500 bp and 40 s for those over 500 bp (Table 3.2). All primers except for those specific to *β-actin* were designed to span introns.

Table 3.2 - Primers for RT-PCR reactions

Gene	Sequence (5'-3')	Annealing Temperature	Size (bp)	Accesion #	Reference
Cytokines					
IL-8	F-ATGAGCATCAGAATGTCAGCCAG R-TCATTTGTTGTTGGCCAGCATC	64°C	294	AY160982.1	N/A
HMGB1	F-ATGGGGAAAGATCCTAGGAAACCGAGAG R-CTACTCGTCATCATCCTCATCCTCATCGTC	54°C	585	BT073488.1	N/A
MH I					
UBA (MH Iα)	F-TCTCCTCCTGGTCATTGTTGC R-CAGAGTCAGTGTCGGAAGTGCT	61°C	101	AY523665.1	(Landis et al. 2008)
β2m	F-TGTCAATCGTTGTACTTGGG R-CTTCAGGTGGCGGACTCTGC	58°C	300	AY217450.1	(Kales et al. 2006)
Control					
β-actin	F-ATGGAAGATGAAATCGCC R-TGCCAGATCTTCTCCATG	60°C	259	NM_001124235.1	(Komatsu et al. 2009)

Electrophoresis

PCR amplicons were size-separated through 1% (w/v) agarose gels embedded with 300 x GelRed nucleic acid stain (Biotium, Hayward, CA) and visualized under UV using an Alpha Innotech Fluorochem 8000 (Cell Biosciences, Santa Clara, CA).

3.2.6 Enzyme-linked Immunosorbent Assay (ELISA) for β2m

An ELISA was used to quantify differences in levels of β2m protein in the media of WGA-treated RTgutGC cultures.

Preparation of conditioned media

Media was taken from cells exposed or not to WGA under different conditions over time. RTgutGC cells (P81) in 25 cm^2 flasks were treated with 10 μg/mL of WGA or an equal volume of DPBS in 2.5 mL of L-15 – FBS after the removal of their complete media and a PBS wash. Flasks were kept at 4°C or room temperature (RT) and 100 μL of conditioned media was taken from each flask at 1, 3, 7, 10 and 13 days post-treatment. Media was stored at −20°C.

Set-up of assay

Soluble β2m in the conditioned media was analyzed using an indirect ELISA that incorporated rβ2m as a standard for quantification. First, wells of 96-well Nunc plates (Thermo Fisher Scientific) were coated with 100 μL of either conditioned media diluted 1:30 or rβ2m ranging in concentration from 1 μg/mL to 0.1 ng/mL for 2 h at 37°C. After washing 3 times with "ELISA TBS-T" (0.8% NaCl, 0.02% KCl, 0.3% Tris, and 0.05% Tween-20; pH 8.0), wells were blocked for 1 h at 37°C or overnight at 4°C with 200 μL of blocking solution (5% (v/v) skim milk in ELISA TBS-T) and washed again (3x). After this, samples were incubated at 37°C for 1 h with 100 μL of either 1:100 anti-β2m antiserum or 1:100 preimmune serum from the same rabbit. This was followed by another 3 washes and incubation with 100 uL of 1:5000 anti-rabbit IgG antibodies (Sigma-Aldrich) for 30 min at

37°C. After a final series of washes, samples were incubated with 100 μL of p-NPP for 30 min after which the colour development was read using a VersaMax™ microplate reader (Molecular Devices).. Everything was added in triplicate. The rβ2m standard curves used to calculate the concentrations of β2m are found in Appendix I.

3.2.7 Statistics

A one-way ANOVA was run on the cytoxicity data followed by Sheffe's Post Hoc analysis to compare the different means. This was done using SPSS version 17.0.

3.3 Results

3.3.1 WGA induces morphological changes in RTgutGC and other cell lines while other lectins and ANFs do not

RTgutGC cells drastically lost their connections with each other after treatment with 10 µg/mL WGA for 24 h. They also became more dendritic in shape and accumulated vesicles (Figure 3.1). These changes were seen after as little as 4 h of treatment. No other lectin (SBA, Con A, or DSA) or ANF (Bowman-Birk or Kunitz SBTIs) at any of the concentrations used (1-100 µg/mL) induced this or any other kind of significant morphological changes in this cell line. WGA-induced morphological changes in RTgutGC were largely abrogated in the presence of 10% FBS: monolayers appeared normal but some cells still possessed intracellular vesicles not seen in the controls (Figure 3.1; black arrows). This may indicate that RTgutGC is more sensitive to the induction of vesicle formation than the other morphological changes as carbohydrates present in FBS likely bind to WGA thereby lowering its effective concentration.

WGA also caused changes in the appearance of two other rainbow trout cell lines after 24 h of treatment. RTgill-W1 cells, when exposed to 10 µg/mL WGA in L-15 – FBS showed an accumulation of vesicles and separation of cells, akin to what happened in RTgutGC (Figure 3.2). The vesicles, however, were obvious in a much higher percentage of cells Morphological changes in all cell lines became more pronounced with increased exposure time or WGA concentration. This was seen as a greater separation of cells and vesicular formation in both RTgutGC, and RTgill-W1.

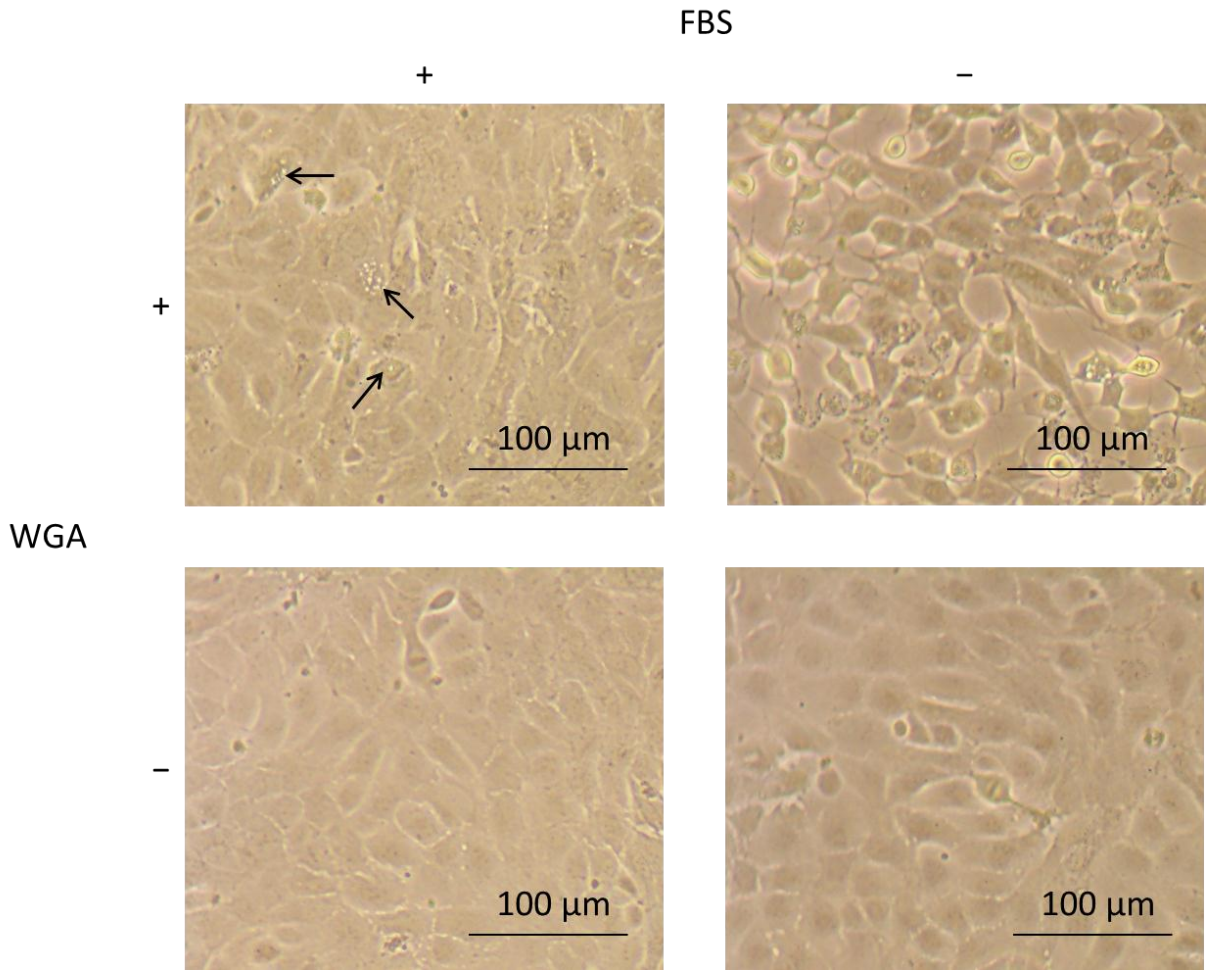

Figure 3.1 - Morphological changes caused by WGA in the presence of absence of FBS.
Confluent 75 cm^2 flasks of RTgutGC cells were exposed or not to 10 µg/mL of WGA for 24 h in L-15 medium that was either supplemented with 10% (v/v) FBS or was not. The WGA (+), FBS (–) morphology is representative of what was observed in the other experiments. Black arrows in the WGA (+), FBS (+) panel highlight cells showing abnormal vesicle formation.

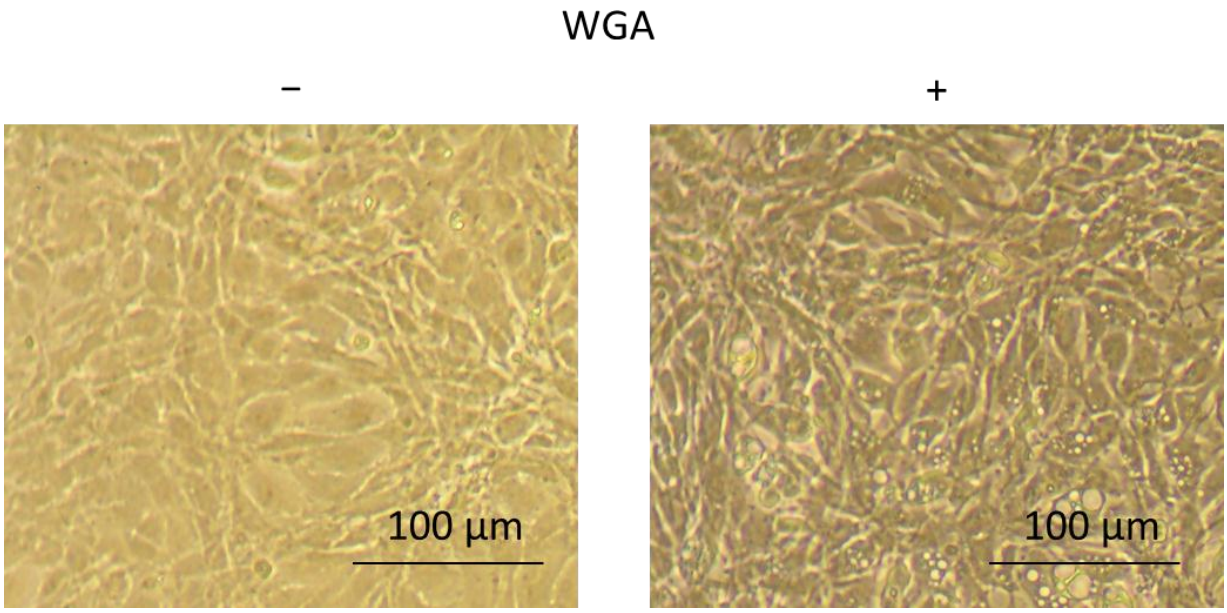

Figure 3.2 - Morphological outcomes of RTgill-W1 treated with WGA.
Pictures of control or WGA-treated (10µg/mL) RTgill-W1 (P51) cells in L-15 – FBS in 25 cm^2 flasks. Taken after 24 h.

3.3.2 WGA is slightly cytotoxic, Con A and DSA are not

In both trials, when cells were treated with 10, 50, or 100 µg/mL of WGA for 24 h they showed significantly lower fluorescence with AB, indicating a lower overall metabolism (Figure 3.3 A and B). CFDA-AM fluorescence also detected a downward trend in those cells treated with WGA but the differences were not significant. Cells treated with similar concentrations of both Con A or DSA for 24 h did not differ from controls in the fluorescence of either dye and were therefore considered nontoxic.

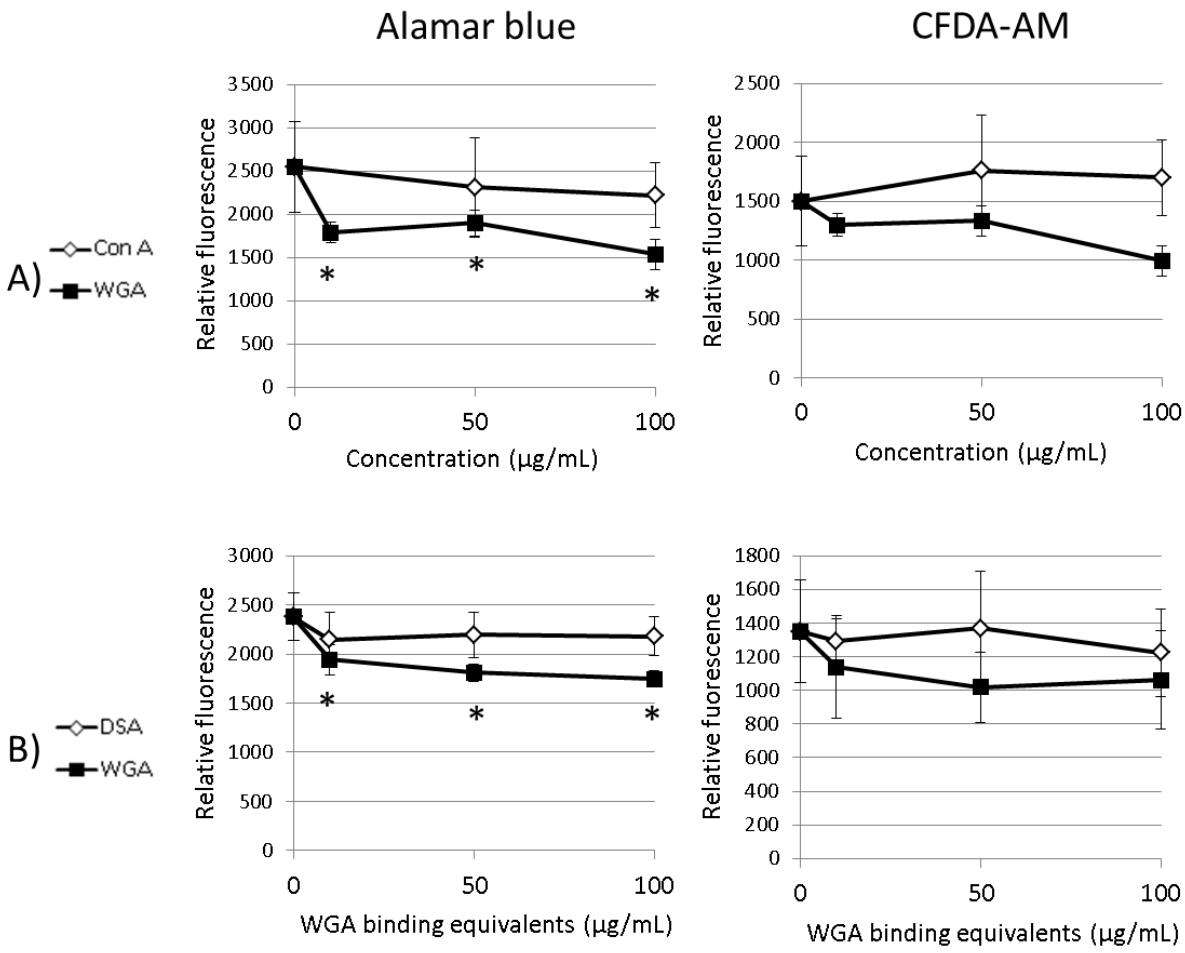

Figure 3.3 - Cytotoxic effects of WGA, Con A, and DSA on RTgutGC.
A) RTgutGC cells (P86) were treated with different concentrations of lectins which contained equal proportion of DPBS for 24 h. The graphs show the relative fluorescence of Alamar blue and CFDA-AM that was obtained following the treatments. **B)** Same as in A) except that P89 RTgutGC cells were used and the DSA concentrations used were equimolar to those used for WGA to keep the total number of binding sites the same between lectin treatments. Some concentrations of WGA showed significant ($p < 0.05$) deviation from the controls (*). All error bars represent ± 1 SD.

3.3.3 WGA and Con A increase the levels of MH I proteins (β2m and MH Iα) in RTgutGC while SBA and SBTIs do not

β2m protein expression in RTgutGC was heightened with increasing exposure to WGA (Figure 3.4 A and D). This effect appeared to plateau somewhat after 50 µg/mL and a similar dose response to WGA was observed for MH Iα expression as well (Figure 3.4 B and E). It is

important to note that, SBA did not cause any discernable change in the brightness of bands corresponding to β2m (~11.7 kDa) or MH Iα (~ 45 kDa) in either of the western blots (Figure 3.4 A and B) but interestingly, Con A caused an increase in β2m levels when cells were treated with the two highest concentrations of the lectin (50 or 100 µg/mL) though this was not as large an increase as that seen with WGA (Figure 3.4 C). β2m levels were also unaltered in RTgutGC cells exposed to SBTIs (Kunitz and Bowman-Birk types) alone (data not shown).

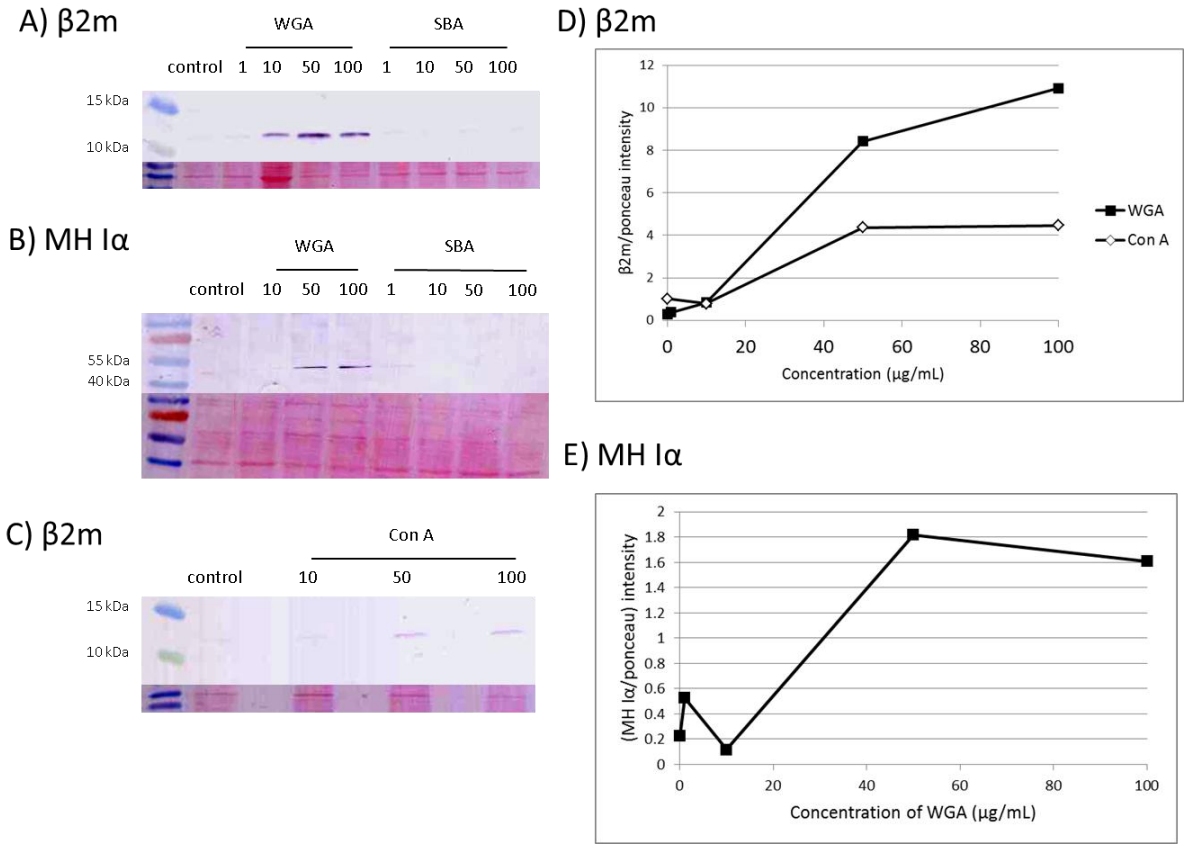

Figure 3.4 – MH I protein expression in RTgutGC exposed to lectins.
A) Western blot (above) and its Ponceau stain (below) showing β2m levels (~ 11.7 kDa band) in RTgutGC lysates (P76) exposed to increasing concentration of either WGA or SBA (µg/mL) for 24 h in L-15 – FBS. **B)** Same as A) but using anti-MH Iα as the 1° antibody. **C)** Same as A) but cells were P84 and treated with different concentrations of Con A. **D)** A graphical representation of the relative band intensities from A) and C) after normalization with the prominent Ponceau stain bands. **E)** A graph of the average values of normalized band intensities from two western blots (B) and one not shown) that measured MH Iα levels (~ 45 kD bands) in the lysates of cells dosed with varying concentrations of WGA.

3.3.4 WGA has no effect on MH I protein levels when attached to agarose beads

WGA's upregulation of β2m and MH Iα protein expression in RTgutGC was completely ablated when the lectin was bound to 6% agarose macrobeads. This was true even though the cells were exposed to double the concentration of WGA that showed an effect in soluble form (Figure 3.5 A and B).

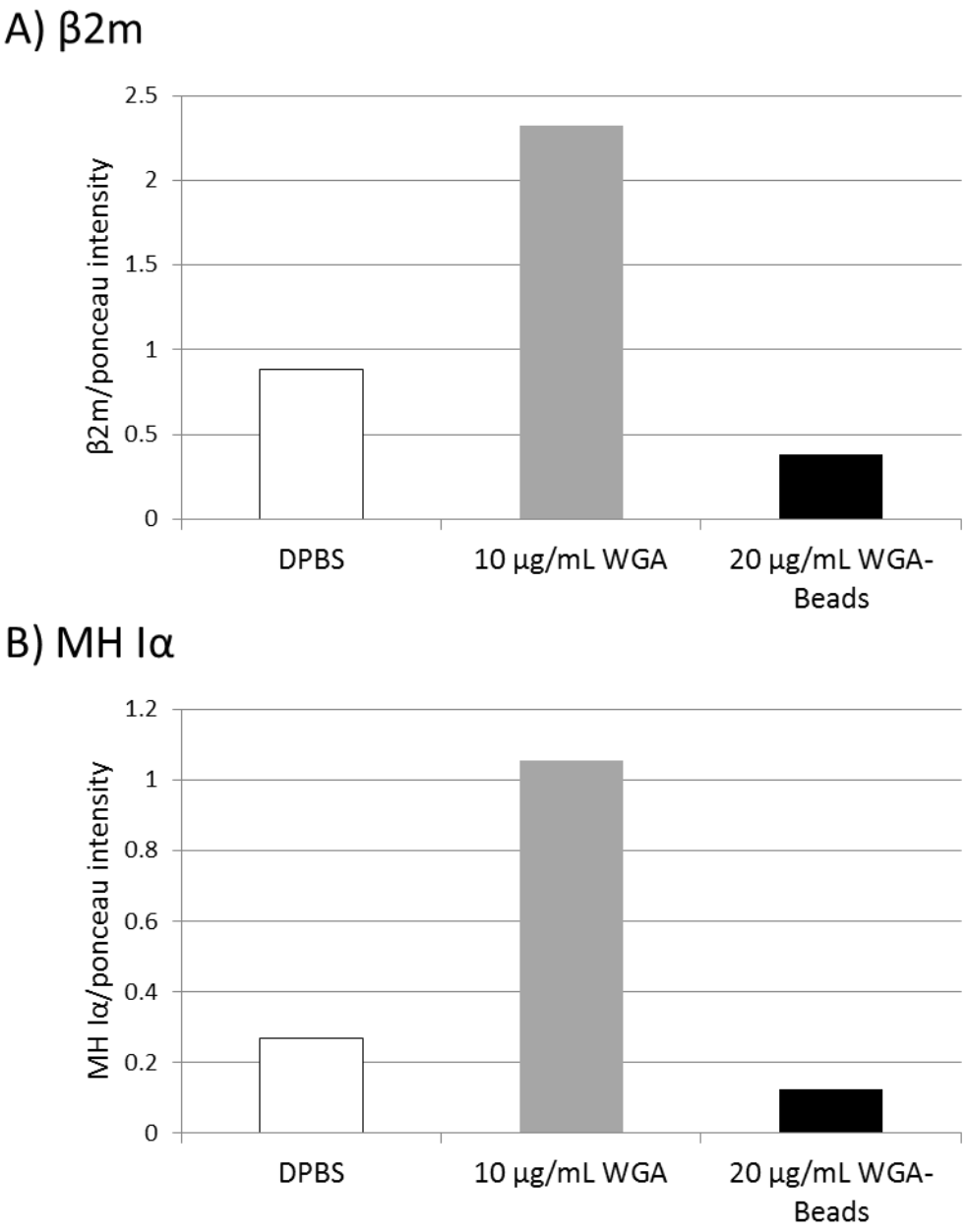

Figure 3.5 – The effect of WGA attached to beads on MH I protein expression in RTgutGC compared to that of soluble WGA.

A) Confluent 75 cm^2 flasks (P79) were treated with 10 µg/mL soluble WGA or 20 µg/mL of WGA bound to 6% agarose beads. Values represent the average intensity of the western bands over the intensity of their prominent Ponceau band from duplicate blots. **B)** The same as A) but from a single blot using the polyclonal antiserum against trout MH Iα.

3.3.5 WGA's effects are inhibited by N-acetylglucosamine (GlcNAc)

When RTgutGC cells were incubated with 10 µg/mL WGA and 20 mM GlcNAc (the monosaccharide it specifically binds to) the usual increase in β2m protein expression was no longer observed while an increase was seen when WGA was introduced with a lower concentration of the sugar or by itself (200 µM; Figure 3.6). Also, GlcNAc alone had no influence on β2m protein levels at either concentration. The same pattern was seen under the microscope: only WGA by itself or in combination with the lower concentration of sugar (not shown) displayed the characteristic WGA-induced morphology changes.

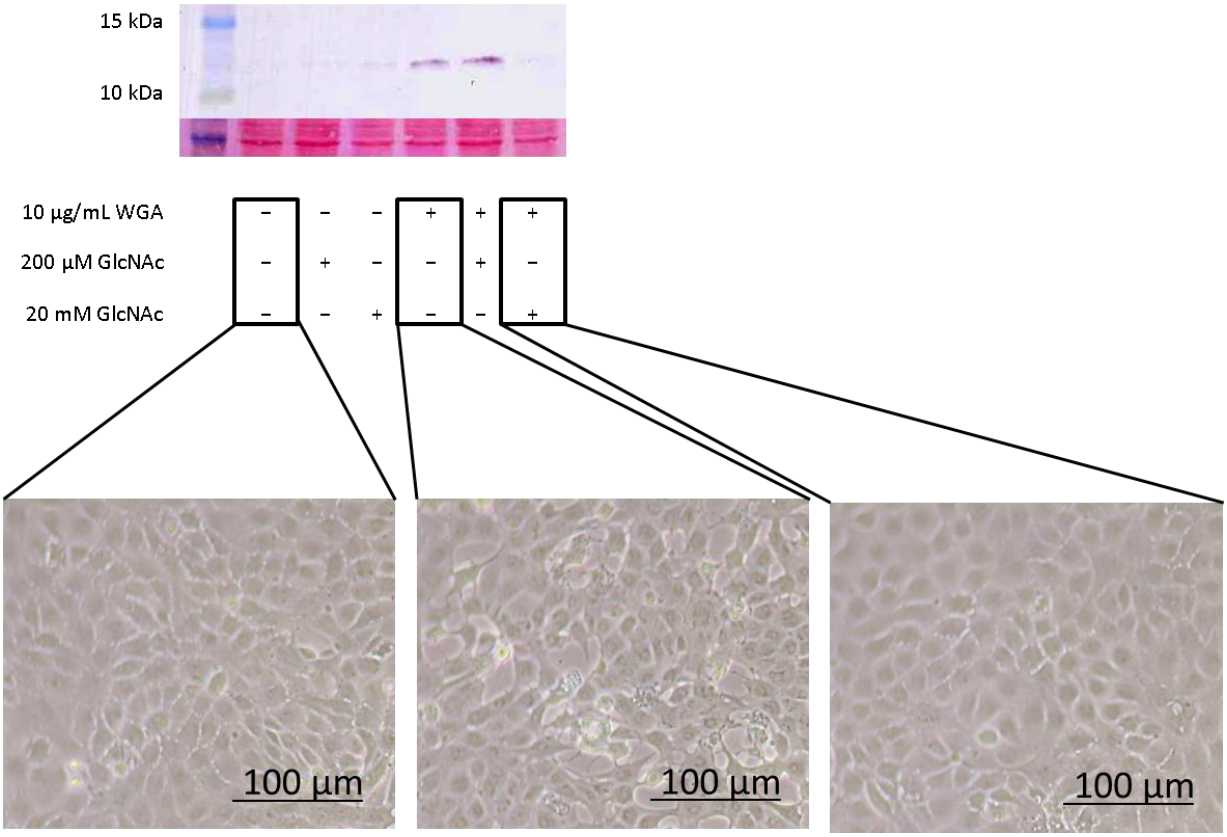

Figure 3.6 – Effect of N-acetylglucosamine sugar (GlcNAc) on β2m protein expression and morphology of RTgutGC treated with WGA.
Above picture shows an anti-β2m western blot, with its Ponceau stain underneath, of lysates of RTgutGC exposed to different combinations of GlcNAc and WGA. The pictures below show the appearance of control cells from this experiment as well as those exposed solely to WGA and those given 20 mM GlcNAc along with WGA.

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3.3.6 WGA does not dramatically change *β2m* or *MH Iα* transcript levels or those of the cytokine genes *IL-8* and *HMGB1*

RTgutGC cells exposed to WGA over time did not show substantial differences in levels of mRNA transcribed from *β2m*, *UBA*, *IL-8*, and *HMGB1* genes nor the *β-actin* gene which served as a loading control when compared to control flasks using RT-PCR (Figure 3.7). These results are likely not attributable to contamination with genomic DNA as every primer set except for those for β-actin were designed to span at least 1 intron and size of the PCR

products were the sizes expected if those introns had been spliced out (see Table 3.2). Also, the "no template" controls did not show bands following any PCR reaction confirming a lack of outside contamination. To ensure that DNA contamination was not causing the appearance of bands when using the β-actin primers, PCR was carried out using these primers on control tubes that did not receive RT during cDNA synthesis. Bands of the expected size were not seen confirming a lack of intervening genomic DNA.

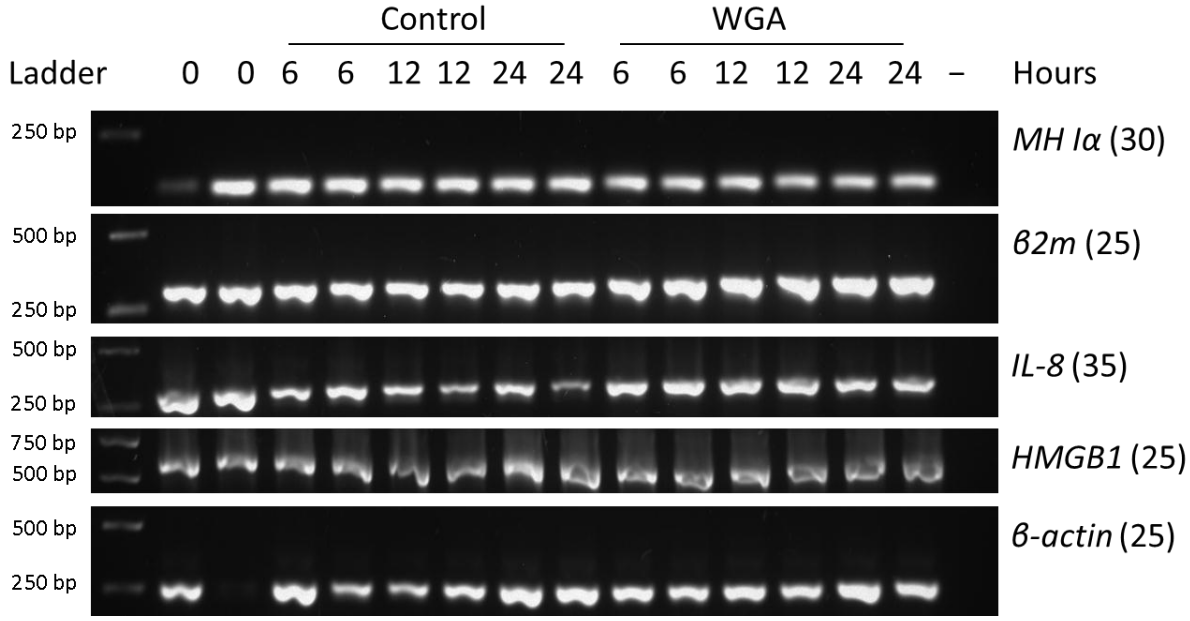


Figure 3.7 - RT-PCR analysis of various immune genes in RTgutGC exposed to WGA over time. Confluent 75 cm^2 flasks of RTgutGC (P87) were treated with 10 µg/mL WGA or not and RNA was extracted from different flasks at different times over a 24 h period. Duplicate PCR reactions were done on cDNA from each time point. A no template control (−) was run for each primer set. The genes analyzed are listed to the right with numbers representing the number of cycles used in their respective PCR reactions.

3.3.7 WGA stimulates β2m release at room temperature but not at 4°C

ELISAs performed on the conditioned media of RTgutGC cultures indicated that more β2m was released from cells treated with 10 µg/mL WGA at room temperature than controls

(Figure 3.8). When incubated at 4°C cultures released less β2m over time. There was also no consistent difference between exposed and unexposed cultures at this lower temperature.

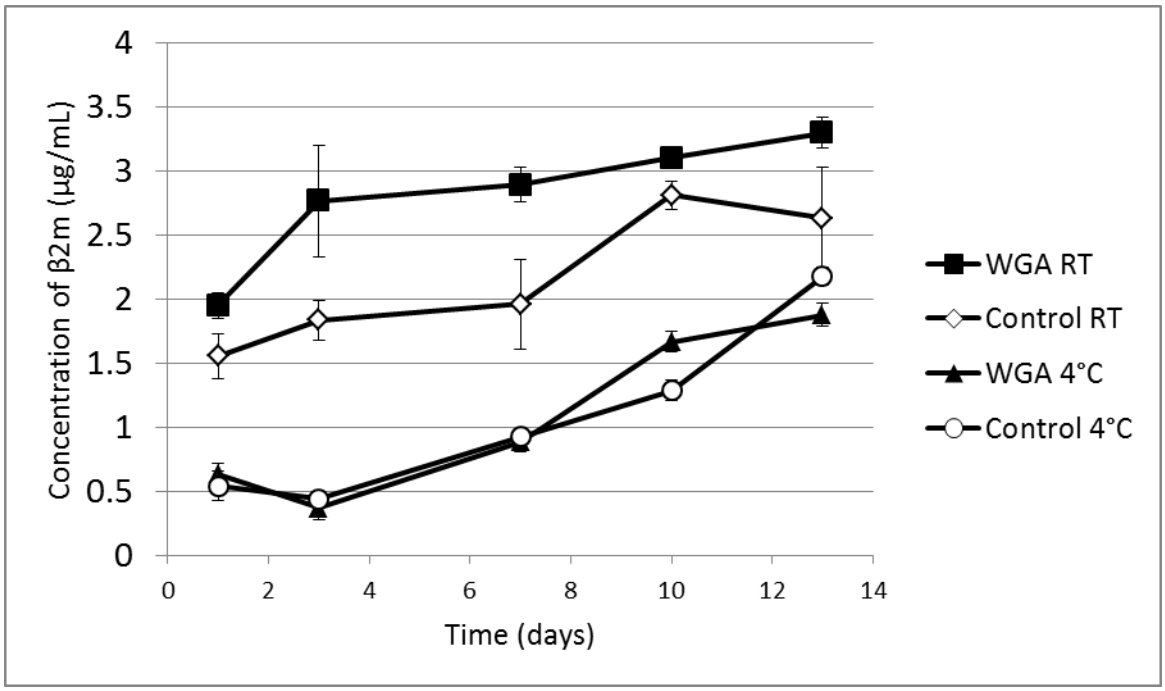


Figure 3.8 - Soluble β2m protein accumulation in condition media from WGA-treated RTgutGC cultures over time and at different temperatures.
25 cm^2 flasks were treated with 10 µg/mL WGA or an equal volume of DPBS (control) and held at 4°C or room temperature (RT). Conditioned media was collected over time and tested in triplicate for its β2m protein concentration using an ELISA. Separate plates were used for 4°C and RT samples. To control for nonspecific antiserum interactions, pre-immune serum controls were included for each experimental well and their values were averaged and subtracted from experimental values. The declining volume of media over each day was also accounted for and the appropriate adjustments made. The error bars represent ± 1 SD.

3.4 Discussion

Throughout this investigation WGA showed that it had numerous effects on RTgutGC and other cell lines while, for the most part, exposure to the other lectins and the SBTIs did not appear to have any real repercussions. In terms of morphology of RTgutGC and RTgill-W1, WGA was the sole ANF to cause any differences from controls: cells lost connections with one another, gained vesicles and became more dendritic (Figure 3.1 and Figure 3.2). These

changes likely require WGA to bind to carbohydrate receptors as co-treatment with 20 mM GlcNAc prevented any morphological changes from occurring (Figure 3.6). Also, the fact that both cell lines showed similar changes suggests that the molecular mechanisms used by WGA may be conserved across different cell types.

The morphological transformation of these cells by WGA might be caused by depolymerization of their actin cytoskeletons due to receptor-ligand binding reactions. Indeed, a change to the cytoskeleton could cause a dendritic appearance in cells and a loosening of junctions between cells (Koninkx 1995, Draaijer et al. 1989). Moreover, WGA has been shown to disturb the actin cytoskeleton of rat enterocytes *in vivo* (Koninkx 1995) as well as decrease transepithelial resistance in Caco-2, a human enterocyte-like cell line (Ohno et al. 2006). Also, when Caco-2 is treated with SBA the amount of cytoplasmic, unpolymerized G-actin was increased (Draaijer et al. 1989).These effects of SBA occur rapidly (after as little as 2 min) and are subject to specific sugar inhibition suggesting rapid biochemical changes (potentially in pH, calcium concentration, or phosphorylation of cytoskeleton-associated protein) mediated by receptor-ligand binding (Koninkx 1995, Draaijer et al. 1989). To test this hypothesis, the actin cytoskeletons of RTgutGC and RTgill-W1 could be stained using NBD phallacidin or anti-actin antibodies and microscopically examined for deformities following WGA treatment over short time intervals and with GlcNAc addition.

WGA was also the only plant factor to show any cytotoxicity towards RTgutGC although it was slight and the effect was seen only in terms of AB (Figure 3.3); Con A and DSA did not have any effects on RTgutGC and in similar experiments done at similar concentrations

for 7 and 31 days respectively, SBA and SBTIs (Kunitz) were not observed to be cytotoxic (Atsushi Kawano, unpublished data). WGA has long been known to have cytotoxic effects in cell lines (reviewed in De Mejía and Prisecaru 2005) and it is known to cause both necrosis and apoptosis (Dalla Pellegrina et al. 2004). However, how it and other lectins mediate cell killing is not well understood. The fact that the decrease in AB fluorescence undergoes a plateau might indicate that there is a rate limiting step in this process such as a limited number of surface receptors or a slow internalization of the lectin. Binding to surface receptors through carbohydrate groups is likely an important step in lectin-mediated cytotoxicity as cell lines that do not bind WGA or other lectins are also not lysed by them (Kim et al. 1993). Internalization may also be indispensible. This was demonstrated with $GS1B_4$ lectin (derived from *Griffonia simplicifolia*) that when attached to agarose beads could bind to but not kill cells that it normally would (Kim et al. 1993). Furthermore, cell killing by WGA was shown to be potentiated by monensin which inhibits the acidification of endosomal vesicles, implying that cellular transport and therefore internalization are significant in WGA-mediated cytotoxicity as well (Dalla Pellegrina et al. 2004). Therefore, it seems evident that future experiments should test the necessity of binding and internalization on WGA's RTgutGC cytotoxicity.

Instead of a reduction in viability however, WGA may be simply arresting the growth of RTgutGC cells because only AB fluorescence was significantly lowered, which indicates a lowered metabolism, and cell membranes remained intact according to CFDA-AM readings. One study which looked at the effect of WGA on mouse fibroblast cells supports this idea since instead of causing cell death WGA caused these cells to undergo cell cycle arrest (Liu

et al. 2004). These researchers also noticed the appearance of intracellular vesicles in treated cells which perfectly match those found in RTgutGC and RTgill-W1 cells exposed to WGA in this study (Figure 3.1and Figure 3.2) providing further evidence that rather than death WGA might be impeding the cell cycle in RTgutGC. More work is obviously needed to come to a definitive conclusion on this matter. This could include measuring, following WGA treatment, the proliferation of RTgutGC cells (e.g. by ^{3}H-thymidine incorporation) or changes in levels of proteins involved in the cell cycle in RTgutGC for which trout antisera exist (i.e. p53, Hus1, and Rad1).

Outside of its effects on morphology and cytotoxicity WGA also increased levels of the β2m and MH Iα proteins found in RTgutGC (Figure 3.4) and caused a greater release of β2m from RTgutGC over time (Figure 3.8). The protein expression of β2m was also found to be increased by Con A but to a lesser extent (Figure 3.4 C). To this author's knowledge, this is the first time MHC expression has been unquestionably linked to a plant lectin. These effects were not seen with SBA (Figure 3.4 A and B) or either type of SBTI, showing that this modulation is not a general property of lectins or other ANFs. Interestingly, WGA did not cause a similar increase in the transcript levels of these genes (Figure 3.7) showing that an increased transcription cannot account for the higher levels of MH I proteins found in RTgutGC cells. The greater release of β2m from RTgutGC also implies that higher intracellular levels of β2m do not result from increased retention of this protein. WGA's effect on MH I protein expression was negated by the presence of 20 mM GlcNAc (Figure 3.6) and also when WGA was attached to agarose macrobeads (Figure 3.5) which suggests that WGA must bind to glycoreceptors and then enter cells to wield its influence. However,

one issue with this latter result is that on top of preventing lectin from getting into cells, conjugation to beads might also prevent WGA from dispersing as it would in soluble form and so even by using double the concentration of lectin, it may still be difficult to disentangle the importance of these two effects. A possible solution to this problem might be to increase the concentration of conjugated WGA even more and monitor what happens.

Assuming WGA binds to cell-surface receptors and is then taken up by RTgutGC cells, as the results would suggest, it is possible that the lectin might mediate its effects on MH I protein levels through altering the normal recycling of these molecules (Figure 3.9). In mammalian cells, the MHC I receptor cycles between the endoplasmic reticulum (ER) and the cis-golgi (a suborganelle of the golgi apparatus, Hsu et al. 1991). Calreticulin (CRT), an important MHC I chaperone, binds to unstable MHC I receptors in the ER with poorly matched peptides through carbohydrate groups attached to their α-chains and follows these molecules into the cis-golgi to ensure they return to the ER to obtain a more stable peptide antigen (Howe et al. 2009). Once this is accomplished CRT is released due to changes in the α-chain's carbohydrate side-chain and the receptor can go to the surface of the cell (Howe et al. 2009). Expectedly, cells lacking CRT have deficient recycling of MHC I between compartments and have a greater turnover of MHC I receptors on the surface of cells likely due to the inferior stability of these receptors (Howe et al. 2009, Gao et al. 2002). WGA is known to bind to the golgi of enterocytes in fish (Pedini et al. 2002) and trout α-chain is also known to be glycosylated (Dijkstra et al. 2003, Kales 2006) and so it is possible that WGA might outcompete CRT for MH I binding and cause an increased turnover of this receptor. This would of course explain the greater release of β2m as this molecule is shed from the

surface of cells with MH I turnover. The increased levels of these proteins within cells might then be rationalized by an as yet unknown negative feedback loop which leads to an increase in translation of MH I proteins when turnover of the receptor is increased.

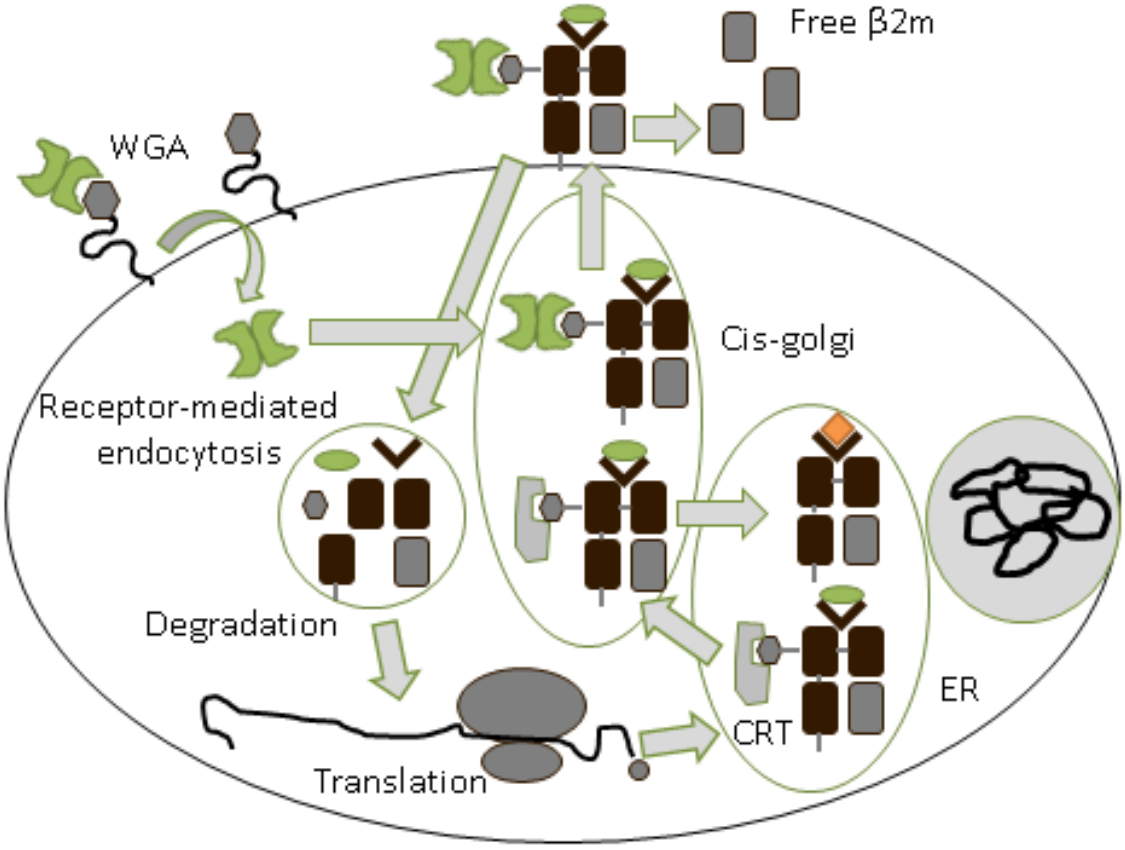


Figure 3.9 – Possible explanation for how WGA changes MH I protein expression
As explained in the body text, MH I receptors with poorly bound antigens cycle between the endoplasmic reticulum (ER) and cis-goli until they bind to an antigen to which they are better suited with the help of calreticulin (CRT). WGA, being a lectin, might interfere with CRT's ability to bind MH I which could lead to increased surface expression of unstable MH I receptors that are quickly degraded. The effects of this could be increased shedding of β2m and potentially an increase in translation due to a lack of proper MH I surface expression which would explain the higher release of β2m in the conditioned media of RTgutGC cultures and the greater MH I protein expression found in these cells after treatment with WGA.

To further explore this and other possibilities a number of future experiments could be performed. For one, fluorescent microscopy could be done staining for WGA, MH I proteins,

and CRT (for which a trout antiserum also exists, Kales 2006). This would ascertain for certain whether or not WGA is getting into RTgutGC and could show lectin co-localization with these proteins as might be expected. Also, flow cytometry could be performed to look for differences in MH surface expression which might change with turnover of the receptor. Finally, to test the idea of an increased translation, cyclohexamide could be added alongside WGA to see if the increase in MH I protein levels in RTgutGC is abrogated or not.

One other interesting result that is worthy of comment is the decreased release of β2m and the disappearance of WGA's effect on soluble β2m levels with RTgutGC cells held at 4°C compared to room temperature. Teleosts are known to be immunosuppressed at lower temperatures, with the immune system of salmonids usually being downregulated at 4°C and below (Bly and Clem 1992, Nath et al. 2006). However, trout maintain protein levels of β2m in a number of tissues including intestine as well as β2m surface expression on leukocytes at temperatures as low as 2°C (Kales et al., 2006). The release of β2m from fish cells has not been investigated until now and it is possible that there exists some active form of release of this protein which is abolished at colder temperatures and is independent of MH I surface expression. The negation of WGA's effect seen at the colder temperature might be due to its reliance on energy intensive processes of the cells such as endocytosis.

One vital question remains unanswered: What is responsible for WGA's high *in vitro* activity and the other lectins' low or nonexistent effects? All of the lectins used in this study can likely bind to RTgutGC since SBA can bind to trout enterocytes *in vivo* (Buttle et al. 2001), DSA has a binding specificity towards the same sugar groups as WGA, and Con A showed some upregulation of β2m in RTgutGC at higher concentrations (Figure 3.4 C) so the

most likely reason is differences in lectin receptor binding due to their unique binding affinities. SBA binds mostly to terminal N-acetylgalactosamine residues and therefore could bind to entirely different cell-surface receptors than WGA accounting for its lack of activity. Con A strongly binds mannose and glucose residues but can also bind weakly to carbohydrate groups that contain terminal N-acetylglucosamine in α-anomeric linkage (Goldstein and Poretz 1986) and so it might bind to a subset of the same molecules as WGA allowing it to share some of WGA's activity. Although this is complicated by the facts that in Chinese hamster ovary cells ConA and WGA show little overlap in surface binding (Emerson and Juliano 1982) and that curiously Con A does not change the morphology of RTgutGC cells along with β2m levels. DSA's lack of activity would at first glance, appear to invalidate this different receptor rationale because it shares WGA's sugar specificity. However, although they do share a preference for similar carbohydrate groups they often show differences in binding and therefore activity. For example, in rats DSA does not bind to the intestinal epithelium as well as WGA and thus has milder intestinal effects (Pusztai et al. 1993). It is also important to keep in mind too that the influence of DSA on the expression of β2m or MH Iα has not yet been assessed. To tease out if lectins are binding to similar receptors or not, competition assays could be performed where surface binding of a labeled lectin is measured with co-incubation of increasing concentrations of a different unlabeled lectin.

Taken together the results of this study suggest that WGA in the feed might be a silent problem for trout in aquaculture. For instance, WGA's morphological effects on RTgutGC could translate *in vivo* to reduced intestinal integrity and might lead to an influx of bacterial

antigens and therefore intestinal inflammation. WGA might also increase surface expression of the MH I receptor in RTgutGC which could also predispose fish to inflammation if WGA had a similar effect on trout enterocytes *in vivo*. Moreover, RTgutGC's increase in MH I protein levels may indicate that WGA is causing these cells to switch into a proinflammatory mode since amplified levels of β2m protein were seen before in RTgutGC when cells were exposed to poly(I:C), a synthetic double-stranded RNA that mimics viral infection (Kawano et al. 2010). However, this argument is weakened somewhat by the fact that the mRNA expression of IL-8 and HMGB1, two known immunostimulatory cytokines, was not altered in RTgutGC exposed to WGA (Figure 3.7).

Alternatively, WGA could compromise the immune system of the trout gut. This could occur by an increase in β2m secretion, without a corresponding increase in MH I surface expression, since soluble β2m was shown to prevent the maturation of dendritic cells *in vitro* (Xie et al. 2003). Poorer immunity would also result if WGA exposure leads cells to display a greater proportion of MH I receptors that are poorly bound to their antigens and therefore turnover more quickly, as hypothesized above.

These effects, although they may not influence fish growth, could predispose fish to disease or impair the quality of the filets and could therefore still be economically detrimental to trout aquaculture. The *in vitro* results of this study could be applicable to the real world as enterocytes of trout would be exposed to approximately 10 μg/mL WGA if wheat flour (30-50 μg/mL WGA) made up only a fifth of their diet. And, the fact that SBA and SBTI which are thought not to play a major role in soybean-induced salmonid

gastroenteritis (discussed in introduction and reviewed in Krogdahl et al. 2010) did not show any effect here further legitimizes the relevance of these results.

In conclusion, WGA has a number of effects on the rainbow trout intestinal epithelial cell line RTgutGC, above and beyond those of other lectins including SBA. These *in vitro* results suggest that wheat in the diets of trout may not be benign and reconfirms SBA's relative unimportance in the effects of SBM on the intestines of salmonids. Further *in vitro* and *in vivo* studies are urgently needed to assess whether the presence of WGA in feed causes harm to these fish and whether this has significance to rainbow trout aquaculture.

Chapter 4
General discussion

Some interesting insights come out of evaluating the results of both chapters 2 and 3 together rather than singly. One major one is that the release of β2m from RTgutGC seen in chapter 3 supports the notion that enterocytes are responsible for the soluble β2m found in the gut of rainbow trout from chapter 2. Both results also validate one another, as release of β2m from RTgutGC seems possible in light of its presence in the intestine and vice versa. If, in fact, the intestinal epithelial cells serve as the source of β2m in the intestines of these fish then ingested WGA may raise β2m levels found in the intestinal lumen. Also, if WGA can cause increased β2m release in other cells and can bypass the gut barrier in fish as it does in mammals then it might increase the level of β2m seen in other body fluids as well.

As a follow up to this work then, an *in vivo* test of WGA's effect on β2m would be invaluable. To do this, groups of trout could be fed diets containing purified WGA while others receive control diets. They could then have their plasma, feces and epidermal mucus analyzed for β2m content by ELISA or western blot. This experiment would not only test the *in vivo* validity of WGA's *in vitro* results, it could also confirm β2m's usefulness as a biomarker. If WGA is found to have harmful effects on trout than it might be possible to use β2m to indicate at what level WGA is deleterious, potentially allowing the use of wheat to be better optimized in trout aquaculture.

Increased β2m caused by WGA ingestion could also have effects on trout rather than simply denoting their presence and these effects could provide clues for understanding the

function of this protein *in vivo*. One ramification of perturbing the balance of β2m might be a disturbance in the normal microflora as soluble β2m can agglutinate bacteria *in vitro* (Ericson 1984) and therefore at higher levels in epidermal or intestinal mucus it might abnormally select against certain bacteria. In accordance with this, rats fed WGA and other lectins are known to show increases in coliform bacteria in their guts (Pusztai et al. 1993). Therefore, as a first step, *in vivo* experiments could also be done to look for changes in the fish gut or skin microbiota when WGA is orally administered. This could be done comprehensively using 16 s rRNA sequences to identify the constituents of the microbiota or narrowly by approximating the numbers of certain bacterial groups using selective media.

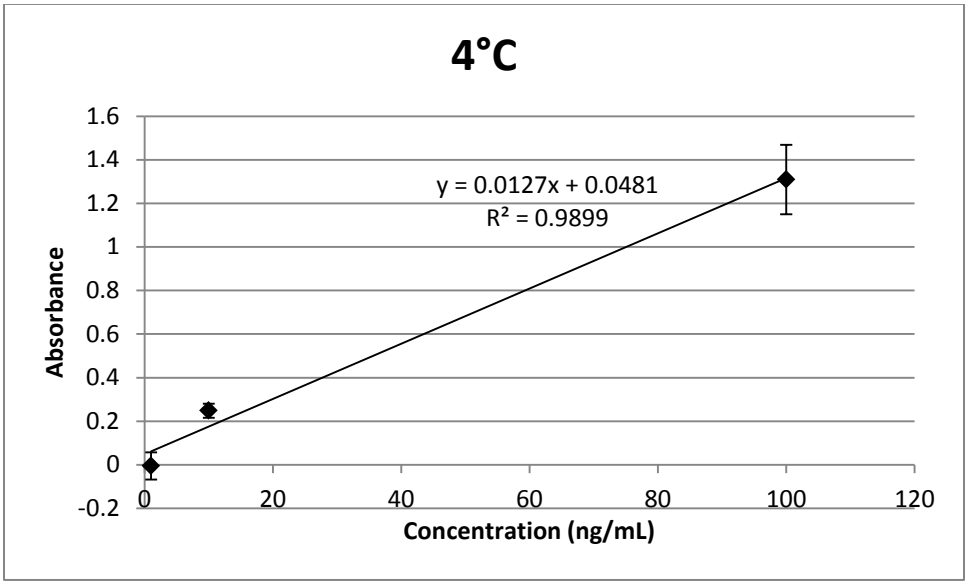
In conclusion, this thesis had two disparate goals: to elucidate the distribution of free β2m in the body of trout and to evaluate the consequences of plant lectins on the intestines of these fish, and both produced interesting preliminary findings and conclusions. However, the results of these two investigations converged on the idea that β2m is released from the intestines and WGA might influence this *in vivo*. This potential interplay between free β2m in the bodily fluids of trout and WGA treatment led to some intriguing hypotheses and possible experiments which should also be considered going forward with these two subjects in the future.

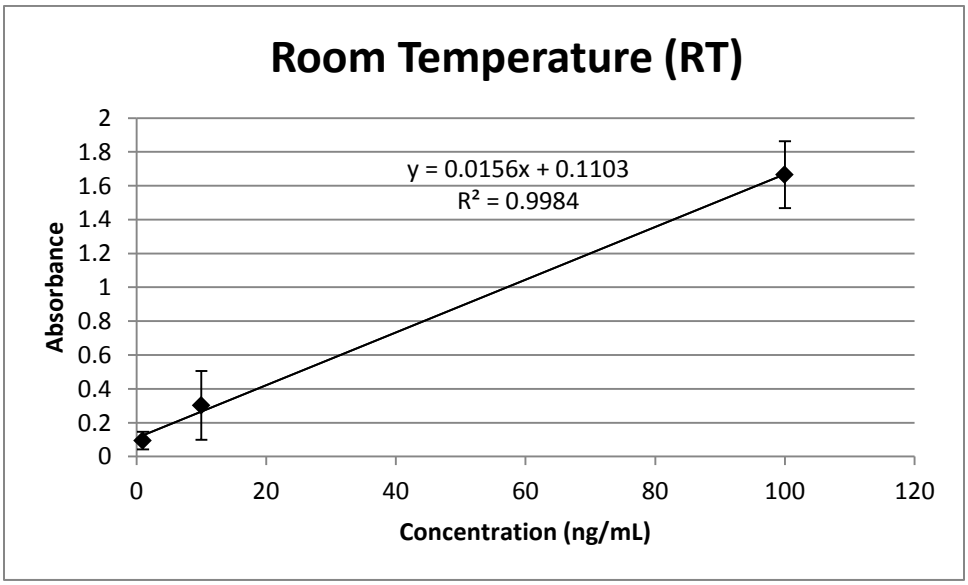
4.1 Final conclusions

Overall, both studies accomplished what they set out to and, in the process, uncovered interesting phenomena which not only forwarded basic knowledge on rainbow trout, β2m and plant lectins but also had implications and possible applications for the trout aquaculture industry. The first study, whose goal was to discern the presence of absence of β2m in trout

bodily fluids, found this protein was present in the blood, reproductive fluids and the mucus, both epidermal and intestinal, of trout. This established for the first time that fish, like mammals, have soluble β2m and that β2m can be released into the intestinal lumen possibly from enterocytes in the proximal intestine. This work also paved the way for β2m's potential use as a biomarker in trout aquaculture or research. The second study examined finding out what effects plant lectins, particularly those from soybean (SBA) and wheat (WGA), have on the rainbow trout intestinal epithelial cell line RTgutGC as a way of understanding the potential gastrointestinal consequences of feeding trout plant products. Intriguingly, WGA altered the morphology of RTgutGC, lowered the metabolism of these cells (possibly indicating a slight toxicity or cell cycle arrest), and caused an increase in β2m and MH Iα proteins in these cells while also causing the former to be released from RTgutGC in greater quantities. SBA, on the other hand, had no effects. These results suggest that wheat in the diets of cultured trout could be having harmful intestinal effects that may not necessarily impair growth but may nevertheless be important (e.g. an increased susceptibility to pathogens of the gut) and provided further evidence that SBA is not a major player in soybean-induced gastroenteritis in trout. Finally, since WGA was found to increase β2m release from RTgutGC and β2m was found in the intestinal lumen it is possible that any *in vivo* effects that WGA may have on trout might be accompanied by an increase in β2m and so it could potentially be used in this case as a biomarker. Alternatively, this relationship might make WGA a useful tool for studying the effects of higher levels of β2m on trout which could hint at its normal physiological role in these fish. Future research then should include more *in vivo* work on top of investigations into the mechanisms of the *in vitro* effects

Appendix I – Standard curves of rβ2m used in ELISA





A triplicate dilution series of rβ2mwas coated to the same plates as conditioned media from 4°C and RT cultures. Only those dilutions whose averages showed linearity were used. The values shown represent these averages less the averages calculated from an additional rβ2m dilution series also done in triplicate that was subjected to preimmune serum rather than anti-β2m antiserum. The formulae of each line were used to calculate the concentration of β2m from absorbance values obtained from the different conditioned media. These concentrations were finally corrected the decrease in volume that occurred in flasks due to removal of media at previous timepoints.

References

Adamashvili, I., Wolf, R., Aultman, D., Milford, E., Jaffe, S., Hall, V., Pressly, T., Minagar, A. & Kelley, R. (2003) "Soluble HLA-I (s-HLA-I) synthesis in systemic lupus erythematosus", *Rheumatology international,* vol. 23, pp. 294-300.

Aeschlimann, P.B., Haberli, M.A., Reusch, T.B.H., Boehm, T. & Milinski, M. (2003) "Female sticklebacks Gasterosteus aculeatus use self-reference to optimize MHC allele number during mate selection", *Behavioral Ecology and Sociobiology,* vol. 54, pp. 119-126.

Antao, A.B., Chinchar, V.G., McConnell, T.J., Miller, N.W., Clem, L.W. & Wilson, M.R. (1999) "MHC class I genes of the channel catfish: sequence analysis and expression", *Immunogenetics,* vol. 49, pp. 303-311.

Aoyagi, K., Dijkstra, J.M., Xia, C., Denda, I., Ototake, M., Hashimoto, K. & Nakanishi, T. (2002) "Classical MHC class I genes composed of highly divergent sequence lineages share a single locus in rainbow trout (Oncorhynchus mykiss)", *Journal of immunology,* vol. 168, pp. 260-273.

Armour, J.C., Perera, R.L.C., Buchan, W.C. & Grant, G. (1998) "Protease inhibitors and lectins in soya beans and effects of aqueous heat-treatment", *Journal of the science of food and agriculture,* vol. 78, pp. 225-231.

Banner, B.F., Allan, C., Smith, L., Savas, L. & Bonkovsky, H.L. (1996) "Effect of interferon therapy on bile duct inflammation in hepatitis C", *Virchows Arch,* vol. 428, pp. 253-259.

Bardocz, S., Ewen, S.W.B., Grant, G. & Pusztai, A. (1995) "Lectins as growth factors for the small intestine and the gut" in *Lectins biomedical perspectives*, eds. A. Pusztai & S. Bardocz, Taylor \& Francis Ltd, London, pp. 103-116.

Bauer, A., Hüttinger, R., Staffler, G., Hansmann, C., Schmidt, W., Majdic, O., Knapp, W. & Stockinger, H. (1997) "Analysis of the requirement for β2-microglobulin for expression and formation of human CD1 antigens", *European journal of immunology,* vol. 27, pp. 1366-1373.

Berggard, I. & Bearn, A.G. (1968) "Isolation and Properties of a Low Molecular Weigt B2-Globulin Occurring in Human Biological Weight Fluids", *The Journal of biological chemistry,* vol. 243, pp. 4095-4103.

Bernier, G.M. & Conrad, E.M. (1969) "Catabolism of human B2 -microglobulin by the rat kidney", *American journal of Physiology,* vol. 217, pp. 1359-1362.

Bhattacharya, S., Das, H. & Bose, A.N. (1988) "Effect of extrusion process variables on in-vitro protein digestibility of fish-wheat flour blends", *Food Chemistry,* vol. 28, pp. 225-231.

Bingulac-Popovic, J., Figueroa, F., Sato, A., Talbot, W.S., Johnson, S.L., Gates, M., Postlethwait, J.H. & Klein, J. (1997) "Mapping of *Mhc* class I and class II regions to different linkage groups in the zebrafish, *Danio rerio*", *Immunogenetics,* vol. 46, pp. 129-134.

Bly, J.E. & Clem, L.W. (1992) "Department of Microbiology , University of Mississippi Medical Center , Jackson , MS 39216-4505 , U . S . A", *Fish & shellfish immunology,* vol. 2, pp. 159-171.

Bols, N.C., Barlian, A., Chirino-Trejo, M., Caldwell, S.J., Goegan, P. & Lee, L.E.J. (1994) "Development of a cell line from primary cultures of rainbow trout, Oncorhynchus mykiss (Walbaum), gills", *Journal of Fish Diseases,* vol. 17, pp. 601-611.

Bonneaud, C., Chastel, O., Federici, P., Westerdahl, H. & Sorci, G. (2006) "Complex Mhc-based mate choice in a wild passerine", *Proceedings.Biological sciences / The Royal Society,* vol. 273, pp. 1111-6.

Bradford, M.M. (1976) "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", *Analytical Biochemistry,* vol. 72, pp. 248-254.

Brenning, G., Wibell, L. & Bergström, R. (1985) "Serum B2-microglobulin at remission and relapse in patients with multiple myeloma", *European journal of clinical investigation,* vol. 15, pp. 242-247.

Buttle, L.G., Burrells, a.C., Good, J.E., Williams, P.D., Southgate, P.J. & Burrells, C. (2001) "The binding of soybean agglutinin (SBA) to the intestinal epithelium of Atlantic salmon, Salmo salar and Rainbow trout, Oncorhynchus mykiss, fed high levels of soybean meal", *Veterinary immunology and immunopathology,* vol. 80, pp. 237-244.

Caspi, R.R. & Avtalion, R.R. (1984) "The mixed leukocyte reaction (MLR) in carp: Bidirectional and unidirectional MLR responses", *Developmental & Comparative Immunology,* vol. 8, pp. 631-637.

Cheng, Z.J., Hardy, R.W. & Blair, M. (2003) "Effects of supplementing methionine hydroxy analogue in soybean meal and distiller's dried grain-based diets on the performance and nutrient retention of rainbow trout [Oncorhynchus mykiss (Walbaum)]", *Aquaculture Research,* vol. 34, pp. 1303-1310.

Cresswell, P., Springer, T., Strominger, J.L., Turner, M.J., Grey, H.M. & Kubo, R.T. (1974) "Immunological identity of the small subunit of HL-A antigens and B2-microglobulin and its turnover on the cell membrane", *Proceedings of the National Academy of Sciences,* vol. 71, pp. 2123-2127.

Cuzick, J., Cooper, E.H. & Maclennan, I.C.M. (1985) "The prognostic value of serum , B2 microglobulin compared with other presentation features in myelomatosis", *Br.J.Cancer,* vol. 52, pp. 1-6.

Dabrowski, K., Poczyczynski, P., Kock, G. & Berger, B. (1989) "Effect of partially or totally replacing fish meal protein by soybean meal protein on growth, food utilization and proteolytic enzyme activities in rainbow trout (Salmo gairdneri). New in vivo test for exocrine pancreatic secretion", *Aquaculture,* vol. 77, pp. 29-49.

Dalla Pellegrina, C., Matucci, A., Zoccatelli, G., Rizzi, C., Vincenzi, S., Veneri, G., Andrighetto, G., Peruffo, A.D.B. & Chignola, R. (2004) "Studies on the joint cytotoxicity of Wheat Germ Agglutinin and monensin", *Toxicology in vitro,* vol. 18, pp. 821-7.

Dargemont, C., Dunon, D., Deugnier, M., Denoyelle, M., Girault, J., Lederer, F., Lê, K.,Ho Diep, Godeau, F., Thiery, J.P., Imhof, B.A. & Imhof, A. (1989) "Thymotaxin, a chemotactic protein, is identical to B2-microglobulin", *Science,* vol. 246, pp. 803-806.

Davies, S.J., Morris, P.C. & Baker, R.T.M. (1997) "Partial substitution of fish meal and full-fat soya bean meal with wheat gluten and influence of lysine supplementation in diets for rainbow trout, Oncorhynchus mykiss (Walbaum)", *Aquaculture Research,* vol. 28, pp. 317-328.

Dayeh, V.R., Bols, N.C., Schirmer, K. & Lee, L.E.J. (2003) "The use of fish-dericed cell lines for investigation of environmental contaminants" in *Current Protocols in Toxicology*, ed. K. Morgan, John Wiley & Sons, Inc, New York, pp. 1-17.

De Mejía, E.G. & Prisecaru, V.I. (2005) "Lectins as bioactive plant proteins: a potential in cancer treatment", *Critical reviews in food science and nutrition,* vol. 45, pp. 425-45.

Dijkstra, J.M., Kollner, B., Aoyagi, K., Sawamoto, Y., Kuroda, A., Ototake, M., Nakanishi, T. & Fischer, U. (2003) "The rainbow trout classical MHC class I molecule Onmy-UBA*501 is expressed in similar cell types as mammalian classical MHC class I molecules", *Fish & shellfish immunology,* vol. 14, pp. 1-23.

Dixon, B., Stet, R.J.M., Erp, S.H.M. & Pohajdak, B. (1993) "Characterization of β_2-microglobulin transcripts from two teleost species", *Immunogenetics,* vol. 38, pp. 27-34.

Dobbe, L.M.E., Stam, N.J., Neefjes, J.J. & Giphart, M.J. (1988) "Biochemical complexity of serum HLA class I molecules", *Immunogenetics,* vol. 27, pp. 203-210.

Draaijer, M., Koninkx, J., Hendriks, H., Kik, M., Van Dijk J & Mouwen, J. (1989) "Actin cytoskeletal lesions in differentiated human colon carcinoma Caco-2 cells after exposure to soybean agglutinin", *Biology of the cell,* vol. 65, pp. 29-35.

Emerson, D. & Juliano, R.L. (1982) "Interactions of lectins with CHO cell surface membranes. I. competition studies indicate concanavalin A and WGA bind to discrete populations of sites", *Journal of cellular physiology,* vol. 111, pp. 171-176.

Ericson, D. (1984) "Agglutination of Streptococcus mutans by low-molecular-weight salivary components: effect of B2-microglobulin", *Infection and immunity,* vol. 46, pp. 526-530.

Evrin, P.E., Peterson, P.A., Wide, L. & Berggård, I. (1971) "Radioimmunoassay of B2 -microglobulin in human biological fluids", *Scandinavian journal of clinical and laboratory investigation,* vol. 28, pp. 439-43.

Evrin, P. & Wibell, L. (1973) "Serum B2-microglobulin in various disorders", *Clinica Chimica Acta,* vol. 43, pp. 183-186.

Evrin, P.-. (1972) "The Serum Levels and Urinary Excretion of β2-Microglobulin in Apparently Healthy Subjects", *Scandinavian Journal of Clinical & Laboratory Investigation,* vol. 29, pp. 69-74.

FAO (2010) *The State of World Fisheries and Aquaculture*, FAO, Rome.

FAO (2009a) *Fish as feed inputs for aquaculture*, FAO, Rome.

FAO (2009b) *Impact of rising feed ingredient prices on aquafeeds and aquaculture production*, FAO, Rome.

Farina, A., Dumonceau, J., Delhaye, M., Frossard, J., Hadengue, A., Hochstrasser, D.F. & Lescuyer, P. (2011) "A step further in the analysis of human bile proteome", *Journal of proteome research,* vol. 10, pp. 2047-2063.

Forsberg, L.a., Dannewitz, J., Petersson, E. & Grahn, M. (2007) "Influence of genetic dissimilarity in the reproductive success and mate choice of brown trout - females fishing for optimal MHC dissimilarity", *Journal of Evolutionary Biology,* vol. 20, pp. 1859-69.

Gao, B., Adhikari, R., Howarth, M., Nakamura, K., Gold, M.C., Hill, A.B., Knee, R., Michalak, M. & Elliott, T. (2002) "Assembly and antigen-presenting function of MHC class I molecules in cells lacking the ER chaperone calreticulin", *Immunity,* vol. 16, pp. 99-109.

Goldstein, I.J. & Poretz, R.D. (1986) "Isolation, physiochemical characterization, and carbohydrate-binding specificity of lectins" in *The lectins properties, functions and applications in biology and medicine*, eds. I.E. Liener, N. Sharon & I.J. Goldstein, Academic Press, Inc, Orlando, pp. 33-247.

Gordon, J., Wu, C., Rastegar, M. & Safa, A.R. (2003) "Beta2-microglobulin induces caspase-dependent apoptosis in the CCRF-HSB-2 human leukemia cell line independently of the caspase-3, -8 and -9 pathways but through increased reactive oxygen species", *International journal of cancer.Journal international du cancer,* vol. 103, pp. 316-27.

Grommé, M. & Neefjes, J. (2002) "Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways", *Molecular immunology,* vol. 39, pp. 181-202.

Gronborg, M., Maitra, A. & Pandey, A. (2007) "Proteomics of Human Pancreatic Juice" in *Proteomics of human body fluids: principles, methods, and applications*, ed. V. Thongboonkerd, Humana Press, Totowa, pp. 377-397.

Haas, H., Falcone, F.H., Schramm, G., Haisch, K., Gibbs, B.F., Klaucke, J., Pöppelmann, M., Becker, W.M., Gabius, H.J. & Schlaak, M. (1999) "Dietary lectins can induce in vitro release of IL-4 and IL-13 from human basophils", *European journal of immunology,* vol. 29, pp. 918-27.

Haga, J.A., She, J.X. & Kao, K.J. (1991) "Biochemical characterization of 39-kDa class I histocompatibility antigen in plasma. A secretable membrane protein derived from transmembrane domain deletion", *J.Biol.Chem.,* vol. 266, pp. 3695-3701.

Hansen, J.D. & La Patra, S. (2002) "Induction of the rainbow trout MHC class I pathway during acute IHNV infection", *Immunogenetics,* vol. 54, pp. 654-661.

Hardy, R.W. (2010) "Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal", *Aquaculture Research,* vol. 41, pp. 770-776.

Hashimoto, K., Nakanishi, T. & Kurosawa, Y. (1990) "Isolation of carp genes encoding major histocompatibility complex antigens", *Proceedings of the National Academy of Sciences,* vol. 87, pp. 6863-6867.

Heikkinen, J., Vielma, J., Kemilainen, O., Tiirola, M., Eskelinen, P., Kiuru, T., Navi-apaldanius, D. & Wright, A. (2006) "Effects of soybean meal based diet on growth performance, gut histopathology and intestinal microbiota of juvenile rainbow trout (Oncorhynchus mykiss)", *Aquaculture,* vol. 261, pp. 259-268.

Hendriks, H., Van, d.I., Krogdahl, A., Olli, J. & Koninkx, J. (1990) "Binding of soybean agglutinin to small intestinal brush border membranes and brush border membrane enzyme activities in Atlantic salmon (Salmo salar)", *Aquaculture,* vol. 91, pp. 163-170.

Hildemann, W.H. (1957) "Scale Homotransplantation in goldfish (Carassius auratus)", *Annals of the New York Academy of Sciences,* vol. 64, pp. 775-791.

Howe, C., Garstka, M., Al-Balushi, M., Ghanem, E., Antoniou, A.N., Fritzsche, S., Jankevicius, G., Kontouli, N., Schneeweiss, C., Williams, A., Elliott, T. & Springer, S. (2009) "Calreticulin-dependent recycling in the early secretory pathway mediates optimal peptide loading of MHC class I molecules", *EMBO J,* vol. 28, pp. 3730-44.

Hsu, V.W., Yuan, L.C., Nuchtern, J.G., Lippincott-Schwartz, J., Hammerling, G.J. & Klausner, R.D. (1991) "A recycling pathway between the endoplasmic reticulum and the Golgi apparatus for retention of unassembled MHC class I molecules", *Nature,* vol. 352, pp. 441-444.

IFFO (2011) *Fishmeal and Fish Oil – The Facts, Figures, Trends, and IFFO 's Responsible Supply Standard*.

Iger, Y., Balm, P.H.M., Jenner, H.A. & Bonga, S.E.W. (1995) "Cortisol Induces Stress-Related Changes in the Skin of Rainbow Trout (Oncorhynchus mykiss)", *General and comparative endocrinology,* vol. 97, pp. 188-198.

Index-Mundi (2011) *Commodity Price Indices*.

Iwashita, Y., Suzuki, N., Yamamoto, T., Shibata, J., Isokawa, K., Soon, A.H., Ikehata, Y., Furuita, H., Sugita, T. & Goto, T. (2008) "Supplemental effect of cholyltaurine and soybean lecithin to a soybean meal-based fish meal-free diet on hepatic and intestinal morphology of rainbow trout Oncorhynchus mykiss", *Fisheries Science,* vol. 74, pp. 1083-1095.

Iwashita, Y., Yamamoto, T., Furuita, H., Sugita, T. & Suzuki, N. (2009) "Influence of certain soybean antinutritional factors supplemented to a casein-based semipurified diet on intestinal and liver morphology in fingerling rainbow trout Oncorhynchus mykiss", *Fisheries Science,* vol. 74, pp. 1075-1082.

Jonasson, L., Evrin, P. & Wibell, L. (1974) "Content of β2-Microglobulin and Albumin in Human Amniotic Fluid: A Study of Normal Pregnancies and Pregnancies Complicated by Haemolytic Disease", *Acta Obstetricia et Gynecologica Scandinavica,* vol. 53, pp. 49-58.

Kaastrup, P., Nielsen, B., set]rlyck, V.H. & Simonsen, M. (1988) "Mixed Lymphocyte Reactions (MLR) in rainbow trout (Salmo gairdneri) sibling", *Developmental & Comparative Immunology,* vol. 12, pp. 801-808.

Kales, S.C. (2006) *A Functional Study of Major Histocompatibility Expression and Immune Function in Rainbow Trout , (Oncorhynchus mykiss)*, University of Waterloo.

Kales, S., Parks-Dely, J., Schulte, P. & Dixon, B. (2006) "Beta-2-microglobulin gene expression is maintained in rainbow trout and Atlantic salmon kept at low temperatures", *Fish & shellfish immunology,* vol. 21, pp. 176-86.

Karlsson, A. (1999) "Wheat Germ Agglutinin Induces NADPH-Oxidase Activity in Human Neutrophils by Interaction with Mobilizable Receptors", *Infection and immunity,* vol. 67, pp. 3461-3468.

Karlsson, F., Groth, T., Sege, K., Wibell, L. & Peterson, P.A. (1980) "Turnover in humans of β2-microglobulin: the constant chain of HLA-antigens", *European journal of clinical investigation,* vol. 10, pp. 293-300.

Kawai, K., Kawashima, S., Miyazaki, T., Tajiri, E., Mori, M., Kitazaki, K., Shirotani, T., Inatome, T., Yamabe, H., Hirata, K. & Yokoyama, M. (2010) "Serum beta2-microglobulin concentration as a

novel marker to distinguish levels of risk in acute heart failure patients", *Journal of cardiology,* vol. 55, pp. 99-107.

Kawano, A., Haiduk, C., Schirmer, K., Hanner, R., Lee, L.E.J., Dixon, B. & Bols, N.C. (2011) "Development of a rainbow trout intestinal epithelial cell line and its response to lipopolysaccharide", *Aquaculture Nutrition,* vol. 17, pp. e241-e252.

Kawano, A., Kales, S.C., Fujiki, K., DeWitte-Orr, S.J., Dixon, B., Lee, L.E.J. & Bols, N.C. (2010) "A comparison of rainbow trout cell lines for their expression of the major histocompatibility complex genes and the induction of beta-2-microglobulin by dsRNA", *Fish & shellfish immunology,* vol. 29, pp. 312-318.

Kilpatrick, D.C. (1995) "Lectins in Immunology" in *Lectins biomedical perspectives* Taylor & Francis Ltd, London, pp. 155-182.

Kim, M., Rao, M.V., Tweardy, D.J., Prakash, M., Galili, U. & Gorelik, E. (1993) "Lectin-induced apoptosis of tumour cells", *Glycobiology,* vol. 3, pp. 447-453.

Knudsen, D., Jutfelt, F., Sundh, H., Sundell, K., Koppe, W. & Frøkiaer, H. (2008) "Dietary soya saponins increase gut permeability and play a key role in the onset of soyabean-induced enteritis in Atlantic salmon (Salmo salar L.)", *The British journal of nutrition,* vol. 100, pp. 120-129.

Knudsen, D., Urán, P., Arnous, A., Koppe, W. & Frøkiaer, H. (2007) "Saponin-containing subfractions of soybean molasses induce enteritis in the distal intestine of Atlantic salmon", *Journal of Agricultural and Food Chemistry,* vol. 55, pp. 2261-2267.

Koelman, C.A., Coumans, A.B.C., Nijman, H.W., Doxiadis, I.I.N., Dekker, G.A. & Claas, F.H.J. (2000) "Correlation between oral sex and a low incidence of preeclampsia: a role for soluble HLA in seminal fluid?", *Journal of reproductive immunology,* vol. 46, pp. 155-166.

Komatsu, K., Tsutsui, S., Hino, K., Araki, K., Yoshiura, Y., Yamamoto, A., Nakamura, O. & Watanabe, T. (2009) "Expression profiles of cytokines released in intestinal epithelial cells of the rainbow trout, Oncorhynchus mykiss, in response to bacterial infection", *Developmental and comparative immunology,* vol. 33, pp. 499-506.

Koninkx, J.F.J.G. (1995) "Enterocyte -like CACO-2 cells as a tool to study lectin interaction" in *Lectins biomedical perspectives*, eds. A. Pusztai & S. Bardocz, Taylor & Francis Ltd, London, pp. 81-101.

Krogdahl, Å., Penn, M., Thorsen, J., Refstie, S. & Bakke, A.M. (2010) "Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids", *Aquaculture Research,* vol. 41, pp. 333-344.

Landis, E.D., Purcell, M.K., Thorgaard, G.H., Wheeler, P.a. & Hansen, J.D. (2008) "Transcriptional profiling of MHC class I genes in rainbow trout infected with infectious hematopoietic necrosis virus", *Molecular immunology,* vol. 45, pp. 1646-1657.

Landry, C., Garant, D., Duchesne, P. & Bernatchez, L. (2001) "'Good genes as heterozygosity': the major histocompatibility complex and mate choice in Atlantic salmon (Salmo salar)", *Proc.R.Soc.Lond.B,* vol. 268, pp. 1279-1285.

Lavelle, E.C., Grant, G., Pusztai, A., Pfüller, U. & O'Hagan, ,D.T. (2000) "Mucosal immunogenicity of plant lectins in mice", *Immunology,* vol. 99, pp. 30-37.

Leinders-Zufall, T., Brennan, P., Widmayer, P., S, P.C., Maul-Pavicic, A., Jäger, M., Li, X., Breer, H., Zufall, F. & Boehm, T. (2004) "MHC class I peptides as chemosensory signals in the vomeronasal organ", *Science (New York, N.Y.),* vol. 306, pp. 1033-7.

Liu, W.K., Sze, S.C.W., Ho, J.C.K., Liu, B.P.L. & Yu, M.C. (2004) "Wheat germ lectin induces G2/M arrest in mouse L929 fibroblasts", *Journal of cellular biochemistry,* vol. 91, pp. 1159-1173.

Lopes, F.C., Cavada, B.S., Pinto, V.P.T., Sampaio, A.H. & Gomes, J.C. (2005) "Differential effect of plant lectins on mast cells of different origins", *Brazilian journal of medical and biological research,* vol. 38, pp. 935-941.

Lorenz-meyer, H., Roth, H., Elsasser, P. & Hahn, U. (1985) "Cytotoxicity of lectins on rat intestinal mucosa enhanced by neuraminidase", *European journal of clinical investigation,* vol. 15, pp. 227-234.

Magor, K.E., Shum, B.P. & Parham, P. (2004) "The B2-microglobulin locus of rainbow trout (Oncorhynchus mykiss) contains three polymorphic genes", *Journal of immunology,* vol. 172, pp. 3635-3643.

Matucci, A., Veneri, G., Pellegrina, C.D., Zoccatelli, G., Vincenzi, S., Chignola, R., Peruffo, A.D.B. & Rizzi, C. (2004) "Temperature-dependent decay of wheat germ agglutinin activity and its implications for food processing and analysis", *Food Control,* vol. 15, pp. 391-395.

Milinski, M., Griffiths, S., Wegner, K.M., Reusch, T.B.H., Haas-Assenbaum, A. & Boehm, T. (2005) "Mate choice decisions of stickleback females predictably modified by MHC peptide ligands", *Proceedings of the National Academy of Sciences,* vol. 102, pp. 4414-8.

Mori, M., Terui, Y., Ikeda, M., Tomizuka, H., Uwai, M., Kasahara, T., Kubota, N., Itoh, T., Mishima, Y., Douzono-Tanaka, M., Yamada, M., Shimamura, S., Kikuchi, J., Furukawa, Y., Ishizaka, Y., Ikeda, K., Mano, H., Ozawa, K. & Hatake, K. (1999) "B2-microglobulin identified as an apoptosis-inducing factor and its characterization", *Blood,* vol. 94, pp. 2744-53.

Mori, M., Terui, Y., Tanaka, M. & B, N.F. (2001) "Antitumor Effect of β2-Microglobulin in Leukemic Cell-bearing Mice via Apoptosis-inducing Activity : Activation of Caspase-3 and Nuclear Factor-κB", *Cancer research,* vol. 61, pp. 4414-4417.

Morris, P., Gallimore, P., Handley, J., Hide, G., Haughton, P. & Black, A. (2005) "Full-fat soya for rainbow trout (Oncorhynchus mykiss) in freshwater: Effects on performance, composition and flesh fatty acid profile in absence of hind-gut enteritis", *Aquaculture,* vol. 248, pp. 147-161.

Nagafuchi, Y. & Scheuer, P.J. (1986) "Hepatic B2-microglobulin distribution in primary biliary cirrhosis", *Journal of hepatology,* vol. 2, pp. 73-80.

Nakamuro, K., Tanigaki, N. & Pressman, D. (1973) "Multiple common properties of human B2-microglobulin and the common portion fragment derived from HL-A antigen molecules", *Proceedings of the National Academy of Sciences,* vol. 70, pp. 2863-2865.

Nakanishi, T., Fischer, U., Dijkstra, J.M., Hasegawa, S., Somamoto, T., Okamoto, N. & Ototake, M. (2002) "Cytotoxic T cell function in fish", *Dev Comp Immunol,* vol. 26, pp. 131-139.

Nath, S., Kales, S., Fujiki, K. & Dixon, B. (2006) "Major histocompatibility class II genes in rainbow trout (Oncorhynchus mykiss) exhibit temperature dependent downregulation", *Immunogenetics,* vol. 58, pp. 443-53.

Nowell, P.C. (1960) "Phytohemagglutinin : An Initiator of Mitosis in Cultures of Normal Human Leukocytes*", *Cancer research,* , pp. 462-466.

Ober, C., Weitkamp, L.R., Cox, N., Dytch, H., Kostyu, D. & Elias, S. (1997) "HLA and mate choice in humans", *American Journal of Human Genetics,* vol. 61, pp. 497-504.

Ohno, Y., Naganuma, T., Ogawa, T. & Muramoto, K. (2006) "Effect of lectins on the transport of food factors in caco-2 cell monolayers", *Journal of Agricultural and Food Chemistry,* vol. 54, pp. 548-553.

Oliva-teles, A., Gouveia, A., Gomes, E. & Rema, P. (1994) "The effect of different processing treatments on soybean meal utilization by rainbow trout, Oncorhynchus mykiss", *Aquaculture,* vol. 124, pp. 343-349.

Ono, H., Figueroa, F., O'hUigin, C. & Klein, J. (1993) "Cloning of the β_2-microglobulin gene in the zebrafish", *Immunogenetics,* vol. 38, pp. 1-10.

Ostaszewska, T., Dabrowski, K., Palacios, M., Olejniczak, M. & Wieczorek, M. (2005) "Growth and morphological changes in the digestive tract of rainbow trout (Oncorhynchus mykiss) and pacu (Piaractus mesopotamicus) due to casein replacement with soybean proteins", *Aquaculture,* vol. 245, pp. 273-286.

Painter, R.H., Yasmeen, D., Assimeh, S.N. & Poulik, M.D. (2009) "Complement Fixing and Macrophage Opsonizing Activities Associated with β2 Microglobulin", .

Pedini, V., Scocco, P., Gargiulo, A.M., Ceccarelli, P. & Lorvik, S. (2002) "Glycoconjugate characterization in the intestine of Umbrina cirrosa by means of lectin histochemistry", *Journal of fish biology,* vol. 61, pp. 1363-1372.

Peterson, P.A., Rask, L. & Lindblom, B. (1974) "Highly Purified Papain-Solubilized HL-A Antigens Contain B2-microglobulin", *Proceedings of the National Academy of Sciences,* vol. 71, pp. 35-39.

Peterson, P.A., Evrin, P.E. & Berggård, I. (1969) "Differentiation of glomerular, tubular, and normal proteinuria: determinations of urinary excretion of B2-macroglobulin, albumin, and total protein", *The Journal of clinical investigation,* vol. 48, pp. 1189-1198.

Peumans, W.J. & Van Damme, E.J.M. (1996) "Prevalence, biological activity and genetic manipulation of lectins in foods", *Trends in Food Science & Technology,* , pp. 132-138.

Pezzilli, R., Billi, P., Fiocchi, M., Beltrandi, E., Cappelletti, O., Sprovieri, G. & Miglioli, M. (1995) "Serum B2-microglobulin in chronic diseases of the pancreas", *International journal of pancreatology : official journal,* vol. 17, pp. 161-166.

Plesner, T. & Bjerrum, O.J. (1980) "Distribution of 'Free' and HLA-associated Human β2-Microglobulin in Some Plasma Membranes and Biological Fluids", *Scandinavian journal of immunology,* vol. 11, pp. 341-351.

Praetor, A. & Hunziker, W. (2002) "B2-microglobulin is important for cell surface expression and pH-dependent IgG binding of human FcRn", *Journal of cell science,* vol. 115, pp. 2389-2397.

Pusztai, A., Grant, G., Ewen, S.W.B., Peumans, W.J., Damme, E.J.M.V. & Bardocz, S. (1995) "Infection of the gut by pathogenic bacteria is inhibited by dietary lectins. Chemical probiosis" in *Lectins biomedical perspectives*, eds. A. Pusztai & S. Bardocz, Taylor \& Francis Ltd, London, pp. 313-322.

Pusztai, B.A., Ewen, S.W., Grant, G., Brown, D.S., Stewart, J.C., Peumans, W.J., Van Damme, ,E.J. & Bardocz, S. (1993) "Antinutritive effects of wheat-germ agglutinin and other N-acetylglucosamine-specific lectins", *British Journal of Nutrition,* vol. 70, pp. 313-21.

Radulescu, M., Bucur, B., Bucur, M. & Radu, G. (2009) "Quality control method based on quartz crystal microbalance and WGA for flour milled from germinated wheat", *European Food Research and Technology,* vol. 229, pp. 833-840.

Ramadori, G., Mitsch, A., Rieder, H. & Büschenfelde, K.,Meyer Zum (1988) "Alpha- and gamma-interferon (IFNα, IFNγ) but not interleukin-1 (IL-1) modulate synthesis and secretion of β 2 -microglobulin by hepatocytes", *European journal of clinical investigation,* vol. 18, pp. 343-351.

Rammensee, H., Friede, T. & Stevanović, S. (1995) "MHC ligands and peptide motifs: first listing", *Immunogenetics,* vol. 41, pp. 178-228.

Refstie, S., Korsen, O.J., Storebakken, T., Baeverfjord, G., Lein, I. & Roem, A.J. (2000) "Differing nutritional responses to dietary soybean meal in rainbow trout (Oncorhynchus mykiss) and Atlantic salmon (Salmo salar)", *Aquaculture,* vol. 190, pp. 49-63.

Reusch, T.B., Häberli, M.a., Aeschlimann, P.B. & Milinski, M. (2001) "Female sticklebacks count alleles in a strategy of sexual selection explaining MHC polymorphism", *Nature,* vol. 414, pp. 300-2.

Rock, K.L., Gamble, S., Rothstein, L. & Benacerraf, B. (1991) "Reassociation with 2-Microglobulin is Necessary for Db Class I Major Histocompatibility Complex Binding of an Exogenous Influenza Peptide", *Proceedings of the National Academy of Sciences,* vol. 88, pp. 301-304.

Romarheim, O., Skrede, A., Gao, Y., Krogdahl, A., Denstadli, V., Lilleeng, E. & Storebakken, T. (2006) "Comparison of white flakes and toasted soybean meal partly replacing fish meal as protein source in extruded feed for rainbow trout (Oncorhynchus mykiss)", *Aquaculture,* vol. 256, pp. 354-364.

Rumsey, G.L., Siwicki, A.K., Anderson, D.P. & Bowser, P.R. (1994) "Effect of soybean protein on serological response, non-specific defense mechanisms, growth, and protein utilization in rainbow trout", *Veterinary immunology and immunopathology,* vol. 41, pp. 323-39.

Sealey, W.M., Barrows, F.T., Smith, C.E., Overturf, K. & LaPatra, S.E. (2009) "Soybean meal level and probiotics in first feeding fry diets alter the ability of rainbow trout Oncorhynchus mykiss to utilize high levels of soybean meal during grow-out", *Aquaculture,* vol. 293, pp. 195-203.

Setchell, J.M. & Huchard, E. (2010) "The hidden benefits of sex: Evidence for MHC-associated mate choice in primate societies", *BioEssays,* vol. 32, pp. 940-948.

Shand, R. & Dixon, B. (2001) "Teleost Major Histocompatibility Genes: Diverse but not complex", *Modern Aspects of Immunology,* vol. 2, pp. 66-72.

Shibasaki, M., Sumazaki, R., Isoyama, S. & Takita, H. (1992) "Interaction of lectins with human IgE: IgE-binding property and histamine-releasing activity of twelve plant lectins", *Int Arch Allergy Immunol,* vol. 98, pp. 18-25.

Shum, B.P., Azumi, K., Zhang, S., Kehrer, S.R., Raison, R.L., Detrich, H.W. & Parham, P. (1996) "Unexpected beta2-microglobulin sequence diversity in individual rainbow trout", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 93, pp. 2779-84.

Shum, B.P., Guethlein, L., Flodin, L.R., Adkison, M.A., Hedrick, R.P., Nehring, R.B., Stet, R.J., Secombes, C. & Parham, P. (2001) "Modes of salmonid MHC class I and II evolution differ from the primate paradigm", *Journal of immunology (Baltimore, Md.: 1950),* vol. 166, pp. 3297-308.

Singh, P.B., Brown, R.E. & Roser, B. (1987) "MHC antigens in urine as olfactory recognition cues", *Nature,* vol. 327, pp. 161-4.

Skarstein, F., Folstad, I., Liljedal, S. & Grahn, M. (2005) "MHC and fertilization success in the Arctic charr (Salvelinus alpinus)", *Behavioral Ecology and Sociobiology,* vol. 57, pp. 374-380.

Skonberg, D.I., Hardy, R.W., Barrows, F.T. & Dong, F.M. (1998) "Color and flavor analyses of fillets from farm-raised rainbow trout (Oncorhynchus mykiss) fed low-phosphorus feeds containing corn or wheat gluten", *Aquaculture,* vol. 166, pp. 269-277.

Statistics-Canada (2009) *Aquaculture Statistics*, Ottawa.

Stefanovic, V., Mitic-Zlatkovic, M., Ignjatovic, I., Vlahovic, P. & Hadzi-Djokic, J. (1999) "β2-Microglobulin in patients with urothelial cancer", *World journal of urology,* vol. 17, pp. 319-323.

Stolka, R., Kashchak, N., Lutsik-Kordovsky, M., Boyko, M., Barska, M. & Tsyrulnyk, A. (2001) "In vitro response of phagocytic cells to immunomodulating agents", *Med Sci Monit,* vol. 7, pp. 652-658.

Strominger, J.L. (2002) "Human histocompatibility proteins Past and future: a memoir", *Immunological reviews,* vol. 185, pp. 69-77.

Subramanian, S., Ross, N.W. & MacKinnon, S.L. (2008) "Comparison of antimicrobial activity in the epidermal mucus extracts of fish", *Comparative biochemistry and physiology, part B,* vol. 150, pp. 85-92.

Sugiura, S.H., Dong, F., Rathbone, C.K. & Hardy, R.W. (1998) "Apparent protein disgetibility and mineral availabilities in various feed ingredients for salmoid feeds", *Aquaculture,* vol. 159, pp. 177-202.

Tacon, a. & Metian, M. (2008) "Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects", vol. 285, pp. 146-158.

Talal, N., Grey, H.M., Zvaifler, N., Michalski, J.P. & Daniels, T.E. (1975) "Elevated salivary and synovial fluid B2-Microglobulin in Sjogren's syndrome and Rheumatoid Arthritis", *Science,* vol. 187, pp. 1196-1198.

Tchernychev, B. & Wilchek, M. (1996) "Natural human antibodies to dietary lectins", *FEBS letters,* vol. 397, pp. 139-142.

van den lngh, T.S.G.A.M., Olli, J.J. & Krogdahl, A. (1996) "Alcohol-soluble components in soybeans cause morphological changes in the distal intestine of Atlantic salmon, Salmo salar L.", *J Fish Dis,* vol. 19, pp. 47-53.

Vasconcelos, I.M. & Oliveira, J.T.A. (2004) "Antinutritional properties of plant lectins", *Toxicon,* vol. 44, pp. 385-403.

Vitiello, A., Potter, T.A. & Sherman, L.A. (1990) "The role of B2-microglobulin in peptide binding by class I molecules", *Science,* vol. 250, pp. 1423-1426.

Watzl, B., Neudecker, C., Hansch, G.M., Rechkemmer, G. & Pool-Zobel, B. (2001) "Dietary wheat germ agglutinin modulates ovalbumin-induced immune responses in Brown Norway rats", *British Journal of Nutrition,* vol. 85, pp. 483-490.

Wedekind, C., Seebeck, T., Bettens, F. & Paepke, A.J. (1995) "MHC-dependent mate preferences in humans", *Proc.R.Soc.Lond.B,* vol. 260, pp. 245-249.

Xie, J., Wang, Y., Iii, M.E.F., Barlogie, B. & Yi, Q. (2003) "B2-Microglobulin as a negative regulator of the immune system: high concentrations of the protein inhibit in vitro generation of functional dendritic cells", *Blood,* vol. 101, pp. 4005-4012.

Yamazaki, B.Y.K., Boyse, E.A., Mike, V., Thaler, H.T., Mathieson, B.J., Abbott, J., Boyse, J., Zayas, Z. & Thomas, L. (1976) "Control of mating preferences in mice by genes in the major histocompatibility complex*", *Journal of Experimental Medicine,* vol. 144, pp. 1324-1335.

Zhu, Y., Su, Y., Cheng, T., Chung, L.W.K. & Shi, C. (2009) "B2-microglobulin as a potential factor for the expansion of mesenchymal stem cells", *Biotechnology Letters,* vol. 31, pp. 1361-1365.

Ziegler, A. & Pink, J.R.L. (1978) "Structural similarity of major histocompatibility antigens on leukocytes and erythrocytes", *Immunochemistry,* vol. 15, pp. 515-516.

Zissis, M., Afroudakis, a., Galanopoulos, G., Palermos, L., Boura, X., Michopoulos, S. & Archimandritis, a. (2001) "B2 microglobulin: is it a reliable marker of activity in inflammatory bowel disease?", *The American Journal of Gastroenterology,* vol. 96, pp. 2177-2183.