# Diplomarbeit

# Autoimmunity as a possible limiting selection pressure for the individual MHC IIB allele diversity in the three-spined stickleback *Gasterosteus aculeatus*?


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**Anja Krause**


Vorgelegt an der Mathematisch-Naturwissenschaftlichen Fakultät der Christian-Albrechts-Universität zu Kiel


Angefertigt in der Abteilung für Evolutionsökologie
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Plön, Februar 2011

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# Autoimmunity as a possible limiting selection pressure for the individual MHC IIB allele diversity in the three-spined stickleback *Gasterosteus aculeatus*?

- An explorative study about the association of the MHC IIB allele diversity and changes in immunological parameters -

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Kiel, den ..... .....

Anja Krause

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## 1. Zusammenfassung

Genetische Diversität ist eine Voraussetzung für Evolution. Aufgrund von Polygenie und stark polymorpher Loci ist der Haupt- Histokompatibilitätskomplex (MHC) genetisch variabel. MHC-Moleküle sind ein wichtiger Bestandteil des adaptiven Immunsystems. Sie sind in der Lage verschiedene Antigene zu binden und präsentieren diese den T-Lymphozyten. Allerdings beinhaltet die hohe Spezifität auch eine Gefahr: je besser die Erkennung verschiedener Antigene, umso wahrscheinlicher ist die Ähnlichkeit zwischen fremden und Autoantigenen. Anhand von sogenannten Cross-reactions während des Molecular-mimicry-Prozesses kann es zu einem Verlust der Selbsttoleranzmechanismen kommen. Der MHC könnte ein Schlüsselmolekül für Selektion, adaptive Prozesse und Artbildung sein.

Der dreistachlige Stichling *Gasterosteus aculeatus* weist ein intermediäres Optimum bezüglich der MHC II-Allelzahl auf. Einerseits bieten zu wenig verschiedene MHC II-Allele eine nicht-ausreichende Diversität für die Entdeckung vieler unterschiedlicher Parasitenantigene. Andererseits muss es einen limitierenden Selektionsdruck geben, der dieses intermediäre Optimum hervorruft. Zu viele verschiedene Allele könnten nachteilig sein, da sie das Risiko auto-aggressiver Reaktionen erhöhen. Deshalb untersucht die vorliegende Studie einen möglichen Zusammenhang zwischen Veränderungen immunologischer Parameter, die mit Autoimmunreaktionen assoziiert werden, und der individuellen MHC II-Alleldiversität des dreistachligen Stichlings. Dies ist eine Pilotstudie über das Auftreten auto-aggressiver Reaktionen als ein möglicher limitierender Selektionsdruck der MHC II-Alleldiversität. Ich habe verschiedene Methoden angewandt, um unterschiedliche Aspekte des Themas zu untersuchen.

Zunächst wurde die MHC II-Allelzusammensetzung der Elterntiere 14 verschiedener Fischfamilien bestimmt. Fünf dieser Familien erfüllen die Voraussetzung dieser Studie: sie beinhalten Individuen mit zwei, bzw. vier verschiedenen MHC II-Allelen. Die MHC-Typisierung aller Fische wurde gemäß der “Reference Strand-mediated Conformation Analysis” (RSCA) durchgeführt. Als nächstes wurden bestimmte Entzündungsgene im Genom von *Gasterosteus aculeatus* untersucht. Verschiedene Primerkombinationen sind mittels einer Standard-PCR und Agarosegelelektrophorese getestet worden. Danach wurde ihre Spezifität mit Hilfe direkter Sequenzierung und der “quantitative Real Time PCR” überprüft. Interleukin 1 beta, MIF, IgM und MHC II wurden im Genom des dreistachligen Stichlings isoliert. Anschließend erfolgte die Sektion von Kopfniere, Milz, Leber, Niere, Darm, Gonaden, Herz und Gehirn, sowie eine Blutentnahme aus der caudalen Vene. Der Gesamtimmunoglobulinlevel des Plasmas ist mit Hilfe des “Enzyme-linked immunosorbent assays” (ELISA) bestimmt worden. RNA wurde aus Kopfniere, Darm, Niere und Milz isoliert und in komplementäre DNA (cDNA) umgeschrieben.

Die Expression von IL 1 beta, MIF, IgM und MHC II ist relativ zur Expression der Housekeeping-Gene L13 und Ubiquitin gemessen worden. Hierzu ist die Methode der "quantitative Real Time PCR" benutzt worden, basierend auf dem nicht-spezifischen Reportermolekül SYBR Green I.

Die Normalisierung der Rohdaten der Genexpression erfolgte mittels der Software qBase Plus. Zusammen mit weiteren Parametern, wie z.B. dem Gesamtimmunoglobulinlevel sind die Daten anhand einer "permutational multivariate analysis of variance" analysiert worden.

Die Familienzugehörigkeit und das Geschlecht scheinen einen Einfluss auf die analysierten Parameter zu haben. Die Bedeutung des Sektionstages hat keinen biologischen Hintergrund, sondern stellt ein Artefakt dar. Das Immunsystem verschiedener Familien könnte sich aus unterschiedlichen Faktoren zusammensetzen. Diese könnten aufgrund von Unterschieden im genetischen Hintergrund entstanden sein. Es wird angenommen, dass Autoimmunität beim Menschen mit weiblichen Sexualhormonen assoziiert sein könnte. Deshalb wäre es möglich, dass auch die weiblichen Sexualhormone des dreistachligen Stichlings einen Einfluss auf das Auftreten von Autoimmunreaktionen haben könnten. Es wäre interessant herauszufinden, ob und inwiefern weibliche Sexualhormone das adaptive Immunsystem beeinflussen. Aufgrund eines niedrigen P-Wertes von 0.074 könnte es sein, dass die Anzahl der verschiedenen MHC II-Allele auch einen Effekt auf die gemessenen immunologischen Parameter hat. Die Zucht und Untersuchung von Fischen mit sehr hohen, bzw. sehr niedrigen MHC II-Allelzahlen wäre ein weiteres interessantes Projekt. Die Fische könnten einer hohen Parasitenbelastung ausgesetzt werden. Beim Vorhandensein eines Trade-offs wären die Kosten, das Auftreten von Autoimmunität, geringer als der Nutzen. Eine höhere Resistenz gegenüber Parasiten könnte also mit einer Akzeptanz von auto-aggressiven Reaktionen einhergehen.

Interleukin 1 beta scheint ein wichtiger Bestandteil der adaptiven Immunantwort zu sein. Die Ergebnisse der Genexpressionsstudie zeigen eine stärkere Expression von IL 1 beta in der Kopfniere der Individuen, die vier verschiedene MHC II-Allele aufweisen. Außerdem wurde eine positive Korrelation zwischen der Expression von IL1 beta und MHC II in Kopfniere, Niere und Milz beobachtet. Das Etablieren weiterer Entzündungsgene wäre hilfreich, um ein besseres Verständnis über das komplexe Netzwerk zahlreicher Zytokine des adaptiven Immunsystems zu erhalten. Einige Studien über die Untersuchung von Zytokinen in Knochenfischen beinhalten die Stimulation des adaptiven Immunsystems mit Hilfe von LPS, FCA und ATH. Die Ergebnisse zeigen einen Anstieg der Expression bestimmter Zytokine in verschiedenen Organen. Ein weiteres interessantes Projekt wäre deshalb die künstliche Induktion von Autoimmunreaktionen aufgrund der Stimulation des Immunsystems des dreistachligen Stichlings *Gasterosteus aculeatus*.

## 2. Summary

Genetic diversity is a prerequisite for evolution. The genes of the Major Histocompatibility Complex (MHC) show genetic variation. They are polygenic and contain highly polymorphic loci. MHC molecules are an important part of the adaptive immune system due to their ability to bind and present different antigens to the T-lymphocytes. But this high specificity also implies a risk: the higher the number of recognized antigens, the more likely the similarity of foreign and auto antigens. This can cause a loss of self tolerance mechanisms due to cross reactions during molecular mimicry. The MHC might be a key molecule for selection, adaptive processes and speciation.

*Gasterosteus aculeatus* shows an intermediate number of MHC IIB alleles. On the one hand, too few different MHC IIB alleles demonstrate an insufficient diversity to detect a large number of parasite antigens. But on the other hand, there must be a limiting selection pressure, which causes this intermediate optimum of MHC IIB alleles. A possible disadvantage assumes that too many alleles might lead to self-immunoreactivity. Therefore, this thesis focuses on a possible correlation between certain immunological changes, which might be associated with autoimmune reactions, and the individual MHC IIB allele diversity of the three-spined stickleback. It is a pilot study about auto aggressive reactions as a possible limiting selection pressure on the evolution of the MHC IIB allele diversity. Thus, I used different methods to examine different aspects of this topic.

First, the MHC class IIB allele configuration of parental fish of 14 different families was determined. The results indicate five families, whose offspring showed a MHC class IIB allele diversity of either two or four different alleles. The MHC genotyping of parents and offspring was done via the Reference Strand-mediated Conformation Analysis (RSCA). The next step was the isolation of several inflammatory genes. Different primer combinations were tested via standard PCR and Agarose gelelectrophoresis. After that, primer specificity was checked via direct sequencing and quantitative Real Time PCR.

I was able to isolate IL 1 beta, MIF, IgM and MHC II in the genome of *Gasterosteus aculeatus*. After that, head kidney, spleen, liver, kidney, gut, gonads, heart and brain were dissected and blood was taken from the caudal vein. The total immunoglobulin level of the plasma was measured with the help of an Enzyme-linked immunosorbent assay (ELISA).

RNA of head kidney, gut, kidney and spleen was isolated and reverse transcribed into cDNA. The expression levels of the established inflammatory genes MIF, IL 1 beta, IgM and MHC class II were measured, relative to those of the housekeeping genes L13 and Ubiquitin. This was done via the quantitative Real Time PCR, based on the non-specific reporter molecule SYBR Green I. The raw gene expression data was normalized with the software qBase Plus and a permutational multivariate analysis of variance was performed with the help of the software R 2.11.1.

The results indicate the family of origin and sex as influencing factors. The influence of the dissection day is due to an artefact. Maybe different families contain different repertoires of immunological factors. This might be due to differences in their genetic background. In humans, it has been assumed that autoimmunity is correlated with female sex hormones. Therefore, it might be possible that female sex hormones of teleost fish are correlated with the occurrence of autoimmune diseases. It would be interesting to examine, whether and how female sex hormones can influence the adaptive immune system.

Due to a low p-value ($p = 0.074$), it might be possible that the number of MHC IIB alleles also affects certain immunological parameters. Therefore, it might be interesting to breed fish with very high and low numbers of MHC IIB alleles and to expose them to many parasites and many different types of parasites. Maybe the costs, namely the occurrence of auto aggressive reactions, are lower than the benefits, namely a high resistance towards parasites. It might be possible that there is a trade-off between the resistance towards parasites and auto aggressive reactions

The results of the expression assay indicate that IL 1 beta seems to be an important component of the adaptive immune response in *Gasterosteus aculeatus*. It shows a higher expression in the head kidney of individuals with four MHC IIB alleles. Apart from that, a positive correlation of IL 1 beta- and MHC II expression is observed in head kidney, spleen and kidney. It might also be helpful to detect more possible inflammatory genes for a better understanding of the complex network of associations between several cytokines of the adaptive immune system. Some studies about cytokines in teleost fish include stimulations of the adaptive immune system with LPS, FCA and ATH. They resulted in an increase of expression levels in several organs. Therefore, another interesting project might deal with the artificial induction of autoimmune reactions due to stimulation of the immune system of *Gasterosteus aculeatus*.

## 3. Introduction

### 3.1 Evolution and the MHC polymorphism

"It takes all the running you can do to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as you can." (Hamilton, 1980), (Ebert, Hamilton, 1996). The content of the so called Red Queen Hypothesis (Barton, 2007), (Stearns, 2005) describes the advantage of genetic diversity due to sexual reproduction during host-parasite coevolution. But genetic diversity can also imply disadvantages, resulting in self-immunoreactivity, for example (Woelfing *et al.*, 2009).

Genetic diversity is a prerequisite for evolution (Reed, Frankham, 2003). It supplies the basic material for adaptation and speciation. Factors, which affect genetic variation, are mutations, recombinations due to sexual reproduction, gene flow, natural and sexual selection (Amos, Harwood, 1998). Recombination arises during meiosis, due to crossing over and gene conversion (Barton, Charlesworth, 1998). This can result in a break-down of associations between gene variants at different loci and thus novel gene combinations occur. This newly created genetic variability can be advantageous for hosts and it is supposed to reduce virulence during host-parasite coevolution (Ebert, Hamilton, 1996). Virulence seems to be the driving force in the dynamic balance of host-parasite coevolution. Parasites try to optimize their trade-off between virulence and other fitness components in a certain host, while the host tries to minimize this virulence (Ebert, Hamilton, 1996). This reciprocal genetic change in interacting organisms is owing to natural selection, imposed by each other. The genes of the Major Histocompatibility Complex (MHC) show genetic variation. They consist of α- and β-chains and present extracellular pathogens like bacteria and parasites to T-lymphocytes (Martin, 2009). The MHC contains several loci per MHC class. Hence, there are different possibilities of α- and β-chain- combination. These individual compositions of MHC molecules are called haplotypes. Therefore, the molecules are able to present a wider spectrum of peptides and antigens (Martin, 2009). This remarkable genetic diversity is supplemented by MHC gene polymorphism (Milinski, 2006). Most MHC genes possess various alleles per locus within a population (Martin, 2009). Two major hypotheses exist, which explain the MHC polymorphism. The first hypothesis is based on negative frequency-dependent selection (Borghans *et al.*, 2004). This selection type is a characteristic of host-parasite coevolution and favors rare MHC genotypes. Individuals, who contain this rare genotype, show an enhanced resistance towards parasites (Barton, 2007). Hence, the frequency of this rare genotype increases. The second hypothesis, the heterozygote advantage, assumes disassortative mating preferences to increase heterozygosity at MHC loci. Hence, a higher diversity of antigens can be detected, resulting in an increase of resistance against diseases (Milinski, 2006). This leads to the assumption, that MHC-heterozygote genotypes are more resistant towards infections than homozygotes.

In three-spined sticklebacks, maximal resistance towards parasite infections is associated with an intermediate allele diversity (Wegner *et al.*, 2003), (Kalbe *et al.*, 2009). Therefore, this heterozygote advantage results in a higher individual fitness (Penn, 2002). Apart from that, the MHC allele configuration of the offspring can be optimised by female behavioural strategies during mate choice (Milinski, 2003). In *Gasterosteus aculeatus,* it is predicted that MHC-dependent mating preferences cause an offspring with an intermediate number of MHC alleles, resulting in an enhanced immunocompetence (Wegner *et al.*, 2003), (Milinski *et al.*, 2005). Apart from that, disassortative mating preferences are supposed to prevent kin matings with the help of specific odours, which are presented by or associated with the MHC. Heterozygosity is also positively correlated with population fitness (Reed, Frankham, 2003). The existence of an extraordinary genetic diversity highlights the MHC as potential key molecule for selection, adaptive processes and speciation. Many studies in different organisms show evidence for a correlation between MHC allele diversity and the susceptibility and resistance towards pathogens (Stearns, 2008), (Apanius *et al.*, 1997). According to the Red Queen hypothesis, an individual “must run at least twice as fast as it can”, to struggle for a better resistance towards parasites, based on an increasing number of MHC alleles. Hence, selection might be assumed to favor individuals with a high MHC allele diversity. But in *Gasterosteus aculeatus,* the optimum of an intermediate number of MHC alleles (Wegner *et al.*, 2003) indicates that there might be a possible risk of this high polymorphism. On the one hand, too few different MHC alleles account for an insufficient diversity to detect a large number of parasite antigens. But on the other hand, there must be a limiting selection pressure, which causes this intermediate optimum of MHC alleles. A possible disadvantage of a high number of MHC alleles seems to be an enhanced loss of T-lymphocytes during negative selection (Woelfing *et al.*, 2009). The MHC initiates an adaptive immune response by antigen presentation to the T-cell receptors. It presents antigens, which derived from pathogens as well as from self-proteins (Woelfing *et al.*, 2009). Hence, a high number of different MHC alleles might cause the recognition and elimination of too many T-lymphocytes. This leads to a reduction of the T-cell repertoire, resulting in a decreased immunocompetence (Woelfing *et al.*, 2009). Another possible selective force against a high number of MHC alleles assumes that too many alleles might lead to self-immunoreactivity (Woelfing *et al.*, 2009). Therefore, this study focuses on the consequences, which a high genetic diversity of the MHC might present for the adaptive immune system. Changes in immunological parameters, which might give hints for the occurrence of auto aggressive reactions, are examined in correlation with the MHC polymorphism. Hence, the study focuses on auto aggressive reactions as a possible limiting selection pressure on the evolution of an increasing number of MHC IIB alleles, concerning to adaptation and speciation.

## 3.2 Immune system and autoimmunity

The immune system is an important component of higher organisms. Its major task is the defense against diseases due to its fast recognition of foreign structures and the induction of first defense mechanisms (Martin, 2009), (Bergstrom, Antia, 2006). In vertebrates, the immune system is organized into innate and adaptive immunity.

The innate immune system is able to distinguish between self and foreign molecules. It is responsible for fast recognition of foreign material within the first four hours after infection (Martin, 2009). Apart from that, it induces an immune response at the location of infection due to processes like phagocytosis. Components of the innate immune system are mast cells for recognition and macrophages and neutrophil granulocytes for the abatement of pathogens, for example (Martin, 2009). The innate immune system is fast, but is not able to deal with modified pathogens. The more specific part of the immune system is called the adaptive immune system. It is specific for vertebrates and it evolved during evolution of chondral fish (Martin, 2009). The adaptive immune system is composed of lymphocytes, antibodies and the Major Histocompatibility Complex (MHC). T-lymphocytes mature in the thymus. They are able to recognize different antigens due to their T-cell-receptor on the surface. An antigen is defined as a molecule, which is recognized by T- and B-cell receptors of the immune system (Male, 2005). The whole process of an immune reaction can be separated into two phases: the recognition phase and the differentiation phase. A pathogen is noticed and phagocytised by macrophages and dendritic cells during the recognition phase. Dendritic cells present an intersection between innate and adaptive immunity (Martin, 2009). After phagocytosis, they degrade antigens into short peptides. This process is called antigen reprocessing. The resulting peptides are bound to MHC II molecules and represented on the surface of dendritic cells, macrophages and B-lymphocytes. Hence, dendritic cells are called antigen presenting cells (APCs) (Martin, 2009). The MHC acts as a peptide-presenting molecule. Epitopes of an antigen can only be recognized by the T-cell receptor of the T-lymphocytes, if they are presented by the MHC. This process is called MHC-restriction (Male, 2005). An epitop is described as the part of the antigen, which binds to the T-cell receptor (Male, 2005). MHC class I molecules are responsible for the presentation of intracellular pathogens, while MHC class II molecules react on extracellular pathogens like bacteria and parasites (Martin, 2009). On the one hand, T-lymphocytes do not only require MHC restriction to differentiate to T-killer- and T-helper cells. They are also reliant on so called co-stimulatory signals, presented by cytokines (Male, 2005). These cytokines are released by APCs and decide, whether a T-lymphocyte proliferates to a T-helper cell 1 or T-helper cell 2 (Martin, 2009). Cytokines are responsible for the activation of macrophages and their differentiation into antigen presenting cells (T-helper cell 1).

The differentiation phase starts with the activation of T-lymphocytes. If they are activated, T-helper cells secrete Interleukins to stimulate B-lymphocytes in antibody- and memory cell production. Apart from that, they also rely on co-stimulatory signals. B-lymphocytes differentiate to plasmatic cells after getting in contact with an antigen. The B-cell receptor is a membrane linked antibody, which is able to bind the presented antigen (Male, 2005). An antibody is a protein, which is able to bind a specific antigen. Finally, the antigen-antibody complex is phagocytised by T-killer cells. Memory cells arrange for a faster immune response after getting in contact with the same pathogen a second time (Janeway, 2005). Figure 2.1 shows the different steps of a typical adaptive immune reaction.

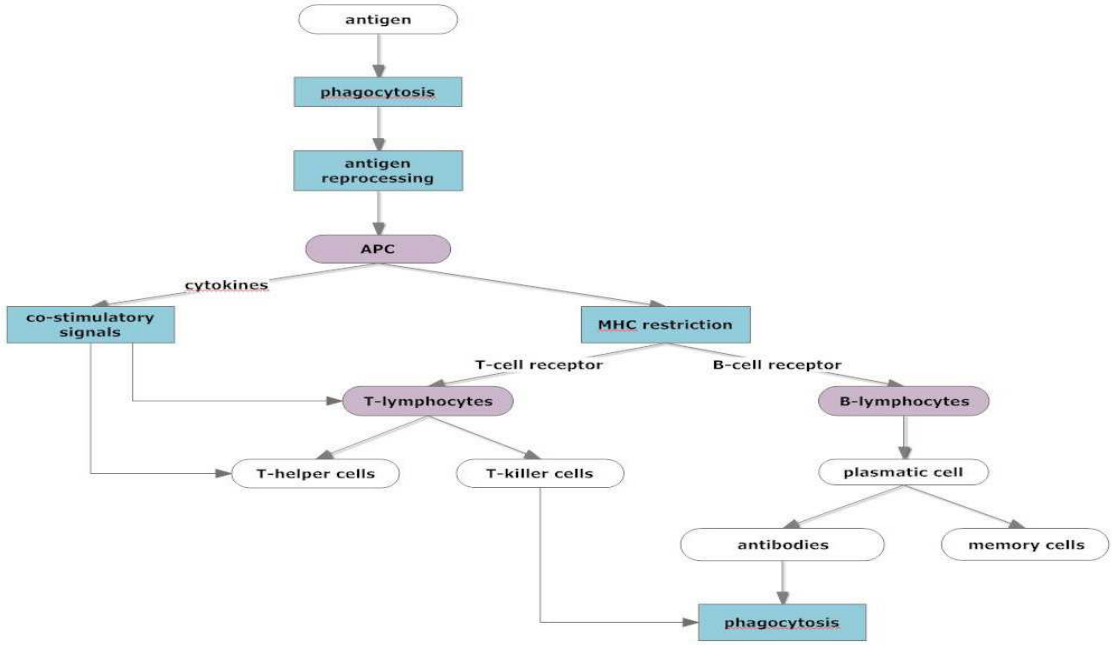


Fig. 2.1: Adaptive immune reaction pathway, created with Smart Draw

The big advantage of the adaptive immune system is its high specificity. Due to this characteristic, the adaptive immune system is able to respond to many different kinds of pathogens with a huge number of different antigens. However, this fact also implies a risk: the higher the number of recognized antigens, the more likely the similarity of foreign and self antigens. This can lead to the existence of auto aggressive reactions (Martin, 2009).

**Self tolerance and autoimmunity:**

The diversity of T- and B-cell receptors is independent of the existing antigens and based on genetic mechanisms. The risk occurs due to antigen receptors, which are able to detect naturally produced structures. In worst case, this can result in damage of the own body (Martin, 2009). Usually, self tolerance avoids this process during the development of T- and B-lymphocytes in the primary lymphatic organs. The lymphoid precursor cells start separating in the thymus and differentiate to T-lymphocytes with different antigen receptors (Martin, 2009). Several selection steps of central tolerance control for this process by checking the specificity of the antigen receptor. The first step is called positive selection. T-lymphocytes, which are able to interact with own MHC molecules, are selected and proliferate. Incompatible T-lymphocytes are apoptized (Janeway, 2005). The second step is called negative selection (Janeway, 2005). Positive selected T-lymphocytes are checked for their affinity to self antigens. These self antigens are called auto antigens. T-lymphocytes with a high affinity are auto reactive. Their antigen receptors bind to auto antigens and would start an immune reaction against own cells, tissues or organs. Dendritic cells of the thymus present own peptides, which are associated with MHC class I and II molecules. If T-lymphocytes interact with these auto antigens, they will die because of apoptosis (Martin, 2009). Finally, self tolerance is the acquirement of the immune system to identify molecules and structures, which belong to the own body, and to discriminate them from foreign molecules (Janeway, 2005).

Apart from these central tolerance mechanisms in the thymus, peripheral tolerance occurs in the peripheral lymphatic organs. It limits the activation of auto reactive lymphocytes, which were not eliminated by central tolerance (DeFranco, 2007). High affinity to auto antigens is a requirement for negative selection in the thymus. T-lymphocytes with a lower, but existing affinity to auto antigens, will escape this selection step. These T-lymphocytes will be eliminated in the periphery due to processes like anergy (Martin, 2009). Anergy is described as the missing reaction to an auto antigen due to the termination of an immune response. A weak auto reactive T-lymphocyte detects an antigen in the periphery. One important requirement is the binding to the antigen. But if the second important assumption, the co-stimulatory signals, is missing, the T-lymphocyte won't be activated. It will lose its competence to proliferate and die of apoptosis (Martin, 2009). Regulatory T-lymphocytes comprise another part of peripheral tolerance. They show a suppressive function for auto reactive T-lymphocytes, caused by the production of suppressive cytokines like interleukin 10 and TGF beta (Martin, 2009).

These self tolerance mechanisms seem to minimize the danger of auto aggressive T-lymphocytes, which could result in autoimmune diseases. This leads to the question why autoimmunity does nevertheless exist? There is no clear answer to this question yet, but some speculations exist (Martin, 2009). Infections might cause a defect in the system of self tolerance. A high affinity to a microbial antigen, which is similar to an auto antigen, paired with an up-regulation of co-stimulatory signals, could induce the activation of an auto reactive T-lymphocyte. This process is called molecular mimicry (Janeway, 2005), (Nepom, Erlich, 1991). The produced antibodies will interact with auto antigens. This interaction is called a cross reaction (Janeway, 2005). During the differentiation of B-lymphocytes, auto antibodies and memory cells are produced. The latter are responsible for a lifelong continuity of autoimmune diseases (Martin, 2009). Memory cells are characterised by their lower activation threshold during a secondary infection. Apart from that, they have no need for co-stimulatory signals. Now, a lower affine interaction with an auto antigen can induce an immune reaction. This immune reaction will produce new memory cells (Martin, 2009). Cytokines are involved in another back-coupling process. The autoimmune reaction is kept up by an increasing synthesis of co-stimulatory molecules. Some of them lead to a higher expression of MHC class II molecules (Martin, 2009).

More than 40 different autoimmune diseases exist in humans. Genetic and environmental factors seem to be the reasons for autoimmunity (DeFranco, 2007). In total, autoimmune diseases don't affect more than eight percent of the human population per disease (Ferencik, 2006). Autoimmune diseases can either be organ specific or systemic. Diabetes mellitus type I, Morbus Crohn, Colitis ulcerosa and Multiple sclerosis are organ specific autoimmune diseases (Ferencik, 2006). They affect just one organ. Systemic autoimmune diseases affect more than one organ and two examples are Rheumatoid Arthritis and Systemic Lupus Erythematodes (SLE) (Ferencik, 2006).

In 1985, first evidence for the occurrence of autoimmunity in a poikilothermal organism was shown in the testis of mature male rainbow trout *Salmo gairdneri* (Secombes *et al.*, 1985). Ten years later, Mochida et al. induced testicular autoimmunity experimentally in the Nile Tilapia *Oreochromis niloticus* by immunization with a mixture of allogeneic testis homogenate (ATH) and FCA (Mochida *et al.*, 2009). Recently, systemic autoimmunity was induced in the farmed Atlantic salmon (Koppang *et al.*, 2008). Fish were injected with an oil-adjuvant vaccine. These studies indicate the potential to produce autoimmunity artificially in teleost fish species.

### 3.3 Cytokines – multifunctional regulators of adaptive immune responses

Cytokines comprise different molecules like Interleukins, Interferons and Tumor-necrosis-factor (Male, 2005). They play an important role for the activation of T-lymphocytes during an immune response, due to their function as co-stimulatory signals. Apart from that, they also regulate differentiation and separation of ancestral cells and mediate inflammatory reactions (Male, 2005). Cytokines are also known in teleost fish and their diversification and type seem to be similar to mammalian cytokines (Savan, Sakai, 2006). This can lead to the assumption, that the basic structure of the fish adaptive immune system is similar to the immune system of mammals (Savan, Sakai, 2006). Cytokines, which are involved in the adaptive immune response, are Interleukin 12, Interleukin 4, Interleukin 10, Tumor-necrosis-factor beta and Interferon gamma. Some of these molecules are also known from teleost fish (Bird *et al.*, 2006).

Interleukin 1 is produced by lymphocytes. It conveys inflammations due to activation of cortison distribution and the increase of MHC class II molecules in APCs. It also acts as a co-stimulatory signal for T-lymphocyte activation (Böcker, 2004), (Male, 2005). Interleukin 1 belongs to the “maste cytokines of inflammation”. It is one of the molecules, which induces inflammation first after an infection or tissue damage (Martin, 2009). Stimulation of B-lymphocyte activation, growth and development of T-helper cells, production of antibodies, Interferon gamma and Interleukin 1 are important functions of Interleukin 4 (Böcker, 2004), (Bird *et al.*, 2006). It results in an up-regulation of MHC class II molecules during B-lymphocyte differentiation (Martin, 2009). Interleukin 12 is secreted by APCs and represents an important role in differentiation of type 1 T-helper cells and the synthesis of Interferon gamma. Hence, it is also involved in adaptive immune responses (Male, 2005), (Bird *et al.*, 2005). It is divided into the two subunits Interleukin 12 p40 (IL12 p40) and Interleukin 12 p35 (IL12 p35), which are “linked by disulphide bonds” (Bird *et al.*, 2005). IL 12 p40 shows increased expression in mucosal tissues of Chinese patients with Colitis ulcerosa (Pang *et al.*, 2007). Hence, it is a possible candidate gene, which might contribute to autoimmune diseases. Interleukin 10, the cytokine synthesis inhibitory factor, is a cytokine with a suppressive effect on adaptive immunity (Savan, Sakai, 2006), (Graham *et al.*, 2005). Therefore, it belongs to the T-regulatory cell cytokines (Bird *et al.*, 2006). It is produced by type 2 T-helper cells and affects type 1 T-helper cells due to inhibition of the production of Interleukin 1, Interleukin 12 and Tumor-necrosis-factor (Male, 2005), (Martin, 2009). It also reduces the antigen presentation in dendritic cells, due to inhibition of MHC class II molecules. Apart from that, it induces the secretion of Immunoglobulin G, A, E and M (Martin, 2009), (Bird *et al.*, 2006). In humans, a study about Systemic Lupus Erythematodes and Rheumatic Arthritis showed an association between an Interleukin 10 promotor polymorphism and autoimmunity (Lard *et al.*, 2003).

Therefore, Interleukin 10 is another gene, which might be important for the existence of auto aggressive reactions. Interleukin 21 is produced by T-lymphocytes and affects T-lymphocytes and B-lymphocytes due to its co-stimulatory function (Male, 2005). It regulates the proliferation and maturation of natural killer cells and the immunoglobulin production by B-lymphocytes (Bird *et al.*, 2005). A study in humans highlighted a positive correlation between an increased expression level of Interleukin 21 and the autoimmune inflammatory diseases Rheumatoid Arthtritis and Morbus Crohn (Pelletier, Girard, 2007). Thus, it is an interesting candidate gene for the examination of autoimmune diseases. Another pro-inflammatory cytokine, the Migration-Inhibiting-Factor (MIF), is secreted by activated T-lymphocytes and it is increased during chronical inflammations (Male, 2005). A polymorphism in the human MIF gene is responsible for an increased risk of systemic-onset Juvenile Idiopathic Arthritis, "the most common chronic arthritic condition of childhood" (Donn *et al.*, 2001). This correlation might highlight the importance of MIF for the occurrence of autoimmune diseases. Finally, Hong et al. demonstrated, that these inflammatory cytokines of fish release inflammatory responses equivalent to their mammalian counterparts (Hong *et al.*, 2001).

### 3.4 Immunoglobulins – the organisms arsenal against antigens

Immunoglobulin genes are expressed in B-lymphocytes and encode antibodies in the blood (Warr, 1995). They are able to bind specific antigens (Male, 2005). Immunoglobulin genes evolved during the evolution of jawed fish (Martin, 2009). Mammalian antibodies are organized into five classes, due to their different heavy chains: IgG, IgM, IgA, IgD and IgE (Male, 2005). IgM seems to be the only antibody class, which is present in all species and it mainly exists in the blood (Wilson *et al.*, 1997). It is produced after getting in contact with an antigen for the first time (Martin, 2009). Therefore, it is used as an indicator for acute inflammation. Only three classes of antibodies exist in bony fish: IgM, IgD and the special IgT/Z (Bird *et al.*, 2006). IgD is also expressed on the surface of B-lymphocytes with the same antigen specificity as IgM. The mammalian function of IgD still remains unclear and the same goes for fish (Martin, 2009).

### 3.5 Lymphatic organs – origin of organisms immunity

The immune system consists of a variety of molecules, cells, organs and tissues. Lymphatic organs are classified into primary and secondary lymphatic organs. In mammals, the differentiation of ancestral cells into T- and B-lymphocytes occurs in the primary lymphatic organs thymus and bone marrow (Martin, 2009). Secondary lymphatic organs like lymph nodes, spleen, mucosa associated lymphoid tissue and skin associated lymphoid tissue contain mature T- and B-lymphocytes. All these organs are linked by lymphatic vessels.

The spleen acts as a filter and control organ for blood circulation and leads to an immune response against antigens in the blood (Martin, 2009). The mucosa associated lymphoid tissue includes plasma cells, which secrete IgM. The gut associated lymphoid tissue is composed of the appendix and the so called Peyer plates. The latter are accumulations of lymphocytes in the septum of the small intestine. Some cells of the gut are also able to secrete cytokines (Martin, 2009). In teleost fish, lymphoid organs are represented by thymus, spleen and head kidney (Yoshiura *et al.*, 2003). Spleen and head kidney are the main sources of antibody production in fish (Ram *et al.*, 2005), (Zapata *et al.*, 1996), (Kurtz *et al.*, 2007). Additionally, the mucus producing goblet cells of the intestine express numerous immunoglobulins.

### 3.6 The Major Histocompatibility Complex (MHC) – initial element of the adaptive immune response

The three-spined stickleback is an important model organism for host parasite interactions. It is a heavily infected fish species, concerning parasite number and parasite variety (Östlund-Nilsson, 2007). Within and between populations and depending on sex, differences in the infection level can be observed in the three-spined stickleback. Possible reasons for this observation might be differences in exposure or resistence to infections (Östlund-Nilsson, 2007). But how do these differences arise? The existence of the Major Histocompatibility Complex (MHC), especially its diversity in the adaptive immune system of *Gasterosteus aculeatus*, might lead to an answer to this question. The MHC gene loci of the three-spined stickleback seem to be located on chromosome 3 and 7, but there are also some fragments, which could not be assigned yet. Two sets of paralogous MHC class II α- and β-genes have already been identified on a 99.5 kb genomic segment (Reusch *et al.*, 2004). As already mentioned (see Chapter 3.2), MHC molecules play an important role during the immune reaction, due to their binding and presentation of different antigens to T-lymphocytes. The consequences of the MHC restriction range from immunodeficiency to autoimmunity (Stearns, 2008). Too few different MHC alleles demonstrate an insufficient diversity to detect a large number of parasite antigens. In turn, too many alleles might either lead to a reduced T-cell repertoire or to self-immunoreactivity (Woelfing *et al.*, 2009). In humans, certain MHC allele variants are responsible for an enhanced risk of disease (Jones *et al.*, 2006), (McDevitt, 1998), (Nepom, Erlich, 1991). This is due to the function of MHC genes to be the most predisposing genetic factor for autoimmune diseases (Fernando *et al.*, 2008), (Klein *et al.*, 2000), (Davies, 1994). This predisposing effect is a result of MHC gene-involvement in elimination of strong self-reactive T-lymphocytes and MHC restriction.

The association between certain MHC haplotypes and the occurrence of autoimmune diseases has been studied for 25 years (McDevitt, 1998). A linkage of the MHC region and diseases like Diabetes mellitus type I, Rheumathoid Arthritis, Pemphigus vulgaris, SLE and Multiple sclerosis has already been demonstrated in humans (Rioux *et al.*, 2009), (Todd *et al.*, 1987), (Tisch, McDevitt, 1996), (Gregersen *et al.*, 1986), (Todd *et al.*, 1988), (Wucherpfennig, Strominger, 1995), (Grumet *et al.*, 1971). This leads to the question, why autoimmune-predisposing MHC alleles are still present? Several reasons are possible. Maybe, benefits of these alleles are stronger than the costs, namely the risk of autoimmunity. This would be a trade-off (Bergstrom, Antia, 2006). It may also be possible, that the fact of arising late in lifetime, allows autoimmunity to add just low costs to total fitness. The relatively recent occurrence of autoimmunity in human history could also indicate that there has not been enough time for selection, to eliminate autoimmune-predisposing MHC alleles (Graham *et al.*, 2005).

### 3.7 The three-spined stickleback *Gasterosteus aculeatus* – an emerging model organism of evolutionary genetics

The three-spined stickleback *Gasterosteus aculeatus* is a teleost species, which belongs to the Gasterosteida (Östlund-Nilsson, 2007). Due to many advantages, it has become a model system for evolutionary processes like host-parasite interactions, adaptive radiation, sexual selection or speciation. This fish shows an endemic distribution in aquatic habitats of all common salinities (Östlund-Nilsson, 2007). Apart from that, *Gasterosteus aculeatus* shows a low sensitivity to environmental disturbances. This results in an easy handling under laboratory conditions. Its small body size supports this. This fish has a small genome of 446 Mb, which has been completed in 2006 (Östlund-Nilsson, 2007), (www.ensembl.org).

The three-spined stickleback exhibits an immune system similar to mammals. The existence of immunoglobulins, lymphocytes and the Major Histocompatibility Complex (MHC) are hints for adaptive immune reactions (Yoshiura *et al.*, 2003). Teleosts evolved a protective immunity to a diversity of antigens and show immunological behaviour, which gives hints for the availability of memory cells (Hoar, 1970). Therefore, the three-spined stickleback can be used as a model organism for the examination of the adaptive immune system and even allows comparisons with mammals.

## 3.8 Thesis outline

Pathogens constitute a selection pressure, which convey a higher individual MHC allele diversity: the more different alleles an individual carries, the higher the probability to detect different pathogens. But several studies mentioned above, showed the selection for an intermediate number of MHC class IIB alleles, in nature as well as under laboratory conditions (e.g. (Milinski, 2006)). A reduced immunocompetence due to the depletion of the T-cell repertoire seems to be a possible limiting selective force (Woelfing *et al.*, 2009). Maybe the intermediate optimum of MHC IIB alleles is a trade-off between "the benefit of a high antigen presentation probability and the necessity to avoid negative selection" (Woelfing *et al.*, 2009), (Nowak *et al.*, 1992). Autoimmune reactions are assumed to be another limiting selection pressure for an increasing number of different MHC IIB alleles in three-spined sticklebacks as well as in other vertebrate species (Östlund-Nilsson, 2007). Individuals, who contain a diverse set of MHC IIB alleles, should show a greater risk of autoimmune disorders (Woelfing *et al.*, 2009). Therefore, this study focuses on a possible correlation between certain immunological changes, which might be associated with auto aggressive reactions, and the individual MHC class IIB allele diversity in the three-spined stickleback *Gasterosteus aculeatus*. The working hypothesis is as follows:

$H_1$-hypothesis:

The MHC class IIB allele diversity of the three-spined stickleback *Gasterosteus aculeatus* influences the occurrence of immunological parameters, which might be associated with auto aggressive reactions. Fish, which carry a high number of different MHC class IIB alleles, show stronger autoimmune reactions than fish with a lower MHC IIB allele diversity.

Here, a low MHC class IIB allele diversity is defined by two different alleles, whereas a high number of different MHC IIB alleles comes along with individuals, who contain four different alleles. This corresponds to the natural diversity, found in lake sticklebacks (Eizaguirre *et al.*, 2011).

This is a pilot study about the examination of auto aggressive reactions as a possible limiting selection pressure for the genetic diversity of MHC IIB. It might be difficult to interpret the measured immunological parameters. Hence, I used a multivariate approach to examine different aspects of this topic. I tried to examine several inflammatory genes in the genome of *Gasterosteus aculeatus* and measured their expression in different organs by using quantitative Real Time PCR. Apart from that, the total immunoglobulin level of the plasma was checked via an Enzyme-linked immunosorbent assay (ELISA).

## 4. Material and Methods

### 4.1 Origin and MHC diversity of fish specimens used in the study

Laboratory bred individuals of five families of the three-spined stickleback *Gasterosteus aculeatus* were used in this diploma thesis. They were chosen out of 14 different families. The individuals of each of the five families were spread over two tanks, including 20 – 30 fish each. They showed a MHC IIB allele distribution of two and four different alleles. Due to the late emergence of autoimmune diseases during lifetime (Stearns, 2008), old individuals of *Gasterosteus aculeatus* were analyzed. The parental fish were caught in Northern Germany in the Großer Plöner See (GPS) in winter 2007. The lab bred fish hatched in summer 2008 and were raised under summer conditions since then: the light shined from 6 a.m. to 10 p.m. and the temperature was 18 °C. The fish were free from macro parasites. A total of 118 fish were examined (66 females, 52 males), 24 to 27 of each family. In three fish, the gonads got lost.

### 4.2 Organs, which were analyzed during the study

The lymphatic organs spleen and head kidney were dissected for the gene expression assay. Other organs, whose gene expression was analyzed, are half of the gut and kidney. Gut and kidney were separated because they should also be examined in a histological assay. Some more organs were dissected to be used for the histological assay, including one head kidney of each fish, the liver, gonads and the complete brain and heart. Blood from the caudal vein was taken to determine the total immunoglobulin level of the plasma.

An increase in gene expression is likely to be expected in head kidney and spleen during a disease. In humans, the gut is the target organ for autoimmune diseases like Morbus Crohn and Colitis ulcerosa (Ferencik, 2006). All selected organs for the gene expression assay have already been examined in expression studies about inflammatory genes. Interleukin 4, 12, 10 and MIF were examined in the pufferfish *Tetraodon nigroviridis* (Li *et al.*, 2007), (Jin *et al.*, 2007) and *Takifugu rubripes* (Yoshiura *et al.*, 2003), (Zou *et al.*, 2003). These studies showed gene expression of Interleukin 4 in head kidney, spleen and liver (Li *et al.*, 2007). Interleukin 12 was expressed in head kidney, spleen and gut (Yoshiura *et al.*, 2003). Interleukin 10 showed expression in liver, kidney and gut (Zou *et al.*, 2003) and MIF was expressed in liver, spleen, gonads and head kidney (Jin *et al.*, 2007).

In humans, tissue changes are observable in the gut due to Morbus Crohn or Colitis ulcerosa (Böcker, 2004). The myelin of the brain is reduced due to multiple sclerosis (Böcker, 2004). Considering this background, morphological abnormalities in histological assays could also be used as a hint for the occurrence of changes in immunological parameters in fish.

4.3 MHC class IIB - Genotyping

**RSCA-Genotyping:**

The Reference Strand-mediated Conformation Analysis (RSCA) is a method to determine the MHC class IIB allele repertoire of the three-spined stickleback *Gasterosteus aculeatus*. Compared to other techniques like cloning and sequencing, this is a fast, sensitive and reliable way (Lenz *et al.*, 2009). The RSCA-Genotyping is based on DNA isolation, amplification via PCR and hybridization with a fluorescent labeled reference strand (FLR). After that, the resulting heteroduplices are separated via capillary electrophoresis. Changes in conformation of the heteroduplices, due to mismatches, affect their velocity of migration in a gel. Different heteroduplices indicate different MHC class IIB alleles and are analyzed in the software GeneMarker 1.90 Net (Snyder-Leiby, 2009), (He, 2009). The allele peaks are compared to a plasmid library of MHC class IIB alleles to name the detected alleles (Lenz *et al.*, 2009).

Here, three different reference alleles from Canadian sticklebacks were used to determine the different MHC class IIB alleles (Lenz *et al.*, 2009). The number of MHC class IIB alleles of the parents of the three-spined sticklebacks was determined, to make sure that the families consist of fish with two and four alleles. RSCA-Genotyping of the offspring was done after dissecting the fish, in order not to hurt them and to avoid infections.

**DNA isolation:**

The DNA of the parental fish had already been isolated from the tail fin by Christophe Eizaguirre. DNA of the offspring was isolated from the tail fin with the DNeasy Blood and Tissue kit (Qiagen) and prepared as mentioned in the protocol “purification of total DNA from animal tissues (DNeasy 96 protocol) of the DNeasy Blood & Tissue handbook (July 2006), except step 16. Instead of 200 µl Buffer AE, 50 µl were used for two times.

**PCR amplification:**

After isolating DNA from tail fin, the exon 2 of MHC class IIB loci of each individual was amplified via a Polymerase Chain Reaction (PCR). The produced fragment consisted of 247 bp. The PCR was done on a Sensoquest labcycler and the “RSCA27LG”-PCR-program was used. It consists of 27 cycles. Each cycle starts with 10 minutes at 94°C to denature the strands, followed by 30 seconds at 94°C, 30 seconds at 58°C to let the primers anneal, 60 seconds at 72°C and 5 minutes at 72°C to elongate the sequences. After that, the samples stayed at 4°C to keep them stable. GAIIEx2startF was used as a forward primer and GAIIExon2R_RSCA as a reverse primer (Lenz *et al.*, 2009). The composition of the PCR reaction is shown in part 1.1 of the supplement.

**DNA-Hybridization:**

DNA-Hybridization is a process, which makes sequences observable with the help of probes like fluorescent labeled sequences. Here, fluorescent labeled references (FLR) from Canadian sticklebacks were used. Three FLRs hybridized with the PCR products in three different reactions, so that each fish was examined in three different hybridization reactions. The following table shows the names and amounts of the FLRs.

Tab. 4.3.1: FLRs, used for hybridization of RSCA-Genotyping

| name | amount FLR (µl) | amount PCR product (µl) |
|---|---|---|
| FLR3_1_4 | 1 | 6 |
| FLR2_2_8 | 1 | 6 |
| FLR2_2_12 | 1 | 8 |

Samples were hybridized on a thermal cycler (Sensoquest labcycler). First, the samples stayed at 95°C for 10 minutes to denature the strands. After that, they were cooled down 2°C per second until they reached 55°C. The samples were kept at this temperature for 20 minutes to form heteroduplices, followed by 15 minutes at 4°C. They stayed at 4°C to keep the heteroduplices stable.

**Fragment analysis:**

A capillary gel electrophoresis had to be done, to make the different MHC class IIB alleles of exon 2 observable. The different alleles are represented by different heteroduplices of different conformation. This property affects the velocity of migration of the products.

The analysis of the products was done on a sequencer (3130 xl Genetic Analyzer, Applied Biosystems Hitachi) with the GeneMapper modul “RSCA36_CAP_GA10x_IT15_IV15”. A run temperature of 18°C and a current of 10 kV were used for 60 minutes. The following table shows the composition of the samples for the sequencer.

Tab.4.3.2: Composition of sequencer reaction

| Ingredient | amount (µl) |
|---|---|
| Hybridization product | 1.5 |
| GS1000 Rox size standard | 0.3 |
| HPLC water | 9.7 |

The results of the sequencer data were analyzed with the software GeneMarker 1.90 Net. The data was compared with the current MHC class IIB allele library for exon 2 (Lenz *et al.*, 2009) to determine the different MHC class IIB alleles of each stickleback.

4.4 Examination of inflammatory genes by PCR, sequencing and sequence analysis

One aim of this study is the identification of several inflammatory genes in the three-spined stickleback *Gasterosteus aculeatus*, which might be associated with auto aggressive reactions. Primers were designed and checked via standard PCR, Agarose gelelectrophoresis and quantitative Real Time PCR.

**Primer design:**

The first step was to check, whether my target genes have already been sequenced in the three-spined stickleback. They could be compared with other teleost species, like the two pufferfish species *Tetraodon nigroviridis* and *Takifugu rubripes* or the Japanese medaka *Oryzias latipes* and the zebrafish *Danio rerio*. This way, we got some information about the conservation of the inflammatory genes. These steps were done with the NCBI Genbank (Pruitt *et al.*, 2007), (http://www.ncbi.nlm.nih.gov/) and Ensembl (Hubbard *et al.*, 2002), (http://www.ensembl.org/index.html) via the Basic Local Alignment Search Tool (BLAST). The resulting sequences of genomic DNA and their appendant exons were aligned with the help of the software BioEdit (Hall, 1999) to determine exon-intron boundaries. Alignments of EST-sequences of other teleost species were done to find conserved homologous regions. Primers were designed manually, by using BioEdit and the primer analysis program Oligo Calc (http://www.basic.northwestern.edu/biotools/oligocalc.html). They should have a length of 18-30 bp and produce a fragment of about 120 bp, so that they can be used for the quantitative Real Time PCR assay. Apart from that, the primers should contain 20-70% C- and G-bases and should be free from hairpins or other secondary structures. To increase the binding to the cDNA, they should not include many wobble-bases. The primers were ordered from the concern Metabion (http://www.metabion.com/home/index.php). The MHC class II gene has already been sequenced by Thorsten Reusch (Reusch *et al.*, 2004). The primers for Immunoglobulin M were taken from Sascha Hibbeler, who has already designed and generously provided them. I also checked alignments between the stickleback genome and primers for Interleukin 4, 10, 12 and MIF, which were used in gene expression studies in the pufferfish species *Tetraodon nigroviridis* and *Takifugu rubripes* (Li *et al.*, 2007), (Zou *et al.*, 2003), (Yoshiura *et al.*, 2003). Primers for Interleukin 12 were taken from a gene expression study in the pufferfish *Takifugu rubripes* (Yoshiura *et al.*, 2003).

The primer combinations IL10_F_1_Chris / IL10_R_1_Chris and IL10_F_2_Chris / IL10_R_2_Chris were designed and generously provided by Christophe Eizaguirre and Sandra Vogel. Properties of the different primers, designed for the inflammatory genes and the two housekeeping genes Ubiquitin and the ribosomal protein L13, are shown in table 4.4.

Tab. 4.4: Primer properties

| name | Sequence | length (bp) | GC (%) | Tm (°C) |
|---|---|---|---|---|
| MHC_GA11 | 5'-AACTCCACTGAGCTGAAGGACATC-3' | 24 | 50 | 65 |
| MHC_Reusch_Ex3_R | 5'-CGTCTCAGAGTGCAGCCTGACGT-3' | 23 | 61 | 68 |
| IL12_p35_F1_Fugu | 5'-TGTGTCTCTCAGGCCCAGGCGCT-3' | 23 | 65 | 70 |
| IL12_p35_R2_Fugu | 5'-GTACTTCACAGCTCTGTTGATGGT-3' | 23 | 46 | 64 |
| IL12_p40_F1_Fugu | 5'-TTTCAACGGTGACTTTCGCTGCTCTTGG-3' | 28 | 50 | 70 |
| IL12_p40_R1_Fugu | 5'-CAGAGGGCATCCTTGGCTCGAACACA-3' | 26 | 58 | 71 |
| GA_IL10_F_1 | 5'-CTGCAACAACCGGTGCTGYMGC-3' | 22 | 64-68 | 66-70 |
| GA_IL10_F_2 | 5'-GCCGCTTCGTGGAGAGCTTYCC-3' | 22 | 64-68 | 68-70 |
| GA_IL10_R_1 | 5'-GAACAAATCCAGCTCYCCCATGGC-3' | 24 | 54-58 | 67-69 |
| GA_IL10_R_2 | 5'-CGATGTAGTTGAAGAACARRTCCAGCTC-3' | 28 | 43-50 | 67-70 |
| GA_IL10_TT_F1 | 5'-GGCTCTCTGCTCTCCGTCCTGCTGC-3' | 26 | 69 | 67.4 |
| GA_IL10_TT_F2 | 5'-CGTGGAGGGCTTCCCTGCGCGCC-3' | 23 | 78 | 67.8 |
| GA_IL10_TT_R1 | 3'-CGTCCATAAGGTGGCAGGCAAACGGCG-5' | 27 | 63 | 65.8 |
| GA_IL10_TT_R2 | 3'-GGTTCCTGGTTTCCGCCGTGACCGTGG-5' | 27 | 67 | 67.3 |
| GA_IL10_TT_R3 | 3'-CCATCTTGGTGTAAGTCGAGTTCAGGAC-5' | 28 | 50 | 61.3 |
| GA_IL10_DR_F1 | 5'-GGCACCCCAAGGAGCTCATCTGTACATC-3' | 28 | 57 | 64.3 |
| GA_IL10_DR_R1 | 3'-CGGAGCTCCCTCAGTCTTAAAGGAAAGCCC-5' | 30 | 57 | 65.7 |
| GA_IL10_DR_R2 | 3'-GAGTGCGAATAACTGCTGGTGTTGTGC-5' | 27 | 52 | 61.3 |
| GA_IL10_For_1 | 5'-CTCATTTGTGGAGGGCTTTCC-3' | 21 | 52 | 54.4 |
| GA_IL10_For_2 | 5'-GGGAGCTCCGTTCTGCATAC-3' | 20 | 60 | 55.9 |
| GA_IL10_Rev_1 | 3'-GCAGGATCTCGTTCATGACGTG- 5' | 22 | 55 | 56.7 |
| GA_IL10_Rev_2 | 3'-GCTGTTGGCAGAATGGTCTCC-5' | 21 | 57 | 56.3 |
| GA_IL10_Rev_3 | 3'-CATTTCACCATATCCCGCTTG-5' | 21 | 48 | 52.4 |
| IL10_F_1_Chris | 5'-GCTCATCTYTACATVTTCTCACTTG-3' | 25 | 36-44 | 52.8-56 |
| IL10_R_1_Chris | 3'-GTCYAACCCARCAACATCCTA-5' | 21 | 43-52 | 50.5-54.4 |
| IL10_F_2_Chris | 5'-ACGCTACTTCGTTGCGACTG-3' | 20 | 55 | 53.8 |
| IL10_R_2_Chris | 3'-CACCAGATCCCTCTTGAGTT-5' | 20 | 50 | 51.8 |
| IgM_For | 5'-AAGGCAGGAGAATGAAACCTTGG-3' | 23 | 48 | 63 |
| IgM_Rev | 5'-CCGAGTGAGCAGACAGGACTGG-3' | 22 | 64 | 68 |
| IL1_beta_For | 5'-ACGGCTTAGTGGAAGAGGAGATCG-3' | 24 | 54 | 67 |
| IL1_beta_Rev | 5'-ACATGGTCATGAGCACTTTTTTGG-3' | 24 | 42 | 62 |
| IL1_beta_R_2 | 5'-GGTACGCCGACATGGTCATGAGC-3' | 23 | 61 | 68 |

| | | | | |
|---|---|---|---|---|
| GA_IL1_beta_F1 | 5’-GTGGAAGAGGAGATCGTGACGCTGTGCAG-3’ | 29 | 59 | 65.7 |
| GA_IL1_beta_F2 | 5’-GCAGTTCGCCGCCACATCTCCAGATCAG-3’ | 28 | 61 | 65.8 |
| GA_IL1_beta_R1 | 3’-CGCAGGGTGCAGGTACGCCGACATGGTC-5’ | 28 | 68 | 68.7 |
| GA_IL1_beta_R3 | 3’-CCGCCTCCAGGTGCAGAGTGGGCTC-5’ | 25 | 72 | 67.5 |
| GA_IL4_F_1 | 5’-GGGCGCCATGAGGATCCCCAG-3’ | 21 | 71 | 69 |
| GA_IL4_F_2 | 5’-GGCTGGTACGCMGAMSTGGGC-3’ | 21 | 67-76 | 67-71 |
| GA_IL4_R_2 | 5’-CGCTTGACCTTGGAGTTGARGAGG-3’ | 24 | 54-58 | 67-69 |
| GA_IL4_R_3 | 5’-TCGGAGCAGGTGTAGGAGGCCAG-3’ | 23 | 65 | 70 |
| GA_MIF_F_2 | 5’-AAACCTGCACAGTATATTGCTGTGC-3’ | 25 | 44 | 64 |
| GA_MIF_F_3 | 5’-CCGATGTTCGTGGTGAACACC-3’ | 21 | 57 | 63 |
| GA_MIF_R_1 | 5’-GCCAATACTGTGGAGGGAGCAG-3’ | 22 | 59 | 66 |
| GA_MIF_R_3 | 5’–GTTAATGTAAATCCTGTBAGGAGAG–3’ | 25 | 36-40 | 61-63 |
| GA_MIF1_F | 5’-GGCCAAAAGCGACGTGCCCGCGTCTC-3’ | 26 | 69.2 | 76 |
| GA_MIF1_R | 3’-GTAGTGTTGTTCCAGGCCACATTGGCT-5’ | 27 | 51.9 | 70 |
| GA_IL21_F_1 | 5’-GGAAGCTGGAAGAGGTCCTGGG-3’ | 22 | 64 | 68 |
| GA_IL21_F_2 | 5’-CCGCTGCTGTGTGTCAACGCTG-3’ | 23 | 61-65 | 68-70 |
| GA_IL21_R_1 | 5’-GGATGCGAGTTGYAGGTTTGGCAG-3’ | 24 | 54-58 | 67-69 |
| GA_MHCI_For_mod | 5’-CCAAACTTCCCAGAGTTTGTG-3’ | 21 | 48 | 60 |
| GA_MHCI_F_5 | 5’-CGGGAGGAGCTCTCTGATGAG-3’ | 21 | 62 | 65 |
| GA_MHCI_R_4 | 5’-CCTCCTCCAGTCTTCAGCAGGG-3’ | 22 | 64 | 68 |
| GA_MHCI_Rev | 5’-GTGGACACCTCCAGTTTGG-3’ | 19 | 58 | 60 |
| Ubi_For | 5'-AGACGGGCATAGCACTTGC-3' | 19 | 58 | 60 |
| Ubi_Rev | 5'-CAGGACAAGGAAGGCATCC-3' | 19 | 58 | 60 |
| L13_For | 5'-CACCTTGGTCAACTTGAACAGTG-3' | 23 | 48 | 63 |
| L13_Rev | 5'-TCCCTCCGCCCTACGAC-3' | 17 | 71 | 60 |

**Verification of primer specificity:**

The primers, mentioned in table 4.4, were checked to be present in the genome of the three-spined stickleback *Gasterosteus aculeatus*. Therefore, a broad range of DNA from *Gasterosteus aculeatus* was tested. Genomic DNA of head kidney and spleen of three-spined sticklebacks from river and lake was used. RNA of head kidney and spleen of fish from the Großer Plöner See, the Neustädter Binnenwasser and from Norwegian fish was examined. Before examination, the RNA was transcribed into cDNA. The RNA was generously provided by Noemie Erin.

### 4.4.1 Reverse Transcription PCR

First, RNA of head kidney and spleen was transcribed into cDNA via a Reverse Transcription PCR (RT-PCR). The Reverse Transcription was done with the Qiagen kit Omniscript Reverse Transcriptase for First-strand cDNA synthesis, as mentioned in the Omniscript Reverse Transcription Handbook (May 2004). It was a 20 µl reaction. More detailed information about the composition of the reaction is shown in table 2.1 in the supplement. The samples were incubated for 60 minutes at 37°C on a Sensoquest labcycler. After that, they were diluted 1:10 in HPLC water.

### 4.4.2 Standard PCR

First, a stock solution of each primer was prepared as mentioned in the synthesis report. These stock solutions were diluted in HPLC water, in a concentration of 1:20. After that, different master mixes for each possible primer combination were prepared for each gene. The composition of the master mixes is shown in the supplement in table 3.1. The PCR was done on a Sensoquest labcycler. It started with an incubation temperature at 95°C for 10 minutes, followed by a down-regulation to 94°C for 1 sec. After that, 40 cycles, containing 20 sec at 94°C and 45 sec at 68°C for annealing and elongation, were done for fragments of a size about 200 bp. For longer fragments, an elongation time of 2 minutes was used to make the amplification possible. In the end, the samples were cooled down at 4°C. The exact cycling conditions are shown in the supplement (part 3.2).

A 1.5% Agarose gel was cast and electrophoresis was carried out to check the PCR products. 1 µl of DNA Loading dye (Fermentas 6x Loading dye) was added to each sample and then 4 µl of each sample was used to load the gel. A 100 bp DNA ladder (Fermentas Gene ruler, 0.1µg/µl) was used as a size standard. The gel electrophoresis was run at a voltage of 80 volt. After that, the gel was stained in an Ethidiumbromid solution for 35 minutes and washed in a water bath for some minutes to clear the surface from Ethidiumbromid. The gel was irradiated with ultraviolet light to make the different PCR products observable. If there were PCR products at the expected size, another PCR with 20 µl per reaction was done and the whole resulting product was used for a gel electrophoresis and subsequent sequencing. In this case, 5 µl of the DNA Ladder (100 bp, Fermentas Gene ruler, 0.1 µg/µl) and 3 µl of DNA Loading dye were used. After running at a voltage of 80 volt, the gel was stained with a SybrGold solution for 35 minutes. 10 ml TAE-Buffer were mixed with 2 µl SybrGold (Invitrogen) and the mixture was distributed on the surface of the gel with the help of a shot. After 35 minutes, the gel was cleaned with Millipore water and irradiated with blue light. Blue light has a wave length of about 500 nm to make the PCR products observable. Products of the expected size were cut out of the gel with the help of X-Tracta (Biozym), placed into 1.5 ml tubes and stored in the fridge until DNA extraction.

### 4.4.3 DNA Gel extraction

The DNA had to be eluted from the gel to sequence the expected PCR products. It was isolated from the gel via a NucleoSpin Extract II kit (Macherey-Nagel) for PCR clean-up and Gel extraction. The instruction followed the PCR clean-up and Gel extraction user manual (Macherey-Nagel, July 2009). Instead of 15-50 µl, 10 µl were used for two times to elute the DNA.

### 4.4.4 Direct Sequencing

Another method to verify the amplification of a target gene, is direct sequencing of a PCR product. Sequencing is a method to determine the progression of nucleotides of a gene and it is similar to a standard PCR (Mülhardt, 2002). It has the same ingredients like DNA-template, primer, Taq-polymerase, dNTPs, HPLC water and buffer. But it contains another ingredient, the so called didesoxynucleotides (ddNTPs, stopnucleotides). These nucleotides miss an oxygen atom at the 3'-position of the ribose. This missing atom is responsible to stop the polymerase during a PCR reaction, because the binding site for nucleotides is absent. Four didesoxynucleotides are available, each of them represents one base (Mülhardt, 2002). The polymerase cannot differentiate between dNTPs and ddNTPs. Hence, ddNTPs are used randomly and a lot of fragments of different sizes arise. These fragments are separated in size with the help of a capillary electrophoresis. The four ddNTPs are labelled with four different fluorescent dyes, which are activated via laser light during the capillary electrophoresis. The different fragments run through the gel with different speeds and occur in different colours, due to the fluorescence label of their appendant didesoxynucleotides. An electropherogramm with different curves in different colours arises and each peak presents one base in the nucleotide sequence of the examined gene (Mülhardt, 2002).

The cycle sequencing PCR was done on a MJ Research PTC 200 Peltier Thermal Cycler. The composition of the reaction is shown in table 4.1 in the supplement. The program starts with 1 minute at 96°C, followed by 25 cycles, containing 96°C for 10 seconds and then 60°C for 4 minutes in each cycle. In the end, the temperature was run down to 4°C. After that, the PCR products were cleaned with the BigDye X-Terminator Purification kit (Applied Biosystems) to remove all PCR substances but the DNA. For each 10 µl reaction, 48 µl SAM-solution and 10 µl X-Terminator-solution are needed. The samples were mixed on a Micromixer E36 for 30 minutes at 2000 rpm, followed by two minutes at 1000 x *g* on a centrifuge. The capillary electrophoresis and sequencing were done on a 3730 DNA Analyzer (Applied Biosystems Hitachi) and followed standard protocols. The data were transferred into an electropherogramm with the help of the software Data Collection Version 3.0. The analysis of the resulting sequences was done with the software BioEdit (Hall, 1999).

The resulting sequences were checked for similarity with the ones, which already exist from Genbank, by using multiple sequence alignment (Clustal W) and the Basic Local Alignment Search Tool (BLAST).

4.5 Dissection of the examined three-spined sticklebacks

Fish had to be dissected to collect the organs for the gene expression assay and the histological assay. Before starting the dissection, the three-spined sticklebacks must not be fed one day before dissection. This had to be done to make the gut as empty as possible.

The instructions for the dissection are based on the parasite assay of the three-spined stickleback, written by Martin Kalbe (Kalbe *et al.*, 2002).

118 fish, 24 to 27 of each family, were randomly chosen and killed with the neurotoxin MS 222 (1.5 g/L, in a dilution of 1:100). Dissections were conducted over several days and three fish of each family were dissected at a time. 0.5 ml tubes were filled with 100 µl RNAlater solution (RNAlater Soln., Tissue collection RNA stabilization solution, Ambion) to collect the organs for the gene expression assay. RNAlater is a solution, which keeps RNA stable because of its high concentration of salt. This stops the activity of the RNase. Due to their larger size, 200 µl were used for gut and liver. 2 ml tubes were filled with 1.5 ml Paraformaldehyd in a concentration of 4% in a PBS-solution. These tubes were prepared for the organs of the histological assay. Each organ is kept in a separate tube.

Before killing, fish had to be weighed and the standard length and height had to be measured. The weight is important to analyze the correlation between the bodyweight and the weight of the spleen – the so called splenosomatic index ((spleen weight/body weight) x 100). The ratio between bodyweight and liver weight is called the hepatosomatic index ((liver weight/body weight) x 100) (Vijayan, Leatherland, 1988), (Chellappa *et al.*, 1995). The hepatosomatic index is a hint for the energy status of three-spined sticklebacks (Chellappa *et al.*, 1995). The splenosomatic index assumes, that a bigger spleen is used as a hint for an immune system, which is more active (Macnab *et al.*, 2009).

Fish were killed and after that, the tail was cut off with a scalpel. The blood of the caudal vein was collected in a 18 µl haematocrit capillary (Hirschmann 9100418, Ammonium-heparinisiert). Fresh blood had to be centrifuged within the first hour for ten minutes at 12500 rpm (Hettich Zentrifugen, Haematokrit 210). The centrifuged blood is stored in the fridge until further processing. The tail fin was cut off and frozen at -20 °C to do the RSCA-Genotyping. The head was cut off directly behind the gill covers with the help of scissors. The body was opened with two long cuts on both lateral sides from anterior to posterior. The following picture shows these steps.

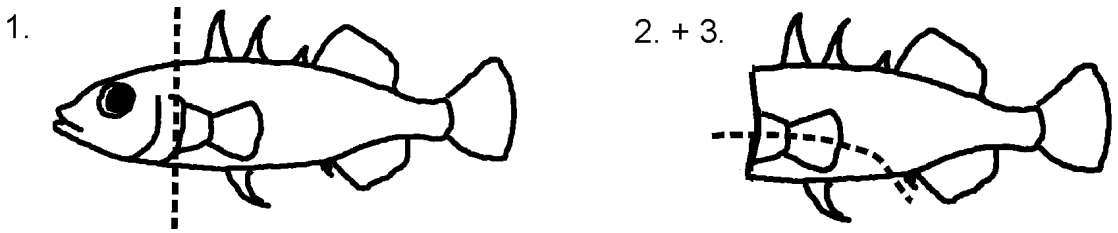


Fig. 4.5.: First dissection steps (Kalbe, Parasitologische Untersuchung von Stichlingen)

First, the lymphatic organs head kidney and spleen were dissected and put into tubes with RNAlater solution with tweezers. After that, these organs were weighed. The next organ, which had to be dissected, was the liver. It was also weighted. Kidney, gut and gonads were dissected. One half of them was conserved in RNAlater solution for about 2 hours at room temperature. After that, the samples were stored at 4°C over night and then frozen at -20°C until further processing. The other halfs were used for the histological assay and dissected as shown in table 4.5. Each of them was transferred into a 2 ml tube, containing Paraformaldehyd. For the dissection of the brain, the eyes must be resected by destroying the skin above the eye lens with the help of fine tweezers. After that, they were taken out of the head. Two lateral cuts in the skull were done with the help of scissors and the skull plate was folded up. The white brain was carefully released from the bottom side and also conserved in Paraformaldehyd. The samples were stored at room temperature in the dark until subsequent treatment. Finally, the remaining organs and tissues of each fish were kept in a tube, filled with 85% ethanol.

Tab. 4.5: Disposal of dissected organs ("+" = yes; "-" = no)

| organ | gene expression: RNAlater solution | histology: Paraformaldehyd | total immunoglobulin level: capillary |
|---|---|---|---|
| spleen | + | - | - |
| head kidney | + | + | - |
| gut | + | + | - |
| kidney | + | + | - |
| brain | - | + | - |
| blood | - | - | + |
| gonads | + | + | - |
| liver | + | + | - |
| heart | - | + | - |

After finishing dissection, the blood had to be converted. First, the length of the haematocrit and the plasma were measured. After that, sterile 0.5 ml tubes were used to store the plasma. The capillary was scored with the help of a nail file at the border between blood cells and plasma and then broken. A pipette helped to push the plasma into the tube. Each plasma weight was taken. The samples were centrifuged and stored at –20°C.

4.6 Enzyme-linked immunosorbent assay (ELISA)

The Enzyme-linked immunosorbent assay is an immunological examination (Janeway, 2005). A possible inflammation due to changes in immunological parameters, which are associated with autoimmunity, might cause changes in the immunoglobulin level of the blood. Here, an ELISA was used to determine the amount of total immunoglobulin values in the plasma of the three-spined stickleback. Plasma of 118 fish was examined in three replicates per fish. The blood of four fish got lost.

The principle of the ELISA is as follows. The plasma, including immunoglobulin M, was added to a 384 well micro titer plate. The surface of the plate shows an affinity to proteins. Here, a total of 25 µl per well was used, including 0.2 µl of plasma, filled up with PBS buffer. The plate was incubated at 4°C over night to enable the antigens to bind to the plate (Male, 2005). After purification with 3x 100 µl TBST buffer, remaining free space on the plate was blocked by adding 100 µl of 1% BSA/TBST. The plate was incubated for one hour at 37°C. Another purification with 100 µl TBST buffer was done for five times. The last purification step was incubated for five minutes at room temperature. 50 µl of the mouse-anti-stickleback-antibody F22, used in a concentration of 1:250 in a 1% BSA/PBS solution, was added to bind to the immunoglobulin antigens of the three-spined stickleback. After one hour at 37°C, excessive antibodies were removed by another purification step. It was the same procedure as mentioned before adding the first antibody. 50 µl of another antibody, a so called secondary layer, was added and incubated for one hour at 37°C (Male, 2005). Here, the rabbit-anti-mouse-antibody 9044 was used in a concentration of 1:2500 in a 1% BSA/PBS solution. This antibody is linked to an enzyme, which results in a fluorescent product, after getting in contact with a chromogen substrat (Janeway, 2005). The samples were washed again, as mentioned in the last purification steps. The chromogen substrat Sigma T0440 was used in 50 µl of TMB. The plate was incubated for ten minutes at room temperature to induce the fluorescent reaction. The reaction stopped after adding 25 µl of 2M H2SO4. The amount of fluorescence was detected with the help of a photometer at a wave length of 450 nm within the first hour after termination of the reaction.

## 4.7 Quantitative Real Time PCR

One aim of this study is the measurement of the expression of immune genes in head kidney, spleen, kidney and gut of *Gasterosteus aculeatus*.

First, RNA was isolated and converted to cDNA with the help of a Reverse Transcription PCR. The resulting cDNA was used as basic material for the quantitative Real Time PCR.

### 4.7.1 RNA isolation

mRNA is needed to measure the expression of a certain gene. This molecule shows the activity of a gene, due to its transcription at a certain time in a certain tissue of an organism. The extraction of RNA from head kidney, spleen, kidney and gut was done with the Qiagen kit NucleoSpin 96 RNA by Macherey-Nagel. The protocol followed the user manual for total RNA isolation (January 2009, p.36-38).

First, the organs were transferred into collection tubes to do the cell lysis. 300 µl of buffer RA1 and 3 µl Mercaptoethanol (Sigma M7 154-25ml) were added to each sample. The lysis buffer inactivated RNases. The cells were crushed with the help of a tissue lyser (Qiagen Tissue Lyser II, Retsch) for two minutes at a frequency of 30 Hz for two times. Before starting the RNA extraction, the gut was cut into pieces because of its bigger size. The resulting samples, lysis buffer and Mercaptoethanol were added to a NucleoSpin 96 RNA Filterplate for nucleic acid and protein purification (Macherey-Nagel), in order not to block the Binding plate. The cell crushing time was elongated to 8 minutes for two times. After that, the lysat was transferred to a NucleoSpin RNA-Binding plate and mixed with 300 µl RA4-buffer. Salts were removed from the silica membrane by adding 500 µl of buffer RA3. A DNase reaction mixture was used to eliminate contaminating DNA within the samples. Remaining salts and metabolites were removed by three washing steps with different buffers. Finally, the pure RNA was eluted in RNase free water, which was added in an amount of 50 µl for two times.

### 4.7.2 RNA purity test

The Qiagen kit NucleoSpin 96 RNA (Macherey-Nagel) contains a purification step to get rid of contaminating DNA. But the purity of RNA should nevertheless be checked.

Therefore, five RNA samples each of head kidney and gut were examined. Three DNA samples from tail fin were used as a positive control. Exon 1 to 2 of MHC II was amplified via a standard PCR. The conditions were the same as for the RSCA genotyping. GA_II_Ex1F was used as a forward primer and GA_II_Exon2R_RSCA as a reverse primer. After that, 4 µl of each sample was tested on a 1.5% Agarose gel. The 100bp DNA Ladder (Fermentas Gene ruler, 0.1µg/µl) was used as a size standard. The gel was stained for 45 minutes in an Ethidiumbromid solution.

### 4.7.3 Reverse Transcription PCR

The Reverse Transcription Polymerase Chain Reaction (RT-PCR) provides the starting material for the quantitative Real Time PCR assay. The transcription of RNA into cDNA was performed with the Qiagen Omniscript RT kit, the Qiagen RNase inhibitor (15.000 units) and the Qiagen Oligo dT-Primer. The protocol followed the Omniscript Reverse Transcription Handbook (May 2004). A 20 µl reaction was prepared. The composition of the reaction is shown in table 2.1 in part 2 of the supplement. 10 µl of template RNA was used and incubated with the master mix at 37°C for 60 minutes on a Sensoquest labcycler.

### 4.7.4 The quantitative Real Time PCR

The quantitative Real Time PCR (qRT-PCR) is a technique to quantify the amount of PCR product in real time, due to monitoring of the reaction with the help of fluorescent molecules (Sluijter *et al.*, 2006). It examines alterations in mRNA expression levels of certain genes during the exponential phase of the reaction (Sluijter *et al.*, 2006) and is a combination of a standard PCR and fluorescent dyes (Ramakers *et al.*, 2003b). Therefore, the following ingredients are needed: buffer, dNTPs, Mg, DNA-Polymerase, primers and template-DNA. Furthermore, a reporter molecule is needed. Here, the nonspecific reporter molecule SYBR Green I was used to detect the amount of amplification product. The amplification product will be doubled after each PCR cycle. This increase of the amplified product is proportional to the increase of the fluorescence signal of SYBR Green I. During the first phase of the reaction, there is too little template. Thus, the possibility is low, that all ingredients will come together. The fluorescence signal will be below the so called threshold cycle. The threshold cycle or Ct-value represents the cycle number “when the fluorescent signal rises above the background levels” (Sluijter *et al.*, 2006). It occurs during the second phase of amplification, the exponential phase. The third phase of amplification is called the plateau phase. It is characterised by an inhibition of the amplified product, due to hybridization of the fragments with each other rather than with the primers. Hence, there will be less substrate, which can be used as starting material for the next amplification cycle (Karlen *et al.*, 2007). The fluorescent dye SYBR Green I inserts in double-stranded DNA molecules, but in each kind of double-stranded DNA. It is not specific for a certain gene. Therefore, the primers need to be designed as specific as possible and a melting curve analysis has to be done as a control for primer specificity. The amplification product is heated until the double-stranded DNA denatures. This gives hints for a specific gene, which shows one clear peak, or unspecific products and primer-dimers. The latter will occur at lower temperatures than the specific gene.

Here, a relative quantification of the expression levels of several immune genes was done. Relative quantification is described as the alteration in expression of the target genes relative to that of the reference genes, by a comparison of the threshold cycles with the help of the ΔΔCt-method (Livak, Schmittgen, 2001). The housekeeping genes L13 and Ubiquitin were used as reference genes for gene expression analysis in *Gasterosteus aculeatus* (Hibbeler *et al.*, 2008). A housekeeping gene is synthesized in every nucleic cell and is stably expressed in a given organ (Thellin *et al.*, 1999), (Hibbeler *et al.*, 2008). To date, L13 and Ubiquitin are established reference genes in head kidney and spleen of the three-spined stickleback (Hibbeler *et al.*, 2008).

**The quantitative Real Time PCR requirements:**

The qRT-PCR method needs several requirements to be achieved. First, the efficiency of target and reference genes should be approximately equal during amplification (Livak, Schmittgen, 2001). This was calculated with the help of the software LinReg PCR (Karlen *et al.*, 2007). The efficiency test was performed with cDNA of three different individuals and their four organs head kidney, spleen, gut and kidney. Different cDNA dilutions (1:10, 1:100), primer dilutions (1:5, 1:10) and primer combinations were analyzed. Another requirement to make use of the qRT-PCR is the stable expression of the reference genes L13 and Ubiquitin in head kidney, kidney, spleen and gut. Hence, a reference gene stability test was done. 20 individuals of each assay group, containing either two or four MHC IIB alleles, were used. First, the cDNA concentration of each sample was measured with the help of a photometer (NanoDrop 1000 Spectrophotometer, Peqlab). After that, the cDNA concentration of each sample was equalized to a concentration of 100 ng per µl to make the gene expression results of the samples comparable. The quantitative Real Time PCR was performed on a Roche Light Cycler 480. The cycling conditions were as followed.

| Program Name | Target (°C) | Acquisition Mode | Hold (mm:ss) | Ramp Rate (°C/sec) | Acquisitions (per °C) | Cycles | Analysis Mode |
|---|---|---|---|---|---|---|---|
| Incubation | 95 | None | 10:00 | 4.4 | | 1 | None |
| Down | 94 | None | 1:00 | 2.2 | | 1 | None |
| PCR | 94 | None | 0:20 | 4.4 | | 45 | Quantification |
| | 68 | Single | 1:00 | 2.2 | | | |
| Melting | 95 | None | 1:00 | 4.4 | | 1 | Melting Curves |
| | 60 | None | 0:30 | 1.5 | | | |
| | 95 | Continuous | | 0.11 | 10 | | |
| Cool | 37 | None | 0:10 | 1.5 | | 1 | |

384-well plates were used. To minimize the influence of bias, three replicates of each sample were performed and for the main assay, the replicates were performed on different days. The replicates were located on different plates and were arranged in different orders.

Each plate contains samples, which were tested for all six genes. Hence, this assay followed the gene maximization method (Derveaux *et al.*). The different genes were also rearranged for each replication plate. For each sample, 4 µl of diluted cDNA, 1 µl of forward and reverse primer, 4 µl of RNase free water and 10 µl of SYBR Green I Mastermix were used. RNase free water and the SYBR Green I Mastermix were used from the Light Cycler 480 SYBR Green I Master kit (Roche).

## 4.8 Statistical analysis

### 4.8.1 PCR efficiency calculation by LinReg PCR

The approximate equality of the amplification efficiency of target and reference genes during the qRT-PCR is required to make the RNA concentrations of target and reference genes comparable (Bustin *et al.*, 2009), (Livak, Schmittgen, 2001). Amplification efficiency can either be determined by doing a serial dilution of a sample, or by using a program, which "associates an efficiency value with each PCR reaction" (Karlen *et al.*, 2007). Amplification efficiency values should range from 1.8 to 2.0 (Ramakers *et al.*, 2003a). Here, the amplification efficiencies of the immune genes and the reference genes L13 and Ubiquitin were calculated via the software LinReg PCR (Karlen *et al.*, 2007). First, the raw Light Cycler amplification data was converted into an Excel file with the help of the software LC480 Converter (http://HFRC.nl). After that, the efficiencies were calculated via the software LinReg PCR (http://HFRC.nl) by plotting the Ct-values of each sample against the number of cycles on a logarithmic scale. A linear regression was performed to determine the efficiency of each reaction (Karlen *et al.*, 2007). Using LinReg PCR is a common method to estimate the efficiencies of a quantitative Real Time PCR assay accurately (Karlen *et al.*, 2007).

### 4.8.2 Normalization of gene expression data using qBase Plus

Before doing statistical analysis, the raw gene expression data was handled with the software qBase Plus (Hellemans *et al.*, 2007), (www.biogazelle.com). This software provides tools for normalization of quantitative Real Time PCR data (Hellemans *et al.*, 2007). It takes into account multiple reference genes and corrects gene specific amplification efficiencies. Apart from that, a quality control can be done by calculating the reference target stability.

For each sample, the mean value of the three replicates was calculated. Gene specific amplification efficiencies, which were determined via the software LinReg, were added to the qBase Plus data sets. After that, a normalization was performed by transforming the differences in the Ct-values of target and reference genes into relative quantities (Hellemans *et al.*, 2007). The model is based on the $\Delta\Delta$Ct-method, developed by Michael W. Pfaffl (Pfaffl, 2001), but it takes into account more than one reference gene.

The use of more than one reference gene also implies the evaluation of the stability of these genes. The coefficient of variation (CV-value) acts as an indicator for the expression stability of a gene. Values should be lower than 25% for homogeneous data and lower than 50% for heterogeneous data, respectively (Hellemans *et al.*, 2007). The M-value is another value to detect the most stable reference gene. It is derived from the software GeNorm (Etschmann *et al.*, 2006) and represents the variation of the expression of a gene compared to all other candidate genes. The variation should be lower than a value of 0.5 for homogeneous data and lower than 1 for heterogeneous data, respectively.

### 4.8.3 Descriptive statistics

The Shapiro-Wilk test is used to check for normality of variables. Here, the Shapiro-Wilk test was performed before doing the reference gene stability test and the permutational multivariate analysis of variance. Gene expression values of L13, Ubiquitin and the immune genes of different organs, the total immunoglobulin values and the values of the splenosomatic and hepatosomatic indices were examined for normality.

The homoscedasticity was analysed with the help of Levene's test. This test determines the homogeneity of variances of different groups. The variables, which were used to do the Shapiro-Wilk test, were also used to do the Levene's test. The Shapiro-Wilk test and the Levene's test examine the requirements of parametric tests: normality and homoscedasticity.

An independent samples t-test was performed to analyze the reference gene stability. This test examines differences in means between two groups. Here, the mean values of the gene expression of L13 and Ubiquitin in the organs head kidney, gut, kidney and spleen were compared. The mean expression values of individuals, carrying either two or four different MHC IIB alleles, were compared.

Differences between a dependent variable and groups of a factor are determined with the help of the Kruskal-Wallis test. This test is based on the principle of an ANOVA, but it is used for non-parametric data (Dytham, 2003). Here, it was used to examine the influence of one factor (for example the family of origin) on a dependent variable (for example the total immunoglobulin value). For parametric data, a one-way ANOVA is performed. Here, the influence of one factor on one dependent variable was determined.

The relationship between two categorical variables is determined with the help of a Chi-square test. Differences between expected and observed frequencies are analyzed with the help of this test (Dytham, 2003). Here, the influence of the family of origin on the number of different MHC IIB alleles was determined. All these statistical tests were done either with the statistical software SPSS (PASW Statistics 18) or with the software R 2.11.1.

Correlations between two continuous variables were done with the help of Spearman's correlation for non-parametric data. Here, a correlation between the gene expression values of Interleukin 1 beta and MHC II was analyzed. I used a level of significance of 5% ($p = 0.05$) for all statistical tests, to reject the null-hypothesis.

### 4.8.4 Permutational multivariate analysis of variance (permutational MANOVA)

The permutational multivariate analysis of variance is based on the principle of an ANOVA, taking into account multiple variables, combined with the process of permutation (Anderson, 2001). This approach has been successfully applied to expression data (Zapala, Schork, 2006). A raw data matrix, containing each individual and its values of the different dependent variables, is converted into a dissimilarity matrix. A pair wise comparison of distances among the variables of individuals within the same group and those of different groups is done. Here, the different groups are composed of individuals of the three-spined stickleback *Gasterosteus aculeatus,* who contain either two or four different MHC IIB alleles. Hence, the number of different MHC IIB alleles is assumed to be the grouping factor. For the model, the number of MHC IIB alleles, family of origin, tank, sex and dissection day were used as potentially influencing factors and the gene expression values, total immunoglobulin value and splenosomatic and hepatosomatic indices as dependent variables. Following the null-hypothesis, there should be no reduction of the total variance compared to the resulting variances, when individuals were partitioned into two groups, based on the number of different MHC IIB alleles. The permutational multivariate analysis of variance is a non-parametric test and the only assumption is that all observations are exchangeable under the null-hypothesis. The second part of the permutational multivariate analysis of variance is the process of permutation. This is a test for the assessment of statistical significance of the multivariate analysis of variance (Zapala, Schork, 2006). The higher the number of permutations, the higher the precision of the p-value (Anderson, 2001). If groups do not really differ from each other, it should be possible to randomly shuffle the rows of the dissimilarity matrix. This is what a permutation test does: it randomly combines and recalculates possible rearrangements of the rows. 1000 replicate permutations were used in this analysis.

The permutational multivariate analysis of variance was performed with the statistical software R 2.11.1. The dissimilarity matrix was created with the amap-package (Dist(x, method="pearson")). The pearson-correlation is recommended to analyze gene expression data (D'Haeseleer, 2005). The permutational multivariate analysis of variance was done with the vegan-package of R, especially the function "adonis" (adonis(y~A+B+C)). "y" represents the dissimilarity matrix and "A", "B" and "C" are different factors, which might influence the variables.

According to the family of origin, permutations were constrained to individuals within one family. This was done to exclude the variance, which arises due to the family of origin. The command “strata = family” was used within the function “adonis”. In conclusion, the permutational multivariate analysis of variance is a combination of analysing and partitioning variation according to an ANOVA, based on a dissimilarity measure, and providing a p-value due to the process of permutation. To locate the influence of different factors to certain dependent variables, the coefficients have to be checked. A coefficient is a constant and it is a measurement for the strength and direction of the effect, which a factor has on a dependent variable. According to the hypothesis of this study, influences, which might be caused by the number of MHC IIB alleles, were checked. Therefore, the extreme values of the measured variables were checked. Certain individuals with extreme coefficient values of the influenced dependent variables were identified by checking the resulting data from qBase Plus. Fish with extremely high and low expression values were selected. These individuals can be used in the histological assay.

## 5. Results

## A. Previous requirements for the actual data analysis

### 5.1 MHC class IIB-Genotyping

Three-spined sticklebacks with two and four different MHC class IIB alleles on exon 2 are needed to define the two assay groups of this study. Table 5.1.1 presents the possible haplotype combinations in the selected five families. All haplotypes in this experiment are composed of two linked MHC IIB alleles. More detailed information about all individual haplotype combinations of parental fish of the 14 different families and the offspring of the five selected families is shown in the supplement in part 5.1.

Tab. 5.1.1: Possible MHC class IIB haplotype combinations on exon 2 of the offspring

| family | haplotype combination 1 | haplotype combination 2 | haplotype combination 3 | haplotype combination 4 |
|---|---|---|---|---|
| L006 x L106 | No18, No13 | No18, No13, No08, SCX15 | | |
| L012 x L119 | No15, No16 | No07, No31 | No15, No16, No07, No31 | No15, No16, No07, No31 |
| L001 x L103 | No01, No12 | No01, No12, No15, No16 | No13, No18, No01, No12 | No13, No18, No15, No16 |
| L002 x L106 | No13, No18 | No13, No18, No16, No15 | No13, No18, No08, SCX15 | No16, No15, No08, SCX15 |
| L013 x L128 | No13, No18 | No13, No18, No15, No16 | No18, No13, No07, No31 | No15, No16, No07, No31 |

Table 5.1.1 shows five available families, which achieve the requirement of the two assay groups. The best ones are presented by family L006 x L106 and L012 x L119. Choosing fish randomly resulted in a chance of 50% to catch individuals with either two or four different MHC IIB alleles in these families. The female L106 was used in two different families. The RSCA-Genotyping of the offspring resulted in 118 fish, containing 66 females and 52 males. The different assay groups contain 45 fish with two and 73 fish with four different MHC IIB alleles. 27 fish originate from family L001 x L103. Family L002 x L106 is represented by 25 fish. Family L013 x L128 includes 23 examined fish and 18 fish are derived from family L012 x L119. Family L006 x L106 is represented by 25 fish. All possible haplotype combinations, presented in table 5.1.1, are found in the offspring.

5.2 Examination of inflammatory genes by PCR and DNA sequencing

Inflammatory genes, which are associated with autoimmune diseases in humans, were identified in the genome of the three-spined stickleback *Gasterosteus aculeatus*. Different combinations of forward and reverse primers were designed, tested via standard PCR and Agarose gel electrophoresis, sequenced and finally checked via qRT-PCR. Table 5.2.1 shows the resulting primer combinations.

Tab. 5.2.1: Inflammatory genes and their primer combinations (for primer sequences see methods part 4.4, Tab.4.4; "-" = no possible primer combination detected)

| **Gene** | **forward primer** | **reverse primer** |
|---|---|---|
| Migration Inhibiting Factor (MIF) | GA_MIF_F_3 | GA_MIF_R_3 |
| | GA_MIF1_F | GA_MIF1_R |
| Interleukin 4 (IL 4) | - | - |
| Interleukin 21 (IL 21) | - | - |
| Interleukin 10 (IL 10) | - | - |
| Interleukin 12 p40 (IL12) | - | - |
| Interleukin 12 p35 (IL12) | - | - |
| Interleukin 1 beta (IL 1 beta) | IL1_beta_F2 | IL1_beta_R1 |
| Immunoglobulin M (IgM) | IgM_For | IgM_Rev |

The melting curve analysis is a control for primer specificity (Bustin *et al.*, 2009). After performing the qRT-PCR, the amplification product is heated until the double-stranded DNA denatures. One clear peak at a certain temperature indicates the presence of just one amplification product. Two possible primer combinations were detected for the Migration Inhibiting Factor (MIF). The primers GA_MIF_F_3 and GA_MIF_R_3 bound to the examined cDNA samples and showed gel bands of the expected size. The melting curve analysis of the MIF gene showed a double peak. This might be due to a duplication in the MIF gene during evolution. Another primer combination (GA_MIF1_F / GA_MIF_1_R) was designed for one of the possible duplicates and showed gel bands at a size of 309 bp. The data was supported by direct sequencing. The resulting nucleotide sequence was compared with the one, which already exists from Genbank, by using multiple sequence alignment (Clustal W) and the Basic Local Alignment Search Tool (BLAST).

The following figures show the melting curve analyses of the MIF gene, which were detected with the primer combination GA_MIF_F_3 / GA_MIF_R_3 (figure 5.2.1) and the specific primer combination GA_MIF1_F / GA_MIF_1_R (figure 5.2.2).

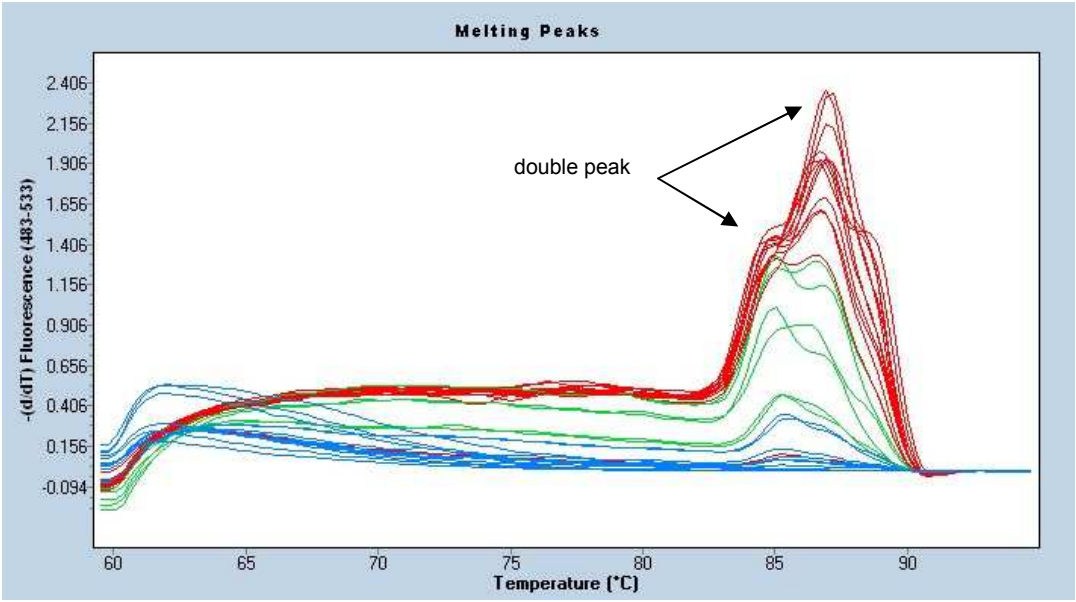


Fig. 5.2.1: Melting curve analysis of MIF in all four organs (blue lines = negative control, red / green lines = cDNA samples), primer combination GA_MIF_F_3 / GA_MIF_R_3

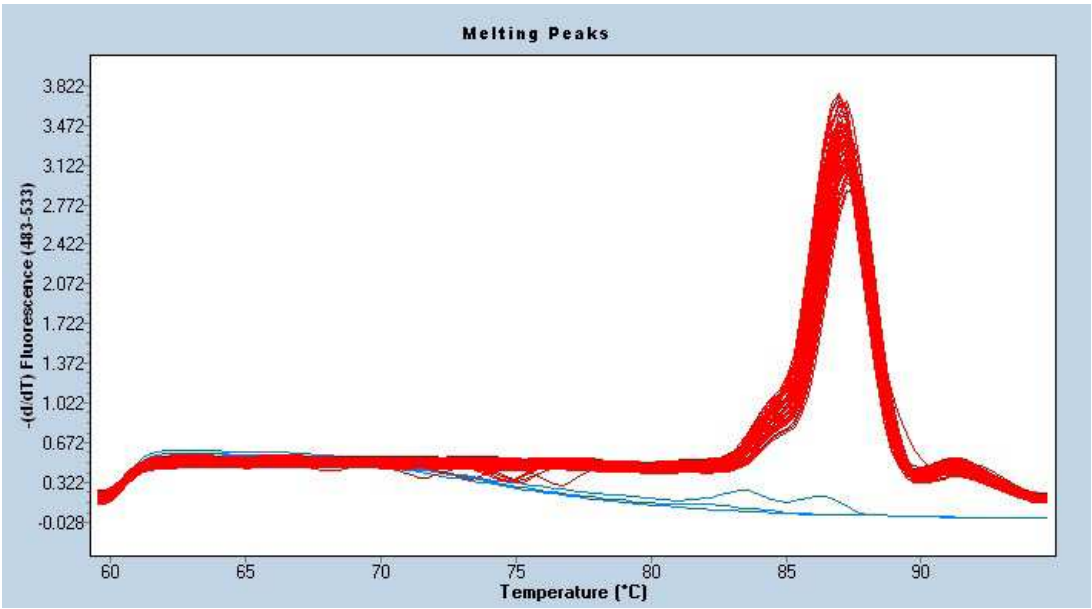


Fig. 5.2.2: Melting curve analysis of MIF in the kidney (blue lines = negative control; red lines = cDNA samples), primer combination GA_MIF1_F / GA_MIF_1_R

The specific primer combination GA_MIF1_F / GA_MIF_1_R was used for the gene expression assay.

Products of Interleukin 1 beta were detected in the genome of the three-spined stickleback at a size of 126 bp and supported by direct sequencing of these PCR products. Figure 5.2.3 shows an example for the melting curve analysis of Interleukin 1 beta in the kidney.

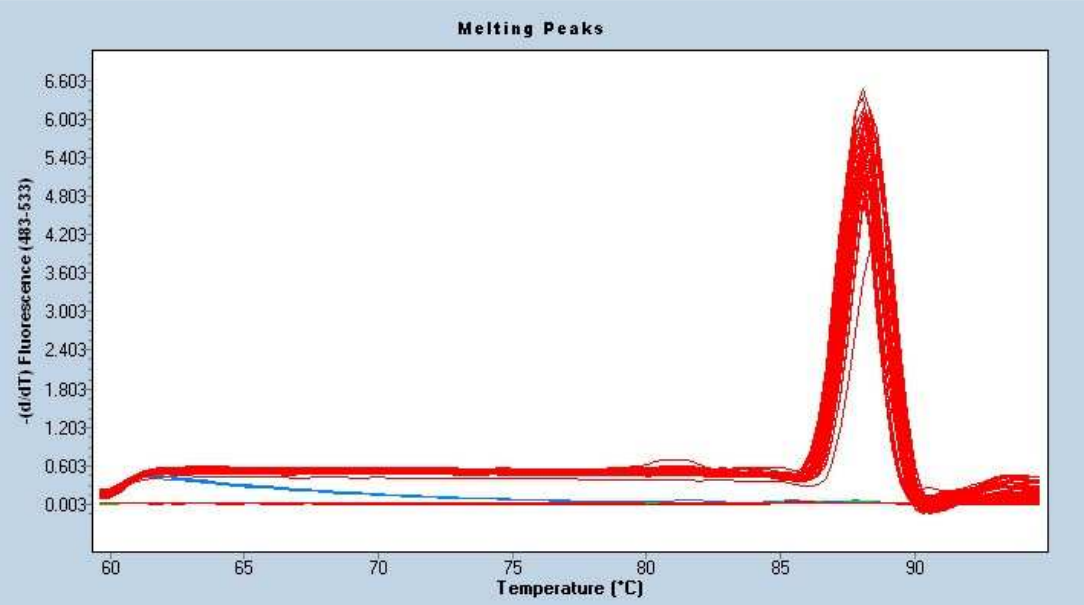


Fig. 5.2.3 : Melting curve analysis of Interleukin 1 beta in the kidney
(blue lines = negative control; red lines = cDNA samples)

No possible primer combination was detected for Interleukin 4 because of missing PCR products on an Agarose gel and so it is for Interleukin 12 p40 and Interleukin 12 p35. These primers, taken from *Takifugu Rubripes,* also did not show a product in the cDNA of *Gasterosteus aculeatus*. The PCR products of Interleukin 21 and Interleukin 10 showed gel bands, but not at the expected size. The gel bands were still cut out and the extracted DNA was used for direct sequencing to check, whether the right genes can be determined. But there were no satisfying results for Interleukin 10 and Interleukin 21 because of missing congruence of the PCR products with the genome sequence of the three-spined stickleback *Gasterosteus aculeatus*. The PCR products of Immunoglobulin M, with the primer combination, designed and generously provided by Sascha Hibbeler, were of the expected size of 200 bp.

5.3 RNA purity test

Five RNA samples of head kidney and gut each were checked for contamination with DNA. Genomic DNA from tail fin was used as a positive control. Figure 5.3 shows the resulting Agarose gel picture of this test. Two of the three genomic DNA samples show PCR products on an Agarose gel at the expected size of about 500 bp. RNA samples should not show any gel bands. In fact, no gel bands were observed for the RNA samples of the head kidney, but the gut samples show unspecific bands of a low fragment size, which may be degraded RNA.


Fig. 5.3: Agarose gel for DNA-contamination test

(1.slot: 100 bp DNA ladder, 2.- 6. slot: RNA samples of the head kidney,

7.-11. slot: RNA samples of the gut, 12.-14. slot: genomic DNA samples of the tail fin)

5.4 Quantitative Real Time PCR efficiency

The similarity of amplification efficiencies is an important requirement for the qRT-PCR assay. It enables a comparison of mRNA concentrations of target and reference genes (Bustin *et al.*, 2009). To ensure the approximate equality of efficiency of target and reference genes, the amplification efficiencies were calculated via the software LinReg PCR (http://HFRC.nl). This was done by plotting the Ct-values of each sample on a logarithmic scale. After that, a linear regression was performed (Karlen *et al.*, 2007).

For this, cDNA of head kidney, gut, kidney and spleen of three individuals was examined. Different primer combinations for the target genes were used in concentrations of 1:5, 1:10 and 1:20. cDNA was diluted 1:10 and 1:100. This was done to determine the concentration of primer and cDNA of the different target genes, which is needed for the gene expression assay. The results show that cDNA should be used in a concentration of 1:10 and for primers a 1:5 dilution is ideal. A lower concentration causes an amplification, which starts within the last five cycles of the qRT-PCR. This is too late, to give reliable conclusions about the amplification of a certain gene. The following table shows the efficiencies of the examined genes in the four different organs.

Tab. 5.4: qRT-PCR efficiencies of the target and reference genes, determined by LinReg PCR

| organ | MHC II | MIF | IgM | IL 1 beta | L 13 | Ubiquitin |
|---|---|---|---|---|---|---|
| head kidney | 1.903 | 1.858 | 1.88 | 1.817 | 1.872 | 1.883 |
| spleen | 1.919 | 1.834 | 1.915 | 1.828 | 1.902 | 1.913 |
| gut | 1.949 | 1.863 | 1.899 | 1.843 | 1.928 | 1.9 |
| kidney | 1.902 | 1.867 | 1.898 | 1.832 | 1.897 | 1.895 |

The amplification efficiencies should contain values between 1.8 and 2.1 to make the expression of different genes comparable (Pfaffl, 2001), (Bustin *et al.*, 2009). Table 5.4 shows, that all efficiency values achieve this assumption.

5.5 Stability of reference gene expression

A stable expression of the reference genes L13 and Ubiquitin had to be checked for the examined organs. This was done to ensure a comparison of the resulting Ct-values of target and reference genes. For this, 20 individuals of each assay group, containing either two or four different MHC IIB alleles, were used. The expression of L13 and Ubiquitin was measured in the organs head kidney, gut, kidney and spleen. After an equalization of the cDNA to 100 ng/µl, a qRT-PCR was performed, according to the description in part 4.7.4. Three replicates were done for each sample and the replicates were located on different plates. For each sample, the mean Ct-value of the replicates was calculated. The data were checked for normality via the Shapiro-Wilk test. The Levene's test was used to determine homoscedasticity. An independent samples t-test was performed to analyze the similarity of the mean gene expression values of either L13 or Ubiquitin between the two study groups. Each organ was analyzed separately. The following table shows the results of these tests.

Tab. 5.5.1: Results of the independent samples t-test for reference gene expression stability

| **organ** | **gene** | **Shapiro-Wilk test (p-value)** | **Levene's test (p-value)** | **Independent samples t-test (t-value)** | **Independent samples t-test (p-value)** |
|---|---|---|---|---|---|
| head kidney | L13 | 0.195 | 0.186 | -0.199 | 0.844 |
| | Ubiquitin | 0.067 | 0.145 | -0.063 | 0.950 |
| gut | L13 | 0.052 | 0.759 | -0.237 | 0.814 |
| | Ubiquitin | 0.569 | 0.732 | -0.062 | 0.951 |
| kidney | L13 | 0.737 | 0.251 | -0.737 | 0.455 |
| | Ubiquitin | 0.888 | 0.145 | -0.063 | 0.950 |
| spleen | L13 | 0.325 | 0.836 | -0.102 | 0.919 |
| | Ubiquitin | 0.136 | 0.939 | -0.118 | 0.907 |

The results of the Shapiro-Wilk test indicate normality for the data of the reference gene stability test and the Levene's test shows homoscedasticity of these data. Hence, the assumptions were achieved to make use of the independent samples t-test. The results of this test indicate that the null-hypothesis cannot be rejected for these data. The reference genes show similar gene expression levels in the four organs.

Individuals, containing two different MHC IIB alleles, show similar expression values of L13 in the gut, as the ones, who contain four different allele variants. The same goes for the expression of L13 in the other three organs. The results for Ubiquitin indicate similar expression patterns. The following two figures show these results.

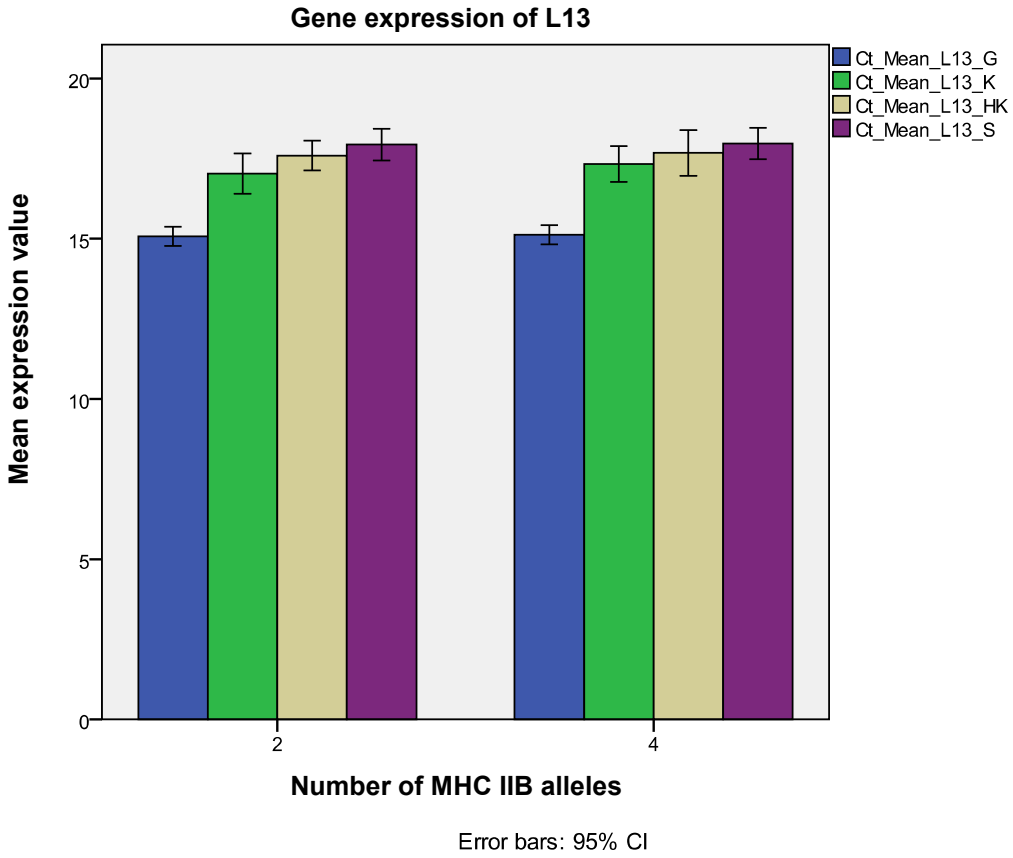


Fig. 5.5.1: Gene expression values of L13 in the four organs gut (G), kidney (K), head kidney (HK) and spleen (S), (x-axis = number of MHC IIB alleles, y-axis = mean Ct-value of expression of L13)

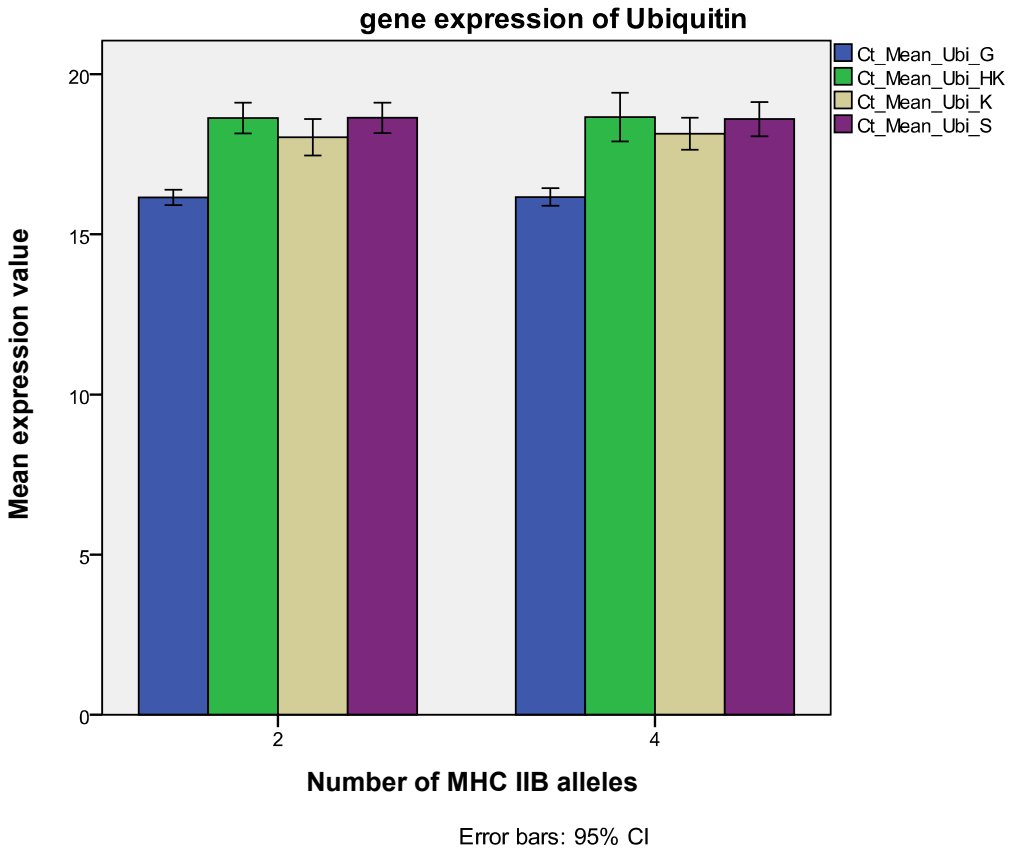


Fig. 5.5.2: Gene expression of Ubiquitin in the four organs gut (G), kidney (K), head kidney (HK) and spleen (S); (x-axis = number of MHC IIB alleles, y-axis = mean Ct-value of Ubiquitin)

The software qBase Plus also analyzed the stability of reference gene expression and the results confirm the use of L13 and Ubiquitin. The following table shows the resulting M- and CV-values for the gene expression of L13 and Ubiquitin in the four different organs gut, head kidney, kidney and spleen.

Tab. 5.5.2: Resulting M- and CV-values of the gene expression of L13 and Ubiquitin, qBase Plus

| **organ** | **L13** | | **Ubiquitin** | |
|---|---|---|---|---|
| | M-value | CV-value | M-value | CV-value |
| gut | 0.442 | 0.145 | 0.442 | 0.165 |
| head kidney | 0.391 | 0.140 | 0.391 | 0.134 |
| kidney | 0.274 | 0.088 | 0.274 | 0.106 |
| spleen | 0.309 | 0.104 | 0.309 | 0.113 |

As already described in the methods, the M- and CV-values should be lower than 0.5 and 0.25, respectively. Table 5.5.2 shows that the data achieves this assumption.

## B. Main statistical analysis

The normalization method enables comparisons of mRNA concentrations among different samples due to the control of variation, caused by extraction- and reverse transcription yield and amplification efficiency (Bustin *et al.*, 2009). The normalization of the raw quantitative Real Time PCR data of 111 individuals was done via the software qBase Plus. 111 individuals were used, because some of the 118 individuals miss data about the total immunoglobulin value or some organs are missing. The resulting data was used in the following statistical analysis.

### 5.6 Descriptive statistics

The normality of all measured variables, including body length, body weight, gene expression values, total immunoglobulin value, hepatosomatic and splenosomatic indices, was determined via the Shapiro-Wilk test. The following table was calculated with the help of SPSS (PASW Statistics 18).

Tab. 5.6.1: Results of the Shapiro-Wilk test for normality, red = p < 0.05

| | Shapiro-Wilk | | |
|---|---|---|---|
| | **Statistic** | **df** | **p-value** |
| body length (mm) | .971 | 111 | .017 |
| body weight (g) | .990 | 111 | .572 |
| immunoglobulin value | .889 | 111 | .000 |
| splenosomat. index | .691 | 111 | .000 |
| hepatosomat. index | .990 | 111 | .572 |
| IL 1 beta Gut | .400 | 111 | .000 |
| IgM Gut | .670 | 111 | .000 |
| MHC II Gut | .906 | 111 | .000 |
| MIF Gut | .905 | 111 | .000 |
| IL 1 beta Head kidney | .581 | 111 | .000 |
| IgM Head kidney | .740 | 111 | .000 |
| MHC II Head kidney | .928 | 111 | .000 |
| MIF Head kidney | .884 | 111 | .000 |
| IL 1 beta Kidney | .664 | 111 | .000 |
| IgM Kidney | .707 | 111 | .000 |
| MHC II Kidney | .986 | 111 | .322 |
| MIF Kidney | .971 | 111 | .017 |
| IL 1 beta Spleen | .848 | 111 | .000 |
| IgM Spleen | .679 | 111 | .000 |
| MHC II Spleen | .955 | 111 | .001 |
| MIF Spleen | .966 | 111 | .007 |

The results of the Shapiro-Wilk test indicate that almost every measured variable, except the body weight, hepatosomatic index and the gene expression value of MHC II in the kidney, is not normal distributed. Therefore, the use of the permutational multivariate analysis of variance as a non-parametric test is required.

## 5.7 Permutational multivariate analysis of variance

### 5.7.1 Main analysis

A permutational multivariate analysis of variance was performed, including the number of MHC IIB alleles on exon 2, the family of origin, sex, tank and the dissection day as influencing factors. Dependent variables are the different gene expression values of IgM, MIF, IL 1 beta and MHC II, the total immunoglobulin value and the hepatosomatic and splenosomatic indices. The data of all four organs was analyzed together and the command strata = family was used to exclude the variance, which is produced by the family of origin. The following table shows the intensity of influence of each factor on the dependent variables.

Tab. 5.7.1: Resulting p-values of the permutational MANOVA with strata = family, performed with R 2.11.1, red = $p < 0.05$

| **factor** | **p-value** |
|---|---|
| family | 0.000999 |
| sex | 0.045954 |
| tank | 0.369630 |
| dissection date | 0.000999 |
| number of MHC IIB alleles | 0.073926 |

The results of the permutational multivariate ANOVA indicate that the null-hypothesis can be rejected for the factors family, sex and dissection date. These factors might explain significant parts of the variance. The p-value of the number of MHC IIB alleles, shows a low probability to accept the null-hypothesis. This p-value of 0.074 might indicate a trend of the number of MHC IIB alleles to be also an influencing factor on the dependent variables. Influences, caused by the dissection date, might rather be due to external reasons than to a biological background. To avoid bias, all families were dissected over several days and several families were dissected per day. Differences in the lighting period or the feeding might be possible external reasons. Therefore, the influencing factor dissection date can be described as an artefact. It is described in more detail by plotting the values of the dependent variables against the different dates of dissection.

The resulting data indicate variation due to differences in gene expression values on different days. But there is no clear structure to allocate variation to one special day. Variation is shown among different days of dissection and also among organs and genes. The following two plots are examples for variation due to the dissection date. The remaining plots are shown in the supplement in part 5.2.

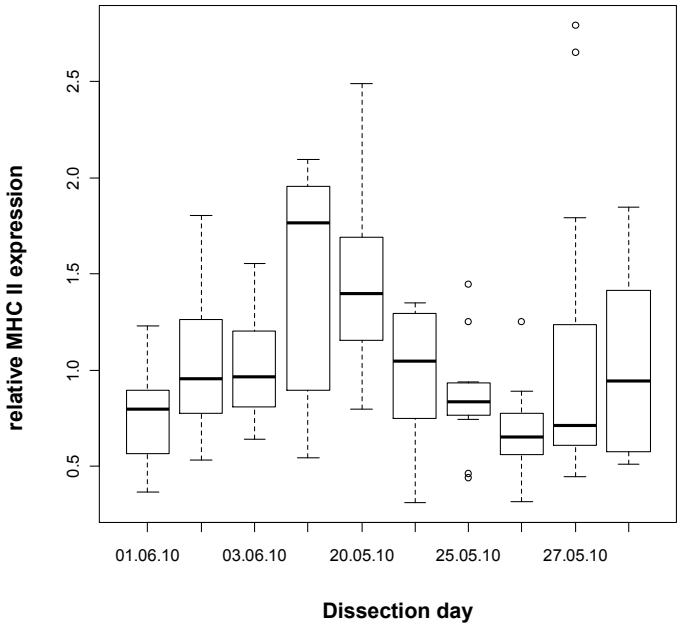


Fig. 5.7.1: Relative MHC II expression in the gut
according to the dissection date
(x-axis = dissection day, y-axis = expression value of MHC II)

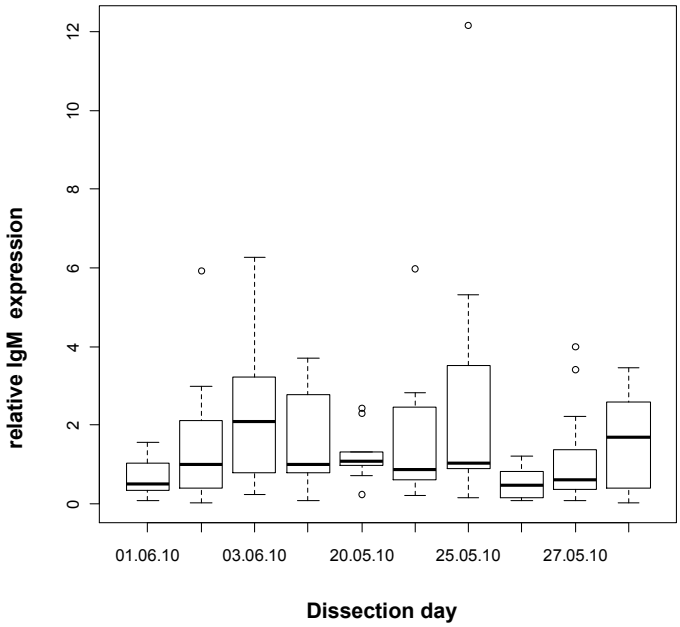


Fig. 5.7.2: Relative IgM expression in the kidney
according to the dissection date
(x-axis = dissection day, y-axis = expression value of IgM)

### 5.7.2 Permutational multivariate ANOVA according to the dissection day

A permutational MANOVA, containing a randomisation of the individuals within the dissection day, was performed to compensate for this artefact. The variance, which is produced by the dissection day, was excluded by using the command strata = dissection date within the function “adonis”. The following table shows the results of this analysis.

Tab. 5.7.2: Resulting p-values of the permutational MANOVA with strata = dissection.date, performed with R 2.11.1, red = $p < 0.05$

| factor | p-value |
|---|---|
| family | 0.02498 |
| sex | 0.03197 |
| tank | 0.45554 |
| number of MHC IIB alleles | 0.11688 |

The results indicate that the null-hypothesis cannot be rejected for the factors tank and number of MHC IIB alleles. But it can be rejected for the family of origin and sex.

### 5.7.3 Coefficients of the main analysis

As already described in the main analysis, the factors family, sex and dissection date might influence all dependent variables. To allocate the influence of these factors to certain dependent variables, the coefficients have to be checked. The coefficient gives an estimate for the strength and direction of the effect on a dependent variable. According to the hypothesis of this study, influences, which might be caused by the number of MHC IIB alleles, were checked. Therefore, the extreme values of the measured variables were checked. The coefficients of the permutational MANOVA indicate an influence on the total immunoglobulin value and the expression of Interleukin 1 beta and Immunoglobulin M. The following table shows the extreme values of the coefficients.

Tab. 5.7.3: Resulting influenced dependent variables and their coefficient values

| variable | coefficient value |
|---|---|
| immunoglobulin value | 0.123 |
| IL 1 beta Head kidney | 0.149 |
| IL 1 beta Gut | -0.289 |
| IgM Gut | -0.276 |
| IgM Kidney | -0.248 |
| IgM Head kidney | -0.211 |
| IgM Spleen | -0.305 |

The coefficients indicate that fish, containing four different MHC IIB allele variants, show higher total immunoglobulin values and expression values of Interleukin 1 beta in the head kidney than fish, which contain two different MHC IIB alleles. But Interleukin 1 beta and IgM are lower expressed in the gut (IL1 beta) and in all four organs (IgM) in individuals with four MHC IIB allele variants compared to those, containing two different MHC IIB alleles. Certain individuals with extreme coefficient values of the influenced dependent variables were identified by checking the expression values of Interleukin 1 beta and IgM and the total immunoglobulin value of each individual. Fish with extremely high and low expression values were selected. These individuals and their expression values of Interleukin 1 beta and immunoglobulin M and the values of the total immunoglobulins are shown in table 5.3.1 and 5.3.2 in part 5.3 of the supplement. These fish can be used to do the histological assay.

### 5.7.4 Gene expression analysis

One aim of this study is the examination of a possible effect of the number of MHC IIB alleles on the gene expression of certain immune genes. Therefore, a permutational multivariate analysis of variance was also done only for the gene expression values of IL 1 beta, IgM, MIF and MHC II as dependent variables. The following table shows the results of this analysis.

Tab. 5.7.4: Resulting p-values of the permutational MANOVA of the gene expression values with strata = family, performed with R 2.11.1, red = $p < 0.05$

| **factor** | **p-value** |
|---|---|
| family | 0.003996 |
| sex | 0.021978 |
| tank | 0.325674 |
| dissection date | 0.004995 |
| number of MHC IIB alleles | 0.040959 |

The results indicate the family of origin, sex, the dissection day and the number of MHC IIB alleles as influencing factors on the gene expression values. Now, the number of different MHC IIB alleles shows a lower p-value compared to the analysis, which includes all dependent variables. This significant difference might arise because of the omitted variables hepatosomatic and splenosomatic index and the total immunoglobulin value in this analysis. The coefficients indicate that the hepatosomatic and splenosomatic index are not as much influenced by the factors of the analysis as the total immunoglobulin value. This variable might explain the lower p-value.

According to the aim of this study, a heat map was designed. A heat map is a two-dimensional visualization, which represents gene expression values across a number of samples (Pryke, 2007). The values are shown as colours, whereas a high value is represented by a lighter colour and a small value by a darker colour (Pryke, 2007). In figure 5.7.3a, the individuals of the two assay groups were plotted against their expression values of the target genes IL 1 beta, IgM, MHC II and MIF in the four organs. Individuals, containing different numbers of MHC IIB alleles, are presented on the y-axis, while the gene expression values are located on the x-axis. Individuals, who contain two different alleles are located above the white line, while those, containing four alleles, are shown below this line. Low gene expression values are dark red, while high values appear yellow. The heat map was designed with the software Mathematica 7.0 (Wolfram Research, Champaign, IL). Figure 5.7.3a shows no clear structure of gene expression between the two study groups. The gene expression pattern of the individuals, who contain two different MHC IIB alleles, is not that different from the one of those fish, containing four different alleles. Maybe the influence of the factors family of origin and sex are stronger than those of the number of MHC IIB alleles. Therefore, these heat maps were also designed. The heat map, according to the different sexes (Fig. 5.7.3b), is not that different from the one, which separates the individuals according to the number of MHC IIB alleles. The gene expression pattern is similar. Hence, the strongest influence on the gene expression values might be due to the family of origin. Figure 5.7.3c shows this heat map. Compared to the other heat maps, this one shows the strongest differences in gene expression values between the individuals. Family L001 x L103, L012 x L119 and L013 x L128 show lower gene expression values than family L002 x L106 and family L006 x L106. The latter two families contain the same female. Maybe this female influences the expression values of the target genes.

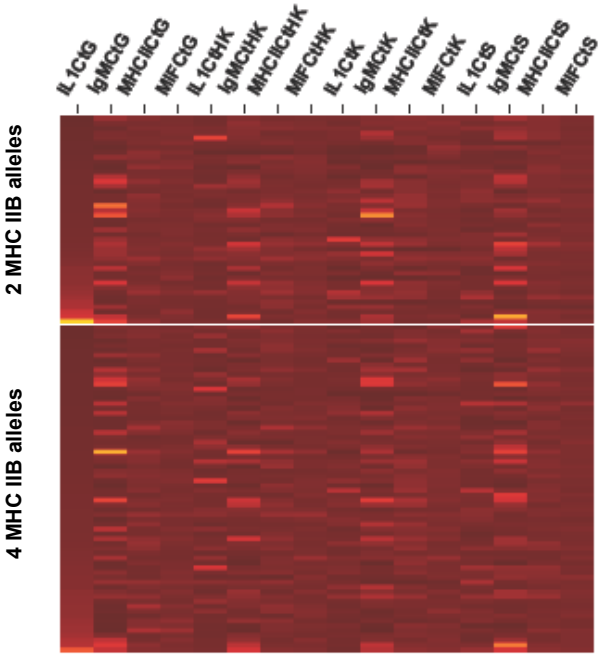


Fig. 5.7.3a: Heat map according to the number of MHC IIB alleles;
range: 0-1, 0 = dark red, 1 = yellow;
above the white line: individuals,
containing 2 different MHC IIB alleles,
below the white line: individuals,
containing 4 different MHC IIB alleles

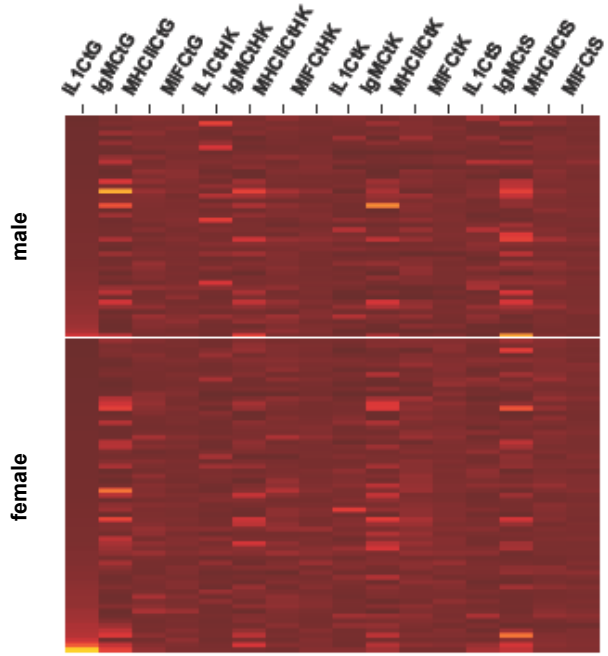


Fig. 5.7.3b: Heat map according to the sex;
range: 0-1, 0 = dark red, 1 = yellow;
above the white line: males
below the white line: females

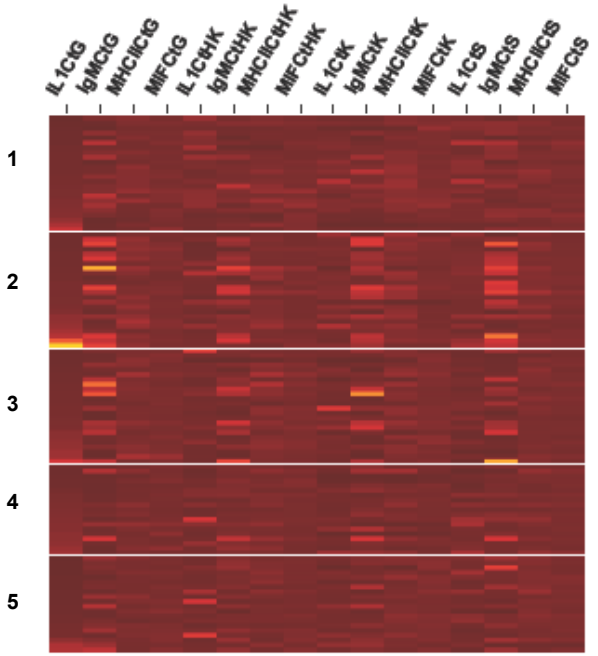


Fig. 5.7.3c: Heat map according to the family;
range: 0-1, 0 = dark red, 1 = yellow;
1 = L001 x L103, 2 = L002 x L106,
3 = L006 x L106, 4 = L012 x L119,
5 = L013 x L128

5.7.5 Permutational multivariate ANOVA for different organs

The main analysis of the permutational multivariate ANOVA takes into account the gene expression values of all four organs. But maybe there are differences between the organs. Therefore, the influence of the factors only on the expression of the different target genes in different organs was checked via a permutational multivariate ANOVA. Each organ was analyzed separately. Hence, the Bonferroni correction was used to neutralize the accumulation of Type I errors during multiple pair wise comparisons. The level of significance changed from 0.05 to 0.0125, due to a division of the previous p-value by the number of tests, which were performed (0.05 divided by 4). Table 5.7.5 shows the resulting influences.

Tab. 5.7.5: Resulting effect of factors on gene expression values in different organs, red = $p < 0.05$

| **organ** | **factor** | **p-value** |
|---|---|---|
| head kidney | number of MHC IIB alleles | 0.021 |
| | family | 0.657 |
| | sex | 0.125 |
| | tank | 0.205 |
| | dissection date | 0.415 |
| gut | number of MHC IIB alleles | 0.252 |
| | family | 0.255 |
| | sex | 0.288 |
| | tank | 0.207 |
| | dissection date | 0.017 |
| kidney | number of MHC IIB alleles | 0.768 |
| | family | 0.111 |
| | sex | 0.419 |
| | tank | 1.000 |
| | dissection date | 0.001 |
| spleen | number of MHC IIB alleles | 0.208 |
| | family | 0.121 |
| | sex | 0.823 |
| | tank | 0.985 |
| | dissection date | 0.208 |

The day of dissection is the only factor, which shows an effect on the gene expression values in the kidney (p = 0.001).

5.7.6 Correlation between number of MHC IIB alleles and the expression of MHC II

A negative correlation between the individual number of MHC IIB allele sequence variants and the MHC II expression was detected by Wegner et al. in 2006 (Wegner *et al.*, 2006). The MHC II expression in the spleen of parasitized fish, originating from six families, was examined. A higher expression of MHC II was observed in fish with a low MHC IIB allele diversity and supposed to compensate for a weaker immune system in these fish (Wegner *et al.*, 2006). Therefore, it is interesting to check, whether the number of MHC IIB alleles influences the MHC II expression in fish of this study. Hence, a Mann-Whitney-test was performed. The following table shows the results of the analysis.

Tab. 5.7.6: Resulting effect of the number of MHC IIB alleles on MHC II expression in different organs

| **organ** | **p-value** |
|---|---|
| gut | 0.937 |
| head kidney | 0.611 |
| kidney | 0.573 |
| spleen | 0.694 |

Containing either two or four different MHC IIB alleles does not seem to have an influence on the MHC II expression in different organs under the conditions of this study.

5.7.7 Correlation between expression of Interleukin 1 beta and MHC II

As already mentioned in the introduction, Interleukin 1 causes the increase of MHC class II molecules in antigen presenting cells during an inflammation. Hence, the correlation between the expression of Interleukin 1 beta and MHC II was checked via a Spearman's rank correlation, performed with R 2.11.1. The results indicate positive correlations in the organs head kidney, kidney and spleen. A strong positive correlation is shown within kidney and spleen. The correlation between the expression of Interleukin 1 beta and MHC II is shown by the Spearman's rank correlation coefficient rho by a value of 0.5885 (p < 2.2e-16) for the kidney and 0.1855 (p < 0 3.770e-05) for the spleen. The correlation between the expression of Interleukin 1 beta and MHC II in the head kidney is weaker (rho = 0.1855, p = 0.0514).The following figures represent the correlation of the expression of Interleukin 1 beta and MHC II in all four organs.

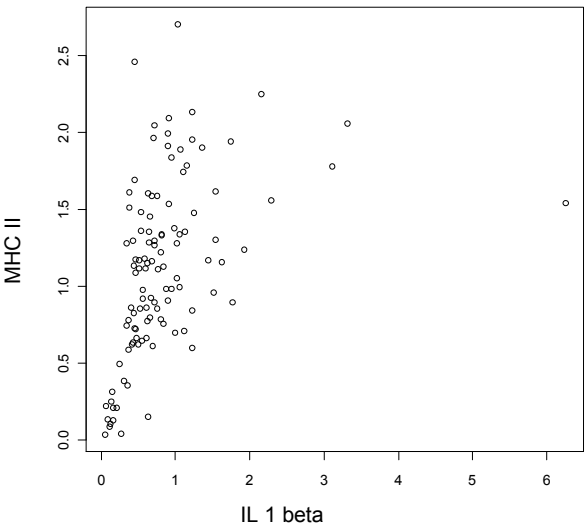


Fig. 5.7.5: Correlation between expression of Interleukin 1 beta and MHC II in the kidney, x-axis = expression of Interleukin 1 beta, y-axis = expression of MHC II

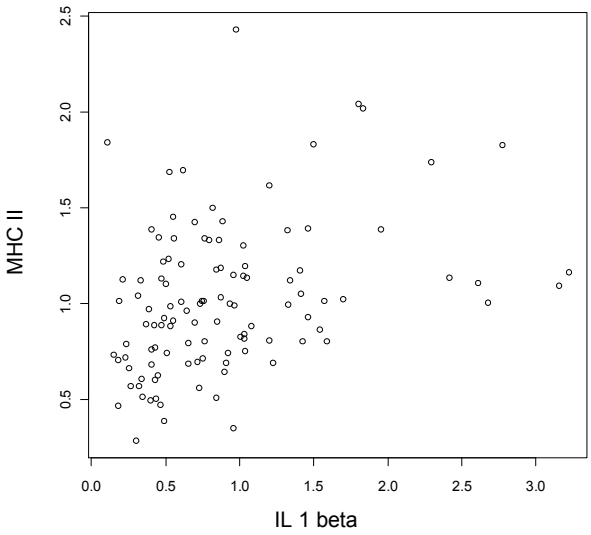


Fig. 5.7.6: Correlation between expression of Interleukin 1 beta and MHC II in the spleen, x-axis = expression of Interleukin 1 beta, y-axis = expression of MHC II

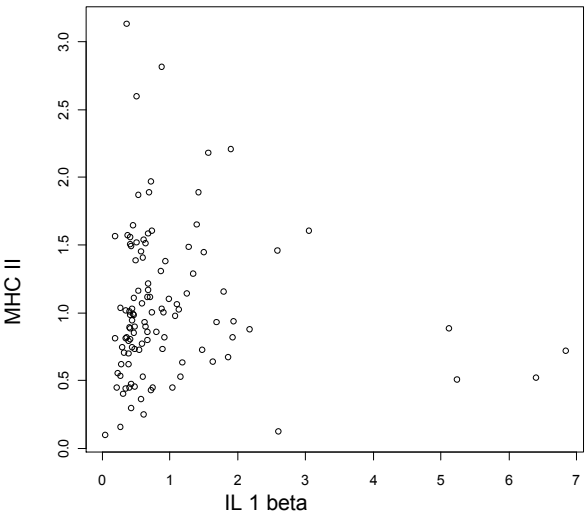


Fig. 5.7.7: Correlation between expression of Interleukin 1 beta and MHC II in the head kidney; x-axis = expression of Interleukin 1 beta, y-axis = expression of MHC II

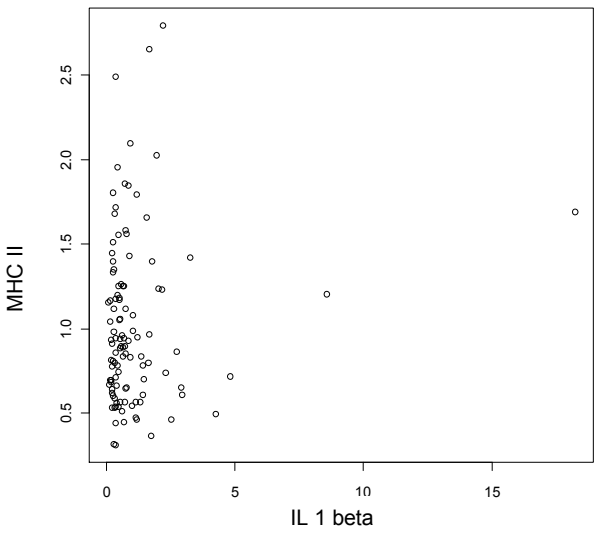


Fig. 5.7.8: Correlation between expression of Interleukin 1 beta and MHC II in the gut; x-axis = expression of Interleukin 1 beta, y-axis = expression of MHC II

## 6. Discussion

### 6.1 Methodological discussion

#### Examination of target genes – PCR and Sequencing

One aim of this study was the identification of the target genes MHC II, IL 1 beta, IL 4, IL 10, IL 12, IL 21, MIF and IgM in the genome of the three-spined stickleback *Gasterosteus aculeatus*. As mentioned in the results, IL 1 beta, MIF, IgM and MHC II could be detected.

Interleukin 1 beta has been characterised in the common carp and trout and it shows a similar intron-exon structure as the mammalian Interleukin 1 beta (Savan, Sakai, 2006). Therefore, it might be possible that Interleukin 1 beta is a highly conserved gene within teleost fish. Jin et al. cloned and identified homologues of the MIF gene in seven different teleost fish species like the pufferfish *Tetraodon nigroviridis*, the rainbow trout *Onchorhynchus mykiss*, the zebrafish *Danio rerio* and the Atlantic salmon *Salmo salar* (Jin *et al.*, 2007). Structure and organisation of teleost fish MIF genes are similar to other vertebrate MIF genes. They are composed of three exons and two introns. Apart from that, they are identical in length. This might be a hint for a high conservation of MIF genes during evolution. But phylogenetic analysis showed, that MIF genes of teleost fish describe an exclusive group compared to other vertebrate MIF genes (Jin *et al.*, 2007). The immunoglobulin isotypes IgM, IgD, IgZ and IgT have already been identified in bony fish. The locus of the immunoglobulin heavy chain gene of teleosts is similar to that of mammals (Ram *et al.*, 2005), (Danilova *et al.*, 2005). However, the organization of the immunoglobulin light chain gene of teleost fish shows an organization, which resembles the one of cartilaginous fish (Bao *et al.*). Therefore, teleost fish immunoglobulins might present an intermediate position between mammalian immunoglobulins and those of cartilaginous fish (Danilova *et al.*, 2005). Apart from that, the immunoglobulin heavy chain gene locus of *Gasterosteus aculeatus* shows a duplication, which is not known from any other bony fish species. This results in a diversity of immunoglobulin organization and might be a hint for a rapid evolution of immunoglobulin gene organization in teleost fish (Bao *et al.*). The stickleback IgM resembles the structure of already analyzed vertebrate IgMs (Bao *et al.*). However, the fish IgD conformation differs from human isotype and the fish specific IgZ and IgT have already been discovered in the zebrafish *Danio rerio*, the rainbow trout *Oncorhynchus mykiss* and the common carp *Cyprinus carpio* (Ram *et al.*, 2005). The primers for the MHC class II gene in the three-spined stickleback *Gasterosteus aculeatus* have already been designed and established by Thorsten Reusch (Reusch *et al.*, 2004).

It still remains unclear, whether the Interleukins 4, 10, 12 and 21 are present in the genome of the three-spined stickleback *Gasterosteus aculeatus*. Maybe the primers for these genes built secondary structures and thus were not able to hybridize with the cDNA template. Apart from that, several primer combinations might have formed primer dimers due to self-annealing. This could have resulted in unspecific products of low fragment sizes. This was observed for some primer combinations, resulting in a smear on the Agarose gel and unspecific amplification products during the qRT-PCR assay. Either no clear peak or a lot of different peaks occured in the melting curve analysis of Interleukin 10, for example. The analysis of direct sequencing showed fragments of short length for Interleukin 10 with either double peaks or no peaks. The reason for this might be suboptimal primer pairs.

Interleukin 4 has already been cloned and characterised in the pufferfish *Tetraodon nigroviridis* (Li *et al.*, 2007). It shows "the same organization as the mammalian and birds" Interleukin 4 and "consists of four exons and three introns" (Li *et al.*, 2007). This might be a hint for a high conservation of Interleukin 4. The primers, I used to isolate Interleukin 4 in the genome of the three-spined stickleback, were based on the genomes of the pufferfish *Tetraodon nigroviridis* and *Takifugu rubripes*. Maybe Interleukin 4 is more variable between the genomes of the pufferfish species and the three-spined stickleback. Hence, it seems to be difficult to detect Interleukin 4 in *Gasterosteus aculeatus*. Interleukin 10 has already been identified within teleosts like *Takifugu rubripes*, *Tetraodon nigroviridis*, *Cyprinus carpio* and *Onchorhynchus mykiss* (Bird *et al.*, 2006). In *Takifugu rubripes,* Interleukin 10 consists of five exons and four introns, similar to the human, mouse, carp, trout and chicken gene (Bird *et al.*, 2006). This seems to be a hint for a highly conserved gene (Zou *et al.*, 2003). The primers of this study were designed on the basis of the genomes of *Tetraodon nigroviridis*, *Takifugu rubripes* and *Danio rerio*. Apart from that, primers of the study by Zou et al. and some other primers, designed by Christophe Eizaguirre, were also tested to detect Interleukin 10. But none of them was successful in this study. The subunits IL 12 p40 and IL 12 p35 have already been identified in the Japanese pufferfish *Takifugu rubripes*. They showed conserved homology to Interleukin 12 subunits of mammals (Yoshiura *et al.*, 2003). They seem to be orthologues because of their "conserved linkage of several genes at specific human chromosomal regions" (Savan, Sakai, 2006). This might be a hint for conservation of Interleukin 12 within vertebrates. But nevertheless, the primers of the study of the Japanese pufferfish (Yoshiura *et al.*, 2003) did not amplify in the genome of the three-spined stickleback.

First evidence for the existence of Interleukin 21 homologues in bony fish species was given by Bird's study in the Japanese pufferfish *Takifugu rubripes* (Bird *et al.*, 2005). They discovered a conservation of gene organisation between the human and pufferfish Interleukin 21 during evolution. The primers, which I used in my study, were designed on the basis of the genome of *Takifugu rubripes*. But they were not successful in the genome of *Gasterosteus aculeatus*.

These results might indicate that Interleukins were not as conserved as expected from previous studies in teleost fish species. Maybe the conservation refers to a comparison of different vertebrate species like humans, chicken and teleost fish. But it might be possible that Interleukins are variable within different teleost species. Therefore, a comparison of the structure of Interleukins of different teleost species might be helpful to give assumptions about the conservation of these genes. After that, primers can be designed on the basis of other available genomes of teleost fish, which might be more similar to that of the three-spined stickleback. Apart from that, a gradient PCR can be done to optimise the annealing temperature of primers.

## 6.2 Interpretation of the results

### 6.2.1 Main statistical analysis – permutational multivariate ANOVA

A permutational multivariate analysis of variance was performed to determine the influence of different factors on several dependent variables, including the expression values of the target genes and the total immunoglobulin value of the plasma. The general aim of this study was the examination of a possible association between the MHC IIB allele diversity and certain immunological parameters, which might be correlated with auto aggressive reactions. The results of this analysis indicate the family of origin and sex as influencing factors. The influence of the dissection day is due to an artefact. Individuals of five different families were examined in this thesis. Maybe the different families contain diverse repertoires of immunological factors. Their adaptive immune systems might be composed of different immune cells in variable concentrations. This might be due to differences in the genetic background. Maybe the parental fish were infected with diverse parasite species. They reacted to this parasite load by modifying their adaptive immune system to optimize their immune response. This process could have resulted in different modifications between the families. These modifications might have been passed on to the next generation, resulting in different immune repertoires.

As already shown by Wegner et al. (Wegner *et al.*, 2006), different families of the three-spined stickleback showed differences in the expression of MHC class IIB after an exposure to three different kinds of parasites. These different expression patterns also existed in the next two generations (Wegner *et al.*, 2006). Hence, this demonstrates the genetic component of the immune repertoire. Infections, due to other microbial organisms like bacteria and viruses, might cause a similar effect. Maybe some of the fish families experienced defects in self tolerance mechanisms due to an infection in the past. This might have caused a mutation, which could have been passed on to the next generation. In humans, mutations can occur in genes, which are important for self tolerance regulation like Fas, AIRE and FoxP3, and change their functions (DeFranco, 2007). The Fas-gene is responsible for the deletion of auto reactive B-cells and FoxP3 acts as a transcriptional factor for regulatory T-lymphocytes, for example (DeFranco, 2007), (Janeway, 2005). Mutations might also have an effect on apoptosis, co-stimuli and cytokines (Janeway, 2005). Hence, this might also happen in teleost fish families and influence their repertoires of the adaptive immune system. These changes could be responsible for the strong influencing effect of the family of origin.

There is also the assumption, that autoimmunity is correlated with certain HLA- and MHC class II- and I molecules or with female sex hormones. The latter is due to the fact that autoimmune diseases occur in a proportion of 2:1 to 9:1 in a comparison of females and males (Ferencik, 2006). This assay assumes sex to be also an influencing factor on certain immunological parameters. It might be possible that female sex hormones of teleost fish are correlated with the occurrence of auto aggressive reactions. Maybe they are linked to certain immune genes and thus regulate the expression of these genes. An influence of the 11-Ketotestosterone on the innate immune system of male three-spined sticklebacks has already been shown (Kurtz *et al.*, 2007). Here, a hormone-implant experiment showed that a higher level of testosterone suppressed the immune function. It resulted in a decrease of respiratory burst upon phagocytosis. A negative correlation between 11-Ketotestosterone levels and the size of the head kidney has also been shown (Kurtz *et al.*, 2007). If differences in the occurrence of auto aggressive reactions between the different sexes also exists in the three-spined stickleback *Gasterosteus aculeatus*, it might be a possible explanation for the influence of sex in this assay.

The day of dissection also seems to affect the examined immunological parameters. The results indicate variation due to differences in gene expression on different days, but also among organs and genes. Therefore, it is not possible to allocate variation to one special day. To minimize the effect of bias, several families had been dissected per day and all families were dissected over several days.

A permutational multivariate ANOVA, including strata = dissection date, was done. Here, individuals were randomly shuffled only within the factor dissection day. The results indicate the family of origin to be the only influencing factor. The influence of the dissection day cannot be due to a biological reason, but it can be produced artificially. External reasons can occur due to changes in environmental conditions. Differences in the light period or feeding occurred during this assay, because of a mistake in the lighting system. Hence, the night-period was elongated. Unfamiliar noises, which might have occurred during the open house day, could have also influenced the fish. Too many fish per tank could have caused competition between individuals. This might have occurred, because two fish showed a missing tail and one fish missed an eye. For future assays, less fish per tank should be used to minimize stress due to competition between individuals. Apart from that, other environmental conditions like the lighting period and feeding should be kept more stable.

### 6.2.2 Gene expression assay

The main aim of this thesis was to estimate, whether different numbers of MHC IIB alleles influence the gene expression of Interleukin 1 beta, MIF, IgM and MHC II. The results of the permutational multivariate ANOVA indicate a trend according to the MHC IIB allele diversity because of a low p-value ($p = 0.074$). Hence, it might be possible that this factor also has an effect on certain immunological parameters. In humans, a correlation between autoimmunity and certain HLA- and MHC II molecules is known (Ferencik, 2006), (Todd *et al.*, 1988), (Wucherpfennig, Strominger, 1995), (Davies, 1994), (Nepom, Erlich, 1991). A high MHC allele diversity provides the ability to respond to many different kinds of antigens. But it also includes the risk to detect self antigens and respond to them. This can occur during a cross reaction in the molecular mimicry process (Martin, 2009). Hence, the probability to initiate an auto aggressive reaction may increase with a higher number of different MHC alleles. The resulting trend of a possible difference between the two assay groups, according to the number of MHC IIB alleles, might indicate this fact. Maybe molecular mimicry, which results in an auto aggressive reaction, might also arise in the adaptive immune system of the three-spined stickleback *Gasterosteus aculeatus*. This might be a possible limiting selection pressure for the MHC IIB allele diversity.

The results of the permutational multivariate ANOVA, which takes into account only the gene expression values, shows significant differences between the two assay groups due to the MHC IIB allele diversity ($p = 0.041$). The coefficients indicate the total immunoglobulin level and the expression of Interleukin 1 beta and IgM as the most influenced variables of this assay. Interleukin 1 beta is synthesized by lymphocytes. It belongs to the co-stimulatory signals during an inflammation (Böcker, 2004). Hence, it might show a high expression in the lymphatic organs head kidney and spleen.

In fact, the coefficients of the permutational multivariate ANOVA indicate that fish, containing four different MHC IIB alleles, show higher expression values of Interleukin 1 beta in the head kidney than those, who contain just two different alleles. But the expression of IL 1 beta in the gut is lower in individuals with four different alleles. Maybe the gut is not as important as the head kidney for defense against an inflammation. It might also be possible that IL 1 beta has not been activated yet or it has been inhibited by other cytokines. Interleukin 10 inhibits the production of Interleukin 1 beta, for example (Male, 2005). A study about Interleukin 1 in the rainbow trout examined gene expression values in lymphoid tissues with the help of quantitative Real Time PCR (Secombes *et al.*, 1999). The results showed no essential expression. But expression could be increased, if the head kidney was stimulated with LPS (Secombes *et al.*, 1999). This might be a hint for the importance of Interleukin 1 in fish immune response. It has been shown that IL 1 beta might be responsible for an increase in antibody titres in the carp (Savan, Sakai, 2006). In *Gasterosteus aculeatus*, the total antibody level of the plasma shows higher values in individuals, containing four different MHC IIB alleles. Hence, the higher expression of Interleukin 1 beta in the head kidney might be associated with this increase. Interleukin 1 beta causes an increase of MHC II expression in antigen presenting cells (APCs) during an inflammation (Böcker, 2004). This positive correlation has been shown for the organs head kidney, kidney and spleen of this study. A strong correlation is detected within kidney and spleen. For the spleen, this might be due to its immune response against blood-antigens (Martin, 2009). Maybe the high antibody titre of the plasma indicates the presence of an immune reaction. This could be due to an auto aggressive reaction. Therefore, IL 1 beta might be activated in the spleen and increases the production of MHC II in APCs to detect more different kinds of antigens. The Migration-Inhibiting Factor (MIF) is secreted by T-lymphocytes and it is activated during chronical inflammations (Male, 2005). The coefficients of the permutational multivariate ANOVA indicate that the expression of MIF does not seem to be influenced by the number of MHC IIB alleles. Maybe MIF is not expressed in the organs head kidney, gut, kidney and spleen. It might be possible that its role in teleost fish is not as important as in humans during inflammations. Jin et al. assumed a high conservation of the MIF gene (Jin *et al.*, 2007). But they also pointed out that the MIF of teleost fish describes an exclusive group compared to other vertebrate MIF genes. Therefore, the function of the teleost MIF gene might be different from that of other vertebrates. This might explain the low expression values in the examined organs. Jin et al. also performed expression assays of MIF in the pufferfish *Tetraodon nigroviridis* (Jin *et al.*, 2007). In healthy fish, gills, gonads, heart and brain showed higher expression levels than liver, skin, muscle, intestine, spleen and head kidney. But expression levels caould be increased in the lymphatic organs head kidney and spleen after stimulation with LPS (Jin *et al.*, 2007).

This might be a hint that MIF plays a role in the adaptive immune response of teleost fish. But maybe it needs a stimulus to be activated. Another explanation for the gene expression results of the MIF gene might be associated with the possible duplication event in the gene. The melting curve analysis of this study indicated two loci for the MIF gene in *Gasterosteus aculeatus*. Primers were designed for one of these loci and maybe this locus has already lost or changed its function in the adaptive immune system. Therefore, it might be helpful to design primers for the other locus and check its importance for gene expression. The immunoglobulin M is one of three different fish antibodies (Bird *et al.*, 2006). It mainly exists in the blood and is an indicator for an acute inflammation (Martin, 2009), (Wilson *et al.*, 1997). Immunoglobulin genes also exist in B-lymphocytes, where they encode antibodies (Warr). Hence, an up-regulation during an inflammation might be possible in the lymphatic organs. The results of the permutational multivariate ANOVA indicate that the total immunoglobulin level is one of the influenced variables. A down-regulation of IgM is shown in individuals, who contain four different MHC IIB alleles. But the results of the ELISA show a high immunoglobulin level in the blood of fish with four different alleles compared to those, who contain two different alleles. Maybe because of this high blood antibody titre, IgM was already produced by the B-lymphocytes of the lymphatic organs and released to the blood. Hence, the expression of IgM might still have been down-regulated during the gene expression assay. This might explain the low gene expression results for IgM in head kidney, spleen, kidney and gut and the high blood antibody level in individuals with four different alleles. MHC class II genes are expressed in cells of the immune system, for example APCs and B-lymphocytes. MHC II is a polygenic and polymorphic gene (Martin, 2009). This MHC diversity results in the detection and presentation of a wide spectrum of antigens. But it also increases the possibility to detect self-antigens and label them as foreign ones. Therefore, a high expression of MHC II might enhance the chance of the occurrence of auto aggressive reactions. Here, the MHC II expression might show higher values in individuals with four different MHC IIB alleles. However, the results of the permutational multivariate ANOVA show no significant differences in the MHC II expression, according to the number of MHC IIB alleles. The number of different MHC IIB alleles does not seem to influence the expression of MHC II in different organs of this study. Maybe the expression of MHC II is influenced by a cytokine. As already mentioned in the introduction, IL 10 inhibits MHC II molecules (Martin, 2009). This suppressive effect reduces the antigen presentation in dendritic cells.

## 6.3 Final conclusions

This diploma thesis is a pilot study about a possible association between the genetic diversity of MHC IIB and certain immunological parameters, which might be correlated with auto aggressive reactions. These auto aggressive reactions are assumed to be a limiting selection pressure for an increasing MHC IIB allele diversity. I used different methods to examine multiple aspects of this topic. I tried to discover and examine the expression of several inflammatory genes in the genome of the three-spined stickleback, which are involved in human autoimmune diseases. Apart from that, I checked the total antibody titer of the plasma. This might be a hint for the activation of the adaptive immune system.

I managed to identify the cytokines Interleukin 1 beta, MIF, the immunoglobulin M and MHC II in the genome of *Gasterosteus aculeatus*. Interleukin 1 beta seems to be highly conserved within teleost fish (Savan, Sakai, 2006) and the results of the expression assay indicate that IL 1 beta is influenced by the number of MHC IIB alleles. Interleukin 1 beta seems to be an important component of the adaptive immune response in *Gasterosteus aculeatus* because of its higher expression in the head kidney of individuals with four MHC IIB alleles. Apart from that, a positive correlation of IL 1 beta- and MHC II expression is shown in head kidney, spleen and kidney. There might also be an association between an increased antibody titer in the blood and the expression of IL 1 beta. On the one hand, MIF genes seem to be highly conserved within vertebrate species (Jin *et al.*, 2007). But on the other hand, teleost fish present an exclusive group (Jin *et al.*, 2007). This outstanding position might be due to a possible duplication event in the gene, which could be observed in the melting curve analysis. Locus-specific primer design might explain the low expression of the MIF gene. Maybe the examined locus has already lost or changed its function. Hence, it might be not that important anymore in the adaptive immune response. The up-regulation of the total immunoglobulin level in the plasma of fish with four different MHC IIB alleles might indicate an acute inflammation (Martin, 2009). The fish are free from macro parasites and should not be infected. Therefore, the reason for this up-regulation might be due to auto aggressive reactions of the adaptive immune system.

These results about the changes of immunological parameters, associated with the number of MHC IIB alleles, might be hints for auto aggressive reactions in the three-spined stickleback *Gasterosteus aculeatus*. But the examination of only four possible immune genes does not give enough evidence for this assumption. Much more work has to be done to find evidence for the importance of auto aggressive reactions as a limiting selection pressure for the individual MHC IIB allele diversity of the three-spined stickleback.

## 6.4 Future prospects

This pilot study shows interesting results about the influence of the family of origin and sex on the role of certain inflammatory genes. These inflammatory genes might be associated with auto aggressive reactions as a possible limiting selective force for the MHC IIB allele diversity of the three-spined stickleback. Apart from that, the low p-value might indicate a trend of the number of MHC IIB alleles to be also an influencing factor. Differences in the occurrence of autoimmune diseases due to female sex hormones are assumed for humans (Ferencik, 2006). Maybe the observed differences in gene expression patterns and the total immunoglobulin level in *Gasterosteus aculeatus* are associated with this. Hence, it would be interesting to examine, whether and how female sex hormones can influence the adaptive immune system of the three-spined stickleback. Maybe female sex hormones can be stimulated and gene expression patterns can be examined.

It might also be helpful to identify more possible inflammatory genes. This can lead to a better understanding of the complex network of associations between several cytokines of the adaptive immune system. As already mentioned, Interleukins maybe more variable within teleost species as expected. Hence, comparisons of the structure of Interleukins of different fish species might help to give assumptions about the conservation of these genes. The possible duplication event in the MIF gene indicates another locus, which might be important for the immune system of *Gasterosteus aculeatus*. Maybe locus-specific primer design and gene expression analysis could give assumptions about the structure and function of this gene locus. Because of its suppressive effect on other cytokines like IL 1 beta and MHC II (Male, 2005), (Martin, 2009), the detection of Interleukin 10 might be interesting. It has already been identified in other teleost species (Bird *et al.*, 2006). The expression assay showed, that Interleukin 10 was expressed at a low level in liver, gut, kidney and spinal cord of healthy fish. This agrees with human studies, where the expression of Interleukin 10 seems to be under strong control (Zou *et al.*, 2003). In contrast, Interleukin 10 was strongly expressed in head kidney and intestine of the carp (Bird *et al.*, 2006). In humans, a study about Systemic Lupus Erythematodes and Rheumatic Arthritis showed an association between an Interleukin 10 promotor polymorphism and autoimmunity (Lard *et al.*, 2003). Patients with this polymorphism showed higher auto antibody production by B-lymphocytes and joint damage in Rheumatoid Arthritis. Due to their co-stimulatory function and the synthesis of other cytokines (Male, 2005), (Böcker, 2004), Interleukin 21, 4 and 12 also play an important role in the adaptive immune response and the occurrence of autoimmune diseases in humans (Pelletier, Girard, 2007), (Pang *et al.*, 2007). Several expression studies about these cytokines exist in *Takifugu rubripes* and *Tetraodon nigriviridis* (Bird *et al.*, 2005), (Yoshiura *et al.*, 2003), (Savan, Sakai, 2006), (Li *et al.*, 2007).

Expression levels of IL 21 in tissues of healthy fish only appeared in the head kidney and no other examined tissues (Bird *et al.*, 2005). Increased expression levels were detected in gills and gut after stimulation (Bird *et al.*, 2005). This might be a hint for the importance of Interleukin 21 in bony fish immune reactions. Expression assays of Interleukin 12 showed an increase of the subunit IL 12 p35 in head kidney and spleen after stimulation and a consistent expression in brain, kidney, stomach, intestine and thymus. IL 12 p40 was expressed in all examined tissues, but did not show an increase during stimulation (Yoshiura *et al.*, 2003). Gene expression of Interleukin 4 was determined via Real Time PCR and resulted in an essential expression in head kidney, spleen, liver, brain, gill, muscle and heart. Stimulation with LPS caused a significant increase of Interleukin 4 expression in head kidney, spleen, liver and muscle (Li *et al.*, 2007). All these assays included stimulations of the adaptive immune system with LPS, which resulted in an increase of expression levels in several organs. This was also shown for the expression of Interleukin 1 in the head kidney of the rainbow trout *Onchorhynchus mykiss* (Secombes *et al.*, 1999) and MIF in head kidney and spleen of the pufferfish *Tetraodon nigroviridis* (Jin *et al.*, 2007). Studies about a stimulation of the immune system of the rainbow trout, the Nile Tilapia and the farmed Atlantic salmon with Freund's complete adjuvant (FCA) and allogeneic testis homogenate (ATH) indicated the induction of autoimmune reactions in testis, liver and blood (Secombes *et al.*, 1985), (Mochida *et al.*, 2009), (Koppang *et al.*, 2008). In the rainbow trout, autoimmunity was induced due to a transfer of anti-sperm antibodies. Fish were pre-treated with Freund's complete adjuvant (FCA). This combination of pre-treatment and passive immunization elicited autoimmune orchitis, characterised by sperm-agglutinating auto antibodies in the serum, accumulation of lymphocytes, an enhanced phagocytosis of spermatozoa and severe granulomatous lesions (Secombes *et al.*, 1985). Eight weeks after the injection of a mixture of allogeneic testis homogenate (ATH) and FCA, histo-pathological changes were observed in the interstitium of the testis of the Nile Tilapia (Mochida *et al.*, 2009). Irregulatory myoid cells, many different kinds of leukocytes, macrophages phagozytosing spermatozoa and degeneration of testicular somatic cells were detected. Apart from that, several kinds of auto antigens were present in the seminal plasma, which might induce inflammatory reactions (Mochida *et al.*, 2009). The results of the study in the farmed Atlantic salmon indicated the production of antinuclear antibodies (ANAs) in the serum (Koppang *et al.*, 2008). This is a characteristic of systemic autoimmunity. Oil-adjuvant vaccinated fish showed high titers of auto antibodies to salmon blood cells compared to unvaccinated fish. Apart from that, serum total IgM levels were increased in vaccinated salmons and the pathology of liver and kidney showed thrombosis, granulomatous inflammation and glomerulonephritis (Koppang *et al.*, 2008). These studies indicate the occurrence of autoimmunity in teleost fish species.

Therefore, another interesting project might deal with the artificial induction of autoimmune reactions due to stimulation of the immune system of *Gasterosteus aculeatus* with certain chemicals. As mentioned above, several expression studies of Interleukins also showed expression values in other organs. Hence, the expression of certain cytokines could be examined in other organs of the three-spined stickleback.

The number of MHC IIB alleles is the grouping factor for the individuals of this thesis. The low p-value indicates a trend that this factor might also influence the dependent variables. Therefore, it might be interesting to breed fish with very high and low numbers of MHC IIB alleles to make this trend more obvious. Maybe a comparison between individuals, who contain either 1 or 5-6 different alleles, might be possible. It might be possible that there is a trade-off between the resistance towards parasites and auto aggressive reactions. Parasites are a selection pressure, which increases the number of MHC IIB alleles. Hence, it seems to be advantageous to contain a high number of alleles to increase the individual fitness. But this increase is limited by another selection pressure, which leads to the optimum of an intermediate number of MHC IIB alleles (Wegner *et al.*, 2003). This is supposed to be the occurrence of autoimmunity (Woelfing *et al.*, 2009). Therefore, it might be interesting to breed fish with high numbers of MHC IIB alleles and to expose them to many parasites and to many different types of parasites. Maybe the costs, namely the occurrence of auto aggressive reactions, are lower than the benefits, namely a high resistance towards parasites. If this is the case, auto aggressive reactions should occur in fish with a high number of MHC IIB alleles. This approach might show a trade-off between the resistance towards parasites and the avoidance of autoimmunity.

As already mentioned, this is a pilot study about certain immunological parameters in *Gasterosteus aculeatus*, which might be associated with auto aggressive reactions. Therefore, the next step should be a histological assay of the remaining tissue samples. This might be another way to detect auto aggressive reactions in the three-spined stickleback. Therefore, individuals of the two assay groups, who show most extreme gene expression and immunoglobulin values, should be chosen. Different tissues will be stained and checked for morphological abnormalities between the two study groups. Tissues of healthy fish should also be examined as a negative control. Apart from that, a staining with the green fluorescent polyclonal goat-anti-rabbit antibody (Alexa 488, Molecular Probes) can be done (Scharsack *et al.*, 2007). This specific antibody detects MHC class I and II molecules of the three-spined stickleback.

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## 8. References


Amos, W.; Harwood, J. (1998): Factors affecting levels of genetic diversity in natural populations, *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* , 177-186.

Anderson, M.J. (2001): A new method for non-parametric multivariate analysis of variance, *Austral Ecology*, 32-46.

Apanius, V.; Penn, D.; Slev, P.R.; Ruff, L.R.; Potts, W.K. (1997): The nature of selection on the major histocompatibility complex, *Critical Reviews in Immunology*, 179-224.

Bao, Y.; Wang, T.; Guo, Y. *et al.* (2010)*:* The immunoglobulin gene loci in the teleost Gasterosteus aculeatus, *Fish & Shellfish Immunology*, 40-48.

Barton, N.H.; Charlesworth, B. (1998): Why sex and recombination?, *Science*, 1986-1990.

Barton, N.H.B.; Derek, E.G. (2007): *Evolution*, 1 edn, CSHL Press

Bergstrom, C.T.; Antia, R. (2006): How do adaptive immune systems control pathogens while avoiding autoimmunity?, *Trends in Ecology & Evolution*, 22-28.

Bird, S.; Zou, J.; Kono, T. *et al.* (2005): Characterisation and expression analysis of interleukin 2 (IL-2) and IL-21 homologues in the Japanese pufferfish, Fugu rubripes, following their discovery by synteny, *Immunogenetics*, 909-923.

Bird, S.; Zou, J.; Secombes, C.J. (2006): Advances in Fish Cytokine Biology Give Clues to the Evolution of a Complex Network, *Current Pharmaceutical Design*, 3051-3069.

Böcker, W.D. (2004): *Pathologie,* Elsevier.

Borghans, J.A.M.; Beltman, J.B.; De Boer, R.J. (2004): MHC polymorphism under host-pathogen coevolution, *Immunogenetics*, 732-739.

Bustin, S.A.; Benes, V.; Garson, J.A. *et al.* (2009): The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments, *Clin Chem*, 611-622.

Chellappa, S.; Huntingford, F.A.; Strang, R.H.C.; Thomson, R.Y. (1995): Condition factor and hepatosomatic index as estimates of energy status in male three-spined stickleback, *Journal of Fish Biology*, 775-787.

D'Haeseleer, P. (2005): How does gene expression clustering work?, *Nature Biotechnology*, 1499-1501.

Danilova, N.; Bussmann, J.; Jekosch, K.; Steiner, L.A. (2005): The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z, *Nature Immunology*, 295-302.

Davies, J.L.; Kawaguchi, Y. (1994): A genome-wide search for human type I diabetes susceptibility genes, *Nature*

DeFranco, A.L.L.; Richard, M.; Robertson, Miranda (2007): *Immunity - The Immune Response in Infectious and Inflammatory Disease,* Primers in Biology.

Derveaux, S.; Vandesompele, J.; Hellemans, J. (2009): How to do successful gene expression analysis using real-time PCR, *Methods*, 227-230.

Donn, R.P.; Shelley, E.; Ollier, W.E.R.; Thomson, W. (2001): A novel 5prime-flanking region polymorphism of macrophage migration inhibitory factor is associated with systemic-onset juvenile idiopathic arthritis, *Arthritis & Rheumatism*, 1782-1785.

Dytham, C. (2003): *Choosing and using statistics - A biologist's guide*, second edn., Blackwell Publishing.

Ebert, D.; Hamilton, W.D. (1996): Sex against virulence: The coevolution of parasitic diseases, *Trends in Ecology and Evolution*, 79-82.

Eizaguirre, C.; Lenz, T.; Sommerfeld, R. *et al.* (2011): Parasite diversity, patterns of MHC II variation and olfactory based mate choice in diverging three-spined stickleback ecotypes, *Evolutionary Ecology*, 1-18.

Etschmann, B.; Wilcken, B.; Stoevesand, K.; von der Schulenburg, A.; Sterner-Kock, A. (2006): Selection of Reference Genes for Quantitative Real-time PCR Analysis in Canine Mammary Tumors Using the GeNorm Algorithm, *Veterinary Pathology Online*, 934-942.

Ferencik, M.R. et al. (2006): *Kompendium der Immunologie - Grundlagen und Klinik,* Springer.

Fernando, M.M.A.; Stevens, C.R.; Walsh, E.C. *et al.* (2008): Defining the Role of the MHC in Autoimmunity: A Review and Pooled Analysis, *PLoS Genet* , e1000024.

Graham, A.L.; Allen, J.E.; Read, A.F. (2005): EVOLUTIONARY CAUSES AND CONSEQUENCES OF IMMUNOPATHOLOGY, *Annual Review of Ecology, Evolution, and Systematics*, 373-397.

Gregersen, P.K.; Shen, M.; Song, Q.L. *et al.* (1986): Molecular diversity of HLA-DR4 haplotypes, *Proceedings of the National Academy of Sciences of the United States of America*, 2642-2646.

Grumet, F.C.; Coukell, A.; Bodmer, J.G.; Bodmer, W.F.; McDevitt, H.O. (1971): HISTOCOMPATIBILITY (HL-A) ANTIGENS ASSOCIATED WITH SYSTEMIC LUPUS ERYTHEMATOSUS - POSSIBLE GENETIC PREDISPOSITION TO DISEASE, *New England Journal of Medicine* , 193-&.

Hall, T.A.(1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, *Oxford University Press*.

Hamilton, W.D. (1980): SEX VERSUS NON-SEX VERSUS PARASITE, *Oikos*, 282-290.

He, H.S.-L. Teresa; Qi, Rensheng; Liu, Jonathan C.S. (2009): GeneMarker HID - Analysis of DNA mixtures in GeneMarker HID Software: with or without single source reference samples.

Hellemans, J.; Mortier, G.; De Paepe, A.; Speleman, F.; Vandesompele, J. (2007): qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data, *Genome Biology*, R19.

Hibbeler, S.; Scharsack, J.; Becker, S. (2008): Housekeeping genes for quantitative expression studies in the three-spined stickleback Gasterosteus aculeatus, *BMC Molecular Biology*, 18.

Hoar, W.S.R. (1970): *Fish Physiology Volume IV - The nervous system, circulation and respiration,* Academic Press.

Hong, S.; Zou, J.; Crampe, M. *et al.* (2001): The production and bioactivity of rainbow trout (Oncorhynchus mykiss) recombinant IL-1 beta, *Veterinary Immunology and Immunopathology*, 1-14.

Hubbard, T.; Barker, D.; Birney, E. *et al.* (2002): The Ensembl genome database project,. *Nucleic Acids Research*, 38-41.

Janeway, C.A.T. Paul; Walport, Mark; Shlomchik, Mark J.; (2005): *Immunobiology - The immune system in health and disease,* Garland Science.

Jin, H.-J.; Xiang, L.-X.; Shao, J.-Z. (2007): Molecular cloning and identification of macrophage migration inhibitory factor (MIF) in teleost fish, *Developmental & Comparative Immunology*, 1131-1144.

Jones, E.Y.; Fugger, L.; Strominger, J.L.; Siebold, C. (2006): MHC class II proteins and disease: a structural perspective, *Nat Rev Immunol*, 271-282.

Kalbe, M.; Eizaguirre, C.; Dankert, I. *et al.* (2009): Lifetime reproductive success is maximized with optimal major histocompatibility complex diversity, *Proceedings of the Royal Society B: Biological Sciences*, 925-934.

Kalbe, M.; Wegner, K.M.; Reusch, T.B.H. (2002): Dispersion patterns of parasites in 0+ year three-spined sticklebacks: a cross population comparison, *Journal of Fish Biology*, 1529-1542.

Karlen, Y.; McNair, A.; Perseguers, S.; Mazza, C.; Mermod, N. (2007): Statistical significance of quantitative PCR, *BMC Bioinformatics*, 131.

Klein, J.; Sato, A.; Mayer, W.E. (2000): Jaws and AIS. In: *Major Histocompatibility Complex - Evolution, Structure and Function* (ed. Kasahara M), pp. 3-26, Springer-Verlag Tokyo, Tokyo.

Koppang, E.O.; Bjerkas, I.; Haugarvoll, E. *et al.* (2008): Vaccination-induced systemic autoimmunity in farmed Atlantic salmon, *Journal of Immunology*, 4807-4814.

Kurtz, J.; Kalbe, M.; Langefors, S. *et al.* (2007): An experimental test of the immunocompetence handicap hypothesis in a teleost fish: 11-ketotestosterone suppresses innate immunity in three-spined sticklebacks, *American Naturalist*, 509-519.

Lard, L.R.; Gaalen, F.A.v; Schonkeren, J.J.M. *et al.* (2003): Association of the -2849 interleukin-10 promoter polymorphism with autoantibody production and joint destruction in rheumatoid arthritis, *Arthritis & Rheumatism*, 1841-1848.

Lenz, T.; Eizaguirre, C.; Becker, S.; Reusch, T. (2009): RSCA genotyping of MHC for high-throughput evolutionary studies in the model organism three-spined stickleback Gasterosteus aculeatus, *BMC Evolutionary Biology*, 57.

Li, J.-H.; Shao, J.-Z.; Xiang, L.-X.; Wen, Y. (2007): Cloning, characterization and expression analysis of pufferfish interleukin-4 cDNA: The first evidence of Th2-type cytokine in fish, *Molecular Immunology*, 2078-2086.

Livak, K.J.; Schmittgen, T.D. (2001): Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the Delta-Delta-CT Method, *Methods*, 402-408.

Macnab, V.; Katsiadaki, I.; Barber, I. (2009): Reproductive potential of Schistocephalus solidus-infected male three-spined stickleback Gasterosteus aculeatus from two U.K. populations, *Journal of Fish Biology*, 2095-2107.

Male, D. (2005): *Immunologie auf einen Blick,* Elsevier.

Martin, M.R.; Resch, K. (2009): *Immunologie,* UTB.

McDevitt, H.O. (1998): The role of MHC class II molecules in susceptibility and resistance to autoimmunity, *Current Opinion in Immunology*, 677-681.

Milinski, M. (2003): The function of mate choice in sticklebacks: optimizing Mhc genetics, *Journal of Fish Biology*, 1-16.

Milinski, M. (2006): The Major Histocompatibility Complex, Sexual Selection, and Mate Choice, *Annual Review of Ecology, Evolution, and Systematics*, 159-186.

Milinski, M.; Griffiths, S.; Wegner, K.M. *et al.* (2005): Mate choice decisions of stickleback females predictably modified by MHC peptide ligands, *Proceedings of the National Academy of Sciences of the United States of America*, 4414-4418.

Mochida, K.; Adachi, S.; Yamauchi, K. (2009): Initial Phase of an Inflammatory Reaction in Testis Caused by Experimental Testicular Autoimmunity in the Nile Tilapia, Oreochromis niloticus, *Zoological Science*, 543-550.

Mülhardt, C. (2002): *Der Experimentator: Molekularbiologie/Genomics,* Spektrum Akademischer Verlag.

Nepom, G.T.; Erlich, H. (1991): MHC Class-II Molecules and Autoimmunity, *Annual Review of Immunology*, 493-525.

Nowak, M.A.; Tarczy-Hornoch, K.; Austyn, J.M. (1992): The optimal number of major histocompatibility complex molecules in an individual, *Proceedings of the National Academy of Sciences of the United States of America*, 10896-10899.

Östlund-Nilsson, S.M.; Huntingford, Felicity Anne (2007): *Biology of the three-spined Stickleback,* CRC Press.

Pang, Y.H.; Zheng, C.Q.; Yang, X.Z.; Zhang, W.J. (2007): Increased expression and activation of IL-12-induced Stat4 signaling in the mucosa of ulcerative colitis patients, *Cellular Immunology*, 115-120.

Pelletier, M.; Girard, D. (2007): Biological Functions of Interleukin-21 and Its Role in Inflammation, *TheScientificWorldJOURNAL*, 1715-1735.

Penn, D.J. (2002): The scent of genetic compatibility: Sexual selection and the major histocompatibility complex, *Ethology*, 1-21.

Pfaffl, M.W. (2001): A new mathematical model for relative quantification in real-time RT-PCR, *Nucl. Acids Res,* e45-.

Pruitt, K.D.; Tatusova, T.; Maglott, D.R. (2007): NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins, *Nucleic Acids Research* , D61-D65.

Pryke, A.M.; Nazemi, Alireza (2007): Heatmap visualization of population based multi objective algorithms.

Ram, S.; Azumi, A.; Kenji, S.; Ryoji, Y.; Masahiro, S. (2005): Discovery of a new class of immunoglobulin heavy chain from fugu, *European Journal of Immunology*, 3320-3331.

Ramakers, C.; Ruijter, J.M.; Deprez, R.H.; Moorman, A.F. (2003): Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data, *Neurosci Letters*, 62 - 66.

Reed, D.H.; Frankham, R. (2003): Correlation between Fitness and Genetic Diversity, *Conservation Biology*, 230-237.

Reusch, T.B.H.; Schaschl, H.; Wegner, K.M. (2004): Recent duplication and inter-locus gene conversion in major histocompatibility class II genes in a teleost, the three-spined stickleback, *Immunogenetics*, 427-437.

Rioux, J.D.; Goyette, P.; Vyse, T.J. *et al.* (2009): Mapping of multiple susceptibility variants within the MHC region for 7 immune-mediated diseases, *Proceedings of the National Academy of Sciences*, 18680-18685.

Savan, R.; Sakai, M. (2006): Genomics of fish cytokines, *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, 89-101.

Scharsack, J.P.;Kalbe, M.; Schaschl, H. (2007): Characterization of antisera raised against stickleback (Gasterosteus aculeatus) MHC class I and class II molecules, *Fish & Shellfish Immunology*, 991-1002.

Secombes, C.J.; Bird, S.; Cunningham, C.; Zou, J. (1999): Interleukin-1 in fish, *Fish & Shellfish Immunology*, 335-343.

Secombes, C.J.; Lewis, A.E.; Laird, L.M.; Needham, E.A.; Priede, I.G. (1985): ROLE OF AUTOANTIBODIES IN THE AUTOIMMUNE-RESPONSE TO TESTIS IN RAINBOW-TROUT (SALMO-GAIRDNERI), *Immunology*, 409-415.

Sluijter, J.P.G.; Pasterkamp, G.; Kleijn, D.P.V. (2006): Quantitative Real-Time PCR, In: *Cardiovascular Research*, pp. 75-83.

Snyder-Leiby, T.H.; Haiguo; Hulce; David (2009): GeneMarker - New technology improves size calling of kilobase DNA fragments.

Stearns, S.C.; Hoekstra, R. F. (2005): *Evolution: an introduction*, 2 edn. Oxford University Press.

Stearns, S.C.; Koella, J. C. (2008): *Evolution in health and disease,* Oxford Biology.

Thellin, O.; Zorzi. W.; Lakaye, B*. et al.* (1999): Housekeeping genes as internal standards: use and limits, *Journal of Biotechnology*, 291-295.

Tisch, R.; McDevitt, H. (1996): Insulin-dependent diabetes mellitus, *Cell*, 291-297.

Todd, J.A.; Acha-Orbea, H.; Bell, J.I. *et al.* (1988): A molecular basis for MHC class II--associated autoimmunity, *Science*, 1003-1009.

Todd, J.A.; Bell, J.I.; McDevitt, H. (1987): HLA-DQ[beta] gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus, *Nature*, 599-604.

Vijayan, M.M.; Leatherland, J.F. (1988): Effect of stocking density on the growth and stress-response in brook charr, Salvelinus fontinalis, *Aquaculture*, 159-170.

Warr, G.W. (1995): The immunoglobulin genes of fish, *Developmental & Comparative Immunology*, 1-12.

Wegner, K.M.; Kalbe, M.; Rauch, G. *et al.* (2006): Genetic variation in MHC class II expression and interactions with MHC sequence polymorphism in three-spined sticklebacks, *Molecular Ecology*, 1153 - 1164.

Wegner, K.M.; Reusch, T.B.H.; Kalbe, M. (2003): Multiple parasites are driving major histocompatibility complex polymorphism in the wild, *Journal of Evolutionary Biology*, 224-232.

Wilson, M.; Miller, N.W. *et al.* (1997): A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD, *Proceedings of the National Academy of Sciences of the United States of America*, 4593-4597.

Woelfing, B.; Traulsen, A.; Milinski, M.; Boehm, T. (2009): Does intra-individual major histocompatibility complex diversity keep a golden mean?, *Philosophical Transactions of the Royal Society B: Biological Sciences*, 117-128.

Wucherpfennig, K.W.; Strominger, J.L. (1995): SELECTIVE BINDING OF SELF PEPTIDES TO DISEASE-ASSOCIATED MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) MOLECULES - A MECHANISM FOR MHC-LINKED SUSCEPTIBILITY TO HUMAN AUTOIMMUNE-DISEASES, *Journal of Experimental Medicine*, 1597-1601.

Yoshiura, Y.; Kiryu, I.; Fujiwara, A. *et al.* (2003): Identification and characterization of Fugu orthologues of mammalian interleukin-12 subunits, *Immunogenetics*, 296-306.

Zapala, M.A.; Schork, N.J. (2006): Multivariate regression analysis of distance matrices for testing associations between gene expression patterns and related variables, *Proceedings of the National Academy of Sciences*, 19430-19435.

Zapata, A.; Chiba, A.; Varas, A. (1996): Cells and tissues of the immune system of fish, *The Fish Immune System*, 1 - 53.

Zou, J.; Clark, M.S.; Secombes, C.J. (2003): Characterisation, expression and promoter analysis of an interleukin 10 homologue in the puffer fish, Fugu rubripes, *Immunogenetics*, 325-335.

## 9. Supplement

### 1. MHC class IIB-Genotyping

1.1 Composition of the PCR reaction

25 µl reaction:

| ingredient | amount (µl) |
|---|---|
| DNA template | 3 |
| GeneAmp 10X PCR Buffer II | 2.5 |
| 25mM MgCl2 Solution | 5 |
| dNTP | 2.5 |
| ForPrimer: GAIIEx2startF | 2.5 |
| RevPrimer: GAIIExon2R_RSCA | 2.5 |
| HPLC water | 6.8 |
| AmpliTaq Gold (HotStart taq) | 0.2 |

### 2. RT-PCR

2.1 Composition of the Reverse Transcription PCR

20 µl reaction:

| ingredient | amount (µl) |
|---|---|
| 10x Buffer | 2 |
| dNTP | 2 |
| Oligo dT | 1 |
| RNase inhibitor | 1 |
| Omniscript RT | 1 |
| HPLC water | 3 |
| RNA | 10 |

## 3. Standard PCR

### 3.1 Composition of master mixes for the standard PCR

10 µl reaction

| ingredient | amount (µl) |
|---|---|
| 10x Buffer | 1 |
| MgCl2 | 2 |
| dNTP | 1 |
| Primer I | 1 |
| Primer II | 1 |
| Taq | 0,1 |
| HPLC water | 2.9 |
| cDNA / gDNA template | 1 |

### 3.2 Cycling conditions

a) short fragments:

| Program Name | Target (°C) | Acquisition Mode | Hold (mm:ss) | Ramp Rate (°C/sec) | Acquisitions (per °C) | Cycles | Analysis Mode |
|---|---|---|---|---|---|---|---|
| Incubation | 95 | None | 10:00 | 4,4 | | 1 | None |
| Down | 94 | None | 0:01 | 2,2 | | 1 | None |
| PCR | 94 | None | 0:20 | 4,4 | | 40 | Quantification |
| | 68 | Single | 0:45 | 2,2 | | | |
| PCR | 4 | | forever | | | | |

b) long fragments:

| Program Name | Target (°C) | Acquisition Mode | Hold (mm:ss) | Ramp Rate (°C/sec) | Acquisitions (per °C) | Cycles | Analysis Mode |
|---|---|---|---|---|---|---|---|
| Incubation | 95 | None | 10:00 | 4,4 | | 1 | None |
| Down | 94 | None | 0:01 | 2,2 | | 1 | None |
| PCR | 94 | None | 0:20 | 4,4 | | 40 | Quantification |
| | 68 | Single | 02:00 | 2,2 | | | |
| PCR | 4 | | forever | | | | |

## 4. Direct Sequencing

4.1 Composition of the cycle sequencing reaction

10 µl reaction

| ingredient | amount (µl) |
|---|---|
| HPLC water | 6 |
| BigDye Terminator v1.1, v3.1, 5x Sequencing Buffer (Applied Biosystems) | 1 |
| BigDye Terminator v3.1 Cycle (Applied Biosystems) | 1 |
| Primer | 1 |
| Template (DNA) | 1 |

## 5. Results

5.1 MHC class IIB haplotype combinations

a) MHC class IIB haplotype combinations of the parents

| individual | MHC IIB allele combination |
|---|---|
| L001 | No01, No12, No18, No13 |
| L002 | No16, No15, No18, No13 |
| L003 | No15, No16 |
| L004 | No25, No27 |
| L005 | No01, No12, No18, No13 |
| L006 | No18, No13 |
| L007 | No18, No13, No05 |
| L008 | No01, No12, No05 |
| L012 | No15, No16, No07, No31 |
| L013 | No15, No16, No18, No13 |
| L102 | No15, No16 |
| L103 | No01, No12, No15, No16 |
| L106 | No18, No13, No08, SCX15 |
| L108 | No01, No12, new, new |
| L109 | No15, No16, No08, SCX 15 |
| L113 | No15, No16, No18, No13 |
| L116 | No07, No31, No08, SCX15 |
| L119 | No15, No16, No07, No31 |
| L121 | No15, No16, No07, No31 |
| L127 | No18, No13 |
| L128 | No18, No13, No07, No31 |

b) MHC class IIB haplotype combinations of the offspring

| individual | family | MHC IIB allele combination |
|---|---|---|
| 001 | L001 x L103 | No18, No13, No15, No16 |
| 002 | L001 x L103 | No01, No12 |
| 003 | L001 x L103 | No01, No12, No15, No16 |
| 004 | L002 x L106 | No08, SCX15, No15, No16 |
| 005 | L002 x L106 | No18, No13 |
| 007 | L013 x L128 | No18, No13 |
| 008 | L013 x L128 | No07, No31, No15, No16 |
| 009 | L013 x L128 | No18, No13, No15, No16 |
| 010 | L006 x L106 | No08, SCX15, No18, No13 |
| 013 | L012 x L119 | No15, No16 |
| 014 | L012 x L119 | No07, No31 |
| 015 | L012 x L119 | No07, No31, No15, No16 |
| 016 | L002 x L106 | No08, SCX15, No15, No16 |
| 017 | L002 x L106 | No18, No13 |
| 018 | L002 x L106 | No18, No13 |
| 019 | L006 x L106 | No08, SCX15, No18, No13 |
| 020 | L006 x L106 | No18, No13 |
| 021 | L006 x L106 | No08, SCX15, No18, No13 |
| 022 | L013 x L128 | No18, No13 |
| 023 | L013 x L128 | No07, No31, No15, No16 |
| 024 | L013 x L128 | No18, No13, No15, No16 |
| 025 | L001 x L103 | No01, No12, No15, No16 |
| 026 | L001 x L103 | No18, No13, No15, No16 |
| 027 | L001 x L103 | No01, No12, No15, No16 |
| 028 | L012 x L119 | No15, No16 |
| 029 | L012 x L119 | No07, No31, No15, No16 |
| 030 | L012 x L119 | No07, No31, No15, No16 |
| 031 | L002 x L106 | No18, No13 |
| 032 | L002 x L106 | No08, SCX15, No15, No16 |
| 033 | L002 x L106 | No08, SCX15, No18, No13 |
| 034 | L013 x L128 | No07, No31, No18, No13 |
| 035 | L013 x L128 | No18, No13, No15, No16 |
| 036 | L013 x L128 | No18, No13, No15, No16 |
| 037 | L006 x L106 | No18, No13 |
| 038 | L006 x L106 | No18, No13 |
| 039 | L006 x L106 | No18, No13 |
| 040 | L001 x L103 | No01, No12, No18, No13 |
| 041 | L001 x L103 | No01, No12 |
| 042 | L001 x L103 | No01, No12, No15, No16 |
| 043 | L012 x L119 | No07, No31, No15, No16 |
| 044 | L012 x L119 | No07, No31, No15, No16 |
| 045 | L012 x L119 | No07, No31, No15, No16 |
| 046 | L006 x L106 | No08, SCX15, No18, No13 |
| 047 | L006 x L106 | No08, SCX15, No18, No13 |
| 048 | L006 x L106 | No18, No13 |
| 049 | L013 x L128 | No18, No13 |
| 050 | L013 x L128 | No18, No13, No15, No16 |

| | | |
|---|---|---|
| 051 | L013 x L128 | No18, No13, No15, No16 |
| 052 | L002 x L106 | No18, No13, No15, No16 |
| 053 | L002 x L106 | No08, SCX15, No18, No13 |
| 054 | L002 x L106 | No18, No13, No15, No16 |
| 055 | L001 x L103 | No01, No12, No18, No13 |
| 056 | L001 x L103 | No01, No12, No15, No16 |
| 057 | L001 x L103 | No01, No12, No18, No13 |
| 058 | L002 x L106 | No18, No13, No15, No16 |
| 059 | L002 x L106 | No18, No13 |
| 060 | L002 x L106 | No18, No13 |
| 061 | L013 x L128 | No18, No13, No15, No16 |
| 062 | L013 x L128 | No07, No31, No18, No13 |
| 063 | L013 x L128 | No18, No13 |
| 064 | L012 x L119 | No07, No31, No15, No16 |
| 065 | L012 x L119 | No07, No31 |
| 066 | L012 x L119 | No07, No31, No15, No16 |
| 067 | L006 x L106 | No08, SCX15, No18, No13 |
| 068 | L006 x L106 | No08, SCX15, No18, No13 |
| 069 | L006 x L106 | No18, No13 |
| 070 | L001 x L103 | No01, No12 |
| 071 | L001 x L103 | No01, No12 |
| 072 | L001 x L103 | No01, No12 |
| 073 | L002 x L106 | No18, No13 |
| 074 | L002 x L106 | No18, No13, No15, No16 |
| 075 | L002 x L106 | No08, SCX15, No15, No16 |
| 076 | L012 x L119 | No07, No31, No15, No16 |
| 077 | L012 x L119 | No07, No31 |
| 078 | L012 x L119 | No15, No16 |
| 079 | L006 x L106 | No18, No13 |
| 080 | L006 x L106 | No08, SCX15, No18, No13 |
| 081 | L006 x L106 | No18, No13 |
| 082 | L002 x L106 | No18, No13, No15, No16 |
| 083 | L002 x L106 | No08, SCX15, No18, No13 |
| 085 | L001 x L103 | No18, No13, No15, No16 |
| 086 | L001 x L103 | No01, No12, No18, No13 |
| 087 | L001 x L103 | No01, No12 |
| 088 | L013 x L128 | No07, No31, No18, No13 |
| 089 | L013 x L128 | No18, No13 |
| 090 | L013 x L128 | No07, No31, No18, No13 |
| 091 | L006 x L106 | No18, No13 |
| 092 | L006 x L106 | No18, No13 |
| 093 | L006 x L106 | No08, SCX15, No18, No13 |
| 094 | L012 x L119 | No15, No16 |
| 095 | L012 x L119 | No07, No31, No15, No16 |
| 096 | L012 x L119 | No15, No16 |
| 097 | L006 x L106 | No08, SCX15, No18, No13 |
| 098 | L006 x L106 | No18, No13 |
| 099 | L006 x L106 | No18, No13 |
| 100 | L001 x L103 | No01, No12 |
| 101 | L001 x L103 | No18, No13, No15, No16 |
| 102 | L001 x L103 | No01, No12, No18, No13 |

| | | |
|---|---|---|
| 103 | L002 x L106 | No08, SCX15, No18, No13 |
| 104 | L002 x L106 | No18, No13, No15, No16 |
| 105 | L002 x L106 | No08, SCX15, No15, No16 |
| 106 | L013 x L128 | No18, No13 |
| 108 | L013 x L128 | No07, No31, No18, No13 |
| 109 | L006 x L106 | No18, No13 |
| 110 | L006 x L106 | No18, No13 |
| 111 | L006 x L106 | No18, No13 |
| 112 | L001 x L103 | No01, No12, No15, No16 |
| 113 | L001 x L103 | No18, No13, No15, No16 |
| 114 | L001 x L103 | No01, No12, No18, No13 |
| 115 | L001 x L103 | No01, No12 |
| 116 | L001 x L103 | No01, No12, No18, No13 |
| 117 | L001 x L103 | No18, No13, No15, No16 |
| 118 | L013 x L128 | No07, No31, No18, No13 |
| 119 | L013 x L128 | No18, No13 |
| 120 | L013 x L128 | No07, No31, No18, No13 |
| 121 | L006 x L106 | No18, No13 |
| 122 | L002 x L106 | No18, No13, No15, No16 |
| 123 | L002 x L106 | No18, No13, No15, No16 |
| 124 | L002 x L106 | No08, SCX15, No15, No16 |

## 5.2 Permutational MANOVA: plots, describing the influence of the dissection date

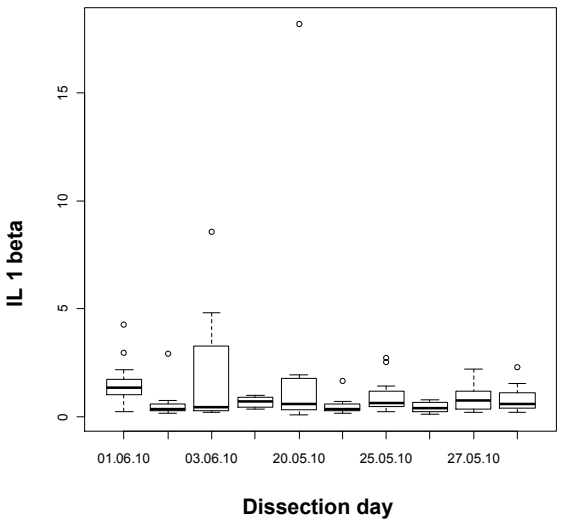


Fig. 5.2.1: Relative expression of IL 1 beta in the gut, according to the dissection date

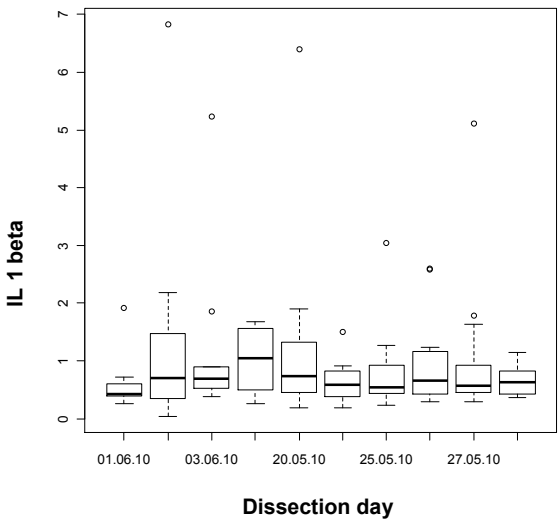


Fig. 5.2.2: Relative expression of IL 1 beta in the head kidney, according to the dissection date

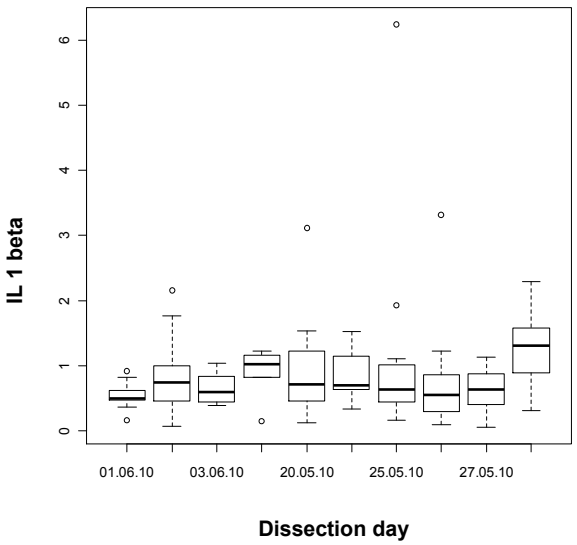


Fig. 5.2.3: Relative expression of IL 1 beta in the kidney, according to the dissection date

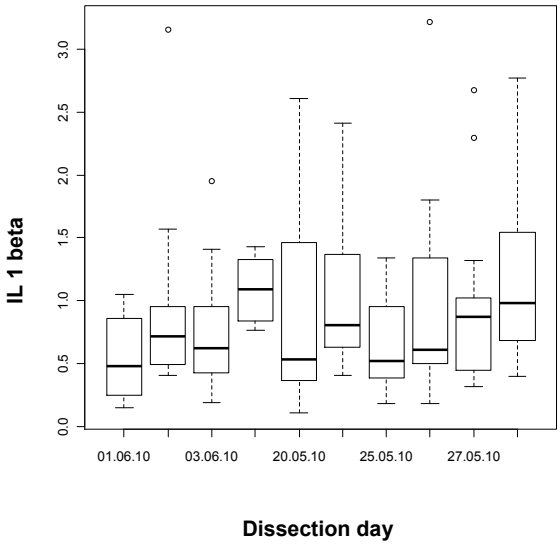


Fig. 5.2.4: Relative expression of IL 1 beta in the spleen, according to the dissection date

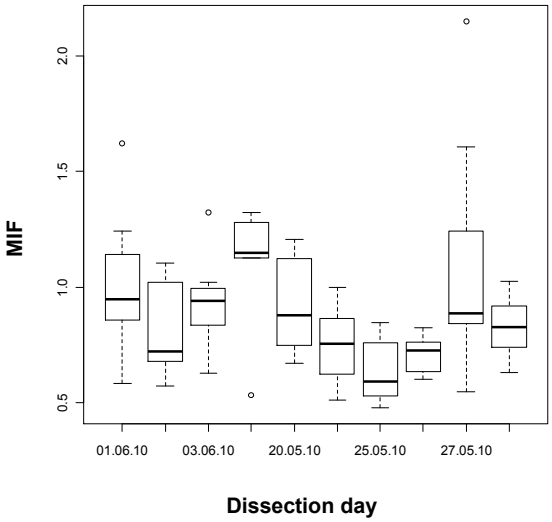


Fig. 5.2.5: Relative expression of MIF in the gut, according to the dissection date

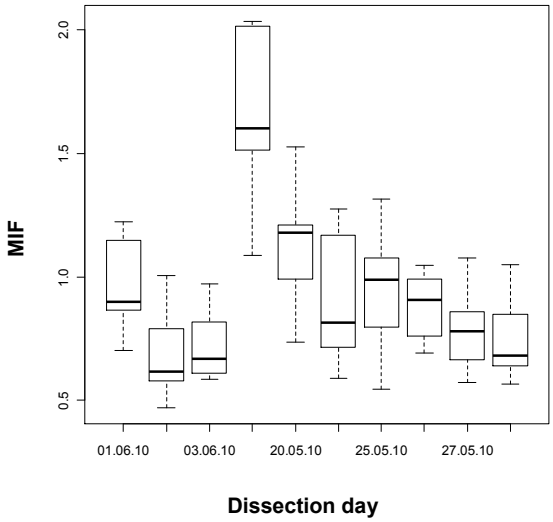


Fig. 5.2.6: Relative expression of MIF in the head kidney, according to the dissection date

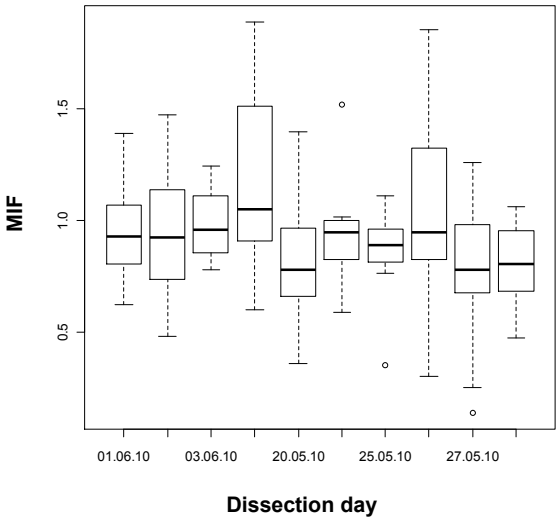


Fig. 5.2.7: Relative expression of MIF in the kidney, according to the dissection date

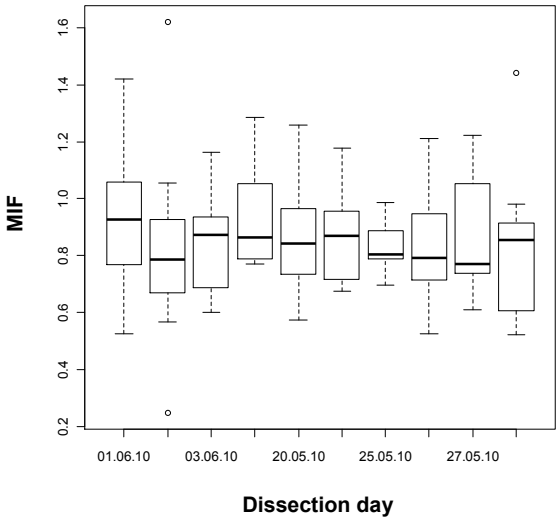


Fig. 5.2.8: Relative expression of MIF in the spleen, according to the dissection date

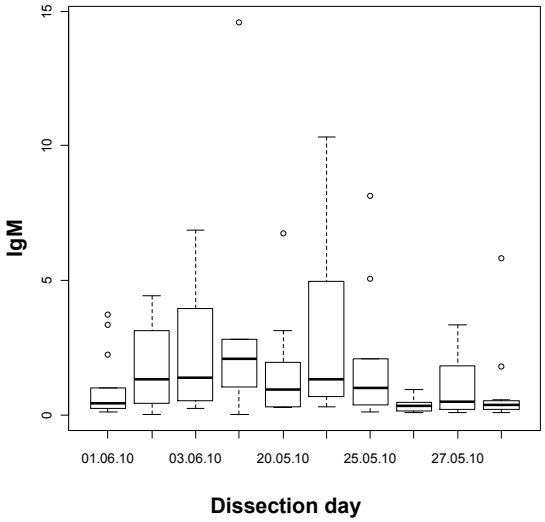


Fig. 5.2.9: Relative expression of IgM in the gut, according to the dissection date

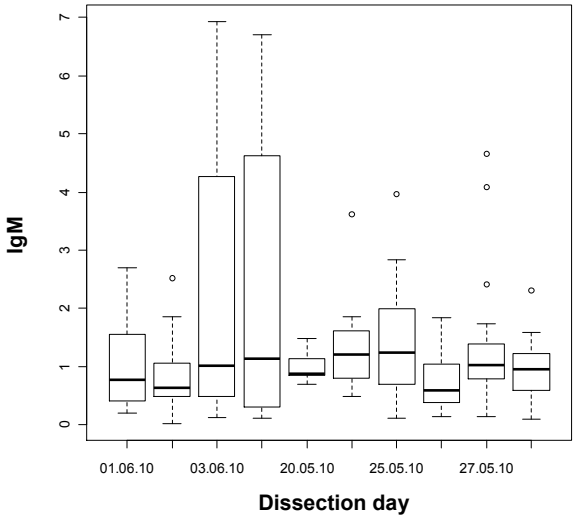


Fig. 5.2.10: Relative expression of IgM in the head kidney, according to the dissection date

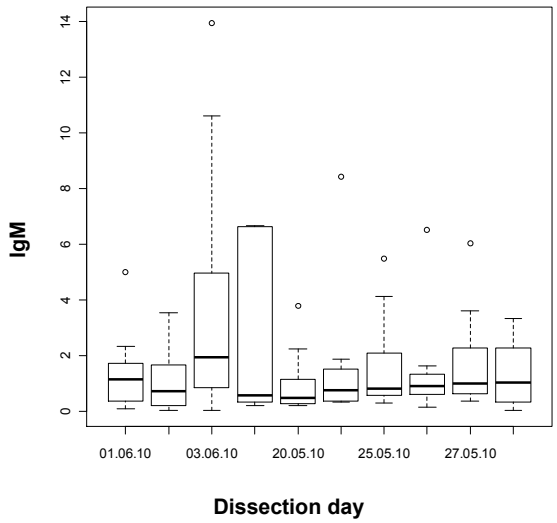


Fig. 5.2.11: Relative expression of IgM
in the spleen,
according to the dissection date

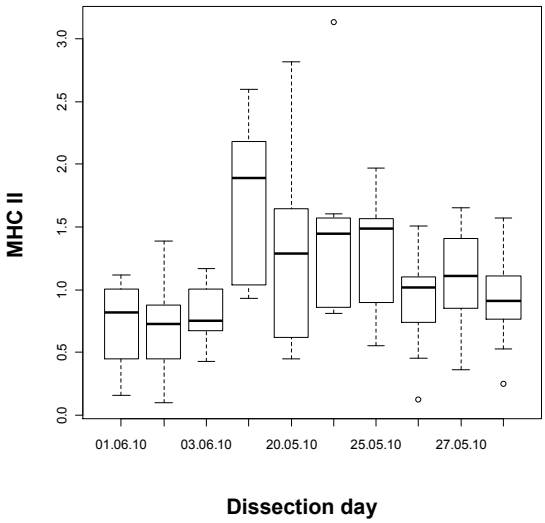


Fig. 5.2.12: Relative expression of MHC II
in the head kidney,
according to the dissection date

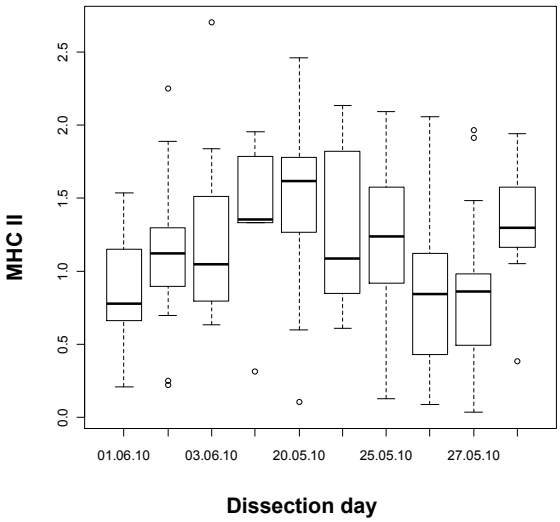


Fig. 5.2.13: Relative expression of MHC II
in the kidney,
according to the dissection date

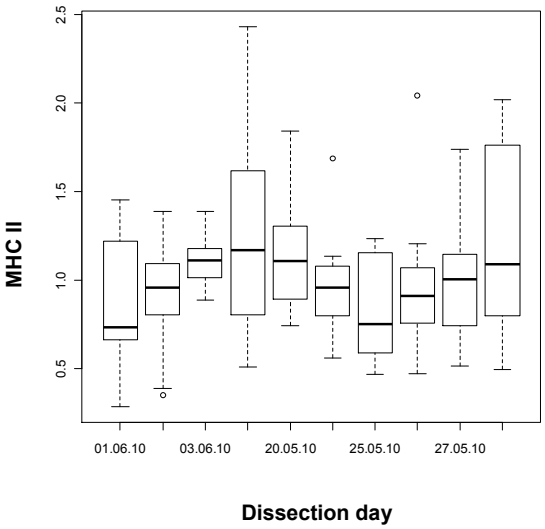


Fig. 5.2.14: Relative expression of MHC II
in the spleen,
according to the dissection date

## 5.3 Permutational MANOVA: coefficient values and the appendant individuals

Tab. 5.3.1: Expression values of the dependent variables according to certain individuals, containing four different MHC IIB allele variants (color = same individual)

| variable | individual | family | value |
|---|---|---|---|
| | 40 | L001 x L103 | 2.955666667 |
| | 85 | L001 x L103 | 3.071333333 |
| | 53 | L002 x L106 | 3.016666667 |
| | 54 | L002 x L106 | 2.941666667 |
| | 83 | L002 x L106 | 3.134333333 |
| | 104 | L002 x L106 | 3.073333333 |
| | 10 | L006 x L106 | 3.349666667 |
| | 21 | L006 x L106 | 3.239333333 |
| | 67 | L006 x L106 | 2.989333333 |
| | 93 | L006 x L106 | 3.022666667 |
| immunoglobulin value | 15 | L012 x L119 | 3.132333333 |
| | 40 | L001 x L103 | 2.6 |
| | 33 | L002 x L106 | 3.04 |
| | 55 | L012 x L119 | 5.12 |
| | 8 | L013 x L128 | 6.4 |
| | 50 | L013 x L128 | 2.59 |
| IL 1 HK | 118 | L013 x L128 | 5.24 |
| | 101 | L001 x L103 | 0.15 |
| | 19 | L002 x L106 | 0.27 |
| | 103 | L002 x L106 | 0.27 |
| | 105 | L002 x L106 | 0.23 |
| | 15 | L012 x L119 | 0.26 |
| | 64 | L012 x L119 | 0.26 |
| | 24 | L013 x L128 | 0.14 |
| | 36 | L013 x L128 | 0.22 |
| | 50 | L013 x L128 | 0.22 |
| | 51 | L013 x L128 | 0.13 |
| | 61 | L013 x L128 | 0.2 |
| IL 1 G | 62 | L013 x L128 | 0.2 |
| | 86 | L001 x L103 | 0.12 |
| IgM G | 102 | L001 x L103 | 0.15 |

| | | | |
|---|---|---|---|
| | 32 | L002 x L106 | 0.11 |
| | 58 | L002 x L106 | 0.23 |
| | 82 | L002 x L106 | 0.18 |
| | 105 | L002 x L106 | 0.02 |
| | 10 | L006 x L106 | 0.29 |
| | 46 | L006 x L106 | 0.1 |
| | 47 | L006 x L106 | 0.1 |
| | 67 | L006 x L106 | 0.09 |
| | 93 | L006 x L106 | 0.14 |
| | 29 | L012 x L119 | 0.23 |
| | 43 | L012 x L119 | 0.17 |
| | 55 | L012 x L119 | 0.24 |
| | 64 | L012 x L119 | 0.24 |
| | 66 | L012 x L119 | 0.2 |
| | 8 | L013 x L128 | 0.27 |
| | 50 | L013 x L128 | 0.26 |
| | 62 | L013 x L128 | 0.19 |
| | 120 | L013 x L128 | 0.23 |
| | 102 | L001 x L103 | 0.15 |
| | 32 | L002 x L106 | 0.37 |
| | 82 | L002 x L106 | 0.03 |
| | 105 | L002 x L106 | 0.13 |
| | 21 | L006 x L106 | 0.20 |
| | 46 | L006 x L106 | 0.14 |
| | 67 | L006 x L106 | 0.06 |
| | 80 | L006 x L106 | 0.08 |
| | 93 | L006 x L106 | 0.17 |
| | 43 | L012 x L119 | 0.08 |
| | 45 | L012 x L119 | 0.28 |
| | 50 | L013 x L128 | 0.18 |
| | 61 | L013 x L128 | 0.28 |
| IgM K | 120 | L013 x L128 | 0.23 |
| | 40 | L001 x L103 | 0.13 |
| IgM HK | 85 | L001 x L103 | 0.36 |

| | | | |
|---|---|---|---|
| | 102 | L001 x L103 | 0.13 |
| | 32 | L002 x L106 | 0.11 |
| | 82 | L002 x L106 | 0.10 |
| | 105 | L002 x L106 | 0.12 |
| | 21 | L006 x L106 | 0.49 |
| | 46 | L006 x L106 | 0.17 |
| | 67 | L006 x L106 | 0.14 |
| | 80 | L006 x L106 | 0.17 |
| | 93 | L006 x L106 | 0.19 |
| | 30 | L012 x L119 | 0.47 |
| | 44 | L012 x L119 | 0.40 |
| | 50 | L013 x L128 | 0.46 |
| | 118 | L013 x L128 | 0.30 |
| | 120 | L013 x L128 | 0.13 |
| | 101 | L001 x L103 | 0.21 |
| | 102 | L001 x L103 | 0.03 |
| | 58 | L002 x L106 | 0.38 |
| | 82 | L002 x L106 | 0.04 |
| | 105 | L002 x L106 | 0.09 |
| | 21 | L006 x L106 | 0.42 |
| | 46 | L006 x L106 | 0.15 |
| | 67 | L006 x L106 | 0.39 |
| | 80 | L006 x L106 | 0.32 |
| | 93 | L006 x L106 | 0.10 |
| | 15 | L012 x L119 | 0.23 |
| | 30 | L012 x L119 | 0.44 |
| | 76 | L012 x L119 | 0.37 |
| | 8 | L013 x L128 | 0.28 |
| | 118 | L013 x L128 | 0.27 |
| IgM S | 120 | L013 x L128 | 0.05 |

Tab. 5.3.2: Expression values of the dependent variables according to certain individuals, containing two different MHC IIB alleles (blue = low expression value; red = high expression value)

| variable | individual | family | value |
|---|---|---|---|
| IL 1 HK | 110 | L006 x L106 | 0.04 |
| IL 1 HK | 6 | L002 x L106 | 0.27 |
| Plasma | 106 | L013 x L128 | 0.341 |
| IL 1 HK | 22 | L006 x L106 | 0.36 |
| IL 1 HK | 7 | L013 x L128 | 0.39 |
| Plasma | 14 | L012 x L119 | 0.408666667 |
| Plasma | 78 | L012 x L119 | 0.411333333 |
| Plasma | 2 | L001 x L103 | 0.452666667 |
| IL 1 HK | 13 | L006 x L106 | 0.46 |
| Plasma | 28 | L001 x L103 | 0.456333333 |
| IL 1 HK | 28 | L001 x L103 | 0.50 |
| IgM HK | 59 | L002 x L106 | 2.41 |
| IL 1 G | 28 | L001 x L103 | 2.73 |
| IgM HK | 37 | L006 x L106 | 2.83 |
| IL 1 G | 106 | L013 x L128 | 2.92 |
| IgM S | 73 | L002 x L106 | 3.34 |
| IgM S | 59 | L002 x L106 | 3.52 |
| IgM S | 109 | L006 x L106 | 3.54 |
| IgM S | 17 | L002 x L106 | 3.79 |
| IgM HK | 31 | L012 x L119 | 3.97 |
| IgM K | 69 | L006 x L106 | 3.98 |
| IgM HK | 69 | L006 x L106 | 4.08 |
| IL 1 G | 87 | L001 x L103 | 4.25 |
| IgM HK | 5 | L002 x L106 | 4.62 |
| IL 1 G | 121 | L006 x L106 | 4.83 |
| IgM K | 39 | L006 x L106 | 4.95 |
| IgM S | 91 | L006 x L106 | 5.02 |
| IgM G | 31 | L012 x L119 | 5.05 |
| IgM K | 31 | L012 x L119 | 5.31 |
| IgM S | 31 | L012 x L119 | 5.48 |
| IgM G | 73 | L002 x L106 | 5.82 |
| IgM S | 5 | L002 x L106 | 6.67 |
| IgM G | 17 | L002 x L106 | 6.73 |
| IgM HK | 121 | L006 x L106 | 6.94 |
| IgM G | 37 | L006 x L106 | 8.15 |
| IgM G | 22 | L006 x L106 | 10.33 |
| IgM K | 37 | L006 x L106 | 12.18 |
| IgM S | 121 | L006 x L106 | 13.95 |