# Infection-dependent MHC expression in the three-spined stickleback, Gasterosteus aculeatus

## Diplomarbeit

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# 1. Summary

The study focused on two main topics. On the one hand primers and a PCR protocol were developed to find a suitable housekeeping gene for quantitative real-time PCR. On the other hand this study explored the expression of genes related to an immune response in cell cultures and organs of living fish. The main focus lay on the genes of the major histocompatibility complex (MHC).

The MHC has been studied for several years. This is mainly because of the central role of MHC molecules in the adaptive immune response and the high number of different alleles in vertebrate populations. Recent research suggests that – in addition to the variation in sequences of the genetic code – genetically based variations in expression of MHC genes do play a crucial role in evolutionary change. Up to now it has been shown that expression is heritable, i.e. expression has a genetic basis in three-spined stickleback (*Gasterosteus aculeatus*). Furthermore a negative correlation, which suggests a compensatory regulation of MHC genes, between expression levels and number of different MHC alleles has been shown. A positive correlation between MHC expression and parasite load has been shown as well.

To date, research has focused on beta-actin as a housekeeping gene in expression analysis on *Gasterosteus aculeatus*. Recent research in other taxa suggests that beta-actin is not the most stable gene depending on the tissue and the taxa of the survey.

In the study primers were developed in order to measure gene expression of candidate housekeeping genes and the immune response genes. Therefore sequences from the EST-library and

the whole genome shotgun library of the stickleback have been used to find exon-intron boundaries. The mRNA has been amplified in a PCR reaction and the product has been sequenced to check the identity of the PCR product.

Ten candidate genes including beta-actin were tested for stable gene expression in three different tissues and under several treatments. The data showed that beta-actin is a good housekeeping gene in most of the tissues and treatments, but other genes proved to be more stably expressed. As it was one of the most stable genes in all examined tissues, especially the L13a ribosomal binding protein seems to be a good candidate to replace beta-actin as a general housekeeping gene for expression analysis.

In another experiment the expression of MHC genes was stimulated in cell cultures with lysates of two widespread parasites of the three-spined stickleback, i.e. *Diplostomum pseudospathacaeum* and *Camallanus lacustris*. In order to enhance comparability, cells from the same organ have been infected in these cultures. Therefore RNA-Extraction and real-time PCR were improved in a way that allowed us to measure gene expression and controls in 15 different PCR runs from only $10^5$ cells. While MHC expression was higher in cells of formerly infected fish, no effect of cell treatment could have been shown. However the cell's survival rate was higher in cultures that were infected with parasite lysate.

Furthermore gene expression was measured at five times after being infected with *Diplostomum pseudospathacaeum* to explore the expression of genes correlated to immune response during the first eleven days.

# 2. Deutsche Zusammenfassung

Die folgende Arbeit zielt in zwei verschiedene Richtungen. Einerseits wurden Primer für verschiedene Gene und ein PCR protocol entwickelt, um ein geeignetes Haushaltsgen für die quantitative real-time PCR zu finden. Andererseits wurde, sowohl in Zellkulturen als auch in lebenden Fischen, die Expression verschiedener, für die Immunabwehr relevanter, Gene untersucht. Dabei wurden hauptsächlich die Gene des Haupthistokompatibilitätskomplex (MHC) analysiert.

Der MHC ist schon seit einigen Jahren Gegenstand intensiver Forschung. Dies ist einerseits seiner zentralen Rolle in der Immunabwehr und andererseits der überraschend hohen Zahl an unterschiedlichen Allelen in Wirbeltierpopulationen zurückzuführen. In letzter Zeit mehren sich Hinweise, dass nicht nur Variation des genetischen Codes, sondern auch die Expressionsraten der MHC Gene eine Rolle in der Evolution spielen. Bisher konnten eine genetische Basis der Genexpressionsraten, eine kompensatorische Regulation bei geringer Zahl an MHC Allelen und eine Expressionserhöhung bei Infektion mit Parasiten in unserem Modellorganismus, dem Dreistacheligen Stichling (*Gasterosteus aculeatus*), gezeigt werden. An diese Ergebnisse möchte ich mit dieser Arbeit anknüpfen.

Alle bisherigen Arbeiten an *Gasterosteus aculeatus* haben für die Expressionsmessung der MHC-Gene Beta-Aktin als Haushaltsgen verwendet. Nachdem jedoch schon bei anderen Organismen gezeigt wurde, dass es bessere Kandidaten gibt, wurden diverse Kandidatengene in verschieden Organen (Milz, Kopfniere und Kiemen) und unter unterschiedlichen Bedingungen (Infektion von Fischen oder Zellkulturen)

gemessen, um das jeweils stabilste Gen zu bestimmen. Zu diesem Zweck wurden Primer konstruiert, um die Genexpression der Kandidaten- und Immunabwehrgene in der quantitativen real-time PCR zu bestimmen. Sequenzen der EST-library und der whole genome shotgun library wurden verwendet, um Exon-Intron Grenzen zu bestimmen und die Produkte der Amplifikation wurden sequenziert, um sicher zu gehen, dass das gewünschte Produkt erzeugt wurde.

Dabei zeigte sich, dass Beta-Aktin zwar häufig ein gutes Haushaltsgen ist, es aber andere Gene gibt, die je nach Bedingungen und Organ stabiler exprimiert werden. In den Experimenten hat sich insbesondere das L13A ribosomal binding protein als Haushaltsgen geeignet gezeigt. Erfreulicherweise war es auch in allen Organen immer eines der stabilsten Gene, so dass das L13 durchaus Beta-Aktin als Haushaltsgen ersetzen könnte.

In einem weiteren Experiment wurde versucht die Expression von MHC-Genen mit Lysaten zweier weitverbreiteter Parasiten, *Diplostomum pseudospathacaeum* und *Camallanus lacustris* in Zellkulturen zu stimulieren, um das Problem der Vergleichbarkeit von Messungen zu lösen. Es ist gelungen, die RNA-Extraktion und die real-time PCR zu verbessern. So konnten aus der RNA von 100000 Zellen in 15 PCR-Läufen die verschiedenen Gene gemessen werden. Während die MHC II Expression in Zellen vorher infizierter Fische höher war, konnte kein Effekt der Lysat-Infektion gezeigt werden. Allerdings hat diese die Überlebensrate der Zellen in den Kulturen erhöht.

Weiterhin wurde der Verlauf der Expression von Immunabwehrgenen in den ersten Tagen nach der Infektion charakterisiert. Auch hier konnte kein signifikanter Effekt der Infektion bei der Expression der MHC Gene beobachtet werden.

# 3. Introduction

## 3.1. Major histocompatibility complex

The major histocompatibility complex (MHC) is a large cluster of genes coding for peptide-binding glycoprotein. These proteins are presented to T cells and play a major role in the adaptive immune response of vertebrates. For several decades the MHC has been studied due to its outstanding genetic polymorphism (Apanius et al. 1997; Sato et al. 1998). Two major hypotheses try to explain the high number of MHC alleles in natural populations.

On the one hand it is assumed that balancing selection is responsible for the maintenance of the high number of alleles. Significant association between MHC alleles and diseases have been demonstrated (Grimholt et al. 2003; Langefors et al. 2001). As most animals have to deal with a number of parasites and diseases, the best allele depends on the specific environment (Kalbe and Wegner 2004).

On the other hand advantages of MHC heterozygosity (Penn et al. 2002) have been shown. More MHC alleles can express a higher variety of MHC molecules. At first sight it seems to be an advantage for an individual fish to possess the maximum number of MHC alleles. However Wegner et al. (2003) stressed the superiority of an intermediate number of alleles. The reason for an optimum rather than a maximum of MHC alleles is as follows. With a higher number of alleles more self-reactive T cell lines get eliminated (Nowak et al. 1992). Consequently the advantage of a higher number of MHC alleles is outweighed by the disadvantage of the eliminated T cell lines. Thus the efficiency of the immune

system is reduced when the number of alleles exceeds the optimal number. Both hypotheses, the advantages of heterozygosity and balancing selection, contribute to the extraordinary number of MHC alleles.

Recent research has shown that genetic components in gene transcription as well as genetic variability in coding sequences are of evolutionary importance (Oleksiak et al. 2002; Wegner et al. 2006). Therefore fitness is a function of both the quality (sequence) and the quantity (expression level) of an allele or its transcript. It has been worked out that gene expression is also important in the case of MHC genes. Wegner et al. (2006) demonstrated that the expression of MHC class IIB (MHC II) alleles in natural populations of three-spined stickleback (*Gasterosteus aculeatus*) was positively correlated with parasite load. This indicated that the immune system response was increased with frequent infections. The same study suggested that the expression of MHC alleles had a genetic basis. A negative correlation between the average number of different MHC alleles per fish and the MHC expression level has also been found. This indicated a compensatory up-regulation in fish with a low number of MHC allele variants. These results implied that MHC expression levels are relevant for the immune response in natural populations of *Gasterosteus aculeatus*.

## 3.2. Real-time PCR

Gene expression levels can either be measured at a protein or at an mRNA level. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) is a technique for the quantification of gene expression at the mRNA level that combines the advantages of specificity, sensitivity, speed, throughput and

reproducibility. Therefore it is a powerful tool in experimental research (Haller et al. 2004).

With the SYBR-Green chemistry it is possible to follow the duplication of the amplicon at every cycle of the PCR, because SYBR-Green binds to double-stranded DNA. Therefore mRNA has to be transcribed to complementary DNA (cDNA) by reverse transcription. Then the cDNA is amplified during the PCR. Fluorescence of SYBR-Green increases with the amount of cDNA that is used as a template. Because SYBR-Green binds to any DNA in the PCR, including primer-dimers, false positives might occur (Peters et al. 2004). This can be avoided with a dissociation analysis of the amplified product. Furthermore the efficiency of the PCR has to be taken into account to calculate the amount input cDNA.

The mRNA level itself is not only influenced by regulation of gene expression. Many other traits, e.g. nutrition, differences in size and components of the tissue, etc. can influence the mRNA level of the target gene. Therefore it is a straightforward solution to measure the mRNA of the target gene relative to a housekeeping gene that is supposed to reflect the status of the fish or tissue and not to be regulated in the selected treatments (Bustin 2002).

## 3.3. Thesis outline

In the first part of this study the PCR assay was developed. Primers were designed to amplify cDNA, while genomic DNA (gDNA) was not amplified. Several annealing temperatures and primer concentrations were tested to optimise specificity and efficiency of the PCR.

In the following experiments the relative-quantification-method was used to measure the expression of the MHC genes. Therefore it is necessary to measure MHC expression relative to a suitable housekeeping gene. In the case of *Gasterosteus aculeatus,* beta-actin was used as the housekeeping gene (Wegner et al. 2006) and until now no other genes have been tested for stability and compared with beta-actin. Recent studies in other taxa (Bas et al. 2004; Radonic et al. 2004; Szabo et al. 2004) suggest that beta-actin is probably not the best choice. Furthermore it has been found (Bustin 2000) that the most stable gene is not necessarily the same in different organs. Therefore the second part of this study was to test diverse genes for their suitability as housekeeping genes in different tissues and under different treatments.

Most of these genes had not been sequenced before in *Gasterosteus aculeatus*. Thus homologous genes in other teleostei were used to find the sequences of the genes in the three-spined stickleback. The homologous genes were aligned with the expressed sequence tags (EST)-library and the whole genome shotgun (WGS) library of the three-spined stickleback. Then EST and WGS sequences were aligned to determine exon-intron boundaries.

The third part of the thesis was supposed to solve another problem of the measurement of MHC expression, i.e. the comparability of the expression value. When fish are infected with parasites it is always necessary to compare the MHC expression of one fish with the expression of another fish. Differences in genetical or physiological traits may contribute to additional variation in gene expression between fish. One way to overcome this problem is to inbreed fish to keep genetic background as similar as possible (Wegner et al. 2006).

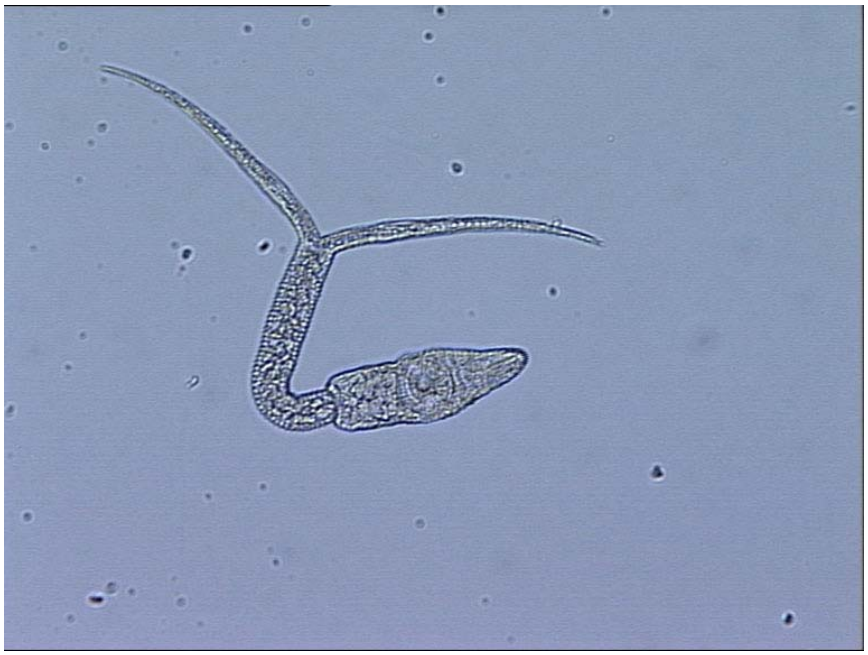

**Figure 1**: Cercaria of *Diplostomum pseudospathacaeum* before infection of *Gasterosteus aculeatus*; M. Kalbe.

In this study another path was followed. Instead of infecting fish, a system was developed that was based on the measurement of MHC expression of cell cultures. Cells of the same organ were infected with different parasites and the cell's MHC expression was measured in the cell cultures.

So far Wegner et al. (2006) found an increase of MHC II expression 15 days after infection. In the fourth part of the study the time course of an immune response was the main focus. Therefore the kinetics of MHC expression was explored. Two other genes that are part of the immune response were included in this experiment to study variation of gene expression within the first eleven days after infection.

The study focused on three organs of *Gasterosteus aculeatus*. The first is the spleen, which is the main organ of the acquired immune response in sticklebacks. Therefore it is the organ of choice to measure an increase of MHC expression after an infection. The second is the head kidney, which is the main organ

of the innate immune response. A cell proliferation after an infection should be strongest in this organ. The third organ is the gills. Wegner et al. (2006) found very high rates of MHC expression in the gills. It is the first line of defence against free floating parasites and therefore the gills are an obvious candidate for an immune response (Koppang et al. 1998)

# 4. Material and methods

## 4.1. Fish for the infection experiment

Lab-bred sticklebacks for the infection experiment were full siblings. Experimental fish were about two years old and had been raised as described for family LL5 in Rauch et al. (2006). Half of the fish were infected five times with *Diplostomum pseudospathacaeum* (30 individuals per infection and fish) in summer 2005, while the control group had never been infected before. Both the formerly infected group and the control group (35 fish per group) were kept in separate aquaria at 18°C and 16 hours of daylight until the start of the experiment.

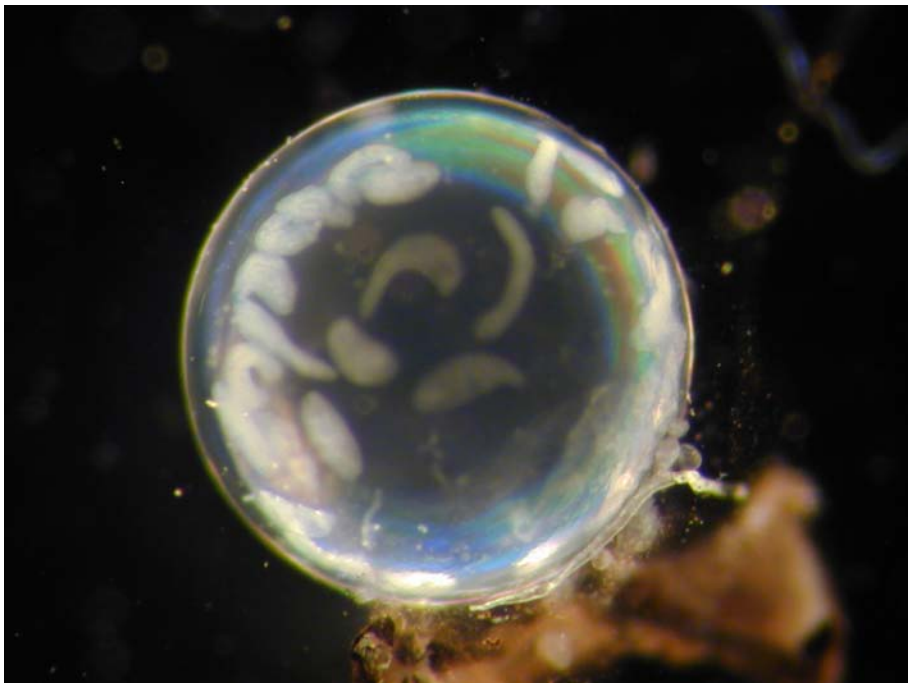

**Figure 2**: *Diplostomum pseudospathacaeum* in an eye lense of *Gasterosteus aculeatus*; M.Kalbe.

Before infection, each fish was transferred to a separate, small aquarium. Each of the two groups was divided into a treatment

and a control group. The two groups to be treated were infected separately with 50 individuals of *Diplostomum pseudospathacaeum* per fish. After 6, 21, 43, and 140 hours three fish of each group were killed by a cut through the neck, while the group killed after 260 hours consisted of four fish each.

**Figure 3**: *Camallanus lacustris*; M.Kalbe.

The spleen, the head kidneys and the gills were dissected from the fish and kept separately in 150 µl of RNA-later (Ambion) for instant preservation of mRNA.

The eye lenses were taken from each fish, and living parasites were counted to confirm former infection (Figure 2) and to make sure fish were successfully infected during the experiment.

Two days after infection the remaining fish were put back together into the aquaria they were kept until the start of the experiment.

## 4.2. Treatment of cell cultures

Lab-bred fish used in the experiment were full siblings. The parents were wild caught fish from the Großer Plöner See (Schleswig-Holstein, Germany). Half of the fish had been infected twice before the experiment. In December 2004 these fish were infected with 20 *Diplostomum pseudospathacaeum*, 11 *Anguilicola crassus* and 5 *Camallanus lacustris* each. In May 2005 another infection followed with 20 *Diplostomum pseudospathacaeum*, 7 *Anguilicola crassus* and 5 *Camallanus lacustris* per fish, while the other half of the fish was a control group and was not infected before the experiment. Cell cultures were prepared from two fish of the control group and two fish that had been infected before. During the dissection the fish were screened for parasites to ensure former infection.

**Figure 4**: *Anguilicola crassus* (200x); M.Kalbe.

All steps for cell culture preparation were performed on ice and only refrigerated media and cooled centrifuges were used. The cell suspensions from head kidneys and spleens were prepared

by forcing the tissues through a 40µm nylon screen (BD-Falcon, USA). Then cells were washed twice (4°C, 10 min 550 x g) with RPMI 1640 (Sigma-Aldrich) diluted with 10% (v/v) distilled water. Numbers of viable cells (exclusion of propidium iodide positive cells) were enumerated by means of flow cytometry. Total cell numbers were determined with the standard cell dilution assay (SCDA), (Pechhold et al. 1994) in a modified form (Scharsack et al. 2004). Washed cells were transferred to individual flow cytometer tubes; 2 x $10^5$ green fluorescent standard particles (4µm, Polyscience, USA) and propidium iodide (2 mg/L, Sigma Aldrich) were added to each tube. Fluorescence intensities at wavelengths of 530 nm and 585 nm were acquired at log scale with a flow cytometer (FACSCalibur, Becton and Dickinson, USA). Flow cytometric data were analysed with the CellQuest Pro 4.02 software for acquisition and analysis. Standard particles (green fluorescence positive) were discriminated from viable cells (propidium iodide-negative, green fluorescence negative). Absolute numbers of cultivated cells in individual wells were calculated according to: N [vital cells] = Events [vital cells] x Number [standard beads] / events [standard beads]. The cell suspensions were diluted to a density of 4 x $10^6$ vivid cells/ml.
Every well (TPP tissue culture test plate 96) contained 150 µl cell medium, 25µl cell suspension ($10^5$ cells) and 25 µl parasite lysate (3mg/ml for *Diplostomum pseudospathacaeum*, 50 and 5µg/ml for *Camallanus* lacustris). Furthermore a positive control was included in the experiment by adding 25µl pokeweed mitogen (PWM, 16µg/ml) per well, while the negative control contained an extra 25µl of cell medium. The cell cultures were kept at 18°C and 2% $CO_2$ (v/v) after infection. After 19 and 40 hours replicates of cell cultures were held on ice for 15 minutes to stop cell activity. Then the cell

cultures were resuspended and transferred to single PCR tubes (Biozym, 0.5 ml). An aliquot of 25µl had been taken off to determine the number of vivid cells by means of flow cytometry. Cell suspension was centrifuged at 550 x g for 10 minutes, the supernatant was discarded and the cells were kept in 175 µl of RNA-later (Ambion) for instant preservation of mRNA.

## 4.3. Primer design

The MHC I and MHC II genes of *Gasterosteus aculeatus* have already been sequenced in our lab (Reusch and Langefors 2005; Schaschl and Wegner). The exon-intron boundaries were determined by aligning the sequences with the EST-library of *Gasterosteus aculeatus* (GenBank, http://www.ncbi.nlm.nih.gov/). Then the genomic sequences and the EST-sequences (mRNA) were aligned with the software BioEdit (Hall 1999).

All other sequences were retrieved from GenBank. Homologous genes in other teleostei (e.g. zebra fish or salmon) were used to find the corresponding sequences in *Gasterosteus aculeatus*. Then the homologous sequences were blasted against the stickleback sequences in the EST-library to find the mRNA sequences. These EST-sequences were blasted to find the corresponding sequences in the whole genome shotgun (WGS) library of the three-spined stickleback http://www.ncbi.nlm.nih.gov/blast/mmtrace.shtml). Finally the EST-sequences and the sequences of the WGS-library of the stickleback were aligned with the program BioEdit to determine exon-intron boundaries.

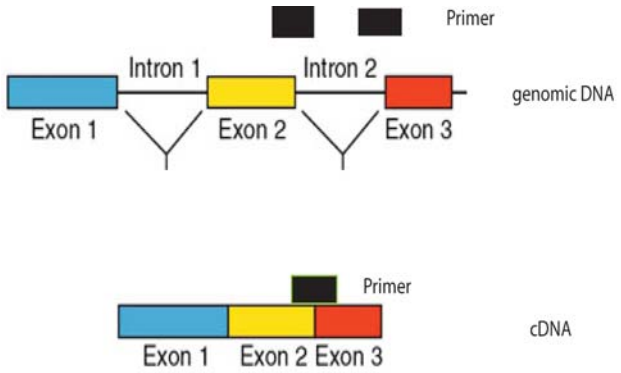


**Figure 5:** Primer design to avoid amplification of genomic DNA; Biology learning center, University of Arizona.

Primers were designed by using the primer analysis software Primer Express 2.0 (Applied Biosystems). One of the primers (MWG BIOTECH, Ebersberg, Germany) of every pair was designed to cover an exon-intron boundary, as outlined in (Figure 5). Since there are no introns in the cDNA, both primers can bind to the cDNA. The introns in the gDNA prevent the primer from binding to the gDNA and therefore from amplifying the product. Amplicon length was between 210 and 240 base pairs to avoid different efficiencies of PCR reactions due to different length of the amplicon. Primer specifications are summarised in Table 1.

## 4.4. Sequencing of genes

The amplification of the cDNA was performed on a LightCycler 480 Instrument (Roche) with a 384-well block. The PCR assay contained a final volume of 20µl, including 2µl 10x PCR Buffer II (Applied Biosystems), 2.4 ng $MgCl_2$, 0.5 pg 10xdNTP, 0.1µl Ampli Taq Gold (Applied Biosystems), 1µl (1000nM final concentration) of each primer and 2µl cDNA template. PCR was carried out with initial 10 min at 95 °C followed by 45 cycles of 20 sec denaturation at 94 °C and 1 min annealing/extension at 68 °C. 10 µl of PCR product was mixed with 10µl SYBR-Green Master Mix (Roche) and dissociation analysis (60 °C – 95 °C) was performed to assure specificity of amplification product.

**Table 1**: Primer specifications of target genes and housekeeping candidate genes.

| Immune response genes | | | | | | |
|---|---|---|---|---|---|---|
| Gene | Abbreviation | | 5´- Sequence – 3´ | Bp | Tm (°C) | %GC |
| MHC class I | MHC I | For | CCAAAGTTCCCAGAGTTTGTG | 21 | 56 | 48 |
| | | Rev | GTGGACACCTCCAGTTTGG | 19 | 55 | 58 |
| MHC class II | MHC II | For | CTGAAGGACATCGAGTACATCG | 22 | 56 | 50 |
| | | Rev | GGCTCAGCGGACTTAGTCAG | 20 | 56 | 60 |
| Caspase 3 | Cas3 | For | AGCGCCAGGATACTACTCATG | 21 | 59 | 52 |
| | | Rev | GGGGAGAAATACATCTCTTTGG | 22 | 58 | 46 |
| Mangan superoxid dismutase | Mn | For | TGTGCTGGTGGCCTCG | 16 | 57 | 69 |
| | | Rev | GGTCACATCTCCCTTTGCG | 19 | 58 | 58 |
| Candidates for housekeeping gene | | | | | | |
| Gene | Abbreviation | | 5´- Sequence – 3´ | Bp | Tm (°C) | %GC |
| Beta-Actin | BA | For | GCGTGGCTACTCCTTCACC | 19 | 57 | 63 |
| | | Rev | AGGACTTCATACCGAGGAAGG | 21 | 56 | 52 |
| Elongation Factor | EF | For | CCACCGTTGCCTTTGTCC | 18 | 58 | 61 |
| | | Rev | TGGGACTGTTCCAATACCTCC | 21 | 57 | 52 |
| Hypoxanthine phosphoribosyltransferase 1 | HPRT | For | GACGCAGATATGGTTCAGATCTC | 23 | 57 | 48 |
| | | Rev | GTCTTGATTGTGGAGGATATTATCG | 25 | 57 | 40 |
| L13A ribosomal binding protein | L13 | For | CACCTTGGTCAACTTGAACAGTG | 23 | 58 | 48 |
| | | Rev | TCCCTCCGCCCTACGAC | 17 | 58 | 71 |
| RNA-Polymerase II | RNA | For | TTAACAGGTGGGGGGTGC | 18 | 58 | 61 |
| | | Rev | AGCTCAAGAGCAGAAAGATCCC | 22 | 58 | 50 |
| TATA-Box binding protein | TATA | For | GGAGTTCATGTTGCAGGTTTTC | 22 | 57 | 45 |
| | | Rev | CGTTCCTCTTCCTATTGAAGGC | 22 | 58 | 50 |
| Ubiquitin | Ubi | For | AGACGGGCATAGCACTTGC | 19 | 58 | 58 |
| | | Rev | CAGGACAAGGAAGGCATCC | 19 | 57 | 58 |
| Glyceraldehyd-3-phosphate dehydrogenase | GAPDH | For | TGGTCAGCTTACCGTTGAGC | 20 | 58 | 55 |
| | | Rev | CCCCCTGGCTAAAGTCATCC | 20 | 59 | 60 |
| Beta-2-microglubolin | B2M | For | AGACTATGCCTGGGAATCAAAC | 22 | 56 | 45 |
| | | Rev | GAAGATGTGTTGAATAGAAGCTGG | 24 | 56 | 42 |
| 18S rRNA | 18S | For | GACTCCGGTCCTATTTTGTGG | 21 | 57 | 52 |
| | | Rev | GCTAGTTGGCATCGTTTATGG | 21 | 56 | 48 |
| Glucose-6-phosphate dehydrogenase | G6PDH | For | GGACGGTGTTCCTTTCATCC | 20 | 58 | 55 |
| | | Rev | TAGGTGAGGTCCAGCTCGG | 20 | 57 | 63 |
| Peptidylprolyl isomerase A | PPIA | For | CCCCTGGCTGGACGG | 15 | 58 | 80 |
| | | Rev | TAAAAATGACGGGAGGGGG | 19 | 58 | 53 |

10 µl of PCR product was purified with the QIAquick PCR Purification Kit (Qiagen), before it was sequenced with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to manufacturer's protocol. The following cycle sequencing PCR contained a final volume of 10µl, including 2µl Cycle Sequencing Mix, 1µl of forward or reverse primer (final concentration 1000 nM), 1µl of Buffer and

2µl of PCR product. It was carried out with an initial 1min at 96°C followed by 30 cycles of 10 seconds denaturation at 96°C and 4 min annealing/extension at 60 °C. The sequencing of the PCR products followed standard protocols (ABI 3100 capillary sequencer). The sequences obtained were analysed with the software Codoncode-Aligner (Codon Code Company 2000-2004) and were finally blasted in GenBank to check the identity of the amplicon.

## 4.5. RNA extraction and reverse transcription

The RNA was extracted with the NucleoSpin RNA Kit (Macharey-Nagel). While the extraction of organs followed manufacturer's protocols, the extraction from cell cultures was performed with 175µl buffer RA1, 1.75µl ß-mercaptoethanol and 175µl of ethanol. Total RNA was eluted in 2*18µl per sample. The reason for this was the low amount of cells. All other steps of extraction of the cell cultures were performed according to manufacturer's protocol.

The synthesis of cDNA was performed with the First-Strand cDNA Synthesis Kit (Amersham Biosciences) according to the manufacturer's protocol. The synthesis of cDNA was performed with 2.5 fold of the components to increase cDNA yield.

The cDNA was precipitated for purification, because some of the components, especially the Reverse Transcriptase, were shown to inhibit the following PCRs (Becker et al. 2005; Liss 2002). After the reverse-transcription, the following components (all molecular biology grade) were added to a total volume of 192.25 µl according to the protocol of Becker et al. (2005):

- 37.5 µl of cDNA
- 0.75 µl Glycogen (Ambion)
- 937.5 ng Poly dA (Amersham Pharmacia)
- 2.5 µl 2 mM Sodium-Acetate (pH 4)
- Mixing, then add
- 150 µl cold (-20°C) Ethanol (100%)
- Mixing again.

The samples were kept at -20°C over night. On the following day samples were centrifuged at 13,000 x g for 1 hour, followed by removal of the supernatant and an addition of 375 µl cold (-20°C) Ethanol (75%). Then the samples were incubated for 10 min at -20°C and centrifuged at 13,000 x g for 15 min. After the removal of the supernatant the samples were incubated at 45°C for at least 15 min to dry the pellet.
Afterwards the pellet was redissolved in 100 µl nuclease-free water (Sigma) and the samples were incubated at 45°C for 15 min to enhance pellet redissolution. The cDNA was stored at -20°C until use in quantitative real-time PCR. The remainder of the mRNA was stored at -20°C until it was used as a –RT control to check for residuals of gDNA after DNAse reaction. So the –RT control was supposed to contain neither gDNA nor cDNA.

## 4.6. Real-time PCR

The quantitative real-time PCR was performed on a LightCycler 480 Instrument (Roche) with a 384-well block. Before the PCR, cDNA template of whole organs was diluted 10-fold. Every PCR assay contained a final volume of 20µl, including 10µl SYBR-Green Master Mix (Roche), 5µl diluted cDNA template, 3µl water and 1µl (1000nM final concentration) of each primer.

PCR was carried out with an initial 10 min hot start polymerase activation at 95 °C followed by 45 cycles of 20 sec denaturation at 94 °C and 1 min annealing/extension at 68 °C. Dissociation analysis (60 °C – 95 °C) directly after PCR were performed to assure specificity of amplification products. Afterwards all PCR product curves were analysed with the software LinReg PCR (Ramakers et al. 2003) to determine the efficiency of every single PCR reaction by linear regression. Then $N_0$ was calculated with LinReg PCR and had given an absolute amount of the number of cDNA copies in the reaction before the PCR started. So the relative expression is given as the ratio of $N_{0\,(\text{target gene})}$ / $N_{0\,(\text{housekeeping gene})}$.

Each run included two no template controls per sample, one no reverse transcription control per sample to identify residual gDNA in samples after DNAse reaction and a one positive PCR control per plate.

The MHC I, MHC II and BA primers were tested with final concentrations of 333 nM, 667 nM and 1000 nM each. Every pair of primer was run with three cDNA templates to optimise efficiency and specificity of the PCR assay.

## 4.7. Housekeeping genes

In order to find suitable housekeeping-genes for the study 10 candidate genes were evaluated (Table 1). The first procedure for addressing housekeeping genes was implemented in the software Bestkeeper (Microsoft excel sheet available at http://www.gene-quantification.de/bestkeeper.html#download). Bestkeeper calculates the standard deviation between the whole data set and the gene with the lowest standard deviation (SD) is

proposed most suitable (Pfaffl et al. 2004). Every gene with a SD higher than 1 is rejected as a housekeeping gene. Bestkeeper calculates the Bestkeeper´s Index, which is a virtual housekeeping gene. The virtual Ct value is given as the geometric mean of the remaining housekeeping candidates. Instead of using raw Ct values for the calculation, Bestkeeper was run with the logarithmic $N_0$ values to consider PCR´s efficiency.

The second programme that was used to determine the best candidate was the software GeNorm (Microsoft excel programme available at http://medgen.ugent.be/~jvdesomp/genorm/). The underlying assumption of this particular programme is that ratios between samples of uniformly expressed, non-normalised housekeeping genes should remain regular. Vandesompele´s (2002) software determines the pair-wise variation between all other housekeeping genes as the standard deviation of the logarithmically transformed ratios of expression levels. In iterative steps the gene with the most irregular expression is excluded from further analysis. Therefore the last two genes cannot be resolved from each other

The third programme to select the housekeeping gene was the software NormFinder (Andersen et al. 2004). (Microsoft excel sheet available at http://www.mdl.dk/publicationsnormfinder.htm). NormFinder calculates the stability of the candidate genes based on an estimate of the inter- and intra-group variation. It calculates the most stable genes and recommends a combination of two genes as a housekeeping gene.

## 4.8. Data analysis and statistical methods

All statistical analysis was done with the software SPSS 11.5 (SPSS Inc 1989 – 2005). In the cell culture experiment gene expression and the number of cells were analysed. The relative MHC expression data was normally distributed (Kolmogorov-Smirnov Test) after log transformation; number of cells was normally distributed as well. Hence the prerequisites of the ANOVA and a univariate analysis of variance are fulfilled.

An effect of the treatment in the infection experiment was analysed with a one-sample ANOVA. The analysis of an effect of the time after infection had to take into account that data points are sorted. Therefore the data was analysed with a univariate analysis of variance. To achieve normal distribution of the data, all relative expression values were log transformed. Normal distribution was tested with the Kolmogorov-Smirnov Test. Only the data of the Mn gene in spleens and head kidneys was still significantly different from normal distribution, but the Levene´s test confirmed homogeneity of the distribution. Therefore prerequisites of the ANOVA were fulfilled. The significance level used for all statistical tests was $p < 0.05$.

# 5. Results

## 5.1. Evaluation of PCR conditions and primers

All primer pairs were tested in PCRs with cDNA and gDNA. They were supposed to amplify a single product with a cDNA-template, but not to amplify a gDNA-template, as shown in Figure 6 and 7 for the Cas3 gene.

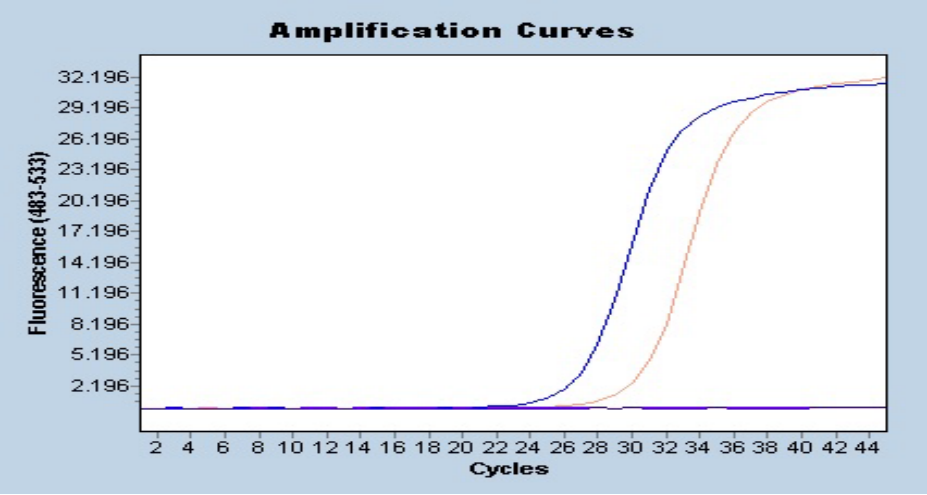


**Figure 6**: Cas3 primers amplify two cDNA templates, while there is no amplification of the two gDNA templates.

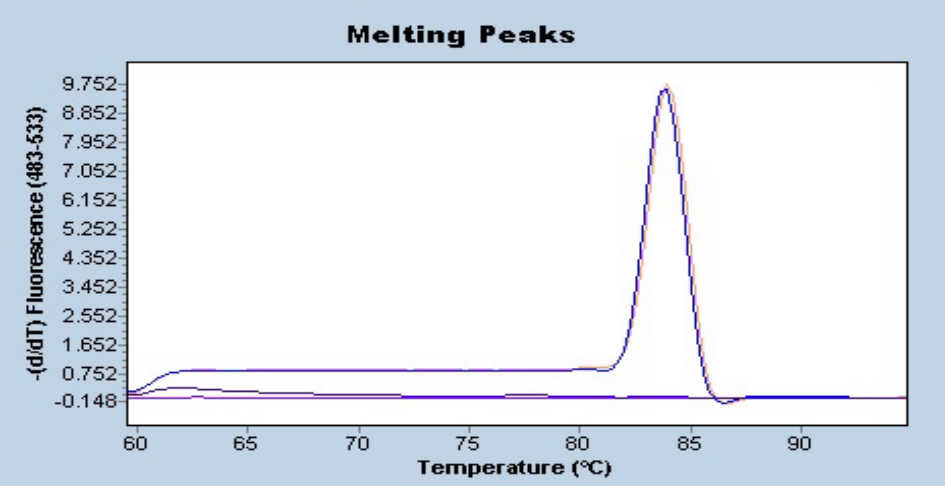


**Figure 7**: Melting curves of the amplification product of the Cas3 primers show single products from cDNA templates, while gDNA templates are not amplified.

All primers tested did not amplify gDNA except the 18S primers. The reason for this is that no exon-intron boundaries were found in the 18S gene. Consequently the 18S primers amplify both cDNA and gDNA. The no-reverse-transcription control showed that the DNAse reaction during mRNA extraction was not always successful (data not shown). Therefore some of the samples contained cDNA and gDNA during PCRs. The PPIA and the G6PDH gene were excluded from further analysis, because no product was amplified during the PCR.

In order to optimise specificity and efficiency of the PCR, the assay was tested (MHC I, MHC II, BA) with both several concentrations of forward and reverse primer (Table 2) and a variety of annealing temperatures (60 °C – 95 °C, data not shown).

Table 2: Efficiency +- SD of the PCR reaction (MHC I) with three cDNA samples.

| Efficiency of PCR (MHC I) | | Concentration of reverse primer (nM) | | |
|---|---|---|---|---|
| | | 1000.00 | 667.00 | 333.00 |
| Concentration of forward primer (nM) | 1000.00 | 1.91 +- 0.02 | 1.88 +- 0.01 | 1.88 +- 0.01 |
| | 667.00 | 1.89 +- 0.04 | 1.84 +- 0.02 | 1.87 +- 0.02 |
| | 333.00 | 1.85 +- 0.01 | 1.84 +- 0.05 | 1.81 +- 0.03 |

Furthermore all amplification products were successfully sequenced and blasted in GenBank. The most similar genes were always the stickleback genes or homolog genes in other fish.

## 5.2. Selection of housekeeping genes in cell cultures

As mentioned above there was no reason to expect a single gene to be the best universal housekeeping gene in all tissues and under all treatment conditions. Therefore data for every organ and every experiment was tested separately.
While it is possible to include all data to the software of Bestkeeper, GeNorm and NormFinder do not accept any missing data in the sample sheet. In this case either the sample or the gene had to be excluded from the analysis.

### 5.2.1. Cultures of head kidney cells

**Table 3:** Results of NormFinder with cultures of head kidney cells (n=28).

| NormFinder cell cultures head kidney | | |
|---|---|---|
| Gene name | Stability value | Standard error |
| L13 | 0.128 | 0.127 |
| BA | 0.396 | 0.091 |
| GAPDH | 0.422 | 0.092 |
| 18S | 0.496 | 0.097 |
| Ubi | 0.573 | 0.104 |
| B2M | 0.644 | 0.111 |
| HPRT | 0.723 | 0.120 |
| TATA | 0.725 | 0.120 |
| EF | 0.824 | 0.131 |
| RNA | 1.136 | 0.169 |
| MHC I | 1.671 | 0.239 |
| MHC II | 2.395 | 0.335 |

The MHC I and MHC II genes were always considered as the least stable genes cultures of head kidney cells. NormFinder´s favourite was the L13 gene (Table 3), which was second best of the Bestkeeper (Table 4) and third in the calculation by the GeNorm software (Figure 8). It was the only gene that had been considered to be within the three most stably expressed genes in all three approaches and therefore it was the most reliable candidate for cell cultures of head kidneys. While Bestkeeper´s favourite gene was the GAPDH, this gene is listed third in NormFinder´s

calculation, but only seventh in GeNorm´s ranking. The 18S and the BA gene, GeNorm´s most stable pair, were also considered to be stably expressed by NormFinder (fourth and second best). But Bestkeeper considered them only as sixth (BA) and seventh (18S) in its ranking. The three approaches agreed that the other genes were more or less an intermediate group, but the RNA gene was an interesting candidate for further discussion. While NormFinder and GeNorm agreed that it is the least stable of the candidate genes it was considered to be the third best gene by the Bestkeeper software.

**Table 4:** Results of Bestkeeper with cultures of head kidney cells.

| Bestkeeper cell cultures head kidney | | |
|---|---|---|
| Gene | n | std dev [± CP] |
| GAPDH | 34 | 0.93 |
| L13 | 34 | 1.08 |
| RNA | 32 | 1.18 |
| TATA | 33 | 1.18 |
| Ubi | 34 | 1.25 |
| BA | 34 | 1.33 |
| 18S | 34 | 1.47 |
| B2M | 34 | 1.58 |
| HPRT | 30 | 1.69 |
| EF | 34 | 2.03 |
| MHC I | 34 | 2.25 |
| MHC II | 34 | 2.90 |

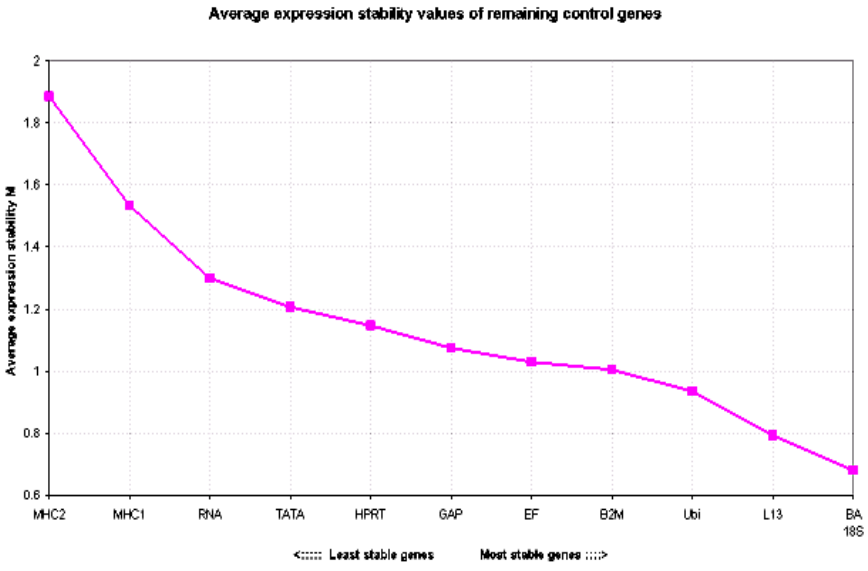


**Figure 8**: Results of GeNorm with cultures of head kidney cells (n=28).

### 5.2.2. Cultures of spleen cells

**Table 5:** Results of NormFinder with cultures of spleen cells (n=27).

| NormFinder Cell cultures Spleen | | |
|---|---|---|
| Gene name | Stability value | Standard error |
| L13 | 0.238 | 0.151 |
| BA | 0.461 | 0.131 |
| GAPDH | 0.526 | 0.134 |
| Ubi | 0.643 | 0.143 |
| 18S | 0.649 | 0.144 |
| RNA | 0.826 | 0.162 |
| B2M | 1.001 | 0.184 |
| EF | 1.192 | 0.210 |
| TATA | 1.268 | 0.220 |
| MHC I | 1.569 | 0.263 |
| HPRT | 2.415 | 0.390 |
| MHC II | 2.555 | 0.411 |

**Table 6:** Results of Bestkeeper with cultures of spleen cells.

| Bestkeeper Cell cultures Spleen | | |
|---|---|---|
| Gene | N | std dev [± CP] |
| GAPDH | 35 | 1.08 |
| BA | 35 | 1.27 |
| L13 | 35 | 1.48 |
| Ubi | 35 | 1.61 |
| HPRT | 29 | 1.62 |
| RNA | 32 | 1.72 |
| 18S | 35 | 1.85 |
| B2M | 35 | 1.85 |
| EF | 35 | 1.96 |
| MHC I | 35 | 2.04 |
| TATA | 28 | 2.06 |
| MHC II | 35 | 2.90 |

The housekeeping candidate genes were divided in three groups and the softwares did even more agree on their results in the cell cultures of spleens as they did in the cell cultures of the head kidneys. The MHC genes and the TATA gene were always considered to be the least stably expressed genes. NormFinder´s favourite candidate was the L13 (Table 5). It was third in the calculation of the Bestkeeper and fourth in GeNorm´s ranking. Bestkeeper (Table 6) considered GAPDH as the most stably expressed gene, which was third in NormFinder´s list and shared first position in the GeNorm calculation (Figure 9), where the GAPDH was ranked first. The BA gene shared first rank and was considered to be the second best in the calculations of NormFinder and Bestkeeper. All the other genes were more or less the

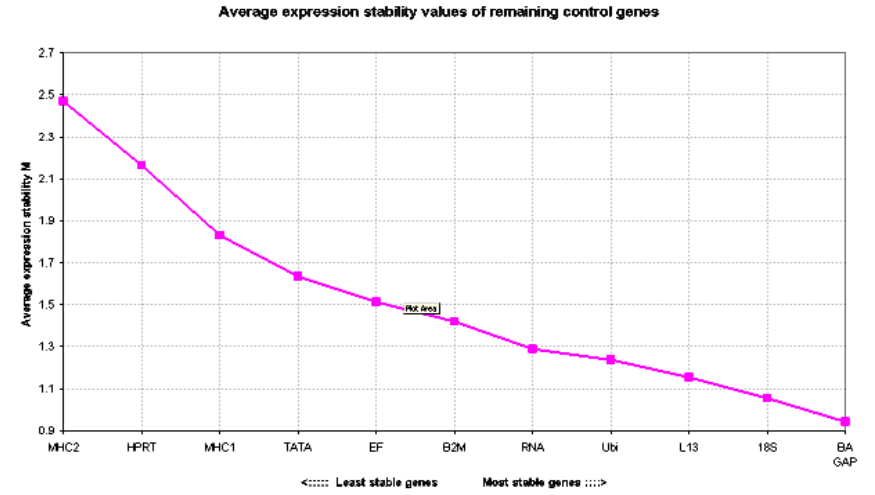


**Figure 9**: Results of GeNorm with cultures of spleen cells (n=27).

intermediate group. But there was an interesting result, which was similar to the results of the RNA gene in the cell cultures of the head kidneys. While the HPRT gene was considered to be the least stable gene by NormFinder and GeNorm, it was again the Bestkeeper software that ranked the HPRT in fifth position of its calculation.

## 5.3. Selection of housekeeping genes in fish

### 5.3.1. Expression in head kidneys

**Table 7:** Results of NormFinder (without BA, GAPDH and TATA), expression in head kidneys (n=60).

| Gene name | Stability value | Standard error |
|---|---|---|
| Ubi | 0.277 | 0.073 |
| L13 | 0.446 | 0.071 |
| MHC I | 0.594 | 0.077 |
| B2M | 0.626 | 0.079 |
| EF | 0.640 | 0.080 |
| HPRT | 0.726 | 0.085 |
| RNA | 0.735 | 0.086 |
| 18S | 0.917 | 0.099 |
| Mn | 1.378 | 0.135 |
| MHC II | 2.696 | 0.250 |

**Table 8:** Results of Bestkeeper, expression in head kidneys.

| Gene | n | std dev [± CP] |
|---|---|---|
| L13 | 64 | 2.24 |
| Ubi | 64 | 2.29 |
| 18S | 64 | 2.45 |
| MHC I | 64 | 2.56 |
| RNA | 63 | 2.61 |
| EF | 64 | 2.96 |
| B2M | 64 | 2.98 |
| HPRT | 64 | 3.01 |
| MHC II | 64 | 3.07 |
| TATA | 54 | 3.21 |
| Mn | 61 | 3.40 |
| GAPDH | 48 | 3.58 |
| BA | 42 | 4.19 |

The three approaches agreed on two genes, Ubi and L13, to be the most stably expressed genes of the ten candidates. While NormFinder (Table 7) favoured Ubi to L13, Bestkeeper (Table 8) ranked L13 first and Ubi second best. GeNorm (Figure 10) calculated L13 and Ubi to be the most stably expressed pair of genes. Surprisingly the BA did not seem to be a good candidate for a housekeeping gene. Bestkeeper even considered it as the least stable housekeeping genes of the ones tested. The BA was not included in the results of NormFinder (Table 7) and GeNorm

(Figure 10), because a group of 24 samples was not amplified during the PCR. If the BA was still included into the analysis,

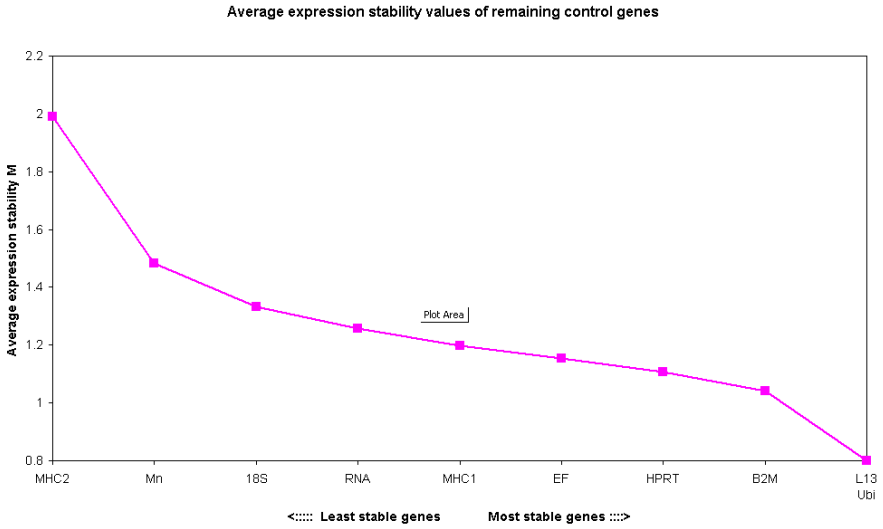


**Figure 10**: Results of GeNorm, expression of head kidneys during infection experiment (n=60, BA and TATA excluded).

the sample size was reduced (n=27). In this case BA was considered to be the fifth stable gene by GeNorm and seventh by NormFinder (data not shown).

### 5.3.2. Expression in spleens

**Table 9:** Results of NormFinder, expression in spleens (n=41).

| Gene name | Stability value | Standard error |
|---|---|---|
| EF | 0.524 | 0.092 |
| Ubi | 0.530 | 0.092 |
| L13 | 0.551 | 0.093 |
| GAPDH | 0.666 | 0.100 |
| RNA | 0.745 | 0.106 |
| MHC I | 0.784 | 0.109 |
| B2M | 0.836 | 0.114 |
| 18S | 0.874 | 0.117 |
| TATA | 0.923 | 0.122 |
| HPRT | 1.052 | 0.134 |
| BA | 2.411 | 0.275 |
| Mn | 2.768 | 0.313 |

Again all programs rejected the BA as a housekeeping gene in spleens as they did in samples from head kidneys. BA is always considered to be the least stable gene of all candidates. Ubi and L13 were the favourite pair of genes calculated by GeNorm

(Figure 10). Ubi was ranked second by NormFinder (Table 9) and fourth by Bestkeeper (Table 10) while L13 was ranked third by NormFinder and first by Bestkeeper. According to the NormFinder program the most stable candidate gene was the EF, but it was only ranked sixth in GeNorm and seventh in Bestkeeper´s calculation.

**Table 10:** Results of Bestkeeper, expression in spleens.

| Bestkeeper Spleen infection | | |
|---|---|---|
| Gene | n | std dev [± CP] |
| L13 | 62 | 1.69 |
| MHC I | 62 | 1.88 |
| GAPDH | 62 | 2.37 |
| RNA | 60 | 2.45 |
| Ubi | 64 | 2.46 |
| HPRT | 61 | 2.54 |
| TATA | 54 | 2.56 |
| EF | 62 | 2.62 |
| 18S | 64 | 2.72 |
| B2M | 64 | 2.82 |
| BA | 55 | 3.11 |
| Mn | 49 | 3.92 |
| MHC II | 38 | 4.17 |

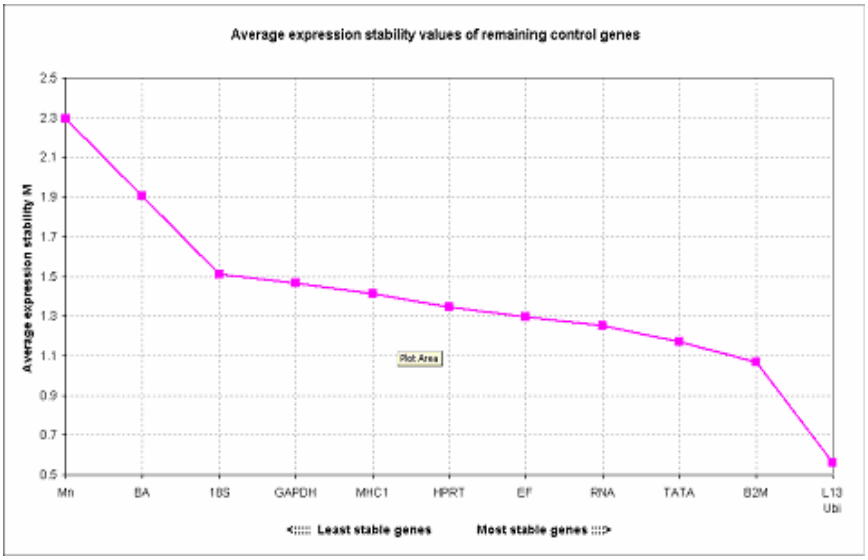


**Figure 11**: Results of GeNorm, expression of spleens (n=41).

### 5.3.3. Expression in gills

**Table 10:** Results of NormFinder (without BA and TATA), expression in gills (n=58).

| Gene name | Stability value | Standard error |
|---|---|---|
| Cas3 | 0.473 | 0.058 |
| Ubi | 0.484 | 0.059 |
| MHC I | 0.528 | 0.062 |
| HPRT | 0.604 | 0.067 |
| L13 | 0.622 | 0.068 |
| B2M | 0.697 | 0.074 |
| GAPDH | 0.703 | 0.074 |
| 18S | 0.903 | 0.090 |
| RNA | 0.953 | 0.094 |
| EF | 1.566 | 0.146 |

**Table 11:** Results of Bestkeeper, gene expression in gills.

| Gene | n | std dev [± CP] |
|---|---|---|
| GAPDH | 58 | 1.46 |
| Ubi | 60 | 1.53 |
| 18S | 60 | 1.53 |
| MHC I | 60 | 1.53 |
| EF | 58 | 1.61 |
| Cas3 | 58 | 1.61 |
| L13 | 60 | 1.65 |
| TATA | 55 | 1.79 |
| RNA | 59 | 1.91 |
| HPRT | 60 | 1.92 |
| B2M | 60 | 1.93 |
| MHC II | 60 | 1.93 |
| BA | 35 | 3.34 |

Concerning gills, the Ubi gene was the only one that had been considered to be one of the most stably expressed genes by all the three approaches. It was first in NormFinder´s ranking (Table 10), second in Bestkeeper´s (Table 11) and it was one of the two favourite genes of GeNorm (Figure 11).

According to the Bestkeeper software, the GAPDH was the most stable gene. But it was only fifth in both NormFinder´s and in GeNorm´s ranking.

The second gene of GeNorm most stable pair of genes was the L13. It was ranked third (NormFinder) and fifth (Bestkeeper) if calculated by the other approaches.

Again I excluded the BA gene from the analysis because of the missing data sets. If the analysis was done with the BA data included, it was the least stable gene according to the

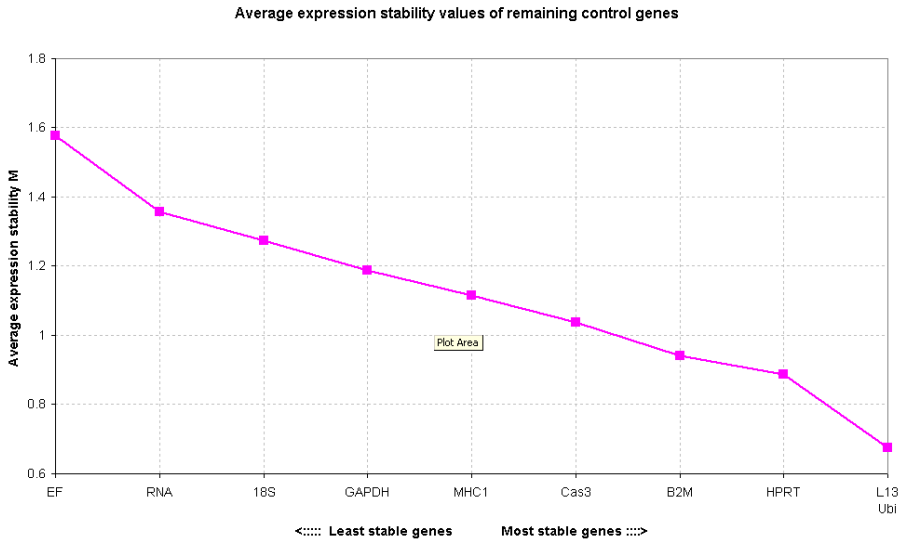


**Figure 12**: Results of GeNorm, expression of gills during infection experiment (n=60).

Bestkeeper software and ninth in the other two approaches (n=34, data not shown).

## 5.4. Cell cultures

### 5.4.1. MHC II-expression in cell cultures

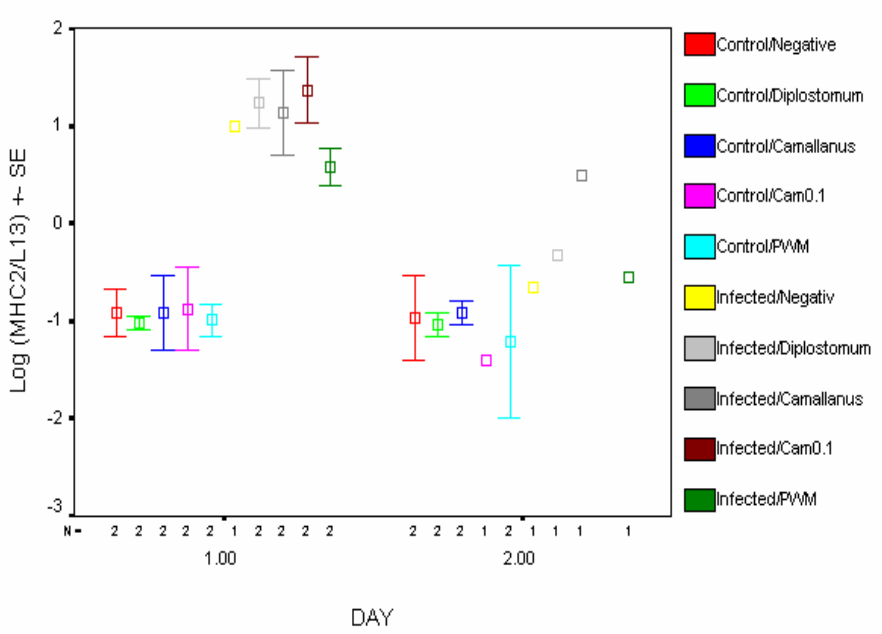


**Figure 13**: Relative MHC II expression in cultures of spleen cells. Group names indicate treatment before/during the experiment.

The MHC II expression in cultures of spleens cells (Figure 13) decreased during the experiment. The univariant analysis of variance revealed that the expression was significantly lower after two days than it had been after the first day ($F_{1,31}$=11.005, p=0.006). Furthermore a difference between the treatments was found ($F_{9,31}$=7.783, p=0.001). A post hoc Bonferroni test showed that the MHC II expression of all fish in control groups was significantly lower than the expression in formerly exposed fish treated with lysates of *Diplostomum pseudospathacaeum* and *Camallanus* lacustris. No significant interaction between time and treatment was found.

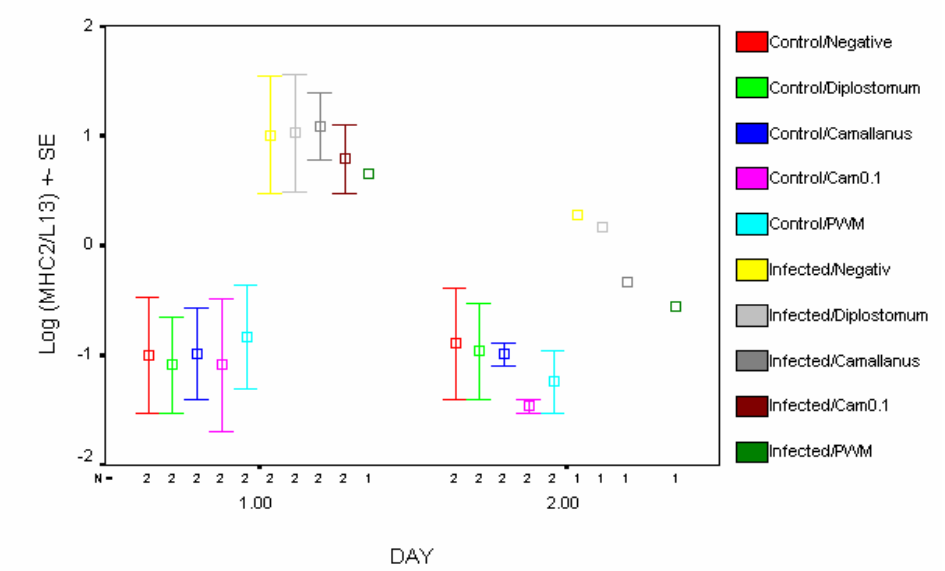


**Figure 14**: Relative MHC II expression in cultures of head kidney cells. Group names indicate treatment before/during the experiment.

The results for the MHC expression of head kidney cells in cell cultures (Figure 14) were similar to the results in spleen cells, even though the differences within days and groups are less marked. The univariate analysis of variance has shown both, a significant difference in expression between the two days ($_{F1,31}$=5.457, p=0.035) and between groups ($F_{9,31}$=5.268, p=0.003). The post hoc Bonferroni test has shown that the expression of the Control/CAM0.1 group differed significantly from the groups Infected/Negativ, Infected/Diplostomum and Infected/Camallanus.

## 5.4.2. Number of cells

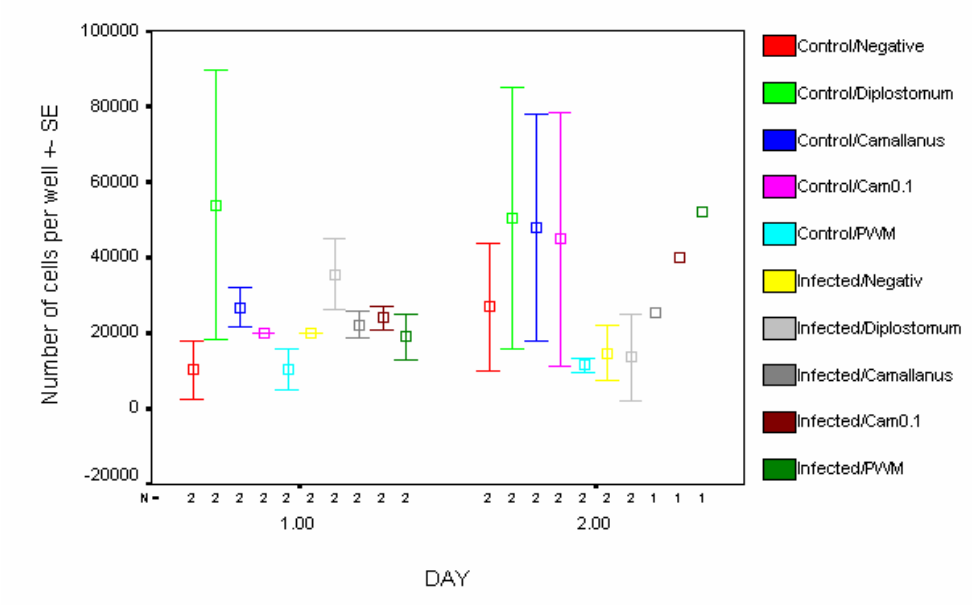


**Figure 15**: Number of cells per well in culture of spleen cells. Group names indicate treatment before/during the experiment.

No significant effect was found in the analysis of the number of cells in spleen cell cultures. Even though the number of cells per well was higher on the second day, the effect was not significant. Neither an effect of treatment was found, but the data suggested that the number of cells was higher in those

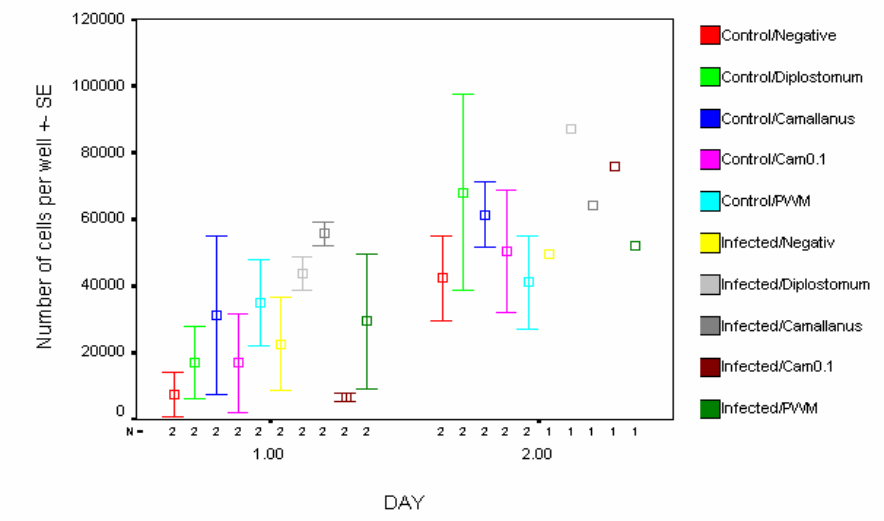


**Figure 16**: Number of cells per well in culture of head kidney cells. Group names indicate treatment before/during the experiment.

wells treated with lysates, than in negative and positive controls. A significant increase in the number of cells in cultures of head kidneys (Figure 16) was found on the second day ($F_{1,34}$=19.663, p<0.001), but no difference in the number of cells could be shown between groups. Furthermore the data suggested that more cells survived in wells treated with parasite lysate than in control cell cultures.

## 5.5 Infection experiment

### 5.5.1. Immune response genes in spleens

The statistical analysis did not show any difference between the treatments in neither of the immune response genes. While the expression of the Mn (Figure 19) and the Cas3 (Figure 20) gene varied significantly during the time of the experiment (Mn, $F_{4,47}$=8.348, p<0.001; Cas3, $F_{4,56}$=3.466 p=0.017), no significant variation was observe in the expression of MHC I

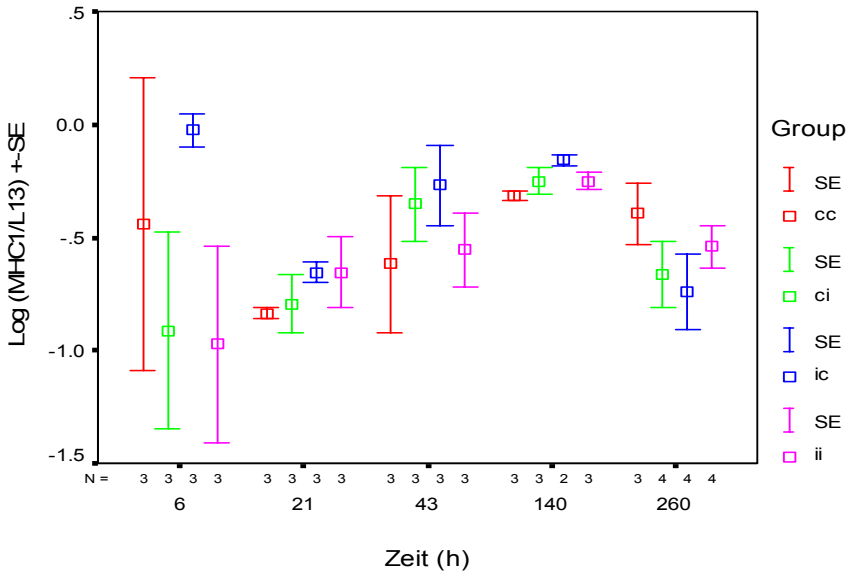


**Figure 17**: Relative MHC I expression in spleens. First letter in group name indicates treatment before; second letter indicates treatment during the experiment.

(Figure 17) and MHC II (Figure 18). Furthermore no significant interaction between time and treatment was found in neither of the genes.

MHC II expression seemed to be higher in formerly infected fish than in control groups, while the Mn expression seemed to be lower, even though effects were not significant.

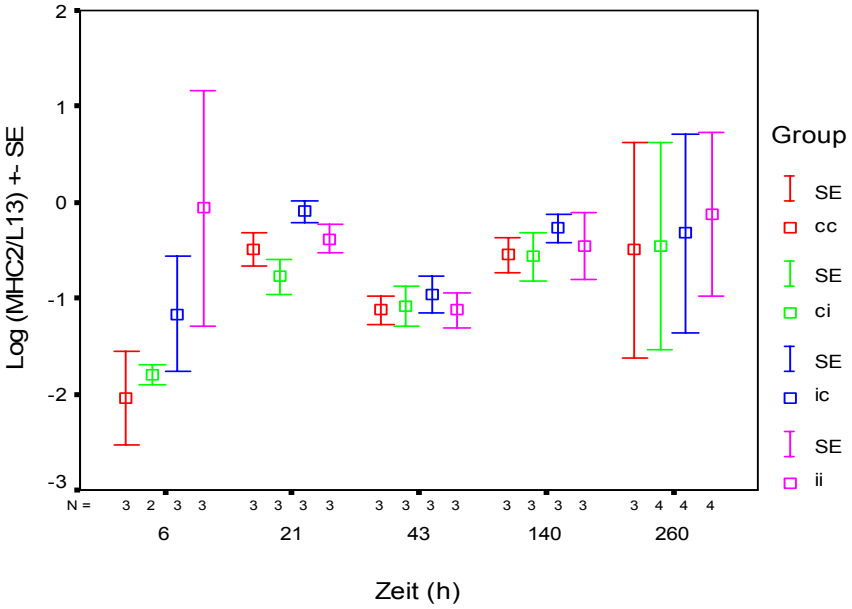


**Figure 18**: Relative MHC II expression in spleens. First letter in group name indicates treatment before; second letter indicates treatment during the experiment.

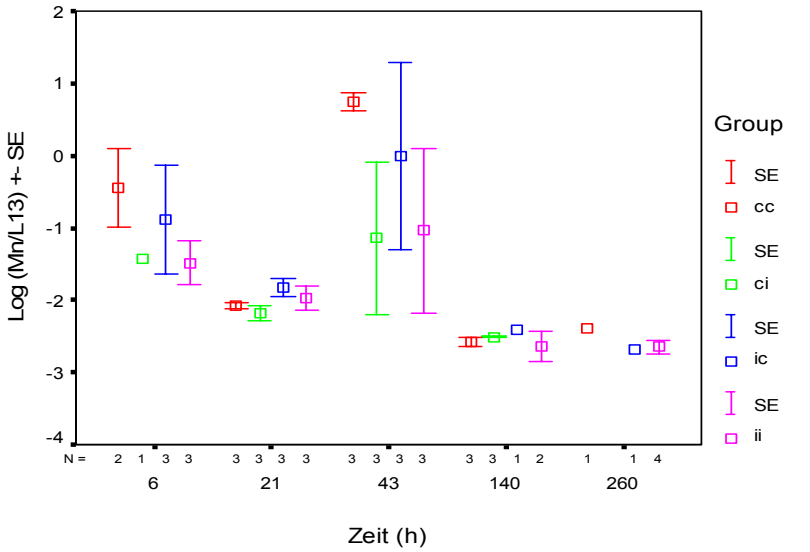


**Figure 19**: Relative Mn expression in spleens. First letter in group name indicates treatment before; second letter indicates treatment during the experiment.

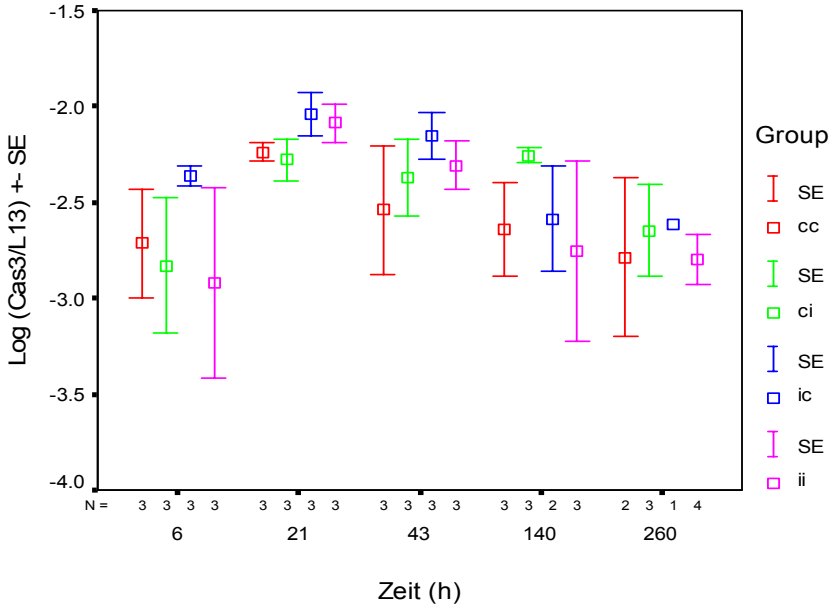


**Figure 20**: Relative Cas3 expression in spleens. First letter in group name indicates treatment before; second letter indicates treatment during the experiment.

### 5.5.2 Immune response genes in head kidneys

While the gene expression of all immune response genes varied significantly during the experiment (MHC I: $F_{4,63}$=3.513, p=0.014; MHC II: $F_{4,63}$=9.184, p<0.001; Mn $F_{4,61}$=44.646,

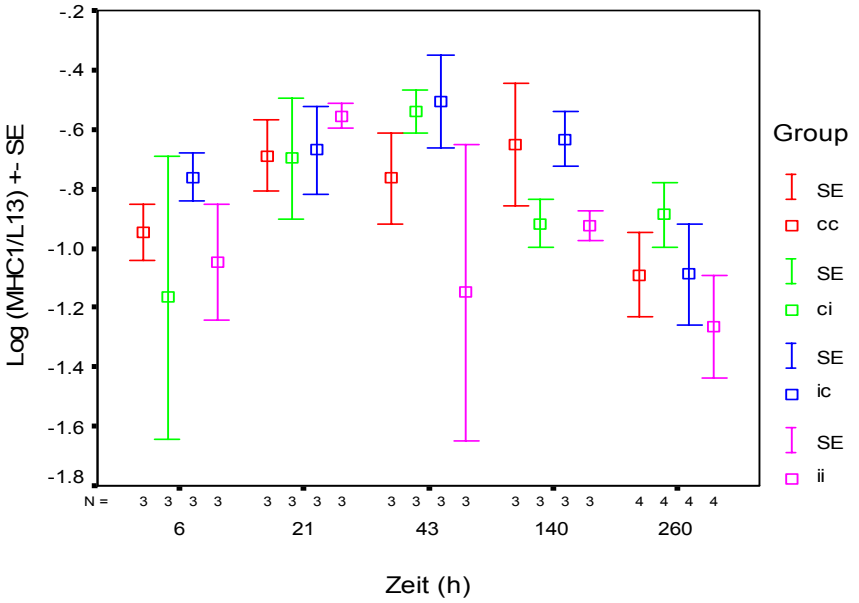


**Figure 21**: Relative MHC I expression in head kidneys. First letter in group name indicates treatment before; second letter indicates treatment during the experiment.

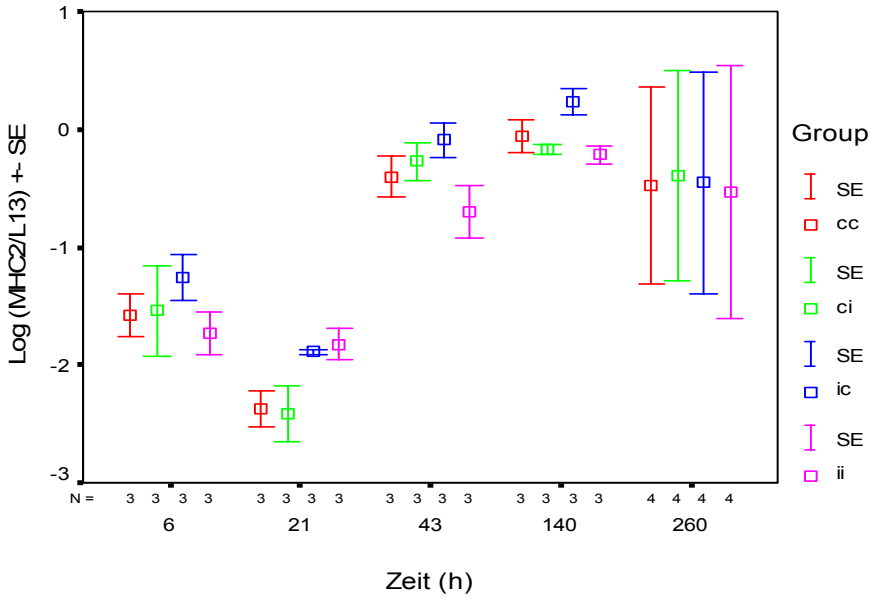


**Figure 22**: Relative MHC II expression in head kidneys. First letter in group name indicates treatment before; second letter indicates treatment during the experiment.

p<0.001; Cas3: $F_{4,59}$=2.827, p=0.037), there were no difference between the treatments and no interaction was found between the factors of time and treatment. Cells from infected fish seemed to express less Cas3 than naïve fish (Figure 24).

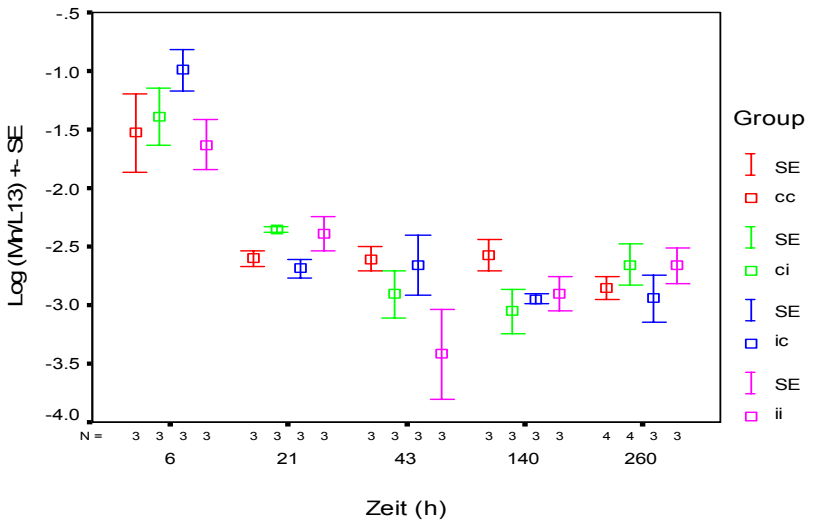


**Figure 23**: Relative Mn expression in head kidneys. First letter in group name indicates treatment before; second letter indicates treatment during the experiment.

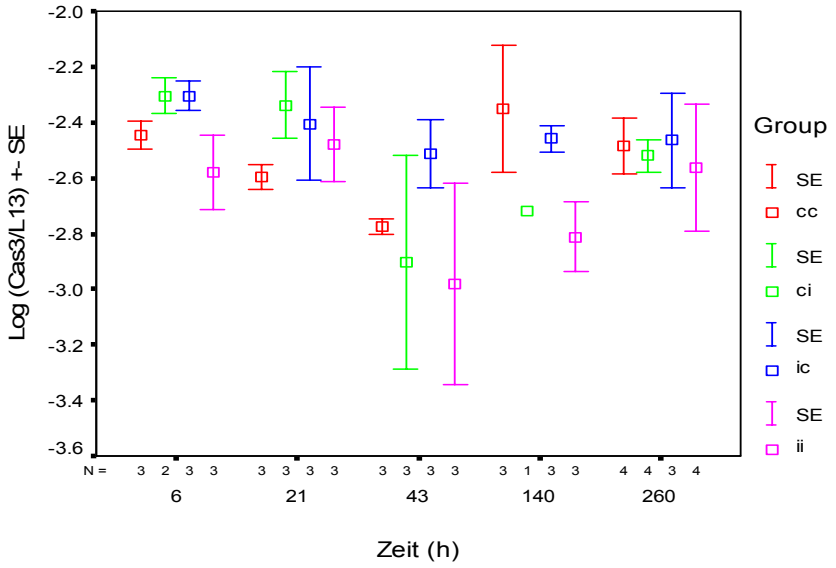


**Figure 24**: Relative Cas3 expression in head kidneys. First letter in group name indicates treatment before; second letter indicates treatment during the experiment.

### 5.5.3 Immune response genes in gills

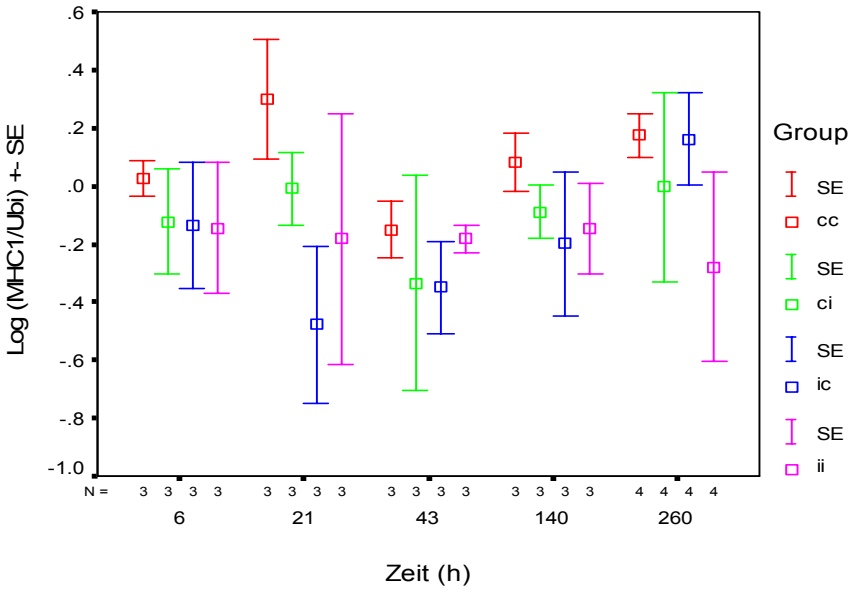


**Figure 25**: Relative MHC I expression in gills. First letter in group name indicates treatment before; second letter indicates treatment during the experiment.

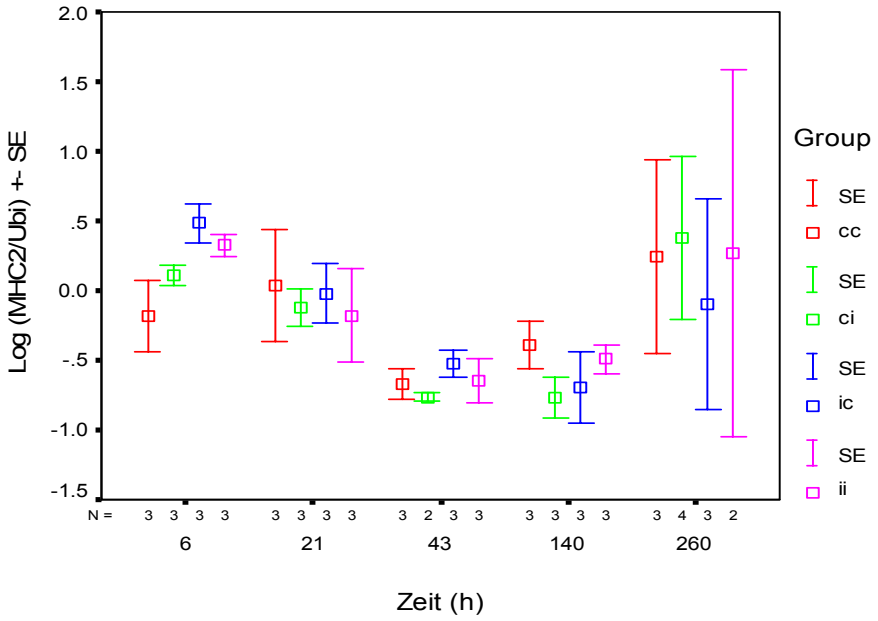


**Figure 26**: Relative MHC II expression in gills. First letter in group name indicates treatment before; second letter indicates treatment during the experiment.

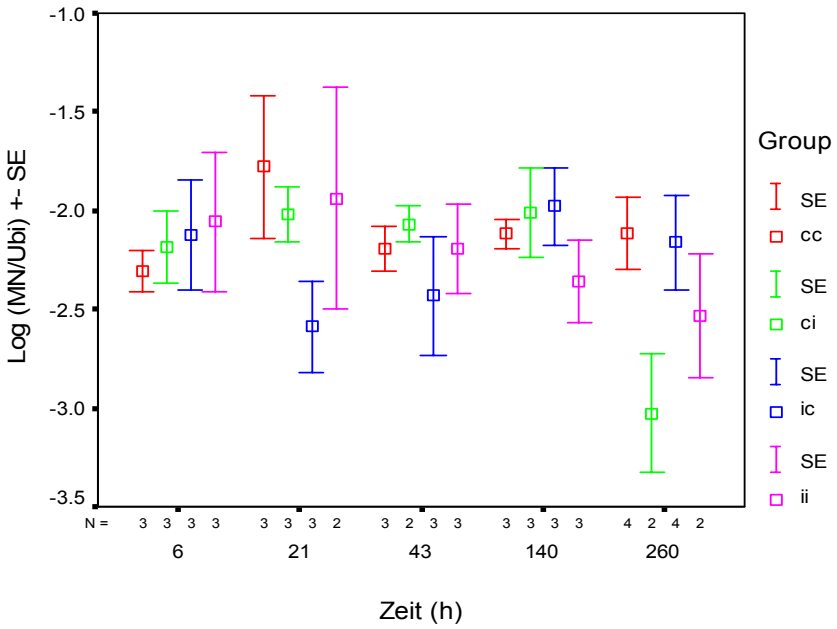


**Figure 27**: Relative Mn expression in gills. First letter in group name indicates treatment before; second letter indicates treatment during the experiment.

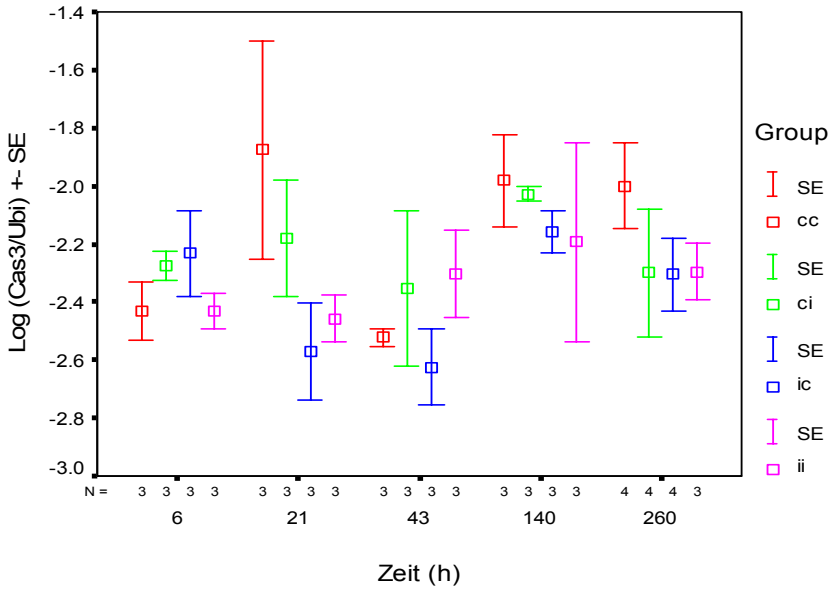


**Figure 28**: Relative Cas3 expression in gills. First letter in group name indicates treatment before; second letter indicates treatment during the experiment.

As one can see in Figure 26 the MHC II expression varied significantly during the time of the experiment ($F_{4,58}$=4.305, p=0.006). No other significant effect of the factors time or treatment was observed in neither of the immune response

genes. And no interaction between time and treatment was found as well.

# 6. Discussion

## 6.1. Primer design and PCR

Except for the primer design for the MHC I and MHC II genes, which had been sequenced in our lab (Reusch and Langefors 2005; Schaschl and Wegner), the primer design for the other genes faced a general problem. The primers were designed using sequences from north-american sticklebacks in order to amplify the cDNA of sticklebacks from the local populations. A difference between the sequences of the north-american and the local stickleback could cause two problems. On the one hand efficiency could be reduced or total failure of PCRs could be caused by mismatches between primer and cDNA sequence. This might be the reason why it was not possible to amplify all the candidate genes. While the PPIA gene cannot be expected to be one of the best housekeeping genes, more efforts should be taken to include the G6PDH gene in the analysis (Radonic 2004). On the other hand primers could bind at the beginning of the intron and amplify both, cDNA and gDNA (Figure 29). The -RT controls showed that the DNAse treatment did not eliminate all the gDNA in samples. Thus all primers that amplify gDNA could not

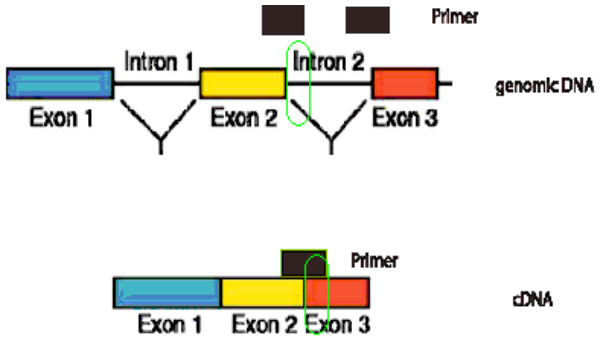


**Figure 29**: The Primer should not bind to gDNA. However a similarity between the intron of the gDNA and the exon of the cDNA (green circles) might lead to amplification of gDNA.

be used to measure gene expression in my PCR assay. Therefore the 18S gene, in which no introns could be found, could not be used as a housekeeping gene in my assay.

The primers for the Interleukin-1 gene amplified both, cDNA and gDNA as well. The Interleukin-1 is a very promising candidate gene to measure an immune reaction on the mRNA level, the increase of gene expression should be very strong within the first hours after infection (~ 50 – 100 fold), so regulation of gene expression should be detectable even with a very small sample size (Bridle et al. 2006; Huising et al. 2006; Huttenhuis et al. 2006). Therefore more efforts should be made to develop suitable primers.

M. Kalbe (personal communication) drew the attention to another problem. It might be possible that bacterial DNA was extracted from organs and homologous genes were amplified in PCRs. This assumes that the homologous genes are conserved in a way that primers bind to bacterial DNA and amplify a PCR product that cannot be distinguished from the product of the cDNA in the melting curves. Even though this possibility cannot be ruled out, it does not seem to be likely.

Final primer concentration in the PCR runs was very high (1000nM). This was supposed to outweigh the high annealing/extension temperature of the PCR. Therefore the assay focused on the specificity rather than on the efficiency of the PCR reaction. So it was possible to measure the expression of 14 genes in 14 PCR runs from about $10^5$ cells. Therefore the assay seems to be suitable even for low amount of mRNA. Due to the high specificity of the assay, the extraction of the mRNA from total RNA was not necessary. The loss of mRNA during this additional extraction did not outweigh the increased efficiency of the following PCRs (data not shown).

It has already been shown (Becker et al. 2005; Liss 2002) that PCR is inhibited by the Reverse Transcriptase. Therefore a precipitation step was included to purify the reverse transcripted cDNA. That was another reason why PCR efficiency could be increased in the assay of this study (data not shown).

## 6.2. Housekeeping gene

Unfortunately, there is no universal reference gene that is stably expressed in all tissues and under all biological conditions (Haller et al 2004). It is therefore necessary to ensure that no significant regulation occurs before a gene can be chosen as standard for relative expression analysis. But the chosen gene has itself to be standardised. Thus there is a circular problem to be solved. One possible solution is to take more than one gene as the housekeeping gene. Pfaffl´s solution (Pfaffl et al. 2004) is to calculate a Bestkeeper´s index, which is the geometric mean of those genes, that are expressed with a SD lower than 1. Probably this condition is too strict. Hardly any of the genes in the cell culture experiment was expressed with a SD lower than 1. In the infection experiment, the SD of the candidate genes was by far too high to include any of them into the calculation of the Bestkeeper´s Index. All other sources of variation, e.g. amount of template, would have to be highly controlled. I doubt that this is possible or even desirable. In the infection experiment, the expression of the whole organs was measured. This led to a SD too high to calculate a Bestkeeper´s Index according Pfaffl´s conditions. Bestkeeper´s prerequisites seem to require a very limited experimental setup. The Bestkeeper´s Index can

only be used under limited conditions, whereas a housekeeping gene should be stably expressed under a variety of conditions.
The software GeNorm (Vandesompele J et al. 2002) suffers from another problem. In this approach the stability of a candidate gene is determined by pair-wise comparison of variation of expression ratios. Therefore the quality of a housekeeping gene depends on the set of candidate genes that are included in the analysis. GeNorm´s calculation is only reliable, if either most of the other candidate genes are already known to be good housekeeping genes or the candidate genes are not co-regulated. Instead of the most stably expressed gene, GeNorm tends to select the gene with the highest degree of similarity to the expression in the data set. It has already been shown (Ransbotyn and Reusch 2006) that the elimination of genes from the analysis changed the ranking of the candidate genes. In the data of this study similar observations were made (data not shown)
The NormFinder software (Andersen et al. 2004) combines the advantages of the two other approaches by an estimation of both the intra- and the inter-group expression variation. NormFinder directly and robustly evaluates gene expression stability (Ransbotyn and Reusch 2006). It should therefore be preferred to the other two approaches.
The L13 was the gene that was ranked best by NormFinder in both organs in the cell culture experiment, head kidney and spleen. The other two approaches did not give any reason to suggest that L13 should not be the best housekeeping gene under these circumstances. BA, GAPDH and Ubi were also good candidates that should be taken into account when more than one housekeeping gene is desired or required.
In the infection experiment results were less straightforward. NormFinder prefered the Ubi to the L13 as the housekeeping

gene for head kidneys, but the other programmes suggested that both are more or less equally suitable.
In spleens NormFinder calculated EF to be the most stable gene, but Bestkeeper and GeNorm ranked EF only in intermediate position. Again Ubi and L13, NormFinder´s second and third choice, seemed to be the best candidates.
In the gills Ubi and L13 were still the best pair of housekeeping genes, but here Ubi seemed to be the better candidate than L13.
The study showed that Ubi and L13 were the best pair of genes whenever a pair of gene is desired as a housekeeping gene in the three analysed organs. In spleens the L13 was the better housekeeping candidate while the Ubi was the better one in gills. In head kidneys it was neck and neck. The BA was a quite good housekeeping candidate in cell cultures. Yet it was one of the worst candidate genes in the analysis of the infection experiment.
Variation in gene expression was lower in the gills than in spleens or head kidneys. This might be caused by high expression levels at all genes, as has already been shown for MHC II (Wegner et al. 2006). MHC II expression has to be at high level, because defence against parasites by mechanical means is limited due to the functioning of the gills.

## 6.3. Cell culture experiment

The MHC II expression was significantly higher in those cells taken from formerly exposed than from naïve fish. Though this increase was shown in spleens and head kidney cells, the effect was stronger in spleen cells.

Wegner et al. (2006) found an increase of MHC expression in sticklebacks 15 days after infection with parasites. Even though infection led to an increase of MHC II expression in spleen cells (Figure 13) no significant effect was observed after infecting cells with the parasite lysates. There are three possibilities to explain these results.

The first possibility is that there was an increase of MHC expression but it could not be measured. However there are several reasons why the assay should be able to measure gene expression correctly. The products of the PCRs were sequenced and the amount of mRNA was fairly stable for the housekeeping genes while it was less stable in the MHC genes. Ratios of the expression rates were as they could be expected. Despite the amplification of residual gDNA, 18S was always expressed at high levels, while TATA and HPRT were expressed at low levels.

The second possibility is that there is an immune reaction and it could be measured, but the increase of MHC expression did not start within the 40 hours of the cultivation of cells. To verify this hypothesis, other genes of the immune response could be included into the analysis. Interleukin-1 is a gene that is known to be regulated within a few hours after infection. An increase of Interleukin-1 expression is a signal for the start of the immune response (Huising et al. 2006; Huttenhuis et al. 2006). So far Interleukin-1 could not be included into the analysis.

The third possibility is that there was no immune response either because the parasite lysates could not induce an immune response at all or there was a missing link in the signal cascade that leads to the increase of MHC expression. The induction of an immune response requires complex cell-to-cell interactions. Therefore it is likely that the immune

response cannot be induced in cell cultures, but the induction requires more than the cells of an organ (J. Kurtz, personal communication). This hypothesis could be ruled out with a positive control that definitely induces an immune response. Unfortunately the PWM failed to do so. Recently Zheng et al. (2006) have shown that poly I:C regulated gene expression in a number of immune genes including MHC II. The Interleukin-1 is another possible positive control, but so far I was not able to run the PCR properly.

The number of cells per well was higher after the second day (Figures 15 and 16), even significantly in head kidney cell cultures. This did not only seem to be an effect of the infection, because the number of cells was higher in controls as well. Even though cells might have started to proliferate during the second day, it is not likely that proliferation caused this result (J. Scharsack, personal communication). Reaggregation of cells on the second day, and therefore a higher proportion of cells in aliquots for flow cytometry, might be an explanation.

The number of cells was higher in wells with parasite lysate than in negative and the positive controls (Figure 16). This might be caused by an activation of cells during the infection and led to a higher survival rate in these cultures.

The results of the cell culture experiment reflected the differences between the two selected organs. The spleen is the main organ of adapted immune response in fish. Therefore the proportion of MHC II presenting leucocytes is higher than in the head kidneys. Any effect concerning the expression of MHC II should be stronger in spleens than in head kidneys.

Whilst the head kidney is the main organ of the innate immune response in fish, an immune response should rather lead to a proliferation of cells than to an increase of MHC II expression.

The results of the cell culture experiment confirmed these hypotheses.

## 6.4. Infection experiment

The infection of sticklebacks with *Diplostomum pseudospathacaeum* did not show any significant effect on the expression of the MHC I gene. This was within the scope of expectations. An infection with macroparasites should not have an effect on MHC I expression. It should be assumed that the immune system of the sticklebacks was continuously stimulated by bacteria, because fish were continuously exposed to bacteria from the water, food or air (open aquaria). A significant variation of the MHC I expression during the experiment was only found in the head kidneys (Figure 21). As the expression in the controls varied in the same way, this effect seemed to be a result of the fish's keeping during the experiment.

Whereas it could be expected that MHC II expression should be affected by the infection with *Diplostomum pseudospathacaeum.* Wegner et al. (2006) have shown a significant increase of 27% in MHC II expression three weeks after an infection. This difference might be too small to be detected with just three fish per group as measured in this survey. But then the study showed that former infection caused an increase of MHC II expression in spleens (Figure 18), whilst the significant variation seemed to be an effect of the keeping.

The results of the expression data suggested that the infection with *Diplostomum pseudospathacaeum* decreased the expression of Mn in spleens (Figure 19) and Cas3 in head

kidneys (Figure 24). In the spleens an interaction between time and group seemed to happen. While the Mn expression of the naïve fish decreases continuously over the time of the experiment, the expression of the infected fish decreases strongly until 43 hours after infection. At the end of the experiment Mn expression level of infected fish showed a slight increase (interaction group x time; $F=1.923$, $p=0.059$). The decrease in Mn and Cas3 might be caused by a trade-off between the adaptive and the innate immune system (Kurtz et al. 2006).

Again variation of expression during the time of the experiment seemed to be caused by the treatment of the fish, because controls varied the same way as the infected fish.

In this study a PCR assay has been established to measure a variety of different genes. Even though many of the genes have never been sequenced in *Gasterosteus aculeatus* before, the products of the PCRs were specific and the efficiency was satisfactory. The improvement of the mRNA extraction made it possible to measure gene expression from an aliquot of less than 5000 vivid cells.

A system based on the measurement of cell cultures was successfully established as well. Since results were in scope of expectations, the cells seemed to be in good shape for at least two days. The cell cultures system might help to gain new insights in the course of an immune response within the first days of an infection.

# 7. Literature

Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research 64:5245-5250

Apanius V, Penn D, Slev PR, Ruff LR, Potts WK (1997) The nature of selection on the major histocompatibility complex. Critical Reviews in Immunology 17:179-224

Bas A, Forsberg G, Hammarstrom S, Hammarstrom ML (2004) Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. Scandinavian Journal of Immunology 59:566-573

Becker S, Hayes PK, Walsby AE (2005) Different gvpC length variants are transcribed within single filaments of the cyanobacterium Planktothrix rubescens. Microbiology-Sgm 151:59-67

Bridle AR, Morrison RN, Cunningham PMC, Nowak BF (2006) Quantitation of immune response gene expression and cellular localisation of interleukin-1 beta mRNA in Atlantic salmon, Salmo salar L., affected by amoebic gill disease (AGD). Veterinary Immunology and Immunopathology 114:121-134

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 25:169-193

Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. Journal of Molecular Endocrinology 29

Grimholt U, Larsen S, Nordmo R, Midtlyng P, Kjoeglum S, Storset A, Saebo S, Stet RJM (2003) MHC polymorphism and disease resistance in Atlantic salmon (Salmo salar); facing pathogens with single expressed major histocompatibility class I and class II loci. Immunogenetics 55:210-219

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser.:95-98

Haller F, Kulle B, Schwager S, Gunawan B, von Heydebreck A, Sultmann H, Fuzesi L (2004) Equivalence test in quantitative reverse transcription polymerase chain

reaction: confirmation of reference genes suitable for normalization. Analytical Biochemistry 335:1-9

Huising MO, van Schijndel JE, Kruiswijk CP, Nabuurs SB, Savelkoul HFJ, Flik G, Verburg-van Kemenade BML (2006) The presence of multiple and differentially regulated interleukin-12p40 genes in bony fishes signifies an expansion of the vertebrate heterodimeric cytokine family. Molecular Immunology 43:1519-1533

Huttenhuis HBT, Taverne-Thiele AJ, Grou CPO, Bergsma J, Saeij JPJ, Nakayasu C, Rombout J (2006) Ontogeny of the common carp (Cyprinus carpio L.) innate immune system. Developmental and Comparative Immunology 30:557-574

Kalbe M, Wegner KM (2004) Different parasite communities in parapatric host populations of three-spined sticklebacks (Gasterosteus aculeatus). International Journal of Medical Microbiology 293:56-56

Koppang EO, Lundin M, Press CM, Ronningen K, Lie O (1998) Differing levels of Mhc class II beta chain expression in a range of tissues from vaccinated and non-vaccinated Atlantic salmon (Salmo salar L.). Fish & Shellfish Immunology 8:183-196

Kurtz J, Wegner KM, Kalbe M, Reusch TBH, Schaschl H, Hasselquist D, Milinski M (2006) MHC genes and oxidative stress in sticklebacks: an immuno-ecological approach. Proceedings of the Royal Society B-Biological Sciences 273:1407-1414

Langefors A, Lohm J, Grahn M, Andersen O, von Schantz T (2001) Association between major histocompatibility complex class IIB alleles and resistance to Aeromonas salmonicida in Atlantic salmon. Proceedings of the Royal Society of London Series B-Biological Sciences 268:479-485

Larkin P, Knoebl I, Denslow ND (2003) Differential gene expression analysis in fish exposed to endocrine disrupting compounds. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 136:149-161

Liss B (2002) Improved quantitative real-time RT-PCR for expression profiling of individual cells. Nucleic Acids Research 30

Nowak MA, Tarczyhornoch K, Austyn JM (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 89:10896-10899

Oleksiak MF, Churchill GA, Crawford DL (2002) Variation in gene expression within and among natural populations. Nature Genetics 32:261-266

Pechhold K, Pohl T, Kabelitz D (1994) Rapid Quantification of Lymphocyte Subsets in Heterogeneous Cell-Populations by Flow-Cytometry. Cytometry 16:152-159

Penn DJ, Damjanovich K, Potts WK (2002) MHC heterozygosity confers a selective advantage against multiple-strain infections. Proceedings of the National Academy of Sciences of the United States of America 99:11260-11264

Peters IR, Helps CR, Hall EJ, Day MJ (2004) Real-time RT-PCR: considerations for efficient and sensitive assay design. Journal of Immunological Methods 286:203-217

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations. Biotechnology Letters 26:509-515

Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochemical and Biophysical Research Communications 313:856-862

Ramakers C, Ruijter JM, Deprez RHL, Moorman AFM (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neuroscience Letters 339:62-66

Ransbotyn V, Reusch TBH (2006) Housekeeping gene selection for quantitative real-time PCR assays in the seagrass Zostera marina subjected to heat stress. Limnology and Oceanography: Methods 4

Rauch G, Kalbe M, Reusch TBH (2006) Relative importance of MHC and genetic background for parasite load in a field experiment. Evolutionary Ecology Research 8:373-386

Reusch TBH, Langefors A (2005) Inter- and intralocus recombination drive MHC class IIB gene diversification in a teleost, the three-spined stickleback Gasterosteus aculeatus. Journal of Molecular Evolution 61:531-U45

Sato A, Figueroa F, O'Huigin C, Steck N, Klein J (1998) Cloning of major histocompatibility complex (Mhc) genes from threespine stickleback, Gusterosteus aculeatus. Molecular Marine Biology and Biotechnology 7:221-231

Scharsack JP, Kalbe M, Derner R, Kurtz J, Milinski M (2004) Modulation of granulocyte responses in three-spined sticklebacks Gasterosteus aculeatus infected with the tapeworm Schistocephalus solidus. Diseases of Aquatic Organisms 59:141-150

Schaschl H, Wegner KM (2006) Polymorphism and signature of selection in the MHC class I genes of the three-spined stickleback (Gasterosteus aculeatus). Journal of Fish biology (in press)

Szabo A, Perou CM, Karaca M, Perreard L, Quackenbush JF, Bernard PS (2004) Statistical modeling for selecting housekeeper genes. Genome Biology 5

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, F. S (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3:research0034

Wegner KM, Kalbe M, Kurtz J, Reusch TBH, Milinski M (2003) Parasite selection for immunogenetic optimality. Science 301:1343-1343

Wegner KM, Kalbe M, Rauch G, Kurtz J, Schaschl H, Reusch TBH (2006) Genetic variation in MHC class II expression and interactions with MHC sequence polymorphism in three-spined sticklebacks. Molecular Ecology 15:1153-1164

Zheng W, Liu G, Ao J, Chen X (2006) Expression analysis of immune-relevant genes in the spleen of large yellow croaker (Pseudosciaena crocea) stimulated with poly I:C. Fish & Shellfish Immunology 21:414-430

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# Stellungnahme

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig angefertigt und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe.

Mit der Einstellung dieser Arbeit in die Fachbibliothek des Biologiezentrums sowie die Zentralbibliothek der Christian-Albrechts-Universität bin ich einverstanden.

Kiel, _____ _____

Sascha Hibbeler