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A study on the peroxisome proliferator-activated receptors in the three-spined stickleback (*Gasterosteus aculeatus*)

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PREFACE

This Master's thesis was done as the final part of the Ecotoxicology graduate studies program at the Department of Environmental Toxicology, Uppsala University, Uppsala, Sweden.

First I want to thank my two supervisors professor Björn Brunström for all the help and for letting me get this research opportunity and doctor Jan Olsson for all the guidance, optimism and motivation.
I also want to thank Director of studies Jan Örberg for making the Ecotoxicology graduate studies program an enjoyable journey.
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And last but not least my girlfriend Hanna Eriksson who always is there for me and supporting me.

ABSTRACT

The consumption of pharmaceuticals is steadily increasing all over the world. Some receptors are conserved during evolution and are quite similar in different animals. This means that pharmaceuticals that effect our body probably also effect animals. If a drug will work properly it has to be fairly stable and lipophilic, which means that there will be a risk that drugs contaminating the environment accumulate in animals. Pharmaceuticals are taken in doses higher than the body can accumulate and metabolize and some amount is released into the wastewater through urine and faeces. Many of our sewage treatment plants cannot purify the water from all substances so some is released into the environment. Fibrates are drugs used to lower cholesterol and triglyceride levels in the blood plasma. In this process they bind to peroxisome proliferator-activated receptors (PPARs).

The first aim of this study was to identify various organs in the three-spined stickleback that express the different PPARs, using Polymerase Chain Reaction (PCR). To determine levels of expression of PPARs, quantitative real-time PCR (qRT-PCR) was used. First primers for the genes of interest were designed and constructed. Samples from gills, heart, kidney, liver and muscle were taken from adult three-spined stickelbacks. The RNA was extracted from the tissue samples and this RNA was then used to synthesise cDNA. The primers and cDNA were used in the different PCR reactions. The result from the PCRs suggests that the different PPARs are expressed in all tissues and that the expression is significantly lower in kidney than in liver.

The second aim was to establish cell-lines from different organs in the three-spined stickleback. The first step was to manage to isolate primary cell cultures from liver and kidney. The protocol was altered many times by changing parameters like temperature, growth medium, serum concentration and CO_2-concentration. We did not manage to optimise the protocol, but some progress was made. The incubation conditions that worked best were a temperature of 18 $^{\circ}$C and air without extra CO_2. A serum concentration of 1% gave a better result than 10% serum. I found five types of 96-well plates with different pre treatment and from different companies that the cells attached to. The problem in the end was that we had problems to identify which type of cells that was isolated.

1. AIMS

The first aim of this study was to determine in which tissues PPARs are expressed in the three-spined stickleback.

The second aim was to develop cell-lines from different organs in three-spined stickleback that could be used in further studies.

2. INTRODUCTION

Medical substances are developed to perform some kind of biological function, for example regulate a biological process. This means that they have to be lipophilic so that they can pass membranes and stable so that they are not inactivated before they have carried out the effect. These characteristics imply that many pharmaceuticals have a tendency to bioaccumulate and induce effects in the aquatic and terrestrial ecosystem (Halling-Sorensen et al. 1998). One example of effects in the environment is from England where roach (*Rutilus rutilus*) in a river with high content of estrogenic compounds from sewage effluent showed a high frequency of hermaphrodites (Jobling et al. 1998). Pharmaceuticals are often taken in doses higher than the body can accumulate and metabolize and this leads to that some amount leaves the body through urine or faeces and subsequently enters the wastewater. It has been shown that the majority of pharmaceuticals are excreted unmetabolised and enter the wastewater as biologically active substances. Unmetabolised pharmaceuticals are very hard to degrade in the nature (Stuer-Lauridsen et al. 2000). The consumption of pharmaceuticals steadily increases every year (WHO homepage 2008). In Sweden for example, the total sale of pharmaceuticals increased with 5 % from 2005 to 2006 and 6.1% the year after (Apoteket homepage 2007a; Apoteket homepage 2008).

Fibrates are drugs used to lower the concentration of cholesterol and triglycerides in the blood plasma. Fibrates bind to receptors called peroxisome proliferator-activated receptors (PPARs) and thereby stimulate the expression of different proteins. One of these proteins, lipoprotein lipase, is responsible for the conversion of very low density lipoproteins (VLDL) to high

density lipoprotein (HDL) and thereby a decreased plasma triglyceride concentration (Stael et al. 1998). In Sweden the defined daily doses (DDD) of fibrates have decreased from 2004 (4035492 DDD) to 2006 (3515119 DDD) (Apoteket homepage 2007b).

Clofibric acid (2-[4]-chlorophenoxy-2-methyl propanoic acid) is an active metabolite from clofibrate and several other fibrates and was the first prescribed drug found in sewage effluent. The half life in the environment has been estimated to 21 years (Buser et al. 1998). Buser et al. (1998) found clofibric acid in various Swiss waters at concentrations ranging from 1 to 9 ng/L. This drug is not manufactured in Switzerland so the origin must be through medical use and subsequent excretion. A study made in Brazil by Stumpf et al. (1999) shows that the removal efficiency from Brazilian sewage treatment plants for clofibric/fenofibric acids, benzofibrate and gemfibrozil ranged from 6 to 50 %. This indicates that sewage treatment plants have limited capacity to remove for these compounds and a large amount is released into the recipient. Clofibric acid is one of the most frequently reported pharmaceuticals in monitoring studies. Clofibric acid has been found in tap water at 270 ng/l (Heberer 2002), 103 ng/l in Detriot River water (Boyd et al. 2003), from 0.28 to 1.35 ng/l in the North Sea (Weigel et al. 2002) and up to 0.5 µg/l in German streams (Ternes 1998). Clofibric acid is one of the most common and widely reported drugs found in open waters (Daughton and Ternes 1999).

Clofibric acid has shown a number of different toxicological effects in various organisms. Some examples of these are: oxidative damage in several biological structures in mouse liver (Qu et al. 2001), induction of oxidative stress in primary cultures of rainbow trout hepatocytes (Laville et al. 2004), several oxidation alterations in gills and hepatic tissues in *Gambusia holbrooki* (Nunes et al. 2008) and effects on the speramtogenes is in Fathead minnow (Runnalls et al. 2007). Another fibrate, gemfibrozil, has been shown to affect *Daphnia magna* with a no-observed effect level of 30 µM (Zurita et al. 2007).

Toxicological tests have been carried out for a number of pharmaceuticals but these tests are limited to standard endpoints in a few species. Pharmaceuticals have specific modes of action and the limited number of test species used and the small set of endpoints studied are not able to unravel all effects of pharmaceuticals. Because many receptor systems and enzymes are evolutionary conserved it is possible that many pharmaceuticals act in the same way in humans as in other vertebrates including fish.

The PPARs belong to the protein super family of nuclear receptors, and are activated by fatty acids and derivatives. They were first identified in 1990 when the isoform α (PPARα) was found to be a receptor of xenobiotics capable of inducing peroxisome proliferation in rodent liver (Isseman and Green 1990). Shortly thereafter two more receptors where found: PPARβ and PPARγ. PPARα is involved in peroxisome proliferation and plays a central role in the control of hepatic lipid metabolism (Schoonjans et al. 1996). PPARβ has diverse roles in basic lipid metabolism and is the main subtype expressed during oogenesis and embryogenesis (Ibabe et al. 2005). PPARγ plays a key role in the differentiation of adipocytes and is implicated in the inflammatory response (Batista-Pinto et al. 2005). When PPARs are activated they first form a heterodimer with another nuclear receptor called retinoid X receptor alfa (RXRα) (Peraza et al. 2006). This complex then binds to a specific DNA sequence called peroxisome proliferator response element (PPRE) and this binding results in transcription of target genes (Figure 1). PPARs have been identified in a wide range of fish species including zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*) and *Pleuronectes platessa* (Buser et al. 2002). In adult frogs (*Xenopus laevis*) it has been shown that the α and β subtypes are evenly expressed in various organs whereas PPARγ is mostly expressed in adipose tissue, kidney and liver (Ibabe et al. 2005).

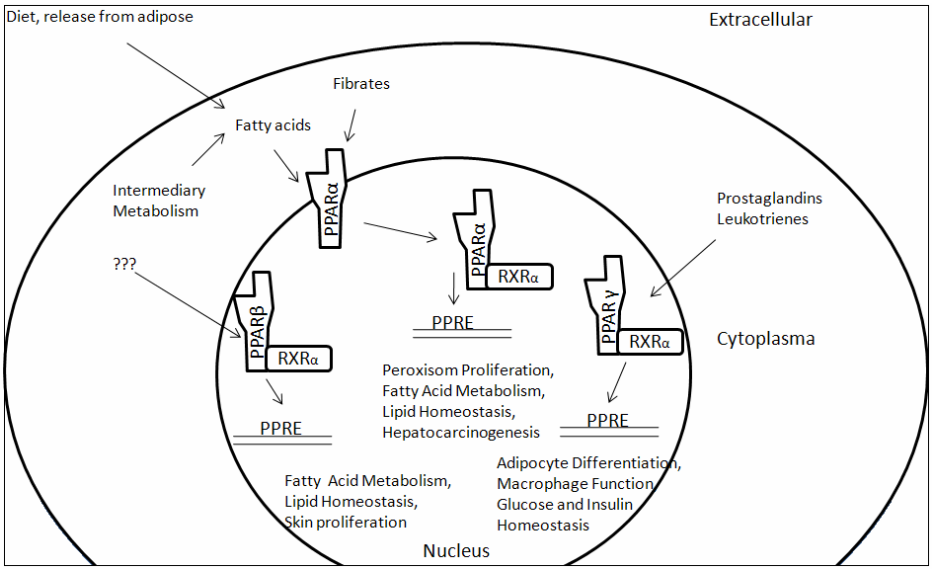

Figure 1. A map over how the different PPARs are activated and what kind of effects this activation can lead to. The picture which was used as model can be found on http://ppar.cas.psu.edu/pparrfront.htm.

The three-spined stickleback is a small shoal-living fish that lives both in fresh and salt waters in the northern hemisphere. It has been widely used in different biological studies. The use of fish in aquarium experiments for assessing effects of different toxic substances is a common procedure. To obtain a more rapid response it is better to do in vitro assays using cell cultures (Björkblom et al. 2007). It is also easier to do many replicates in vitro and therefore many in vitro assays are more cost efficient than in vivo experiments.

3. MATERIALS AND METHODS

3.1 Animals

Adult non-breeding male and female three-spined stickleback (*Gasterosteus aculeatus*) were kept in aquaria at the Evolutionary Biology Centre at Uppsala University, Sweden. They were caught in Öresund on the Swedish south-west coast and were kept in a reproductive quiescent state (8 h light/day and 8°C).

3.2 Detection of PPARs in different tissues by PCR amplification

3.2.1 Design of primers

Sequences for the different PPARs were found on the Ensembl homepage (http://www.ensembl.org/Gasterosteus_aculeatus/index.html). Primers for PCR and real-time PCR were constructed so that at least one primer covered an exon-exon-junction or if this was not possible the forward and the reverse primer were on different exons. The primers were constructed with Primer 3 (v 0.4.0) (http://frodo.wi.mit.edu/) and then analyzed with Netprimer (http://www.premierbiosoft.com/netprimer). All primers had a rating above 88 which was acceptable. Primers were bought from Invitrogen. The following primers (table 1) were used.

Table 1. The sequences and product size of the different primer pairs used. The sequences were retrieved from ensemble homepage and the primers were constructed with Primer 3. FW = Forward; RV = Reverse.

Primer	Sequence	Product size (bp)
SpiggPPARα1-FW1	CTGAAGGCGGAGATGGTAAC	185
SpiggPPARα1-RV1	AAGGAGGTGTGGTGGTCTTG	
SpiggPPARα2-FW1	CGCACTGGACACTTTGACC	229
SpiggPPARα2-RV1	GTTGCGGTTCTTCTTTTGGA	
SpiggPPARβ-FW1	CCAGACACGCACAATTTGAT	245
SpiggPPARβ-RV1	AAACCCTTACAGCCCTCACA	
SpiggPPARγ-FW1	CAGACTTGGGGGAGTGAAAC	190
SpiggPPARγ-RV1	CCTCACTTTCCTTGCCCTTT	

3.2.2 Sample preparation

Samples of gill, heart, kidney, liver and muscle tissues were taken from one male and one female adult three-spined stickleback and stored in -80°C. RNA was extracted from the tissues with the Invisorb® Spin Tissue RNA Mini Kit and then stored in -80°C. The RNA samples were analysed with 1% agarose gel-electrophoresis to see if the samples contained traces of genomic DNA. RNA concentrations and quality were measured using a Nanodrop (NZ 3012033B). From the RNA, cDNA was synthesized with SuperScript™ III First-Strand Synthesis System for RT-PCR.

3.2.3 PCR reaction and analysis of PCR products

The designed primers were diluted to concentrations of 100 pmol/µl and 10 pmol/µl. Primers (10 pmol/µl) (2*0.5 µl), cDNA (1 µl), ddH_2O (5.5 µl) and Fideli Taq™ PCR Master Mix 2x (USB) (7.5 µl) were mixed in a PCR-tube to give a total volume of 15 µl. The DNA polymerase was activated with a hot start (10 min at 95 °C). Thereafter the PCR-reaction was run (40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s) and the reaction was ended by a final extension step (2 min at 72 °C). The PCR-products were analyzed with gel-electrophoresis to see if the size of the product corresponded with the expected size.

3.3 Analysis of the level of expression on PPARs in different tissues by quantitative real-time PCR

3.3.1 Design of primers

Primers for the quantitative real-time PCR were designed as described in chapter 3.3.1 but more genes were studied. Except the PPARs we also wanted to study the expression of RXRs and the reference gene ACTB. The product size varied from 80 to 110 base pairs. Two primer pairs were constructed for each gene. During the optimization of the quantitative real-time PCR the primer pairs were tested. For each gene, the pair with the highest efficiency is listed below (Table 2).

Table 2. The sequences, product size and the efficiency of the different primer pairs used. The sequences were retrieved from ensemble homepage, the primers were constructed with Primer 3 and the efficiency was given from the quantitative real-time PCR. FW = Forward; RV = Reverse.

Primer	Sequence	Product size (bp)	Efficiency
QSpiggPPARα1-FW2	CAATACTGCCGCTTCCAGA	110	1.16
QSpiggPPARα1-RV2	GTTACCATCTCCGCCTTCAG		
QSpiggPPARα2-FW1	CGCACTGGACACTTTGACC	102	1.65
QSpiggPPARα2-RV1	CGGCACTCCAGGTTGAGG		
QSpiggPPARβ-FW1	CAGACTTGGGGGAGTGAAAC	91	1.00
QSpiggPPARβ-RV1	GGTCACGCTGCTTGGTACTT		
QSpiggPPARγ-FW1	AGGGTTTCTTCAGACGGACA	91	0.78
QSpiggPPARγ-RV1	CATTTGTTGCGGGACTTCTT		
QSpiggRXRα-FW2	ACATGCCCGTGGAGAAGAT	104	1.00
QSpiggRXRα-RV2	GTCGTTAGGCGAATTGGATG		
QSpiggACTB-FW1	GCCCTCTACCAACCTTCCTT	104	0.97
QSpiggACTB-RV1	TAGAGGTCCTTGCGGATGTC		

3.3.2 Sample preparation

Samples of kidney and liver tissues were taken from seven male and ten female adult three-spined sticklebacks and stored in -80°C. The extraction of RNA was described in 3.2.2. From the measurement on nanodrop the six cleanest samples from male liver, female liver, male kidney and female kidney were taken. The DNA-*free*™ Kit (Ambion) was used to digest the

genomic DNA to make sure that it would not effect the qRT-PCR. To equalize the variation in concentration of RNA in the different samples the same amount of RNA was taken from samples of the same tissues. From the RNA, cDNA was synthesised with SuperScript™ III First-Strand Synthesis System for RT-PCR. In this step the difference in concentration between the liver and kidney samples was equalized so that the amount of cDNA would be the same in all the samples. The samples were then diluted ten times with nuclease-free water (Ambion).

3.3.3 Analysis

The designed primers were diluted to concentrations of 100 pmol/µl and 10 pmol/µl. Primers (10 pmol/µl, 2*0.6 µl or 0.2 µl), cDNA (4 µl), Brilliant® SYBR® Green QPCR Master Mix (Strategene) (10µl) and ddH_2O (Ambion) were added to a total volume of 20 µl in qRT-PCR tubes. Before the analysis was made, the qRT-PCR reactions were optimized for each gene. A dilution series was made with five different concentrations of cDNA (1:10, 1:80, 1:640, 1:5120 and 1:40960). For each primer pair, qRT-PCR was run with triplicates of each concentration of cDNA and two controls (one to test that the cDNA synthesized was not contaminated and one to test if the q-RT PCR reaction was contaminated). From the dilution series the efficiency for each reaction was calculated (Table 3). Primer concentration and annealing temperature were altered to get the highest for each gene. The DNA polymerase was activated with a hot start (10 min at 95 °C). Thereafter the PCR-reaction was run with triplicates of the samples (45-50 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s) and the reaction was ended by a melting curve analysis to see whether the samples contained unspecific PCR-products or primer-dimers.

3.3.4 Calculations

The mean CT-values were calculated for each sample (N=1-3). The expression relative to ACTB was calculated for each gene. The efficiency (Table 2) was also taken in account for the genes and for ACTB (eq.1).

$$\text{Relative expression of GENE} = (2^{\text{Efficiency}_{ACTB}})^{Ct_{ACTB}}/(2^{\text{Efficiency}_{GENE}})^{Ct_{GENE}} \qquad \text{(eq.1)}$$

3.3.5 Statistical analyses

To analyse if there was differences in the data set of the genes a one-way ANOVA (p=0.05) was used. Because the Bartlett's test for equal variances (p=0.05) gave a significant difference in variance between groups the CT-values were log-transformed. Bonferoni post test (p=0.05) was used to determined the difference in expression of the genes.

3.4 Primary culture of liver and kidney cells

The protocol was based on a number of earlier studies (Björkblom et al 2007; Cao et al 1996; Lannan 1994; Liu et al 2005; Vallone et al 2007). One fish was killed by decapitation and was first washed in 70 % ethanol and then in phosphate-buffered saline (PBS). The liver and kidney were removed and washed tree times with PBS containing fungicide (50 μg/ml gentamicin (Sigma) or 50 μg/ml amphotercinin B (Sigma)), and then stored on ice for one hour. The tissues were then sliced into smaller pieces and 6 ml trypsin (Sigma-Aldrich) was added. The cells were trypsinised for different times (5 or 30 minutes) and some were trypsinised on a shaker (200 rpm) at room temperature. The suspension was either filtered first through a nylon mesh (size 100 μm) and then through another nylon mesh (size 40 μm) or only through a 100 μm nylon mesh. To remove the trypsin solution the solutions were centrifuged (at different g, at room temp, 10 min) and the supernatant was removed. The cells were washed one time with 10 ml cell medium. The cell medium used for culturing cells consisted of phenol red-free Leibovitz's L-15 medium (Sigma), 0.5 μg/ml gentamicin (Sigma) or 2.5 μg/ml amphotercinin B (Sigma), 100 units/ml penicillin, 0.1 mg/ml streptomycin (Sigma), 0.01 μg/ml basic fibroblast growth factor (bFGF, Sigma), 15 mM Hepes (Sigma) and 10 or 1% foetal calf serum (FCS, Sigma). The cells were then finally resuspended in 5 ml cell medium and 200 μl suspension was added to each well on different brands of 96-well plates.

4. RESULTS

4.1 Detection of PPARs by PCR

Table 1 shows expression of PPARs in different organs of males and females. PPARα was expressed in all organs and all PPARs were expressed in muscle and liver.

Table 1. Results from PCR on different organs of males and females in three-spined stickleback (*Gasterosteus aculeatus)*. The PCR-products were observed on gel-electrophoresis and compared with the expected size. An X in the table represents that a PCR product of the right size was indentified.

	PPARα1	PPARα2	PPARβ	PPARγ
Heart Female	X		X	X
Heart Male	X			X
Gill Female	X		X	
Gill Male	X		X	X
Kidney Female	X			X
Kidney Male	X			
Liver Female	X	X	X	X
Liver Male	X	X	X	X
Muscle Female	X	X	X	X
Muscle Male	X	X	X	X

4.2 Quantitative real-time PCR analysis

The optimal primer concentration for two genes (PPARβ and RXRα) was 0.1mM. For the other genes the optimal primer concentration was 0.3 mM. The optimal temperature was the same for all samples and is described in chapter 3.2.3. The results from the q-RT PCR are shown in figures 2-6. All genes showed a significant difference in expression between liver and kidney in females and males except PPARα2. There was a significant difference in expression of PPARα1 between male and female liver and this was the only gender-related difference.

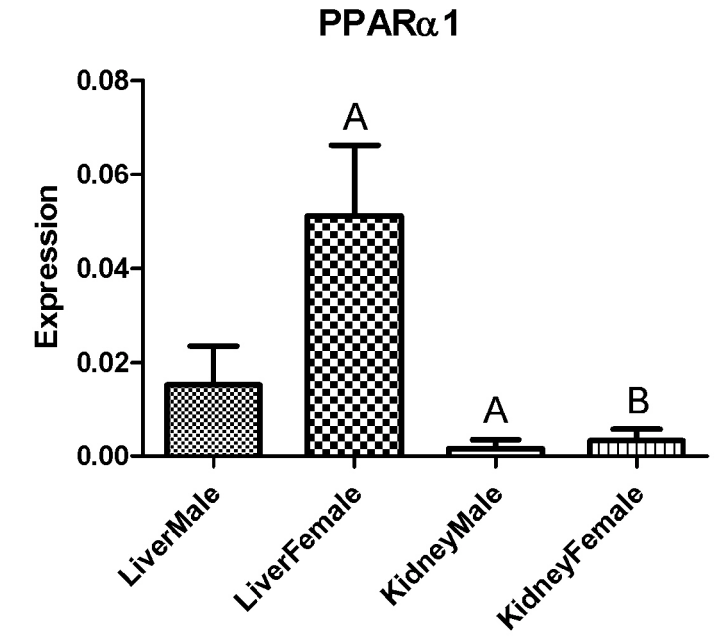

Figure 2. Gene expression (mean + SD) of PPARα1 in liver and kidney of female and male three-spined stickleback (*Gasterosteus aculeatus*). The gene expression was measured by quantitative real-time PCR and is normalized to the reference gene ACTB. All the groups were tested against each other, except LiverMale versus KidneyFemale and LiverFemale versus KidneyMale, with Bonferoni post test (p=0.05). A=significantly different from LiverMale and B=significantly different from LiverFemale.

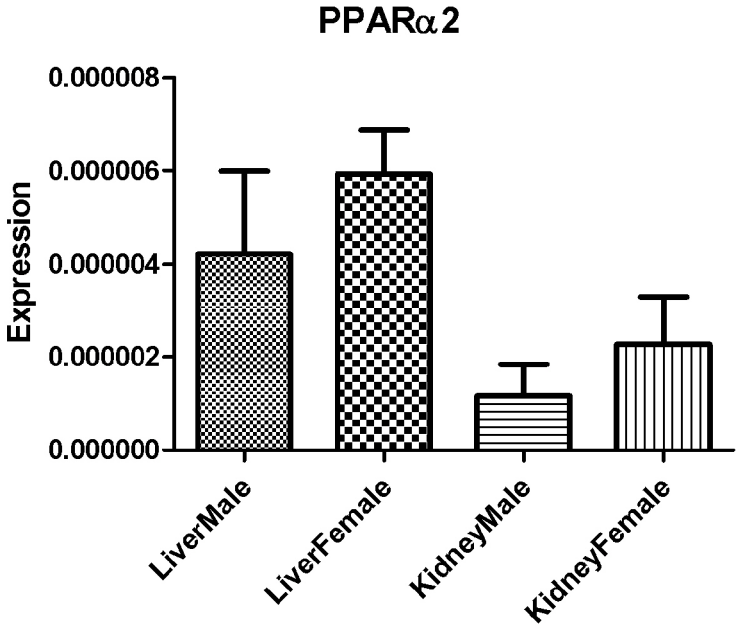

Figure 3.Gene expression (mean + SD) of PPARα2 in liver and kidney of female and male three-spined stickleback (*Gasterosteus aculeatus*). The gene expression was measured by quantitative real-time PCR and is normalized to the reference gene ACTB. All the groups were tested against each other, except LiverMale versus KidneyFemale and LiverFemale versus KidneyMale, with Bonferoni post test (p=0.05).

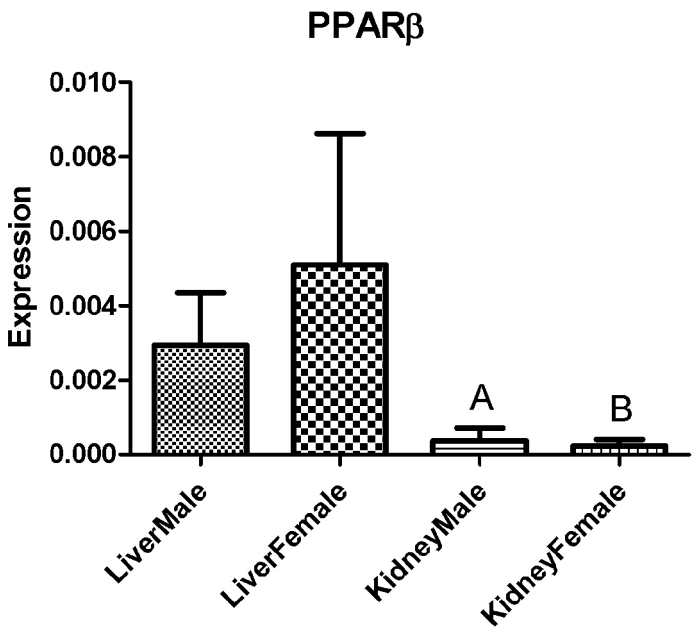

Figure 4. Gene expression (mean + SD) of PPARβ in liver and kidney of female and male three-spined stickleback (*Gasterosteus aculeatus*). The gene expression was measured by quantitative real-time PCR and is normalized to the reference gene ACTB. All the groups were tested against each other, except LiverMale versus KidneyFemale and LiverFemale versus KidneyMale, with Bonferoni post test (p=0.05). A=significantly different from LiverMale and B=significantly different from LiverFemale.

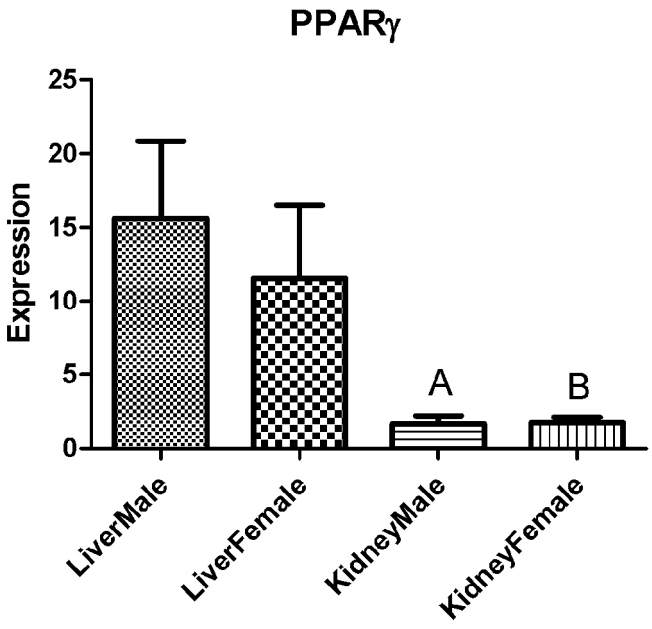

Figure 5. Gene expression (mean + SD) of PPARα1 in liver and kidney of female and male three-spined stickleback (*Gasterosteus aculeatus*). The gene expression was measured by quantitative real-time PCR and is normalized to the reference gene ACTB. All the groups were tested against each other, except LiverMale versus KidneyFemale and LiverFemale versus KidneyMale, with Bonferoni post test (p=0.05). A=significantly different from LiverMale and B=significantly different from LiverFemale.

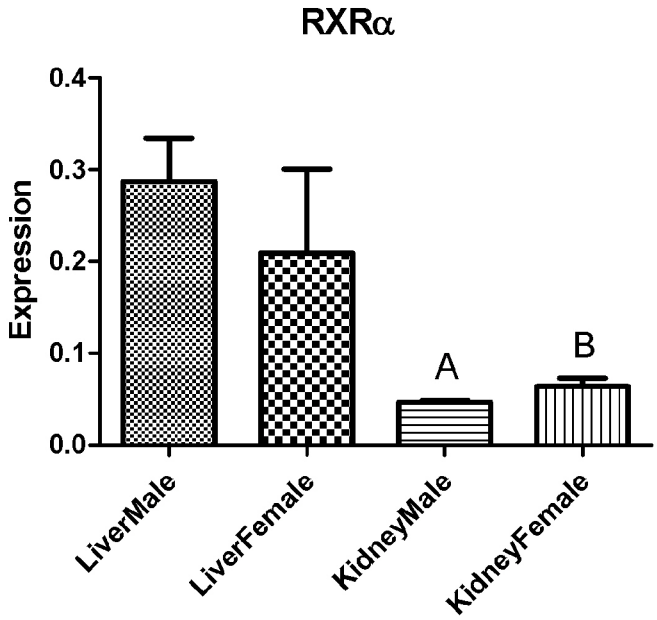

Figure 6. Gene expression (mean + SD) of RXRα in liver and kidney of female and male three-spined stickleback (*Gasterosteus aculeatus*). The gene expression was measured by quantitative real-time PCR and is normalized to the reference gene ACTB. All the groups were tested against each other, except LiverMale versus KidneyFemale and LiverFemale versus KidneyMale, with Bonferoni post test (p=0.05). A=significantly different from LiverMale and B=significantly different from LiverFemale.

4.3 Establishing primary cultures

No cell culture could be established, but some progress was made. The optimal incubation temperature was 18 °C. To culture in an atmosphere with increased CO_2 concentration did not give a better result than using air. Five different types of plates were tested with good result. The plates were Cell+ (Sarstedt), mircrotest primaria (Becton Dickinson), poly-D lysine (Bio-one), poly-L lysine (Bio-one) and collagen typ 1 (Bio-one). The serum level in the medium was reduced from 10 to 1 % with a higher amount of cells attached to the plate. Two different fungicides (gentamicin and amphotercinin B) were tested with good result. The best centrifuge speed was 300 g at room temperature.

5. DISCUSSION

The result from the PCR shows that PPARα1 is expressed in all tissues and that all PPARs are expressed in liver and muscle. Whether PPAR beta and gamma were expressed in gill, heart and kidney differed between males and females when analyzed by PCR. Why some samples did not show expression can be because the concentration of transcript was too low to be detected in the PCR. Using qRT-PCR all genes were found expressed in kidney and this supports the idea that the detection level was too low in the PCR. Taken together the results from the PCR and the qRT-PCR indicate that all PPARs are expressed in gill, heart, kidney, liver and muscle.

A significant difference in expression was shown for all studied genes except PPARα2 between liver and kidney. However, the efficiency of the primers for PPARα2 was rather low which indicates that with better primers the result could be the same as for the other PPARs. That the levels of expression of the different PPARs are lower in kidney than in liver is expected because the receptors are involved in a number of fat regulatory processes. Liver is the main organ for fat metabolism. A study made in grey mullet (*Chelon labrosus*) showed that the expression of PPARα was highest in liver (Raingeard et al. 2006).

PPARα1 showed a significant difference between males and females. We found a gender difference in only this case. In previous studies it has been shown that PPAR expression is not depending on gender in zebrafish (Ibabe et al. 2005).

In our initial cell culture experiment we isolated liver and kidney cells but they did not attach to the surface of the plates. Cao et al. (1996) showed that a serum concentration of 1% gave a higher amount of attached cells than a serum concentration of 10%. We did this alteration and got more cells attached to the surface. We also tested different incubation conditions and found that 18°C was the optimal temperature in accordance with findings by Björkblom et al. (2007). To get more cells we extended the trypsination time from 5 to 30 minutes and also did the trypsination in a shaker (200 rpm). We excluded the finest of the nylon filters when preparing the cells and tried treated plates from different companies. After all these alterations we got a lot more cells which all attached to the surface. We compared our cells with those shown in Björkblom et al. (2007) and found that our cells had a different shape. We then used

fishes from another aquarium and a new fungicide (gentamicin) was used in the medium. This did however not change our results and we still had cells of a type that we could not identify. Due to lack of time the study had to end here. The next step would be to find out which type of cells that was isolated. This could be done by PCR for specific genes for different cell types. Also the cells might be given a longer time to grow on the plates. The shape of the cells can change during growth so perhaps the cells could be identified after longer time of growth.

As conclusion of this study I recommend to continue the cell study on liver cells because the PPARs are expressed significantly higher in liver cells than in kidney cells. It is also easier to dissect a liver than a kidney and you get more cells per fish from the liver.

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