

Stress in Fish: The use of Serum Amyloid A as a Biomarker in Farmed Fish

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Abstract: The stress response is a non-specific mechanism by the host organism in response to exposure to a stressor, physical damage or infection. It involves the Acute Phase Reaction (APR) which is activated by the immune system, and involves the expression of Acute Phase Proteins (APPs) which include Serum Amyloid A (SAA) and C-Reactive Protein (CRP). SAA is a major APP which increases in concentration by up to 1000-fold during the APR. Stress and disease is of major economic and welfare concerns in farmed fish such as Rainbow Trout (*Oncorhynchus mykiss*), and the development of a fast molecular based bioassay to monitor this biomarker would be of benefit to the industry. The level of SAA expression was measured using mRNA extracted from the internal organs (liver, spleen, kidney, reproductive organs, heart and fatty tissue) of *O. mykiss*, as the initial test subject, using both standard PCR and qRT-PCR. A baseline level of SAA expression has been determined for the different organs/tissue and will be used as a comparator for SAA expression levels in fish obtained from sites considered polluted or under stress.

Keywords: SAA, APR, APP, *Oncorhynchus mykiss*, qPCR

1. Introduction

Fisheries and Aquaculture play an important role not just in food and nutrition but also in the livelihood and income of millions of people around the globe (FAO, 2016). As the population of Earth increases so too does the demand for a greater yield of food to help sustain the increasing global human population. According to the FAO, 2016 fish is one of the most traded food sources worldwide.

Fish farming is becoming a more common practice for breeding fish for consumption due to the increased demand for fish. This makes it vital that the fish being raised in these farms are healthy and not subject to unnecessary stresses caused by their living conditions.

Stress is a fundamental mechanism all organisms possess which is activated in response to any demand placed upon it (Selye, 1973 cited in Barton, 2002). The types of stress found in fish will depend on whether they are wild type fish or fish bred in fish farms. The most common types of stress in fish bred in fish farms include: the size and shape

of the enclosure, the material used to construct the enclosure, the number of fish per enclosure, the quality of the water, how often the fish are fed, competition from other fish for food, space or mating, how the fish are monitored, if they need to be transported between enclosures or to be treated when sick and how they are euthanized (Tort, 2011 and Nardocci *et al*, 2014).

The cause of the stress will also effect whether the fish is exposed to acute (short term) or chronic (long term) stress and both acute and chronic stress will have various effects on the system (Tort, 2011). Once exposed to a stressor the immune system is switched on to help the body deal with the stress and revert it to its normal physiological state. This involves the activation of a process known as the Acute Phase Reaction (APR) which according to Jensen *et al*, 1997 is a "rapid systemic reaction" the immune system has in response to tissue damage, infection, burns, surgery and even some advanced forms of cancer (Gabay and Kushner, 1999). During the APR, plasma proteins (Manley *et al*, 2005) known as Acute Phase Proteins (APP's) are synthesised by the liver to help the APR to combat the stress or inflammation. These proteins include Serum Amyloid A (SAA) which raises in concentration by up to 1000 fold during an APR.

This study will aim to use SAA as a biomarker to measure stress and disease in fish in a fast molecular bioassay.

2. Methodology

2.1. Dissection of Fish

Rainbow Trout, *Oncorhynchus mykiss* (*O. mykiss*), fish samples were obtained from a commercial fish farm in Co. Carlow, Ireland. The fish had been euthanized prior to arrival. In order to obtain the necessary organs the trout was opened from the vent to throat on the underside of the fish using slow shallow cuts to prevent any damage occurring to the internal organs. Once the opening was large enough the internal organs (Heart, Liver, Kidney's, Reproductive Organs and Spleen) were removed by following protocols as described on Trout Unlimited, 2006 and Salmonids in the Classroom. Once organ was removed, they were placed into individual storage bags and kept on ice. A section of fatty tissue was also removed from just below the rib cage. All organs were stored in the -20°C freezer for further analysis.

2.2. RNA Extraction, DNase Treatment and cDNA Synthesis

Using the E.Z.N.A. Total RNA Kit I, Omega bio-tek, an RNA extraction was performed on 30mg of each target organ (Liver, Heart, Kidney, Reproductive Organs, Spleen and Fatty tissue) as per the manufacturers' protocol. Any genomic DNA contamination of the RNA samples was removed by carrying out DNase treatment (RNase-Free DNase, Promega). The RNA samples were run on a 1% agarose gel to observe the effectiveness of the DNase treatment and the integrity of the RNA. The concentration of RNA extracted was measured using the DeNovix DS-11 Spectrophotometer from Mason Technology. The RNA was stored at -70°C for further testing. cDNA synthesis was carried out to a total volume of 20µl using the qScript cDNA Synthesis Kit provided by VWR. These samples were stored at -20°C until required for PCR analysis.

2.3. PCR and qPCR

PCR was carried out on the cDNA samples to test for the presence of the gene of interest (A-SAA) along with a housekeeping gene (β -actin). The primers used were designed from the SAA (Gene Bank Reference: AM422446.1) and β -actin (Gene Bank Reference: AJ438158.1) sequences of Rainbow Trout from the National Center for Biotechnology Information (NCBI) database (Geer *et al*, 2009) using Primer3 (Untergasser *et al*, 2012, Koressaar and Remm, 2007), an online tool which is used to design PCR primers from gene sequences, and taken from Villarroel *et al*, 2007 as seen in Table 1. The gene sequence, in FASTA format, was input into Primer3 and using this software the PCR primers were designed. The samples were run using a master mix, primers and PCR grade water according to manufacturer's instructions (Promega) and then optimised to give the most defined band. The PCR was optimised by altering the annealing temperature and the volume of the components used in the reaction mixture. The samples were run on a 2% agarose gel at 70 Volts for 60 minutes, then visualised

using the Compact Gel Transilluminator Instrument. Distinct and clear bands were observed after optimisation.

Once the genes of interest were observed on the 2% agarose gel, PCR amplicons were chosen representing each organ and were purified and sequenced by Eurofins MWG to confirm that the bands being detected were in fact SAA and β -actin from *O. mykiss*.

Gene expression was measured using the Lightcycler Nano and 480 (Roche). The protocol used included an initial holding step of 95°C for 15 minutes, followed by 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. The components used include 8µl master mix, 0.5µl of each primer (forward and reverse), 2µl sample (concentration measured using the DeNovix DS-11 Spec.) and made up to a final volume 20µl using PCR grade water (Roche). A final Melt Curve Analysis step was also carried out to determine gene expression levels. In order to measure gene expression a 10 fold serial dilution standard curve was designed using mRNA taken from the heart (636ng/µl of RNA). The cycle threshold (Cp) values of the unknown samples was compared to the standard curve which allows for the concentration of the unknowns to be determined (Wong and Medrano, 2005).

3. Results

When determining which organs were to be tested in this study it was essential to include the liver as this is where the APPs are synthesised (Manley *et al*, 2005), this could then be used as a baseline for SAA levels in the other organs. The other organs chosen were the heart, kidneys, spleen, reproductive organs and some fatty tissue. Once the samples were prepared, PCR was carried out using the designed primer sets Table 1, to determine if SAA and β -actin transcripts were present in the tissue and organs.

After multiple PCR runs it was determined that the primers in Table 2 would be the most suitable to continue with in this study.

Table 1. SAA and β -actin Primer Sets tested using PCR

Primer Set	Primer Name	Sequence (5' – 3')	Source
SAA 1	SAAF1	TGAAGCTGCTCAAGGTGCTA	This Study - designed using Primer3
	SAAR1	GTGTCCTCGACCATTGGAAC	This Study - designed using Primer3
SAA 2	SAAF2	GACGCCAACTGGAAAACTC	This Study - designed using Primer3
	SAAR2	TTACGTCCCCAGTGGTTAGC	This Study - designed using Primer3
SAA 3	SAAF	TAAAGACATGTGGCGTGCAT	Villarroel <i>et al</i> , 2007
	SAAR	TTACGTCCCCAGTGGTTAGC	Villarroel <i>et al</i> , 2007
β -actin 1	B-actinF1	CCCGACTACCACTTCAGCTC	This Study - designed using Primer3
	B-actinR1	CTTTAATCCGCTGCTTCACC	This Study - designed using Primer3
β -actin 2	B-actinF2	AAGGACCTGTACGCCAACAC	This Study - designed using Primer3
	B-actinR2	TCCACATCTGTTGGAAGGTG	This Study - designed using Primer3
β -actin 3	B-actinF	ATGGAAGATGAAATCGCC	Villarroel <i>et al</i> , 2007
	B-actinR	TGCCAGATCTTCTCCATG	Villarroel <i>et al</i> , 2007

Table 2. SAA and β -actin Primer Sets chosen for use in this study

Primer Set	Primer Name	Sequence (5' - 3')	Source
SAA 3	SAAF	TAAAGACATGTGGCGTGCAT	Villarroel <i>et al</i> , 2007
	SAAR	TTACGTCCCCAGTGGTTAGC	Villarroel <i>et al</i> , 2007
β -actin 1	B-actinF1	CCCGACTACCACTTCAGCTC	This Study – designed using Primer3
	B-actinR1	CTTTAATCCGCTGCTTCACC	This Study – designed using Primer3

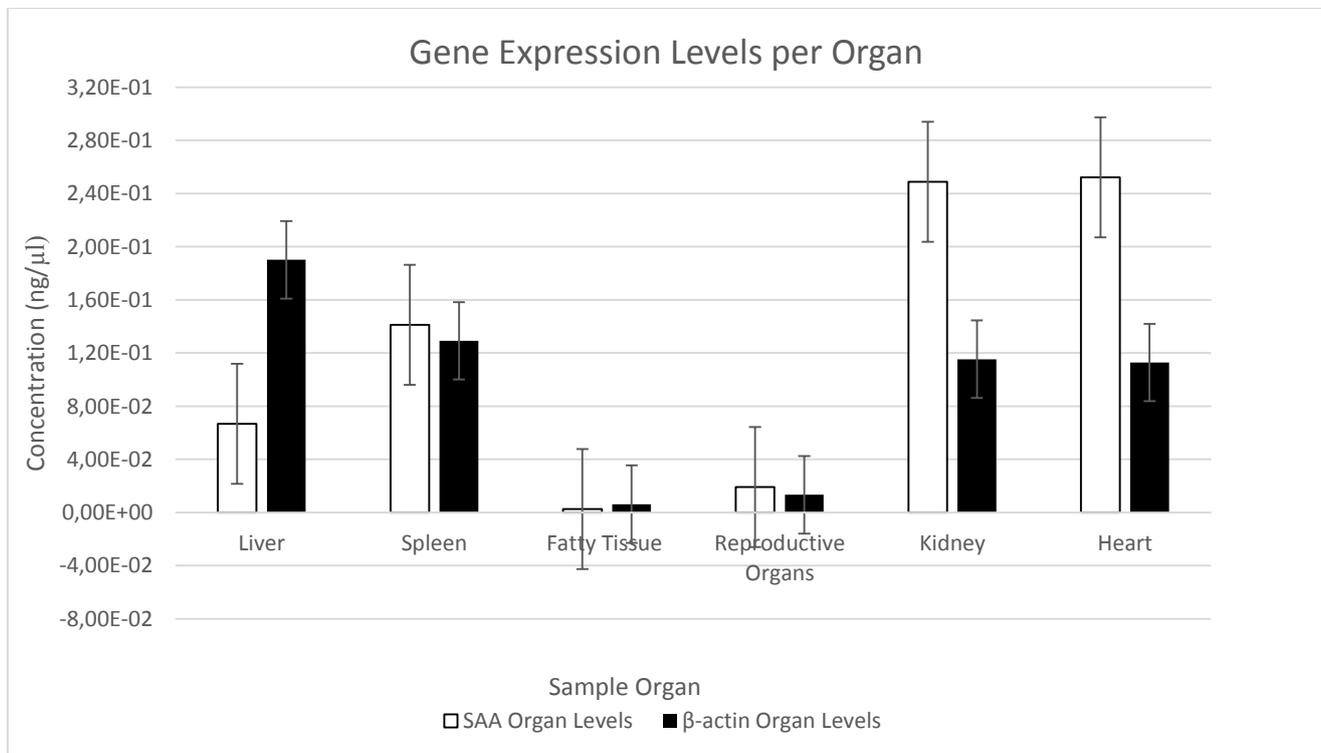


Figure 1. Pooled comparison of measured SAA and β -actin concentration levels per organ using the LightCycler 480.

Once SAA and β -actin bands were observed using PCR, the samples could then be quantified using qPCR. A 10-fold serial dilution was performed on heart RNA, the concentration of which was measured on a Microvolume Spectrophotometer (DeNovix ds-11). This standard curve was then run on the LightCycler 480 (Roche) along with the unknowns to determine the concentration SAA and β -actin present in each sample organ.

The levels of SAA and β -actin from four *O. mykiss* were analysed as observed in figure 1. By measuring SAA and β -actin concentrations in non-stressed fish, it is possible to use the data obtained as a baseline for SAA levels in fish after removal from the tank and being euthanized. This will allow for a direct comparison of SAA levels in response to other stressors such as confinement, overcrowding, environmental conditions such as pollutants.

This study has focused on measuring SAA levels in various organs of the fish while previous studies (Bayne *et al*, 2001 and Talbot *et al*, 2009) have only focused on measuring SAA levels in the liver as the liver is the primary site of APP synthesis (Manley *et al*, 2005). It was expected then that the liver would yield the greatest level of SAA expression however after analysis of preliminary

results this does not seem to be the case. The heart (2.52E-01ng/μl), kidney (2.49E-01ng/μl) and spleen (1.41E-01ng/μl) are giving a higher SAA concentration than that of the liver (6.68E-02ng/μl). It is unclear why this has occurred and further analysis is required to determine the reason for the high concentration of SAA in the heart and kidneys and the low concentration of SAA in the liver.

In this study β -actin is used as a housekeeping gene in this study as a comparison for SAA gene expression due to it being ubiquitous in nature. The β -actin levels measured (liver; mean 1.90E-01 \pm SD 0.52ng/μl; spleen: mean 1.29E-01 \pm SD 0.02ng/μl; fatty tissue: mean 6.27E-03 \pm SD 0.01ng/μl; reproductive organs: mean 1.33E-02 \pm SD 0.01ng/μl; kidney: mean 1.15E-01 \pm SD 0.09ng/μl and heart: mean 1.13E-01 \pm SD 0.15ng/μl) in this study are lower than the SAA levels measured (liver; mean 6.68E-02 \pm SD 0.06ng/μl; spleen: mean 1.41E-01 \pm SD 0.03ng/μl; fatty tissue; mean 2.69E-03 \pm SD 0ng/μl; reproductive organs: mean 1.92E-02 \pm SD 0.02ng/μl; kidney: mean 2.49E-01 \pm SD 0.18ng/μl and heart: mean 2.52E-01 \pm SD 0.32ng/μl) in all organs except the liver. It was expected that β -actin levels would be lower than SAA as it does not increase in concentration when exposed to a stressor.

4. Conclusion

Fish farming is becoming a more common practice as a way to maintain a steady supply of fish for consumption in a world whose population is continuing to increase. As the industry continues to grow it is vital that the fish being bred are safe and healthy for human consumption. If the fish are not maintained in healthy environments they can become ill and could pass on illnesses to those who consume them.

By developing a bioassay that can detect miniscule levels of stress or infection in fish, the situation can be monitored to determine the cause of the stress before it becomes a major problem. This will not only protect the fish but also anyone who would consume them.

Further analysis of *O. mykiss* stress levels will be measured using fish from fish farms as well as cell lines grown in a sterile environment. Once the cell lines are grown various stressors will be applied to the lines to measure SAA gene expression levels in response to each stressor. These levels can then be compared to those obtained from the fish farms to observe any correlation between the levels of the cell lines and the fish bred in fish farms.

Acknowledgments

This study is supported by the Institute of Technology Carlow Presidents Research Fellowship Award

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