Deliverable Report

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Deliverable Title	Determine a developmental stage ability to synthesize key enzymes in Tau and bile acid synthesis in grey mullet		
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Lead Scientist preparing the Deliverable: B. Koven (IOLR)

Objective: Determine the ability to synthesize key enzymes in Tau and bile acid synthesis in grey mullet.

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1. Introduction

The β-amino sulfonic acid taurine, which is not incorporated into proteins, plays an array of critical roles in its free form. These include involvement in bile salt synthesis, anti-oxidative defence, cellular osmoregulation, as well as contributing to visual, neural and muscular function (Fang et al., 2002; Omura and Inagaki, 2000). Taurine cannot be synthesized in carnivorous teleosts and therefore must be provided in the diet. The yellowtail (*Seriola quinqueradiata*), Atlantic bluefin tuna (*Thunnus thynnus*) and skipjack tuna (*Katsuwonus pelamis*), as well as the Japanese flounder (*Paralichthys olivaceus*) were found deficient in cysteine sulfinate decarboxylase (CSD), a key enzyme in the main taurine synthesis pathway (Yokoyama et al., 2001; Goto et al., 2003; Chen et al., 2005; Takagi et al. 2006). However, it is unclear if herbivorous and omnivorous fish, where vegetation is a major component of their diet and characteristically deficient in taurine or contains low levels of this nutrient, have taurine synthesis capability. It has been reported that common carp (*Cyprinus carpio*) do not have a dietary taurine requirement and no significant expression of CSD but retain taurine in the tissues suggesting that this species synthesizes taurine through a different pathway (Kim et al., 2008).

Taurine is mainly synthesized either from the oxidation of cysteine via cysteine dioxygenase (CDO), which generates cysteine sulfinate that is decarboxylated by cysteine sulfinic acid decarboxylase (CSD), or less commonly from the oxidation of cysteamine by cysteamine (2-aminoethanethiol) dioxygenase (ADO). Both pathways generate hypotaurine, which is oxidized to taurine. This ability may attenuate or possibility eliminate the need for dietary taurine in some species. Studies carried out on juvenile grey mullet at the IOLR (P4), which is omnivorous, demonstrated a significant dietary taurine requirement (see **Sub-task 13.3.2**), at the 0.5% DW dietary level. Nevertheless, it remains unclear if this species is able to synthesize taurine through the CSD and/or ADO pathways.

One of the major roles for taurine is to conjugate with bile acids such as cholic acid or chenodeoxycholic acid in the liver, which is then stored in the gall bladder. Conjugated bile acids, when released into the lumen of the intestine after feeding, emulsifies fats to make them more accessible for digestion and absorption. In fish, bile acid is conjugated not with glycine but with taurine only by the bile acid- coenzyme A (CoA)–amino acid N-acyltransferase in the liver and implies that taurine is critical in fish for lipid digestion and absorption (Huxtable, 1992). Moreover, 7α-hydroxylase (CYP7A1) is the rate limiting enzyme in bile salt synthesis (Fukuda et al., 2011) and has been associated with dietary taurine and its growth promoting properties. Fish liver samples taken in **Sub-task 13.3.2** for analysis of the gene expression of CSD and ADO, were also analysed for CYP7A1 in order to determine if dietary taurine up regulated bile salt synthesis.

2. Materials and Methods

Total RNA was extracted from grey mullet (*Mugil cephalus*) liver tissue by the guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987) using Bio-Tri RNA reagent (Bio Lab Ltd., Jerusalem, Israel). The concentration of RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer (Synergy HT, BioTek, Winooski, VT, USA). The purity of each sample was assessed for proteins by the 260 vs. 280 nm ratio. After residual DNA removal, a qScript™ cDNA Synthesis Kit (Quantabio, Beverly, MA, USA) was used for the synthesis of cDNA from RNA samples according to manufacturer's protocol. Degenerate primers were designed in order to sequence the desired genes. The PCRs were carried out in a final volume of 25 µl using the GoTaq® Green Master Mix (Promega, Madison, WI, USA) and 25 pmol of each Primer. PCR products were purified using QIAquick PCR Purification Kit (QIAGENE, Hilden, Germany) and sequenced by Hy- Labs (Rehovot, Israel). Gene identity was confirmed by comparing the obtained sequences with those available at the Genebank (http://www.ncbi.nlm.nih.gov/Genbank/). Real time PCR was performed using PerfeCTa® SYBR® Green FastMix®, Low ROX™ (Quanta Biosciences, Inc. MD, USA). Gene specific primers (Table X) were designed and synthesized by Agenktek (Tel-Aviv, Israel).

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed (in triplicates) in a total reaction volume of 10 µl, consisting of the respective primer set (300 nM), cDNA template and PerfeCTa® SYBR® Green FastMix®, Low ROX™ (QuantaBioSciences, Inc. MD, USA). Since the fluorophore, Fast SYBR Green®, binds in a nonspecific manner to double strand DNA, it is necessary to ensure that the amplified PCR product is homogenous. Hence, the presence of a single amplicon was verified at the end of each run via a dissociation analysis (Melting curve), by which fluorescence was quantified in regard to temperature rise. When temperature increases, DNA strands separate and the DNA bounded fluorophore releases. A single peak in a gaussian curve, which outlines the change in fluorescence as a function of temperature (-dF/dT), will indicate a homogenous PCR product.

To normalize the levels of target genes, qPCR for rRNA 18S was also performed with the sample cDNAs. A negative control with sterile water as template was included in order to check for possible reagent contamination. In addition, in order to rule out the presence of contaminating genomic DNA, our qPCR experiments included minus-reverse transcriptase controls (i.e., PCR amplification using DNAse-treated total RNA samples without reverse transcription as a template). The results were analyzed by 7500 Fast Real-Time PCR System software (Applied Biosystems). Gene expression levels were calculated by: relative expression = $2^{-\Delta\Delta Ct}$, Ct – threshold cycle (Livak and Schmittgen, 2001).

3. Results

In **Fig. 1a** the results show a taurine dose dependent and significant (P<0.05) response in the gene expression of cysteine sulfinic acid decarboxylase (CSD) in fish fed the control (0% taurine) to 1% taurine treatments and then a decrease of the gene expression of these enzymes in fish fed the highest dietary taurine level (2% DW diet). Fish fed the 0.5% taurine diet demonstrated almost 8 times the expression level of this gene compared to the control. The general level of gene expression of cysteamine (2-aminoethanethiol) dioxygenase (ADO) in the less common taurine synthesis pathway was much lower, where 0.5% dietary taurine stimulated the highest but non-significant (P>0.05) ADO response (**Fig. 1b**). Moreover, the results show considerable CSD synthesis when the fish are not consuming any taurine although the ADO pathway is not stimulated until fish are ingesting 1%. In **Fig. 2** there was no clear, significant (P>0.05) response by dietary taurine on the expression of fish liver CYP7A1.

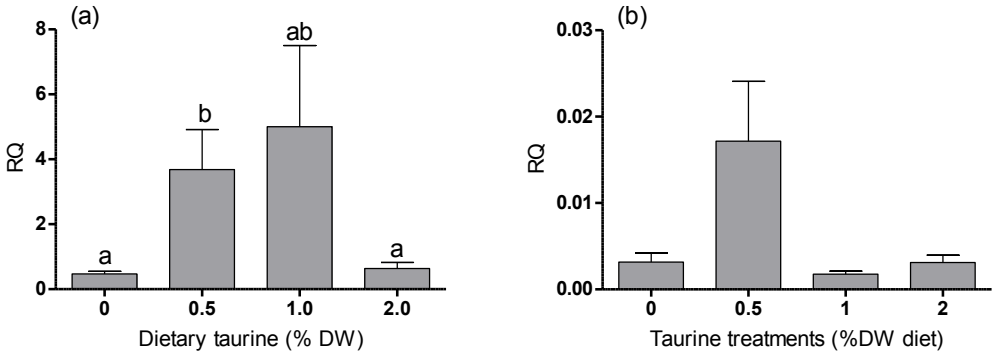

Figure 1. The effect of dietary taurine (0, 0.5, 1.0, 2.0% DW diet) on the gene expression of (a) cysteine sulfinic acid decarboxylase (CSD) and (b) cysteamine (2-aminoethanethiol) dioxygenase (ADO) in juvenile fish. Bars represent average values ± SEM (N=15). Values having different letter(s) were significantly (P<0.05) different.

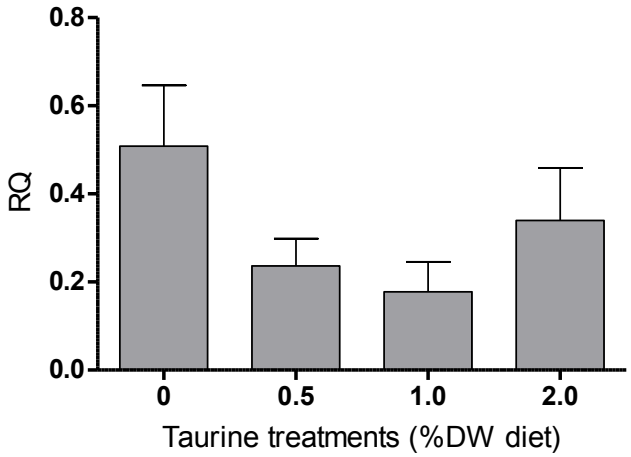

Figure 2. The effect of dietary taurine (0, 0.5, 1.0, 2.0% DW diet) on the gene expression of CYP7α1 in juvenile fish. Bars represent average values ± SEM (N=15).

4. Discussion

The results suggest not only that the CSD pathway is active in the main taurine synthesizing pathway in the absence of dietary taurine but that the expression of this key gene increases with increased levels of dietary taurine until 1% where CSD expression drops at the high dietary taurine level. The synthesis of taurine in the liver when levels of this nutrient are increasing in the diet seems counter intuitive. However, taurine can function as an osmolyte to maintain cell volume. Conceivably, increased taurine in the blood circulation of the liver, due to higher dietary taurine, may stimulate increased synthesis within liver cells to reduce osmotic pressure across the membrane in order to prevent cell shrinkage and changes in intracellular hydro-mineral balance. The cell taurine content of an astrocyte primary culture varied with extracellular taurine concentration through endogenous synthesis via the CSD pathway (Reymond et al. 1996). However, Bitoun and Tappaz (2000) argued that taurine synthesis would not be sufficient to explain taurine intracellular content and is likely accompanied by biosynthesis of taurine transporter (TauT) that allows more extracellular taurine to enter the cell. On the other hand, the capacity of endogenous taurine synthesis in liver cells may be greater than brain astocyte cells investigated in that study. Although there was a clear but non-significant ($P>0.05$) increase of ADO gene expression in fish feeding on the 1% taurine diet, the RQ values were generally very low, suggesting that this pathway represented a minor contribution to endogenous taurine synthesis. The sharp decrease in CSD in the 2% taurine diet may indicate a negative feedback mechanism and/or a strategy to increase TauT synthesis and increased transport of taurine into the cell as more effective than intracellular biosynthesis at this dietary concentration.

Taurine dietary supplementation of a number of species resulted in increased bile salt production (Kim et al. 2007, 2015). This was supported by Yun et al. (2012) who found a correlation between dietary taurine and CYP7a1 synthesis in juvenile turbot, *Scophthalmus maximus*. In addition, a taurine deficiency in fish has been associated with green liver syndrome caused by decrease in production of bile pigments (Sakai et al. 1990). However, in our study, there was no clear effect of dietary taurine on CYP7α1 and bile salt. In fact, the highest expression of bile salt synthesis was in the control where there was no dietary taurine suggesting that CSD endogenous taurine synthesis was supplying sufficient levels of this nutrient. On the other hand, **Sub-task 13.3.2** found that a diet containing at least 0.5% taurine resulted in significantly ($P<0.05$) improved weight gain in juvenile fish. This suggests that endogenous taurine synthesis, although possibly sufficient in the synthesis of bile salt, may not be adequate in contributing taurine to muscle function and growth. Taurine is very abundant in muscle tissue, where it can represent 53% of the free amino acid pool (Brosnan and Brosnan, 2006). Taurine is required for calcium signaling in vertebrates for normal cardiac, skeletal muscle, nervous, and retinal function (Bouckenooghe et al., 2006). In rainbow trout the muscle ratio (fillet mass x100/fish mass) markedly increased when taurine was supplemented at 0.5 and 1.0% of the diet (Gaylord et al. 2007).

Taken altogether, it appears that grey mullet juveniles have the capacity for endogenous taurine synthesis that may be sufficient for cell volume homeostasis and bile salt production but may fall short in optimizing skeletal muscle function and growth, thereby requiring a minimum of 0.5% of taurine in the diet.

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