# Taurine metabolism and effects of inclusion levels in rotifer (*Brachionus rotundiformis*, Tschugunoff, 1921) on Atlantic bluefin tuna (*Thunnus thynnus*, L.) larvae

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Accepted refereed manuscript of:
Betancor M, Laurent G, Ortega A, de la Gándara F, Tocher D & Mourente G (2019) Taurine metabolism and effects of inclusion levels in rotifer (Brachionus rotundiformis, Tschugunoff, 1921) on Atlantic bluefin tuna (Thunnus thynnus, L.) larvae. *Aquaculture*, 510, pp. 353-363.
DOI: https://doi.org/10.1016/j.aquaculture.2019.05.040

**Abstract**

Taurine appears to be a crucial nutrient for teleosts, especially top predator species such as Atlantic bluefin tuna (*Thunnus thynnus,* L.; ABT). While dietary taurine supplementation has been highly recommended, there is a lack of studies on taurine assimilation and biosynthesis for this iconic species. The present study aims to provide insight into the molecular mechanisms involved in taurine biosynthesis and transport in ABT by studying tissue distribution and ontogenetic development of expression of cysteine dioxygenase (*cdo*), cysteine sulfinic acid decarboxylase (*csad*), 2-aminoethanethiol dioxygenase (*ado*) and taurine transporter (*tauT*) in response to graded levels of dietary taurine supplementation. The full open reading frame (ORF) for *cdo* and partial sequences for *csad*, *ado* and *tauT* were obtained, with the translated polypeptides being 202, 176, 166 and 324 amino acids, respectively. All three showed characteristics such as cupin motifs in Cdo and predicted N-glycosylation sites in Taut that are common to these genes in other species. Phylogenetic analysis showed that the ABT sequences clustered with sequences of other teleosts, and separately from mammals and molluscs. Tissue distribution varied, with adipose tissue, kidney, white muscle and testis/brain showing highest expression of *cdo*, *csad*, *ado* and *tauT*, respectively. Whole larvae expression of *csad* peaked at 15 dah, whereas the other genes generally increased throughout development to show highest expression at 25 dah. The nutritional trial was carried out by feeding ABT larvae from mouth opening to 14 days after hatching (dah) with rotifers (*Brachionus rotundiformis*) enriched with 4 different levels of taurine: 0.0 (tau0), 0.5 (tau0.5), 1.0 (tau1), and 2.0 g taurine per $10^6$ rotifers (tau2). Rotifers effectively accumulated taurine with ABT larvae fed on treatment tau2 attaining the highest concentration of taurine. However, ABT larvae fed tau1 displayed higher growth and survival, and flexion index at 14 dah, than larvae fed the other taurine levels. Larvae fed tau1 also showed generally higher expression of *tauT* and *cdo* and digestive and antioxidant enzyme genes. While this study showed that larval ABT express taurine metabolism genes, suggesting possible synthesis that could contribute to the taurine pool in the fish, larval

47 performance was enhanced by a level of dietary taurine (3.7 mg taurine $g^{-1}$ rotifer) supplied by
48 enrichment of rotifers at 1 g taurine per $10^6$ rotifers.
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50 **Keywords**: bluefin tuna, larvae, taurine, gene expression, rotifer enrichment, cDNA
51
52 **Abbreviations**: aa, amino acids; ABT, Atlantic bluefin tuna (*Thunnus thynnus*); *alp*, alkaline
53 phosphatase; *amy*, amylase; *anpep*, amino peptidase; *bactin*, beta actin; *bal1*, bile salt activated lipase
54 1; *bal2*, bile salt activated lipase 2; *cat*, catalase; *cdo*, cysteine dioxygenase; *csad*, cysteine sulfinic
55 acid decarboxylase; dah, days after hatch; *ef1α*, elongation factor 1 alpha; FC, fold change; *gpx1*,
56 glutathione peroxidase 1; *gpx4*, glutathione peroxidase 4; *myhc*, myosin heavy chain; ORF, open
57 reading frame; *pl*, pancreatic lipase; *pla2*, phospholipase A2; qPCR, quantitative real time PCR; *sod*,
58 superoxide dismutase; *tauT*, taurine transporter; *tropo*, tropomyosin; *tryp*, trypsin; *ubiq*, ubiquitin;
59 UTR, untranslated region.
60

## Introduction

Atlantic bluefin tuna (ABT, *Thunnus thynnus,* L.) is a species with high market value although its closed aquaculture is currently inefficient and far from large-scale commercial production with low survival of larval stages, (De la Gandara *et al*., 2016; Van Beijnen, 2017). In order to optimize the ABT production cycle, further knowledge of the nutritional requirements of the species is pivotal, and understanding biological mechanisms of nutrient assimilation in larvae is a key area. Although some studies have been performed on different aspects of ABT nutrition (Morais *et al*., 2011; Betancor *et al*., 2017a,b; Koven *et al*., 2018) there is limited information regarding requirements for many nutrients that can be critical for larval and juvenile stages of this species.

Taurine is the common name for 2-aminoethanesulfonic acid, an amino sulfonic acid which is not incorporated into proteins but, rather, resides in the free amino acid pool (Hamre *et al.*, 2013). Despite this, taurine is not considered an amino acid since it contains a sulphonyl acid group rather than a carboxyl acid group (Pinto *et al*., 2012). However, taurine plays a critical role in many major biological functions and, in teleosts, is involved in bile salt conjugation, osmoregulation, membrane stabilization, modulation of neurotransmitters, antioxidant function and early development of visual, neural and muscular systems (Huxtable, 1992; Salze and Davis, 2015). In vertebrates, there are two main pathways for biosynthesizing taurine from cysteine with the final step in both pathways being the oxidation of hypotaurine to taurine, with the production of hypotaurine varying (Salze and Davis, 2015). One pathway involves the participation of two enzymes, cysteine dioxygenase (Cdo; EC 1.13.11.20) and cysteine sulfinate decarboxylase (Csad; EC 4.1.1.29), which produce hypotaurine from cysteine. A second route for hypotaurine production is through the action of the enzyme 2-aminoethanethiol dioxygenase (Ado; EC 1.13.11.19), which converts cysteamine, derived from coenzyme A degradation, to hypotaurine. In addition to these enzymes, taurine transporter (Taut), a highly conserved membrane transporter is critical for the transport and recycling of taurine and plays crucial roles in intestinal functions (O'Flaherty *et al*., 1997; Shimizu and Satsu, 2000). Fish have varied taurine biosynthesis capability, possibly reflecting differences in the expression

levels/activities of the key biosynthetic enzymes and the taurine transporter (Liu *et al*., 2017). For instance, Csad activity has been reported to differ among different teleost species (El-Sayed, 2014; Salze and Davis, 2015) and an apparent lack of Csad activity has been reported in fish families such as the *Labridae*, *Scombridae* and *Soleidae* (Salze and Davis, 2015) and ABT (Yokoyama *et al*., 2001).

So far, it is unknown if the metabolic pathway for biosynthetizing taurine using enzymes to transform methionine-derived cysteine is active in ABT. Therefore, if ABT is unable to synthesize taurine by endogenous metabolism, dietary input would be essential especially for larval stages where biosynthetic functions in general are still developing and incomplete (De la Rosa and Stipanuk, 1985). In the wild, ABT larvae can assimilate taurine from natural food, mainly copepods (Uotani *et al*., 1990; Catalan *et al.*, 2011) that contain high levels of taurine (Van der Meeren *et al*., 2008; Karlsen *et al*., 2015). In farming, taurine would have to be supplied by feed and, given the present trend in aquafeed production, with fish meal and oil being replaced by terrestrial plant sources that are devoid of taurine, it is crucial to determine the taurine biosynthetic capacity of ABT, as a deficiency in this nutrient could appear (Gatlin *et al*., 2007; Barrows *et al*., 2008; Takagi *et al*., 2008). This is particularly important in ABT, a top predator in the trophic chain, suggesting that taurine enrichment of feed might be essential. Some previous studies have indicated the positive effect that dietary taurine can have on teleost larvae, such as enhancement on growth (Matsunari *et al*., 2005a,b, 2008, 2013; Karlsen *et al*., 2015; Kim *et al*., 2016), feed conversation ratio and lipid metabolism (Chatzifotis *et al*., 2007), digestive enzyme activities (Salze *et al*., 2012), and metamorphosis (Pinto *et al*., 2010). Indeed, a recent study in Pacific bluefin (*Thunnus orientalis*) and yellowfin tuna (*T. albacares*) larvae demonstrated that feeding rotifers enriched with 800 mg taurine $L^{-1}$ promoted larval growth and total protein content (Katagiri *et al*., 2017), suggesting that taurine is an important nutrient for the early stages of rapidly growing teleost species.

The aim of the present study was to provide insight into the molecular mechanisms involved in taurine biosynthesis and transport in ABT by studying the tissue distribution, ontogenetic development and response to graded dietary taurine supplementation of *cdo*, *csad*, *ado* and *tauT* genes

For this purpose, the open reading frames (ORF) of the genes were sequenced and their expression determined by real time quantitative PCR (qPCR) in tissues and during development. Additionally, a dose-response nutritional trial was performed by feeding ABT larvae from mouth opening to 14 days after hatching (dah) with rotifers enriched with four increasing levels of taurine (0.0 g taurine per $10^6$ rotifers, tau0; 0.5 g taurine per $10^6$ rotifers, tau05; 1.0 g taurine per $10^6$ rotifers, tau1 or 2.0 g taurine per $10^6$ rotifers, tau2). Moreover, the effects of graded taurine inclusion in rotifers on the expression of larval ABT genes related to antioxidant and digestive enzymes was also investigated.

## 2. Materials and Methods

### *2.1. Isolation of genes of taurine metabolism*

Sequences of genes encoding for taurine metabolism (*tauT*, *cdo*, *ado* and *csad*) were obtained by identifying the sequences from Sequence Read Archives (SRA) SRX2255758, ERX555873 and ERX555874. The set of contiguous sequences were assembled using CAP3 (Huang and Madan, 1999) and identity of the deduced amino acid (aa) sequences confirmed using the BLASTp sequence analysis service of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Primers were designed in order to sequence the open reading frames (ORF) of each gene (Supplementary Table) using cDNA from whole ABT larvae (see below) as template. PCR products obtained were purified using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Little Chalfont, UK) and sequenced to confirm identity (Sanger ABI3730xl, Eurofins Genomics, Konstanz, Germany). Subsequently, primers for qPCR were designed on these PCR fragments using the online software Primer3 (Untergasser *et al*., 2012; Supplementary Table).

The deduced aa sequences of the newly sequenced ABT *tauT*, *cdo*, *ado* and *csad* and sequences of these genes of a variety of species across vertebrate and invertebrate lineages were aligned with the ClustalW tool (BioEdit v7.0.9, Tom Hall, Department of Microbiology, North Carolina State University, USA). Phylogenetic analysis was performed using the neighbour-joining

method with MEGA 5.1 (http://www.megasoftware.net/) (Saitou and Nei, 1987). Confidence in the resulting tree branch topology was measured using bootstrapping through 1,000 replications.

*2.2. Tissue RNA extraction and cDNA synthesis*

Samples of 100 mg of larvae or tissue were homogenized in 1 mL of TRI Reagent (Sigma-Aldrich, Dorset, UK) using a bead tissue disruptor (BioSpec, Bartlesville, OK, USA) before being mixed with 100 µL BCP (Phase separation reagent, 1–bromo–3–chloropropane, Sigma-Aldrich). The upper aqueous phase was transferred to a fresh tube and mixed with RNA precipitation solution (sodium chloride + sodium citrate sesquihydrate, Sigma-Aldrich) and isopropanol. After centrifugation, the RNA pellet was washed twice with ethanol and resuspended in molecular biology grade water. Quantity and quality of the RNA were determined by spectrophotometry using a NanoDrop ND-1000 (Labtech Int., East Sussex, UK), and integrity determined by electrophoresis using 200 ng of total RNA in 1 % agarose gel. cDNA was synthesized using 2 µg of total RNA and random primers in 20 µL reactions and the high capacity reverse transcription kit without RNase inhibitor according to the manufacturer's protocol (Applied Biosystems, Warrington, UK).

*2.3. Quantitative PCR (qPCR) analysis of gene expression*

Primers for qPCR were designed on the above PCR fragments for taurine metabolism genes using the online software Primer3 (Untergasser *et al*., 2012), and were available for ABT genes related to antioxidant enzymes, digestive enzymes and housekeeping from previous studies (Betancor *et al*., 2017a,b) (see Supplementary Table). Three housekeeping genes were tested (elongation factor-1α, *elf1α*, ubiquitin, *ubiq* and β-actin, *bactin*), with *elf1α* and *ubiq* selected as being more stable according to geNorm (Vandesompele *et al.*, 2002; M stability value = 0.165 for both genes). The efficiency of primers for each gene was evaluated by serial dilutions of cDNA pooled from the samples to confirm it was > 85 % for all primer pairs. qPCR was performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate 20 µL reaction

volumes containing 10 µL of Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Hemel Hempstead, UK), 1 µL of the primer corresponding to the analyzed gene (10 pmol concentration), 3 µL of molecular biology grade water, and 5 µL of cDNA (1/20 diluted). In the case of housekeeping genes only 2 µL of cDNA were used increasing the molecular biology grade water to 6 µL. In addition, amplifications were carried out with a systematic negative control (NTC, no template control) containing no cDNA. Standard amplification parameters contained a UDG pre-treatment at 50 °C for 2 min and an initial denaturation step at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 30 s at the annealing temperature (Supplementary Table 1) and 30 s at 72 °C. At the end of the qPCR run, a melt curve of 0.5 °C increments from 75 °C to 90 °C was performed, enabling confirmation of the amplification of a single product in each reaction. For gene expressions in ontogenesis and the dietary trial, the expression levels (gene expression fold change) of the target genes were calculated following the method described by Pfaffl (Pfaffl, 2001). The relative expression of each gene among the tissues was calculated as the logarithm of arbitrary units after normalization against the expression level of the housekeeping gene *elf1α*. One arbitrary unit was equal to the lowest expression level of the gene in each dataset.

*2.4. Tissue distribution of taurine metabolism genes*

Samples of tissues including brain, gills, heart, kidney, spleen, liver, intestine, white muscle, red muscle, adipose tissue, ovary and testis were obtained from broodstock tuna (n = 4; 2 males and 2 females; between 200 - 250 kg total weight and 10 to 15 years old) that were being sacrificed as part of the normal operating procedures to check for maturation stage and gonadal development. Additionally, ovaries and testis from a futher two females and males were collected in order to have an adequate sample size (n = 4). All tissue samples (~ 100 mg) were placed in RNA*Later*® (Sigma-Aldrich, Dorset, UK), left overnight at 4 °C and subsequently stored at -70 °C prior to RNA extraction.

*2.5. Ontogenesis of taurine metabolism genes*

Samples of ABT larvae at 1, 13, 15, 18, and 25 dah were used to determine the expression of taurine metabolism genes during early ontogenesis. The samples were whole larvae (four pools of 50 larvae, n = 4) obtained from a cohort of production fish following the current standard feeding protocol (Ortega, 2015). Sampling points were chosen based on changes in the feeding protocol. Briefly, ABT larvae were fed copepod (*Acartia tonsa*) nauplii from 2 dah (mouth opening) to 13 dah. From 13 dah onwards ABT larvae were fed gilthead sea bream (*Sparus aurata*) yolk-sac larvae at a density of 5 larvae $mL^{-1}$ and from 25 dah onwards inert microdiets were used. Samples at 15 and 18 dah were taken as intermediate points within the piscivorous phase. Prior to the piscivorous phase, a mixture of the microalgae *Isochrysis* sp. (T-Iso) and *Chlorella* (V12 DHA-enriched, Pacific Trading Co., Japan) were added to tanks at a density of 2 - 3 $x10^5$ cells $mL^{-1}$ as green water. Photoperiod was maintained at 14 h / 10 h light/dark (light intensity about 500 lux), temperature ranged between 23 - 25 ºC and daily water renewal was 100-200 % tank volume·$day^{-1}$. Larvae samples were collected and processed in RNA*Later*® as described above.

*2.6. Atlantic bluefin tuna larvae rearing conditions*

All procedures with ABT were carried out according to the current national and EU legislation on the handling of experimental animals. The ABT eggs used in the present study were obtained in June 2018 from ABT broodstock maintained in captivity in a floating net cage located at El Gorguel, off the Cartagena coast, SE Spain. Captive-reared ABT broodstock fish spawned naturally and spontaneously, and floating eggs were collected inside the cage by means of a net of 500 µm mesh screen size. A 1.5 m polyvinyl sheet was also placed around the inside of the cage to avoid eggs drifting away from the cage by means of currents and/or waves. Collected eggs were transported in a 500 L plastic tank supplied with oxygen to the Spanish Institute of Oceanography (IEO) Planta Experimental de Cultivos Marinos (Puerto de Mazarrón, Murcia, Spain) aquaculture facilities and placed in 100 L tanks with gentle oxygenation and flow-through sterilized seawater. After 1 h,

aeration and water flow were stopped to separate buoyant (viable) from non-buoyant (non-viable) eggs. After washing and counting, fertilized eggs were incubated in 1400 L cylindrical tanks at a density of 8.5 eggs $L^{-1}$. Incubation was carried out at a water temperature 24 ºC, 37 ‰ salinity, dissolved oxygen 6.5 mg $L^{-1}$ and continuous photoperiod, with light intensity of 1000 lux as recorded in the centre of the tank. An upwelling current was created to avoid larvae sinking (mainly at night) and maintain oxygen level approaching saturation (Ortega, 2015; De la Gándara *et al*., 2016; Betancor *et al*., 2017a,b). Larvae hatched approximately 32 h after fertilization, with a hatching rate of almost 90 %, and were fed with enriched (Algamac 3050; Pacific Trading LTD, Kent, England) rotifers *Brachionus rotundiformis* until 2 dah. A mixture of the microalgae *Isochrysis* sp. (T-Iso) and *Chlorella* (V12 DHA-enriched, Pacific Trading Co., Japan) were added to tanks at a density of 2 - 3 $x10^5$ cells $mL^{-1}$ as green water. Incoming seawater was filtered at 10 µm and UV sterilized (40 $mJ.cm^2$; SEF2 PE 120, Sefiltra SA, Alcobendas, Spain).

*2.6.1. Dietary treatments*

From 2 dah, larvae were fed with rotifers (*B. rotundiformis*) enriched for 18 h with Algamac 3050 (Pacific Trading LTD, Kent, England) and different levels of taurine (Andres Pintaluba SA, Reus, Spain). Rotifers (500 rotifers $mL^{-1}$) were enriched for 18 h at 28º C in culture medium that was supplemented with taurine at concentrations 0, 250, 500 and 1000 mg taurine $L^{-1}$ medium, which translated to 0.0 g (tau0), 0.5 g (tau05), 1.0 g (tau1) and 2.0 g (tau2) taurine per $10^6$ rotifers, respectively. The taurine contents and amino acid profiles of the experimental rotifers are provided in Table 1. The water temperature for the larval rearing was 29.3 °C (± 1.1), photoperiod was maintained at 14 h / 10 h light/dark (light intensity about 1000 lux, as measured in the centre of the tank), oxygen level was maintained around 6.85 mg $L^{-1}$ (± 0.65), pH ranged between 7.9 - 8.0, and daily water renewal was 100 - 200 % tank volume $day^{-1}$. All parameters were measured daily. The trial was performed in 1,500 L capacity cylindro-conical tanks and triplicate tanks per treatment. The

ABT larvae and rotifers were supplied to tanks at an ABT larval density of 10 larvae $L^{-1}$, and a prey density of 5000 rotifers $L^{-1}$.

*2.6.2. Larval growth, flexion index and survival*

At 1, 2, 3, 6, 8, 12 and 14 dah, twenty-five randomly caught ABT larvae per replicate treatment were anaesthetized (0.02 % 2-phenoxyethanol, Sigma, Spain), and weight, length, and developmental stage determined. Individual larva dry mass was determined on a precision balance (Sartorius R200D) after maintaining samples at 110 °C for 24 h and cooling *in vacuo* for 1 h before weighing. Individual larvae were photographed using a camera (Olympus SC20) connected to a microscope (Olympus SZ61-TR) and the images used to measure total length employing the software Image Pro 6.2 (Media cybernetics; Buckinghamshire, UK). Developmental stage was assessed by counting the number of ABT larvae which had attained full flexion of the notochord by the end of the feeding trial (14 dah) in each replicate set of samples. Final survival (%) was calculated by counting individual live larva at the beginning and the end of the trial (n = 3 per treatment replicate).

*2.6.3. Biochemical and molecular analysis.*

Triplicate samples of rotifers (approximately 1 g) nutritionally boosted with enricher and the corresponding taurine dose were washed and filtered, excess water drained and blotted with filter paper, and immediately frozen in liquid $N_2$ and stored at -80 ºC prior to analysis. Three samples per tank replicate of 14 dah ABT larvae fed the different taurine doses were collected, filtered, washed, dried, frozen in liquid $N_2$ and stored at -80 ºC : i) one sample of 20 ABT larvae per replicate for dry mass determination; ii) a second sample of 50 ABT larvae per replicate for amino acid analysis; and iii) a third sample of 50 ABT larvae per replicate was not frozen but placed in 2 mL cryovials in 1.5 mL of RNA*later*® for RNA extraction and molecular analysis.

*2.7. Taurine and amino acid analyses*

Taurine and total amino acid contents of samples of enriched rotifers *B. rotundiformis* and 14 dah ABT larvae were determined by the AccQ-Tag Ultra Method®, which is part of the Waters UPLC® Amino Acid Analysis (AAA) Solution (AAA for H-Class System Guide, Waters Corporation 2012). The procedure involves the preparation of hydrolysates of samples and their subsequent derivatisation and Ultra-Performance Liquid Chromatography (UPLC) analysis. Hydrolysis and derivatization were performed according to the manufacturer's instructions and amino acid contents (including taurine) were determined by UPLC using a Waters H-Class UPLC fitted with an ACQUITY BEH Phenyl 1.7µ UPLC column. Briefly, approx. 20 mg replicates of sample were hydrolysed at both 190ºC and 150 °C in 10 ml 6 M phenolic HCL by microwave digestion. The hydrolysate was diluted with MilliQ water to 250 ml and filtered prior to derivatisation. In a total recovery vial, 10 µl of hydrolysate was added to 70 µl of borate buffer and 20 µl of derivatisation reagent, mixed by vortex and incubated at 55 °C for 10 min. This solution was then transferred to the UPLC for UV detection at 260 nm. The samples were quantified against the supplied amino acid hydrolysate standard modified to contain taurine at the same concentration as the other amino acids.

*2.8. Statistical analysis*

Results for growth performance were determined as means ± SD (n = 25 per replicate for total length, total weight and flexion index, and n = 3 for survival rates). Taurine and amino acid contents, and lipid class and fatty acid compositions are presented as means ± SD (n = 3), whereas gene expression analysis are means ± SE (n = 4, for ontogeny and tissue distribution; n = 6 for dietary trial). The data were checked for homogeneity of the variances by the Bartlett test and, where necessary, arc-sin transformed before further statistical analysis. Relationships between dietary components and the different variables measured were determined by linear regression (Zar, 1999). Differences between mean values were analyzed by t-test and one-way analysis of variance (ANOVA) followed, when pertinent, by Tukey's multiple comparison test. Differences were reported as statistically significant when $P < 0.05$ (Zar, 1999).

## 3. Results

### *3.1. Taurine metabolism genes of ABT*

The sequence obtained for ABT *tauT* was 1,178 bp long, with a 5' untranslated region (UTR) of 207 bp and an incomplete ORF of 971 bp corresponding to 324 aa. The *T. thynnus* deduced partial Taut showed distinctive structural features of other Taut such as four potential N-glycosylation sites and six transmembrane domains (Supplementary Fig. 1). Subjecting the deduced aa sequence to BLASTp showed it had highest similarity (all 97 %) to Taut-like sequences of other teleost species such as *Stegastes partitus* (XP_008290391.1), *Acanthochromis polyacanthus* (XP_022054458.1) and *Amphiprion ocellaris* (XP_023139396.1). Phylogenetic analysis showed that ABT Taut clustered together with other teleost species forming a separate cluster with Taut from molluscan (*Crassostrea gigas, Mytilus galloprovincialis, Bathymodiolus platifrons* and *Bathymodiolus septemdierum*), mammalian (*Mus musculus* and *Homo sapiens*) and avian (*Gallus gallus*) species (Supplementary Fig. 2).

In the case of ABT *cdo* gene, the full 5' UTR and ORF and partial 3' UTR were obtained, with sequences being 212, 609 and 402 bp long, respectively. The ORF encoded a putative protein of 202 aa and contained the consensus motifs of the cupin family as well as conserved cysteine and histidine residues (Supplementary Fig. 3). Pairwise aa sequence comparisons of ABT Cdo with other Cdo-like proteins showed highest identities (89 - 90 %) with other fish species, *Larimichthys crocea* (XP_010731491.1), *Monopterus albus* (XP_020449407.1) and *Acanthochromis polyacanthus* (XP_022047992.1). Phylogenetic analysis showed the *T. thynnus* Cdo clustered together with other freshwater and marine teleost fish Cdo1-like proteins, whereas salmonid Cdo (*Salmo salar* and *Oncorhynchus mykiss*) clustered together in another branch. Mammalians (*Mus musculus* and *Homo sapiens*) were placed in another branch as well as the only mollusc (*Crassostrea virginica*) included in the analysis (Supplementary Fig. 4).

For the ABT *csad* gene, the partial sequence contained 78 and 529 bp of 5' UTR and ORF, respectively. The partial ORF corresponed to 176 aa and domain analysis revealed the pyridoxal phosphoric acid dependent decarboxylase domain that is highly conserved in Csad (Supplementary Fig. 5). The deduced partial Csad was highly similar (81 – 83 %) to Csad sequences of *Pagrus major* (ALF39405.1), *Kryptolebias marmoratus* (XP_017270483.1) and *Notothenia coriiceps* (XP_010764534.1). The ABT Csad-like aa sequence clustered closely with *Takifugu rubripes* and separately from mammalian Csad (*Mus musculus* and *Homo sapiens*) (Supplementary Fig. 6).

A partial sequence of 166 aa of the ORF was obtained for Ado of ABT (Supplementary Fig. 7) and contained the consensus motifs of the cupin family as well as conserved histidine residues (Supplementary Fig. 8). The partial deduced aa showed high similarity to that of *Larimichthys crocea* (XP_027145465.1; 88 %), as well as *Seriola lalandi* (XP_023274571.1; 87 %) and *S. dumerilii* (XP_022621764.1; 88 %). In agreement, the *T. thynnus* Ado sequence clustered in the same branch as *L. crocea* and closely related to other teleosts, while mollusc were the organisms more distantly related (Supplementary Fig. 7).

### *3.2. Ontogenetic expression of taurine metabolism genes*

The expression of the four taurine metabolism genes (*cdo, csad, tauT* and *ado*) in whole fish was evaluated during the development of ABT from 1 dah to 25 dah (Fig. 1). The expression level of *cdo* increased significantly between 1 dah and 13 dah and then stabilized until 25 dah, when the level peaked. Cysteine sulfonic acid decarboxylase (*csad*) gene expression increased from 0 dah to peak at 15 dah before decreasing at 18 and 25 dah. Average expression level of *tauT* in whole fish was highest at 25 dah, with no differences observed from 1 to 18 dah. Similarly, the expression of *ado* increased in whole fish increased throughout early development up to 25 dah, although the expression levels were not different from 15 to 25 dah.

### *3.3. Expression of taurine metabolism genes in adult ABT tissues*

345 The taurine metabolism genes showed varied tissue distributions (Fig. 2). The highest number
346 of transcripts of *cdo* was found in adipose tissue, followed by liver and intestine. In contrast, the
347 expression level of *csad* was highest in kidney followed by intestine with liver showing the lowest
348 value. The highest number of mRNA copies of *tauT* were found in red muscle, followed by white
349 muscle ≥ spleen, with only a low level found in liver. With *ado*, testis and brain were the tissues with
350 the higher numbers of transcripts whereas expression was much lower in all the other tissues.
351
352 *3.4. Dietary trial*
353 *3.4.1. Taurine content in ABT larvae*
354 ABT larvae effectively accumulated taurine in their bodies as a strong and positive correlation
355 was found between dietary taurine and larval taurine levels (Tables 1 and 2). This relationship was
356 found to be linear with an $R^2$ value of 0.95 ($y = 5.3x – 4.3$) (Table 2).
357
358 *3.4.2. Growth, development and survival of ABT larvae*
359 Growth performance of ABT larvae 14 dah and fed on rotifers *B. rotundiformis* enriched with
360 Algamac 3050 Bio Marine® and different doses of taurine (0.0, 0.5, 1.0 and 2.0 g taurine.$10^{-6}$ rotifer)
361 is shown in Table 3. Total length and weights were significantly highest when ABT larvae were fed
362 diet tau1 (rotifers enriched with 0.5 g taurine per$10^6$ rotifers), which corresponded to 3.7 mg taurine
363 $g^{-1}$ rotifer dry mass based on the measured taurine content of the rotifers (Table 1), and numerically
364 lowest in those fed tau0. Flexion index was significantly higher in ABT larvae fed tau1 compared to
365 larvae fed tau0 and tau0.5, with larvae fed tau2 showing an intermediate value. While ABT larvae
366 fed the tau1 diet showed the numerically highest average survival, there were no statistically
367 significant differences in survival among ABT larvae fed the different taurine doses largely due to
368 variations within treatments.
369
370 *3.4.3. Gene expression in ABT larvae*

371 The expression levels of both *cdo* and csad were both significantly higher in larvae fed diet
372 tau1 compared to larvae fed tau0 and the other levels of dietary taurine (Fig. 3). In contrast, the
373 expression of *ado* showed the opposite pattern to this with expression being lower in larvae fed tau1
374 compared to larvae fed the other diets. The *tauT* expression levels showed a decreasing trend as
375 dietary taurine increased with expression in larvae fed tau0 being significantly higher than in larvae
376 fed the diets supplemented with taurine (Fig. 3).

377 The expression of all the digestive genes measured showed a similar pattern with highest
378 expression in ABT larvae fed tau1 (Fig. 4). The expression of both bile salt-activated lipase 1 (*bal1*)
379 and phospholipase $A_2$ (*pla2*) was significantly higher in ABT larvae fed tau1 compared to larvae fed
380 tau0. While a similar pattern in expression was observed with bile salt-activated lipase 2 (*bal2*) the
381 differences did not reach statistical significance.

382 All the genes of the antioxidant system that were measured showed a similar pattern with the
383 highest expression in ABT larvae fed the tau1 diet (Fig.5). While this was significant for superoxide
384 dismutase *(sod*), glutathione peroxidase 1 (*gpx1*) and glutathione peroxidase 4 (*gpx4*), the differences
385 in expression of catalase (*cat*) were not statistically significant.

386

387 **4. Discussion**

388 The present study aimed to investigate the impacts of dietary taurine level via enrichment of
389 rotifer on growth and metabolism of first feeding ABT larvae. Firstly, key genes of taurine
390 metabolism were cloned, with the full ORF sequence obtained for *cdo*, and partial sequences achieved
391 for *tauT*, *csad* and *ado*. For *tauT* the partial ORF (324 aa) contained potential N-glycosylation sites
392 and six transmembrane domains, which was in agreement with tauT of other species (Wang *et al*.,
393 2017). Phylogenetic analyses showed a clear distinction between teleost and mammal clusters with
394 similarity scores of more than 90 % and 81 %, respectively. Furthermore, molluscs were clearly
395 separated from both mammals and teleosts, which may indicate that taurine transporter developed
396 earlier in evolution as previously suggested (Hui *et al*., 2012). In agreement the phylogenetic trees

for the three genes grouped ABT together with other teleost species indicating high evolutionary conservation.

The full mRNA sequence for Cdo was obtained with an ORF coding for a protein of 202 aa, whereas a partial ORF sequence of 166 aa was acquired for Ado. Alignment of aa from both genes revealed cupin motifs 1 and 2 separated by an intermotif region, which are common characteristics for cupin proteins (Dunwell *et al*., 2001; Stipanuk *et al*., 2011; Wang *et al*., 2016). The partial ORF sequence coding for 176 aa found for *csad* contained the important pyridoxal-dependent decarboxylase conserved domain, an enzyme group which is also present in *csad* of *Pagrus major*, *Seriola quinqueradiata*, *Oreochromis niloticus*, and *Oryzias latipes* (Haga *et al*., 2015). The phylogenetic analyses also revealed high similarity scores for the ABT genes with genes of other teleosts other than salmonids in the case of Csad, and *Salmo salar* and *Anguilla japonica* for Cdo. This highlights interesting differentiation in taurine metabolism genes, on one hand, between freshwater and marinewater species and, on the other hand, between anadromous and catadromous fish. Thus, evolutionary adaptations to different lifestyles, including migrations and transfer between freshwater and marine environments with associated different requirements of osmoregulation may have generated differentiation in genes for taurine assimilation and/or biosynthesis.

The expression levels of the four ABT taurine metabolism genes was evaluated during early ontogenesis from 1 dah to 25 dah. Results showed that, during early larval development, the expression level of the *csad* gene peaked earlier than the expression levels of *cdo*, *ado* and *tauT*. In general, expression of the genes was low 1 dah and increased during development suggesting increasing biosynthesis of taurine, which may reflect that taurine is necessary for larval development of ABT. As the transcript copies could be detected at 1 dah, it is possible that maternal mRNA is present in the egg, as has been observed in zebrafish embryos (Chang *et al*., 2013). The peak of *tauT* transcript copy number at 25 dah was similar to results found in Senegalese sole at 30 dah by Pinto *et al*. (2010), which may indicate that during the intermediate larval stage (18-25 dah), marine fish larvae including ABT have increased capacity to transport taurine. Although the ontogenic analysis

of gene expression was carried out on whole larvae, muscle is the main tissue and, given that *tauT* expression was greatest in ABT muscle tissues, it is likely that the peak in *tauT* expression reflects the enhanced transport of taurine in muscle, where growth potential is very high at this stage of development. In agreement with this, Ado, an enzyme that produces hypotaurine by the oxidation of cysteamine through a pathway different to that of Csad and Cdo (Salze and Davis, 2015), also peaked at 25 dah. However, the highest fold change (FC) for these genes is relatively low (1.8 for *tauT* and 2.4 for *ado*), whereas a FC of 18.3 and 33.3 was observed for *csad* and *cdo*, respectively, both enzymes participating in the same biosynthetic pathway. These high FC indicate that the Csad/Cdo combination is the main pathway for taurine biosynthesis and that *csad* is the rate limiting enzyme for taurine biosynthesis in both mammals (De La Rosa and Stipanuk, 1985) and fish (Chang *et al*., 2013).

The four taurine metabolism genes were expressed to some extent in all tissues of ABT examined, in agreement with other fish species (Pinto *et al*., 2012; Haga *et al*., 2015; Plasus *et al*., 2019). However, in the present study, *tauT* was predominantly expressed in muscle tissue (white > red), which is consistent with fish muscle containing relatively high levels of taurine (Huxtable, 1992). Therefore, the high expression levels observed in this tissue might reflect the physiological function of *tauT*, inducing the uptake of taurine into skeletal muscle cells. Adipose tissue displayed the highest *cdo* transcript copy number, indicating a high potential for taurine biosynthesis in this tissue, as found previously in mice (Ueki and Stipanuk, 2008). However, taurine also plays an important role in osmoregulation and this may be reflected in the high mRNA copy numbers of *csad* in kidney, which has also been observed in other teleost species (Haga *et al*., 2015). In the present study, the highest expression levels of *ado* in ABT were observed in testis and brain. The high level of expression of these genes in gonads is related to the high concentration of taurine in these tissues (Plante *et al*., 2008). Little information is currently available regarding the cysteamine pathway involving *ado*, although a recent study in carp (*Cyprinus carpio*) reported brain to be the main tissue expressing the enzyme, although testis was not included in that study (Plasus *et al*., 2019). Studies in

449 different animal species have also shown that activities of the taurine metabolism enzymes vary
450 among tissues (Kuo and Stipanuk, 1984; Stipanuk and Ueki, 2011). Therefore, it seems that the
451 pattern of tissue expression of the taurine metabolism genes in ABT is related to the biochemical
452 functions each enzyme and the role of different tissues. On the other hand, it should noted that high
453 mRNA levels of these genes have not been correlated to higher enzyme activity (Higuchi *et al*., 2012).
454 This could explain why, for instance, the expression levels of *csad* in kidney were elevated whereas
455 cdo levels were quite low, suggesting that regulation might be at the protein level as opposed to the
456 transcriptional level. Overall though, the presence and expression of these genes indicates that,
457 despite being a top predator, ABT has some capacity to biosynthesize taurine, and does not rely
458 entirely upon dietary intake. However, no taurine was detected in larvae fed tau0, which indicates
459 that although they contain the enzymatic machinery, it is not efficient. In contrast, neither mRNA nor
460 enzyme activity for some of the taurine metabolism enzymes have been identified in some fish species
461 such as cobia (*Rachycentron canadum*; Goto *et al*., 2001a; Watson *et al*., 2014).

462 In order to confirm an active role for taurine metabolism including biosynthesis in ABT, a
463 trial was carried out by feeding larvae from mouth opening to 14 dah with different levels of taurine
464 supplied via rotifers enriched with increasing levels of taurine. Taurine concentration in larvae was
465 strongly correlated to the level of taurine enrichment in rotifer in agreement with previous trials
466 (Matsunari *et al*., 2007; Katagiri *et al*., 2017; Koven *et al*., 2018). This confirms that ABT larvae are
467 able to assimilate dietary taurine into their tissues and may reflect a taurine requirement. The lack of
468 taurine in the enrichment media (tau0) led to poor growth in terms of total length and total dry mass
469 and impaired development indicated by reduced flexion index. In contrast, the highest growth and
470 most rapid development was obtained in larvae fed tau1 that corresponded to 3.7 mg taurine per g
471 rotifer dry mass. These results are consistent to what has been observed in larvae of other tuna
472 (Katagiri *et al*., 2017) and teleost species (Matsunari *et al*., 2005a.b, 2013; Pinto *et al*., 2010;
473 Hawkyard *et al*., 2015; Kim *et al*., 2016), where enrichment of rotifers with taurine promoted larval
474 growth. Nonetheless, the increase of dietary taurine from 3.7 to 9.0 mg $g^{-1}$ rotifers did not further

promote larvae growth, similarly to a study in humpback grouper (*Cromileptes altivelis*), where increasing the levels from 2.7 to 8.5 mg taurine $g^{-1}$ rotifer did not lead to increased larval total length (Ridwan and Haryati, 2017). These results indicate that levels of taurine of around 3.8 mg $g^{-1}$ may satisfy the requirements of ABT larvae for this nutrient. In contrast, survival of larval ABT was not significantly affected by dietary taurine in the present study in contrast to several previous studies in *Pagrus major* and *Paralichthys olivaceus* (Chen *et al.*, 2004a,b), *Seriola dumerili* (Matsunari *et al*., 2013), *Nibea albiflora* (Xie *et al*., 2015) or *Seriola lalandi* (Rotman *et al*., 2017). This is likely due to the large inter-tank variability observed in the present trial, although a lack of effect of dietary taurine has also been reported in other species such as *Atractoscion nobilis* (Rotman *et al*., 2017) and *Solea Senegalensis* (Pinto *et al*., 2010).

While the above confirmed a role for dietary taurine in larval ABT, the present trial also demonstrated a role for endogenous taurine metabolism. The mRNA copy number of *tauT* was regulated by dietary taurine in a dose dependent manner, with the gene being down-regulated as dietary levels of taurine increased. This indicates that when substrate (taurine) levels are low, *tauT* expression is up-regulated to promote and enhance the absorption and transport of taurine. Similar results were observed in turbot (*Scophtalmus maximus*) both *in vitro* (Wang *et al*., 2017) and *in vivo* (Wei *et al*., 2018) as well as in Atlantic salmon smolts (Zarate and Bradley, 2007). Aside from *tauT*, other genes in teleosts have been speculated to take part in taurine homeostasis, participating in the biosynthesis of this amino acid. In this respect, the regulation of taurine biosynthesis is complicated, as it is not only regulated by the product taurine but also the levels of substrate sulfur amino acids, with differential regulation of *csad* and *cdo* (Wang *et al*., 2016). It would be expected that both enzymes would be up-regulated when taurine levels were low/deficient, but this was not the case as peak mRNA copy numbers were observed in larvae fed tau1 with 3.7 mg taurine per g rotifers. Several studies in teleosts have reported the lack of regulation by taurine of *cdo* expression/activity, which was mainly regulated by cysteine and methionine (Gaylord *et al*., 2006; Wang *et al*., 2015, 2016). Therefore, the consistent pattern of expression of both *cdo* and *csad* in ABT could be influenced by

501 the combination/ratio of sulphur amino acids rather than solely by the levels of dietary taurine.
502 Additionally, the lack of regulation by dietary taurine could indicate a low capacity to biosynthesize
503 taurine in ABT, given that in the wild these fish usually consume taurine-rich prey, such as smaller
504 fish. Consistent with this, no Csad activity was found in Pacific bluefin tuna (Yokoyama *et al*., 2001).
505 There is another pathway to produce taurine in teleosts using cysteamine, produced from the
506 breakdown of coenzyme A, which is then the substrate for cysteamine dioxygenase (Ado). Most of
507 the studies in teleosts have focussed on the cysteine sulfinic acid pathway, and paid little attention to
508 the expression and/or activity of *ado*. In the present study, a partial *ado* mRNA was reported for the
509 first time in tuna, and it was shown that its transcript copy number was modulated by dietary taurine
510 level. A dietary taurine level of 3.7 mg $g^{-1}$ rotifer (tau1) lead to down-regulation of *ado* expression
511 although the levels were not statistically different to those in fish fed tau0 or tau2. Previous studies
512 showed no regulation of *ado* expression by taurine in a zebrafish cell line, which could indicate that,
513 similar to *csad* and *cdo*, *ado* could be regulated post-transcriptionally (Liu *et al*., 2017). These results
514 suggest that the cysteamine pathway is not very active in ABT, as has been shown for other
515 carnivorous marine teleosts (Goto *et al*., 2001b).
516 In addition to promoting growth, taurine has also been shown to enhance digestibility in fish
517 (Lunger *et al*., 2007). The digestive enzymes, bile salt-dependant lipases 1 and 2 (*bal1* and *bal2*),
518 have been reported to be the main enzymes involved in lipid digestion in Pacific bluefin tuna
519 (Murashita *et al*., 2014). In the present trial, both *bal1* and *bal2* showed a similar pattern of
520 expression, with highest expression levels in larvae fed tau1 (3.7 mg $g^{-1}$ rotifers). Furthermore, *pla2*,
521 an enzyme involved in intestinal phospholipid digestion (Tocher, 2003), showed the same pattern as
522 *bal1*, again with highest expression level in larvae fed tau1. Taken together these results indicate a
523 digestive promoting effect of taurine at an enrichment level of 3.7 mg taurine $g^{-1}$ rotifer, which was
524 entirely consistent with the impact of dietary taurine on ABT larval growth. However, it is worth
525 noting that the expression levels of the digestive genes could be influenced by growth rather than
526 dietary taurine levels, as previously suggested (Betancor *et al*., 2017b). Indeed, similar results were

found by Sæle *et al*. (2010), where a relationship between *bal* genes and cod (*Gadus morhua*) larvae body size was shown.

Taurine is known to have antioxidant properties, and can serve as a scavenger of some reactive oxygen species (Metayer *et al*., 2008). Indeed, taurine deficiency can have an impact on red-ox balance that can, consequently, result in mitochondrial oxidative stress *in vitro* (Jong *et al*., 2012). A previous study found that Cat, Sod and Gpx activities increased with dietary taurine level in several fish species (Li *et al.*, 2016). In agreement, the expression levels of *sod*, *gpx1* and *gpx4* in ABT in the present study were highest in larvae fed tau1, these larvae also showing the highest growth and rate of development. Indeed, a strong correlation was found between larval total length, dry weight and *gpx1* expression levels (r = 0.6 and 0.5, respectively), which corroborates the role of taurine as an antioxidant. In contrast, another study showed decreased expression of antioxidant enzymes when sea bream larvae were fed increased dietary taurine levels (Izquierdo *et al*., 2019).

In summary, the present study indicated that ABT larvae possess enzymes necessary to biosynthesize taurine through the two main pathways. The three enzymes and the taurine transporter showed differential tissue expression and could be detected before the onset of external feeding. Expression of the biosynthesis enzymes was not obviously regulated by dietary taurine level, possibly indicating a nutritional requirement for this nutrient. In contrast, *tauT* expression was upregulated when dietary levels of taurine were low, indicating a role for this gene in maintaining taurine levels in muscle and taurine homeostasis in ABT. Rotifers supplemented with taurine at 1 g per $10^6$ rotifers improved the growth of ABT larvae, without affecting final survival. In conclusion, despite the presence of taurine biosynthesis genes, ABT larvae required a supply of dietary taurine at around 3.7 mg $g^{-1}$ feed (rotifer) in order to ensure adequate growth and development.

**Acknowledgements**

We wish to thank the technical staff at Laboratory of Marine Aquaculture (IEO), Puerto de Mazarrón (Murcia), Spain and Nutritional Analytical Services (NAS), Institute of Aquaculture, University of

553 Stirling, UK that contributed to this work. This work was supported by the Consejería de Innovación,
554 Ciencia y Empresa de la Junta de Andalucía, Proyecto de Excelencia de Promoción General del
555 Conocimiento [Ref. RNM 733, 2012), and Programa Estatal de Investigación del Ministerio de
556 Economía y Competitividad [Ref. AGL2014-52003-C2-1-R, 2014].
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751

752 **Figure Legends**

753 **Figure 1**. Expression of cysteine dioxygenase (*cdo*), cysteine sulfinic acid decarboxylase (*csad*),
754 taurine transporter (*tauT*) and 2-aminoethanethiol dioxygenase (*ado*) during development of Atlantic
755 bluefin tuna (*Thunnus thynnus*) larvae (1 dah-25 dah) reared under standard procedures. Results
756 represent means ± standard error (n = 4) of relative expression normalized with two housekeeping
757 genes (*ubiquitin* and *elongation factor 1 alpha*). Different letters show significant differences for the
758 expression of each gene during development.

759

760 **Figure 2.** Tissue distribution of *cdo, csad, tauT* and *ado* transcripts in Atlantic Bluefin tuna
761 broodstock. Transcript expression level was determined by qPCR in 12 tissues with values denoting
762 the log-normalized (*ef1α*) relative expression of the target genes in each tissue. Data represent the
763 average of four individuals (n = 4) with standard errors (SEM). B, brain; G, gills; H, heart; K, kidney;
764 S, spleen; L, liver; I, intestine; R, red muscle; W, white muscle; A, adipose tissue; O, ovary; T, testis.

765

766 **Figure 3**. Nutritional regulation of taurine metabolism genes, cysteine dioxygenase (*cdo*), cysteine
767 sulfinic acid decarboxylase (*csad*), taurine transporter (*tauT*) and cysteamine dioxygenase (*ado*) in
768 larvae of Atlantic bluefin tuna (*T. thynnus*). Larvae were fed rotifers (*Brachionus rotundiformis*)
769 enriched with 4 levels of taurine: 0.0 (tau0); 0.5 (tau0.5); 1.0 (tau1); 2.0 (tau2) g taurine.$10^{-6}$ rotifers.
770 Values are normalized expression ratios, corresponding to an average of 6 pools of larvae (n = 6) with
771 standard errors (SEM). Letters denote significate differences as determined by one-way ANOVA ($p$
772 $< 0.05$).

773

774 **Figure 4**. Nutritional regulation of digestive enzymes, bile salt-activated lipase 1 (*bal1*), bile salt-
775 activated lipase 2 (*bal2*) and phospholipase $A_2$ (*pla2*) in larvae of Atlantic bluefin tuna (*T. thynnus*).
776 Larvae were fed rotifers (*Brachionus rotundiformis*) enriched with 4 levels of taurine: 0.0 (tau0); 0.5
777 (tau0.5); 1.0 (tau1); 2.0 (tau2) g taurine.$10^{-6}$ rotifers. Values are normalized expression ratios,

778 corresponding to an average of 6 pools of larvae (n = 6) with standard errors (SEM). Letters denote
779 significate differences as determined by one-way ANOVA ($p < 0.05$).
780
781 **Figure 5**. Nutritional regulation of antioxidant enzymes, glutathione peroxidase 1 (*gpx1*), glutathione
782 peroxidase 4 (*gpx4*), catalase (*cat*), superoxide dismutase (*sod*) in larvae of Atlantic bluefin tuna (*T.*
783 *thynnus*). Larvae were fed rotifers (*Brachionus rotundiformis*) enriched with 4 levels of taurine: 0.0
784 (tau0); 0.5 (tau0.5); 1.0 (tau1); 2.0 (tau2) g taurine.$10^{-6}$ rotifers. Values are normalized expression
785 ratios, corresponding to an average of 6 pools of larvae (n = 6) with standard errors (SEM). Letters
786 denote significate differences as determined by one-way ANOVA ($p < 0.05$).
787

**Table 1.** Total amino acid content including taurine (mg/g dry mass) of rotifers *B. rotundiformis* enriched with Algamac 3050® and increasing doses of taurine (0.0 g/$10^6$ rotifers (tau0), 0.5 g/$10^6$ rotifers (tau0.5), 1.0 g/$10^6$ rotifers (tau1) and 2.0 g/$10^6$ rotifers (tau2).

| | **tau0** | **tau0.5** | **tau1** | **tau2** |
|---|---|---|---|---|
| Taurine | 0.0 ± 0.0$^{e}$ | 2.5 ± 0.2$^{d}$ | 3.7 ± 0.1$^{c}$ | 9.0 ± 0.1$^{a}$ |
| EAA | | | | |
| Valine | 18.5 ± 3.4 | 22.8 ± 0.8 | 20.4 ± 1.2 | 22.2 ± 3.0 |
| Isoleucine | 1.7 ± 0.3 | 2.1 ± 0.1 | 1.9 ± 0.1 | 2.0 ± 0.3 |
| Leucine | 26.8 ± 1.8$^{b}$ | 30.3 ± 0.6$^{a}$ | 26.4 ± 1.6$^{b}$ | 31.3 ± 0.1$^{a}$ |
| Phenylalanine | 17.3 ± 1.2$^{b}$ | 19.5 ± 0.5$^{a}$ | 16.9 ± 1.0$^{b}$ | 20.2 ± 0.3$^{a}$ |
| Histidine | 6.1 ± 0.8$^{b}$ | 7.1 ± 0.2$^{a}$ | 6.0 ± 0.4$^{b}$ | 7.4 ± 0.6$^{a}$ |
| Lysine | 24.0 ± 1.8$^{b}$ | 28.0 ± 0.6$^{a}$ | 23.0 ± 2.1$^{b}$ | 30.1 ± 0.1$^{a}$ |
| Arginine | 17.5 ± 3.2$^{b}$ | 22.1 ± 0.4$^{a}$ | 18.6 ± 1.7$^{ab}$ | 23.0 ± 0.3$^{a}$ |
| Threonine | 11.3 ± 1.6$^{b}$ | 14.7 ± 0.6$^{a}$ | 11.5 ± 0.6$^{b}$ | 14.3 ± 0.3$^{a}$ |
| Methionine | 7.2 ± 0.1$^{b}$ | 8.4 ± 0.1$^{a}$ | 7.1 ± 0.6$^{b}$ | 8.4 ± 0.1$^{a}$ |
| NEAA | | | | |
| Aspartic acid | 33.9 ± 2.1$^{b}$ | 38.1 ± 0.8$^{a}$ | 32.5 ± 1.9$^{b}$ | 38.2 ± 0.2$^{a}$ |
| Glutamic acid | 42.4 ± 2.8$^{b}$ | 49.0 ± 1.4$^{a}$ | 42.3 ± 2.4$^{b}$ | 49.5 ± 0.3$^{a}$ |
| Serine | 12.1 ± 0.4$^{bc}$ | 16.1 ± 0.4$^{a}$ | 10.4 ± 0.6$^{c}$ | 13.3 ± 0.7$^{b}$ |
| Proline | 17.9 ± 1.2$^{ab}$ | 19.7 ± 0.7$^{a}$ | 16.7 ± 1.0$^{b}$ | 19.8 ± 0.3$^{a}$ |
| Glycine | 15.7 ± 1.5$^{b}$ | 17.2 ± 0.4$^{ab}$ | 16.2 ± 1.1$^{b}$ | 18.9 ± 0.3$^{a}$ |
| Alanine | 15.4 ± 1.0$^{bc}$ | 17.1 ± 0.5$^{ab}$ | 15.5 ± 0.7$^{bc}$ | 18.1 ± 0.2$^{a}$ |
| Tyrosine | 13.4 ± 0.8$^{b}$ | 15.6 ± 0.7$^{a}$ | 12.5 ± 0.8$^{bc}$ | 15.3 ± 0.2$^{a}$ |
| Cysteine | 3.4 ± 0.1$^{ab}$ | 4.0 ± 0.1$^{a}$ | 3.4 ± 0.3$^{ab}$ | 3.0 ± 0.2$^{b}$ |

Data are means ± SD (n = 3). Means within a row bearing different superscript letters are significantly different as determined by one-way analysis of variance (ANOVA), and Tukey's multiple comparison test ($P < 0.05$). EAA, essential amino acids; NEAA, non-essential amino acids.

**Table 2.** Total amino acid content including taurine (mg/g dry mass) of Atlantic bluefin tuna (*T. thynnus* L.) larvae 14 days after hatch fed on rotifers *B. rotundiformis* enriched with Algamac 3050 ® and increasing doses of taurine; 0.0 g/$10^6$ rotifers (tau0), 0.5 g/$10^6$ rotifers (tau05), 1.0 g/$10^6$ rotifers (tau1) and 2.0 g/$10^6$ rotifers (tau2).

| | **tau0** | **tau0.5** | **tau1** | **tau2** |
|---|---|---|---|---|
| Taurine | 0.0 ± 0.0$^{d}$ | 1.8 ± 0.1$^{c}$ | 3.8 ± 0.1$^{b}$ | 6.4 ± 0.2$^{a}$ |
| EAA | | | | |
| Valine | 35.6 ± 0.6 | 35.9 ± 0.1 | 32.5 ± 4.6 | 36.8 ± 0.6 |
| Isoleucine | 25.8 ± 0.4$^{a}$ | 26.1 ± 0.2$^{a}$ | 25.7 ± 0.4$^{a}$ | 26.3 ± 0.5$^{a}$ |
| Leucine | 42.4 ± 0.3$^{bc}$ | 43.1 ± 0.2$^{ab}$ | 42.8 ± 0.5$^{b}$ | 44.4 ± 0.4$^{a}$ |
| Phenylalanine | 23.9 ± 0.7 | 24.2 ± 0.7 | 24.1 ± 0.4 | 25.1 ± 0.8 |
| Histidine | 3.2 ± 0.4 | 3.3 ± 0.2 | 3.2 ± 0.2 | 3.1 ± 0.6 |
| Lysine | 45.6 ± 0.5$^{b}$ | 46.5 ± 0.2$^{b}$ | 46.6 ± 0.6$^{b}$ | 48.5 ± 0.7$^{a}$ |
| Arginine | 8.5 ± 0.5 | 9.1 ± 0.2 | 8.9 ± 0.2 | 9.1 ± 0.3 |
| Threonine | 10.2 ± 0.4$^{c}$ | 11.7 ± 0.4$^{ab}$ | 11.5 ± 0.3$^{b}$ | 12.7 ± 0.5$^{a}$ |
| Methionine | 22.0 ± 0.8$^{ab}$ | 22.4 ± 0.3$^{ab}$ | 20.9 ± 1.1$^{b}$ | 23.8 ± 1.3$^{a}$ |
| NEAA | | | | |
| Aspartic acid | 9.4 ± 0.8 | 9.7 ± 0.2 | 9.2 ± 0.5 | 9.7 ± 0.2 |
| Glutamic acid | 18.2 ± 0.6 | 19.4 ± 0.9 | 19.6 ± 0.6 | 20.1 ± 1.7 |
| Serine | 3.3 ± 0.2$^{b}$ | 3.9 ± 0.5$^{ab}$ | 4.7 ± 0.6$^{a}$ | 4.6 ± 0.3$^{a}$ |
| Proline | 15.1 ± 0.6$^{ab}$ | 15.8 ± 0.5$^{ab}$ | 14.8 ± 0.3$^{b}$ | 16.0 ± 0.3$^{a}$ |
| Glycine | 10.2 ± 0.5 | 10.4 ± 0.4 | 9.5 ± 0.8 | 9.2 ± 1.0 |
| Alanine | 13.7 ± 0.6 | 14.8 ± 0.5 | 14.0 ± 0.5 | 14.5 ± 0.2 |
| Tyrosine | 17.4 ± 0.5 | 17.9 ± 0.8 | 17.2 ± 0.6 | 18.3 ± 0.8 |
| Cysteine | 4.1 ± 0.4 | 3.6 ± 0.4 | 3.2 ± 0.8 | 4.1 ± 0.8 |

Data are means ± SD (n = 3). Means within a row bearing different superscript letters are significantly different as determined by one-way analysis of variance (ANOVA), and Tukey's multiple comparison test ($P < 0.05$). EAA, essential amino acids; NEAA, non-essential amino acids.

**Table 3**. Growth performance of 14 days after hatch ABT larvae fed on rotifers Brachionus rotundiformis enriched with Algamac 3050 Bio Marine® and different doses of taurine (0.0, 0.5, 1.0 and 2.0 g of taurine per $10^6$ rotifers).

| | tau0 | tau0.5 | tau1 | tau2 |
|---|---|---|---|---|
| Total length (mm) | 6.6 ± 0.4$^{c}$ | 6.7 ± 0.1$^{bc}$ | 6.9 ± 0.3$^{a}$ | 6.8 ± 0.3$^{b}$ |
| Dry weight (mg) | 0.41 ± 0.04$^{c}$ | 0.45 ± 0.01$^{bc}$ | 0.55 ± 0.06$^{a}$ | 0.46 ± 0.08$^{bc}$ |
| Flexion index | 38.7 ± 16.2$^{b}$ | 40.0 ± 7.2$^{b}$ | 51.0 ± 10.4$^{a}$ | 45.7 ± 9.7$^{ab}$ |
| Survival (%) | 12.4 ± 1.8 | 9.6 ± 2.8 | 14.7 ± 7.8 | 10.5 ± 8.5 |

Results for growth performance are presented as means ± SD (n = 25 per replicate for total length, total weight and flexion index, and n = 3 for survival rates. An SD of 0.0 implies an SD of < 0.05. Means within a row bearing different superscript letters are significantly different as determined by one-way analysis of variance (ANOVA), and Tukey's multiple comparison test ($P < 0.05$).

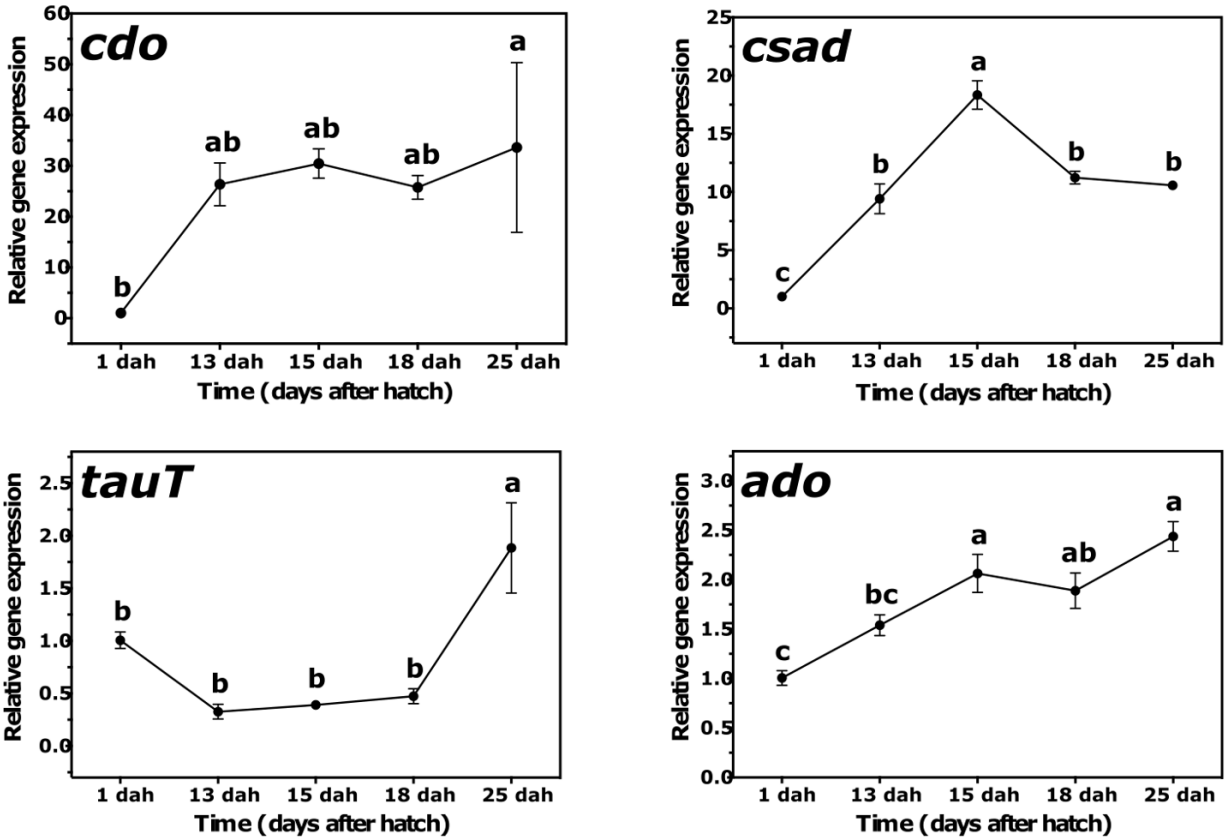
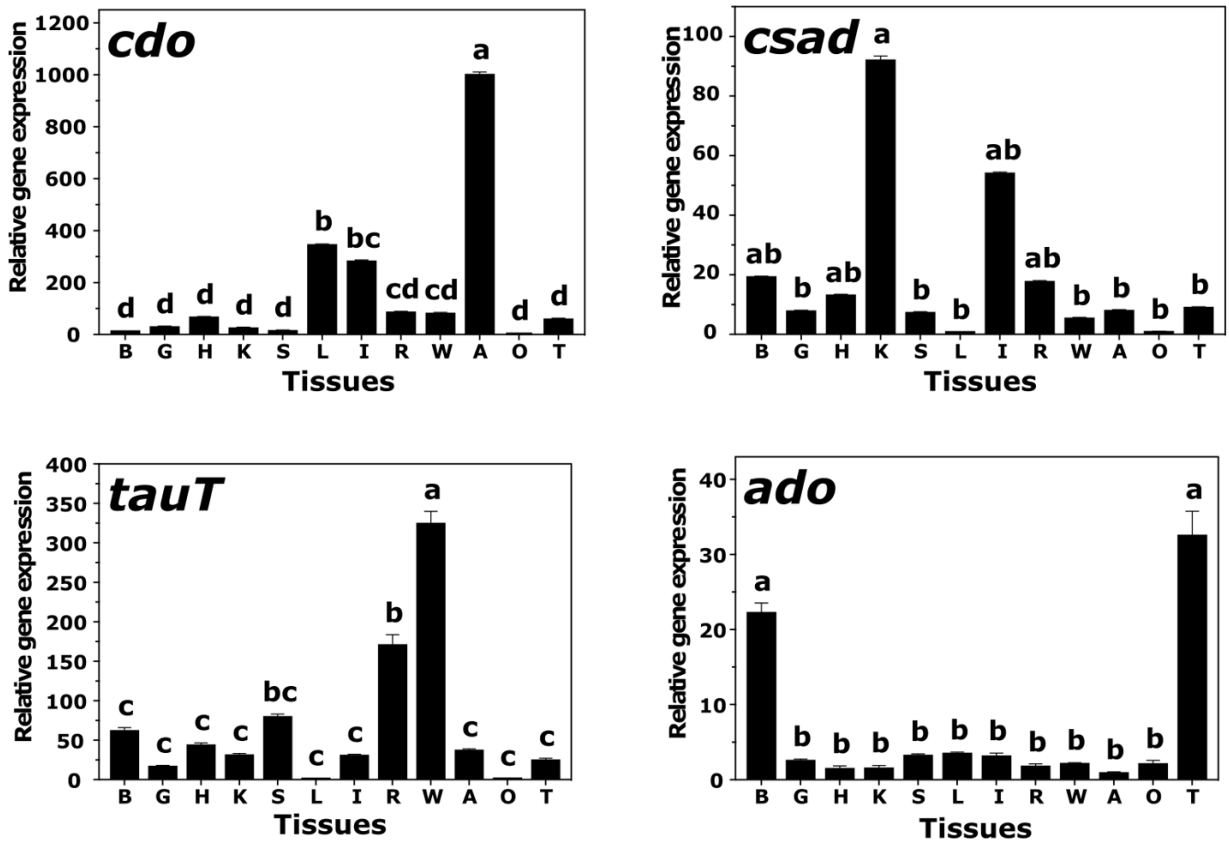

cdo
Relative gene expression
60
50
40
30
20
10
0
b
ab
ab
ab
a
1 dah
13 dah
15 dah
18 dah
25 dah
Time (days after hatch)
csad
Relative gene expression
25
20
15
10
5
0
c
b
a
b
b
1 dah
13 dah
15 dah
18 dah
25 dah
Time (days after hatch)
tauT
Relative gene expression
2.5
2.0
1.5
1.0
0.5
0.0
b
b
b
b
a
1 dah
13 dah
15 dah
18 dah
25 dah
Time (days after hatch)
ado
Relative gene expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
c
bc
a
ab
a
1 dah
13 dah
15 dah
18 dah
25 dah
Time (days after hatch)

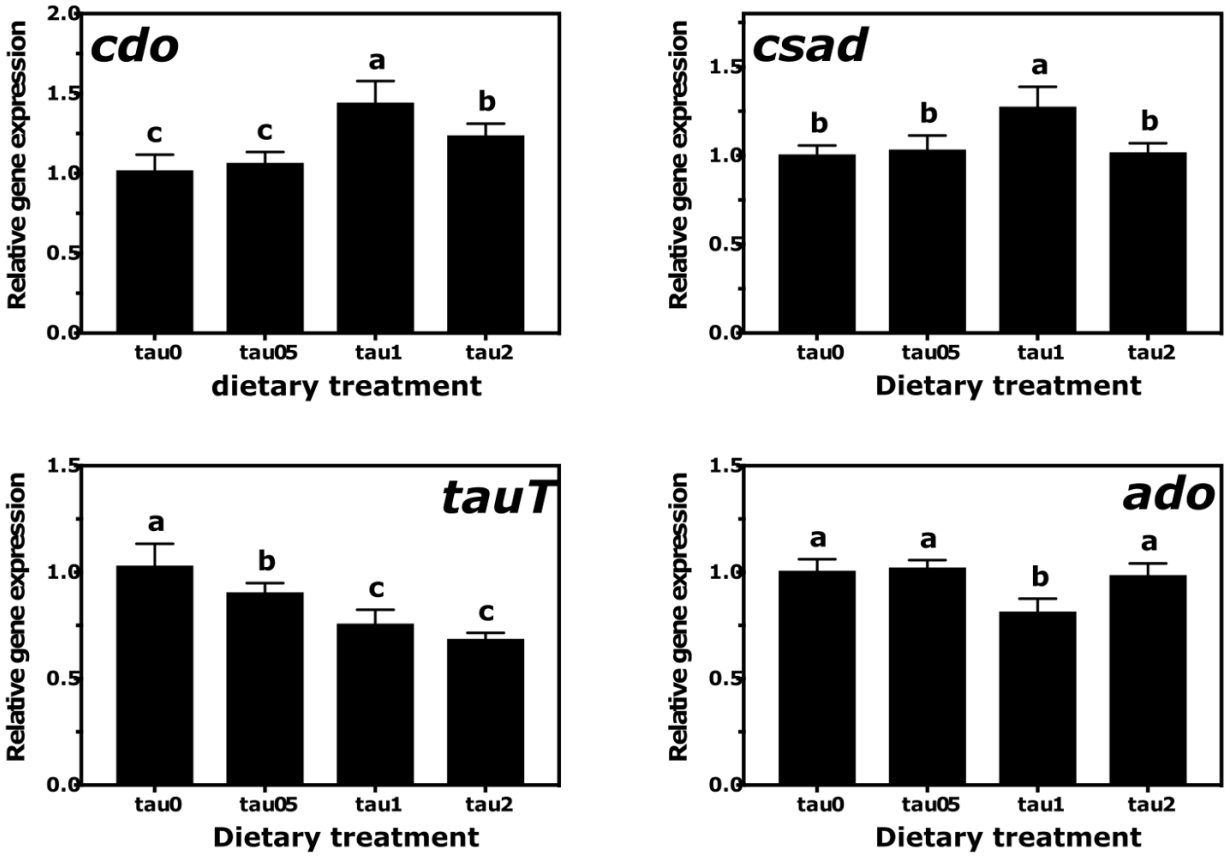
**Figure 1**




819

820 **Figure 2**

821

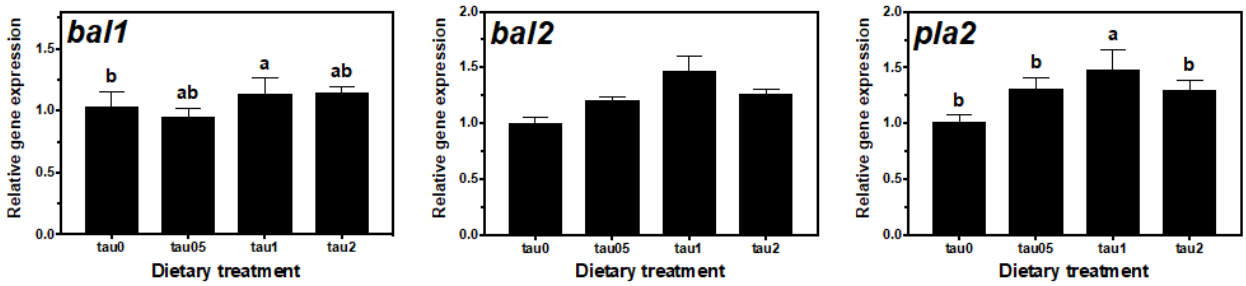



822

823 **Figure 3**

824

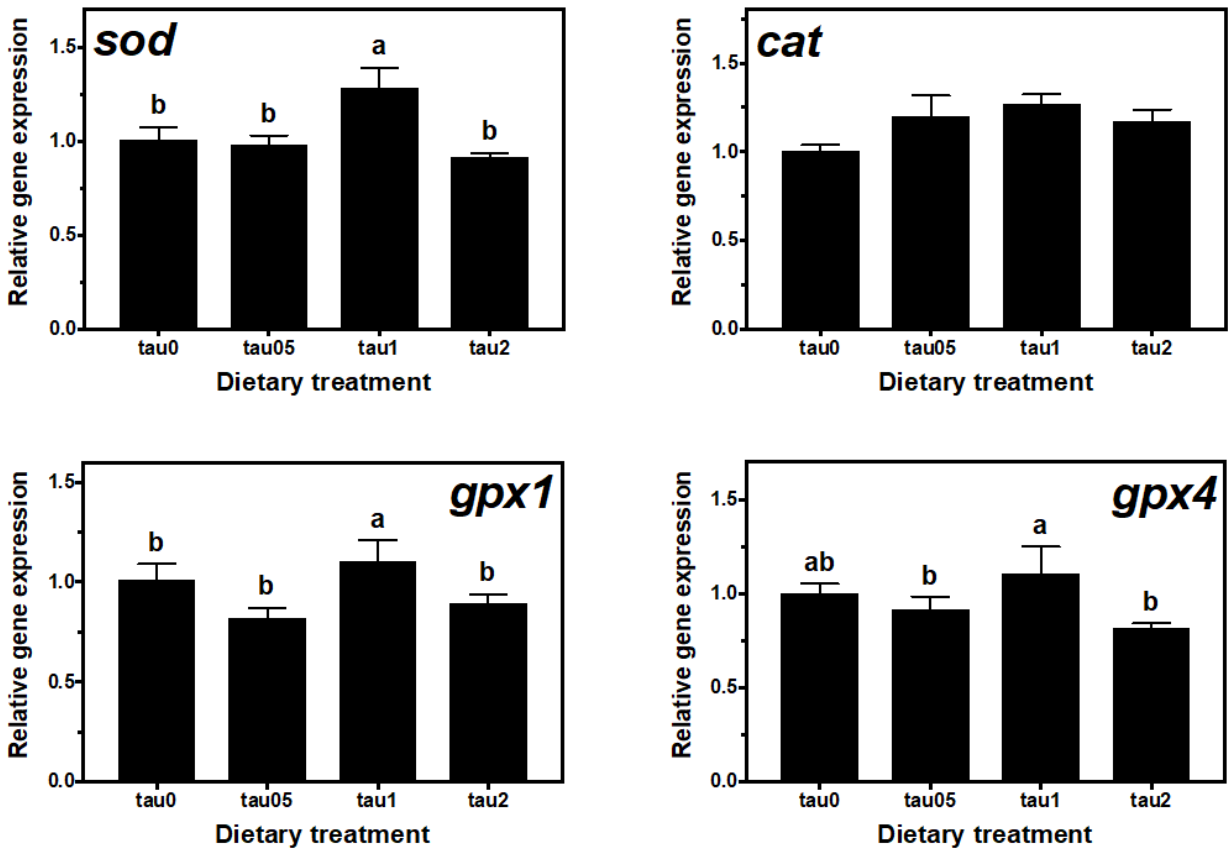
825




826

827 **Figure 4**

828


sod
cat
gpx1
gpx4
Relative gene expression
Dietary treatment
tau0
tau05
tau1
tau2
0.0
0.5
1.0
1.5
a
b
ab

829

830

831

## Supplementary Files

**Supplementary Table**. Sequence, annealing temperature (Tm) and size of the fragment produced by the primer pairs used for quantitative PCR (qPCR).

| Aim | Name | Sequence (5'-3') | Amplicon size (bp) | Tm°C |
|---|---|---|---|---|
| ORF Sequencing | *tauT_ORF* | F: ATGGCTCAAAAAGAGAAACT | 603 | 60 |
| | | R: TGACGTGAACTGACCCAGGG | | |
| | *csd_ORF* | F: ATGAGTCACCAGCTTTTTAA | 529 | 60 |
| | | R: CACCAGGGAAGAAAATACCA | | |
| | *cdo_ORF* | F: ATGGAGCATACCGAGGTGAT | 610 | 60 |
| | | R: TTAGTTGTTCTCTTGTGAGA | | |
| | *ado_ORF* | F: AGCGAGCTCCGGGGCAGCGG | 501 | 60 |
| | | R: TGCTGCTCCTGGTTACCCT | | |
| qPCR | *gpx1* | F: TGGAGAAAGTGGATGTGAACGG | 309 | 55 |
| | | R: GTGCTGTGGAAGCTGTATGATGG | | |
| | *gpx4* | F: TGGGGAATAGCATCAAGTGG | 206 | 55 |
| | | R: CGAGAAAGGAGGGAAACAGG | | |
| | *cat* | F: ATGGTGTGGGACTTCTGGAG | | 60 |
| | | R: ATGAAACGGTAGCCATCAGG | | |
| | *sod* | F: TCCCAGATCACCTACATGCC | 182 | 59 |
| | | R: CTGCGGAGAGTTGCTTGATC | | |
| | *bal1* | F: CATGGATGGACACCTCTTTACTGGT | 126 | 59 |
| | | R: AAACCAGCCTGGCCCTTCTCTTTAG | | |
| | *bal2* | F:GGATGGGCACCTCTTCACATCACAG | 120 | 59 |
| | | R: CCAGCTTGGCCCTTCTCTTTGGTAT | | |
| | *pla2* | F: GGATGATCTGGACAGGTGCT | 217 | 59 |
| | | R: TCTGGCAAAACACTCAACGG | | |
| | *tauT* | F: AGAAGCTCTGCCCCATCTTT | 170 | 60 |
| | | R: GTTTTCGGTGTTCCATGCCT | | |
| | *csd* | F: GTTGCCAAGTACAGCGTCAA | 207 | 60 |
| | | R: ATCACCTTCTGTCCAGCCAA | | |
| | *cdo* | F: GGATGACCTGGTGCAAATCC | 199 | 60 |
| | | R: TCCCCAGCACAGAATCATGA | | |
| | *ado* | F: GAACGGGATGCTGAAGGTTC | 181 | 60 |
| | | R: CCCGCTGTTCTCTGAGTACT | | |
| | *ef1a* | F: CCCCTGGACACAGAGACTTC | 119 | 60 |
| | | R: GCCGTTCTTGGAGATACCAG | | |
| | *bactin* | F: ACCCACACAGTGCCCATCTA | 155 | 61 |
| | | R: TCACGCACGATTTCCCTCT | | |
| | *ubiq* | F: CTGATCTTCGCTGGCAAACA | 215 | 60 |
| | | R: TTCTTCTTGCGGCAGTTGAC | | |

*ado*, cysteamine dioxygenase; *bal1*, bile salt activated lipase 1; *bal2*, bile salt activated lipase 2; *cat*, catalase; *csd*, cysteine sulfinic acid decarboxylase; *gpx1*, glutathione peroxidase 1: *gpx4*, glutathione peroxidase 4; *pla2*, phospholipase $A_2$; *sod*, superoxide dismutase; *tauT*, taurine transporter; *ef1α*, elongation factor 1 alpha; *bactin*, beta actin.

**Supplementary Figure 1.** ClustalW alignment of deduced amino acid sequences of Atlantic bluefin tuna (*Thunnus thynnus*) partial taurine transporter gene (*tauT*) with those of other species. Identical amino acids and similar amino acids are indicated with black backgrounds and are shaded, respectively. Asterisks show potential N-glycosylation sites and the box shows the transmembrane domain. Predicted N-glycosylation sites identified using a CDD search (Marchler-Bauer *et al*., 2011). Accession numbers for the sequences are as follows: *Solea senegalensis* (ADM88612.1); *Scophthalmus maximus* (ALX34943.1); *Lateolabrax japonicus* (AFC36524.1); *Salmo salar* (AAM90737.1); *Danio rerio* (AAX55331.1); *Siniperca chuatsi* (AKA27597.1); *Mus musculus* (NP_033346.2) and *Homo sapiens* (CAA79481.1).

**Supplementary Figure 2**. Phylogenetic tree comparing the Atlantic bluefin tuna (*Thunnus thynnus*) taurine transporter gene (*tauT*) to other vertebrates and invertebrates. The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) using Mega 5.1. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 1000 iterations. GenBank Accession Numbers: *Epinephelus coioides* (APW83833.1); *Siniperca chuatsi* (AKA27597.1); *Lateolabrax japonicus* (AFC36524.1); *Solea senegalensis* (ADM88612.1); *Salmo salar* (AAM90737.1); *Oreochromis mossambicus* (BAB18038.1); *Scophthalmus maximus* (ALX34943.1); *Anguilla japonica* (BAM16279.1); *Cyprinus carpio* (BAA89537.1); *Danio rerio* (AAX55331.1); *Gallus gallus* (NP_001025771.2); *Mus musculus* (NP_033346.2); *Homo sapiens* (CAA79481.1); *Crassostrea gigas* (BAE80716.1); *Mytilus galloprovincialis* (BAD91313.1); *Bathymodiolus platifrons* (BAI66658.1); *Bathymodiolus septemdierum* (BAF95543.1).

**Supplementary Figure 3.** ClustalW alignment of deduced amino acid sequences of Atlantic bluefin tuna (*Thunnus thynnus*) cysteine dioxygenase gene (*cdo*). Identical amino acids and similar amino acids are indicated with black backgrounds and are shaded, respectively. Dashes indicate gaps. The

boxes with black line and dashed line show the cupin motif 1 and 2, respectively. The red and the green dashes show the histidine residues and the cysteine residues, respectively. All the predicted sites identified using a CDD search (Marchler-Bauer *et al*., 2011). Accession numbers for the sequences are as follows: *Epinephelus bruneus* (AEM37687.1); *Larimichthys crocea* (XP_010731491.1); *Seriola lalandi dorsalis* (XP_023276921.1); *Danio rerio* (NP_957035.2); *Salmo salar* (NP_001134993.1); *Mus musculus* (AAK53364.1) and *Homo sapiens* (BAA12873.1).

**Supplementary Figure 4**. Phylogenetic tree comparing the Atlantic bluefin tuna (*Thunnus thynnus*) cysteine dioxygenase gene (*cdo*) to other vertebrates. The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) using Mega 5.1. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 1000 iterations. GenBank Accession Numbers: *Oreochromis niloticus* (XP_003451108.1); *Haplochromis burtoni* (XP_005919158.1); *Monopterus albus* (XP_020449407.1); *Amphiprion ocellaris* (XP_023120211.1); *Acanthochromis polyacanthus* (XP_022047992.1); *Larimichthys crocea* (XP_010731491.1); *Paralichthys olivaceus* (ALX34909.1); *Seriola lalandi dorsalis* (XP_023276921.1); *Clupea harengus* (XP_012691379.1); *Cyprinus carpio* (BAE73111.1); *Danio rerio* (NP_957035.2); *Epinephelus bruneus* (AEM37687.1); *Lates calcarifer* (XP_018529918.1); *Anoplopoma fimbria* (ACQ58703.1); *Mus musculus* (AAK53364.1); *Homo sapiens* (BAA12873.1); *Salmo salar* (NP_001134993.1); *Oncorhynchus mykiss* (XP_021460917.1); *Crassostrea virginica* (XP_022308727.1).

**Supplementary Figure 5.** ClustalW alignment of deduced amino acid sequences of Atlantic bluefin tuna (*Thunnus thynnus*) for cysteine sulfinic acid decarboxylase gene (*csad*). Identical amino acids and similar amino acids are indicated with black backgrounds and are shaded, respectively. Dashes indicate gaps. The box show the pyridoxal-dependent decarboxylase conserved domain. The predicted pyridoxal-dependent decarboxylase conserved domain was identified using a CDD search

(Marchler-Bauer *et al*., 2011). Accession numbers for the sequences are as follows: *Seriola quinqueradiata* (ALF39406.1); *Kryptolebias marmoratus* (XP_017270483.1); *Notothenia coriiceps* (XP_010777688.1); *Pagrus major* (ALF39405.1); *Monopterus albus* (XP_020472686.1); *Poecilia reticulata* (XP_017161080.1); *Mus musculus* (AAK60398.1) and *Homo sapiens* (AAI05919.1).

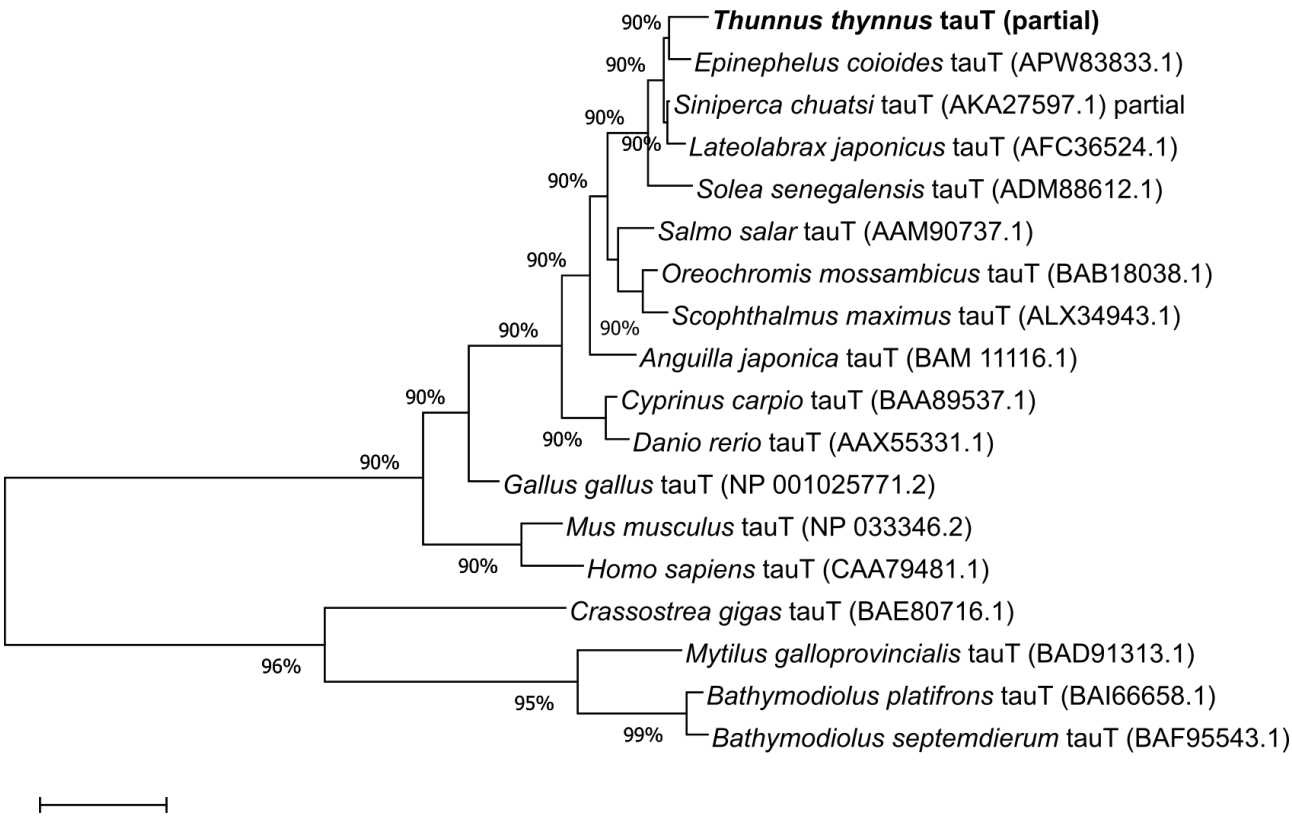
**Supplementary Figure 6**. Phylogenetic tree comparing the Atlantic bluefin tuna (*Thunnus thynnus*) cysteine sulfinic acid decarboxylase gene (*csad*) to different vertebrates and invertebrate. The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) using Mega 5.1. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 1000 iterations. GenBank Accession Numbers: *Kryptolebias marmoratus* (XP_017270483.1); *Austrofundulus limnaeus* (XP_013873699.1); *Oryzias latipes* (XP_011475423.1); *Larimichthys crocea* (XP_010745581.2); *Fundulus heteroclitus* (XP_012722036.1); *Xiphophorus maculatus* (XP_014329813.1); *Poecilia reticulata* (XP_017161080.1); *Boleophthalmus pectinirostris* (XP_020785267.1); *Neolamprologus brichardi* (XP_006785741.1); *Oreochromis niloticus* (XP_003448309.1); *Haplochromis burtoni* (XP_005914768.1); *Maylandia zebra* (XP_004558875.1); *Seriola quinqueradiata* (ALF39406.1); *Notothenia coriiceps* (XP_010777688.1); *Pagrus major* (ALF39405.1); *Monopterus albus* (XP_020472686.1); *Takifugu rubripes* (ABF22453.1); *Salmo salar* (XP_014009644.1); *Anguilla japonica* (BAL22277.1); *Mus musculus* (AAK60398.1) and *Homo sapiens* (AAI05919.1).

**Supplementary Figure 7.** ClustalW alignment of deduced amino acid sequences of Atlantic bluefin tuna (*Thunnus thynnus*) for cysteamine dioxygenase gene (*ado*). Identical amino acids and similar amino acids are indicated with black backgrounds and are shaded, respectively. Dashes indicate gaps. The boxes with black line and dashed line show the cupin motif 1 and 2, respectively. The red dashes show the histidine residues. All the predicted sites identified using a CDD search (Marchler-Bauer *et*

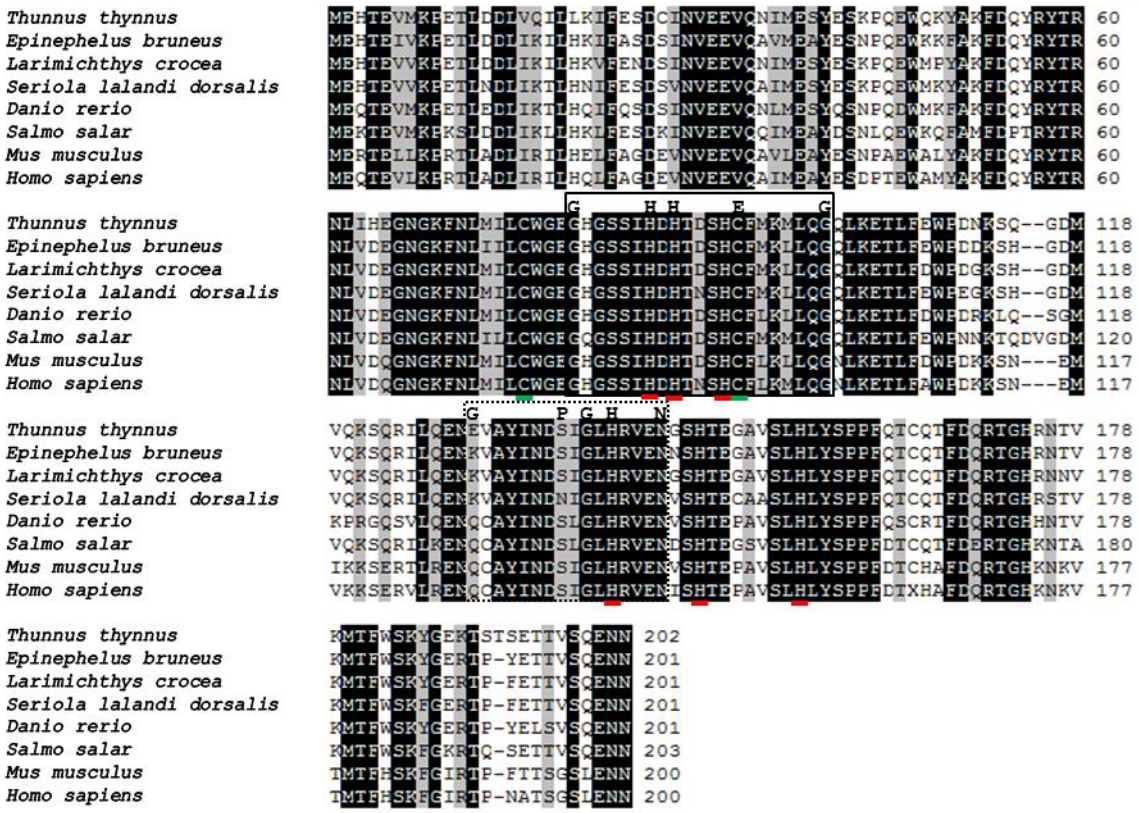
*al*., 2011). Accession numbers for the sequences are as follows: *Larimichthys crocea* (XP_027145465.1); *Seriola lalandi* (XP_023274571.1); *Paralichthys olivaceus* (XP_019939118.1); *Scophthalmus maximus* (AWP17167.1); *Stegastes partitus* (XP_008277525.1); *Poecilia reticulata* (XP_017166122.1) *Mus musculus* (AAH58407.1) and *Homo sapiens* (NP_116193.2).

**Supplementary Figure 8**. Phylogenetic tree comparing the Atlantic bluefin tuna (*Thunnus thynnus*) cysteamine dioxygenase gene (*ado*) to other vertebrates and invertebrates. The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) using Mega 5.1. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 1000 iterations. GenBank Accession Numbers: *Seriola lalandi* (XP_023274571.1); *Seriola dumerilii* (XP_022621764.1); *Paralichthys olivaceus* (XP_019939118.1); *Scophthalmus maximus* (AWP17167.1); *Mastacembelus armatus* (XP_026172273.1); *Monopterus albus* (XP_020467340.1); *Poecilia reticulata* (XP_017166122.1); *Oreochromis niloticus* (XP_003454087.1); *Larimichthys crocea* (XP_027145465.1); *Anabas testudineus* (XP_026206300.1); *Stegastes partitus* (XP_008277525.1); *Acanthochromis polyacanthus* (XP_022056185.1); *Fundulus heteroclitus* (XP_021164333.1); *Asatatotilapia calliptera* (XP_026033338.1); *Maylandia zebra* (XP_004570438.2); *Crassostrea gigas* (EKC26413.1); *Pornacea canaliculata* (XP_025093516.1); *Gallus gallus* (XP_015143625.1); *Mus musculus* (AAH58407.1) and *Homo sapiens* (NP_116193.2).


**Supplementary Figure 1**




**Supplementary Figure 2**

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953 **Supplementary Figure 3**

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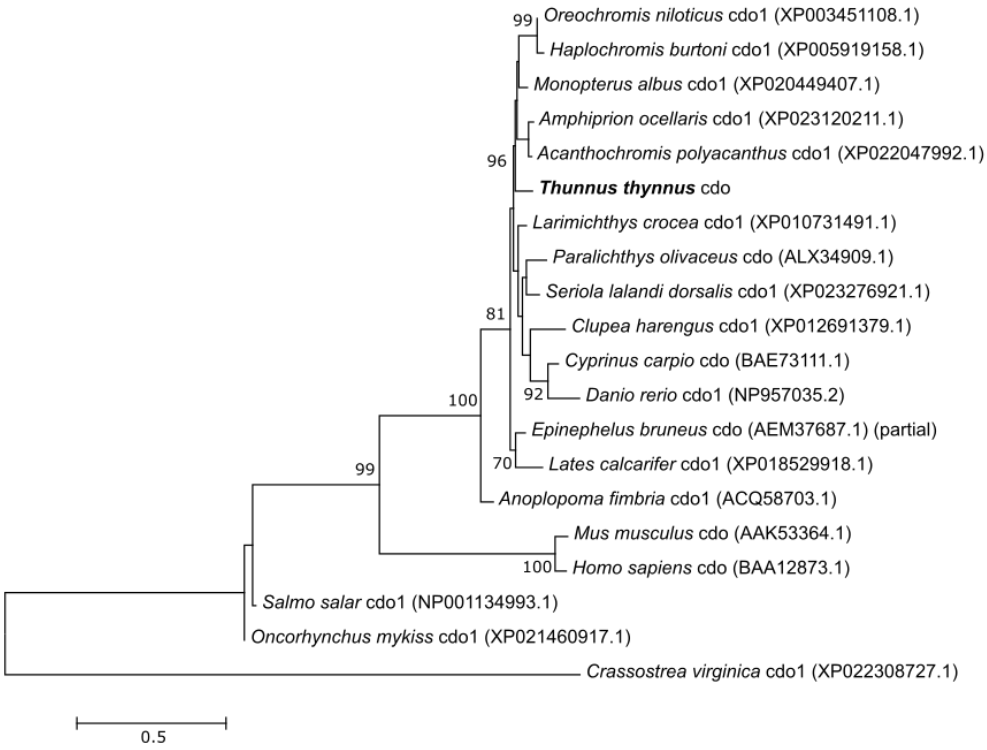
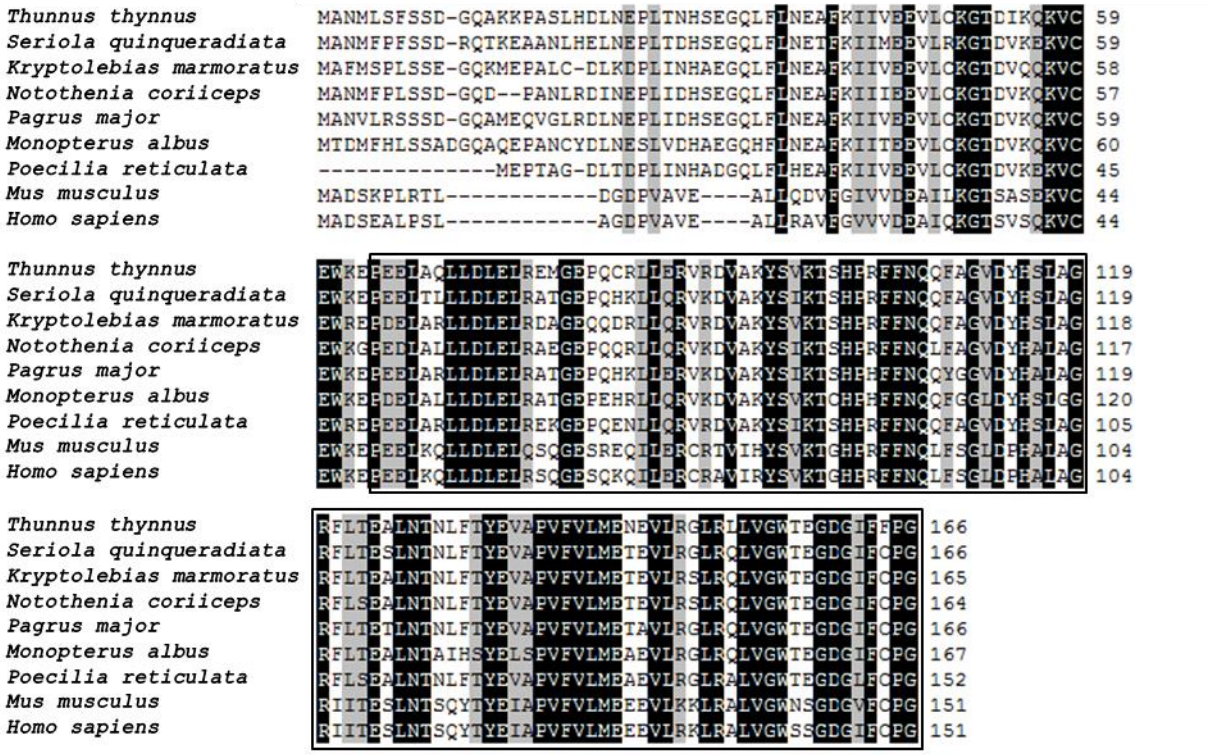
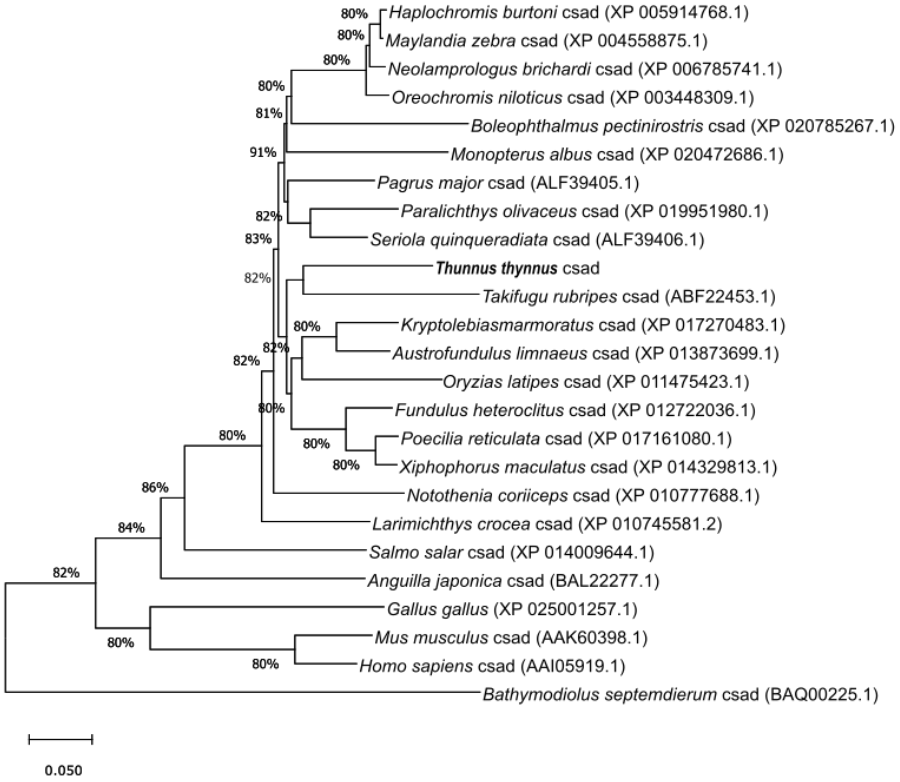



**Supplementary Figure 4**




**Supplementary Figure 5**

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963 **Supplementary Figure 6**

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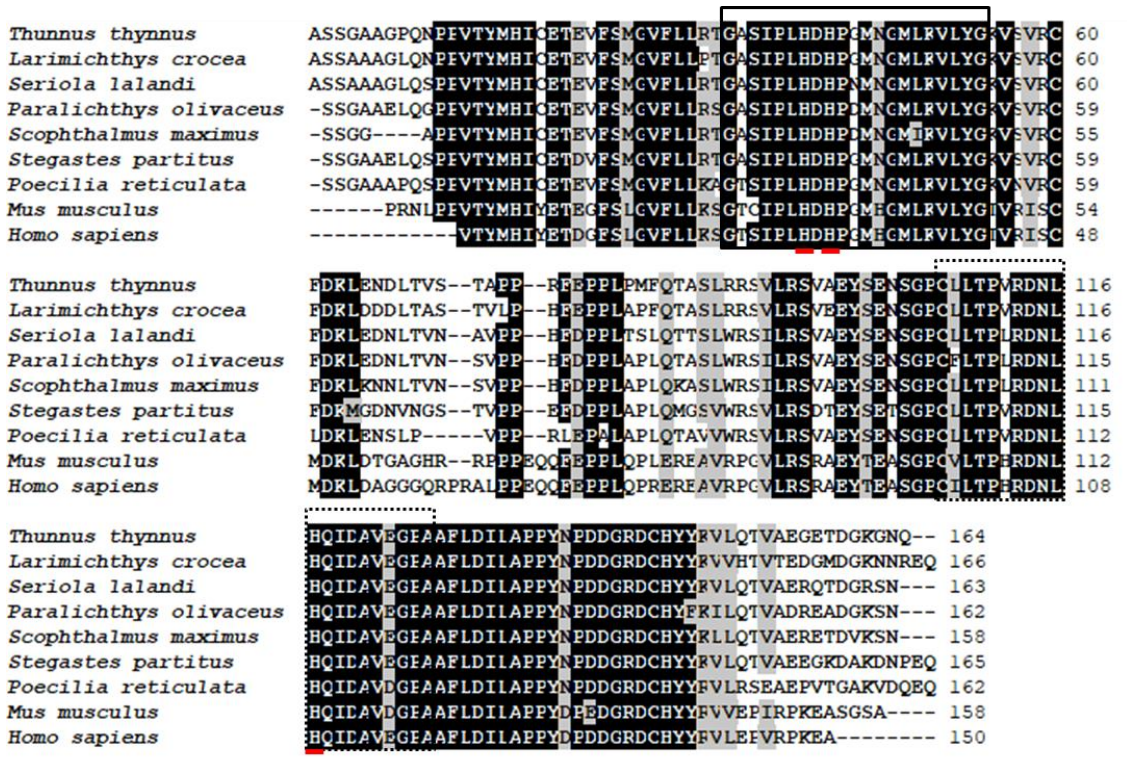
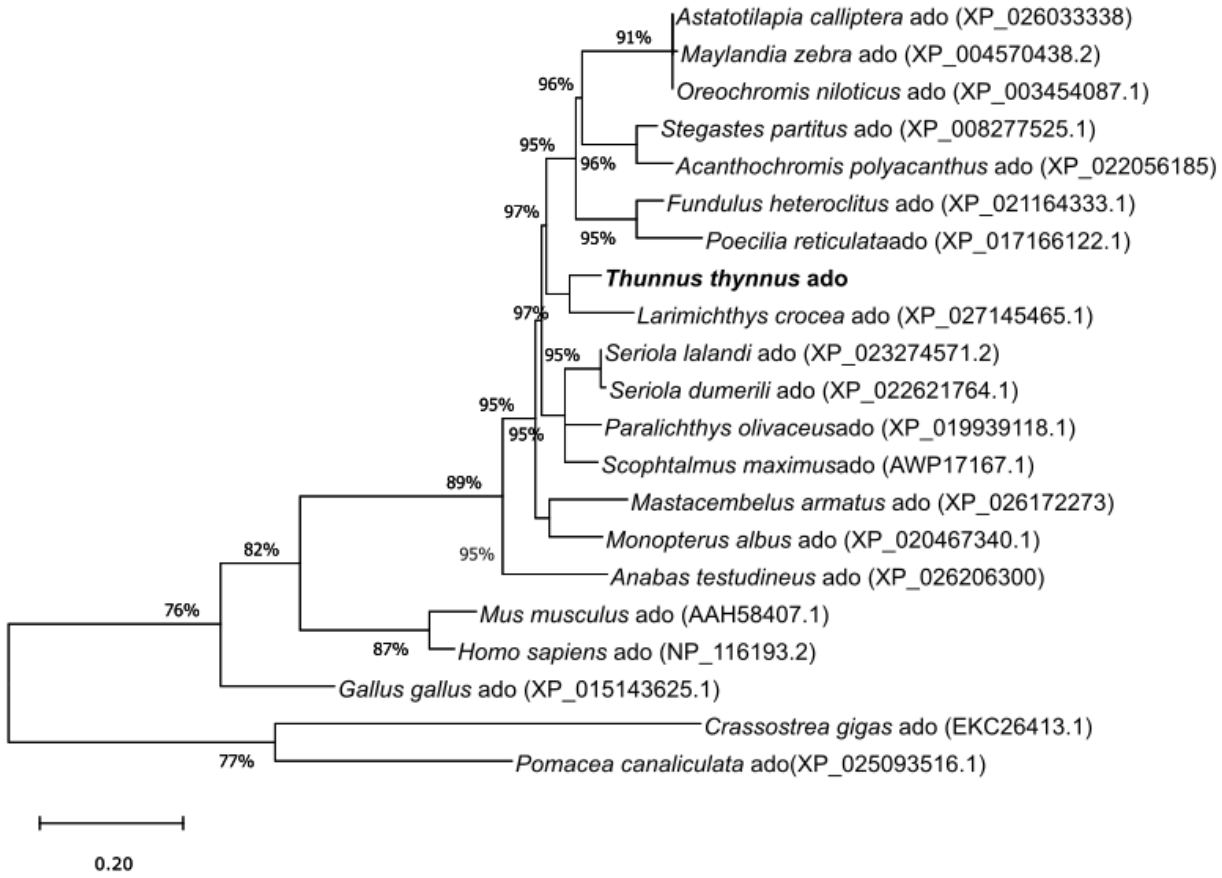



**Supplementary Figure 7**




**Supplementary Figure 8**