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# THE EFFECTS OF STOCKING DENSITY ON CYP 450 1A GENE EXPRESSION AND CARBONIC ANHYDRASE ENZYME ACTIVITY IN RAINBOW TROUT (*Oncorhynchus mykiss*)

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## ABSTRACT

It is well-known that environmental factors cause some changes in metabolisms of all living organisms. Such metabolic changes may take place in genetic structure, antioxidant mechanism, certain enzyme activities etc. The aim of this study was to assess the effects of increasing stock density on the activity of carbonic anhydrase (CA) enzyme in liver, muscle, gill and kidney tissues, and CYP 450 1A gene expression in muscle tissue of rainbow trout.

For this purpose, we prepared four groups of trout: the control group (15 kg/m$^3$) and three experimental groups of 20, 25, 30 kg/m$^3$. CA activities were determined from tissue homogenates. Interestingly, an increase was observed in gill CA activity. The studies, particularly those on gill CA activity, showed that there could be an important correlation between CA activity and stocking density, and oxygen consumption of rainbow trout. The results about CYP 450 1A gene expression demonstrated that expression of the gene significantly increases with stocking density.

**KEYWORDS:** Stocking density; gene expression; multiplex real time PCR; cytochrome P450; carbonic anhydrase; rainbow trout.

## 1. INTRODUCTION

Recently, public, governmental and commercial interest in the welfare of intensively farmed fish has witnessed a global increase. Stocking density has been emphasized as an area of particular concern since the effects of stocking density are known to be complex and appeared to comprise of numerous interacting and case-specific factors [1]. Studies on these factors are important to improve the welfare of intensively farmed fish. It is known that stocking density, diet, feeding techniques, and management procedures may influence on fish welfare. In general, stocking density can be defined as the weight of fish per unit volume [1, 2]. Therefore, stocking density is crucial in the welfare of farmed fish including rainbow trout. It could be argued that rainbow trout is the most-cultured fish species in aquaculture industry around the world. Therefore, there are many studies showing the effects of stocking density on production and physiological parameters of some aquacultures including rainbow trout. For instance, Montero and colleagues [3] investigated the effect of high stocking density on one of the most important marine fish species for Mediterranean aquaculture: gilthead seabream (*Sparus aurata*). They found that stocking density affected growth, biochemical composition, immune status and hematology. In addition, North et al. [4] examined the impact of stocking density on the welfare of rainbow trout. They stocked juvenile rainbow trout in triplicate in 1.82 m$^3$ flow-through tanks at densities of 10, 40, and 80 kg m$^{-3}$ over a 9-month period, and they demonstrated that stocking density did not significantly affect growth or mortality, but it had a significant effect on fin condition. Besides, there are some studies associated with effects of stocking density on fish growth and survival [5-7]. Furthermore, some other articles reported that on certain alterations of fish behavior, metabolism etc., in relation to high stocking density [8, 9]. As mentioned above, environmental conditions including stocking density have influence on genetic, some stress factors, enzyme activities and oxygen consumption etc. of living beings including fish metabolism. Particularly, some factors ranging from metal toxicity to density are important as a target in critical functions of living metabolism [5-7, 10-14]. Among these factors, stocking density is well-known as a biological stress factor in aquaculture. Such biological stress directly or indirectly influences metabolic molecules, such as DNA, RNA, and a variety of proteins and enzymes including cytochrome P450 and carbonic anhydrase.

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Cytochromes P450 (CYPs), heme-thiolate protein family, play important roles in oxidative metabolism of endogenous and exogenous compounds. CYPs have been described in both eukaryotic and prokaryotic organisms, such as vertebrate and invertebrate animals, plants, fungi, yeasts and bacteria. It is known that CYPs have 74 gene families [15]. A CYP gene family known in fish metabolism includes CYP1A, CYP1B, CYP1C, and CYP1D. CYP1A monooxygenase constitutes a ubiquitous family of proteins that plays a critical role in the xenobiotic metabolism. This metabolism can lead to detoxification or activation of reactive intermediates [16]. There are few studies about the effects of rearing density on the expression of stress-related genes including CYP4501A and HSP70 in fish metabolism [17, 18].

In the present study, we also described that stocking density was not effective only on CYP 450 1A but also on carbonic anhydrase (CA) activity, a vital enzyme in respiration. However, our literature review did not yield any studies about the effect of stocking density on CA enzyme activity in the muscle, gill, liver and kidney of rainbow trout. Nevertheless, we described that there is a close relationship between stocking density, oxygen consumption and CA enzyme activity. The most interesting result obtained concerns gill CA activity. It is well-known that carbonic anhydrase is expressed in almost all tissues of rainbow trout and other organisms. The enzyme is basic for respiration and transformation mechanism of $CO_2$ to $HCO_3^-$ for all living beings. So the enzyme is known to be a target for many environmental factors, such as those mentioned above. In view of this fact, CA enzyme has been increasingly studied in various sources by scientists around the world. Our laboratory is also specialized in this subject [19-22].

Consequently, this study focused on determining the effects of stocking density on CYP 450 1A gene expression in muscle and CA activity in the muscle, gill, liver and kidney of rainbow trout. Furthermore, the present study revealed a close relationship between stocking density, oxygen consumption and CA enzyme activity.

## 2. MATERIALS AND METHODS

### 2.1. Materials

TRIzol reagent for total RNA isolation and ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis were purchased from Invitrogene Co. FastStart TaqMan Probe Master using real time applications was purchased from Applied Biosystems. Chemicals used for enzyme activity experiments were purchased from Sigma-Aldrich or Merck. All other chemicals were of analytical grade and obtained from either Sigma-Aldrich or Merck.

### 2.2. Fish Husbandry and Experimental Design

Rainbow trouts were obtained from the Fisheries Department of the Agricultural Faculty at Atatürk University in Erzurum and weighed 130±20 g. They were fed twice a day with a commercial pelleted trout feed (at 1% body weight). Trout feed was purchased from Pınar Yem Company, İzmir-Turkey. Fish were fed a commercial pellet diet with 49.4% protein, 18.2% fat, 94.3% dry matter, and 9.8% ash at a daily ration of 1% of their wet body mass during the study. Feed was given by hand. Fish treatments were conducted according to Applied Research Ethics National Association (ARENA, 2002).

Prior to the experiment, fish of each group were kept in 1x1.2 m (diameter-deep) fiber-glass tanks for one month. The average water temperature was 10±2 °C during the experiment. Aeration was provided along the experiments. The water quality parameters were measured to be $O_2$ = 8.6 ppm, pH = 7.7, $SO_4^{-2}$ = 0.33 mg/L, $PO_4^{-3}$ = trace, $NO_3^-$ = 3.45 mg/L, $NO_2^-$ = trace, and conductivity = 230 µs/cm. Throughout the experiments, one tank was used as control (15 kg/m$^3$) while the other three experimental groups were 20, 25, 30 kg/m$^3$. After 2 months, three animals from each group were randomly sampled, immediately stunned and sacrificed. Tissue samples were transferred into liquid nitrogen and stored at -80 $^0$C until analysis.

### 2.3. Enzyme Assay

#### 2.3.1. Homogenate Preparation

The samples of muscle, gill, liver and kidney were washed three times with 0.9 % NaCl. Cells were lysed by immersion in liquid nitrogen (approximately -163 $^o$C). The lysed sample was transferred to the buffer solution (25 mM Tris-HCl / 0.1 M $Na_2SO_4$, pH 8.7) and centrifuged at 4 $^o$C and 20000 x g for 30 min. The supernatant was centrifuged again and the second supernatant was taken. In order to remove lipids from the tissues, $CCl_4$ was applied and the solution was centrifuged at 4 $^o$C and 1500 x g for 5 min. Supernatant was used in subsequent studies [23].

#### 2.3.2. Hydratase Activity Assay

Carbonic anhydrase activity was assayed by following the hydration of $CO_2$ according to the method described by Wilbur and Anderson [23]. $CO_2^-$ hydratase activity (enzyme units (EU)) was calculated by using the equation $t_0$-tc/tc where $t_0$ and $t_c$ are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

### 2.4. Real-Time PCR Assay

#### 2.4.1. RNA isolation and cDNA Synthesis

Total RNA was isolated from frozen muscle tissues using TRIzol reagent. RNA was treated with DNase in order to avoid genomic contamination. RNA concentrations and quality were verified by means of a Nanodrop spectrophotometer and gel electrophoresis, respectively. After isolation, cDNA synthesis was performed using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis Kit according to the manufacturer's protocol. All cDNA samples were stored at − 20 °C until use.

#### 2.4.2. Primer and TaqMan Probe Design

Primers and TaqMan probes were designed in Primer3 software (v. 0.4.0) (http://frodo.wi.mit.edu/) using rainbow

**TABLE 1 - Primers and probes set.**

| Genes | Sequences (5′- 3′) | Amplification length |
|---|---|---|
| CYP 1A Forward | AGTGATGAGTTTGGGCAGGT | 179 bp |
| CYP 1A Reverse | TCACGGATGTTGTCCTTGTC | |
| CYP 1A TaqMan Prob | $^{FAM}$- TCGTTACCTGCCCAACCGCAC -$^{TAMRA}$ | |
| GAPDH Forward | ATCAAAGGGGCTGTCAAGAA | 106 bp |
| GAPDH Reverse | AGGAGTGGGTGTCTCCAATG | |
| GAPDH TaqMan Prob | $^{Cy5}$- CGCCGAAGGACCCATGAAGG -$^{BQ2}$ | |

trout CYP-1A (GenBank accession no. AF015660) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (GenBank accession no. NM_001124246) sequences and BLASTed to ensure correct mRNA sequences. GAPDH was used as the housekeeping gene. In order to perform multiplex real time PCR, TaqMan probe of the housekeeping gene was conjugated with Cy5/Blackhole Quencher 2, the fluorophore and quencher molecules, whereas the probe for target gene was conjugated with FAM/TAMRA. The primer and probe sequences are provided in Table 1.

#### 2.4.3. Multiplex Real-Time PCR

Quantification of gene expression by real-time PCR analysis was performed using a thermal cycler Stratagene MxPro3000. The PCR was carried out in a reaction volume of 50 µl containing template DNA, 900 nM of both target and reference forward and reverse primers, 250 nM both target and reference TaqMan probes, and 25 µl Fast-Start TaqMan Probe Master (Applied Biosystems) which consists of AmpliTaq Gold DNA Polymerase, AmpErase uracil N-glycosylase (UNG), dNTP with dUTP, and optimized buffer component. Amplification and detection of samples and standards were performed using the following thermal cycling conditions: 50 °C for 2 min for activation of optical AmpErase UNG enzyme, 95 °C for 10 min as hot start to activate AmpliTaq Gold DNA polymerase, followed by 45 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Real-time PCR data were analysed using the efficiency $(e)^{(-\Delta\Delta Ct)}$ method [24], which is used to determine mean-fold changes in gene expression against control group and housekeeping gene GAPDH. Analytical sensitivity was confirmed by running standard curves. Amplification efficiency (e) was calculated based on the slopes of the curves (slope) using the formula $e = 10^{(-1/slope)}$ [24], and the slope value via Stratagene MxPro3000 software.

#### 2.5. Statistical Analyses

The statistical analysis was performed using SPSS (version 17.0) software. Data were presented as means ± standard error of the mean (SEM), and analyzed by one-way ANOVA. The significant means were compared by Duncan's multiple range tests at $p<0.05$ level ($n = 3$).

## 3. RESULTS

In the present study, muscle, liver and kidney tissues of rainbow trout were homogenized in stocking densities of 20, 25, 30 kg/m$^3$, and 15 kg/m$^3$ density was selected as the control group. CA activity was observed decreasing in the muscle, liver and kidney of rainbow trout (Fig. 1). While the difference between the decrease in CA activity of the control group and that of the 20 kg/m$^3$ group was statistically insignificant, that between others was significant ($p<0.05$). A significant decrease in muscle and kidney CA activities was observed in all groups ($p<0.05$). Interestingly, an increase was seen in gill CA activity (Fig. 1). While this increase was not significant between control and 20 kg/m$^3$ group, it was significant between the other groups. We also determined the effects of stocking density on expression levels of CYP4501A gene in rainbow trout muscle using real time PCR. The results demonstrated that expression of CYP 450 1A gene significantly increased along with stocking density. CYP 450 1A gene expression significantly decreased ($p<0.01$) in the 1$^{st}$ group (20 kg/m$^3$) (0.99±0.016) when compared to the 2$^{nd}$ group (25 kg/m$^3$) (6.32±0.23) (Fig. 2). In a much similar way, this gene expression also significantly decreased ($p<0.01$) in the 1$^{st}$ group (20 kg/m$^3$) (0.99±0.016) with regard to the 3$^{rd}$ group (30 kg/m$^3$) (6.49±0.068) (Fig. 2). However, no statistical difference was found between the 2$^{nd}$ and 3$^{rd}$ groups. We also determined the amplification efficiency to be 1.96.

## 4. DISCUSSION AND CONCLUSIONS

To achieve potential for growth and profit in fish farms, fish must be provided with optimal environmental conditions. Any deviation from these conditions can result in decreased performance, and it will clearly affect the profitability of aquaculture industry. Because high stocking density can lead to reduced performance due to a number of factors, the effect of stocking density on welfare is the key factor in fish production. In this context, there are numerous studies showing that high stocking density has a wide variety of effects on cultured fish populations, such as alterations in physiological parameters and poor feed utilization, results in mortality and poor growth, and inhibition or activation of some vital enzymes.

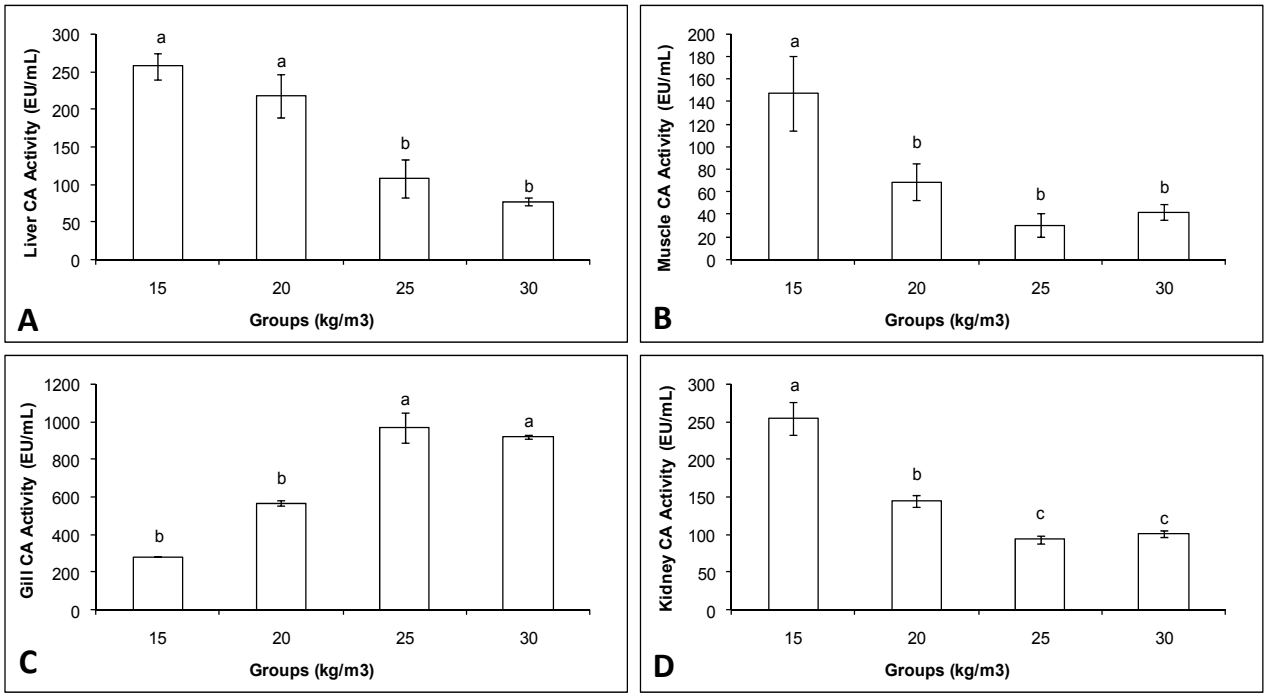


**FIGURE 1 - Stock density-dependent alterations in CA activity (A: liver; B: muscle; C: gill; D: kidney) ($p<0.05$).**

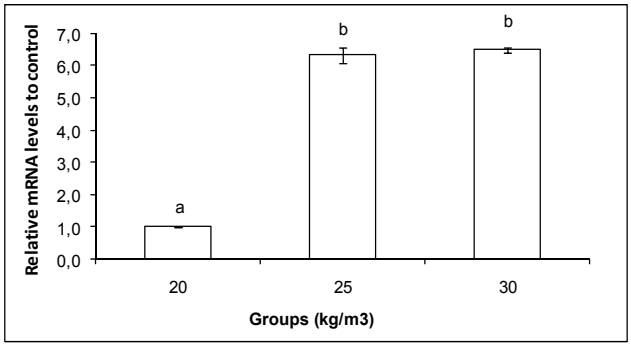


**FIGURE 2 - Relative mRNA levels of CYP 1A to GAPDH and control group (15 kg/m$^3$) in muscle after rear to different stock density ($p<0.05$).**

It is known that CA enzyme, a Zn-containing metalloenzyme, is found in almost all tissues and plays a crucial role in physiological and pathological processes in the living metabolism. CA catalyses the reversible hydration of $CO_2$ to $HCO_3^-$ and $H^+$ [10]. Carbonic anhydrase is also important in osmotic pressure and acid-base regulation in the fish metabolism. Indeed, it is well-known that there is a close relation between CA enzyme activity and oxygen consumption. The results of the present study, particularly those on gill CA activity, revealed the possibility of a critical correlation between CA activity and stocking density, and oxygen consumption of rainbow trout. This may be stress-induced and so might be associated with increased oxygen consumption caused by an attempt to supply the energy demand under these conditions [25]. The fish gill is a complex organ, known to be involved in respiratory gas exchange, ion transport, and acid–base regulation. CA, abundantly present in gill epithelial cells, is assumed to play a role in these processes [26]. In contrast to gill, we observed an inhibitory effect of stock density on liver, muscle and kidney CA enzyme activity. This inhibition effect increased with stocking density in all other tissues. This phenomenon could be attributed to the fact that increasing stock density leads to stress and increased $CO_2$ release, which inhibits CA activity in liver, muscle and kidney, while gill combats to compensate dissolved oxygen concentration. Some common strategies and procedures used in fish farming industry, like variation of stocking density, can be a source of stress for fish. Stocking density is directly related to animal comfort and fish culture productivity, and can be a determining factor in the economic return on production. The ideal density is one that does not cause substantial reduction in growth rates or environmental quality [27]. Specifically, stress is vital for both welfare and productivity in farmed fish and it has been linked to reduction in growth, abnormal behavior and immuno-depression [28]. Although we have not found any study investigating oxygen consumption and CA activity, there are some studies on stocking density and oxygen consumption in the literature. For instance, Li et al. [29] investigated the effects of dissolved oxygen concentration and stocking density on phenoloxidase, superoxide dismutase, and peroxidase of Chinese shrimp (*Fenneropenaeus chinensis*). Their results demonstrated that the activities of these 3 enzymes were significantly affected by dissolved oxygen concentration. While phenoloxidase activity increased with stocking density, it was not affected by dissolved oxygen concentration. On the contrary, no changes were produced in superoxide dismutase activity as a result

of the stocking density treatments; yet, the activity was found to be affected by different dissolved oxygen concentrations, such as low, medium and high. No significant effect was observed in peroxidase activity for dissolved oxygen concentration or stocking density.

In addition to decreased dissolved oxygen concentration, high stocking densities imply more feed input, more metabolites produced by the fish, more feed spills. Thus, more organic matter decomposition will occur. Furthermore, the nutrients that are not digested by the fish are accumulated in the fish pond [30], and then a part of this remnant dissolved in water. For so long, dissolved organic material has been regarded to be of great importance, because of its ability to interact with many xenobiotics [31-33]. In the present study, induction of CYP 4501A in rainbow trout parallel to stocking density may be a result of binding of a foreign compound (xenobiotic) to a cellular receptor [34,35]. This binding triggers the expression of the gene coding for CYP 4501A and leads to increase in mRNA transcription [35, 36]. CYP 4501A is especially sensitive to induction by a wide-range of organic contaminants, such as petroleum hydrocarbons, dioxins, furans, organochlorine pesticides etc. [37]. For instance, Binelli and colleagues [38] studied the effects of several xenobiotics on ethoxyresorufin O-deethylase (EROD), whose activity is considered to be specifically catalyzed by the cytochrome P450 1A1 gene product and AChE activities in Zebra mussel (*Dreissena polymorpha*). They exposed the freshwater bivalve Zebra mussel (*Dreissena polymorpha*) to different pollutants at laboratory conditions. The pollutants were Arochlor 1260, CB 153 and 126, pp'-DDT, chlorpyrifos, and carbaryl. They observed a significant induction of EROD activity when mussels were exposed to 100 ng/L of PCB mixture of Arochlor 1260 and dioxin-like CB 126, but this congener also showed a clear competitive inhibition after 48 h of exposure. Furthermore, they determined a significant decrease of basal EROD activity after only 24 h of exposure after pp'-DDT application. Additionally, Gornati et al. [18] carried out a study on the effects of rearing density on expression of stress-related genes in sea bass (*Dicentrarchus labrax* L.). They found that CYP4501A and HSP70 were influenced only at the higher population density of 100 kg/m$^3$.

In an interesting study about the effects of population density on seabass (*Dicentrarchus labrax,* L.), gene expression showed that population density had an effect at gene level by repressing or enhancing the expression of different genes. The study argues that these genes can be used as rapidly to detect biomarkers [18]. Additionally, Montero and colleagues [3] investigated the effect of high stocking density on juveniles of *Sparus aurata*, and they revealed some information on the alterations in the growth, biochemical composition, immune status and hematology of this fish under the conditions used in their study. Their results revealed that high stocking density produced a chronic stress situation, plasma cortisol levels in low stocking conditions was four times higher than high stocking density. Besides, they observed a decrease in both haemoconcentration and in alternative complement pathway (ACP), an important component of the immune system of fish, and hematocrit; hemoglobin concentration and red blood cell count were significantly higher in high stocking density. They found some alterations in liver fatty acid composition with high stocking density.

Consequently, many environmental conditions influence fish metabolism, including stocking density effect on genetic, some stress factors, enzyme activities and oxygen consumption etc. This study focused on determining the effects of stocking density on CYP 450 1A gene expression in muscle and carbonic anhydrase activity in the muscle, gill, liver and kidney of rainbow trout. Besides, the present study found a close relationship between stocking density, oxygen consumption and CA enzyme activity.

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