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Endocrine Disruption Induced by Sub-Lethal Mercury Chloride on Hormone Indices of Seabream

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Abstract: A number of chemicals released into the Persian Gulf may interfere with the thyroid and testosterone hormones and behave as endocrine disruptors (EDs). Despite of their important for environment assessment, there is little knowledge about role of serum hormones during mercury exposure of fishes, so in this study, a multi factorial approach, involving determining thyroid and testosterone hormones during the *in vitro* exposure of mercury chloride was used. In laboratory, fish maintained in a seawater re-circulatory system and then according to the LC_{50} test, fish divided in 5 treatments. All serum hormones were assayed using diagnostic ELISA direct immuno-enzymatic kits. Both T3 and T4 activities exhibited significant analysis of variance ($P<0.5$) with lower considerable values than those of the control group for T3 and higher considerable values than those of the control group for T4. No significant changes occurred in the activities of the Testosterone, however it was decreased. Results of the present investigation indicated that mercury is a toxic substance in Yellowfin sea bream, with change thyroid hormone activities in serum of fish exposed to various concentrations.

Key words: Persian Gulf % Mercury % Hormone % Endocrine disruption % Yellowfin sea bream

INTRODUCTION

Thyroid hormones (THs) have many physiological roles in fish like growth regulation, development, metabolism and hydromineral balance [1]. A little change in serum concentrations of these hormones, as well as in glucose levels reflects endocrine changes; thereupon, fish physiological competence to cope with ecosystem xenobiotics can be affected. Thus, the hormones biomarkers may also be useful tools in monitoring the impact of heavy metals stressors on fish [2].

Almost all exposed environmental stressor have share structural similarity with THs and if they substitute THs and disrupt the thyroid axis. In fish, thyroid hormones [thyroxine (T4) and triiodothyronine (T3)] have many important roles in maintaining proper physiological function and also in fish growth and early development [3]. When fish are exposed to environmental chemicals the levels of thyroid hormones have been demonstrated to be decreased [4] and it well confirmed that chemical pollutants have more affect thyroidal hormone status in a number of fish species [3, 5].

The thyroid hormones have a small hydrophobic thyronine nucleus that mediates their action by binding to specific nuclear receptors, which act directly on target genes bringing about a cellular response [6]. 3,5,30-L-Triiodothyronine (T3) is the superlative active hormone and binds with high affinity to nuclear receptors (TRs), while L-thyroxine (T4), which is the precursor of T3 binds with low affinity and has few direct application [7]. Glucose is a suitable index that mostly used as secondary stress biomarkers in a many fish species [8].

In recent decade, there is a major issue of concern that chemicals in the marine ecosystems may change steroid hormone levels and affect the reproductive success of the fish. In animals, sex steroid hormones are produced by the endocrine system and control the life cycle stages of an organism including gametogenesis, fertilization, sexual development and reproduction [9]. Based on similar study related to freshwater fish, impacts of contaminants on sex steroid titres might be expected in marine fish, but few have been reported to date.

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In principle, sex steroids alternation in fish serum is because of intervention with the control of steroid synthesis via the pituitary-gonadal axis, or to effects on steroid metabolism and excretion [10].

During chemical exposure, for regaining safe homeostasis fish do many physiological processes and two important physiological processes which are modulated when fish are exposed to stress, are hormonal status and immune function [8]. Whereas it is conspicuous that both of these processes are necessary for an animal survive, but there is few knowledge about role of serum hormones during mercury exposure of marine fish, so in this study, a multi factorial approach, involving determining thyroid and testosterone hormones during the *in vitro* exposure of mercury chloride was used. The information gained from this study may be useful for future strategies in monitoring and predicting the effects of mercury exposure and also in developing indices to measure stress during sea bream culture.

MATERIALS AND METHODS

Experimental Procedures: 15 tanks (300 Litre) with seawater re-circulatory system equipped with physical/chemical filters and aeration. Acclimation period was in a 15 aerated fiberglass tank containing 46 ppt saltwater maintained at 25°C under a constant L12:12D photoperiod.

Conditions within each experimental tank were monitored daily with the temperature 25°C ± 1, pH 7.8 ± 0.1 and salinity 46 ± 1 ppt under a natural photoperiod (12 hr L:12 hr D) in controlled room. Water was oxygen saturated through constant aeration in a static system. Voluntary feed intake was near to maintenance ration at the time of the maintenance; fish were fed two times a day (08:30 and 17:30) but were starved for 48 hr prior to the start of the experiment and throughout its duration. Fecal remains and food residues were removed by suction every other day. The food supply was provided to each predator fish with fresh prawn, collected from creeks without pollutants sources.

Fish: Forty five fish all immature male in the same size (120 gm final body weight average) were maintained in laboratory to the Mariculture Research Station of the South Iranian Aquaculture Research Center, Mahshahr, Iran. All samples were acclimated for one week. Acclimatized fish were fed daily with a live feed (fresh shrimp) and daily we check water quality and water parameters. Fish were randomly divided into five equal groups (15 per group) and each tank was randomly assigned to one of five experimental treatments filled with the appropriate concentration of an aqueous solution of Hg (standard solution for atomic absorbance spectrophotometer) in dechlorinated tap water.

The Yellowfin sea bream were exposed to nominal mercury concentrations of control (tank 1), 10 µg l (tank 2), 20 µg l (tank 3), 40 µg 1 (tank 4), 80 µg l (tank 5) respectively and maintained for three weeks with aeration. These sub-lethal doses were chosen on the basis of preliminary toxicity tests and determinations of LC_{50} 96 hr for this species, suggestive of inducing toxic effects but not lethally so [11].

At sampling time (3 weeks post-exposure), nine fish from control and any confinement tanks were netted into a bucket containing clove, fish were anesthetic and weighed, total lengths were recorded and an external examination for any signs of abnormalities or infestation was conducted [12].

Blood Collecting: To obtain blood samples, fish were quickly taken out from the water and held firmly on a bench with a cloth covering the head and blood was withdrawn from caudal vessels by a vacuum heparinized syringes and needle containing 0.1 ml of anticoagulant (after filling up and expelling about 1.0 ml), to the eppendorf tubes, whole blood withdrawal process took less than 1 min per fish. Handling time was less than 1 min to minimize stress effects. Extracted blood (1.5 ml) was immediately placed in two sets of tubes, one in heparinized tubes for hematology and leukocyte analysis and the remaining blood for biochemical analyses. The first part was maintained in refrigerator (4°C) and the second was placed in non-heparinized tubes and left to clot at 4°C for 15 min. Afterwards, tubes were centrifuged at 3000 rpm (20000 g) using an eppendorf centrifuge for 10 min to obtain serum. The serum was separated into aliquots and was frozen and stored at-80°C until hormone analyses. All samples were immediately immersed in liquid nitrogen and then transferred to a-80°C freezer until analysis.

Testosterone Steroid Analysis: Hormone analyses were done with ELISA, DA 3400, USA and ELISA reader SEAC Italy. Serum testosterone were assay using pre-coated ELISA kits purchased from IBL testosterone enzyme immunoassay kit (RE52151), Hamburg, Germany according to supplier's instructions. Absorbance was measured using a testosterone ELISA (RE52151) instruments at 420 nm for detection. The limit of detection (LOD) of the procedure was 100 pg/ml ml. Intra-assay and inter-assay coefficients of variation were of 9.5% and 11.6% (T) respectively.

Thyroid Analysis: Serum thyroxine T4 and triiodothyronine T3 were assayed using diagnostic ELISA direct immuno-enzymatic kits purchased from Monobind, USA according to supplier's instructions [4]. Absorbance was measured using a Monobind T3 and T4 ELISA instruments at 450 nm for detection of both hormones. T3 and T4 methodology requires immobilized T3 or T4 antibodies, as well as HRP-T3 or HRP-T4 conjugates. Regarding TSH, an antibody specific to the $-chain of TSH molecule is immobilized on microwell plates and other antibodies to the TSH molecule are conjugated with HRP. TSH from the sample is bound to the plates. The enzymatic reaction is proportional to the amount of TSH in the sample.

Statistical Procedure: Significant differences were determined with one-way analysis of variance Anova with Duncan Post Hoc was used. To investigate associations between bioaccumulation and its effects, Pearson correlation coefficients (r) were calculated between mercury concentrations and enzymatic parameters. Multiple regressions were used to determine the relationship between mercury concentration and blood parameters. The differences between means were analyzed at the 5% probability level (p<0.05) was considered as statistically significant). Data are reported as means ± standard deviation.

RESULTS

With respect to *in vitro* raw data, the Kolmogorov-Smirnov normality test was significant at P<0.05, for all our measured parameters. Results of hormone activity analysis are presented in Table (1). Both T3 and T4 activities exhibited significant analysis of variance (P<0.5) with lower considerable values than those of the control group for T3 and higher considerable values than those of the control group for T4. No significant changes occurred in the activities of the Testosterone (P<0.05), however it was decreased.

During *in vitro* results, the correlation between mercury with all hormonal parameters was statistically tested by analyzing the data obtained during the mercury exposed. The testosterone levels had not statistically significant correlation (P<0.05) with mercury exposed and this correlation was negative (P<0.05) (Fig. 1).

Thyroid parameter show significant correlation (P<0.05) with mercury exposed, that T3 correlation was negative but T4 was positive (Table 2). Correlation test of thyroid hormone with each other imply that there have significant negative correlation.

Curve estimation regressions data were used to determine the relationship between mercury concentration and T3, T4 and testosterone. T3 and T4 show significant

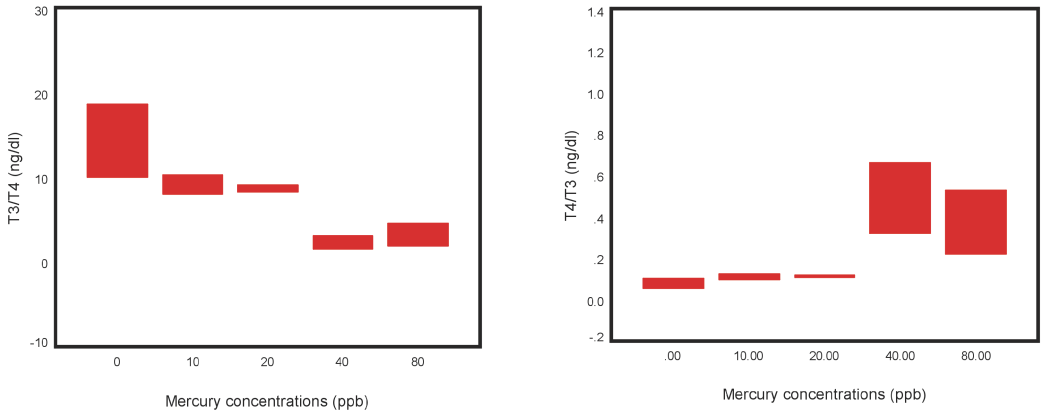

Fig. 1: Portion hormonal response (T3/T4 and T4/T3) of the Yellowfin sea bream during *in vitro* exposed to different concentration of mercury chloride (box plots contain mean and standard deviation). Values of specific hormone activity are expressed in ng/dl

Table 1: *In vitro* hormone activities of Yellowfin sea bream exposed to mercury chloride.

Control	10 µg	l 20 µg	l 40 µg	1 80	µg l
T3 (ng/dl)	5.74±0.67^{a}	4.79±0.41^{b}	4.77±0.73^{b}	2.04±0.21^{d}	3.56±0.36^{c}
T4 (ng/dl)	0.41±0.14^{c}	0.52±0.07^{bc}	0.55±0.08^{bc}	1.10±0.72^{ab}	1.42±0.81^{a}
Testosterone (ng/dl)	0.39±0.05^{ab}	0.41±0.10^{a}	0.36±0.05^{ab}	0.31±0.04^{b}	0.37±0.10^{ab}

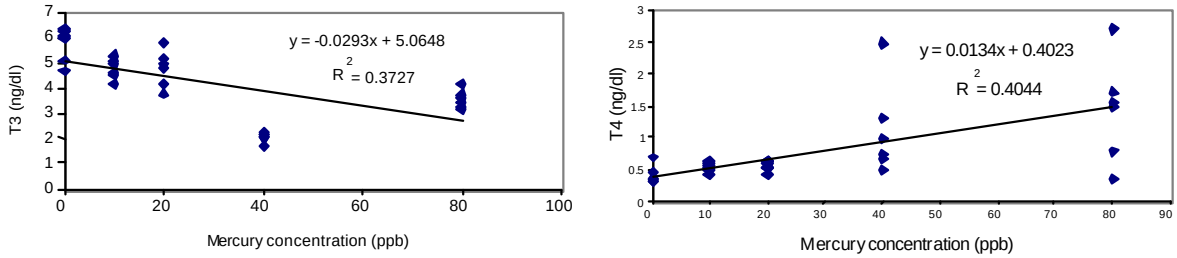

Fig. 2: Regressions model (Y = a ± bX) of T3 and T4 of the Yellowfin sea bream during *in vitro* exposed to different concentration of mercury chloride

Table 2: *In vitro* correlation of hormonal activities of Yellowfin sea bream with mercury chloride

	T3	T4	Testosterone
Pearson correlation (r)	0.74**	0.61**	0.25
sig (p)	0.00	0.00	0.17

Table 3: *In vitro* curve fit linear regression of hormonal activities of Yellowfin sea bream with mercury chloride

	T3	T4	Testosterone
R square (r^2)	0.37**	0.40**	0.02
F	6.6	19.0	0.78
sig (p)	0.0003	0.0002	0.17

linear regression (P<0.05) with mercury, but testosterone had not statistically significant (P<0.05) with mercury (Table 3). Regressions model Y = a ± bX of significant parameter are in Figure (2).

DISCUSSION

Thyroid hormones (Ths) are known to play a crucial role in many metabolic and physiologic processes and are essential for normal growth, differentiation and development of animals [13]. In fish, THs are implicated in reproduction and appear to be important in the regulation of development. Disruption of the thyroid axis may seriously compromise normal development, differentiation, growth or reproduction in many vertebrates like fishes [3].

Chemical pollutants have been reported to affect thyroidal hormone status in many fish species [3, 5] but the effect of mercury on this group of hormones, in fish, has yet to be reported. In the present study it was found that serum T3 significantly decreased upon exposure of Yellowfin sea bream to mercury whereas levels of serum T4 was increased.

A number of chemicals released into the environment share structural similarity to the thyroid hormones (THs), thyroxine (T4) and triiodothyronine (T3) and it is thought that they may interfere with the thyroid axis and behave as endocrine disruptors (Eds). One of the ways by which such environmental contaminants may disrupt the TH axis is by binding to TH transporter proteins (e.g. Transthyretin (TTR) that is responsible for TH transport in the blood).

Serum T3 and T4 levels are considered valuable indicators of thyroid function in animals [14]. Decreased serum triiodothyronine (T3) level was also noticed in fresh water fish (*Clarias batrachus*) after subchronic exposure to cadmium [15]. Teles *et al.* [2] show insignificance different in serum TSH and T4 levels, with a tendency to serum T3 reduction at all exposure sites with significant decrease in pollution source. These results agree with previous studies where a serum T3 decrease was detected after exposure either to heavy metals [16].

Since it was previously observed that heavy metals inhibit the conversion of T4 into T3 by 5'monodeiodinase, the T3 serum decreased level may indicate a lower capability of converting T4 into T3. On the other hand, Thangavel *et al.* [17] justifies the decrease in serum T3 after pesticide exposure with a reduction in fish metabolic rate, indirectly reducing the toxic impact of the pesticide. Additionally, the indirect action of contaminants through the interference of cortisol may also be considered as an explanation for serum T3 decrease.

This was different to findings from a previous study investigating sea bream that were exposed to mercury where a decrease of T3 during exposure was observed and taken together these results indicate that chemical stressors have different effects on thyroid hormone status in sea bream. In animals, enzymatic conversion of T4 to T3 occurs via the action of 5-monodeiodinase and as serum T3 remained unchanged, then it is possible that mercury exposure may have modulated this enzymatic conversion process, but further studies would be needed to verify this.

The levels of circulating thyroid hormones were found to be significantly reduced during environmental stress [18]. Therefore, the progressive decrease in the levels of serum T3 during mercury expose of Yellowfin sea

bream may be indicative of a re-directing of metabolic energy away from anabolic processes and towards more vital catabolic processes which are necessary in order to sustain life. It also remains a possibility that the reduction in thyroid hormones are due to increased activity of the HPI axis as studies on infected sea bream, serum T3 were at reduced levels. Since thyroid hormones are involved in anabolic processes, the coordinated changes in these hormones may influence the overall metabolic and physiological status of sea bream during stress [19].

Study of interference between heavy metals with the thyroid system in marine fish concerns mummichogs (*Fundulus heteroclitus*) living in an estuarine environment contaminated with heavy metals [20]. Compared with a clean reference site, confirmed attributed to interference with the thyroid system, perhaps through its involvement in neurological development, also the fish from pollution site had larger thyroid follicles and follicular cell heights and contain elevated titres of serum thyroxine (T4), but not serum T3 that is like our finding.

Mechanisms of action are still not fully understood, but “cross-talk” from the adrenal system may be partly involved. Normally, the stress hormone cortisol stimulates conversion of T4 to T3, but chronic organic and metallic pollution can nonspecifically “exhaust” the cortisol-secreting cells [21], possibly leading in turn to a build-up of T4. However, there are several alternative (but not necessarily mutually exclusive) explanations, including heavy metal inhibition of the enzyme (5'-monodeiodinase) which catalyses T4 to T3 conversion. The example of thyroid disturbance in *F. heteroclitus* serves well to illustrate the potential complexity of endocrine-disrupting effects in fish and the extent of our ignorance of this subject outside the restricted (but important) field of estrogenic impacts [10].

Testosterone secreted by the testes is the main androgen in males, along with its similarly active metabolite dihydrotestosterone. In principle, altered levels of sex steroids in serum might be due to interferences with the control of steroid synthesis via the pituitary-gonadal axis, or to effects on steroid metabolism and excretion. Thus, the hypothesis of a decrease of circulating testosterone in exposed individuals due to an inhibition of steroid biosynthesis might be considered. Indeed, one might hypothesize increased energy requirements for detoxifying functions and xenobiotic clearance in exposed fish, increased physiological stress and hence, decreased energy for growth and reproduction [22]. Results of the present investigation indicated that mercury is a toxic substance in Yellowfin sea bream, with change hormone activities in serum of fish exposed to various concentrations.

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