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The Effect of Acute Toxicity and Thyroid Hormone Treatments on Hormonal Changes during Embryogenesis of *Acipenser persicus*

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***Abstract*—**Production of high quality fish eggs with reasonable hatching rate makes a success in aquaculture industries. It is influenced by the environmental stimulators and inhibitors. Diazinon is a widely-used pesticide in Golestan province (Southern Caspian Sea, North of Iran) which is washed to the aquatic environment (3 mg/L in the river). It is little known about the effect of this pesticide on the embryogenesis of sturgeon fish, the valuable species of the Caspian Sea. Hormonal content of the egg is an important factor to guaranty the successful passes of embryonic stages. In this study, the fate of Persian sturgeon embryo to 24, 48, 72, and 96-hours exposure of diazinon (LC_{50} dose) was tested. Also, the effect of thyroid hormones (T3 and T4) on these embryos was tested concurrently or separately with diazinon LC_{50} dose. Fertilized eggs are exposed to T3 (low dose: 1 ng/ml, high dose: 10 ng/ml), T4 (low dose: 1 ng/ml, high dose: 10 ng/ml). Six eggs were randomly selected from each treatment (with three replicates) in five developmental stages (two cell- division, neural, heart present, heart beaten, and hatched larvae). The possibility of changing T3, T4, and cortisol contents of the embryos were determined in all treated groups and in every mentioned embryonic stage. The hatching rate in treated groups was assayed at the end of the embryogenesis to clarify the effect of thyroid hormones and diazinon. The results indicated significant differences in thyroid hormone contents, but no significant differences were recognized in cortisol levels at various early life stages of embryos. There was also significant difference in thyroid hormones in (T3, T4) + diazinon treated embryos ($P<0.05$), while no significant difference between control and treatments in cortisol levels was observed. The highest hatching rate was recorded in HT3 treatment, while the lowest hatching rate was recorded for diazinon LC_{50} treatment. The result confirmed that Persian sturgeon embryo is less sensitive to diazinon compared to teleost embryos, and thyroid hormones may increase hatching rate even in the presence of diazinon.

***Keywords*—**Persian sturgeon, diazinon, thyroid hormones, cortisol, embryo**.**

I. Introduction

DIAZINON (O,O-Diethyl O-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate) is a widely organophosphate pesticide used in rice paddy fields of Golestan province, Iran to control insects [30]; then, it is washed to the aquatic environment [20], [28], [34], i.e. enters in the rivers leading to the Caspian Sea [41], [46]. There are evidences on the presence of diazinon in the rivers of Golestan province such as Qara-su River (18.6-22.4 mg/l) and Gorgan-rud River (6.74-6.89 mg/l) [49].

There are some environmental factors that affect the rate of diazinon degradation. Low temperature, low moisture, high alkalinity, and lack of adequate microbial degraders for more than six months cause decrease in diazinon degradation [15], [20], [28]. It also enters into the running water based on the time, concentration, frequency of use, and also rainfall [56]. Diazinon degradation through hydrolysis, photolysis, volatilization, and microbial metabolism occurs in small amount to break down the chemical [2], so it is persistent and makes high contamination. Vast distribution of diazinon in aqua ecosystems affects non-target aquatic organisms such as fish.

Sub-lethal dose of diazinon varies from one species to another [5], [23] and resulted in inhibition of acetylcholinesterase activity [43]. Organophosphates also hamper neurotransmitters activity which then affect thyroid hormone function [3], [36]; it also alters thyroid hormones and cortisol concentrations in fish [31]. It is shown that fishes are the most susceptible to the toxic during the developmental process of embryos and larvae [22], [28], [33].

Maternal hormones such as thyroid hormones (THs) and cortisol are important in the regulation of growth, development, sex determination, and survival of fish embryos and larvae [11], [12], [37], [38]. Usually during the development, the levels of maternal hormones decrease, and endocrine organs start to differentiate. Probably, artificial increase in the levels of maternal hormones will be a key factor in larviculture [53]. Studies showed that THs in the fish oocyte increased by T3 injection to the female breeders [18], and positive effects of increase in maternal T3 level have been proved in larval survival [7], [12], [13].

The present study was initiated to determine acute toxicity of diazinon in the embryos of Persian sturgeon, *Acipenser persicus*; one of the commercial and valuable sturgeon fishes in the Caspian Sea [40], [44]. Persian sturgeon is a suitable species for the study because of long term exposure to toxins in their ecosystems.

There are internal and external factors to affect the quality of the fish eggs [10]. This study is designed *in vitro* to evaluate the effect of sublethal diazinon and also THs on some embryonic hormonal contents in order to contribute the knowledge of the early life stages of fish. The critical period that ultimately makes the fate of an embryo.

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II. Materials and Methods

A. Collection of the Eggs and Artificial Fertilization

Persian sturgeon fish embryos used for acute toxicity tests were obtained from autumn brood stock program of Shahid Marjani Hatchery Complex near Gorgan city (North-Eastern of Iran). All samples were received from artificially fertilized oocytes of one wild female (24 kg in weight and 152 cm length) and sperm of three wild males.

B. Experimental Treatment

Diazinon (95% purity) was purchased from Qingdao Hisigma chemicals Co. Ltd (China). The nominal treatment concentrations (0, 2, 4, 6, 8 mg/L) were used for any of different embryonic stages [6]. The medium was the sterile of well water used at Shahid Marjani Hatchery Complex.

After the second egg cleavage, the fertilized eggs were separated from unfertilized, parthenogenesis, and poly sperm eggs. 300 fertilized eggs (60 for each treatment) were used for the experiment. Samples were transferred to the central lab, University of Tehran within six hours.

C. Acute Toxicity Test

Embryos were incubated in six well-culture dishes (SPL, Korea) with no or different concentrations of diazion and put into CO_2 incubator under 16 °C. Incubation period lasted for 24, 48, 72, and 96 hours. It was semi-static method, and the culture media were changed daily with consideration of the mortalities. The procedure was arranged according to OECD guideline for fish embryo toxicity (FET) test [26].

D. Hormonal Assays

The second part of the test was carried out after determining the LC_{50} value. The fertilized eggs were obtained as described before and transported to the laboratory within six hours. There were 10 groups with three replicates each group in six well-culture dishes. One group was just in medium as control, one group was toxin treatment in medium as LC_{50} dose, two groups were low dose (LD) of T3 (tri-iodothyronine) and T4 (thyroxine) (1 ng/ml) in medium, two groups were high dose (HD) of T3 and T4 (10 ng/ml) in medium, two groups with the mixture of LC_{50} diazinon and LD of T3 or T4 in medium and two other groups were the mixture of LC_{50} diazinon and HD of T3 or T4 in medium.

The concentrations for low dose of T3 and T4 treatments were chosen similar to the results of a previous study by [9] for THs in the fertilized eggs of sturgeon fish, and the high dose were selected more than the result of [39] for THs in plasma of juvenile sturgeon fish. There were 90 embryos in each group. The embryos were kept in CO_2 incubator under desired condition until they hatched and removed only for monitoring the developmental stages at defined time by stereomicroscope (10X). The samples for hormonal assays were removed in five embryonic stages (2 cell-division, neurula, heart present, heart beaten and immediately after hatch) to evaluate the hormonal contents in each stage.

E. THs Analysis

TH extraction was performed according to [52] and [47]. Each three frozen eggs or larvae were homogenized in two volumes of phosphate- buffered saline (PBS; pH=7.5), and the mixtures were sonicated. Then, 0.167 volume of a 10X stock trypsin solution (Inoclon) was added to each tube and was vortexed to release thyroglobulin from thyroid tissues, then incubated in shaking water bath at 38 °C for 1 hour. After that, 2 ml ice-cold ethanol (99%) added to the samples, was vortexed and centrifuged at 3000 rpm for 10 minutes at 4 °C. The supernatant was decanted, and 2 mL ice-cold ethanol (99%) was again added again to the residue, vortexed and centrifuged. The supernatant was extracted and pooled to the previous one. Finally, it was evaporated overnight, then was frozen at -20 °C until later hormone assay.

F. Cortisol Analysis

Cortisol extraction was followed by [35]. Every three embryos were homogenized in 800 µl distilled water. The tubes were vortexed. 8 ml diethyl ether was added to the samples and then vortexed vigorously for 30 seconds. Diethyl ether was applied for the second time about 4 ml and they were vortexed again. Samples centrifuged at 2100 g for 5 minutes at 4 °C. Supernatants were evacuated to other tubes. At the end of the procedure, they were reconstituted with phosphate- buffered saline containing gelatin (PBSG).

G. Enzyme Immunoassay of hormones

T3, T4 (Pishtaz Teb, Iran) and cortisol (DiaMetra, Italy) enzyme immunoassay tests were performed in duplicate using up to 50, 25, and 20 µL, respectively for each sample. The OD values of standards and experimental samples were read within 10 minutes using a microplate reader (BioTek, ELx808) with a 450-nm filter. A standard curve was drawn ranging from 0 to 20 for T3 or T4 and 0 to 40 ng/ml for cortisol. The coefficient in linear range of the curves was almost 0.8 for all of them to calculate the hormone concentrations.

H. Statistical Analysis

After 96 hours, the mortalities were assessed by statistical package SPSS 16.0 (Chicago, IL). The data were subjected to Probit Analysis Statistical Method to determine the 96h LC_{50}.

The data obtained, expressed as means ± SE, were statistically analyzed by multivariate analysis and significant differences were detected. Duncan multiple range test was performed to determine significant differences among groups (P<0.05).

III. Results

A. Determination of Sub-Lethal Dose of Diazinon

The number of dead embryos for diazinon doses of 2, 4, 6, and 8 mg/L were examined for 24, 48, 72, and 96 hours of exposure in Persian sturgeon embryos. The 96LC_{50} value (95% confidence limits) of water-soluble diazinon of Persian sturgeon fish at early life stage was detected as 3.558 mg/L.

B. TH Level

All the embryos were used for hormonal extractions. The whole-body T3 concentration of Persian sturgeon ranged from 8.47±1.07 to 13.38±1.63 pg/mg in control during embryonic stages (after fertilization until immediately after hatch). T3 content also varied significantly by embryonic development ($F_{5,120}$= 18.969, P<0.05; Fig. 1). T3 level increased significantly (P<0.05) in the low dose of T3 (LT3) and high dose of T3- treated group (HT3) compared to the control and other treated groups. In contrast, LC_{50}- treated group showed decrease in T3 level significantly ($F_{9,120}$ =18.126, P<0.05; Fig. 1).

The whole-body T4 concentration of Persian sturgeon embryos ranged from 140.40±9.61 to 262.25±45.36 pg/mg in control (after fertilization until immediately after hatch). The highest T4 level was observed in low dose of T4-treated group (LT4) and the lowest one in LC_{50}- treated group ($F_{9,120}$= 19.954, P<0.05). Like T3, T4 content of the body varied significantly during the embryogenesis ($F_{5,120}$= 198.491, P<0.05; Fig. 2).

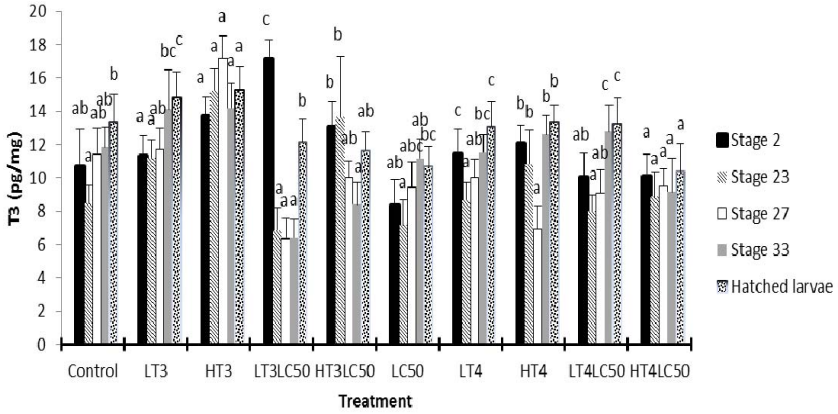

Fig. 1 T3 concentrations (pg/mg) in developing Persian sturgeon embryos following exposure to all treated groups or control. X-axis shows treated groups: LT3: low dose of T3; HT3: high dose of T3; LT3LC50: the mixture of low dose of T3 and LC_{50} of diazinon; HDLC50: the mixture of high dose of T3 and LC_{50} of diazinon; LC50: only diazinon in the amount of LC_{50}; LT4: low dose of T4; HT4; high dose of T4; LT4LC50: the mixture of low dose of T4 and LC_{50} of diazinon; HT4LC50: the mixture of high dose of T4 and LC_{50} of diazinon. The columns also show the developmental stages: stage 2: 2 cell-division; stage23: neurula; stage 27: heart present; stage 33: heart beaten and the larvae immediately after hatching. Data are presented as mean±SEM

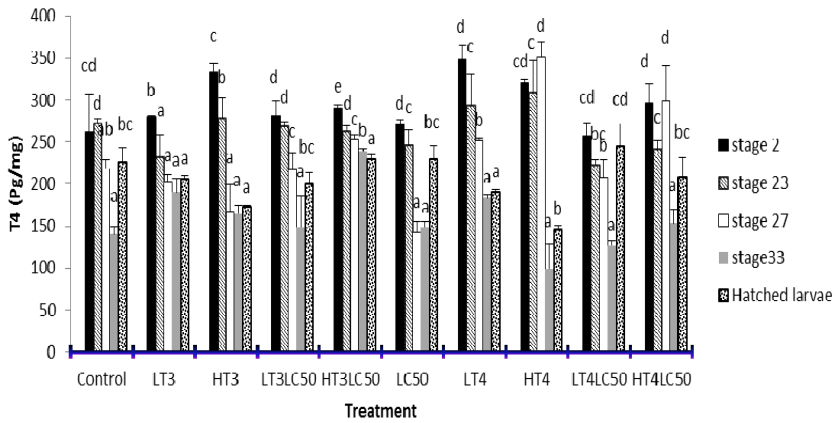

Fig. 2 T4 concentrations (pg/mg) in developing Persian sturgeon embryos following exposure to all treated groups or control

C. Cortisol Level

Although there was fluctuation during embryogenesis and also an increase in cortisol content after hatching but this increase did not become statistically significant in early development stages of control group. There was also no significant difference found among all treated groups in cortisol level (Fig. 3). The whole-body cortisol content in control was from 2.4±1.22 to 3.59±1.18 pg/mg during embryogenesis (after fertilization until immediately after hatch).

D. Hatching Rate

The hatching rate of different treated groups is shown in Fig. 4. The highest hatching rate was recorded in low dose and high dose of T3 and high dose of T4-treated group. The hatching rate revealed significant difference among all treated groups (P=0.000).

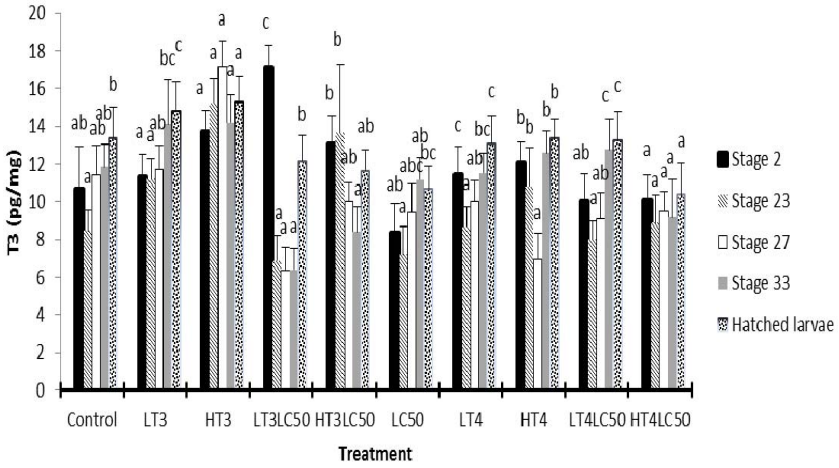

Fig. 3 Cortisol concentrations (ng/mg) in developing Persian sturgeon embryos following exposure to all treated groups or control

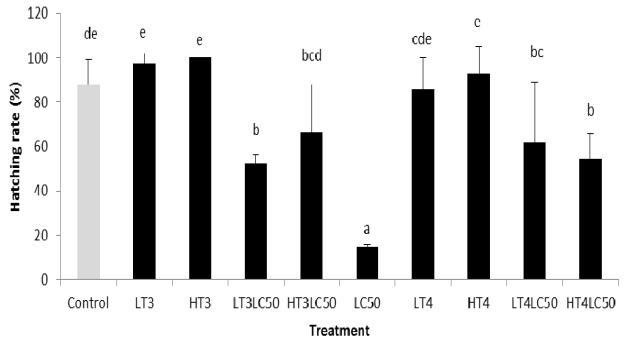

Fig. 4 The hatching rate in Persian sturgeon embryos following exposure to all treated groups or control. Each column represents the mean value of three replicates

IV. Discussion

A. Acute Dose of Diazinon

In this study, the LC_{50} value of organophosphate pesticide diazinon was calculated for Persian sturgeon embryos for the first time. This value showed that sturgeon embryos are more resistant to the diazinon compared to the other fish embryos as it is observed in common carp, *Cyprinus carpio* with 48h LC_{50} value of 0.999 (0.698-1.427) mg/L [6], 96-h LC_{50} value for eyed egg and alevins of chinook salmon, *Oncorhynchus tshawytscha* with 545 and 29.5 ppm, respectively [55] and 96-h LC_{50} value for European cat-fish fingerling, *Silurus glanis* L. was 14.597 (12.985-16.340) mg/L, 9.710 ppm for young Gambusia [48] and 96-h LC_{50} value for Persian sturgeon fish fingerling was 1.8 mg/L [27]. Sturgeon fish embryos have a thick envelope consisting of four distinct layers [16]. It is probably a barrier for low external chemicals diffusion to the inside of eggs.

In this experiment, the sub-lethal dose of diazinon was used to determine the effect of this chemical on sturgeon embryos as the previous studies showed that the presence of Man-built dams especially at Gorganrud and discharges of chemicals to the rivers and estuaries cause the high pollution environment for the fish [4], [32]. The concentration of diazinon is recorded until 3.8 mg/L [49], [50] in the rivers of southern Caspian Sea basin. These higher pesticide concentrations usually happen during spring when the precipitation is low [41], and unfortunately fish restocking performed [1].

The results showed that the all concentrations of media diazinon caused obvious hormonal changes in the whole Persian sturgeon embryo content. THs decreased significantly in diazinon LC_{50} treated group, but no significant difference was recorded in cortisol levels. It was reasonable that the lowest hatching rate was pertained to diazinon LC_{50} treated group. Similar studies confirm decrease of THs against organophosphates [31]. They showed that chlorpyrifos, an organophosphate pesticide, induces decrease in the level of T3 and T4 in Bloch, *Heteropneustes fossilis* weighing 10±0.5 g embryos. Diazinon decreased T3 and T4 significantly in Caspian roach, *Rutilus rutilus* fingerlings [30] also in Persian sturgeon fingerlings [27]. The changes of T3 and T4 levels in our study support the interaction of these hormones during early development. Some amounts of diazinon also caused decrease in plasma cortisol level in spotted scat, *Scatophagus argus* with a mean weight of 137±6 g [24]. Plasma cortisol increased in Persian sturgeon fingerlings during short term exposure of diazinon [27]. Although the developing embryos are more sensitive to endocrine-disrupting chemicals (EDC), but effects on early life stages may not be revealed until adulthood [25]. Sometimes, extremely low-dose exposure of chemicals makes significant effect on developing organisms [57], but it is worth mentioning that effects are often appeared by non-traditional dose-response curves [25], [57], so maybe some doses of lower diazinon exert cortisol alteration.

Higher mortality was recorded in Zebrafish, *Danio rerio* embryos exposed to 3000 µg/L diazinon [42]. Diazinon exposure manifested decrease in hatch success in Medaka, *Oryzias latipes* embryos too [28].

B. THs-Treated Groups and Control

The possibility of passive diffusion of THs in to eggs may lead to the increase of TH levels in embryos significantly compared to the control. There were no significant changes in

cortisol content among T3 and T4 treated groups and the others. The present results demonstrated that the hatching rate increased significantly in low dose and high dose of T3-treated groups and high-dose of T4-treated group. Female brood injection of T3 and maternally transfer to the larvae increases growth and survival [14]. Elevating the T4 level in embryos and larvae probably prevents from heavy mortality in some species [7].

There are many reports of fish eggs THs content such as chum salmon, *Oncorhynchus keta* with 9 ng/g T3 and 15-20 ng/g T4, sockeye salmon, *Oncorhynchus nerka* with 1ng/egg T3 and 6 ng/egg T4, Stripped bass with 4.5 ng/g T3 and 5.1 ng/g Tilapia with 11.4 ng/g T3 and 2.1 ng/g T4, Rabbit fish with 2ng/g T3 and 10-16 ng/g T4, Conger eel, *Conger myriaster* with 0.15 ng/g T3 and 5ng/g T4 [45] and Rassian sturgeon fish, *Acipenser guldenstaedtii* with mean maximum 0.025±0.004 ng/egg T3 and 0.34±0.09 ng/egg T4 [9] while in this case study, the value of T3 in control was 8.47±1.07 to 13.38±1.63 pg/mg. The value of T4 in control was recorded from 140.40±9.61 to 262.25±45.36 pg/mg during early developmental stage. Since these two species of sturgeon fish are so close in phylogenetic, similarity in hormonal content of eggs is expected.

In this study, the cortisol content of control varied from 2.4±1.22 to 3.59±1.18 pg/mg during embryogenesis (after fertilization until immediately after hatch). Data obtained are quite similar to what were recorded by Falahatkar et al., in the same species [21]. Simontacchi et al. expressed that cortisol content of white sturgeon, *Acipenser transmontanus* differed from 0.73±0.22 to 3.76±0.53 ng/g during early development [51]. Cortisol level showed no significant difference among all treated groups in this study. Falahatkar et al. demonstrated that stress exposure to developing eggs had no effect on whole-body cortisol concentration [21]. Most evidences showed that cortisol and THs activities are synergistic during metamorphism in larvae [19], [29]. It seems that HIP axis is not functional at early stages of sturgeon fish life or probably the synergism of cortisol and THs is species specific bioassay for toxicological assessment. Diazinon decreases ACTH and dbcAMP-stimulated cortisol secretion. Adrenotoxic pattern of diazinon in trout, *Oncorhynchus mykiss* adreno cells, was presented by Bisson and Hontela [8]. Diazinon probably is less cytotoxic to sturgeon fish during embryogenesis as it is suggested that diazinon at the endocrine-disrupting dose is not cytotoxic to the rat adrenal cells [17].

C. THs and Acute Toxicity Effects

In this study, T3 content of embryos in the mixed treated groups of LT3+ LC_{50} value of diazinon ($LT3LC_{50}$ of diazinon) or HT3+LC_{50} value of diazinon ($HT3LC_{50}$ of diazinon) was higher compared to $LT4LC_{50}$ of diazinon and $HT4LC_{50}$ of diazinon-treated groups, and all of them were higher than LC_{50} value of diazinon- treated group.

The results showed that T4 content of embryos in the mixed treated groups of LT4+LC_{50} value of diazinon or HT4+LC_{50} value of diazinon was higher than LC_{50} value of diazinon treated group and also $LT3LC_{50}$ of diazinon. But, they are lower than control and $HT3LC_{50}$ of diazinon.

The rate of hatching in hormonal plus LC_{50} of diazinon treated group was higher than LC_{50} of diazinon treated group but lower than control and THs-treated groups. Thangavel et al. demonstrated that reduction of T3 in *Sarotherodon mossambicus* under dimecron (an orghanophosphate pesticide) exposure leads to drop in oxygen uptake of fish [54]. It is suggested that decreasing hatching rate in embryo exposed to the toxin might possibly be compensated by adding external THs.

The present results suggest that sturgeon fish breeding in to the diazinon contaminated Caspian Sea basin may alter the embryos and larvae physiologically and the ability of hatch and endanger their survival and ultimately Persian sturgeon fish stocks in the Caspian Sea.

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