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The effects of diazinon on the cell types and gene expression of the olfactory epithelium and whole-body hormone concentrations in the Persian sturgeon (*Acipenser persicus*)

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ABSTRACT

The olfactory function and imprinting of odorant information of the native stream play a critical role during the homing migration in fish. Pesticides may impair olfactory imprinting by altering olfaction and hormone functions. The present study aimed to determine how diazinon impacts olfactory epithelium morphology and cell composition, as well as hormone concentrations in Persian sturgeon (*Acipenser persicus*) during their lifetime in freshwater and, also during diazinon-free saltwater acclimation. Fingerlings were exposed to 0, 150, 300, and 450 $\mu g \cdot L^{-1}$ of diazinon in freshwater for 7 days and then were transferred to diazinon-free saltwater by gradually increasing salinity up to 12 ppt. After diazinon exposure, the number of olfactory receptor cells (ORCs) and goblet cells (GCs) decreased and increased, respectively, and the expression of G-protein αolf (GPαolf) and calmodulin-dependent kinase II delta (CAMKIId) was down-regulated and up-regulated, respectively. Transferring the fish to diazinon-free saltwater (8 and 12 ppt) raised the number of ORCs, supporting cells (SCs), GCs, and GPαolf expression, and down-regulated CAMKIId without any significant differences among treatments. Exposure to diazinon increased whole-body cortisol at the high concentration, while decreased whole-body thyroxin (T4) and triiodothyronine (T3) in a dose-dependent manner. Although whole-body T4 and T3 increased in all the treatments after saltwater acclimation (8 and 12 ppt), the level of these hormones was lower in fish that had been exposed to diazinon than in the control. These results showed that diazinon can disrupt olfactory epithelium morphology and cell composition as well as hormone concentrations, which in turn may affect the olfactory imprinting in Persian sturgeon fingerlings.

1. Introduction

Persian sturgeon, *Acipenser persicus*, is an anadromous fish species native to the southern Caspian Sea in Iran (Coad, 1980; Soleimani et al., 2012), which migrates into the rivers of southern Caspian Sea for spawning (Billard and Lecointre, 2000). Downstream migration and subsequent formation of olfactory imprinting to odorant cues in natal rivers are among the most important aspects of the anadromous life history, as it will be vital in the reproductive age of the fish for recognizing the natal river during the spawning migration (Hasler and Scholz, 1983). Olfactory imprinting occurs in sturgeon fish at early life stages (Boiko et al., 1993; Boiko and Grigor'yan, 2002); however, it is a continuous process that may last during downstream migration (Ueda et al., 2016) as well as during early seawater entry (Iwata et al., 2003).

Olfactory imprinting is a complex neurophysiological phenomenon, which is controlled at molecular, physiological, and hormonal levels (Ueda, 2016; Ueda et al., 2016). Recent investigations revealed that olfactory functions have a critical role in the imprinting process (Ueda, 2012). The olfactory epithelium in fish includes several cell types, such as: basal cells (BCs), goblet cells (GCs), supporting cells (SCs), and olfactory receptor cells (ORC) (Kasumyan, 2004; Zeiske et al., 2003). New GCs, SCs, and ORC are developed and derived from BCs (Cancalon, 1982). GCs produce olfactory mucus, which in turn protects the olfactory epithelium against small physical particles and chemicals in water

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(Kasumyan, 2004). The function of SCs is to provide metabolic support to the other olfactory epithelium cells and to transport, by microvilli, water containing oxygen and odorant molecules to the ORCs (Døving, 1986; Kasumyan, 2004). The initial odor detection takes place in the olfactory rosettes, where key odorants bind to the ORCs in the olfactory epithelium. Activation of the olfactory receptors initiates a downstream signaling cascade mediated by G-protein coupled receptors and adenylyl cyclase (AC) for the transmission of the information to the olfactory bulb for processing (Pifferi et al., 2010), with subsequent imprinting on the odorants (Nevitt et al., 1994). The interaction between these two proteins is mediated by other important proteins conserved in signaling transduction pathways, such as G-proteins and the members of the calcium signaling pathways. G-proteins are GTPases that are recruited by activated G-protein coupled receptors that act as the initiators of the signaling cascade. Calmodulins are sensors of intracellular levels of Ca2+ which activates a series of Calcium/calmodulin dependent kinases, which may activate or inhibit the AC (Nakamura and Gold, 1987; Laberge and Hara, 2001; Pifferi et al., 2010).

Previous studies have demonstrated that thyroid and cortisol hormones are critical in the formation and control of olfactory imprinting in fish (Boiko and Grigor'yan, 2002; Braithwaite, 2011; Ueda, 2016). There is evidence that thyroid hormone receptors are present in the anterior and middle brain and olfactory epithelium in fish (Kudo et al., 1994), which can indicate the role of these hormones on olfaction and imprinting (Lema and Nevitt, 2004; Ueda, 2016). During olfactory imprinting, thyroid hormones surge and lead to increased expression of imprinting-related genes (Ueda, 2016) as well as induce the proliferation of olfactory cells (Lema and Nevitt, 2004). In addition, in species requiring imprinted odorant memory, cortisol can affect the olfactory receptors and potentiate the formation of olfactory memory (Carruth et al., 2002).

Organophosphate pesticides, such as diazinon, are commonly used in paddy field agriculture in northern Iran (Nouri et al., 2000) and have been detected in surrounding surface waters (Shayeghi et al., 2001; Fadaei et al., 2012). Organophosphate pesticides can impair olfactory processes in fish and, therefore, can disrupt olfactory-related behaviors including imprinting, homing, predator avoidance, and prey selection (Sandhal et al., 2004; Sandahl et al., 2005; Tierney et al., 2010). Previous studies reported that organophosphate pesticides can alter olfactory signal transduction pathways (Tilton et al., 2011; Maryoung et al., 2015) and may even lead to the loss of olfaction in fish (Sandhal et al., 2004, Sandahl et al., 2005; Tierney et al., 2010). Maryoung et al. (2015) showed that exposure to the organophosphorus pesticide, chlorpyrifos, and then to a condition of hypersalinity, up-regulated the inhibitory olfactory signal transduction-related genes in rainbow trout juveniles, *Oncorhynchus mykiss*. Furthermore, exposure to chlorpyrifos changed the expression of genes related to odorant-binding in zebrafish, *Danio rerio*, (Tilton et al., 2011). It is proven that environmental pollutants can affect endocrine systems and cause changes in hormone function and concentration (Cocco, 2002). Moreover, chemicals can also bind to hormone receptors inhibiting their action (Okubo et al., 2004), as well as suppressing their transcription (Iwasaki et al., 2002). Previous studies reported that exposure to organophosphate pesticides reduced thyroxin (T4) and triiodothyronine (T3) and increased cortisol in different fish species (Cocco, 2002; Sugiyama et al., 2005; Leghait et al., 2009; Braithwaite, 2011; Khosravi Katuli et al., 2014; Hajirezaee et al., 2016; Ueda, 2016).

Persian sturgeon fingerlings are mainly produced through artificial propagation centers in Iran (Steinshamn and Alaei Borujeni, 2014; FAO, 2018). Given the occurrence of diazinon in rivers of southern Caspian Sea, where the sturgeon fingerlings are released (FAO, 2018), the current study aims to determine if Persian sturgeon fingerlings exposed to different concentrations of diazinon are affected by changes in gene expression, histology and hormone levels related to the olfactory function during their lifetime in freshwater and, whether the toxic effects induced by diazinon remain, are reversed or changed after transferring the fish to saltwater without diazinon.

2. Material and method

2.1. Experimental fish

Persian sturgeon fingerlings (1.5 ± 0.5 g) were supplied from the Eslami Persian sturgeon Propagation Centre (Hossein Abad, Sari, Northern Iran). Fish were maintained in a 14 h light-10 h dark cycle at 26 °C and pH 8.2 ± 0.2 for one week in a 1000 L freshwater tank to acclimate. Water was continuously aerated and 50% of it was changed daily. During this period, fish were fed with a commercial diet at a rate of 3% of biomass (Commercial concentrates; BioMar; Germany +Artemia) four times per day. All procedures were carried out following the Animal Care and Use Committee guidelines of the Faculty of Sciences at the University of Tehran (357, 8 November 2002).

2.2. Fish exposure

After the acclimation period, fish were randomly divided into four groups and each group consisting in 3 × 60 L tanks as replicates (60 fish per tank) and maintained at the same light cycle and temperature as previously stated. Each group of fish were exposed to one of the four different concentrations of diazinon, control (0 $\mu g \cdot L^{-1}$), low (150 $\mu g \cdot L^{-1}$), medium (300 $\mu g \cdot L^{-1}$) and high (450 $\mu g \cdot L^{-1}$) in freshwater for 7 days. The diazinon concentrations were chosen based on the reports of diazinon concentration in river surface waters entering to the Caspian Sea, *i.e.* 18.88–768.91, 11.59–650.8, and 4.97–248.83 $\mu g \cdot L^{-1}$ (Fadaei et al., 2012). Fifty percent of the water was changed daily and the diazinon exposure concentration was renewed. Since sturgeon fingerlings migrate from freshwater rivers to seawater (Kottelat and Freyhof, 2007), we mimicked this activity by transferring the fish to diazinon-free saltwater. To do this, after the 7-day exposure to diazinon in freshwater, the freshwater was replaced by 4 ppt saltwater without diazinon in each group, and then the salinity was increased 4 ppt every two days until reaching the maximum 12 ppt (Fig. 1) as previously described (Lavado et al., 2009). In this way, the fish were kept at each salinity (4, 8, and 12 ppt) for two days. Caspian Sea water was used to increase the salinity of the laboratory domestic freshwater.

2.3. Sampling

At the end of the diazinon exposure in freshwater and the end of each salinity period (4, 8 and 12 ppt), three fish per tank (9 fish per treatment) were anesthetized in clove oil (150 ppm) for 40–50s (Holloway et al., 2004) and the left olfactory rosette was dissected and fixed in Bouin's fixative for 48 h for histological examination. Four additional fish per replicate were anesthetized and the olfactory rosettes pooled, placed in cryotubes (Greiner Bio-One), and stored at −80 °C until RNA isolation. The carcasses were dried with tissue paper, weighed, frozen immediately in liquid nitrogen, and stored at −80 °C until whole-body hormone extraction.

2.3.1. Histology

After fixation, the samples were washed in distilled water and stored in 70% ethanol. The olfactory rosette was embedded in paraffin and serial transverse cross-sections (3 μm) were made using a microtome. Dewaxed and rehydrated sections were stained with hematoxylin and eosin 1% (Sigma-Aldrich) (Van Denbossche et al., 1995; Liu et al., 2015). For cell counting, five sections per fish were chosen, and the same region of the olfactory epithelium was selected under the microscope. The number of ORCs, SCs, and goblet cells GCs was counted in three 100 μm^2 representative areas for each selected region using an Olympus BH-2 optical microscope equipped with an eyepiece micrometer, Dino-Capture camera and ISCapture Software at a magnification of 100× (Van Denbossche et al., 1995; Hansen, 2007; Namdariyan Rad et al.,

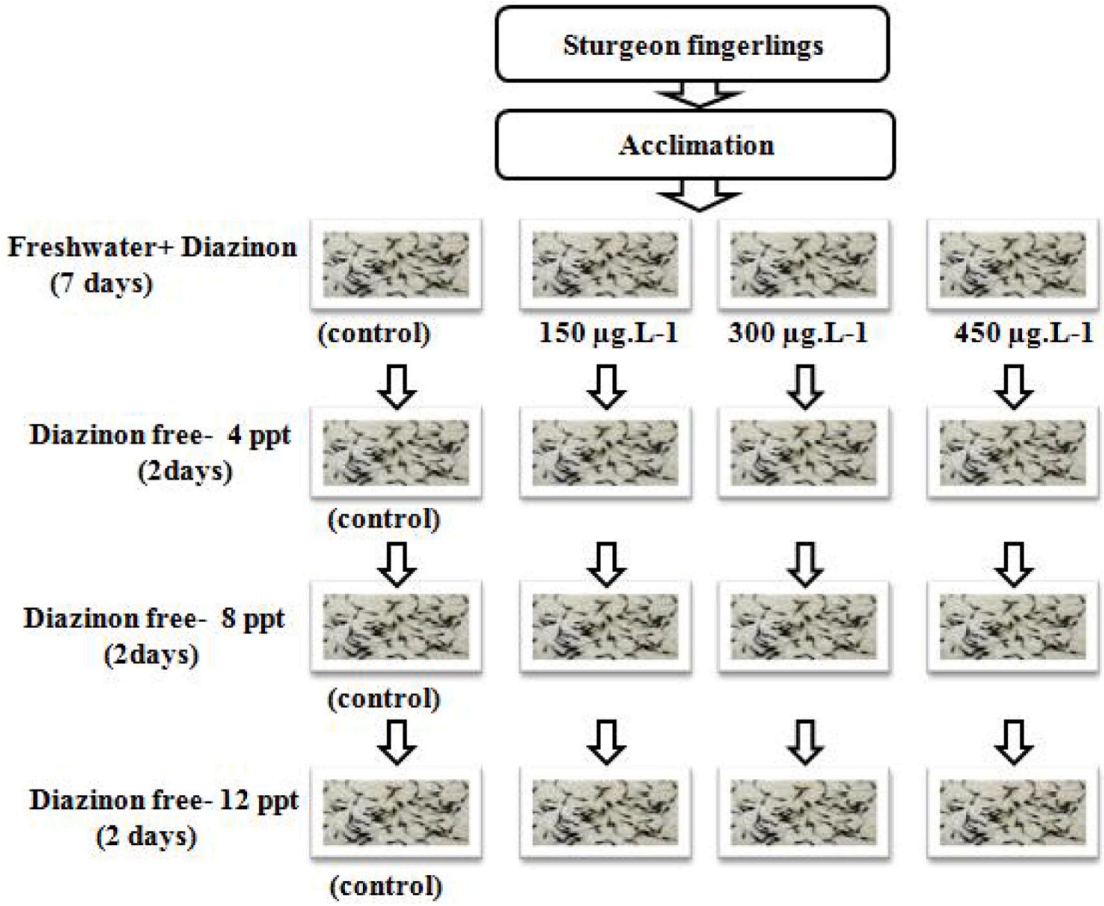

Fig. 1. Experimental design: Fish were exposed to diazinon in freshwater for 7 days and then transferred to diazinon-free saltwater in a stepwise manner with an increase in 4 ppt every two days. $n = 3$ replicates per each treatment.

2017). The images of each section were counted by two blind investigators and the final counting of each cell type is represented as a mean of the counting value reported by both investigators. ORCs, SCs, and GCs were distinguished according to previous reports: ORCs are normally located in different parts of the olfactory epithelium and have a thick dendrite that penetrates the epithelium and possess cilia. SCs are relatively thin, elongated cells that are located between the ORCs. BCs are rounded cells with large nuclei located in the deep part of the olfactory epithelium. GCs are round cells that are located at the apical surface of the olfactory epithelium (Døving, 1986; Van Denbossche et al., 1995; Mokhtar and Abd-Elhafeez, 2014).

2.3.2. Molecular studies

Total RNA was isolated from the olfactory rosettes using Trizol (Gene All, Seoul, Korea). The quantity (ng/µL) of RNA was determined by NanoDrop ND-1000 (Thermo Fisher Scientific) and the RNA purity was assessed measuring OD260/280 and OD260/230 ratios. Total RNA (0.7 µg) was treated with DNase I (Fermentas, USA) following the manufacturer's instructions and retrotranscribed to cDNA using Reverse Transcription System Kit (Fermentas, USA). Primers were designed using Primer3 software (Untergasser et al., 2007) for G-Protein αolf (GPαolf) and calcium calmodulin-dependent protein kinase II delta (CAMKIId) with the obtained cDNA sequences of Persian sturgeon. These sequences were obtained from purified PCR products using the Bigdye Terminator v3.1 cycle sequencing kit (Fermentas, USA) and by Applied Biosystems 3730xl genetic analyzer. The sequences were deposited in GeneBank (NCBI) under accession numbers MN556695 for GPαolf and MN556694 for CAMKIId. The primer sequences are listed in Table 1. qPCR was run using SYBR Green Master Mix (Ampliqon, Denmark). Thermo-cycling parameters were set as follows: 15 min at 95 °C; 40 cycles of 15 s at 95 °C, 20 s at 58 °C, and 20 s at 72 °C; 15 s at 95 °C, 1 min at 70 °C, and 15 s at 95.1 °C. Relative gene expression results for each gene were calculated with RPL-6 as a housekeeping gene using the ΔΔCt method (Livak and Schmittgen, 2001). The stability of the selected housekeeping gene was assessed by confirming that the mean coefficient of variation of the sample group (Mean CV = 0,026)

Table 1
Primer sequences (5′-3′).

Target	Primer Sequence	Length (bp)	Efficiency (%)	R^2
GPαolf	GGACCTTCTCCGATGCAGAG ATGATCGCAGTGACGTCGTT	150	181.5	0.999
CAMKIId	GTGACTCCCGAAGCCAAAGA GTTGACAGATCCACGGGTGT	103	183.8	0.991
RPL-6 (housekeeping gene)	GTGGTCAAACTCCGCAAGA GCCAGTAAGGAGGATGAGGA (Akbarzadeh et al., 2013)	149	164.8	0.999

was lower than 0,5 (Hellemans et al., 2007).

2.3.3. Hormonal studies

Due to inadequate blood volume, whole-body extracts were used to measure hormonal parameters as described elsewhere (Ramsay et al., 2006; Prodocimo et al., 2007; Peterson and Booth, 2010; Khosravi Katuli et al., 2014). Whole-body thyroid hormones (T4 and T3) and thyroid-stimulating hormone (TSH) contents were extracted as described by Tang et al. (2015). Briefly, frozen fish (1 g) from each replicate were homogenized with a glass tissue grinder in 1 mL of PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, and 100 mM NaCl; pH = 7.4) at 0 °C. The homogenate was then centrifuged at 12000*g* for 5 min at 4 °C. The

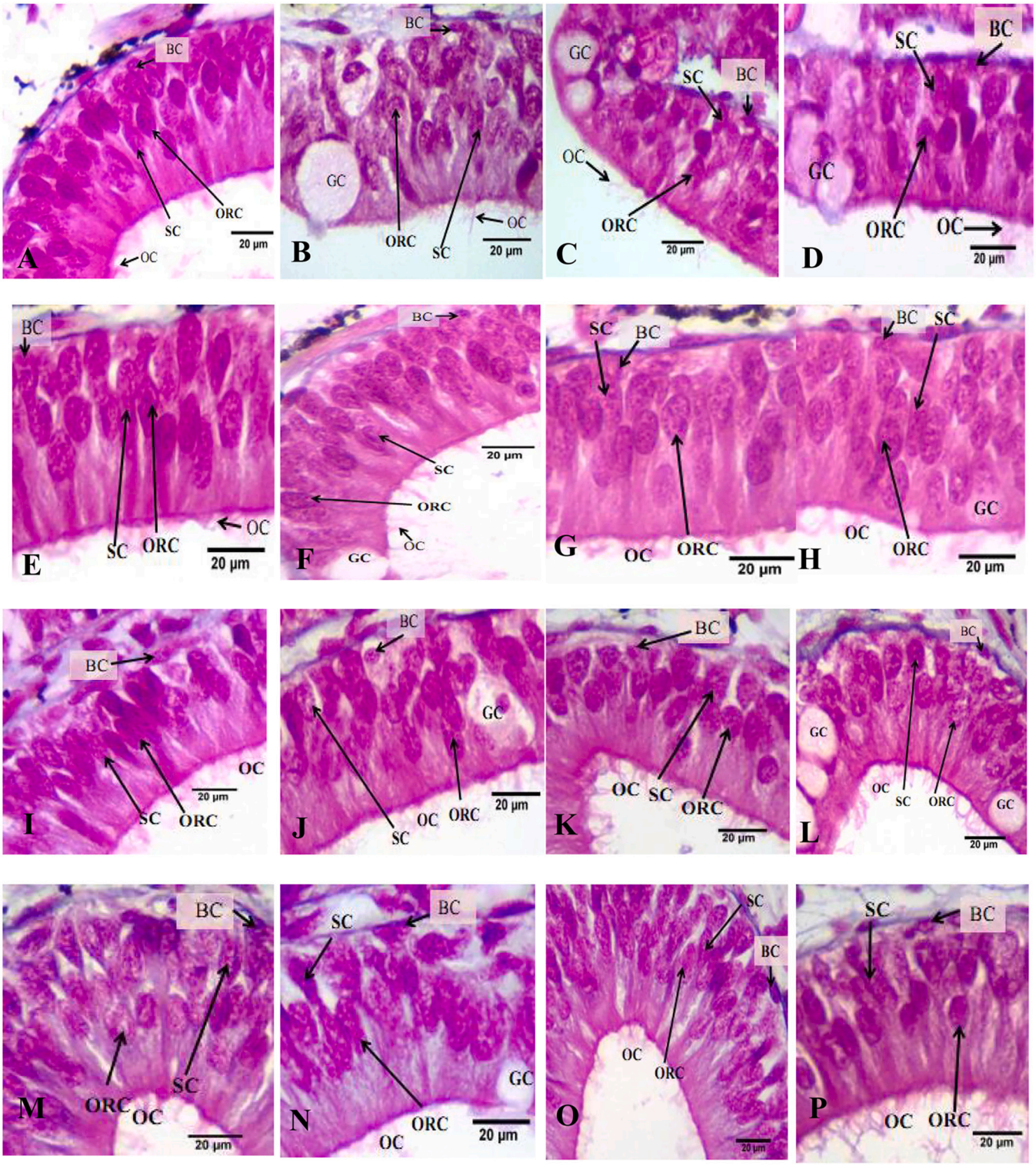

Fig. 2. Histopathological changes in the olfactory epithelium of Persian sturgeon fingerlings exposed to different levels of diazinon either in freshwater (A: 0 µgL-1, B: 150 µgL-1, C:300 µgL-1, D:450 µgL-1) or diazinon-free saltwater (E, F, G, H: Salinity 4 ppt; I. J, K. L salinity 8 ppt; M, N, O, P: Salinity 12 ppt). Light macro-photograph (40× magnification). BC: Basal cell, GC: goblet cell, OC: Olfactory cillia, ORC: olfacrory receptor cell, SC: Supporting cell.

supernatants were collected and stored at −80 °C until the hormone assays. Whole-body cortisol extracts were prepared as stated by Manuel et al. (2014). Frozen fish (1 g) were homogenized with a glass tissue grinder in 1 mL PBS. The homogenate was mixed with 4 mL of methanol and stored at 4 °C for 1 h. Subsequently, the mixture was centrifuged (4 °C, 3000 rpm, 5 min) and the resulting supernatants containing the steroids were stored at −80 °C until cortisol assay. The total T4, T3, TSH, and cortisol levels in whole-body samples were measured by enzyme-linked immunosorbent assay (ELISA) as previously described (Liu et al., 2015).

2.4. Statistical analysis

All data analyses were performed using SPSS 19.0 software (SPSS, Chicago, IL, USA). All the data were tested for normality and homoscedasticity (Kolmogorov-Smirnov and Levene tests, respectively) before conducting statistical analyses. The effects imparted by the exposure to different concentrations of diazinon in freshwater were analyzed by one-way ANOVA and the effects of acclimation to three different salinity levels without diazinon on the previous treated groups were tested in a two-way ANOVA model, where the interaction between both factors was also considered. Tukey test was used as a *post hoc* to

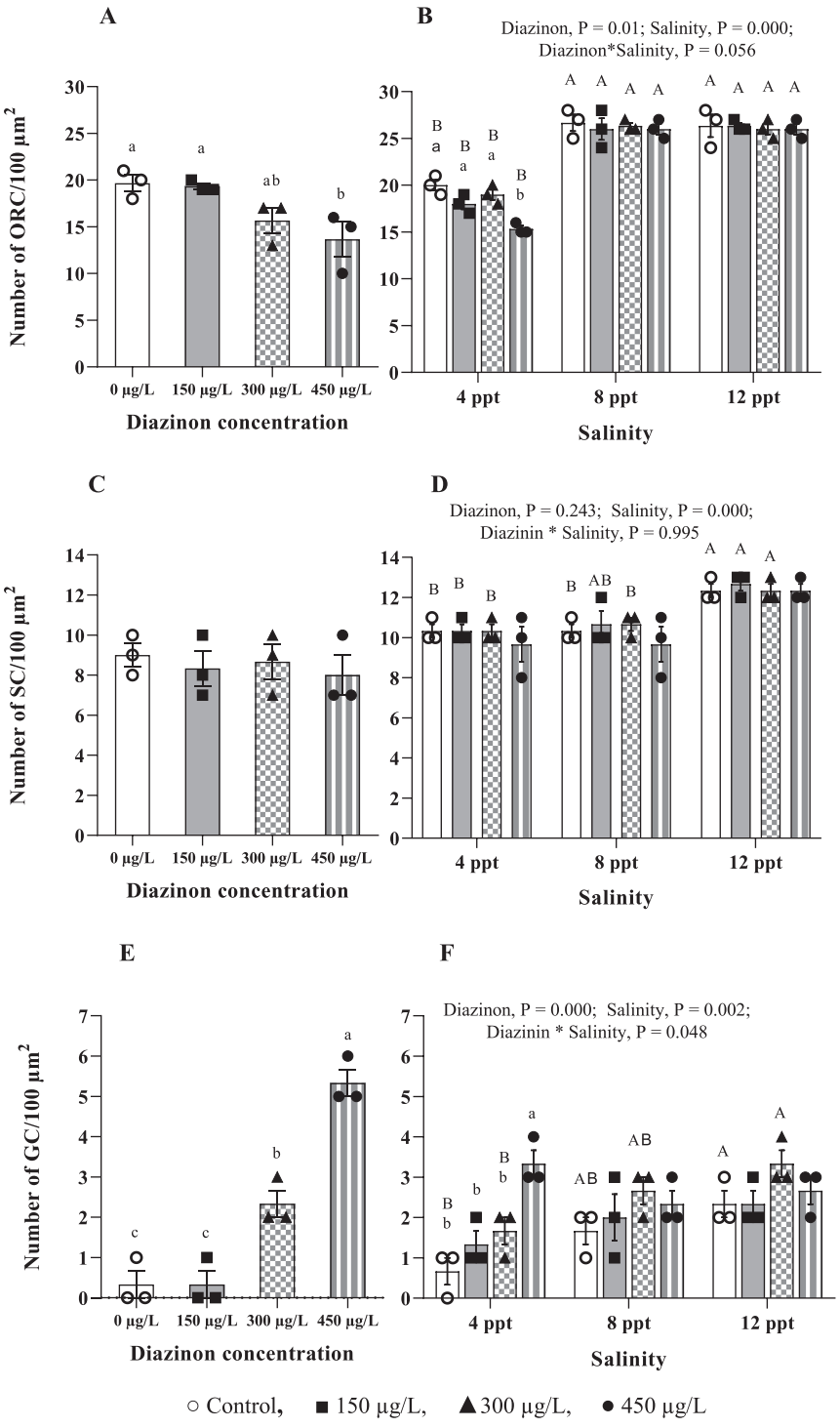

Fig. 3. Significant effects of different levels of diazinon in freshwater for 7 days (A, C, and E; $P < 0.05$, Tukey test, one-way ANOVA) on the number of olfactory receptor cells (ORC), supporting cells (SC) and goblet cells (GC) per 100 µm2 of olfactory epithelium of fingerlings and during diazinon-free saltwater acclimation (B, D, and F; $P < 0.05$, Tukey test, two-way ANOVA). Individual dots are presented as the mean of each tank (3 fish per tank). Column data represent the mean of tanks ± SEM. Different lowercase letters indicate significant differences among diazinon concentrations within the same salinity. Different capital letters indicate significant differences among salinity treatments within the same diazinon concentrations.

assess the significant effects of diazinon concentrations on freshwater and salinity acclimation. A value of $P < 0.05$ was considered statistically significant for all statistical tests.

3. Results

3.1. Histological data

Representative images of olfactory epithelium cross-sections stained with hematoxylin and eosin of the different treatments are shown in Fig. 2. Exposure to the highest concentration of diazinon in freshwater caused a significant decrease in the number of ORCs compared to the control and the low dose of diazinon (Fig. 3-A). A significant change in the number of SCs was not observed after 7 days of diazinon exposure in freshwater (Fig. 3-C). The number of GCs in the olfactory epithelium of fish significantly increased in a dose-dependent manner in response to diazinon 300 and 450 μg·L^{-1} but not to 150 μg·L^{-1}(Fig. 3-E).

The fish that had been exposed to the highest concentration of diazinon in freshwater had lower and higher number of ORCs and GCs, respectively, in 4 ppt saltwater compared to respective control group (Fig. 3-B and D). The number of ORCs in salinities of 8 and 12 ppt were significantly higher than those observed at 4 ppt, whereas a significant increase in the number of SCs was only observed at 12 ppt in comparison to 4 ppt in all the treatments, except fish previously exposed to the highest level of diazinon in freshwater (Fig. 3-B and D). Moreover, being exposed to diazinon in freshwater did not significantly affect the cell number of ORCs, SCs and GCs in the olfactory epithelium after transferring the fish to diazinon-free saltwater at 8 and 12 ppt (Fig. 3-B, D, and F).

3.2. Relative gene expression

The relative gene expression of GPαolf was significantly down-regulated 10-, 12- and 16-fold in fish exposed to 150, 300, and 450 μg·L^{-1} of diazinon, respectively in comparison to control fish (Fig. 4-A). CAMKIId mRNA levels were increased 3-, 7- and 9-fold in fish exposed to 150, 300, and 450 μg·L^{-1} of diazinon in freshwater, respectively, compared to freshwater control fish (Fig. 4-C).

After being transferred to 4 ppt diazinon-free saltwater, the fish previously exposed to 300 and 450 μg·L^{-1} of diazinon in freshwater presented lower (in a dose dependent manner) and higher relative gene expression of GPαolf and CAMKIId, respectively, compared to the respective control group. However, there were no significant differences in GPαolf and CAMKIId mRNA levels among treatments and respective control group either at 8 or 12 ppt saltwater without diazinon (Fig. 4-B and D). Moreover, increasing the salinity up to 12 ppt caused a significant up-regulation of GPαolf transcription in all the treatments (Fig. 4-B), but a down-regulation of CAMKIId in fish previously exposed to 300 and 450 μg·L^{-1} of diazinon (Fig. 4-D) when compared to salinity of 4 ppt.

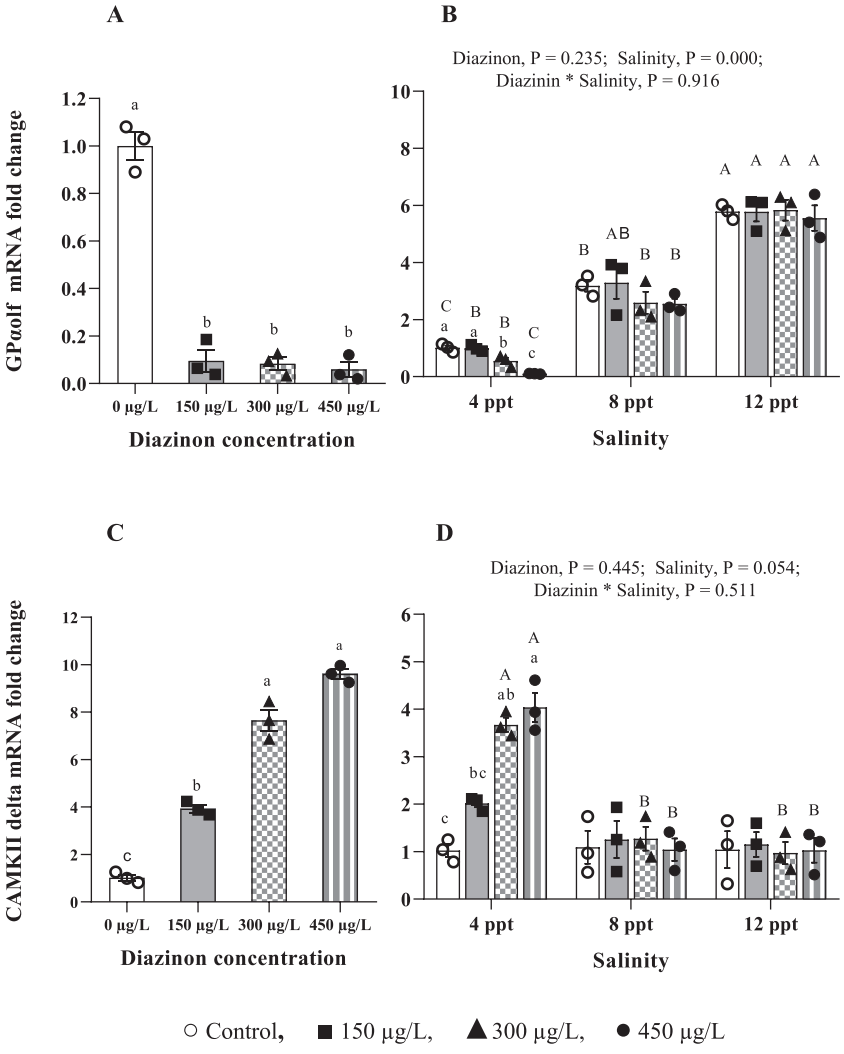

Fig. 4. Effect of different levels of diazinon in freshwater for 7 days (A and C; Tukey test, one-way ANOVA) on relative gene expression of G-protein αolf (GP αolf) and calmodulin-dependent kinase II delta (CAMKIId) and during diazinon-free saltwater acclimation (B and D; Tukey test, two-way ANOVA) in olfactory tissue. Individual dots are presented as the mean of relative gene expression of each tank (3 fish per tank). Column data represent the mean of tanks ± SEM. Different lowercase letters indicate significant differences among diazinon concentrations within the same salinity. Different capital letters indicate significant differences among salinity treatments withen the same diazinon concentrations. RPL-6 was used as the housekeeping gene.

3.3. Whole-body hormone levels

At the end of the exposure to different levels of diazinon in freshwater, whole-body cortisol was significantly elevated only in fish exposed to the highest concentration of diazinon (Fig. 5-A).

After transferring the fingerlings to 4 ppt diazinon-free saltwater, the levels of whole-body cortisol in the previously exposed fish increased in a dose dependent manner until the 300 $\mu g \cdot L^{-1}$ treated fish, which presented similar values than the fish previously exposed to the highest diazinon concentration. Once acclimated to 8 and 12 ppt, the fish previously exposed to the medium and highest diazinon concentrations, presented significantly higher whole-body cortisol levels in comparison to the respective control and diazinon 150 $\mu g \cdot L^{-1}$ pretreated fish groups. In addition, the levels of whole-body cortisol in the control group and the fish that had been exposed to diazinon, elevated progressively by increasing the salinity from 4 to 8 and then 12 ppt (Fig. 5-B).

The 7 days acclimation to diazinon in freshwater provoked a dose-dependent decrease in whole-body T4 and T3 levels compared to the control fish, although there were no significant differences in whole-body T3 levels between control and fish treated with 150 and 300 $\mu g \cdot L^{-1}$ of diazinon. (Fig. 5-C and E).

Subsequently, by transferring the fish to diazinon-free 4 ppt saltwater, the whole-body T4 levels presented the same trend and same differences among groups as observed in freshwater. After acclimation to 8 and 12 ppt, the levels of this hormone were significantly lower in the diazinon pretreated fish in comparison to the control group, with the exception of the fish pretreated with diazinon 150 $\mu g \cdot L^{-1}$ at 12 ppt (Fig. 5-D). In the case of whole-body T3, after acclimation to diazinon-free 4 ppt saltwater, the fish previously exposed to 300 and 450 $\mu g \cdot L^{-1}$ presented lower levels of this hormone in comparison to the control and fish pretreated with diazinon 150 $\mu g \cdot L^{-1}$ group. At 8 and 12 ppt, the levels of this hormone were significantly lower in the diazinon pretreated fish, in a dose dependent manner, than the respective control groups (Fig. 5-F). Moreover, increasing the salinity up to 12 ppt led to an elevation in whole-body T4 in all the treatments and in whole-body T3 in control and fish that had been exposed to low and medium levels of diazinon when compared to 4 ppt (Fig. 5-D and F).

Significant changes in whole-body TSH were not observed among treatments after seven days of diazinon exposure in freshwater (Fig. 5-G). However, there was a significant decrease in whole-body TSH by increasing the salinity up to 12 ppt in the fish that had been previously exposed to 150 $\mu g \cdot L^{-1}$ of diazinon in freshwater (Fig. 5-H).

4. Discussion

Olfactory function and imprinting play a critical role in recalling natal stream during homing migration in fish (Hasler and Scholz, 1983; Ueda, 2016). Imprinting and homing migration have a key role in fish conservation and production, and are economically important for fish propagation centers (Ueda et al., 2016). However, contaminants in the water can negatively affect olfactory functions and even cause loss of olfaction in fish, which in turn can affect the fish population and survival

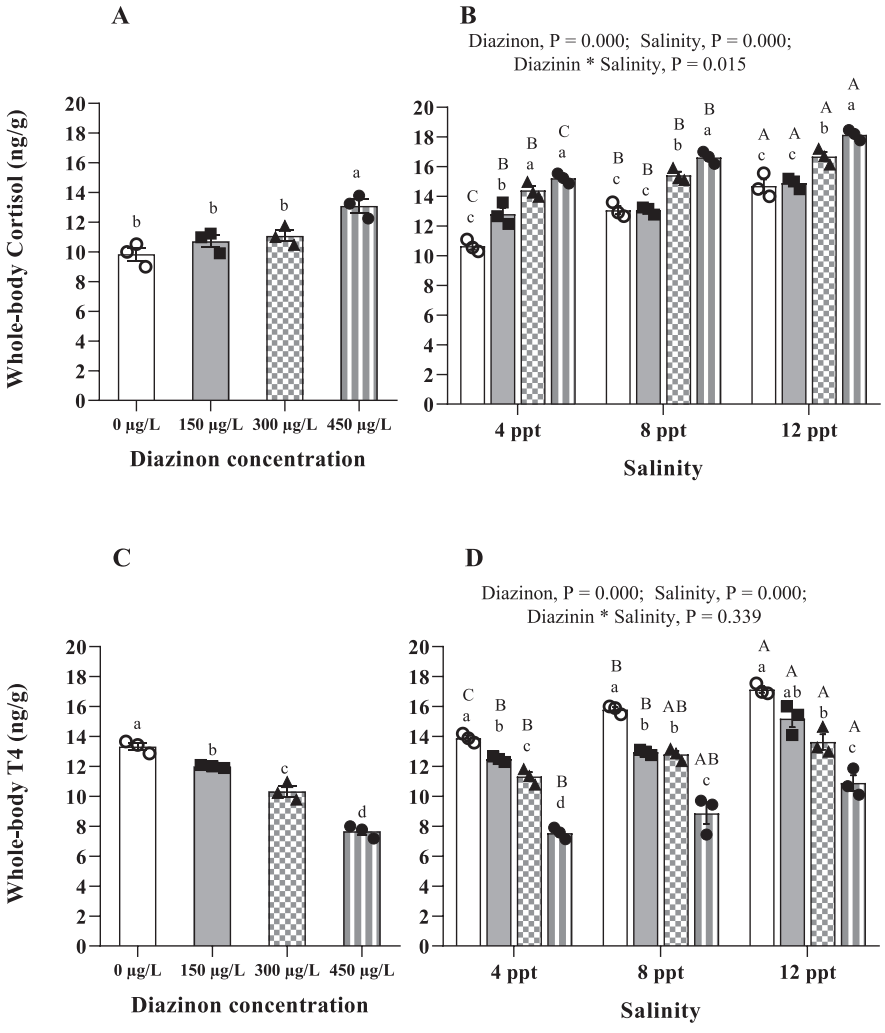

Fig. 5. Effect of different levels of diazinon in freshwater for 7 days (A, C,E, and G; Tukey test, one-way ANOVA) on whole-body cortisol, thyroxin (T4), triiodothyronine (T3) and thyroid-stimulating hormone (TSH) in fish and during diazinon-free saltwater acclimation (B, D, F, and H; Tukey test, two-way ANOVA). Individual dots are presented as the mean of each tank (3 fish per tank). Column data represent the mean of tanks ± SEM. Different lowercase letters indicate significant differences among diazinon concentrations within the same salinity. Different capital letters indicate significant differences among salinity treatments withen the same diazinon concentrations.

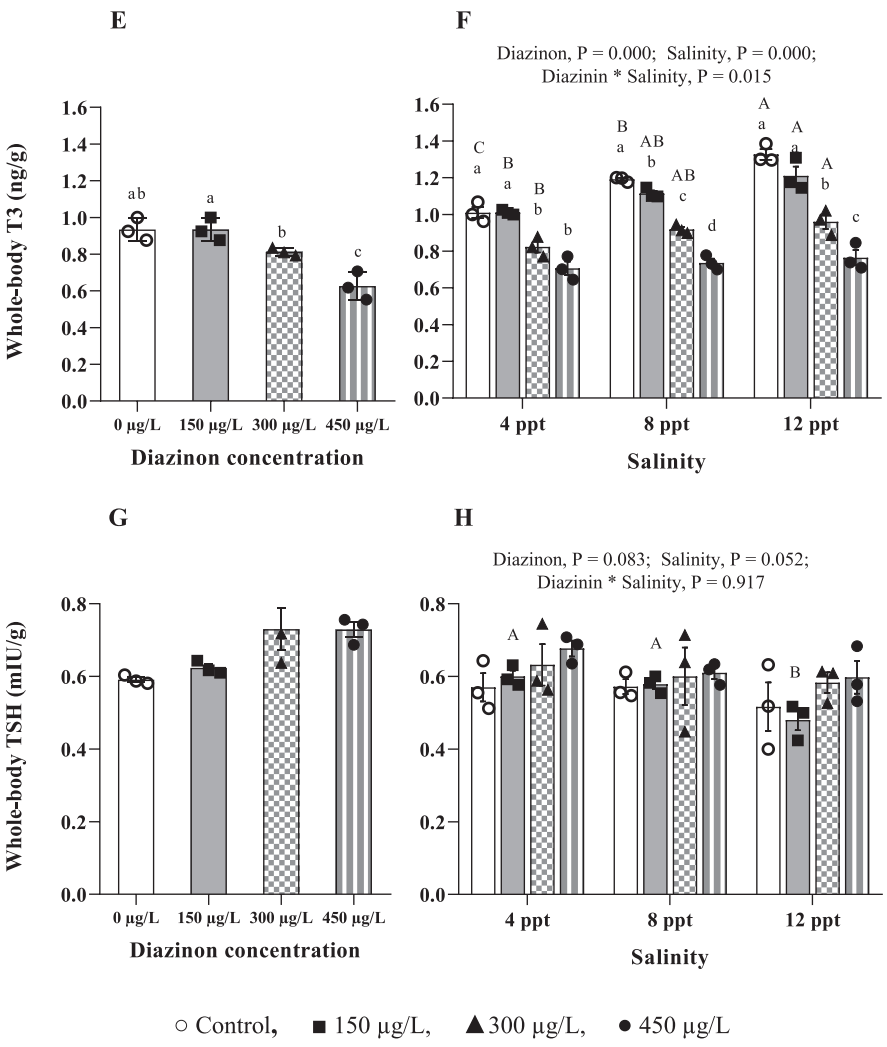

Fig. 5. (*continued*).

(Tierney et al., 2010). Hence, our study assessed the effects of the insecticide diazinon on the olfactory functions and whole-body cortisol and thyroid hormones in Persian sturgeon fingerlings.

4.1. Histological and molecular endpoints

Histological observation of the olfactory epithelium showed that the highest concentration of diazinon caused a decrease in the number of ORCs and an increase in the number of GCs. The decrease in the number of ORCs may be due to cell death in the olfactory epithelium following diazinon exposure. Although few studies have evaluated the histological effects of pesticides on the olfactory epithelium in fish (Tierney et al., 2010), in agreement with the present results, some studies reported that exposure to heavy metals reduced the number of ORCs and increased the number of GCs in rainbow trout (Saucier and Astic, 1995; Hansen et al., 1999). In addition, several studies demonstrated that organophosphate pesticides have toxic effects in different fish organs, such as the gill (Chamarthi et al., 2014; Khosravi Katuli et al., 2014), kidney (Khosravi Katuli et al., 2014), liver (Chamarthi et al., 2014), brain (Chamarthi et al., 2014) and olfactory epithelium (Pourgholam et al., 2013).

In this study, we hypothesized that diazinon can alter the expression of the olfactory signal transduction genes CAMKIId and GPαolf. Calmodulin and G-proteins are involved in many signal transduction pathways related to different physiological processes including, modulation of ion channel activity, learning, and memory, among others (Swulius and Waxham, 2008; Gurevich and Gurevich, 2017). CAMKIId has been shown to modulate neuronal signal transduction through a reduction of the intracellular levels of adenosine monophosphate (AMP) by inhibiting AC (Schulz and Schoneberg, 2003). However, the GPαolf is expressed only in the olfactory tissue (Syrovatkina et al., 2016), where it regulates the signal transduction cascade by activating the formation of cAMP (Nakamura and Gold, 1987; Pifferi et al., 2010).

Our results showed that even the lowest concentration of diazinon down-regulated the GPαolf transcripts and up-regulated the expression of CAMKIId mRNA levels in the olfactory tissues. Maryoung et al., (2015) previously reported that the exposure to another organophosphate insecticide, chlorpyrifos, up-regulated the expression of CAMKIId, chloride intracellular channel 4, and $G\alpha i_1$, inhibiting the olfactory signal transduction in saltwater acclimated rainbow trout. Also, exposure to 35, 88, and 220 $\mu g \cdot L^{-1}$ of chlorpyrifos disrupted the expression of gene pathways related to the odorant-binding in zebrafish (Tilton et al., 2011). Moreover, previous studies reported that exposure to 275 and 675 $\mu g \cdot L^{-1}$ of diazinon decreased the olfactory responses in Persian sturgeon fingerlings (Nabavi et al., 2016), which would be supported by the present findings that diazinon may disrupt olfactory function by affecting the expression of olfactory signal transduction genes and the number of olfactory epithelium cells.

During olfactory signal transduction, odorant molecules bind to ORCs leading to the activation of G-protein and AC pathways to increase the levels of cAMP, a second messenger (Pifferi et al., 2010). In this study, diazinon decreased the number of ORCs, and consequently, may have decreased the G-protein and cAMP activities. Moreover, higher

mucus secretion following the increase in GCs number could also reduce the olfactory sensitivity to detect odorants and therefore decrease the olfactory signal transduction (Elinder and ArhemMetal, 2003; DeMaria and Ngai, 2010; Tierney et al., 2010). In addition, the up-regulation of CAMKIId (inhibitor of the signaling pathway) and down-regulation of GPαolf indicated that the olfactory signal transduction pathway may negatively affect cAMP synthesis and reduce the overall olfactory function in fish treated with diazinon. Thus, potential outcomes may reduce the olfactory sensitivity to odorants and contribute to the impairment of olfactory function in fish, which may affect olfactory imprinting in sturgeon fingerlings.

Since sturgeon fingerlings undergo transition to seawater after the exposure to pesticides in the rivers, we transferred all the fish to higher salinity. The current study demonstrated that, the changes in ORC and GC numbers, as well as gene expression of GPαolf and CAMKIId, which had been induced by diazinon in freshwater, were equalized after diazinon-free 8 and 12 ppt saltwater acclimation. Some studies previously reported that after removing the chemical treatments, the function of the olfactory system could be recovered in zebrafish (Iqbal and Byrd-Jacobs, 2010; Lazzari et al., 2017). Moreover, the olfactory function in yellow perch, *Perca flavescens*, which had been exposed to the metals in a contaminated lake, was recovered after transferring the fish to clean lakes (Azizishirazi et al., 2013). Also, saltwater has been shown to reduce the olfactory copper toxicity in chinook salmon, *Oncorhynchus tshawytscha*, and coho salmon, *O. kisutch*, (Sommers et al., 2016). Khosravi Katuli et al. (2014) showed that the fish can repair the diazinon-induced damage to the gill after being transferred to diazinon-free brackish water. As both, gills and the olfactory epithelium, are in direct contact with the water, the previous results support the idea that the diazinon-induced olfactory disruption observed in the present study may be reverted when the fish are transferred to diazinon-free saltwater.

4.2. Whole-body hormone levels

Environmental pollutions can have different effects on hormone levels depending on the toxicant type, concentrations, time of exposure, fish species, and water quality (Monterio et al., 2005; Jee et al., 2005). It is proven that an increase in cortisol levels is associated with stressful situations in fish to produce energy (Pankhurst, 2011; Saravanan et al., 2011). Previous investigations reported that after 96 h of exposure to diazinon, whole-body cortisol increased in Caspian roach, *Rutilus rutilus*, (Khosravi Katuli et al., 2014) and, also the exposure to another pesticide, atrazine, for 7 days, increased the plasma cortisol levels in Atlantic salmon, *Salmo salar* (Waring and Moore, 2004). However, some studies have shown that when fish are stressed for long periods, the levels of cortisol may return to basal levels as a protective response to prevent tissue damage (Robertson et al., 1987; Wendelaar-Bonga, 1997; Iwama et al., 2006; Mojazi Amiri et al., 2017). This idea is supported by the fact that in Persian sturgeon fingerlings exposed to 180, 540, and 900 $\mu g \cdot L^{-1}$ of diazinon for 96 h, the cortisol levels increased in all the concentrations (Hajirezaee et al., 2016), but in the current trial, after 7 days of exposure, the level of whole-body cortisol was not affected in the 150 and 300 $\mu g \cdot L^{-1}$ concentrations of diazinon. Therefore, these findings suggest that depending on the time of exposure and the diazinon concentrations, the levels of the cortisol in Persian sturgeon fingerlings may return to basal levels. However, the observed increases in whole-body cortisol in the fingerlings treated with high level of diazinon indicated that fish were still undergoing a stressful situation.

It has been demonstrated that cortisol rises in response to increased environmental salinity to facilitate seawater adaptation in many teleost species (Evans et al., 2005; Arjona et al., 2008; McCormick et al., 2008). Similarly, in the present study, after transferring the sturgeon fingerlings to 12 ppt salinity, whole-body cortisol increased in all the treated fish in comparison to the previous salinities (4 and 8 ppt). These results indicated an osmoregulatory function of cortisol to seawater acclimation in Persian sturgeon. In addition, a significant increase in whole-body cortisol was provoked after salinity acclimation in the fish that had been exposed to the medium and high levels of diazinon in freshwater. Therefore, it can be stated that the toxicity induced by prior treatment with diazinon upper than 150 $\mu g \cdot L^{-1}$ in freshwater, may still have effects on cortisol levels after the progressive acclimation of the fish to diazinon-free saltwater. Similar to the present findings on diazinon, freshwater exposure to atrazine led to an increase in plasma cortisol in Atlantic salmon smolts after transference of the fish to atrazine-free seawater (Waring and Moore, 2004).

In addition to saltwater adaptation and osmoregulatory function (McCormick, 2001), cortisol is well known as a stress indicator in fish (Pankhurst, 2011). Besides, it is known that chronic stress can disrupt learning and memory (Luine et al., 1994; Nishimura et al., 1999). Thus, the observed dose-dependent increase in whole-body cortisol in response to diazinon indicates a stress induction that was even maintained after saltwater acclimation in the absence of the stressor (diazinon). Such a long-term cortisol response may have a negative effect on the olfactory imprinting in fish, but additional studies are needed to confirm this hypothesis.

In this study, whole-body thyroid hormone levels (T4 and T3) were altered by diazinon in the freshwater. In this regard, high and medium concentrations of diazinon had stronger effects on T4 and T3. Previous studies demonstrated that organophosphate pesticides such as diazinon and chlorpyrifos disrupt the thyroid hormone concentrations and their functions in fish (Khosravi Katuli et al., 2014; Hajirezaee et al., 2016; Mojazi Amiri et al., 2017). For example, diazinon was shown to suppress thyroid hormone production in Caspian roach, (Khosravi Katuli et al., 2014). Similarly, thyroid hormones decreased in walking catfish, *Clarias batrachus*, exposed to another organophosphate insecticide, malathion (Lal et al., 2012). Pesticides can disrupt the thyroid system by different mechanisms, such as the inhibition of iodine absorption, direct interaction with thyroid hormone receptors, binding to plasma thyroid hormone-binding proteins and changing the expression of genes that regulate hormone biosynthesis or clearance, (Rakitsky et al., 2000; Zoeller, 2007).

The increase observed in whole-body T4 and T3 during the salinity acclimation in the present study is consistent with the previous postulate that thyroid hormones play a role in seawater acclimation (McCormick, 2001; Allen et al., 2009; Khosravi Katuli et al., 2014) and it has been demonstrated that thyroid hormone levels increase after salinity exposure in some anadromous and freshwater fish (Orozco et al., 2000; Peter, 2007) and also during downstream migration (Iwata et al., 2003; Ojima and Iwata, 2007). Moreover, the fingerlings that had been exposed to the highest level of diazinon in freshwater had the lower concentrations of these hormones. Similarly, Khosravi Katuli et al. (2014) reported that whole-body T4 and T3 levels were lower in Caspian roach fingerlings acclimated to brackish water after being exposed to diazinon while in freshwater when compared to control fish. Also, in another study, prior exposure to atrazine in freshwater altered the levels of T4 in Atlantic salmon smolts after being transferred to seawater (Waring and Moore, 2004).

Olfactory imprinting is associated with an increase in thyroid hormones and cortisol in fish (Hasler and Scholz, 1983; Dickhoff et al., 1990; Lema and Nevitt, 2004; Ojima and Iwata, 2007; Ueda, 2016) and these hormones affect olfactory cell proliferation, sensitivity (Lema and Nevitt, 2004) and the expression of genes related to memory forming at the sensitive period of imprinting (Ueda, 2016). Scholz (1980) demonstrated that a decrease in the levels of thyroid hormones at early life stages in coho salmon impair the detection of artificial odorants during homing. Moreover, changes in salinity during downstream migration in fish are essential to promote the thyroid hormone response to complete the olfactory imprinting and memory forming (Kim et al., 2015; Ueda, 2016). Based on the obtained results, the reduction in the concentrations of whole-body T4 and T3 in fish exposed to diazinon may adversely affect the olfactory function and the gene expression associated to olfactory imprinting in fish, which in turn may result in a potential

disruption of the homing migration of sturgeon fish and other anadromous species.

5. Conclusions

In conclusion, a properly functioning olfactory system at physiological and molecular levels is essential for the accurate formation of olfactory imprinting in fish. Our findings indicate that the release of sturgeon fingerlings into diazinon polluted rivers can change the number of ORCs and GCs in the olfactory epithelium and alter the expression of GPαolf and CAMKIId in olfactory tissues of Persian sturgeon fingerlings. Moreover, diazinon also altered the levels of whole-body cortisol, T4, and T3, which in turn may have negative consequences for the formation of olfactory imprinting. Although after acclimation to diazinon-free saltwater the cellular and molecular responses caused by diazinon diminished, the hormone levels were still affected by this pesticide. Therefore, exposure to pesticides such as diazinon in freshwater may affect the hormonal function of the fingerlings during migration to saltwater. With all this, the present study found evidence of the toxic effects of an overused pesticide in Iran, on different physiological and molecular factors related to the olfactory imprinting in Persian sturgeon fingerlings. However, additional studies are needed to determine in more detail how these molecular and hormonal changes are translated into impaired olfactory imprinting or other behavioral responses.

Declaration of Competing Interest

All of the authors declare that they have no conflict of interest.

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